Mechanism of Topoisomerase II Inhibition by Staurosporine and Other Protein Kinase Inhibitors*

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Topoisomerase II is an essential enzyme for proliferation of eukaryotic cells. It is also a target for many antineoplastic drugs that promote stabilization of covalent complexes between topoisomerase II and DNA. Topoisomerase II and protein kinases both catalyze the transfer of phosphoester bonds from nucleotides to proteins. This similarity suggests that inhibitors may affect both classes of enzymes. In the present study, we have examined the mechanism of topoisomerase II inhibition by three different classes of protein kinase inhibitors. We report that staurosporine inhibited the catalytic activity of topoisomerase II by blocking the transfer of phosphodiester bonds from DNA to the active tyrosine site, a mechanism of inhibition not previously reported for this enzyme. In contrast, other kinase inhibitors, such as methyl 2,5-dihydroxycinnamate, most likely inactivated topoisomerase II by alkylation of essential amino acids, whereas the mechanism of inhibition of bis-indolylmaleimide possibly involved a direct interaction with DNA.

Topoisomerase II is a nuclear enzyme that regulates the topology of DNA by passing an intact double strand of DNA through transient double-stranded breaks created in an adjacent DNA segment (1–3). This allows for resolution of topological perturbations that occur during transcription (4), DNA replication, and separation of chromosomes (5). In the past decade, a number of clinically important and structurally diverse antineoplastic agents have been found to exert their cytotoxic mechanism by stabilizing the covalent complex formed between topoisomerase II and DNA (e.g. etoposide, mitoxantrone, and Adriamycin; reviewed in Ref. 6). Accumulation of these covalent protein-DNA intermediates activates apoptosis, resulting in cell death (7–9). Newer inhibitors have recently been described that do not stabilize the covalent complex but nevertheless inhibit the function of this essential enzyme. Some of these inhibitors were shown to have a different spectrum of antitumor activity (10–14) compared to etoposide or Adriamycin. For example, a 2,6-dioxopiperazine found to be effective against adult T-cell leukemia/lymphoma was not cross-resistant to other antitumor drugs and was recently approved for clinical use in Japan (15). Detailed mechanistic studies revealed that 2,6-dioxopiperazines inhibited the catalytic activity of topoisomerase II by interfering with enzyme turnover (16). These findings have renewed interest in identifying topoisomerase II inhibitors with new mechanisms of action.

The catalytic cycle of topoisomerase II can be separated into six discrete steps as reviewed by Osheroff et al. (17): 1) noncovalent binding of topoisomerase II to DNA; 2) establishing pre-strand passage cleavage/religation equilibrium; 3) DNA strand passage upon binding of ATP; 4) establishing cleavage/religation equilibrium following strand passage; and 5) ATP hydrolysis that results in the 6) dissociation of the enzyme from the DNA (i.e. enzyme turnover). Topoisomerase II maintains the integrity of the cleaved DNA (steps 2–4) by forming covalent, O4-phosphotyrosyl bonds with each newly created 5'-phosphate termini of the cleaved DNA segment. This transfer of phosphodiester bonds from DNA to topoisomerase II is similar to the autoprophosphorylation reaction of tyrosine kinases, where the enzyme forms an O4-phosphotyrosyl bond between its tyrosine and the γ-phosphate of ATP. It is possible, therefore, that kinase inhibitors that interact with the active tyrosine site of the kinase may also inhibit topoisomerase II through a similar mechanism. It was also noticed that topoisomerase II shares the second of its two consensus ATP binding motifs, GXXGXXG, with protein kinases (18). When an initial observation was made that some tyrosine kinase inhibitors (e.g. erbstatin, tyrphostin, and genistein) also inhibit topoisomerase II, it was suggested that these compounds inhibit both classes of enzymes by interacting with their ATP sites (18, 19). Subsequent experiments (20) indicated, however, that yeast topoisomerase II uses the first of its two consensus ATP binding motifs, which is not shared with kinases, and the mechanism whereby these compounds inhibit topoisomerase II remained unelucidated. To clarify the mechanism of topoisomerase II inhibition by protein kinase antagonists, we have studied three of them in detail, including an ATP competitive inhibitor (bis-indolylmaleimide) (21); a substrate/ATP-competitive tyrosine kinase inhibitor (methyl-2,5-dihydroxycinnamate) (22), and staurosporine (not competitive with either substrate or ATP) (23).

