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Isodiospyrin as a novel human DNA topoisomerase I inhibitor

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Abstract

Isodiospyrin is a natural product from the plant *Diospyros morrisiana*, which consists of an asymmetrical 1,2-binaphthoquinone chromophore. Isodiospyrin exhibits cytotoxic activity to tumor cell lines but very little is known about its cellular target and mechanism of action. Unlike the prototypic human topoisomerase I (htopo I) poison camptothecin, isodiospyrin does not induce htopo I–DNA covalent complexes. However, isodiospyrin antagonizes camptothecin-induced, htopo I-mediated DNA cleavage. Binding analysis indicated that isodiospyrin binds htopo I but not DNA. These results suggest that isodiospyrin inhibits htopo I by direct binding to htopo I, which limits htopo I access to the DNA substrate. Furthermore, isodiospyrin exhibits strong inhibitory effect on the kinase activity of htopo I toward splicing factor 2/alternate splicing factor in the absence of DNA. Thus, these findings have important implications on naphthoquinone and its derivatives' cellular mode of actions, i.e. these novel DNA topoisomerase I inhibitors can prevent both DNA relaxation and kinase activities of htopo I.
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Keywords: Isodiospyrin; Camptothecin; Human DNA topoisomerase I; *Diospyros morrisiana*; Kinase; Binaphthoquinone

1. Introduction

DNA topoisomerases which catalyze the interconversions of various topological states of DNA were originally discovered as activities that change the superhelical structure of closed circular DNAs [1]. Depending on the nature of the reactants and reaction conditions, topoisomerases can catalyze DNA relaxation/supercoiling, catenation/decatenation and knotting/unknotting reactions [1]. Based on their functional mechanisms, DNA topoisomerases have been classified into two types. Type I DNA topoisomerases break and rejoin only one of the two strands during catalysis, while type II DNA topoisomerases break and rejoin both strands for each DNA strand-passing reaction. Studies in eukaryotes have shown topoisomerase I to be associated with actively transcribed genes [2,3]: whereas, topoisomerase II is required for DNA replication and for successful traverse of mitosis [4–7]. Furthermore, studies have suggested a role for topoisomerases in the control of template supercoiling during RNA transcription [8,9]. Through these two fundamentally different mechanisms, DNA topoisomerases modify the topological states of DNA which facilitate various DNA transactions such as DNA replication, RNA transcription, recombination, chromosome condensation/decondensation, and chromosome segregation.

Perhaps more importantly, studies have identified several human DNA topoisomerases as important therapeutic targets in cancer chemotherapy [10–12]. htopo I is a molecular target of the camptothecin (CPT) class of antitumor drugs (e.g. topotecan and irinotecan) while human

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Abbreviations: htopo I, human topoisomerase I; CPT, camptothecin; m-AMSA, (*N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulphonanilide); SRPK1, SR protein kinase-1; ATP, adenosine triphosphate; SF2/ASF, splicing factor 2/alternate splicing factor.

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DNA topoisomerase II (htopo II) is a molecular target of a number of clinically useful antitumor drugs such as etoposide (VP-16), doxorubicin, mitoxantrone and (*N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulphonanilide) (m-AMSA) [12–17]. Other compounds such as saintopin, intoplicine, indoloquinolinedione derivatives, β-lapachone, and related naphthoquinones, have been shown to target both htopo I and htopo II [18–23].

Although previous works have suggested that htopo I does not require a nucleotide cofactor or any other energy source to relax supercoiled DNA, a recent study have demonstrated that htopo I exhibit an adenosine triphosphate (ATP) binding activity which is associated with altered conformation of the enzyme [24]. htopo I possesses an intrinsic protein kinase activity that phosphorylates members of the SR protein family [25]. For these reasons, htopo I is considered to act as a dual functional enzyme.

Isodiospyrin (Fig. 1), isolated from the root of *Diospyros morrisiana* (a relative of persimmon), exhibits cytotoxic activity against tumor cell lines [26,27] and antibacterial activity [28]. Previous study has shown that isodiospyrin exhibits significant cytotoxicity against the HCT-8 colon tumor, P-388 lymphocytic leukemia [26], HEPA-3B hepatoma, KB nasopharynx carcinoma, CLOL-205 colon carcinoma and HeLa cervical carcinoma [27]. Since isodiospyrin consists of an asymmetrical 1,2-binaphthoquinone chromophore like diospyrin which belongs to a novel class of DNA topoisomerase poisons [29], we have initiated a series of experiments to test the hypothesis that isodiospyrin is an inhibitor of htopo I.

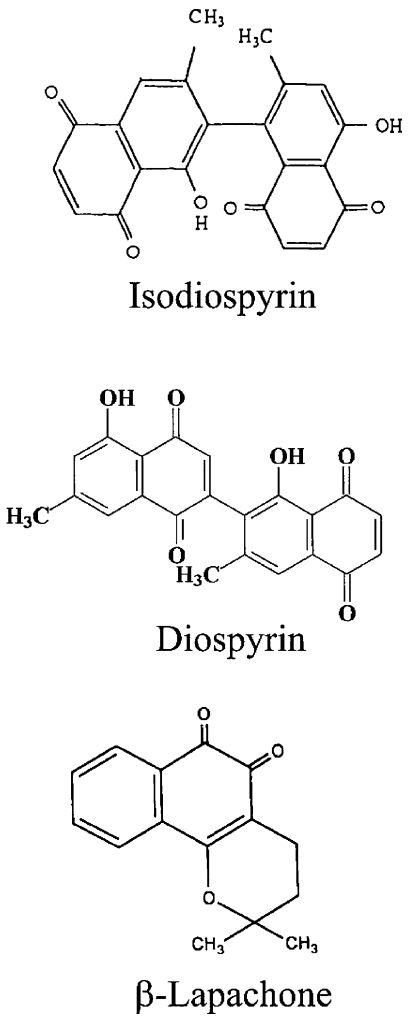


Fig. 1. The chemical structure of isodiospyrin and other naphthoquinone based DNA topoisomerase inhibitors.

