DNA Topoisomerase II as the Target for the Anticancer Drug TOP-53: Mechanistic Basis for Drug Action†

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ABSTRACT: TOP-53 is a promising anticancer agent that displays high activity against non-small cell lung cancer in animal tumor models [Utsugi, T., et al. (1996) *Cancer Res. 56*, 2809−2814]. Compared to its parent compound, etoposide, TOP-53 is considerably more toxic to non-small cell lung cancer cells, is more active at generating chromosomal breaks, and displays improved cellular uptake and pharmacokinetics in animal lung tissues. Despite the preclinical success of TOP-53, several questions remain regarding its cytotoxic mechanism. Therefore, this study characterized the basis for drug action. Results indicate that topoisomerase II is the primary cytotoxic target for TOP-53. Furthermore, the drug kills cells by acting as a topoisomerase II poison. TOP-53 exhibits a DNA cleavage site specificity that is identical to that of etoposide. Like its parent compound, the drug increases the number of enzyme-mediated DNA breaks by interfering with the DNA religation activity of the enzyme. TOP-53 is considerably more efficient than etoposide at enhancing topoisomerase II-mediated DNA cleavage and exhibits high activity against human topoisomerase IIα and IIβ in vitro and in cultured cells. Therefore, at least in part, the enhanced cytotoxic activity of TOP-53 can be attributed to an enhanced activity against topoisomerase II. Finally, TOP-53 displays nearly wild-type activity against a mutant yeast type II enzyme that is highly resistant to etoposide. This finding suggests that TOP-53 can retain activity against systems that have developed resistance to etoposide, and indicates that substituents on the etoposide C-ring are important for topoisomerase II−drug interactions.

Topoisomerase II is an essential enzyme that plays critical roles in a number of DNA processes (*1*−*4*). It is also the target for some of the most widely prescribed and most successful drugs currently used to treat human cancers (*1*, *3*−*8*). These agents kill cells by increasing the concentration of covalent topoisomerase II-cleaved DNA complexes that are normal, but short-lived, intermediates in the catalytic cycle of the enzyme (*1*, *3*−*9*). As a consequence of their action, anticancer drugs targeted to the enzyme generate high levels of double-stranded breaks in the genomes of treated cells and ultimately trigger cell death pathways (*3*−*8*, *10*). Since these agents convert topoisomerase II into a lethal cellular toxin, they are termed "topoisomerase II poisons" to distinguish their mechanism from drugs that act by inhibiting the catalytic activity of the enzyme (*11*).

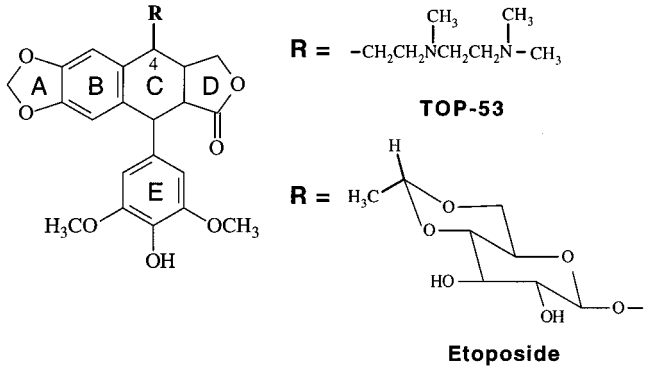

FIGURE 1: Structures of TOP-53 and etoposide. The drugs differ only by the substitution of an aminoalkyl moiety (TOP-53) for a glycoside moiety (etoposide) at the 4-position (denoted by the R substituent) of the C-ring.

One of the most important topoisomerase II-targeted agents currently available is the podophyllotoxin derivative, etoposide (Figure 1) (*3*, *4*, *6*−*8*, *12*). Etoposide is front-line therapy for a variety of systemic malignancies, including several leukemias and lymphomas. It is also effective against selected solid tumors, including small cell lung cancer, sarcomas, and germ cell cancers.

Despite the overall success of drugs such as etoposide, a number of solid tumors respond poorly to current chemotherapy regimens (*13*−*16*). For example, in marked contrast to the high response of small cell lung cancer patients to

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etoposide (40−80%), the response rate of non-small cell lung cancer patients is low (∼10%). Several specific attributes of non-small cell lung cancer cells may explain this difference. When compared to small cell lung cancer cells, non-small cell lung cancers display a 2−3-fold longer doubling time, a lower labeling index, and a decreased nuclear concentration and activity of topoisomerase II (*17*, *18*).

