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INHIBITION OF TYPE II TOPOISOMERASE BY FOSTRIECIN

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Abstract—Fostriecin is a new antitumor antibiotic which is being developed further as an anticancer agent based on its marked activity in murine leukemias. Its mechanism of action, however, has thus far remained unknown. The present study demonstrates that fostriecin inhibits the catalytic activity of partially purified type II topoisomerase from Ehrlich ascites carcinoma. Under the experimental conditions employed, fostriecin completely inhibited the enzyme at 100 μM. A general kinetic analysis showed that fostriecin inhibited topoisomerase in an uncompetitive manner with a $K_{i,app}$ of 110 μM and produced kinetics that were distinctly different from those of VM-26 which exhibited noncompetitive inhibition. Fostriecin did not cause DNA strand breaks in L1210 cells, suggesting that it did not stabilize a cleavable complex as do other known inhibitors of this enzyme. Fostriecin, however, did partially inhibit DNA strand breaks produced by amsacrine. An analysis by flow cytometry showed that L1210 cells exposed to 5 μM fostriecin for 12 hr caused a block in the G_2 phase of the cell cycle. These studies thus suggest that the mechanism by which fostriecin produces its antitumor effects may be through inhibition of topoisomerase II and that the type of inhibition is markedly different from existing antitumor agents which inhibit this enzyme.

Fostriecin is a new antitumor antibiotic that was isolated from the fermentation beer of a subspecies of *Streptomyces pulveraceus* (subspecies *fostreus*, ATCC 31906) [1, 2]. The structure of this novel agent has been characterized [3] and is shown in Table 1 (see Results) along with a number of existing analogues. This drug exhibits antitumor activity against a wide spectrum of tumor cells *in vitro* and has excellent activity against L1210 and P388 leukemias *in vivo* [4–6]. Fostriecin is unique in that it utilizes the reduced folate carrier system to enter cells [7]. Initial biochemical studies have shown that fostriecin rapidly suppresses nucleic acid synthesis, but that this effect is not due to depletion of nucleotide pools, direct inhibition of DNA or RNA polymerases, or damage or binding to the DNA template [8]. These earlier studies which have been summarized by Jackson *et al.* [9] imply that, although the primary target for fostriecin was not DNA or RNA polymerase nor an enzyme within the purine or pyrimidine biosynthetic pathways, it nevertheless affected a process essential for replication and transcription.

The present study demonstrates that fostriecin can directly inhibit the catalytic activity of purified topoisomerase II from Ehrlich ascites carcinoma. Topoisomerase II is very likely a primary target for fostriecin. This enzyme has been identified as a viable target for anticancer agents, and a number of clinically useful drugs appear to function through inhibition of this enzyme including etoposide [10], doxorubicin [11] and amsacrine [12]. In our present experiments, however, fostriecin was shown to be distinctly different from previously described inhibitors of topoisomerase II in that it was cytotoxic, but did not cause protein-associated DNA strand breaks and, therefore, did not appear to stabilize a cleavable ternary complex as has been shown for other inhibitors of this enzyme [13, 14]. Fostriecin appears to be, therefore, the first inhibitor of topoisomerase II that possesses antitumor activity but does not produce protein-associated DNA strand breaks. The significance of these observations is discussed with regard to cytotoxic mechanism.

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MATERIALS AND METHODS

Enzyme purification. Topoisomerase II was purified from Ehrlich ascites tumor cells essentially as described by Miller *et al.* [15] for the HeLa enzyme. Triton X-100 (0.5%) was added to the nuclei extraction buffer to facilitate cell lysis.

Preparation of DNA substrates. Kinetoplast DNA was purified from *Crithidia fasiculata* (provided by Dr. L. Simpson of the University of California, Los Angeles) grown in brain–heart infusion medium (Difco Laboratories, Detroit, MI) with 10 μg/ml hemin. The DNA was extracted and sedimented through sucrose as described by Laurent *et al.* [16], followed by centrifugation through a CsCl block gradient [17]. PBR322 DNA was prepared from *Escherichia coli* K-12/PBR, which was supplied by Dr. N. Waleh, Stanford Research Institute, and isolated by standard procedures [1].