2. Materials and methods

2.1. Materials

Reagents for electrophoresis were obtained from Bio-Rad. Camptothecin and most other chemicals were purchased from Sigma. Isodiospyrin, a natural product possessing (−)-*R* configuration, was isolated from *D. morrisiana* as described [26]. Proteinase K and DMSO were purchased from Merck. Recombinant human DNA topoisomerase I was obtained using the baculovirus expression system as described [30]. Restriction enzymes, Klenow polymerase, T4 DNA ligase and pGEM-5Z plasmid were purchased from Promega. [*methyl*-^{3}H]Thymidine and [α-^{32}P]dATP were obtained from Amersham. Nitrocellulose membrane was purchased from Schleicher & Schuell Inc., 0.45 μm pore size. GST-splicing factor 2/alternate splicing factor (GST-SF2/ASF) and GST-SR protein kinase-1 (GST-SRPK1) protein were expressed in *Escherichia coli* and purified by a GST affinity column [31].

2.2. Plasmid DNA relaxation by human DNA topoisomerase I

The relaxation activity of htopo I was measured using supercoiled plasmid DNA pGEM-5Z [32]. Briefly, each reaction mixture had a total volume of 10 μL containing 1 U of htopo I (approximately 3 ng), 0.2 μg supercoiled DNA, 50 mM Tris–HCl, pH 7.5, 60 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 50 μg/mL BSA, and varying amount of isodiospyrin or CPT in 5% DMSO. The reaction mixture was incubated at 37° for 30 min, terminated by adding 1% SDS, and separated on a 0.8% agarose gel. One unit of htopo I activity was defined as the amount of enzyme necessary to completely relax 0.2 μg of supercoiled DNA in 30 min at 37°.

2.3. 3′-End labeling of EcoRI-digested pGEM-5Z DNA

3′-End labeling was performed as described [24]. Briefly, pGEM-5Z DNA was digested with the *Eco*RI restriction endonuclease and then labeled with [α-^{32}P]dATP using the large fragment of *E. coli* DNA polymerase I (Klenow polymerase) and unlabeled dTTP at 15° for 1 hr. Unincorporated triphosphates were removed by two cycles of

ethanol precipitation in the presence of 2 M ammonium acetate.

2.4. Topoisomerase I-mediated DNA cleavage assay

htopo I-mediated DNA cleavage assay was performed as described previously [24]. Each reaction mixture (20 μL each) containing 40 mM Tris–HCl (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/mL acetylated BSA, 5000 cpm of 3′-end labeled pGEM-5Z DNA, 10 ng of recombinant htopo I, and various concentrations of drugs were incubated at 37° for 30 min. The reaction was terminated by adding 2 μL of 5% SDS and 1.5 mg/mL proteinase K for 1 hr at 37°. Following the addition of sucrose (final concentration, 5%) and bromphenol blue (final concentration, 0.05 mg/mL), DNA samples were heat-denatured (94° for 5 min) and electrophoresed in a 5% urea–polyacrylamide gel with TBE buffer. After electrophoresis, gel was dried onto Whatman 3 MM chromatographic paper and autoradiographed at −80° using Kodak XAR-5 films.

2.5. Isodiospyrin–DNA interactions

Interactions between isodiospyrin and DNA were assessed by two independent techniques. First, the ability of the drug to intercalate into plasmid DNA was determined by unwinding assay [32]. Briefly, plasmid pGEM-5Z (0.5 μg) was linearized with *Eco*RI restriction endonuclease and then ligated with 1 U of T4 DNA ligase in the presence of different drugs. The ligation reaction (20 μL each) was performed under conditions identical to those described for topoisomerase cleavage assays except that 1 mM ATP was added. The reactions were terminated by the addition of 5 μL of a pre-warmed termination solution (5% sarkosyl, 25% sucrose, 50 mM EDTA, and 0.05 mg/mL bromphenol blue). Gel electrophoresis was performed in the cold room (4°) using a 1.0% agarose gel in TBE electrophoresis buffer supplemented with 5 mM $MgCl_2$. The DNA bands were stained with 0.5 μg/mL of ethidium bromide and visualized by UV light. Second, an ethidium-displacement fluorescence assay [33,34] was employed to determine whether isodiospyrin binds DNA. Fluorescence emission spectra ($\lambda_{max} = 600$ nm, excitation wavelength 546 nm) were obtained at 25° on a Hitachi F-4010 fluorescence spectrophotometer. The assays contained 1 μM ethidium bromide, 0–30 μM isodiospyrin or m-AMSA and 100 nM salmon sperm DNA in 2 mL of fluorescence buffer.

2.6. In vitro K-SDS co-precipitation assay

In vitro K-SDS co-precipitation assay was performed as described previously [16]. Briefly, reaction mixtures (50 μL each) containing 5000 cpm of 3′-end labeled pGEM-5Z DNA, 30 ng of recombinant htopo I, and various concentrations of drugs in reaction buffer (40 mM Tris–HCl (pH 7.5). 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/mL acetylated BSA) were incubated at 37° for 30 min. The reactions were terminated by addition of 100 μL of a pre-warmed (37°) denaturing solution (0.2 M NaOH, 2% SDS, 5 mM EDTA, 0.5 mg/mL salmon sperm DNA) and further incubated for 10 min at 37°. The covalent enzyme–DNA complexes were precipitated by the addition of 50 μL of a neutralization solution (0.25 M KCl, 0.4 M HCl, 0.4 M Tris–HCl, pH 7.9). Precipitation was allowed to occur at 0° for 10 min. The precipitate was collected by centrifugation (15 min at 4°) in a microcentrifuge and washed once by heating to 65° for 10 min in 200 μL of a wash buffer (10 mM Tris, pH 7.9, 100 mM KCl, 1 mM EDTA, 100 μg/mL salmon sperm DNA, and 50 μg/mL BSA). After cooling on ice and re-centrifugation, the pellet was suspended in 200 μL of H_2O, dissolved by heating to 65°, and transfer to a vial containing 4 mL of a scintillation cocktail for determination of radioactivity.

