Effect of Antineoplastic Agents on the DNA Cleavage/Religation Reaction of Eukaryotic Topoisomerase II: Inhibition of DNA Religation by Etoposide†

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ABSTRACT: Beyond its essential physiological functions, topoisomerase II is the primary cellular target for a number of clinically relevant antineoplastic drugs. Although the chemotherapeutic efficacies of these drugs correlate with their abilities to stabilize the covalent topoisomerase II–DNA cleavage complex, their molecular mechanism of action has yet to be described. In order to characterize the drug-induced stabilization of this enzyme–DNA complex, the effect of etoposide on the DNA cleavage/religation reaction of *Drosophila melanogaster* topoisomerase II was studied. Under the conditions employed, etoposide increased levels of enzyme-mediated double-stranded DNA cleavage 5–6-fold and single-stranded cleavage ~4-fold. Maximal stimulation was observed at 80–100 μM etoposide with 50% of the maximal effect at ~15 μM drug. By employing a topoisomerase II mediated DNA religation assay [Osheroff, N., & Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303–4309], etoposide was found to stabilize the enzyme–DNA cleavage complex (at least in part) by inhibiting the enzyme's ability to religate cleaved DNA. Moreover, in order for the drug to affect religation, it had to be present at the time of DNA cleavage.

Eukaryotic topoisomerase II is an essential enzyme which is involved in a number of fundamental cellular processes (Vosberg, 1985; Wang, 1985; Osheroff, 1989). Central to the physiological function of the enzyme is its ability to create and religate double-stranded breaks in the backbone of DNA (Vosberg, 1985; Wang, 1985; Osheroff, 1989).

Beyond its normal cellular activities, topoisomerase II is the primary cellular target for a number of clinically relevant antineoplastic drugs (Zwelling, 1985; Glisson & Ross, 1987; Bodley & Liu, 1988). The chemotherapeutic efficacies of these agents correlate with their abilities to stabilize the covalent topoisomerase II–DNA cleavage complex (Zwelling, 1985; Glisson & Ross, 1987; Bodley & Liu, 1988), which is an intermediate in the enzyme's DNA strand passage reaction (Osheroff, 1989; Gale & Osheroff, 1989). Thus, as monitored in vitro, these drugs increase levels of enzyme-mediated DNA cleavage (Zwelling, 1985; Glisson & Ross, 1987; Bodley & Liu, 1988). Unfortunately, despite the important role of topoisomerase II in the treatment of human cancers, virtually nothing is known about the molecular mechanism by which antineoplastic drugs alter enzyme function.

One of the major stumbling blocks that has prevented the detailed mechanism of drug action on topoisomerase II from being delineated has been the inability to uncouple the enzyme's forward DNA cleavage reaction from the reverse religation event. Therefore, while it is possible to demonstrate that antineoplastic agents stimulate enzyme-mediated DNA breakage by shifting the cleavage/religation equilibrium toward cleavage, it is impossible to state with certainty whether these drugs act by enhancing the forward cleavage reaction or by inhibiting DNA religation.

Recently, an assay was developed which uncouples topoisomerase II mediated religation from DNA cleavage (Osheroff & Zechiedrich, 1987). The assay takes advantage of the finding that DNA cleavage intermediates generated by topoisomerase II in the presence of calcium can be trapped in the absence of a protein denaturant (Osheroff & Zechiedrich, 1987). The resulting covalent enzyme–DNA cleavage complex is kinetically competent and provides a substrate for which religation can be examined in the absence of the forward cleavage reaction.

The above assay system has been employed to examine the mechanism by which etoposide, a potent antineoplastic drug (Issell, 1982; Issell et al., 1984; Muggia & McVie, 1987), stabilizes the cleavage complex between DNA and *Drosophila melanogaster* topoisomerase II. Results indicate that the drug acts (at least in part) by inhibiting the enzyme-mediated religation of cleaved DNA. In addition, etoposide has to be present at the time of DNA cleavage in order to affect religation.

A preliminary report of this work has appeared (Osheroff & Gale, 1988).