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Topoisomerase II (from Drosophila melanogaster) was purchased from Amersham Corp. Staurosporine, etoposide, NaCl (molecular biology grade), N-lauroyl sarcosine, and polyethyleneimine-impregnated thin layer cellulose plates were purchased from Sigma. Methyl-2,5-dihydroxycinnamate, genestein, EDTA, CsCl (optical grade), ethidium bromide, and all buffers were obtained from Life Technologies, Inc. Proteinase K, pBR322 plasmid DNA, AMP-PNP, ATP, and BSA were purchased from Boehringer Mannheim, and agarose from EM Science. Bis-indolylmaleimide was from Calbiochem, and mitoxantrone was from Lederle Laboratories; ICRF 193 was a generous gift from Dr. A. M. Creighton, St. Bartholomew’s Hospital, London, United Kingdom. [γ-32P]ATP (2000 Ci/mmol) and [3H]thymidine (86.9 Ci/mmol) were from DuPont NEN.

1H/[K]inetoplast DNA (KDNA)—[1H]Thymidine-labeled kinetoplast DNA.
DNA from Crithidia fasciculata was isolated according to a modified protocol (24). One liter of blood was mixed with 1.25 mL of 5 M NaCl, 250 μM etoposide, and various concentrations of inhibitors. In the absence of inhibitors, the reaction was stopped by the addition of 50 μM proteinase K at 37 °C, reaction products were resolved in 1.2% agarose gels, and the amount of linear DNA was quantitated using the Eagle Eye II imaging system.

ATPase Activity of Topoisomerase II—ATP hydrolysis by topoisomerase II was measured essentially as described by Osheroff et al. (1). Reaction mixture (4 μL) contained 10 mM Tris/HisCl, pH 7.8, 150 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 15 μg/ml BSA, 0.74 μg of KDNA, 200 μM [γ-32P]ATP (4 μCi), 5 ng of topoisomerase II, and various drug concentrations. After incubation at 30 °C for 15 min, 2 μL from each reaction were applied on the polyethyleneimine-impregnated thin layer cellulose plates, and samples were chromatographed in freshly prepared 0.4 M ammonium bicarbonate. Inorganic phosphate was visualized by autoradiography, and areas containing inorganic phosphate were cut out of the chromatograms and quantitated in a Packard liquid scintillation counter. The amount of inorganic phosphate generated by topoisomerase II in the absence of DNA was small and was subtracted from the total to give the DNA-dependent ATP hydrolysis.

RESULTS

Inhibition of the Topoisomerase II-mediated Decatenation by Protein Kinase Inhibitors—The catalytic activity of topoisomerase II was determined using a [3H]KDNA decatenation assay that quantitates the formation of the final products of this multistep reaction. The effect of various protein kinase inhibitors on the catalytic activity of topoisomerase II was evaluated using this assay. Several kinase inhibitors were more effective than etoposide in inhibiting the catalytic activity of topoisomerase II. Staurosporine was the most potent (IC50, 17 μM), followed by methyl-2,5-dihydroxycinnamate (IC50, 32 μM), bisindolylmaleimide (IC50, 84 μM), and genistein (IC50, 160 μM). By comparison, the IC50 of etoposide was 196 μM.

Stabilization of Topoisomerase II-cleaved DNA Complexes—Stabilization of the covalent topoisomerase II-DNA complexes was assayed by measuring the amount of linear DNA generated from KDNA in the presence of topoisomerase II and ATP. Except for genistein, none of the protein kinase inhibitors promoted the stabilization of the topoisomerase II-DNA complex.