2.7. Filter binding assay

Filter binding assay was performed as described previously [24]. Briefly, 3′-end labeled pGEM-5Z DNA (10,000 cpm, approximately 2 ng) and human DNA topoisomerase I (50 ng) was mixed in 50 μL of binding buffer (10 mM Tris–HCl, pH 7.5, 20 μg/mL BSA, and 0.1 mM EDTA) with or without isodiospyrin and incubated for 10 min at 25°, then filtered through a nitrocellulose membrane (Schleicher & Schuell Inc., 0.45 μm pore size). The trapped DNA–protein complexes were washed with 3 mL of binding buffer. The bound radioactivity was measured by liquid scintillation counter. The data were plotted after subtraction of background from control samples prepared in the absence of DNA topoisomerase I.

2.8. Kinase assay

The reaction mixtures for protein kinase activity contained 100 ng of recombinant htopo I or GST-SRPK1, 300 ng of recombinant SF2/ASF protein, 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and isodiospyrin or DMSO (as a solvent control) in 10 μL reaction buffer (50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, and 0.5 mM DTT). The samples were incubated at 30° for 30 min and then mixed with 6 μL of 4× sample buffer and applied to a 10% SDS–polyacrylamide gel. Radioactivity incorporated into SF2/ASF on the dried gel was detected by autoradiography.

2.9. Photocrosslinking of ATP to human DNA topoisomerase I

Photocrosslinking of ATP to htopo I was performed as described previously [24]. Briefly, the reaction mixtures for

photochemical cross-linking contained 100 ng of htopo I, 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol) and isodiospyrin (or DMSO as a solvent control) in 10 μL of 20 mM HEPES (pH 7.0). The reaction mixtures (in 1.5 mL microfuge tubes) were incubated at room temperature for 5 min in the dark and then irradiated with a transilluminator UV Stratalinker-2400 (75 W/cm^2) placed 10 cm beneath the reaction tubes. The irradiated samples were then mixed with 6 μL of 4× SDS sample buffer and analyzed by electrophoresis in a 6% SDS–polyacrylamide gel. Radioactivity incorporated into htopo I was detected by autoradiography.

2.10. UV-Vis spectroscopy

Absorption spectra were collected using a Beckman DU-600 spectrophotometer. The effect of glutathione on the absorbance spectrum of isodiospyrin was measured (250 nm × 700 nm) by adding glutathione (100 μM final) to a buffer solution containing the isodiospyrin (100 μM in 100 mM sodium phosphate, pH 7.4, containing 20% DMSO) in a quartz cuvette. The spectrum of isodiospyrin alone was recorded before reaction, and the reaction was monitored at incubation times of 5, 10, 20 min. The spectrophotometer was blanked on the sodium phosphate buffer pH 7.4 containing 20% of DMSO.

3. Results

3.1. Isodiospyrin inhibits the DNA relaxation activity of human DNA topoisomerase I

CPT, a well-known htopo I inhibitor, was employed as a positive control (Fig. 2A). Inhibition of the DNA relaxation activity of htopo I by CPT was concentration dependent and inhibition of DNA relaxation was undetectable at CPT concentrations below 10 μM. Like CPT and naphthoquinone derivatives (e.g. β-lapachone), isodiospyrin strongly inhibited the DNA relaxation activity of htopo I. As shown in Fig. 2A (middle panel), isodiospyrin at the concentration of 40 μM completely inhibited the relaxation activity of htopo I, while CPT at the same concentration inhibited it only partially. When htopo I was pre-incubated with isodiospyrin prior to the addition of DNA, isodiospyrin exhibited a more prominent inhibitory effect on the DNA relaxation. As shown in Fig. 2A (lower panel), isodiospyrin at the concentration of 20 μM completely inhibited the DNA relaxation activity of htopo I when isodiospyrin was pre-incubated with htopo I for 5 min at 37°. These results suggest that isodiospyrin is a more potent inhibitor of htopo I than CPT.

Two different types of assays (DNA relaxation and DNA cleavage) were used to determine whether isodiospyrin and CPT share the same mechanism of inhibition. In the DNA