Unwinding assay. Relaxation of supercoiled pBR322 DNA by type II topoisomerase was assayed in 400-μl microfuge tubes at 37°. Reaction mixtures contained 50 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), pH 6.8, 50 mM KCl, 0.1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), 1 mM ATP and 0.4 μg pBR322 DNA in a final volume of 20 μl. Reaction mixtures were prepared on ice, and the reaction was started by transfer to 37°. Tubes were incubated for 30 min, and reactions were terminated by addition of 5 μl of 40% sucrose, 5% sodium dodecyl sulfate (SDS), 0.25% bromophenol blue. Electrophoresis was performed on 1% agarose gels for 8 hr at 40 V in Tris–borate buffers [19]. Gels were stained with 5 μg/ml ethidium bromide in electrophoresis buffer for 30 min and photographed using Polaroid type 55 film with a Wratten 3A and 300 nm UV transillumination.

Kinetic assay. For kinetic studies topoisomerase catalytic activity was assayed by measuring the rate of decatenation of kinetoplast DNA [19]. Assays were performed at 37° in 400-μl microfuge tubes. The standard reaction mixture contained 50 mM Hepes, pH 6.8, 10 mM $MgCl_2$, 0.5 mM EDTA, 75 mM NaCl, 25 mM KCl, and 90 μg/ml kinetoplast DNA in a final volume of 20 μl. Tubes were preincubated at 37° for 5 min, and reactions were started by addition of ATP. ATP concentration was varied between 0.2 and 0.8 mM. Reactions were terminated after 20 min by the addition of 5 μl of 40% sucrose, 5% SDS, 0.25% bromophenol blue, and the solution was mixed and placed immediately on ice. Twenty microliters of this solution was electrophoresed for 5 hr at 40 V on 12 cm long 1% agarose gels using Tris–borate buffers [19]. Gels were stained and photographed as for the relaxation assay. Densitometry was performed on film negatives with an LKB Gelscan scanning densitometer, and reaction products were quantified using Gelscan software. Kinetic parameters were determined by the method of Cleland [20].

Alkaline elution. DNA strand scission was assayed by the alkaline elution technique [21] using the conditions described previously [22].

Flow cytometry. L1210 cells were stained with propidium iodide by the method of Taylor [23]. Chicken erythrocytes were included as internal standards, and all samples were analyzed on a Becton Dickinson FACS Analyzer.

Amsacrine uptake. Uptake of 9-acridinyl-[^{14}C]amsacrine (obtained from K. H. Davis, Jr., Research Triangle Institute, NC) was measured in L1210 cells essentially as described by Zwelling *et al.* [24]. L1210 cells (2×10^5 cells/ml) grown in RPMI 1640 medium with 10% fetal calf serum were treated with 50 μM fostriecin for 2 hr and then concentrated to 10^7 cells/ml by centrifugation. Treated cells were suspended in medium containing fostriecin, and [^{14}C]amsacrine was added to a final concentration of 1 μM (sp. act. 20.8 mCi/mmol). After incubation at 37°, 0.8-ml aliquots of cell suspension were layered on top of 0.6 ml silicone oil (84 parts Dow Corning DC 550 fluid and 16 parts Dow Corning DC 200) and centrifuged in an Eppendorf model 5412 microcentrifuge for 10 sec [25]. Tubes were immediately frozen in a dry ice–methanol bath. The tube bottoms were clipped off and placed in scintillation vials. Pellets were dispersed in 0.9% saline and analyzed for radioactivity by liquid scintillation counting after addition of 10 ml Ready-Solv MP (Beckman Instruments Co., Fullerton, CA). Other portions of the cell suspension were incubated with [*carboxy*-^{14}C]inulin and then centrifuged as described above to measure the volume of extracellular buffer that accompanies cells to the bottom layer.

RESULTS

Inhibition of type II topoisomerase by fostriecin and its analogs. Figure 1 shows the inhibitory effects of fostriecin on the relaxation of supercoiled DNA by type II topoisomerase from Ehrlich ascites tumor cells. VM-26 is included for comparison since it has

