Biochemistry

 Volume 38, Number 12 March 23, 1999

Accelerated Publications

Binding of Etoposide to Topoisomerase II in the Absence of DNA: Decreased Affinity as a Mechanism of Drug Resistance†

Paul S. Kingma,‡ D. Andrew Burden,‡ and Neil Osheroff*,‡,§

Departments of Biochemistry and Medicine (Hematology/Oncology), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received December 3, 1998; Revised Manuscript Received January 22, 1999

ABSTRACT: Despite the prevalence of topoisomerase II-targeted drugs in cancer chemotherapy and the impact of drug resistance on the efficacy of treatment, interactions between these agents and topoisomerase II are not well understood. Therefore, to further define interactions between anticancer drugs and the type II enzyme, a nitrocellulose filter assay was used to characterize the binding of etoposide to yeast topoisomerase II. Results indicate that etoposide binds to the enzyme in the absence of DNA. The apparent K_d value for the interaction was ~5 μM drug. Etoposide also bound to ytop2H1012Y, a mutant yeast type II enzyme that is ~3−4-fold resistant to etoposide. However, the apparent K_d value for the drug (~16 μM) was ~3 times higher than that determined for wild-type topoisomerase II. Although it has been widely speculated that resistance to topoisomerase II-targeted anticancer agents results from a decreased drug−enzyme binding affinity, these data provide the first direct evidence in support of this hypothesis. Finally, the ability of yeast topoisomerase II to bind etoposide was dependent on the presence of the hydroxyl moiety of Tyr783, suggesting specific interactions between etoposide and the active site residue that is involved in DNA scission.

Etoposide is one of the most commonly prescribed anticancer drugs in clinical use (*1*−*6*). This agent exerts its chemotherapeutic effects by increasing levels of covalent topoisomerase II-cleaved DNA complexes (*5*, *7*−*10*). Although these complexes are normal intermediates in the DNA strand passage reaction catalyzed by topoisomerase II (*8*, *10*−*13*), when present in high concentrations, they trigger mutagenic and cell death pathways (*5*, *14*−*18*). Thus, etoposide and other chemotherapeutic agents that stimulate topoisomerase II-mediated DNA cleavage are referred to as topoisomerase II "poisons" because they convert this essential enzyme to a potent cellular toxin (*5*, *7*, *8*, *10*, *19*).

While the success of topoisomerase II poisons in the treatment of human malignancies has been significant, the response of different patients and/or cell types varies considerably (*4*, *17*, *20*, *21*). Unfortunately, the basis for altered cellular sensitivity to topoisomerase II-targeted drugs is not well understood. It is often assumed that resistance of catalytically active type II enzymes toward anticancer agents results from a decreased drug binding affinity (*17*, *20*, *21*). While this mechanism has been shown for a quinolone-resistant DNA gyrase from *Escherichia coli* (*22*), altered drug binding has never been demonstrated for any drug-resistant type II topoisomerase from a eukaryotic species. Thus, the "decreased drug binding" hypothesis for the resistance of

† This work was supported by NIH Grant GM33944.
* To whom correspondence should be addressed.
‡ Department of Biochemistry.
§ Department of Medicine.

10.1021/bi982855i CCC: $18.00
Published on Web 03/04/1999

topoisomerase II to anticancer drugs, although attractive, remains unproven.

One of the most well characterized type II topoisomerases with altered drug sensitivity is ytop2H1012Y, a mutant yeast enzyme in which His1012 is converted to Tyr (*23*). This enzyme is ~3−4-fold resistant to etoposide and quinolones, yet is hypersensitive to ellipticine. His1012 is located ~20 Å from the active site of the enzyme that binds DNA (*24*). Consequently, this residue is unlikely to form direct contacts with topoisomerase II poisons. However, since the binding affinity of ytop2H1012Y for DNA is less than that of the wild-type enzyme (*23*), the His → Tyr mutation appears to induce distal conformational changes in topoisomerase II. It is believed that ytop2H1012Y displays altered sensitivity to anticancer drugs because of these conformational changes.