In an effort to develop more effective strategies against non-small cell lung cancer, a novel etoposide derivative, TOP-53 [4β-(aminoalkyl)-4′-*O*-demethyl-4-desoxypodophyllotoxin (Figure 1)], was developed (*19*). This derivative replaces the glycoside moiety at the 4-position of the etoposide C-ring with an amino alkyl residue. TOP-53 displays a greater (∼2-fold) ability to inhibit topoisomerase II-catalyzed decatenation in cell extracts and induces DNA breaks in human non-small cell lung cancer cells with an effective dose that is ∼7-fold lower than that of the parent drug (*19*). Furthermore, at optimal doses, TOP-53 is taken up by normal lung tissue and non-small cell lung tumors at levels that are ≥10-fold higher than that of etoposide, and the drug persists for several hours after etoposide is no longer detectable (*19*). Finally, TOP-53 displays more potent activity than etoposide against a variety of mouse tumors and human non-small cell lung cancer xenographs (*19*).

Despite the preclinical success of TOP-53, the cellular targeting of topoisomerase II by TOP-53 has not been fully addressed. Furthermore, information regarding the mechanistic basis for drug action is minimal. At the present time, it is not clear whether TOP-53 is intrinsically more active against human topoisomerase II, or whether its enhanced efficacy against tumor models specifically reflects its improved pharmacokinetics.

Therefore, to reconcile these important issues, the cellular targeting of topoisomerase II by TOP-53 was confirmed and interactions between TOP-53 and human topoisomerase II were characterized. Results indicate that the drug displays better DNA cleavage-enhancing activity against human topoisomerase IIα and IIβ than etoposide. Furthermore, it appears that TOP-53 increases the level of enzyme-mediated DNA scission primarily by interfering with the ability of topoisomerase II to religate cleaved nucleic acids. Finally, TOP-53 exhibits a DNA cleavage specificity that is identical to that of etoposide, but displays markedly different activity against mutant forms of topoisomerase II with altered drug sensitivity. These results imply that the substituent at the 4-position of the etoposide C-ring plays an impotant role in mediating drug−enzyme interactions.

EXPERIMENTAL PROCEDURES

Wild-type yeast topoisomerase II as well as the mutants ytop2H1011Y[1] (*20*) and ytop2G436S (*21*) were expressed in *Saccharomyces cerevisiae* (*23*) and purified as described by Elsea et al. (*20*). Human topoisomerase IIα and topoisomerase IIβ were expressed in *S. cerevisiae* (*23*) and purified by the protocol of Kingma et al. (*24*). Negatively supercoiled pBR322 DNA was prepared as described previously (*25*). Restriction endonucleases were from New England Biolabs. Etoposide was from Sigma and was stored at 4 °C as a 10 mM stock solution in 100% DMSO. TOP-53 was obtained from Taiho Pharmaceuticals and was made fresh as a 10 mM stock solution in water or 100% DMSO. 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* (*26*). Two strains derived from JN394 also were used: JN394top1$^-$, which has a chromosomal deletion of the topoisomerase I gene (*27*); and JN394t2-1, in which the wild-type topoisomerase II gene (*TOP2*+) is replaced with the temperature-sensitive *top2-1* mutant allele (*26*, *28*, *29*). These two strains are isogeneic to JN394 in all other respects.

Determination of the Primary Cellular Target of TOP-53. The sensitivities of yeast strains JN394, JN394top1$^-$, and JN394t2-1 to TOP-53 were determined as previously described (*26*, *27*, *29*, *30*). Briefly, cells were cultured in YPDA medium at 25 °C or (as appropriate) at 30 °C. After logarithmically growing cells were adjusted to a titer of 1×10^6 cells/mL, TOP-53 (0−100 μM) was added to the cultures. Cells were incubated with drug for 24 h, then diluted into drug-free YPDA medium, and plated in triplicate onto YPDA medium solidified with 1.5% Bacto-agar. In experiments that utilized strain JN394top1$^-$, cells were plated in triplicate onto synthetic complete medium solidified with 1.5% Bacto-agar. Plates were incubated at 25 or 30 °C, and drug sensitivity was determined by counting the number of surviving colonies.