Inhibition of the Topoisomerase II-mediated Decatenation: Competition with ATP—Many protein kinase inhibitors, including bis-indolylmaleimide, inhibit kinases by competing with ATP for binding to the enzyme (20). Since ATP binding is necessary for activity of both kinases and topoisomerase II, these compounds may inhibit both enzymes through a similar mechanism. To address this possibility, we have carried out a series of competition experiments to calculate the IC50 values for protein kinase inhibitors using the topoisomerase II decatenation assay at different ATP concentrations. ATP saturated the reaction at 20 μM, and there was no significant substrate inhibition effect at 5 mM (Fig. 1). The IC50 values for protein kinase inhibitors were calculated at 50 μM, 500 μM, and 5 mM ATP. The nonhydrolyzable ATP analog, AMP-PNP, was used as a positive control, while the intercalating topoisomerase II inhibitor, mitoxantrone, was used as a negative control (2). The IC50 values were expressed as a function of the IC50 obtained at 50 μM ATP to facilitate comparison between compounds (Table I). A 100-fold increase in the ATP concentration resulted in a more than 100-fold increase in the IC50 of AMP-PNP, in agreement...
Inhibition of Topoisomerase II-mediated DNA Cleavage

Inhibition of Topoisomerase II-mediated Decatenation of KDNA: Topoisomerase II (2.5 ng) was used to decatenate 400 ng of [3H]thymidine-labeled KDNA at various ATP concentrations, under conditions described under “Experimental Procedures.”

The effect of staurosporine on the etoposide-induced topoisomerase II-DNA complex is shown on Fig. 3. At equimolar concentrations, staurosporine reduced the amount of linear DNA to background levels. Bis-indolylmaleimide was less potent than staurosporine but also substantially inhibited formation of the complex (78% inhibition at equimolar concentrations). In contrast, inhibitors that interfere with the topoisomerase II-catalyzed reaction at or after the ATP-binding step (ICRF 193, see Table II) had no effect on the level of etoposide-induced complex.

One molecule of the topoisomerase II dimer generates one molecule of topoisomerase II-DNA complex. This contrasts with the decatenation reaction where each topoisomerase II dimer catalyzes the liberation of several molecules of circular DNA. Therefore, more enzyme is required to generate a measurable amount of released linear DNA. The amount of released linear DNA corresponds to the quantity of the topoisomerase II-DNA complex. Inhibitors of topoisomerase II that interfere with any of the reaction steps preceding the binding of ATP would decrease the amount of etoposide-stabilized topoisomerase II-DNA complex.

These findings indicate that staurosporine and bis-indolylmaleimide either directly inhibited formation of the topoisomerase II-DNA complex, or prevented the initial noncovalent binding of the enzyme to DNA. However, staurosporine and bis-indolylmaleimide may simply neutralize etoposide by directly interacting with it. To address that possibility, we have examined the effect of staurosporine on the formation of the pre-strand passage topoisomerase II-DNA complex in the absence of etoposide. In the absence of etoposide, staurosporine inhibited the pre-strand passage topoisomerase II-DNA complex in a dose-dependent manner (Fig. 3). This indicates that the block in the etoposide-stabilized pre-strand passage com-

with an ATP-competitive mode of action. A 100-fold increase in the ATP concentration produced less than a 2-fold increase in the IC50 for mitoxantrone and less than a 3-fold increase in the IC50 values for staurosporine and methyl-2,5-dihydroxycinnaminate. For genistein, bis-indolylmaleimide, and N-ethylmaleimide, the increase in IC50 values was higher but was still less than 7-fold. These results suggest that staurosporine does not inhibit topoisomerase II by competing at the ATP site and possibly interacts with a different domain and inhibits a different step of the catalytic cycle of the enzyme.

Inhibition of the Pre-strand Passage Topoisomerase II-cleaved DNA Complex—In the absence of ATP, topoisomerase II can bind to DNA and establish a cleavage-religation equilibrium prior to strand passage (26). Etoposide shifts the equilibrium toward cleaved DNA (27), resulting in elevated levels of the topoisomerase II-DNA complex. DNA present in the complex is in the form of a broken circle, held together by phosphodiester bonds, linking each terminal 5′-phosphate to a tyrosine residue of topoisomerase II (17). Digestion of topoisomerase II with proteinase K results in release of the cleaved (linear) DNA. The amount of released linear DNA corresponds to the quantity of the topoisomerase II-DNA complex. Inhibitors of topoisomerase II that interfere with any of the reaction steps preceding the binding of ATP would decrease the amount of etoposide-stabilized topoisomerase II-DNA complex.

