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Diospyrin, A Bisnaphthoquinone: A Novel Inhibitor of Type I DNA Topoisomerase of *Leishmania donovani*

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ABSTRACT

Diospyrin is a plant product that has significant inhibitory effect on the growth of *Leishmania donovani* promastigotes. This compound inhibits the catalytic activity of DNA topoisomerase I of the parasite. Like camptothecin, it induces topoisomerase I mediated DNA cleavage *in vitro*. Treatment of DNA with diospyrin before addition of topoisomerase I has no effect. Preincubation of topoisomerase I with diospyrin before the addition of DNA in the relaxation reaction increases this inhibition. Our results suggest that this bis-naphthoquinone compound exerts its inhibitory effect by binding with the enzyme and stabilizing the topoisomerase I-DNA "cleavable complex." Diospyrin is a specific inhibitor of the parasitic topoisomerase I. It does not inhibit type II topoisomerase of *L. donovani* and requires much higher concentrations to inhibit type I topoisomerase of calf thymus. The potent inhibitory effect of diospyrin on type I DNA topoisomerase from *L. donovani* can be exploited for rational drug design in human leishmaniasis.

Leishmaniasis presents as a spectrum of diseases, ranging from benign cutaneous lesions through metastasizing mucocutaneous forms to the often fatal visceralizing form (Walton, 1987). Current therapies are inadequate. The pentavalent antimonials sodium stibogluconate and meglumine antimonate, the first line of drugs for visceral and cutaneous leishmaniasis, have variable efficacy and side effects (Thakur *et al*., 1988). The second line of drugs, amphotericin B and pentamidines, although used clinically, are often of limited efficacy and are very toxic (Iwu *et al*., 1994). Therefore, improved drug therapy of leishmanial infections is still desirable and the need for new molecular targets on which to base future treatment strategies is clear and justified.

Currently DNA topoisomerases have been recognized as potential chemotherapeutic targets for antitumor and antiparasitic agents (Chakraborty and Majumder, 1988; Liu, 1989; Burri *et al*., 1996). DNA topoisomerases are ubiquitous enzymes that control many vital cellular processes by making reversible DNA breaks, enabling a specific tyrosyl residue in the enzyme to covalently link to the phosphoryl group at the DNA break via a phosphodiester bond. They have been classified into two types. The type I enzymes make a transient single stranded nick in absence of any high energy cofactor, whereas the type II enzymes make double-stranded breaks in the presence of ATP, which allows supercoils to be removed from the circular DNAs. Both types of enzymes have been characterized in kinetoplastid hemoflagellated protozoan parasites (Riou, 1983; Chakraborty and Majumder, 1987; Melendy and Ray, 1987; Chakraborty and Majumder, 1991; Chakraborty *et al*., 1993). It has been suggested that topoisomerase I targeting agents may have broad spectrum antiprotozoal activity (Bodley *et al*., 1995). Our own studies have indicated that leishmanial DNA topoisomerases may well provide suitable targets for potential chemotherapy of antileishmanial drugs (Chakraborty and Majumder, 1988; Ray et al., 1996; Ray et al., 1997). Inhibitors of DNA topoisomerases comprise a variety of structurally diverse compounds that interfere with the nicking-closing activities catalyzed by the enzymes. Clinically active antitumor drugs include inhibitors of topoisomerases, such as camptothecin and three of its water soluble derivatives (Slichenmeyer *et al*., 1993), and inhibitors of topoisomerase II, such as acridines, anthracyclines, ellipticines, epipodophyllotoxins, and quinolones, etc. (Liu, 1989). Some of these antitumor drugs (e.g., camptothecin, ellipticin, etoposides) also inhibit trypanosomal topoisomerases (Shapiro and Englund, 1990; Bodley and Shapiro, 1995).

In the present study, we describe the plant-derived bisnaphthoquinonoid compound diospyrin (Fig. 1). Diospyrin is an antitumor compound (Hazra *et al*., 1984) capable of

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis [2-ethane sulfonic acid]; DTT, dithiothreitol, kDNA, kinetoplast DNA.