Experimental Procedures

DNA topoisomerase II was purified from the nuclei of *D. melanogaster* Kc tissue culture cells as described by Shelton et al. (1983). Negatively supercoiled bacterial plasmid pBR322 (Bolivar et al., 1977) DNA was isolated from *Escherichia coli* DH1 by a Triton X-100 lysis procedure followed by a double banding in cesium chloride–ethidium bromide gradients (Maniatis et al., 1982). Etoposide (VePesid, VP-16-23) was obtained from Bristol Laboratories as a sterile 20 mg/mL solution in etoposide diluent [2 mg/mL citric acid, 30 mg/mL benzyl alcohol, 80 mg/mL polysorbate 80/Tween 80, 650 mg/mL poly(ethylene glycol) 300, 30.5% (v/v) ethanol]. The drug was stored at room temperature as per the manufacturer's instructions. Tris and ethidium bromide were obtained from Sigma; analytical reagent grade $CaCl_2 \cdot H_2O$ and $MgCl_2 \cdot 6H_2O$ were from Fisher; and sodium dodecyl sulfate (SDS)[1] and proteinase K were from E. Merck Biochemicals. All other chemicals were of analytical reagent grade.

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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Cleavage of DNA by Topoisomerase II. DNA cleavage reactions employed 75 nM topoisomerase II and 5 nM (0.3 μg) negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin) which contained 5 mM $MgCl_2$. Samples were incubated at 30 °C for 6 min. DNA cleavage products were trapped by the addition of 2 μL of 10% SDS. One microliter of 250 mM EDTA and 2 μL of 0.8 mg/mL proteinase K were added and mixtures were incubated at 45 °C for 30 min to digest the topoisomerase II. Products were mixed with 3 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole FF, and 10 mM Tris-HCl, pH 7.9), heated at 75 °C for 2 min, and subjected to electrophoresis in 1.0% agarose (MCB) gels in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm. Following electrophoresis, gels were stained in an aqueous solution of ethidium bromide (1 μg/mL). DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak Nos. 24A and 12 filters with Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Biomed Instruments Model SL-504-XL scanning densitometer. Under the conditions employed, the intensity of the negative was directly proportional to the amount of DNA present.

The effect of etoposide on topoisomerase II mediated DNA cleavage was examined over a range of 0–100 μM drug. Control samples always contained an amount of etoposide diluent equivalent to that in the drug sample. Over the drug concentration range employed, topoisomerase II activity was not affected by the diluent.

Religation of Cleaved DNA by Topoisomerase II. The religation assay of Osheroff and Zechiedrich (1987) was employed. Topoisomerase II (75 nM) and 5 nM negatively supercoiled pBR322 DNA were incubated for 6 min at 30 °C in 20 μL of cleavage buffer which contained 5 mM $CaCl_2$. Kinetically competent covalent topoisomerase II–DNA cleavage complexes were trapped by the addition of 2 μL of 100 mM EDTA. NaCl (0.8 μL of a 5 M solution) was added to prevent recleavage of DNA during the religation reaction (Liu et al., 1983; Osheroff & Zechiedrich, 1987). Samples were placed on ice to slow reaction rates, and religation was initiated by the addition of cold $MgCl_2$ (7.5 mM final concentration). SDS (2 μL of a 10% solution) was added to terminate religation at various time points up to 30 s. Samples were digested with proteinase K and were analyzed by electrophoresis as described above. The effect of antineoplastic agents on the topoisomerase II mediated DNA religation reaction was determined by including 100 μM etoposide in assays prior to the trapping of enzyme–DNA cleavage complexes. As above, control samples contained an equivalent amount of etoposide diluent equivalent to that in the drug sample. Rates of enzyme-mediated DNA religation were not affected by the diluent.

Results

Effect of Etoposide on the DNA Cleavage/Religation Equilibrium of Drosophila Topoisomerase II. In order to simplify the analysis of etoposide's action on topoisomerase II mediated DNA cleavage/religation, assays were carried out in the absence of ATP. Thus, reactions presented below represent the cleavage and religation events which take place prior to the enzyme's catalysis of DNA strand passage (Hsieh & Brutlag, 1980; Osheroff et al., 1983; Osheroff, 1989).