DNA Cleavage. Topoisomerase II DNA cleavage reactions were carried out as described previously (*31*). When human type II topoisomerases were employed, assays contained 40 or 10 nM enzyme (when carried out in the absence or presence of 1 mM ATP, respectively) and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of human cleavage buffer [10 mM Tris-HCl (pH 7.9), 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, and 2.5% glycerol]. In assays in which topoisomerase IIα was characterized, drug concentrations were varied from 0 to 50 μM. In assays in which topoisomerase IIβ was characterized, drug concentrations were varied from 0 to 100 μM. Reactions were started by the addition of topoisomerase II, and the mixtures were 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). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45 °C to digest the topoisomerase II. 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 45 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in TAE buffer [40 mM Tris-acetate (pH 8.3) and 2 mM EDTA] containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled plasmid to linear 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. Alternatively, DNA

[1] The mutant type II topoisomerases employed in this study formerly were known as ytopH1012Y (*20*) and ytop2G437S (*21*). The names were changed to ytop2H1011Y and ytop2G436S, respectively, to reflect a mistake that was recently discovered in the amino acid sequence of wild-type yeast topoisomerase II (*22*).

bands were quantitated using an Alpha Innotech digital imaging system. In either case, the intensity of bands in the negative was proportional to the amount of DNA present.

When yeast type II topoisomerases were utilized, reaction mixtures contained 150 nM enzyme and 5 nM negatively supercoiled pBR322 DNA in a total volume of 20 μL of 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% glycerol, 10% DMSO, and 0−200 μM drug (*29*). Samples were incubated at 28 °C for 6 min, and cleavage products were trapped by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM EDTA. Enzymes were digested with proteinase K, and DNA products were resolved and analyzed as described above.

Site-Specific DNA Cleavage Induced by TOP-53. Topoisomerase II DNA cleavage sites were identified as described by Burden et al. (*32*). A linear 564 bp fragment (*Eag*I/*Bam*HI) of pBR322 plasmid DNA was prepared and labeled with ^{32}P on a single 5′ terminus. Cleavage reaction mixtures contained 1.4 nM (25 ng) labeled DNA substrate, 60 nM human topoisomerase IIα, and 1 mM ATP in 50 μL of human cleavage buffer. Assays were carried out in the absence of drugs, or in the presence of 0−50 μM TOP-53 or 50 μM etoposide. Reactions were started by the addition of topoisomerase IIα and the mixtures 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 a 0.8 mg/mL solution) 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 a 10% methanol/10% acetic acid mixture and dried. DNA cleavage products were analyzed on a Molecular Dynamics PhosphorImager.

Stimulation of Topoisomerase II-Mediated DNA Cleavage in CEM Cells Treated with TOP-53. Human CEM cells (purchased from ATTC) were cultured at 37 °C in RPMI 1640 medium (Cellgro), containing 10% heat-inactivated fetal calf serum and 2 mM glutamine, under 5% CO_2. The in vivo complex of enzyme (ICE) bioassay (*33*, *34*) was employed to determine the ability of TOP-53 to induce topoisomerase II-mediated DNA cleavage in treated CEM leukemic cells. Briefly, exponentially growing cultures were treated with 100 μM TOP-53 or etoposide for 1 h. Cells (∼4 × 10^6) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% Sarkosyl. Following gentle douncing, cell lysates were layered over CsCl step gradients (containing 2 mL each of 1.82, 1.72, 1.50, and 1.45 g/mL CsCl) and centrifuged at 165000*g* for 24 h at 20 °C. Fractions (0.4 mL) were collected from the tops of the centrifuge tubes. Samples (100 μL) from each fraction were diluted 1:1 with 25 mM sodium phosphate (pH 6.5) and blotted onto nitrocellulose membranes using a Schleicher and Schuell slot blot apparatus. Covalent complexes formed between DNA and either topoisomerase IIα or IIβ were detected by Western blot analysis using monoclonal antibodies directed against human topoisomerase IIα (purchased from Kiamaya; 1:200 dilution) or polyclonal antibodies directed against human topoisomerase IIβ (gift of A. H. Andersen and O. Westergaard; 1:500 dilution). Topoisomerase II was visualized by ECL. DNA-containing fractions were detected by electrophoresis on 0.5% agarose gels in TAE buffer followed by staining with ethidium bromide.

DNA Religation. The DNA religation reaction of human topoisomerase IIα was monitored according to the procedure of Byl et al. (*35*). Topoisomerase II DNA cleavage−religation equilibria were established as described above in the presence or absence of 1 mM ATP. Religation was initiated by shifting reaction mixtures 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.