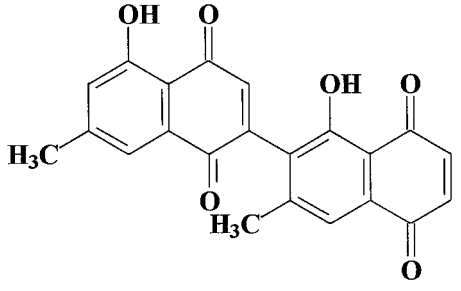
inhibiting the growth of *Leishmania donovani* promastigotes (Yardley *et al*., 1996). It also inhibits the growth of *Trypanosoma brucei*, *Trypanosoma cruzi* (Yardley *et al*., 1996), and *Plasmodium falciparum* (Hazra *et al*., 1995) *in vitro*. We have shown here that it is a potent inhibitor of type I DNA topoisomerase of *L. donovani*; like camptothecin, it also induces stabilization of the "cleavable complex" mediated by topoisomerase I. These observations suggest an important clinical application of the compound, making the present studies highly relevant. There are several naphthoquinones that have been reported to have inhibitory activities toward eukaryotic topoisomerases (Fujii *et al*., 1992; Li *et al*., 1993). This is the first report of a dimeric naphthoquinone acting as a potent inhibitor of type I DNA topoisomerase of *L. donovani*.

Materials and Methods

Isolation of diospyrin. Diospyrin was isolated from the stem bark of *Diospyros montana* Roxb. as described previously (Hazra *et"" al*., 1984). The purity of the compound was attained by repeated crystalization from chloroform [m.p., 270° (d)] and confirmed by the appearance of a single spot in thin layer chromatography [Adsorbent-silica gel G, thin layer chromatography grade (Merck, Mumbai, India); developer, petroleum ether (b.p. 60–80°): ethyl acetate, 4:1 (v/v); indicator, iodine; $R_f = 0.54$] and recording of a single molecular ion peak (M+374) in the mass spectrometry. The structure of the compound was ascertained by analytical and spectroscopic methods (Hazra *et al*., 1995) and confirmed by superimposable IR spectra and undepressed mixed melting point wth an authentic sample of diospyrin. Diospyrin solution was made in 100% DMSO.

Parasite culture and growth conditions. *L. donovani* strain UR6 promastigotes (MHOM/IN/1978/UR6) were grown in Ray's modified media (Ray, 1932) and subcultured at 72-hr intervals.

Enzymes, DNA, and chemicals. Type I and type II DNA topoisomerases were purified from *L. donovani* strain UR6 promastigotes as described previously (Chakraborty and Majumder, 1987; Chakraborty *et al*., 1993). Calf thymus topoisomerase I was purchased from Life Technologies (Gaithersburg, MD). Plasmid pGEM4Z DNA was purchased from Promega (Madison, WI) and plasmid pHOT1 DNA was purchased from TopoGEN (Columbus, OH). pHOT1 DNA contains the high affinity topoisomerase I cleavage site (Bonven *et al*., 1985) that is derived from the tetrahymena ribosomal gene repeat (hexadecameric sequence). kDNA was purified from *L. donovani* strain UR6 as described by Dasgupta *et al*. (1986). Camptothecin (lactone form) and etoposide were obtained from Sigma (St. Louis, MO). Camptothecin was dissolved in DMSO and etoposide in 50% DMSO at 10 mM concentrations. Drug solutions were kept frozen at −20°. Further dilutions were made in distilled water immediately before use. Final DMSO concentrations never exceeded 2%, and all control experiments were carried out with equal volume of drug solvent.



DIOSPYRIN
(bis-Naphthoquinone)

Fig. 1. Structure of diospyrin.

Enzyme assay. The type I DNA topoisomerase was assayed by decreased mobility in an agarose gel of supercoiled pGEM4Z after treatment with the enzyme. The standard topoisomerase I assay mixture (25 μl) contained: 25 mM Tris·HCl, pH 7.5, 5% glycerol, 50 mM KCl, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 0.5 μg of pGEM4Z, and 1 unit of enzyme (one unit of topoisomerase I activity is the amount of enzyme that converts 0.5 μg of superhelical DNA to the relaxed state under the condition of assay). The reaction was carried out at 37° for 30 min. Reactions were stopped by adding 1% SDS, 10 mM EDTA, 0.25 μg/ml bromphenol blue, and 15% glycerol. Samples were applied to a horizontal 1% agarose gel and subjected to electrophoresis in Tris-acetate/EDTA buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0) at 1.5 V/cm for 14–16 hr at room temperature. The gels were stained with ethidium bromide (5 μg/ml), destained in water, and photographed under UV illumination. Percent relaxation was measured by microdensitometry of negative photographs of supercoiled monomer DNA band fluorescence after ethidium bromide staining with a microdensitometer (LKB BROMMA 2202 Ultrascan) and the area under the peak calculated. The standard decatenation assay mixture (25 μl) contained: 25 mM Tris·HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, 10% glycerol, 0.2 μg of kDNA from *L. donovani* strain UR6 and 1 unit of enzyme (1 unit of enzyme activity is defined as the amount of enzyme needed for 50% decatenation of 0.2 μg of kDNA networks into minicircles). The reaction was carried out at 30° for 30 min. Decatenations were monitored in 1% agarose gel as described above. Calf thymus DNA topoisomerase I was assayed according to the conditions specified by the manufacturer.