As previously demonstrated for mammalian topoisomerase II (Chen et al., 1984; Ross et al., 1984; Yang et al., 1985),

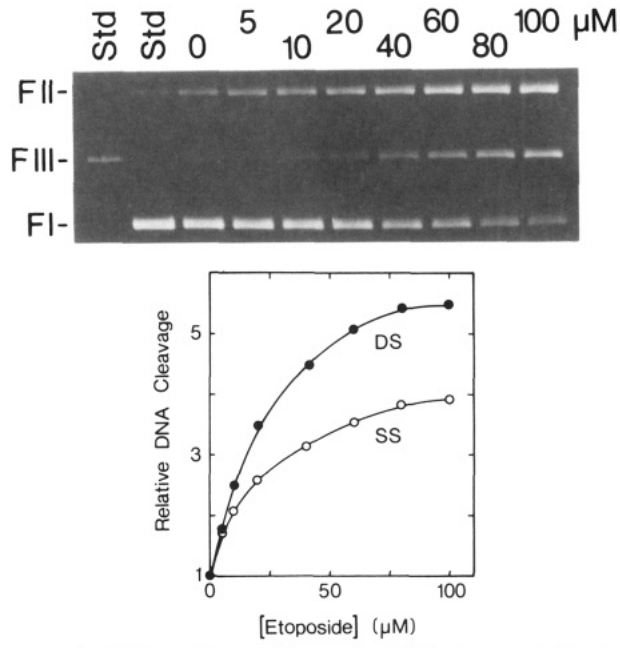

FIGURE 1: Effect of etoposide on the DNA cleavage/religation equilibrium of *D. melanogaster* topoisomerase II. Assays were carried out as described under Experimental Procedures. The top panel shows reaction products that were subjected to electrophoresis on a 1% agarose gel. DNA standards (Std) are shown in the first two lanes. The concentration of etoposide included in assay mixtures is indicated at the top. The positions of fully supercoiled DNA (form I, FI), nicked circular plasmid molecules (form II, FII), and linear molecules (form III, FIII) are indicated. Results are depicted graphically in the bottom panel. The effects of etoposide on the production of double-stranded (DS) and single-stranded (SS) DNA breaks by topoisomerase II are shown.

etoposide had a profound influence on the DNA cleavage/religation equilibrium of the type II enzyme from *D. melanogaster*. Under the conditions employed, the drug shifted the equilibrium markedly toward cleavage, enhancing the formation of enzyme-mediated double-stranded breaks by 5–6-fold and single-stranded breaks by approximately 4-fold (Figure 1). DNA breakage (both double and single stranded) was enhanced 50% at approximately 15 μM etoposide and maximal enhancement was observed at 80–100 μM drug. These values approximate the physiological drug concentrations found in the plasma of human patients immediately following acute chemotherapeutic treatment (Creaven, 1984).

All of the effects of etoposide on DNA were mediated through topoisomerase II. To this point, linear (i.e., doubly cut) and nicked (i.e., singly cut) DNAs generated in the presence of the drug were covalently attached to topoisomerase II (not shown). Cleaved nucleic acids were released from the enzyme only after digestion of the cleavage complex with proteinase K. The covalent linkage of the enzyme to its DNA products is a hallmark of topoisomerase-mediated cleavage (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987). Additionally, in the presence of 100 μM etoposide, no linear or nicked DNA was produced in the absence of topoisomerase II (not shown).

EDTA Reversibility of Topoisomerase II Generated DNA Breaks Formed in the Presence of Etoposide. The topoisomerase II mediated DNA cleavage reaction (carried out in the presence of magnesium) is readily reversed by the addition of salt or EDTA (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987). This is due primarily to the fact that topoisomerase II never releases its cleaved DNA intermediate (Vosberg, 1985; Wang, 1985; Osheroff, 1989). Enzyme-generated DNA breaks formed in the presence

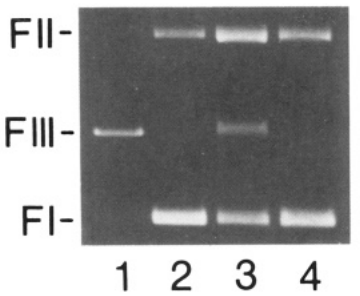

FIGURE 2: EDTA reversibility of DNA breaks mediated by topoisomerase II in the presence of 100 μM etoposide. Assays were carried out as described under Experimental Procedures. Lanes 1 and 2, DNA standards; lane 3, SDS was added prior to EDTA; lane 4, EDTA was added prior to SDS.