RESULTS

TOP-53 Kills Yeast Cells by Enhancing Topoisomerase II-Mediated DNA Cleavage. TOP-53 inhibits the decatenation activity of human topoisomerase II in cell extracts and generates DNA breaks in treated cells (*19*). Together with the fact that TOP-53 is structurally related to etoposide, it is believed that the drug kills cells by increasing levels of topoisomerase II-mediated DNA breaks (*19*). However, the assays originally used to monitor cellular DNA breaks did not determine whether cleaved chromosomal fragments were protein-linked (as would be expected for a drug that enhanced DNA cleavage by either topoisomerase I or II) (*3*, *4*, *6*−*8*, *12*). Therefore, on the basis of the original report, the cytotoxic bases for the actions of TOP-53 are unclear.

To address the cellular targeting of TOP-53, a second study utilized a yeast (*S. cerevisiae*) genetic system that overexpressed varying levels of plasmid-encoded yeast topoisomerase II or human topoisomerase IIα (*36*). The theory underlying these experiments is as follows: If a drug kills cells by enhancing topoisomerase II-mediated DNA cleavage, increased levels of enzyme should lead to higher levels of DNA breaks (*37*). Consequently, a compound such as TOP-53 should be more toxic in a background of higher topoisomerase II concentrations. Since yeast cells became hypersensitive to TOP-53 when the level of topoisomerase II overexpression was increased, it was concluded that the drug killed cells by acting as a topoisomerase II poison (*36*).

An important caveat to this study should be noted. Since the genetic model that was utilized depended on topoisomerase II overexpression, it can only be concluded that topoisomerase II is an important (but not necessarily the only) target for TOP-53. The only way to draw the conclusion that TOP-53 kills cells primarily by acting as a topoisomerase II poison is to decrease normal cellular levels of topoisomerase II and determine whether this leads to drug resistance (*27*, *38*).

Therefore, in the present study, we employed a yeast strain carrying a temperature-sensitive topoisomerase II allele (*top2-1*) (*26*, *28*, *29*). Because of its essential nature, topoisomerase II cannot be deleted from cells (*1*, *2*, *39*, *40*). 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 (*28*).

As seen in Figure 2 (left panel), *top2-1* cells are highly resistant to TOP-53 at the semipermissive temperature. Resistance was not due to a change in drug metabolism at 30 °C, since the sensitivity of the *TOP2* parental strain was

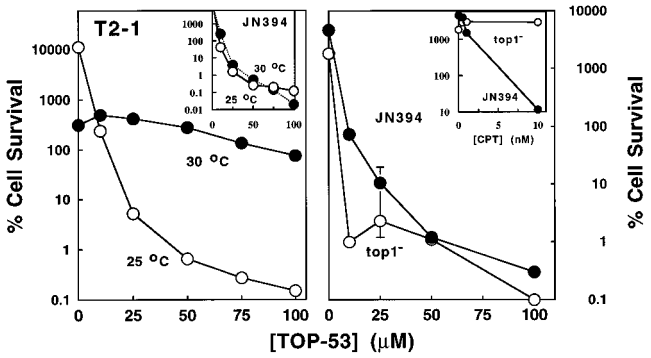

FIGURE 2: Topoisomerase II is the primary cytotoxic target for TOP-53 in yeast cells. (Left) The role of topoisomerase II in TOP-53 cytotoxicity was assessed using yeast strain JN394top2-1, which expresses a temperature sensitive topoisomerase II allele. Top2-1 displays greatly reduced catalytic activity at the semipermissive temperature of 30 °C. The effects of TOP-53 on JN394top2-1 and its parental strain JN394 (inset) at 25 °C (permissive temperature) and 30 °C are shown. (Right) The role of topoisomerase I in TOP-53 cytotoxicity was examined by comparing the drug sensitivities of yeast strains JN394 and JN394top1⁻. JN394top1⁻ is isogenic to JN394 except that the topoisomerase I gene is not functional. The effects of camptothecin (CPT) on these two strains also are shown (inset). In all cases, data represent one of two independent experiments, each carried out in triplicate. Standard deviations are indicated by error bars.

identical at 25 and 30 °C (see the inset). These results indicate that topoisomerase II is the primary cellular target for TOP-53 and that the drug kills cells by acting as a topoisomerase II poison.

To confirm that topoisomerase I plays no role in TOP-53 toxicity, the effects of the drug on a *top1*⁻ strain (devoid of topoisomerase I activity) were determined [Figure 2 (right panel)]. Since topoisomerase I is not an essential enzyme in yeast, strains can be established that completely lack the enzyme (*27*, *28*, *39*, *40*). As seen in the inset, the *top1*⁻ strain is refractory to the topoisomerase I poison camptothecin.