Topoisomerase I cleavage assay. Reaction mixtures (50 μl) containing 50 mM PIPES, pH 6.0, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 5 μg/ml of pHOT1 DNA, 80 units of topoisomerase I enzyme, and drugs were incubated at 37° for 30 min (Hsiang *et al*., 1985). The reactions were terminated by adding 1% SDS and 150 μg/ml proteinase K. After the additional 1 hr incubation at 37°, DNA samples were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Results

Catalytic inhibition of topoisomerase I by diospyrin. Topoisomerase I was assayed by the method described previously by Chakraborty *et al*. (1993) (i.e., the relaxation of supercoiled DNA in a Mg^{+2}-dependent, ATP-independent reaction). While studying the *in vitro* effect of diospyrin on *L. donovani* topoisomerase I, we found that the compound, when added together with DNA and enzyme, inhibited relaxation at 1 μg/ml and more strongly at higher concentrations (Fig. 2A, *lanes 1–6*). *Lane 8* shows the relaxation of supercoiled pGEM4Z (*lane 7*) by 2 units of purified topoisomerase I of *L. donovani*. Complete inhibition of DNA relaxation activity takes place at 15 μg/ml concentration of diospyrin (*lane 5*). Inhibition of enzyme activity is more predominant when the enzyme is preincubated with the compound for 5 min at 37° in the relaxation assay mixture before addition of the DNA substrate. Fig. 2B shows the inhibition of catalytic activity by diospyrin in the above reaction condition. *Lane 4* shows that the compound exerts 50% inhibition only at 0.5 μg/ml concentration. The inhibition by the compound occurs in a highly dose-dependent manner. Densitometric analysis of the agarose gel shows that when the enzyme, DNA, and diospyrin were added simultaneously in the standard relaxation assay mixture, 10% inhibition was found at 1 μg/ml concentration. However, at this concentration, 80% inhibi-

tion was found when the enzyme was preincubated with diospyrin before addition of the supercoiled DNA substrate (Fig. 2C).

Diospyrin by itself did not unwind DNA. To test this, supercoiled pGEM4Z was incubated with diospyrin at concentration up to 200 μg/ml without addition of topoisomerase I. No relaxation and no apparent DNA conformational changes take place because of the unwinding of DNA (data not shown).

Diospyrin is acting reversibly against the enzyme. Because diospyrin inhibits the catalytic activity of DNA topoisomerase I of *L. donovani*, the most important issue is to understand the mechanism of inhibition. It is not clear whether diospyrin is acting reversibly or irreversibly against the enzyme. This critical matter has been sorted out in two ways. In the first experiment, based on the dose response data shown in Fig. 2 one unit of enzyme was preincubated with three different concentrations of diospyrin for 5 min at 37°. After preincubations, supercoiled pGEM4Z DNA was added to each reactions and further incubated at 37° for different times. Fig. 3 shows the time course of inhibitions. Preincubations at noninhibitory (0.1 μg/ml) and inhibitory concentrations (1 and 10 μg/ml) of diospyrin followed by incubations with DNA for different times reveal that diospyrin-mediated inhibition of relaxation is relieved with time. It may be noted here that at 0 min, at all the three concentrations of diospyrin, there is no relaxation of supercoiled DNA (Fig. 3A, *lane 3*; Fig. 3B, *lanes 3* and *8*) as in 0 min the catalytic action of topoisomerase I on the supercoiled DNA substrate has not started. Hence incubation for 0 min is also another experimental control. At 0.1 μg/ml of the compound, there is hardly any inhibition of catalytic activity of topoisomerase I (Fig. 3A). At 1 and 10 μg/ml of the compound, the corresponding relief of inhibitions are 55% and 35%, respectively, after 30 min of incubations (Fig. 3, B and C). The nature of inhibition and its relief followed similar kinetics when diospyrin was used at inhibitory concentrations (Fig. 3C). In the second experiment, topoisomerase I was preincubated with 1 μg/ml of diospyrin, an inhibitory dose (Fig. 4, *lane 5*) as above, and then diluted by 2- and 5-fold, respectively (*lanes 6* and *7*). Drug control reactions showed the expected pattern of inhibition (*lanes 3* and *4*). Dilution from an inhibitory dose (1 μg/ml) to a noninhibitory dose (0.2 μg/ml) resulted in a relief of inhibition (*lane 7*). Taken together, these results indicate that diospyrin is acting reversibly against topoisomerase I.