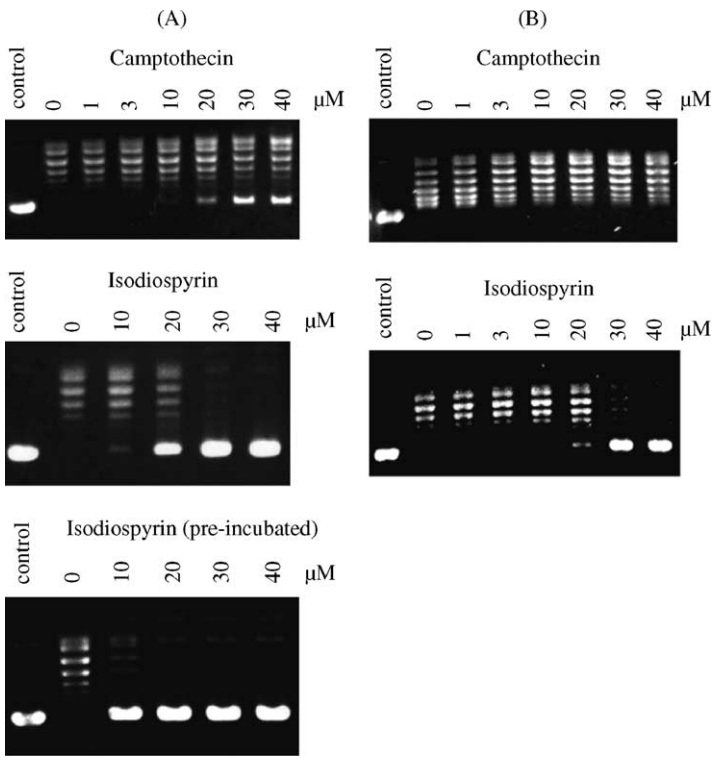


Fig. 2. Isodiospyrin inhibits the catalytic activity of human DNA topoisomerase I. (Panel A) DNA relaxation assay was performed in the presence of CPT (upper panel). Lane 1 (control), 0.2 μg plasmid pGEM-5Z with no enzyme added; lanes 2–7 (0–40 μM), plasmid pGEM-5Z with 1 U of htopo I in the presence of various amounts of CPT as indicated. The inhibition of the catalytic activity of htopo I by isodiospyrin was analyzed by incubating 1 U (3 ng) of htopo I, 0.2 μg plasmid pGEM-5Z and isodiospyrin (middle panel). Pre-incubation of enzyme with isodiospyrin enhanced the inhibitory potency (lower panel). DNA was added after pre-incubation of 1 U (3 ng) of htopo I with isodiospyrin for 5 min at 37°. (Panel B) Inhibition of the catalytic activity of CPT-resistant htopo I (CPT$^{®}$-2000) by CPT (upper panel) and isodiospyrin (lower panel). All reaction samples were electrophoresed in 0.8% agarose gel as described in Section 2.

relaxation assay, isodiospyrin was shown to inhibit CPT-resistant htopo I (CPT®-2000) [35] (Fig. 2B), suggesting that isodiospyrin may inhibit htopo I by a mechanism distinct from that of CPT. In the DNA cleavage assay, isodiospyrin, unlike CPT, did not induce covalent htopo I–DNA complexes (Fig. 4A), further suggesting different modes of htopo I inhibition by these two agents.

3.2. Isodiospyrin exhibits no detectable DNA binding via the intercalative mode

A growing list of compounds (e.g. nitidines, saintopin, morpholinyl doxorubicin, aclacinomycin A, indeno[1,2-*c*]isoquinolines, nogalamycin, actinomycin D and protoberberines) have been identified to be htopo I poisons [21,36–42]. Most if not all of these htopo I poisons are DNA intercalators, suggesting that the intercalative mode of DNA binding may underlie a major htopo I poisoning mechanism [37,40,41,43]. Unwinding of the double strands of the DNA helix is a hallmark feature of DNA intercalators such as chloroquine, ethidium bromide, and m-AMSA. To clarify whether isodiospyrin exerts its inhibitory activity on htopo I by intercalative binding to DNA, plasmid pGEM-5Z was linearized with *Eco*RI and then ligated with T4 DNA ligase in the presence of increasing concentrations of isodiospyrin. The drug was removed, and the DNA products were examined by agarose gel electrophoresis. Intercalation of isodiospyrin into DNA would be expected to unwind the DNA helix resulting in underwound (negatively supercoiled) circular products, CPT and ethidium bromide were used as negative and positive controls, respectively [13]. As shown in Fig. 3, DNA unwinding by isodiospyrin, similar to CPT, was undetectable even at a concentration of 20 μM. In contrast, ethidium bromide was shown to unwind DNA even at a concentration as low as 0.8 μM (Fig. 3A, notice the asterisk). This result indicates that isodiospyrin does not exhibit any significant DNA binding activity through the intercalative mode.

Displacement of ethidium bromide from DNA with concomitant reduction in ethidium fluorescence was used as a second approach to examine whether isodiospyrin binds to DNA. As shown in Fig. 3B, an increasing concentration of the intercalator m-AMSA displaced the ethidium bromide from DNA causing a marked reduction in fluorescence. However, addition of isodiospyrin had little or no effect on ethidium fluorescence. It appears from both the DNA unwinding and ethidium displacement experiments that isodiospyrin does not intercalate into DNA.

3.3. Isodiospyrin is not a human DNA topoisomerase I poison

Topoisomerase poisons such as CPT are characterized by their ability to induce topoisomerase I–DNA covalent cleavage/cleavable complexes. To test whether isodiospyrin is a topoisomerase I poison, the ability of isodiospyrin to induce htopo I-mediated DNA cleavage was investigated. In this study, CPT was used as a positive control. As shown in Fig. 4A, the induction of htopo I-mediated DNA cleavage was detectable when the concentration of CPT was 0.2 μM. DNA cleavage became successively more prominent when higher concentrations of CPT were used in the DNA cleavage assay. In contrast to CPT, isodiospyrin had no effect on htopo I-mediated DNA cleavage even at a concentration of isodiospyrin as high as 125 μM (Fig. 4A, see appropriately labeled lanes).

The effect of isodiospyrin on the formation of protein–DNA covalent complexes was also examined by an *in vitro* K-SDS co-precipitation assay. As shown in Fig. 4B, isodiospyrin did not trap any significant amount of htopo I–DNA covalent complexes, while CPT was highly effective in trapping htopo I–DNA covalent complexes even at 0.2 μM. Furthermore, when htopo I was incubated with isodiospyrin and CPT at the same time, isodiospyrin completely abolished CPT-induced htopo I–DNA covalent complexes (Fig. 4C). A partial inhibition of these complexes was observed when CPT was added 2 and 4 min prior to isodiospyrin addition (Fig. 4C). The longer delay of isodiospyrin addition resulted in less inhibition of CPT-induced htopo I–DNA covalent complexes. These results suggest that binding of isodiospyrin to htopo I blocks htopo I binding to DNA, htopo I binding to CPT, or both. The data add to the understanding that isodiospyrin is a htopo I inhibitor rather than a poison.

3.4. Isodiospyrin inhibits the DNA binding activity of human DNA topoisomerase I

Our results have suggested that isodiospyrin could bind to htopo I, which results in abolition of CPT-induced htopo I–DNA covalent complex formation (Fig. 4C). To examine how isodiospyrin interferes with these complexes, the DNA binding activity of htopo I was measured by the filter binding assay in the presence of various concentrations of isodiospyrin. In the filter binding assay, DNA–protein complexes (but not DNA) are retained by a nitrocellulose membrane and can be quantitated by using a radiolabeled DNA. A prominent decrease in DNA–htopo I binding activity was observed when the htopo I–DNA solution was incubated with isodiospyrin at concentrations up to 20 μM (Fig. 5). The gel retardation assay was also performed and the result was consistent with that of the filter binding assay (data not shown). These results suggest that the inhibitory effect of isodiospyrin on the htopo I DNA relaxation activity may be due to an interference of isodiospyrin on the DNA binding activity of htopo I.