The hypersensitivity of ytop2H1012Y has been addressed using a fluorescence-based assay that exploited the spectral properties of bound ellipticine and analyzed binding by monitoring changes in the emission intensity of the drug (*25*, *26*). As determined by this method, the hypersensitivity of the mutant enzyme correlated with an increased binding affinity for the anticancer agent. However, because the drug−enzyme binding assay utilized was dependent on the unique fluorescent properties of ellipticine, this method is not generally applicable to more widely prescribed drugs such as etoposide.

Therefore, to further describe interactions between etoposide and eukaryotic topoisomerase II and to characterize the mechanistic basis of drug resistance, the binding of etoposide to yeast topoisomerase II was analyzed using a nitrocellulose filter binding assay. Results indicate that etoposide binds to the enzyme in the absence of DNA and that ytop2H1012Y has a decreased affinity for etoposide that is comparable to its level of resistance. Finally, studies with an active site mutant of topoisomerase II suggest that etoposide binds to the enzyme in the vicinity of the active site tyrosine.

EXPERIMENTAL PROCEDURES

[^{3}H]Etoposide (~0.5 Ci/mmol) was obtained from Moravek Biochemicals and stored at −80 °C in ethanol. Nitrocellulose filters (0.45 μm) were from Millipore. All other chemicals were analytical reagent grade.

Purification of Topoisomerase II. Wild-type and mutant (ytop2H1012Y and ytop2Y783F) type II topoisomerases from *Saccharomyces cerevisiae* were overexpressed using the inducible plasmid YepGAL1TOP2 (*27*) and purified from yeast cells using the following modification of the procedure described by Elsea et al. (*23*). Following differential precipitation with $(NH_4)_2SO_4$, the pellet containing topoisomerase II was resuspended in 15 mM sodium phosphate, pH 7.7, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, 0.5 μg of leupeptin/mL, and 1 μg of pepstatin/mL and diluted with this buffer until its conductivity was similar to that of HAP column buffer (15 mM sodium phosphate, pH 7.7, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM dithiothreitol, and 10 mM $Na_2S_2O_5$) containing 150 mM potassium phosphate, pH 7.7. The protein sample was subsequently applied to a 10 mL hydroxylapatite column that had been equilibrated with HAP column buffer containing 150 mM potassium phosphate, pH 7.7. The column was washed with 30 mL of the same buffer, and protein was eluted with a linear 150 mL gradient of HAP column buffer containing 200 mM potassium phosphate, pH 7.7, to 600 mM potassium phosphate, pH 7.7. Fractions containing topoisomerase II (as monitored by gel electrophoresis) were pooled and applied to a phosphocellulose collection column as described by Elsea et al. (*23*).

Nitrocellulose Filter Binding Assay. Etoposide−topoisomerase II binding was monitored by a modification of the nitrocellulose filter binding assay originally described by Higgins and Cozzarelli (*28*). Nitrocellulose filters (0.45 μm, Millipore) were presoaked in wash buffer (10 mM Tris-HCl, pH 7.7, 0.1 mM EDTA, 5 mM $MgCl_2$, and 2.5% glycerol) for ~10 min. Binding mixtures contained wild-type or mutant topoisomerase II (at concentrations ranging from 0.5 to 5 μM) and [^{3}H]etoposide (at concentrations ranging from 1 to 10 μM in 1% ethanol) in 80 μL of 10 mM Tris-HCl, pH 7.7, 100 mM KCl, 35 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, and 3.5% glycerol. Samples were incubated at room temperature for 6 min and applied to the center of nitrocellulose filters at a flow rate of ~2 mL/min. Filters were washed three times with 1 mL of cold wash buffer at a flow rate of ~10 mL/min, dried, and quantified by liquid scintillation counting. Binding of etoposide to the filter in the absence of enzyme was subtracted for binding calculations. Under the conditions employed, wild-type yeast topoisomerase II, ytop2H1012Y, and ytop2Y783F displayed comparable abilities to bind the nitrocellulose filters used in assays. In all cases, data sets represent the average of three to five independent experiments with the average standard error of the mean being equal to ± 0.08 μM drug bound.