Diospyrin is more effective on *L. donovani* topoisomerase I. To understand whether diospyrin exerts its specific inhibitory effect on type I DNA topoisomerase of *L. donovani*, relaxation reaction by calf thymus topoisomerase I and decatenation reaction by *L. donovani* topoisomerase II were carried out in presence of the compound. Fig. 5 shows the inhibition of relaxation of supercoiled plasmid pGEM4Z DNA by calf thymus topoisomerase I in presence of diospyrin. Diospyrin is more effective on *L. donovani* topoisomerase I, because the compound requires much higher concentration

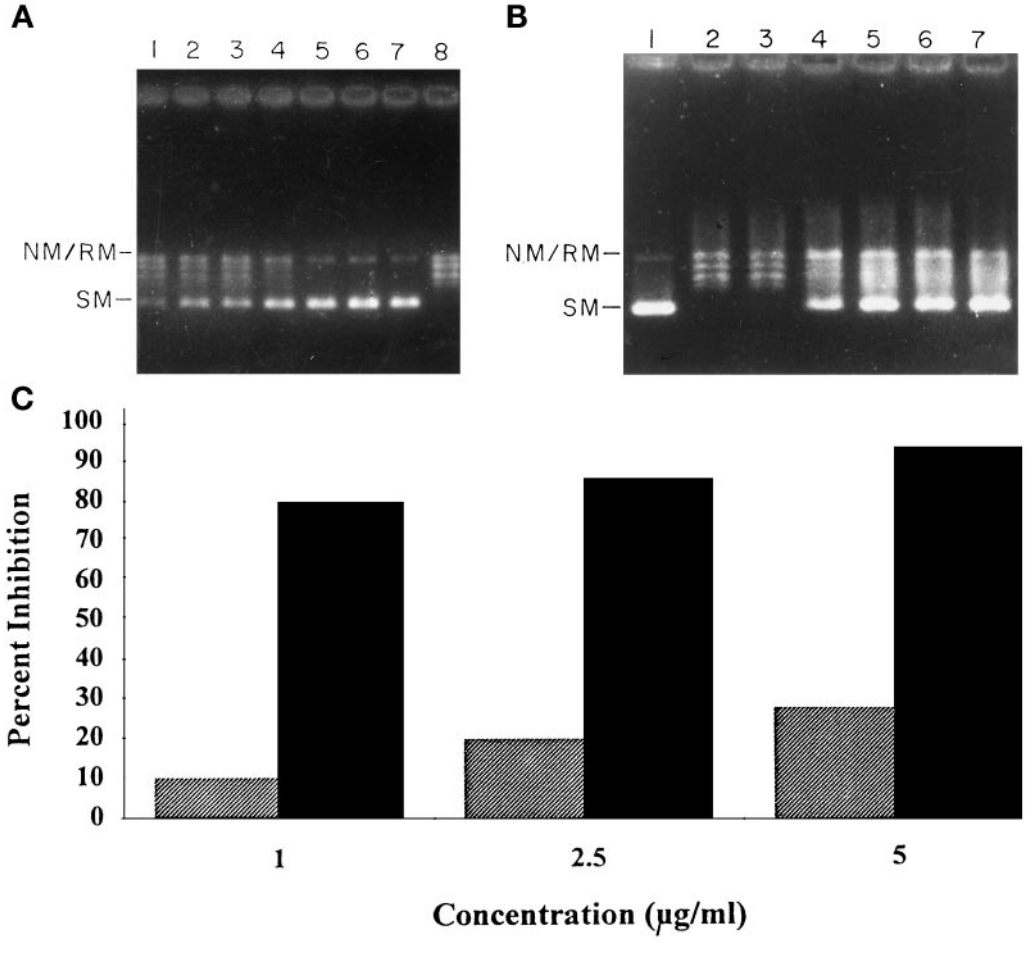


Fig. 2. A, Catalytic inhibition of *L. donovani* topoisomerase I by diospyrin. *Lanes 1–6*, inhibition of 2 units of *L. donovani* topoisomerase I in presence of 1, 2.5, 5, 10, 15, and 20 μg/ml of diospyrin added simultaneously with enzyme and DNA; *lane 7*, 0.5 μg of pGEM4Z DNA alone; *lane 8*, same as *lane 7* but with 2 units of *L. donovani* topoisomerase I. B, Preincubation of enzyme with diospyrin enhanced the inhibitory potency. *Lane 1*, supercoiled pGEM4Z; *lane 2*, DNA was added after preincubation of 2 units of *L. donovani* topoisomerase I with the reaction buffer for 5 min at 37°; *lanes 3–7*, same as lane 2, but the enzyme was preincubated with 0.1, 0.5, 1, 2.5, and 5 μg/ml of diospyrin at 37° for 5 min, before the addition of DNA substrate. Samples were electrophoresed in 1% agarose gel as described in Materials and Methods. Positions of supercoiled monomer (*SM*), nicked monomer (*NM*), and relaxed monomer (*RM*) are indicated. C, Densitometric scanning of inhibition of DNA topoisomerase I of *L. donovani* by diospyrin. ▨, Simultaneous addition of the enzyme, diospyrin, and DNA in the reaction mixture; ■, preincubation of enzyme and the compound.

(i.e., 50 μg/ml to significantly inhibit calf thymus topoisomerase I compared with the inhibition of *L. donovani* enzyme, which is achieved only at 5 μg/ml concentration.

Diospyrin does not inhibit type II topoisomerase. Diospyrin selectively inhibits topoisomerase I and does not inhibit topoisomerase II of *L. donovani*, as determined by decatenation of *L. donovani* kDNA, which contains a large network of interlocked, 830-bp catenated DNA circles, the minicircles. The decatenation assay is a highly specific assay for type II topoisomerases. Fig. 6, *lane 2*, shows the decatenation of kDNA (*lane 1*) by *L. donovani* topoisomerase II. *Lanes 3–6* refer to the decatenation in presence of 5, 25, 50, and 100 μg/ml of diospyrin; *lanes 7–10* refer to the decatenation of kDNA in presence of 3, 15, 30 and 75 μg/ml of etoposide. Etoposide is a known topoisomerase II inhibitor. It also inhibits decatenating activity of type II topoisomerase of *L. donovani* and does so with greater potency at higher concentrations. Diospyrin does not show any inhibition of topoisomerase II of *L. donovani* at concentrations up to 50 μg/ml, which is 10 times higher than the concentration that inhibited topoisomerase I of *L. donovani*. Preincubation of topoisomerase II with diospyrin is also without effect on the decatenating activity of the enzyme (data not shown).