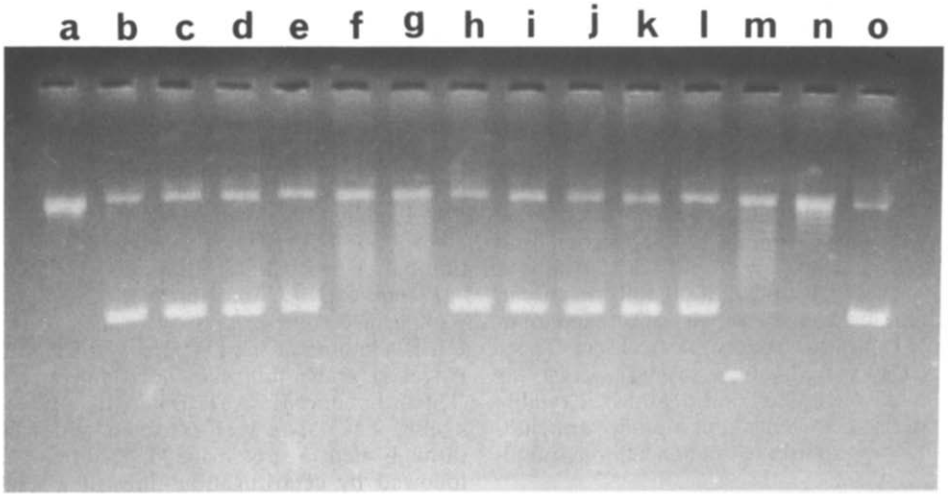
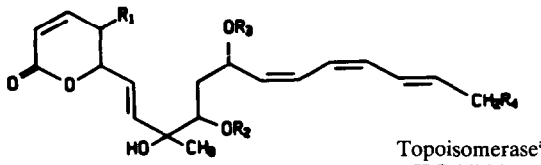


Fig. 1. Inhibition of the supercoiled DNA relaxing activity of Ehrlich ascites topoisomerase II by fostriecin and VM-26. Key: lane a, control; lane b, 400 μM fostriecin; lane c, 200 μM; lane d, 100 μM; lane e, 50 μM; lane f, 25 μM; lane g, 12.5 μM; lane h, 400 μM VM-26; lane i, 200 μM; lane j, 100 μM; lane k, 50 μM; lane l, 25 μM; lane m, 12.5 μm; lane n, control; and lane o, blank (no ATP).

Table 1. Inhibition of topoisomerase II catalytic activity and L1210 growth rates by fostriecin and its analogs



Compound	R_1	R_2	R_3	R_4	Topoisomerase[a] II Inhibition MIC (μM)	Cytotoxicity[b] L1210 leukemia IC_{50} (μM)
Fostriecin	H	$PO_3^=$	H	OH	50.0	0.46[c]
PD 113271	OH	$PO_3^=$	H	OH	12.5	1.8[c]
PD 114631	H	H	H	OH	25.0	5.5[c]
PD 116245[d]	OH	$PO_3^=$	H	OH	25.0	8.1
PD 116250	H	$PO_3^=$	$COCH_3$	$OCOCH_3$	200	>100
VM-26					10	0.01

[a] Topoisomerase catalytic activity was assayed using the supercoiled DNA relaxing protocol described under Materials and Methods. The amount of topoisomerase that was added relaxed 0.4 μg pBR322 DNA in 30 min and was determined in separate experiments. MIC concentrations are the minimum concentration at which topoisomerase inhibition was observed.

[b] IC_{50} is the concentration of drug necessary to reduce the growth rate by 50%.

[c] From Ref. 6.

[d] Opened lactone.

been established previously as an inhibitor of this enzyme [10]. Fostriecin (lanes b–g) prevented the conversion of supercoiled to relaxed DNA by nearly 100% at concentrations of 50 μM or above. Similar results were obtained with VM-26 (lanes h–m) beginning at 25 μM.

Table 1 compares the inhibitory potency of fostriecin to that of a number of structural analogs. The addition of a hydroxyl at position 4 of the lactone ring (PD 113271) increased potency by 4-fold over fostriecin and was comparable to VM-26. The open lactone derivative of PD 113271 (PD 116245) was 2-fold less potent as an inhibitor of topoisomerase II, whereas eliminating the phosphate (PD 114631) from fostriecin increased potency by 2-fold. Acetylation of fostriecin at the 6 and terminal hydroxyl destroyed activity.

The inhibition of topoisomerase II by fostriecin was further examined through a general kinetic analysis. Again VM-26 was chosen for comparison since it is known to form a cleavable complex with the enzyme but does not intercalate into DNA. Panels A and B of Fig. 2 show a double-reciprocal plot of rates for the decatenation of kinetoplast DNA by Ehrlich ascites topoisomerase II versus ATP concentration at different concentrations of fostriecin (A) or VM-26 (B). The $K_{m,app}$ for ATP was 380 μM. Inhibition of topoisomerase II by fostriecin produced parallel line kinetics and suggested an uncompetitive interaction, indicating that the magnitude of inhibition increased with ATP concentration. The $K_{i,app}$ for fostriecin was 110 μM. In contrast, VM-26 produced inhibition kinetics consistent with a noncompetitive interaction which indicates that inhibition was independent of ATP concentration. The $K_{i,app}$ for VM-26 was 7.3 μM. The results suggest distinctly different mechanisms for inhibition by fostriecin and VM-26 possibly due to binding at different sites on the enzyme.