As expected, no resistance was observed when *top1*⁻ cells were treated with TOP-53 [Figure 2 (right panel)]. In fact, the strain was hypersensitive to the drug. This latter effect is commonly seen for topoisomerase II poisons in *top1*⁻ strains, and reflects the fact that topoisomerase II assumes the physiological duties of the missing type I enzyme (*39*−*42*). Taken together, these findings provide strong evidence that TOP-53 kills cells by increasing levels of topoisomerase II-mediated DNA cleavage.

TOP-53 Stimulates DNA Cleavage Mediated by Human Topoisomerase II Isoforms. TOP-53 is more effective than etoposide at increasing levels of DNA breaks in human non-small cell lung cancer cells (*19*). However, it is not clear whether this is due to an intrinsically greater effect on the DNA cleavage activity of topoisomerase II or reflects the improved pharmacokinetics of the drug. Therefore, the ability of TOP-53 to increase DNA cleavage mediated by human topoisomerase II was determined.

Mammalian species contain two closely related isoforms of topoisomerase II, α and *β* (*1*, *4*, *43*−*46*). Topoisomerase IIα is dramatically upregulated during periods of rapid cell proliferation and plays critical roles in mitosis (*47*−*49*). In contrast, topoisomerase II*β* activity is relatively constant across cell and growth cycles, leading to speculaion that the enzyme is involved in ongoing nuclear processes (*46*). Although both topoisomerase II isoforms appear to play a

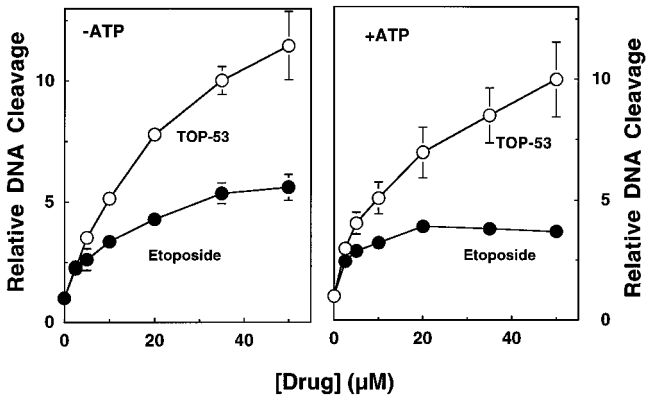

FIGURE 3: TOP-53 stimulates DNA cleavage mediated by human topoisomerase IIα in vitro. Negatively supercoiled pBR322 DNA was used as the cleavage substrate. Assays were carried out in the presence (right) and absence (left) of 1 mM ATP. The effects of TOP-53 (○) and etoposide (●) on DNA cleavage mediated by topoisomerase IIα are shown. Levels of DNA cleavage are reported relative to the amount of cleavage observed in the absence of drug. Error bars represent the standard error of the mean for three independent experiments.

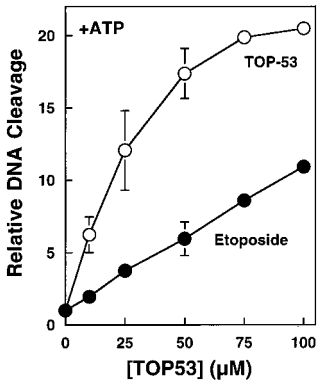

FIGURE 4: TOP-53 stimulates DNA cleavage mediated by human topoisomerase II*β* in vitro. Negatively supercoiled pBR322 DNA was used as the cleavage substrate. The effects of TOP-53 (○) and etoposide (●) on DNA cleavage mediated by topoisomerase II*β* in the presence of 1 mM ATP are shown. Levels of DNA cleavage are reported relative to the amount of cleavage observed in the absence of drug. Error bars represent the standard error of the mean for two independent experiments.

role in mediating the effects of anticancer drugs, topoisomerase IIα is believed to be the more important target for many agents, including etoposide (*7*, *44*, *50*−*55*).

The effects of TOP-53 on the DNA cleavage activity of human topoisomerase IIα and II*β* are shown in Figures 3 and 4. TOP-53 was a potent enhancer of enzyme-mediated DNA cleavage. At every concentration that was tested, TOP-53 generated higher levels of topoisomerase II-mediated DNA cleavage than etoposide. It was impossible to reach plateau levels of cleavage with TOP-53 under the conditions that were employed, because the drug induced multiple cleavage events per plasmid when concentrations were higher than those shown in the figures. However, at the maximal drug doses that were examined, levels of cleavage in the presence of TOP-53 were 2−3 times higher than those induced by etoposide and represented a 10−20-fold enhancement of DNA scission.