Diospyrin induces topoisomerase I mediated DNA cleavage. Topoisomerase I introduces a single-strand nick in the phosphodiester bond of the DNA, allows an intact strand to pass through the nick, and then rejoins the nicked strand of the DNA. A covalent bond is formed between the 3′-OH group of the DNA backbone and the tyrosine group at the active site of topoisomerase I. This covalent enzyme DNA complex is the putative reaction intermediate termed the 'cleavable complex,' which can be detected when the reaction is terminated with a strong detergent such as SDS or alkali. Camptothecin is an established antitumor drug and a well-characterized inhibitor of eukaryotic topoisomerase I. Camptothecin has been shown to stabilize the cleavable complex, which is probably the mechanism of its enzymatic inhibition (Slichenmeyer *et al*., 1993). To understand the mechanism of diospyrin inhibition, we investigated the effect of diospyrin on the cleavable complex formation between the *L. donovani* topoisomerase I and pHOT1 DNA. With increasing concentrations of camptothecin, closed circular pHOT1 DNA (form I) was converted to nicked circular DNA (form II). Fig. 7A, *lanes 3–6*, show the cleavage of supercoiled pHOT1 DNA (*lane 1*) in presence of 80 units of *L. donovani* topoisomerase I (*lane 2*) and the same amount of enzyme plus 5, 10, 20, and 30 μM camptothecin. When the cleavage reaction was carried out with increasing concentrations of diospyrin, closed circular pHOT1 DNA was also converted to nicked circular DNA. Fig. 7B, *lanes 5–7*, shows the cleavage of supercoiled pHOT1 DNA (*lane 1*) in presence of 80 units of topoisomerase I (*lane 3*), and the same amount of enzyme plus 5, 10, and 20 μg/ml of diospyrin. The gradual increase of nicked form II DNA (*lanes 5–7*) indicates that diospyrin stabilizes the "cleavable complex" in a dose dependent manner. Lane 4 is same as lane 3 but in presence of 10 μM camptothecin. Lane 2 shows relaxation of pHOT1 DNA with 80 units of topoisomerase I, but without SDS and proteinase K treatment. There is a marked increase in the formation of nicked product when the covalent complex was trapped with SDS and proteinase K (*lane 3*) compared with untrapped reaction (*lane 2*) and control DNA (*lane 1*). In this experiment, ethidium bromide (0.5 μg/ml) was included in the gel and electrophoresis buffer to resolve the more slowly migrating nicked product (form II)