RESULTS

Previous kinetic studies indicate that interactions between etoposide and topoisomerase II direct the entry of the drug into its site of action in the drug•enzyme•DNA complex (*29*). Despite the wide clinical use of etoposide (*1*−*6*), these interactions are poorly understood.

Therefore, to characterize binding interactions between etoposide and topoisomerase II, a nitrocellulose filter binding assay was employed. Because of the large quantities of highly concentrated protein that were required for this study and the availability of well-characterized mutant enzymes, *S. cerevisiae* topoisomerase II purified from a yeast overexpression system was utilized.

Etoposide−Topoisomerase II Binding. As seen in Figure 1, [^{3}H]etoposide binds to topoisomerase II in the absence of DNA.[1] Binding was concentration dependent and saturable. Furthermore, ~90% of the binding was eliminated by the addition of a 10-fold molar excess of unlabeled etoposide (not shown). As determined by double reciprocal analysis (see Figure 2), the apparent K_d for the etoposide−topoi-

[1] A preliminary account of some of the binding studies that characterized interactions between etoposide and wild-type yeast topoisomerase II was cited previously by Burden et al. (*29*). In this earlier report, [^{3}H]etoposide−topoisomerase II binding was observed at a single fixed concentration of enzyme and drug (5 and 2 μM, respectively). Furthermore, [^{3}H]etoposide binding was decreased dramatically when a 100-fold molar excess of nonradioactive drug was included in binding mixtures.

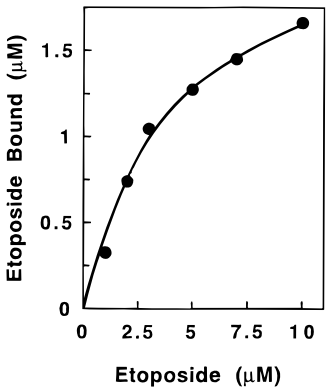

FIGURE 1: Binding of etoposide to topoisomerase II. Yeast wild-type topoisomerase II (5 μM) was incubated with various concentrations of [^{3}H]etoposide, and the free and bound fractions of etoposide were separated by filtration through nitrocellulose membranes. Binding of etoposide to the filter in the absence of enzyme was subtracted as background. Data represent the averages of three to four independent experiments.

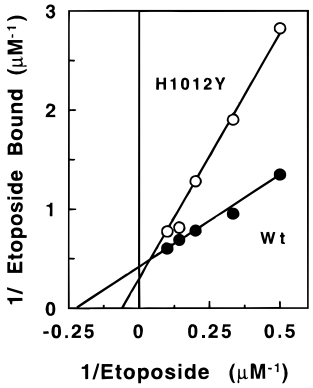

FIGURE 2: Kinetic analysis of etoposide binding to the wild-type enzyme and the etoposide-resistant topoisomerase II mutant, ytop2H1012Y. Reactions contained 5 μM wild-type (●) or mutant (○) enzyme and increasing concentrations of [^{3}H]etoposide (2−10 μM). Levels of total and bound etoposide were plotted in double reciprocal format. Data represent the averages of three to four independent experiments.

somerase II interaction was ~5 μM and the maximum binding was ~0.5 drug molecules per enzyme homodimer. Similar results were obtained by a nonlinear analysis of the binding data (not shown).