As seen in experiments with topoisomerase IIα (Figure 3), similar levels of cleavage enhancement were observed in either the absence or presence of ATP. Although levels

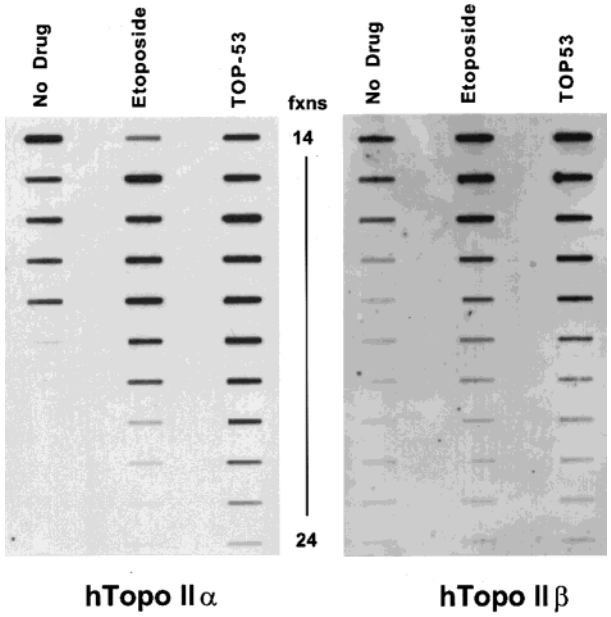

FIGURE 5: TOP-53 enhances DNA cleavage mediated by topoisomerase IIα and IIβ in treated human CEM cancer cells. An ICE bioassay was employed to monitor the effects of TOP-53 on topoisomerase II-mediated DNA scission. DNA-containing CsCl gradient samples from cultures treated with no drug, 100 μM etoposide, or 100 μM TOP-53 are shown. Blots were probed with antibodies directed against human topoisomerase IIα (left) or IIβ (right).

of cleavage in the presence of ATP may have risen slightly faster at low concentrations of TOP-53, no significant differences were observed.

The effects of TOP-53 on the DNA cleavage activity of human topoisomerase IIα and IIβ were similar. Higher levels of drug-induced DNA cleavage were reached in experiments in which the β-isoform was utilized, because it was possible to use greater levels of drug without generating multiple cleavage events per plasmid.

To determine whether these in vitro results are predictive of cellular events, an ICE bioassay (*33*, *34*) was employed to monitor the effects of TOP-53 on topoisomerase II-mediated DNA scission in cultured human cancer cells. In this assay, proteins that are covalently attached to DNA are separated from free proteins by sedimentation through a CsCl gradient. DNA-containing gradient samples from cultures treated with no drug, 100 μM etoposide, or 100 μM TOP-53 are shown in Figure 5. Blots were probed with antibodies directed against human topoisomerase IIα (left) or IIβ (right). As determined by the increased levels of covalent enzyme−DNA complexes, TOP-53 stimulated cleavage mediated by human topoisomerase IIα and IIβ in treated CEM cells. Although the effects of TOP-53 on topoisomerase IIα appeared to be more dramatic, cleavage enhancement by both enzymes was greater than that observed following treatment of cells with etoposide.

Inhibition of Topoisomerase II-Mediated DNA Religation by TOP-53. There are two distinct, but not mutually exclusive, mechanisms by which topoisomerase II-targeted drugs increase levels of enzyme-generated DNA breaks. Some members of the podophyllotoxin family, such as etoposide and teniposide, strongly inhibit the DNA religation activity of the type II enzyme (*4*, *56*, *57*). In contrast, azatoxin, which contains a related ring structure, has virtually

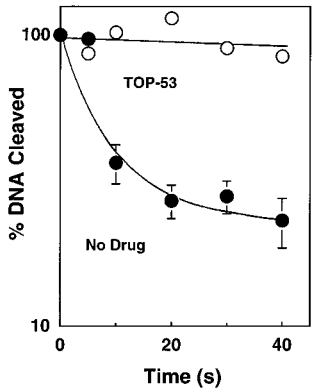

FIGURE 6: TOP-53 enhances topoisomerase II-mediated DNA cleavage primarily by inhibiting religation. The effect of 25 μM TOP-53 on the ability of human topoisomerase IIα to religate pBR322 DNA was compared to the effect of enzyme-mediated DNA religation in the absence of drug. Assays were carried out in the absence of ATP. Religation is expressed as the percent loss of linear DNA, which was set to 100% at time zero. Data represent the average of three independent assays. Error bars indicate the standard error of the mean.

no effect on religation rates and is assumed to act by increasing the forward rate of enzyme-mediated DNA scission (*58*).