3.5. Isodiospyrin inhibits the kinase activity of human DNA topoisomerase I

Both CPT and NB-506 (an indolocarbazole) are htopo I poisons. However, unlike CPT, NB-506 inhibits htopo I

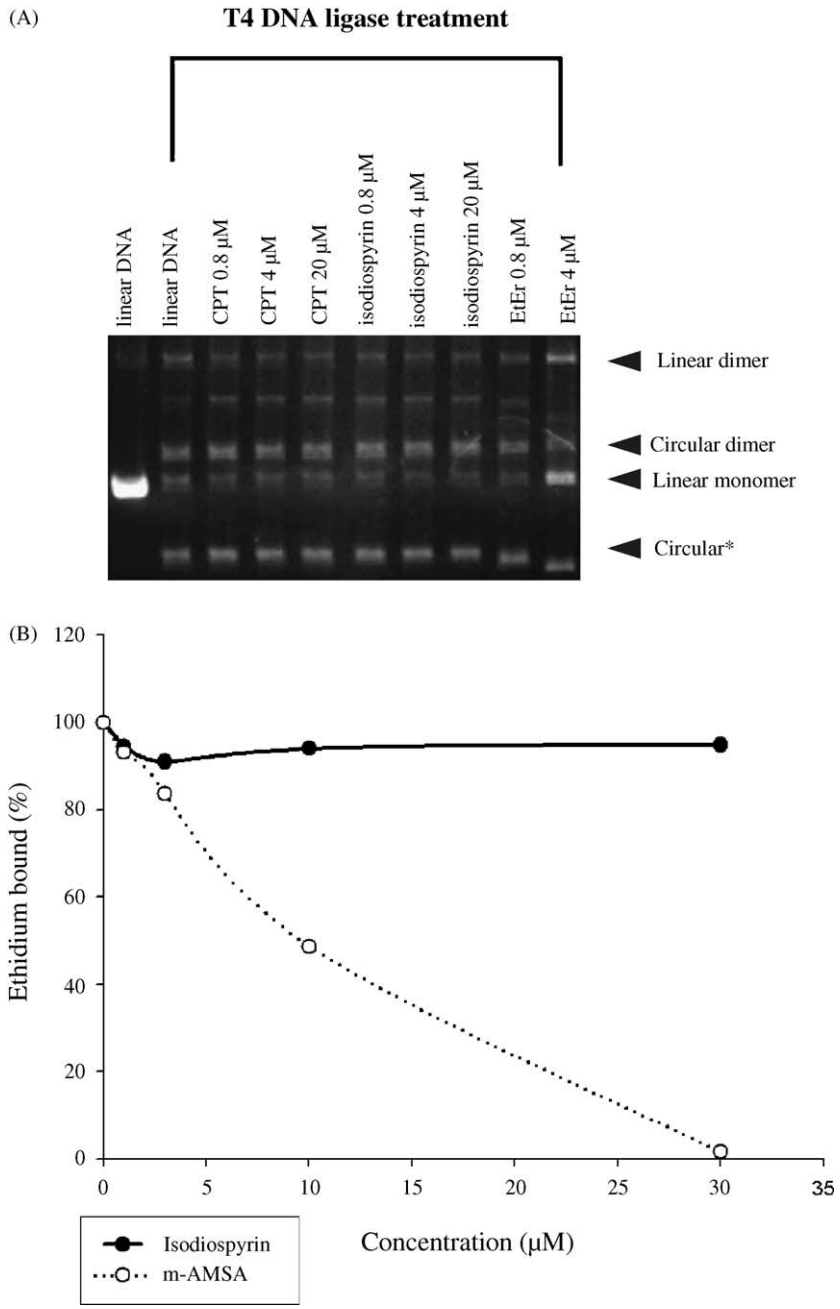
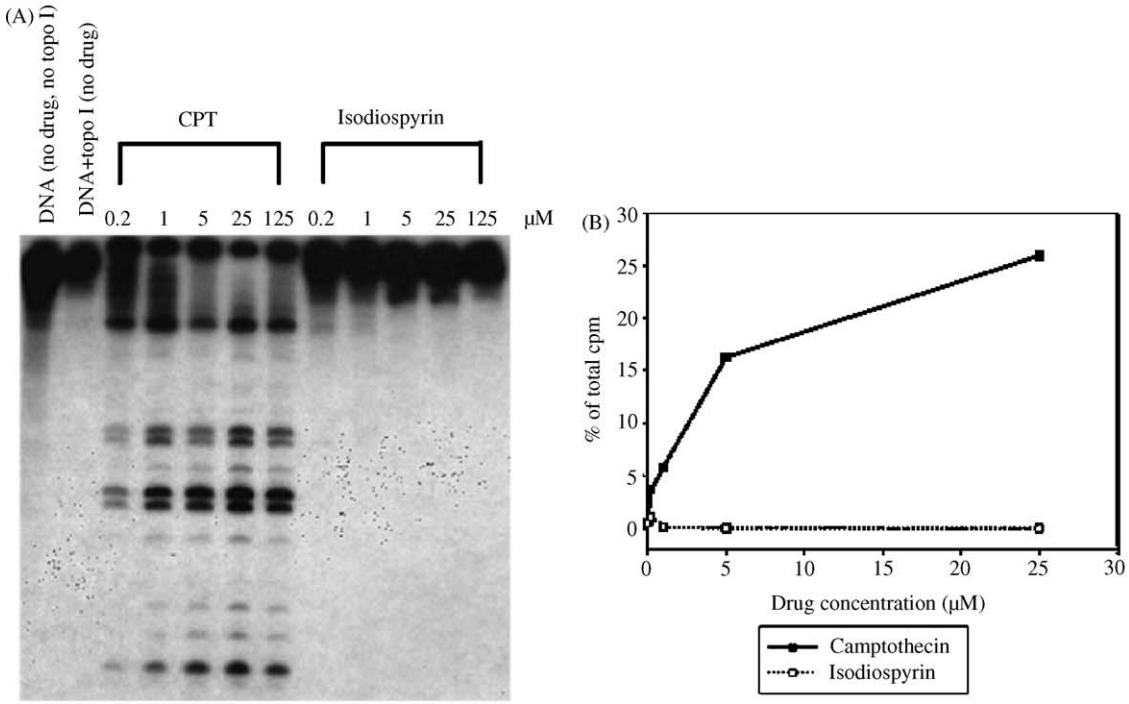