Two caveats regarding the nitrocellulose filter binding assay should be noted. First, the mechanics of the assay, including trapping and washing the drug·enzyme complex on the filter, require binding to be analyzed under nonequilibrium conditions. Given the micromolar K_d value calculated for the etoposide·topoisomerase II complex, it is likely that some drug dissociation occurred during the 5−10 s required for washes (despite the use of cold buffer). Therefore, binding stoichiometries must be considered an underestimate. Second, due to the relatively low specific activity of commercially available [^{3}H]etoposide, enzyme concentrations in the micromolar range were required to achieve an appropriate signal-to-noise ratio. Thus, the apparent K_d values obtained in this assay system are probably artificially high. However, these values appear to be representative of the intrinsic drug−enzyme dissociation constant and can be used for comparative purposes.

The above qualifications regarding quantitation of the nitrocellulose filter assay notwithstanding, it is clear from the data shown in Figures 1 and 2 that etoposide binds to topoisomerase II in the absence of DNA. This finding supports the kinetic studies of Burden et al. (*29*) and provides further evidence that topoisomerase II mediates the entry of etoposide into the ternary drug·enzyme·DNA complex.

Mechanistic Basis for Resistance of ytop2H1012Y to Etoposide. Despite the impact of drug resistance on the efficacy of cancer chemotherapy, the mechanistic basis underlying the intrinsic resistance of mutant type II topoisomerases to anticancer agents has yet to be described (*17, 20, 21*). To address this critical issue, the binding of etoposide to the drug-resistant yeast type II enzyme, ytop2H1012Y (*23*), was determined.

The His → Tyr mutation at position 1012 had little effect on maximal levels of drug−enzyme binding but clearly decreased the affinity of etoposide for topoisomerase II (Figure 2). The apparent K_d value for the interaction of the drug with ytop2H1012Y was ~16 μM, as compared to ~5 μM for the wild-type enzyme. This 3-fold reduction in binding affinity for the mutant enzyme is comparable to the 3−4-fold decrease in the ability of etoposide to stimulate DNA cleavage mediated by ytop2H1012Y (*23*). This finding provides the first direct evidence that resistance-conferring mutations in topoisomerase II can exert their effects by decreasing the affinity of the enzyme for anticancer agents.

Mutation of the Active Site Tyrosine of Topoisomerase II Decreases Etoposide Binding. Although the site of action of anticancer agents on topoisomerase II has not been defined, several lines of evidence suggest that these drugs interact with the DNA at or near the site of scission. First, a photoactivatable form of amsacrine cross-links to DNA in the topoisomerase II cleavage complex within one base of the covalent enzyme−DNA bond (*30*). Second, the specificity of drug-induced DNA cleavage by topoisomerase II generally localizes to the nucleotide immediately 5′ or 3′ to the point of scission (*5, 31*). Third, lesions that distort DNA act as topoisomerase II poisons and stimulate enzyme-mediated DNA cleavage, but do so only when they are located within the four base stagger that separates the two points of cleavage on the opposite strands of the double helix (*32−35*).

When these studies, which suggest that drugs function proximal to the site of DNA scission, are taken together with the binding data (Figures 1 and 2), which indicate that etoposide binds directly to topoisomerase II, they predict that drugs bind to the enzyme in the vicinity of its active site. In yeast topoisomerase II, the residue that attacks the scissile bond in DNA and forms the covalent enzyme−nucleic acid bridge is Tyr783 (*27*). Therefore, we examined the binding of etoposide to ytop2Y783F, a mutant yeast enzyme in which the active site Tyr is converted to Phe.

The level of overexpression of ytop2Y783F in yeast is lower than that of the wild-type enzyme. As a result, the maximal concentration of mutant topoisomerase II that was available for binding assays was 2 μM. Consequently, Figure 3 shows the effects of topoisomerase II concentration (0−2 μM) on drug−enzyme binding at a constant concentration of 5 μM etoposide.