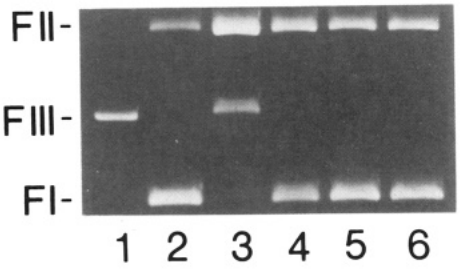

FIGURE 3: DNA religation reaction of *Drosophila* topoisomerase II. Assays were carried out as described under Experimental Procedures. Lanes 1 and 2, DNA standards; lane 3, religation of DNA at zero time; lane 4, religation of DNA at 1 min following the addition of magnesium; lanes 5 and 6, assays (in the absence or presence of 100 μM etoposide, respectively) in which topoisomerase II was left out of reaction mixtures through step 3 of the assay (see text) and was added immediately prior to the addition of magnesium.

of etoposide and mammalian topoisomerase II have also been shown to be reversed (i.e., religated) following the addition of 0.5 M sodium chloride to reaction mixtures (Chen et al., 1984). In order to determine whether the etoposide-enhanced DNA breakage observed with *Drosophila* topoisomerase II was also reversible, the experiment shown in Figure 2 was carried out. In this experiment, the *Drosophila* enzyme was allowed to establish a DNA cleavage/religation equilibrium in the presence of 100 μM etoposide and 5 mM magnesium, but the divalent cation was chelated with 10 mM (final concentration) EDTA prior to the addition of SDS. As seen in lane 4, drug-enhanced double- and single-stranded DNA breaks were reversed by this procedure (compare with lane 3, in which SDS was added prior to EDTA). Taken together with the previous study on salt reversibility (Chen et al., 1984), this result demonstrates that etoposide acts by stabilizing the active topoisomerase II cleaved DNA intermediate rather than by trapping it in a "dead-end" complex. This leads to the simple conclusion that etoposide must enhance the level of the cleavage intermediate either by stimulating the forward rate of enzyme-mediated DNA cleavage or by inhibiting the rate of enzyme-mediated religation (or both).

Inhibition of Topoisomerase II Mediated DNA Religation by Etoposide. As a direct test of the above conclusion, the effect of etoposide on the rate of topoisomerase II mediated religation of cleaved DNA was determined. For this purpose, the DNA religation assay described by Osheroff and Zechiedrich (1987) was employed. This assay, which is shown in Figure 3, takes advantage of the fact that (unlike magnesium) calcium can be used to trap a kinetically competent covalent topoisomerase II–DNA cleavage intermediate. It proceeds as follows. (1) In order to establish a DNA cleavage/religation equilibrium, topoisomerase II was incubated with negatively supercoiled pBR322 DNA in the presence of

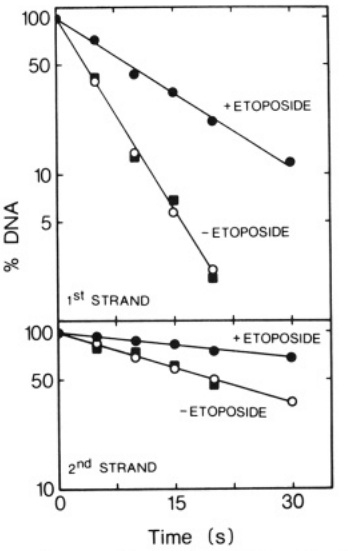

FIGURE 4: Effect of etoposide on the DNA religation reaction of *Drosophila* topoisomerase II. A semilogrithmic plot of percent DNA versus time is shown. The religation of the first DNA strand (i.e., the conversion of linear DNA to nicked circular molecules) is displayed in the top panel. Results are plotted as the loss of linear DNA. The religation of the second DNA strand (i.e., the conversion of nicked circular DNA to negatively supercoiled molecules) is displayed in the bottom panel. Results are plotted as the difference between the percent negatively supercoiled DNA at the completion of the reaction (at t_∞, FI DNA = 100%) and the percent negatively supercoiled DNA at any reaction time (t). The open circles depict reactions that contained no drug; the closed circles depict reactions in which 100 μM etoposide was present at the time of enzyme-mediated DNA cleavage and religation; the closed squares depict reactions in which no drug was present at the time the enzyme–DNA cleavage complex was trapped, but 100 μM etoposide was added prior to the initiation of religation (i.e., immediately after the EDTA).

5 mM calcium and 100 mM salt at 30 °C. (2) EDTA (10 mM final concentration) was added to chelate the calcium and trap the active enzyme–DNA cleavage complex (Osheroff & Zechiedrich, 1987) (lane 3). (3) The salt concentration was increased from 100 to 250 mM to prevent recleavage of the DNA during the course of religation (Liu et al., 1983; Osheroff & Zechiedrich, 1987). (4) Religation was initiated on ice (to slow reaction rates) by the addition of excess magnesium ions. A 1-min time point is shown in lane 4.