Effect of fostriecin on DNA and amsacrine-induced DNA strand breaks in L1210 cells. Since it has been established that many inhibitors of topoisomerase cause protein-associated DNA strand breaks [13, 14], filter elution procedures were used to assess whether fostriecin also caused these lesions. Table 2 shows that, unlike amsacrine, fostriecin caused very little DNA damage even at concentrations ranging from 1 to 100 μM. Similar results were obtained for novobiocin. Previous reports [26, 27] have also shown that novobiocin partially inhibits the accumulation of DNA protein-associated strand breaks produced by amsacrine. Similar results were obtained

Table 2. Effects of fostriecin and novobiocin on amsacrine-induced strand breaks in L1210 cells

Addition	Alkaline elution rate constant*
Control	0.014
1 μM Fostriecin	0.022
10 μM Fostriecin	0.023
50 μM Fostriecin	0.028
100 μM Fostriecin	0.034
250 μM Novobiocin	0.039
1 μM Amsacrine	0.730
100 μM Fostriecin + 1 μM Amsacrine	0.557
250 μM Novobiocin + 1 μM Amsacrine	0.484

* Values were derived from alkaline elution procedures and represent the negative slope from a plot of the fraction of DNA retained on the filter versus time in hours. Cells were preincubated with fostriecin or novobiocin for 1 hr before the 1-hr amsacrine treatment. Values represent the mean of three experiments.

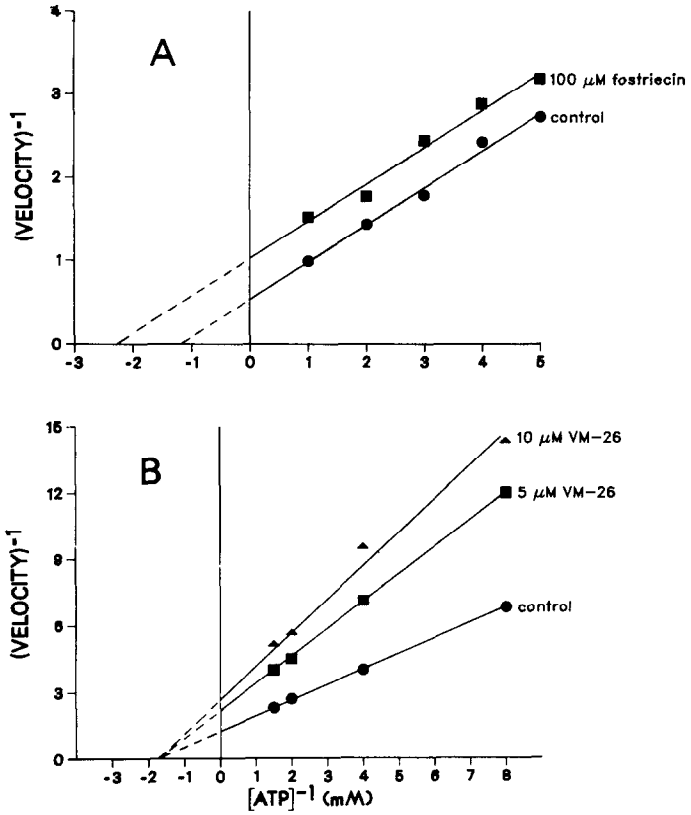


Fig. 2. Double-reciprocal plots of rates (velocity) for the decatenation of kinetoplast DNA by Ehrlich ascites topoisomerase II versus ATP concentration at different concentrations of fostriecin (A) and VM-26 (B) with ATP as the variable substrate. The amount of topoisomerase used in each determination relaxed 0.4 μg pBR322 supercoiled DNA in 30 min.

in this study for both fostriecin and novobiocin. Table 2 shows that, in combination with amsacrine, fostriecin or novobiocin inhibited amsacrine-induced DNA strand cleavage in L1210 cells by 25 and 34% respectively. To ensure that fostriecin was not inhibiting amsacrine-induced strand cleavage by interfering with amsacrine transport, the net uptake of amsacrine was examined in fostriecin-treated L1210 cells. A 2-hr pretreatment with 100 μM fostriecin had no significant effect on the net accumulation of amsacrine in cells (data not shown).