Even though ytop2Y783F and wild-type topoisomerase II differ by the mere loss of an hydroxyl group from Tyr783, the binding of etoposide to the mutant enzyme was decreased nearly 3-fold. This finding is consistent with the DNA studies

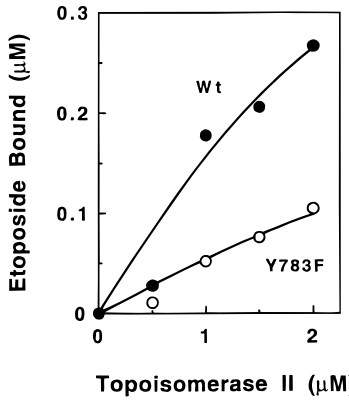

FIGURE 3: Binding of etoposide to wild-type and ytop2Y783F topoisomerase II. Reactions contained 5 μM [^{3}H]etoposide and increasing concentrations of the wild-type (●) or active site mutant (○) enzyme. Data represent the averages of four to five independent experiments.

discussed above and suggests that anticancer agents such as etoposide bind to topoisomerase II in close proximity to its active site tyrosine residue.

Alternatively, it is possible that the conversion of Tyr to Phe at position 783 causes a conformational distortion within the active site of the enzyme that leads to a decrease in drug affinity at a distal site. Unfortunately, no structural studies have been reported for ytop2Y783F, and it is impossible to detect such a distortion based on altered enzymological properties, since the loss of the hydroxyl group from Tyr783 renders topoisomerase II incapable of cleaving DNA or altering its topological state (*29*, *36*). However, since ytop2Y783F binds DNA with an affinity equal to that of wild-type topoisomerase II (*37*), this latter possibility appears to be unlikely.

DISCUSSION

Despite the prevalence of topoisomerase II-targeted drugs in the treatment of human malignancies, interactions between these agents and the enzyme are poorly defined (*1*−*10*). At the present time, no crystallographic structure has been reported for any drug·topoisomerase II complex. Furthermore, while a number of drug-resistant mutant type II enzymes have been identified, there is as yet no evidence that any resistance-conferring mutation actually resides within the drug interaction domain on topoisomerase II (*8*, *17*, *20*, *21*). Finally, the mechanistic basis of drug resistance is largely unknown (*17*, *20*, *21*).

Previous attempts to describe the interactions of anticancer agents with either wild-type or mutant eukaryotic type II topoisomerases have been severely limited by a lack of available drug−enzyme binding assays. Therefore, to further our understanding of drug action, a nitrocellulose filter assay was developed and used to characterize the binding of etoposide to the enzyme. Results indicate that this drug binds to topoisomerase II in the absence of DNA and that the resistance of ytop2H1012Y to etoposide is caused by a decrease in drug−enzyme binding affinity. In addition, etoposide binding is dependent on the hydroxyl moiety of Tyr783 of yeast topoisomerase II. Taken together with studies that place topoisomerase II poisons at or near the site of DNA cleavage, this finding suggests specific interactions between anticancer drugs and the active site residue involved in DNA scission.

In summary, the present study provides novel information regarding the interactions of topoisomerase II with a clinically important anticancer drug. Information such as this greatly extends our current understanding of drug mechanism and may provide a critical catalyst for the development of future, more effective, topoisomerase II-targeted anticancer agents.

ACKNOWLEDGMENT

The authors are grateful to S. Erin Hannah and Jo Ann Byl for their assistance in the preparation of topoisomerase II and to Dr. Janet E. Lindsley (University of Utah) for her generous gift of the overexpression plasmid encoding ytop2Y783F.