A previous kinetic analysis of this assay (Zechiedrich et al., 1989) revealed that topoisomerase II religates cleaved DNA via a sequential two-step mechanism. Linear (i.e., doubly cut) DNA is first converted to nicked (i.e., singly cut) DNA which in turn is converted to the original negatively supercoiled substrate. The first-order rate constant for the first religation step is approximately 6-fold faster than that of the second.

In order to demonstrate that religated DNA was not being recleaved during the course of the assay, topoisomerase II was left out of reaction mixtures through step 3 of the assay. The enzyme was added immediately before the addition of magnesium to see if DNA cleavage was possible under the final reaction conditions. As seen in Figure 3, no DNA cleavage was observed under the final conditions for religation, in either the absence (lane 5) or presence (lane 6) of 100 μM etoposide.

With the assay described above, 100 μM etoposide was found to inhibit the topoisomerase II mediated religation of cleaved DNA. Results are presented as a semilogarithmic plot of percent DNA versus time (Figure 4). This type of plot was employed because it allows the direct quantitation of apparent rate constants for a first-order process (Segel, 1975; Fersht, 1985). Religation of the first strand of DNA (i.e., FIII → FII) is shown in the top panel, while religation of the second

strand (i.e., FII → FI) is shown in the bottom panel. When drug was present at the time of DNA cleavage, rates of religation for both DNA strands were inhibited approximately 3-fold (closed circles). Etoposide lowered the apparent first-order rate constant for the religation of the first strand from 0.20 to 0.07 s^{-1} and that for the religation of the second strand from 0.03 to 0.01 s^{-1}.

Finally, in order for etoposide to inhibit enzyme-mediated religation, it had to be present at the time of DNA cleavage. This conclusion is based on the following experiment. Rather than adding the drug to reaction mixtures prior to trapping the topoisomerase II–DNA cleavage complex with EDTA, 100 μM etoposide was added immediately after the chelator. Subsequent to the addition of salt and magnesium, the rate of DNA religation (Figure 4, closed squares) was indistinguishable from that observed in the absence of drug (open circles).

Discussion

Understanding the molecular mechanism by which a drug mediates its action is critical to rational drug design. Although the mechanism(s) of topoisomerase II targeted drugs has yet to be determined, it has long been assumed that these antineoplastic agents stabilize enzyme–DNA cleavage complexes by interfering with the ability of topoisomerase II to religate its bound DNA intermediates. The data presented above validate this assumption. Indeed, etoposide does inhibit the DNA religation reaction of *Drosophila* topoisomerase II.

It should be noted, however, that the drug-induced 3-fold inhibition of DNA religation is somewhat less than its 5–6-fold enhancement of double-stranded DNA breakage. Since the experimental conditions (i.e., temperature, ionic strength, and divalent cation concentration) employed for the DNA religation assay differed from those utilized in cleavage reactions, it is not necessarily possible to directly compare results generated by the two systems. Thus, while it is clear that etoposide inhibits topoisomerase II mediated religation, it is not yet definitive that this represents its sole mode of action. It is quite possible that etoposide increases levels of enzyme–DNA cleavage intermediates by stimulating the forward rate of DNA cleavage in addition to its inhibitory effects on religation.

Recent studies indicate that etoposide may be able to bind to both DNA (Chow et al., 1988) and topoisomerase II (Osheroff & Gale, 1988). However, the site of drug action has yet to be determined. Since etoposide had to be present at the time of DNA cleavage to inhibit religation, it is tempting to speculate that the drug acts within the enzyme–DNA ternary complex. Hopefully, future studies will be able to address this important mechanistic point.

Finally, in a study similar to that presented above, the topoisomerase II targeted chemotherapeutic drug *m*-AMSA (Nelson et al., 1984; Tewey et al., 1984; Pommier et al., 1984, 1985) was also found to inhibit the DNA religation reaction of the *Drosophila* enzyme (M. J. Robinson & N. Osheroff, unpublished results). This finding suggests that even structurally disparate classes of antineoplastic agents may alter topoisomerase II function through a similar mechanism of action.

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Registry No. Etoposide, 33419-42-0; DNA topoisomerase, 80449-01-0.

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