Fig. 3. Isodiospyrin does not unwind DNA. (Panel A) Linearized pGEM-5Z DNA, 1 U of T4 DNA ligase and various concentrations of isodiospyrin, CPT and ethidium bromide were added to each reaction mixture. The unwinding measurement was performed as described in Section 2. CPT and ethidium bromide were used as negative and positive DNA unwinding controls, respectively (indicated as asterisk). (Panel B) Isodiospyrin does not displace ethidium bromide from the minor groove of DNA. The ability of isodiospyrin to interact with the minor groove of DNA was determined by a fluorescence-based ethidium bromide displacement assay. Samples contained 1 μM ethidium bromide and 100 nM salmon sperm DNA. Increasing concentrations of isodiospyrin or m-AMSA was added, and ethidium fluorescence at 600 nm (λ_{max}) was monitored (546 nm excitation wavelength).

kinase activity [44]. Recent studies have shown that the C-terminal domain of htopo I is important for both the relaxation and kinase activities of htopo I [45]. To test whether isodiospyrin inhibits the kinase activity of htopo I, we performed a kinase assay using GST-SF2/ASF as a substrate in the presence of various concentrations of isodiospyrin. CPT, which does not inhibit htopo I kinase activity, was used as a negative control. As shown in Fig. 6A, isodiospyrin (10 μM) partially inhibited the kinase activity of htopo I, and increased inhibition was observed at higher concentrations of isodiospyrin. Isodiospyrin at a concentration of 40 μM reduced kinase activity by 90%. As expected, CPT (at 40 μM) did not inhibit the kinase activity. We have also tested whether isodiospyrin inhibits other kinase. As shown in Fig. 6B, isodiospyrin (at 40 μM) did not inhibit SF2/ASF phosphorylation by SRPK1 even at an isodiospyrin concentration of 40 μM.



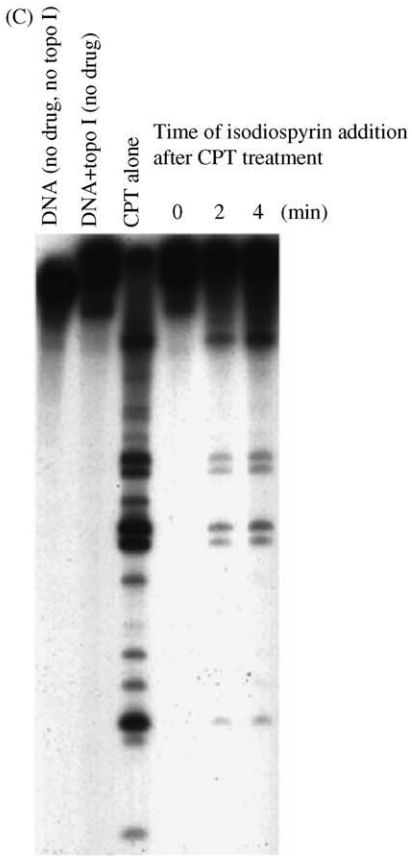


Fig. 4. Isodiospyrin does not induce human DNA topoisomerase I-associated DNA breaks. (Panel A) DNA cleavage assays were performed by incubating 3′-end labeled pGEM-5Z linear DNA and various concentrations of drugs in the presence of 10 ng of htopo I. After the reaction, the samples were separated on a 5% urea–polyacrylamide gel. (Panel B) *In vitro* K-SDS assay were performed by incubating 3′-end labeled pGEM-5Z linear DNA and various concentrations of drugs in the presence of 30 ng of htopo I. The experiment was performed as described in Section 2. (Percentage of total cpm: count of precipitated htopo I–DNA cleavable complex/count of total DNA input). (Panel C) 3′-end labeled pGEM-5Z linear DNA, 10 ng of htopo I and 125 μM CPT were used for the DNA cleavage assay. Isodiospyrin (125 μM) was added to the DNA cleavage reaction mixture at 0, 2, and 4 min after the addition of CPT.