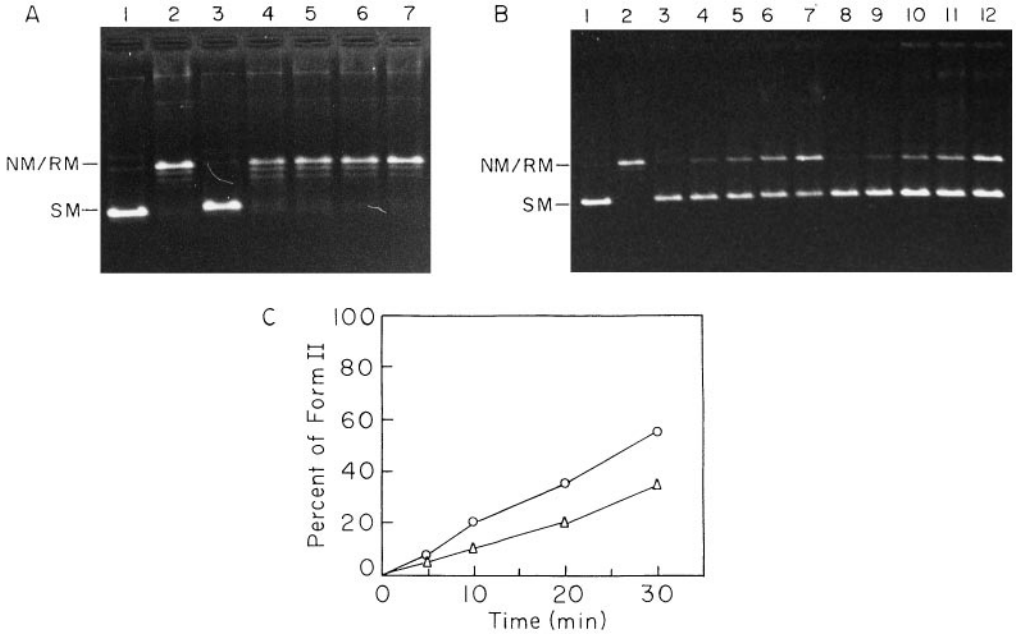


Fig. 3. Reversible inhibition of topoisomerase I-dependent DNA relaxation as examined by time kinetics. The experimental protocol used is described in the text in Materials and Methods. A, One unit of topoisomerase I was preincubated with 0.1 μg/ml of diospyrin for 5 min at 37°. After preincubations, 0.5 μg of supercoiled pGEM4Z DNA was added to the reaction and further incubated for different times. *Lanes 3–7*, relaxation of supercoiled DNA (*lane 1*) at 0, 5, 10, 20, and 30 min respectively; *lane 2*, enzyme control. Positions of supercoiled monomer (*SM*), nicked monomer (*NM*), and relaxed monomer (*RM*) are indicated. B, Reactions were carried out as in A but with 1 and 10 μg/ml of diospyrin. *Lanes 3–7*, relaxation of supercoiled DNA (*lane 1*) at 0, 5, 10, 20, and 30 min, respectively, in presence of 1 μg/ml; *lanes 8–12*, same as *lanes 3–7*, but in presence of 10 μg/ml diospyrin; *lane 2*, control relaxation. C, Percent of Form II (*NM*/*RM*) in each lane were measured by microdensitometry of negative photographs of nicked or relaxed monomer DNA bands fluorescence in comparison to the supercoiled monomer bands as shown in B. ○, 1 μg/ml diospyrin; △, 10 μg/ml diospyrin.

from covalently closed relaxed molecules (form I^r). These results suggest that diospyrin exerts its inhibitory action on *L. donovani* topoisomerase I by a different mechanism. Unlike camptothecin, there is a direct interaction between diospyrin and the enzyme; like camptothecin, however, it stabilizes the topoisomerase I-DNA covalent binary complex.

Discussion

The bisnaphthoquinone compound diospyrin and its derivatives have been found to have antitumor properties against Ehrlich ascites carcinoma (Hazra *et al*., 1984) and sarcoma 180 (Hazra *et al*., 1994) and exhibit antiprotozoal activities toward *L. donovani*, *T. brucei*, *T. cruzi* (Yardley *et al*., 1996) and *P. falciparum in vitro* (Hazra *et al*., 1995). Our studies suggest that diospyrin exerts its action by interacting with type I DNA topoisomerase of leishmania and stabilizing the "cleavable complex". The inhibition by diospyrin is relatively specific as the compound requires 10-fold higher concentrations to inhibit DNA topoisomerase I from calf thymus and it does not inhibit DNA topoisomerase II of *L. donovani* at this concentration.