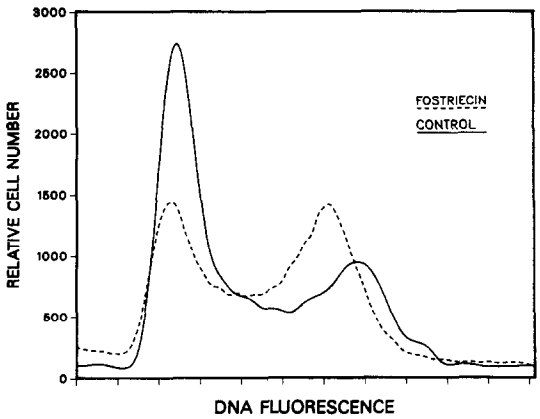


Fig. 3. DNA histograms of control L1210 cells and cells treated with 5 μM fostriecin for 12 hr. Each histogram is derived from 100,000 cells.

Flow cytometry. Previous studies have shown that long-term exposure to inhibitors of topoisomerase II usually causes cells to accumulate in the G_2 phase of the cell cycle [28, 29]. The DNA histogram in Fig. 3 shows that L1210 cells exposed to 5 μM fostriecin for 12 hr had a markedly reduced G_1 population with a corresponding increase in G_2 which is consistent with topoisomerase II functioning as the primary target for fostriecin.

DISCUSSION

The results show that fostriecin inhibited the catalytic activity of isolated type II topoisomerase and confirm data from a previous presentation [30]. This compound represents a novel inhibitor of this enzyme not only structurally but also by virtue of its mechanism of inhibition. Evidence for this distinction is attributed to the fact that in contrast to previously reported inhibitors of this enzyme, i.e. epipodophyllotoxins, amsacrine and doxorubicin [10–12], no protein-associated strand breaks were observed in cells exposed to this drug. A general kinetic analysis showed that fostriecin inhibited this enzyme in an uncompetitive manner with respect to ATP, whereas VM-26 produced noncompetitive inhibition kinetics. Some similarities did exist, how-

ever, between fostriecin and novobiocin. Both compounds inhibited topoisomerase II without causing protein-associated DNA strand cleavage and both partially inhibited the production of those DNA lesions caused by amsacrine. Fostriecin, however, remains distinct from novobiocin in that it possesses antitumor activity whereas the latter does not. Since the structure of fostriecin is uniquely different from all other known inhibitors of topoisomerase II, several analogs were tested in an attempt to gain some insight into what features of this molecule were critical for inhibition. The data indicate that hydroxyl groups at the 4, 6 and terminal positions increased inhibitory potency; there is, unfortunately, no unifying concept that can be made regarding general structural determinants that confer the ability to inhibit topoisomerase II activity especially in view of variations in structural class among other inhibitors of this enzyme.

The first question that arises is whether or not this form of topoisomerase inhibition is cytotoxic. Certain data suggest that simple inhibition of the strand passing activity of topoisomerase II may not be sufficient to cause cytotoxicity. For example, Glisson *et al* [31] found that decatenation activities from wild-type and etoposide resistant cell lines are equally sensitive to inhibition by etoposide and that drug-resistance could be explained only through the significant differences found in sensitivity to cleavable complex formation. Also, Tewey *et al.* [32] reported that the intercalator, ethidium bromide, is a potent inhibitor of strand passage but does not form a cleavable complex. This compound, which has little cytotoxic potency against mammalian cells, markedly reduces the cytotoxicity of etoposide [33]. Other studies, however, provide a counterpoint to these observations and show that this enzyme activity is essential for mitosis and, therefore, cell survival [34, 35]. In addition, other reports have indicated that topoisomerase II is necessary to maintain a transcriptionally active state in chromatin [36–38] and in gene expression [39]. These data thus indicate that suppression of the catalytic activity of this enzyme could indeed result in cell death.

The final question that remains is: while given that fostriecin is an inhibitor of the strand passing activity of topoisomerase II, does this property represent the primary mechanism of its cytotoxicity? The fact that both *in vitro* and *in vivo* potencies for fostriecin analogs [4–6] do not correlate with the degree of topoisomerase inhibition is not a negative consideration. Previous studies have provided strong evidence that fostriecin enters cells via the reduced folate carrier system [7] and, thus, the relative potency could depend on how efficiently the drug enters the cells and not necessarily on the potency with which it interacts with the primary target. Earlier biochemical studies [8] showed that fostriecin inhibits nucleic acid synthesis without reducing nucleotide pools or interacting with DNA. The present study demonstrated that fostriecin, like other type II topoisomerase inhibitors, caused accumulation of cells into the G_2 or M phase of the cell cycle. These results, while not conclusive, are at least consistent with fostriecin functioning as an inhibitor of topoisomerase II *in situ*.

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