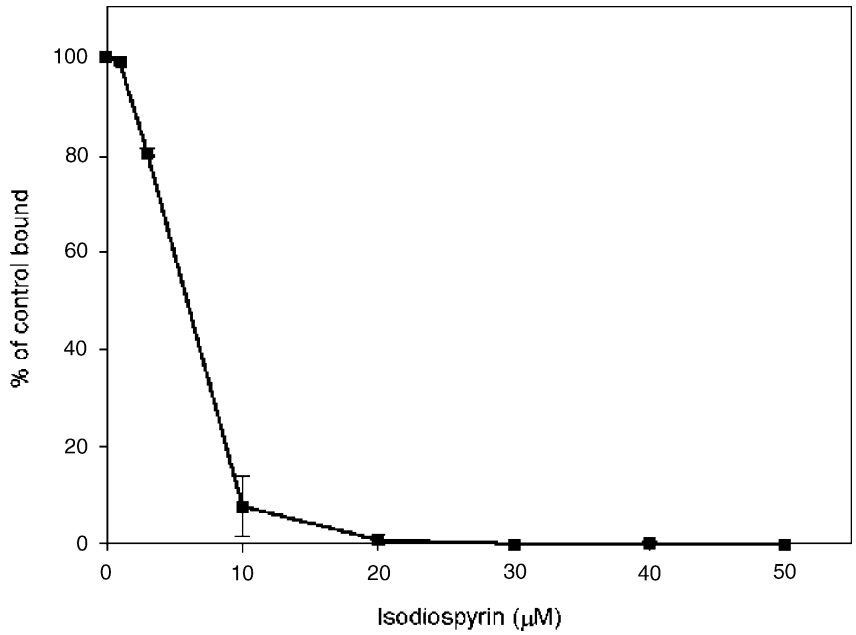


Fig. 5. Isodiospyrin prevents binding of human DNA topoisomerase I to DNA. The filter binding assay was performed as described in Section 2. 3′-End labeled pGEM-5Z DNA (10,000 cpm, approximately 2 ng) and human DNA topoisomerase I (50 ng) was mixed in binding buffer (10 mM Tris–HCl, pH 7.5, 20 μg/mL BSA, and 0.1 mM EDTA) with or without isodiospyrin and incubated for 10 min at 25°, then filtered through a nitrocellulose membrane. The plot is the representative of three independent experiments, using the mixture of DNA and htopo I without isodiospyrin was used as control (100%).

3.6. Isodiospyrin inhibits ATP binding to human DNA topoisomerase I

htopo I shows a strong preference for ATP as the phosphate donor for its kinase reaction. Previous studies have shown that the C-terminal region (amino acids 641–756) of htopo I contains an ATP binding site for its kinase activity [45]. To test whether isodiospyrin inhibits ATP binding to htopo I, a photo-crosslinking assay was performed. In this assay, ATP was crosslinked to htopo I upon UV irradiation. As shown in Fig. 7, isodiospyrin (30 μM) completely inhibited ATP crosslinking to htopo I, suggesting that isodiospyrin, probably through competing with ATP, inhibits binding of ATP to htopo I.

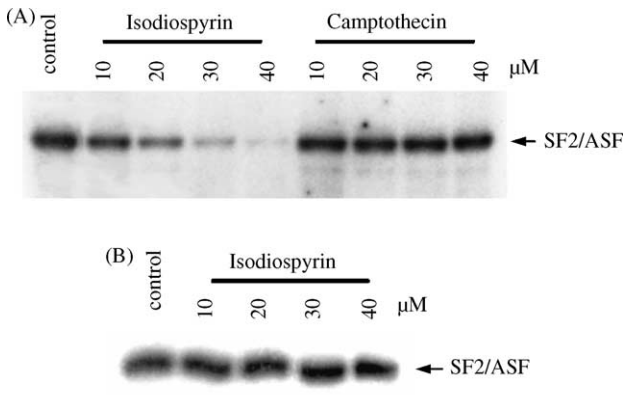


Fig. 6. Inhibition of the kinase activity of human DNA topoisomerase I by isodiospyrin. (Panel A) One hundred nanograms of htopo I, 300 ng of recombinant SF2/ASF protein and 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol) were used in a kinase assay cocktail. Concentrations of isodiospyrin and CPT (negative control) were as indicated. The samples were incubated at 30° for 30 min and then applied to a 10% SDS–polyacrylamide gel. Radioactivity incorporated into SF2/ASF on the dried gel was detected by autoradiography. (Panel B) One hundred nanograms of GST-SRPK1, 300 ng of recombinant SF2/ASF protein and 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol) were used in a kinase assay cocktail. Concentrations of isodiospyrin were as indicated.

3.7. Isodiospyrin inhibits human DNA topoisomerase I via the direct modification mode

Naphthoquinones are well known to interact with sulfhydryl-containing proteins and since isodiospyrin have similar

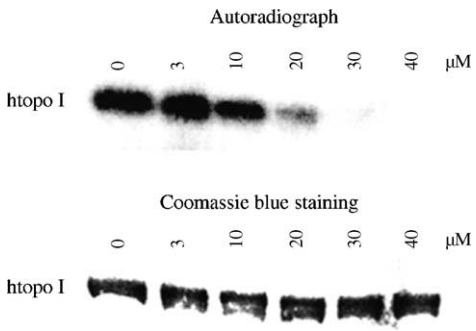


Fig. 7. Isodiospyrin inhibits ATP binding to human DNA topoisomerase I. One hundred nanograms of htopo I, 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and various concentration of isodiospyrin were mixed in a reaction mixture (as described in Section 2) for photocrosslinking assay. The samples were electrophoresed on 6% SDS–PAGE after photocrosslinking reaction. Upper panel shows the autoradiograph and lower panel shows the Coomassie blue-stained gel.

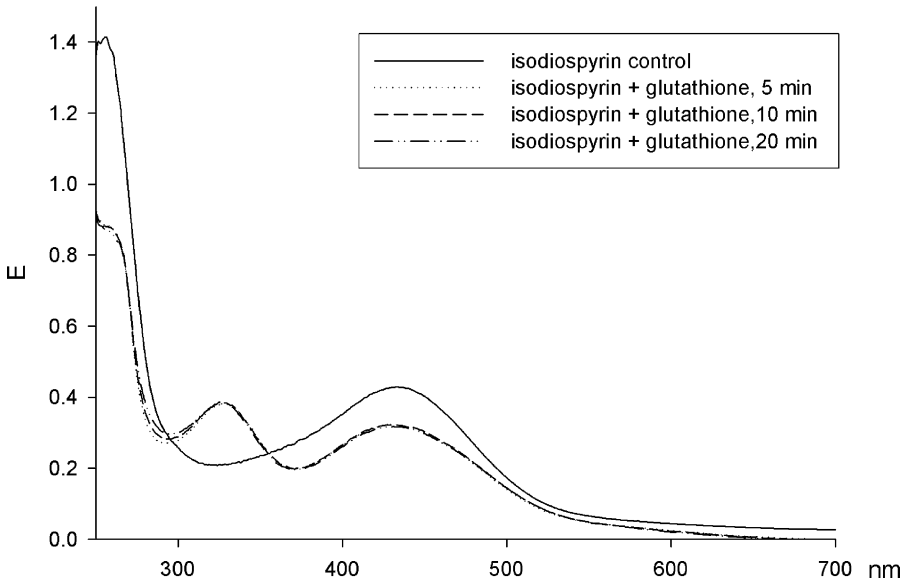


Fig. 8. Isodiospyrin reacts with sulfhydryl containing compound—glutathione. Absorption spectra change of isodiospyrin incubated with glutathione was measured by adding glutathione (100 μM final) to a buffer solution containing the isodiospyrin (100 μM in 100 mM sodium phosphate, pH 7.4, containing 20% DMSO) in a quartz cuvette. The reaction was monitored at 5, 10, 20 min (room temperature), using the absorption of isodiospyrin in the absence of glutathione as control. The spectrophotometer was blanked on the sodium phosphate buffer pH 7.4 containing 20% of DMSO.