The IC50 values for protein kinase inhibitors in the topoisomerase II decatenation assay, normalized to one at 50 μM ATP

### Table I

| Compound                  | IC50 at indicated ATP concentration |
|---------------------------|-------------------------------------|
|                           | 50 μM  | 500 μM  | 5 mM   |
| AMP-PNP                   | 1.0    | 6       | 160    |
| Mitoxantrone              | 1.0    | 1.5     | 1.4    |
| Bis-indolylmaleimide      | 1.0    | 2.5     | 4.8    |
| Methyl-2,5-dihydroxycinnaminate | 1.0    | 2.3     | 2.6    |
| Staurosporine (S.D., experiments.) | 1.0    | 1.2     | 2.7    |
| N-Ethylmaleimide          | 1.0    | 1.3     | 6.5    |
| Genistein                 | 1.0    | 5.8     | 3.1    |

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Inhibition of Topoisomerase II-mediated DNA Cleavage

Inhibition of topoisomerase II-mediated DNA cleavage by staurosporine. Inhibition of the cleavage in the absence of etoposide, 

Topoisomerase II - DNA Complex

- 250 μM Etoposide
- No Etoposide

Staurosporine (μM)

% Free DNA

FIG. 3. Inhibition of the topoisomerase II-mediated, ATP-independent DNA cleavage by staurosporine. ○, inhibition of the cleavage in the absence of etoposide; ●, inhibition of the etoposide-enhanced cleavage. In both cases, the amount of linear DNA produced in the absence of staurosporine was set to 100%. Reactions were carried out as described under “Experimental Procedures” without or with 250 μM etoposide. All linear DNA was covalently associated with topoisomerase II, and the enzyme was digested with proteinase K prior to electrophoresis.

Table II

| Topoisomerase II inhibitors | Relative amount of complex formed |
|----------------------------|----------------------------------|
| None                       | 0                                |
| Etoposide                  | 100                              |
| Etoposide + 7.5 μM ICRF 193| 102                              |
| Etoposide + 15 μM ICRF 193 | 99                               |
| Etoposide + 15 μM AMP-PNP  | 107                              |
| Etoposide + 30 μM AMP-PNP  | 97                               |
| Etoposide + 160 μM staurosporine | 30                          |
| Etoposide + 250 μM staurosporine | 0                           |
| Etoposide + 100 μM bis-indolylmaleimide | 61                          |
| Etoposide + 250 μM bis-indolylmaleimide | 22                          |

our results suggest that staurosporine, methyl-2,5-dihy-droxy-cinnamate, and bis-indolylmaleimide directly inhibited the formation of the pre-strand passage topoisomerase II-DNA complex through different mechanisms.

Effect of Dithiothreitol on Inhibition of Topoisomerase II by Protein Kinase Inhibitors—Methyl-2,5-dihydroxycinnamate represents a class of chemically reactive protein kinase inhibitors called erbstatins (29). These compounds are capable of reacting with sulphydryls and may inactivate enzymes by alkylating their cysteines (30). Topoisomerase II contains essential cysteines, and reaction with N-ethylmaleimide leads to loss of catalytic activity (31). We have examined the possibility that some protein kinase inhibitors inhibit topoisomerase II through that mechanism. Preincubation with dithiothreitol totally abolished inhibition of topoisomerase II by methyl-2,5-dihydroxycinnamate. Similar results were obtained for the standard sulphydryl reagent, N-ethylmaleimide. Surprisingly, bis-indolylmaleimide showed some reactivity with sulphydryls, as judged from a reproducible, 2.3-fold increase in IC_{50} upon treatment with dithiothreitol. In contrast, staurosporine inhibited topoisomerase II with the same potency before (IC_{50}, 16.5 μM) and after (IC_{50}, 15.5 μM) preincubation with dithiothreitol.