Diospyrin is similar to β-lapachone, a naphthoquinone and also a novel inhibitor of type I DNA topoisomerase (Li *et al*., 1993) with respect to its binding with the enzyme and inhibition of catalytic activity of the enzyme. However, unlike β-lapachone, it also induces the formation of a stable cleavable complex.

In contrast with topoisomerase II, there are few specific inhibitors of topoisomerase I-mediated DNA relaxation (Fang *et al*., 1993); camptothecins are the only agents that are known to specifically stabilize the topoisomerase I-DNA covalent binary complex without binding to the enzyme or DNA alone (Hsiang *et al*., 1985). Camptothecin binds to a site that is created when topoisomerase I is covalently bound to specific sequences of DNA.

Recently developed topoisomerase inhibitors are either natural products or are derivatives of natural products; several of these compounds are effective antitumor and antibacterial agents (Drlica and Franco, 1988; D'Arpa and Liu, 1989). Consequently, there is a lot of interest in discovering novel topoisomerase poisons from natural sources as potential lead compounds for drug development. Therefore, as a part of a continuing search for novel topoisomerase I inhibitors, we investigated diospyrin, which has significant inhibitory effect on the growth of *L. donovani* promastigotes. Our results suggest that diospyrin is a novel inhibitor of type I DNA topoisomerase of *L. donovani*. Like camptothecin, a class I inhibitor, it also stabilizes the cleavable complex. However it differs from camptothecin with respect to its mode of action. Camptothecin does not bind with the enzyme alone, but diospyrin does; this interaction is reversible. Topoisomerase inhibitors fall into two general classes: those that stimulate formation of enzyme-DNA covalent complexes, termed as topoisomerase poisons, and those that interfere with this covalent intermediate (Wang, 1994). The experimental results suggest that the compound may belong to the former class. Simultaneous incubation of large molar excess of enzyme, DNA, and diospyrin at inhibitory concentrations (5 μg/ml and above) leads to stabilization of enzyme-DNA covalent complex. Therefore diospyrin is a novel "topoisomerase I poison." Plant metabolites like plumbagin and shikonin (Fujii *et al*., 1992), which have naphthoquinone structure, induce mammalian topoisomerase II-mediated DNA cleavage *in vitro*. On the other hand, lawson and lapachol, which also have naphthoquinone moieties, could not

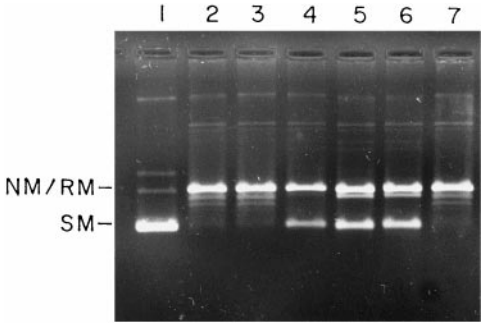


Fig. 4. Reversible inhibition of topoisomerase I-dependent DNA relaxation as examined by preincubation and dilution. The experimental protocol used is described in the text and under Materials and Methods. *Lane 1*, 0.5 μg of pGEM4Z DNA incubated alone; *lane 2*, in presence of 1 unit of topoisomerase I; *lanes 3* and *4*, same as *lane 2*, but in presence of 0.2 and 5 μg/ml of diospyrin added simultaneously with enzyme and DNA. One unit of topoisomerase I was preincubated with 1 μg/ml of diospyrin for 5 min at 37°, then diluted 0-, 2- and 5-fold, while maintaining or diluting final drug concentrations to 1 μg/ml (*lane 5*), 0.5 μg/ml (*lane 6*) and 0.2 μg/ml (*lane 7*). Reactions were carried out at 37° for 15 min after addition of 20 μg/ml of supercoiled pGEM4Z DNA as substrate. Positions of supercoiled monomer (*SM*), nicked monomer (*NM*), and relaxed monomer (*RM*) are indicated.

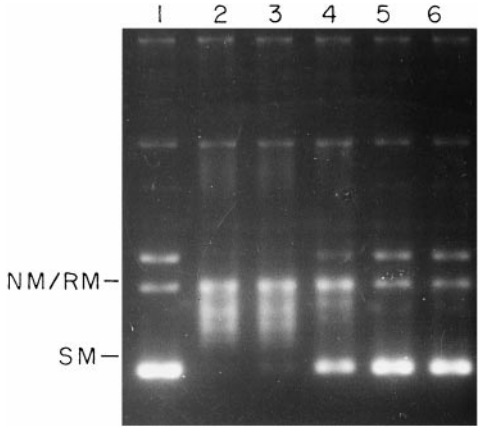


Fig. 5. Effect of diospyrin on calf thymus topoisomerase I. Supercoiled pBR322 DNA was incubated alone (*lane 1*), in the presence of 2 units of calf thymus topoisomerase I (*lane 2*) or in the presence of 2 units of enzyme plus 20, 50, 100, and 200 μg/ml of diospyrin (*lanes 3–6*). Electrophoresis was carried out as described in Materials and Methods. Positions of supercoiled monomer (*SM*), nicked monomer (*NM*), and relaxed monomer (*RM*) are indicated.