REFERENCES

1. Smith, M. A., Rubinstein, L., Cazenave, L., Ungerleider, R. S., Maurer, H. M., Heyn, R., Khan, F. M., and Gehan, E. (1993) *J. Natl. Cancer Inst. 85*, 554−558.
2. Smith, M. A., Rubinstein, L., and Ungerleider, R. S. (1994) *Med. Pediatr. Oncol. 23*, 86−98.
3. Liu, L. (1994) *Adv. Pharmacol. 29*.
4. Sinha, B. K. (1995) *Drugs 49*, 11−19.
5. Pommier, Y., Fesen, M. R., and Goldwasser, F. (1996) in *Cancer Chemotherapy and Biotherapy: Principles and Practice* (Chabner, B. A., and Longo, D. L., Eds.) pp 435−461, Lippincott−Raven Publishers, Philadelphia, PA.
6. Hande, K. R. (1998) *Biochim. Biophys. Acta 1400*, 173−184.
7. Chen, A. Y., and Liu, L. F. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 191−218.
8. Corbett, A. H., and Osheroff, N. (1993) *Chem. Res. Toxicol. 6*, 585−597.
9. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21429−21432.
10. Burden, D. A., and Osheroff, N. (1998) *Biochim. Biophys. Acta 1400*, 139−154.
11. Osheroff, N., Zechiedrich, E. L., and Gale, K. C. (1991) *BioEssays 13*, 269−273.
12. Watt, P. M., and Hickson, I. D. (1994) *Biochem. J. 303*, 681−695.
13. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
14. Hickman, J. A. (1992) *Cancer Metastasis Rev. 11*, 121−139.
15. Anderson, R. D., and Berger, N. A. (1994) *Mutat. Res. 309*, 109−142.
16. Ferguson, L. R., and Baguley, B. C. (1994) *Environ. Mol. Mutagen. 24*, 245−261.
17. Nitiss, J. L., and Beck, W. T. (1996) *Eur. J. Cancer 32a*, 958−966.
18. Kaufmann, S. H. (1998) *Biochim. Biophys. Acta 1400*, 195−211.
19. Kreuzer, K. N., and Cozzarelli, N. R. (1979) *J. Bacteriol. 140*, 424−435.
20. Larsen, A. K., and Skladanowski, A. (1998) *Biochim. Biophys. Acta 1400*, 257−274.
21. Dingemans, A. M. C., Pinedo, H. M., and Giaccone, G. (1998) *Biochim. Biophys. Acta 1400*, 275−288.
22. Willmott, C. J., and Maxwell, A. (1993) *Antimicrob. Agents Chemother. 37*, 126−127.
23. Elsea, S. H., Hsiung, Y., Nitiss, J. L., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 1913−1920.
24. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) *Nature 379*, 225−232.
25. Froelich-Ammon, S. J., Patchan, M. W., Osheroff, N., and Thompson, R. B. (1995) *J. Biol. Chem. 270*, 14998−15005.

26. Froelich-Ammon, S. J., Burden, D. A., Patchan, M. W., Elsea, S. H., Thompson, R. B., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 28018−28021.
27. Worland, S. T., and Wang, J. C. (1989) *J. Biol. Chem. 264*, 4412−4416.
28. Higgins, N. P., and Cozzarelli, N. R. (1982) *Nucleic Acids Res. 10*, 6833−6847.
29. Burden, D. A., Kingma, P. S., Froelich-Ammon, S. J., Bjornsti, M.-A., Patchan, M. W., Thompson, R. B., and Osheroff, N. (1996) *J. Biol. Chem. 271*, 29238−29244.
30. Freudenreich, C. H., and Kreuzer, K. N. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11007−11011.
31. Capranico, G., and Binaschi, M. (1998) *Biochim. Biophys. Acta 1400*, 185−194.
32. Kingma, P. S., and Osheroff, N. (1997) *J. Biol. Chem. 272*, 1148−1155.
33. Kingma, P. S., Greider, C. A., and Osheroff, N. (1997) *Biochemistry 36*, 5934−5939.
34. Kingma, P. S., and Osheroff, N. (1998) *J. Biol. Chem. 273*, 17999−18002.
35. Kingma, P. S., and Osheroff, N. (1998) *Biochim. Biophys. Acta 1400*, 223−232.
36. Tennyson, R. B., and Lindsley, J. E. (1997) *Biochemistry 36*, 6107−6114.
37. Morris, S. K., Harkens, T. T., Tennyson, R. B., and Lindsley, J. E. (1999) *J. Biol. Chem. 274*, 3446−3452.

BI982855I