chemical structure as naphthoquinones, we hypothesize that isodiospyrin inhibits htopo I *via* interaction with free sulfhydryl groups on htopo I. Thus, we used glutathione, a sulfhydryl reagent, to test the reactivity of isodiospyrin to sulfhydryls. Figure 8 shows absorption spectra of isodiospyrin (100 μM) in the absence and in the presence of glutathione (100 μM), incubating in 0.1 M sodium phosphate, pH 7.4. In the presence of glutathione, isodiospyrin absorbance spectrum was altered; absorbance at 430 nm was decreased while a new absorbance band at 325 nm was found to increase. This change was observed at different time points (5, 10, 20 min). The change in absorbance with glutathione indicates that isodiospyrin reacts quickly with sulfhydryl compound. In addition, isodiospyrin, when pretreated with 2-mercaptoethanol, results in a decrease in the potency of isodiospyrin to inhibit htopo I activity (data not shown), suggesting the inhibition of isodiospyrin to htopo I is *via* its interaction with the enzyme.

4. Discussion

Isodiospyrin, a natural product of *D. morrisiana* is an asymmetrical binaphthoquinone [26]. Like many other naphthoquinones, isodiospyrin exhibits a significant cytotoxicity against tumor cells but its molecular targets remains to be identified. In this article, we have determined that isodiospyrin is a novel inhibitor of htopo I. Isodiospyrin inhibits both the kinase activity of htopo I and the ability of htopo I to catalyze DNA relaxation.

As an inhibitor of htopo I, isodiospyrin appears to share similar properties with another known htopo I inhibitor, β-lapachone. Both isodiospyrin and β-lapachone are naphthoquinones. Like β-lapachone, isodiospyrin inhibits htopo I through direct interaction with the enzyme, and neither of them induces htopo I-mediated DNA cleavage. Recent studies have shown that diospyrin, a related binaphthoquinone isolated from the stem-bark of a plant *Diospyros montana* Roxb, exhibits antitumor and antiparasite activity [29]. Diospyrin is an inhibitor of type I DNA topoisomerase I of *Leishmania donovani*, which is consistent with our results on isodiospyrin. However, unlike isodiospyrin, diospyrin induces *L. donovani* topoisomerase I-mediated DNA cleavage.

Like CPT, β-lapachone, and related naphthoquinones, isodiospyrin is able to inhibit the DNA relaxation activity of htopo I. In contrast to CPT and other known DNA topoisomerase I poisons, isodiospyrin does not induce htopo I-mediated DNA cleavage. Rather, isodiospyrin antagonizes htopo I-mediated DNA cleavage induced by CPT. Of note, our unwinding assay showed that isodiospyrin is not a DNA intercalator. These results suggest that the inhibitory effect of isodiospyrin on htopo I catalysis is probably due to the isodiospyrin–htopo I rather than isodiospyrin–DNA interaction. The fact that isodiospyrin inhibits the htopo I kinase activity in the absence of DNA appears to support this conclusion.

Four distinct steps characterize htopo I catalyzed DNA relaxation: (a) htopo I binds to the substrate DNA; (b) scission of one DNA strand with concomitant formation of a covalent protein–DNA adduct; (c) strand passage; and (d) religation of the transiently broken DNA strand. There are two potential explanations for why isodiospyrin does not induce htopo I-mediated DNA cleavage. Firstly, isodiospyrin forms a complex with htopo I thereby preventing htopo I from interacting with its DNA substrate (step a).

Secondly, isodiospyrin's interaction with htopo I potentially blocks the nicking reaction (step b). Results from both filter binding and gel retardation assays suggest that isodiospyrin inhibits binding of htopo I to DNA (step a). It seems possible that binding of isodiospyrin to htopo I, which blocks htopo I binding to DNA, is responsible for the observed inhibitory effects of isodiospyrin on both htopo I-catalyzed DNA relaxation and the htopo I kinase activity.

The most important question is how isodiospyrin inhibits both the htopo I relaxation and kinase activity. Previous study has demonstrated that β-lapachone is a bioactive molecule that was reduced by sulfhydryl group compound, e.g. L-cysteine and 2-mercaptoethanol [46]. Our experiment also reveals that isodiospyrin, also a member of naphthoquinone, reacts quickly with sulfhydryl compound—glutathione (Fig. 8). The similar behavior observed for isodiospyrin and β-lapachone suggests that isodiospyrin may interact with sulfhydryl-containing enzymes such as htopo I by Michael addition reaction. Previous study [45] has shown that ATP binding region of htopo I (amino acids 641–756) contains one cysteine located at residue 733 which is adjacent to the active center of htopo I (tyrosine-723). This cysteine residue 733 could be isodiospyrin's potential binding site to htopo I, where it can confer htopo I kinase and relaxation inhibition. However, htopo I contains eight cysteine residues. For this reason, it is also possible that inhibition of the htopo I kinase and relaxation activities could arise from relative non-specific effect cause by multiple isodiospyrin modification on any one, or all cysteine residues of the protein. The exact site of isodiospyrin–htopo I interaction remains to be elucidated. Importantly, this study has identified (a) htopo I as a molecular target of isodiospyrin and (b) a potential mechanism by which isodiospyrin (or naphthoquinone's family of htopo I inhibitors) inhibit htopo I kinase activity and DNA relaxation.

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