Inhibition of the ATPase Activity of Topoisomerase II by Protein Kinase Inhibitors—All topoisomerase II inhibitors we have investigated interfere with catalytic steps of the enzyme.
mechanism of topoisomerase II inhibition by erbastatin and tyrphostin is identical to that of N-ethylmaleimide. To test this hypothesis, we have studied the inhibition of topoisomerase II by the erbastatin methyl-2,5-dihydroxycinnamate (22) and compared it to N-ethylmaleimide. Both compounds blocked formation of the ATP-independent topoisomerase II-DNA complex.

2 Preincubation of these compounds with an excess of dithiothreitol totally abolished inhibition of the enzyme by N-ethylmaleimide as well as by methyl-2,5-dihydroxycinnamate. In both cases, inhibition of the catalytic activity was partially competitive with ATP. It is known that ATP binding to topoisomerase II changes the conformation of the enzyme from “open” to “closed” (35), possibly rendering target nucleo- side-accessible to N-ethylmaleimide or methyl-2,5-dihydroxycinnamate. Thus, ATP may protect topoisomerase II from inactivation by an allosteric effect. These results strongly suggest that 2,5-dihydroxycinnamate inactivates topoisomerase II by alkylating residues essential for the enzyme activity, most likely cysteines.

Staurosporine was originally thought to be an ATP-competitive inhibitor specific for protein kinase C. More recent studies showed that it is a broad, potent inhibitor of various kinases, including tyrosine kinases, and does not compete with ATP (23, 36, 37). We report here that staurosporine is also a potent inhibitor of the catalytic activity of topoisomerase II, does not seem to compete with ATP, and that it inhibits the ATP-independent transfer of phosphodiester bonds from DNA to the active site tyrosine residues of the enzyme. Staurosporine does not react with thiois, which makes its mechanism of action different from that of the erbastatins. Staurosporine is the first topoisomerase II inhibitor shown to directly interfere with the transfer of phosphodiester bonds from DNA to the enzyme.

Although staurosporine is not selective for topoisomerase II, it may be possible to develop analogs that are selective for this enzyme. Similar efforts to optimize the specificity of staurosporine toward protein kinase C were successful, resulting in the synthesis of bis-indolylmaleimide, a selective and potent, ATP-competitive inhibitor of protein kinase C, that only modestly affects other kinases (21). This compound inhibited topoisomerase II but was less potent than staurosporine and was partially competitive with ATP. Surprisingly, it was shown to interact directly with DNA, suggesting that it may inhibit topoisomerase II by more than one mechanism.

It was recently shown that compounds that inhibit the catalytic activity of topoisomerase II but that do not stabilize the formation of topoisomerase II-DNA complexes are clinically useful in the treatment of cancer (15). Our results indicate that the ATP-independent transfer of the phosphodiester bond from the DNA to the enzyme can constitute a target for the design and development of novel anticancer drugs.

REFERENCES
1. Osheroff, N., Shelton, E. R., and Brutlag, D. L. (1983) J. Biol. Chem. 258, 9536–9543
2. Wang, J. C. (1985) Annu. Rev. Biochem. 54, 665–697
3. Andersen, A. H., Svejstrup, J. Q., and Westergaard, O. (1994) Adv. Pharmacol. 29A, 89–102
4. Crothen, M., Wu, H. Y., and Liu, J. F. (1994) Adv. Pharmacol. 29A, 135–146
5. Nishi, J. L. (1994) Adv. Pharmacol. 29A, 103–114
6. Crosetti, A. H., and Osheroff, N. (1993) Chem. Res. Toxicol. 6, 565–597
7. Roy, C., Brown, D. L., Little, J. E., Valentine, B. K., Walker, P. R., Sikorska, M., Leblanc, J., and Chaly, N. (1992) Exp. Cell Res. 206, 416–424
8. Kastoffmann, S. H. (1989) Cancer Res. 49, 5870–5878
9. Hickman, J. A. (1992) Cancer Metastasis Rev. 11, 121–139
10. Boritzki, T. J., Wolfard, T. S., Besserer, J. A., Jackson, R. C., and Fry, D. W. (1988) Biochem. Pharmacol. 37, 4063–4069
11. Drake, F. H., Hofmann, G. A., Meng, S.-M., O’Leary Bartus, J., Hertzberg, R. P., Johnson, R. K., Mattern, M. R., and Mirabelli, C. (1989) Cancer Res. 49, 2578–2583
12. Jensen, P. B., Sorensen, B. S., Demant, E. J. F., Sehested, M., Jensen, P. S.,