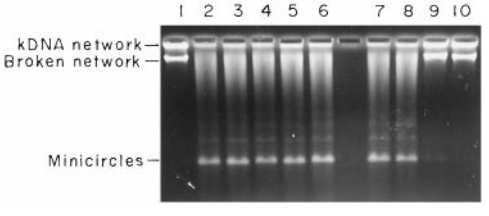


Fig. 6. Effect of diospyrin on DNA topoisomerase II of *L. donovani*. *Lane 1*, 0.5 μg of kDNA network; *lane 2*, plus 2 units of *L. donovani* topoisomerase II in the decatenating assay mixture containing 2 mM ATP; *lanes 3–6*, same as *lane 2*, but in presence of 5, 25, 50, and 100 μg/ml of diospyrin; *lanes 7–10*, same as *lane 2*, but in presence of 3, 15, 30, and 75 μg/ml of etoposide. Electrophoresis was carried out as described in Materials and Methods.

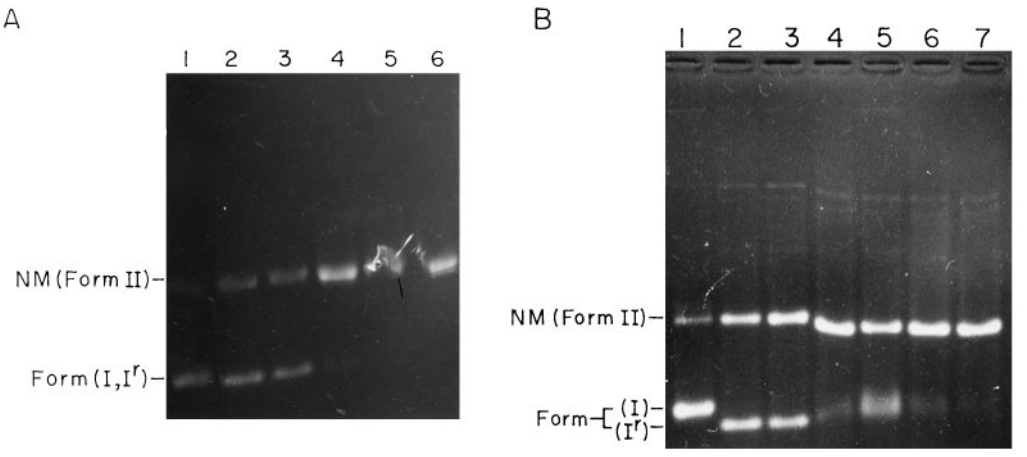


Fig. 7. A, Cleavage of pHOT1 DNA in presence of *L. donovani* topoisomerase I and camptothecin. Cleavage reaction and electrophoresis in agarose gel were carried out as described in Materials and Methods. *Lane 1*, 0.25 μg of supercoiled pHOT1 DNA; *lane 2*, plus 80 units of *L. donovani* topoisomerase I; *lanes 3–6*, same as *lane 2*, but in presence of 5, 10, 20, and 30 μM camptothecin. B, Effect of diospyrin on the trapping of nicked DNA intermediates in presence of *L. donovani* topoisomerase I. *Lane 1*, 0.25 μg of pHOT1 DNA; *lane 2*, plus 80 units of *L. donovani* topoisomerase I but without SDS and proteinase K treatment; *lane 3*, same as lane 2, but SDS- and proteinase K-treated; *lane 4*, same as *lane 3*, but in presence of 10 μM camptothecin; *lanes 5–7*, same as *lane 3*, but in presence of 5, 10, and 20 μg/ml of diospyrin. Cleavage reactions and electrophoresis were carried out as described in Materials and Methods.

induce topoisomerase II mediated DNA cleavage (Fujii *et al*., 1992). This is the first example of a bisnaphthoquinone compound acting as a topoisomerase I inhibitor. The finding that *L. donovani* topoisomerase I is more susceptible to this compound than other eukaryotic topoisomerases may be exploited in developing rational approaches to chemotherapy of Leishmaniasis.

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