As seen in Figure 6, TOP-53 is a strong inhibitor of DNA religation mediated by human topoisomerase IIα. Similar results were observed in the presence or absence of ATP (not shown). These results indicate that TOP-53 increases levels of topoisomerase II-generated DNA breaks primarily by interfering with the DNA religation reaction of the enzyme.

Site-Specific DNA Cleavage Induced by TOP-53. It is possible that the higher levels of DNA scission induced by TOP-53 compared to etoposide reflect a change in the nucleic acid site specificity of the two drugs. Therefore, sites of DNA cleavage mediated by human topoisomerase IIα were mapped in the presence of these two podophyllotoxin derivatives. As seen in Figure 7, sites of DNA cleavage generated in the presence of TOP-53 appear to be identical to those observed in the presence of etoposide. However, the intensity of cleavage at each individual site was greater in reaction mixtures that contained TOP-53. These results imply that drug−DNA interactions are not altered by the different drug substituents; only the affinity of TOP-53 for the enzyme−DNA complex is increased.

Effects of TOP-53 on the DNA Cleavage Activities of Mutant Type II Topoisomerases. Previous studies indicate that topoisomerase II−etoposide interactions direct the formation of the ternary enzyme−drug−DNA complex (*32*, *59*). Together with the finding that the DNA site specificity of TOP-53 is identical to that of etoposide, this suggests that the increased efficacy of TOP-53 may result from an altered interaction with topoisomerase II.

To address this issue, the effects of TOP-53 on the DNA cleavage activities of three yeast type II enzymes with different drug sensitivities were compared to those of etoposide. The three enzymes employed for these experiments were the wild type, ytop2H1011Y (*20*), and ytop2G436S (*21*). As seen in Figure 8 (left panel), the histidine to tyrosine mutation at position 1011 results in an enzyme that is highly resistant to etoposide, while the glycine to serine substitution at position 436 generates an enzyme

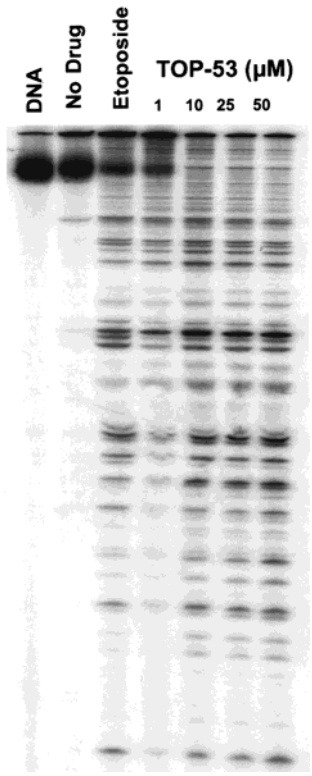

FIGURE 7: Etoposide and TOP-53 enhance DNA cleavage mediated by human topoisomerase IIα at common sites. The DNA cleavage site specificities of etoposide (50 μM) and TOP-53 (0−50 μM) were determined. The DNA cleavage substrate was an end-labeled 564 bp fragment of pBR322. Controls that were included were DNA substrate only (DNA) and topoisomerase II in the absence of drug (No Drug).

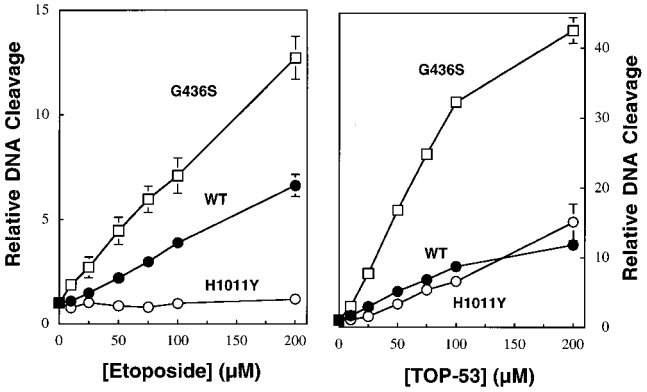

FIGURE 8: Effects of TOP-53 on the DNA cleavage activities of mutant forms of yeast topoisomerase II. Wild-type yeast topoisomerase II (●) and ytop2H1011Y [highly etoposide resistant (○)] and ytop2G436S [slightly etoposide hypersensitive (□)] were employed. Negatively supercoiled pBR322 DNA was used as the cleavage substrate. Assays were carried out in the presence of TOP-53 (right) or etoposide (left). Levels of DNA cleavage are reported relative to the amount of cleavage observed in the absence of drug. Error bars represent the standard error of the mean for three or four independent experiments.