G. Singh and P. Lassota, unpublished result.
Vindeløwe, L., and Hansen, H. (1990) Cancer Res. 50, 3311–3316
13. Tanabe, K., Ikegami, Y., Ishida, R., and Andoh, T. (1991) Cancer Res. 51, 4903–4908
14. Ishida, R., Miki, T., Narita, T., Yui, R., Sato, M., Utsumi, K. R., Tanabe, K., and Andoh, T. (1991) Cancer Res. 51, 4909–4916
15. Ohno, R., Masaoka, T., Shirakawa, S., Sakamoto, S., Hirano, M., Hanada, S., Yasunaga, K., Yokomaku, S., Mitome, Y., Nagai, K., Yamada, K., and Furue, H. (1993) Cancer (Phila.) 71, 2217–2221
16. Roca, J., Ishida, R., Berger, J. M., Andoh, T., and Wang, J. C. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1781–1785
17. Osheroff, N., Zechiedrich, E. L., and Gale, K. C. (1991) BioEssays 13, 269–283
18. Markovits, J., Linassier, C., Fosse, P., Couprie, J., Jacqemin-Sablon, A., Saucier, J.-M., Le Pecq, J.-B., and Larsen, A. K. (1989) Cancer Res. 49, 5111–5117
19. Markovits, J., Larsen, A. K., Segal-Bendirdjian, E., Fosse, P., Saucier, J.-M., Gazit, A., Levitzki, A., Umezawa, K., and Jacqemin-Sablon, A. (1994) Biochem. Pharmacol. 48, 549–560
20. Lindeley, J. E., and Wang, J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 10485–10489
21. Davis, P. D., Elliot, L. H., Harris, W., Hill, C. H., Hurst, S., Keech, E., Kumar, M. K. H., Lawton, G., Nixon, J. S., and Wilkinson, S. E. (1992) J. Med. Chem. 35, 994–1001
22. Umezawa, K., Hori, T., Tajima, H., Imoto, M., Ishuki, K., and Takeuchi, T. (1996) FEBS Lett. 260, 198–200
23. Ward, N. E., and O’Brian, C. A. (1992) Mol. Pharmacol. 41, 387–392
24. Englund, P. T. (1978) Cell 14, 157–168
25. Sahai, B. M., and Kaplan, J. G. (1986) Anal. Biochem. 156, 364–379
26. Osheroff, N. (1986) J. Biol. Chem. 261, 9944–9950
27. Osheroff, N. (1989) Biochemistry 28, 6157–6160
28. Benchekroun, Y., Couprie, J., and Larsen, A. K. (1995) Biochem. Pharmacol. 49, 305–313
29. Umezawa, K., and Imoto, M. (1991) Methods Enzymol. 201, 379–385
30. Kupchan, S. M., Fessler, D. C., Eakin, M. A., and Giacobbe, T. J. (1970) Science 168, 376–378
31. Sander, M., Nolan, J. M., and Hsieh, T.-s. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 6938–6942
32. Robinson, M. J., Corbett, A. H., and Osheroff, N. (1993) Biochemistry 32, 3638–3643
33. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Furakami, Y. (1987) J. Biol. Chem. 262, 5592–5595
34. Corbett, A. H., Hong, D., and Osheroff, N. (1993) J. Biol. Chem. 268, 14394–14398
35. Roca, J., and Wang, J. C. (1992) Cell 71, 833–840
36. Tamaoki, T., Numoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 133, 397–402
37. Ruegg, U. T., and Burgess, G. M. (1989) Trends Pharmacol. Sci. 10, 218–220