that is slightly hypersensitive to the drug. In both cases, altered drug sensitivity has been shown to correlate with changes in the affinity of etoposide for the mutant type II topoisomerase (*20*, *21*, *59*).

In contrast to etoposide, ytop2H1011Y displayed nearly wild-type DNA cleavage activity in the presence of TOP-53. Moreover, ytop2G436S was dramatically hypersensitive to the drug. These results strongly suggest that the substitution of the glycoside portion of etoposide with the amino alkyl residue in TOP-53 significantly enhances interactions between the drug and topoisomerase II.

DISCUSSION

TOP-53 is a promising new anticancer agent that is highly active against non-small cell lung cancer cells in culture and in animal tumor models (*19*). It is identical in structure to etoposide, except that the glycoside moiety at the 4-position of the C-ring has been replaced with an amino alkyl residue. Compared to etoposide, TOP-53 is ≥10 times more cytotoxic to non-small cell lung cancer cells, is more active at generating chromosomal DNA breaks, and displays improved cellular uptake and pharmacokinetics in animal lung tissues (*19*).

Despite the preclinical success of TOP-53, a number of questions remain regarding its mechanism of action. Prior to this study, it was not definitively demonstrated that topoisomerase II was the primary cellular target of this drug. Furthermore, given the ability of TOP-53 to concentrate in non-small cell lung cancer cells, it was not clear whether increases in cytotoxicity reflected an enhanced ability to stimulate topoisomerase II-mediated DNA cleavage, a higher cellular concentration of the drug, or potentially both.

Results of the study presented here indicate that TOP-53 shares many properties with etoposide. First, topoisomerase II is the primary cytotoxic target of TOP-53, and the drug kills cells by acting as a topoisomerase II poison. Second, both topoisomerase IIα and IIβ appear to be targeted by the drug in treated human cells. Third, TOP-53 increases levels of enzyme-mediated DNA scission by interfering with the ability of the enzyme to religate cleaved molecules. Finally, the DNA cleavage site specificity of TOP-53 appears to be identical to that of etoposide.

Similarities aside, TOP-53 is considerably more efficient than etoposide at increasing the level of topoisomerase II-mediated DNA breakage. At every drug concentration that was examined, in vitro or in cultured cells, in assays in which human topoisomerase IIα or IIβ, wild-type yeast topoisomerase II, or mutant forms of the yeast enzyme with altered drug sensitivities were employed, levels of DNA cleavage generated in reaction mixtures that contained TOP-53 were higher than those observed in the presence of etoposide.

Clearly, a number of relevant differences exist between other tumor types and non-small lung cancer cells (*17*−*19*). Furthermore, important and as yet undescribed pharmacokinetic differences may exist between TOP-53 and etoposide. Any of these could influence levels of drug-induced topoisomerase II-mediated DNA cleavage. However, when taken together, the studies described above strongly suggest that the enhanced activity of TOP-53 against non-small lung cancer cells reflects, at least in part, an enhanced activity against topoisomerase II.

On the basis of the activity of TOP-53 against the mutant yeast type II enzymes, TOP-53 probably displays improved DNA cleavage activity because it interacts more tightly with topoisomerase II than does etoposide. This conclusion has two important ramifications. First, it demonstrates that TOP-53 has the ability to retain activity against systems that have developed resistance to etoposide. Second, it suggests that the 4-position of the etoposide C-ring is important for drug

interactions with topoisomerase II. This suggestion is consistent with the fact that teniposide, which is ~10-fold more potent than etoposide, differs in structure only in the nature of its 4-glycoside moiety (*19*). Moreover, podophyllotoxin, which displays no activity against topoisomerase II, contains only a hydroxyl moiety in this position.

In summary, the present work characterized the mechanistic basis for the actions of TOP-53. Although this drug is similar in structure to etoposide, its unique attributes and improved activity against non-small cell lung cancer cells make it a potential candidate for further development.

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