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Selective Inhibition of Topoisomerase I and Various Steps of Spliceosome Assembly by Diospyrin Derivatives

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ABSTRACT

Pre-mRNA splicing is an essential step of the expression of most metazoan protein-coding genes, which is often regulated in a cell type-specific or developmental manner. We have demonstrated previously that human DNA topoisomerase I, an extensively studied target for anticancer drugs, also has an intrinsic protein kinase activity that specifically phosphorylates proteins involved in splice site selection. Therefore, DNA topoisomerase I was recently shown to play a critical role in alternative splicing. Here, we have exploited these novel properties of DNA topoisomerase I to develop entirely novel diospyrin derivatives targeting its protein kinase activity and thereby modulating pre-mRNA splicing. Although some derivatives indeed inhibit kinase activity of topoisomerase I, they did not block reactions of topoisomerase I on DNA. However, these drugs interfere with camptothecin-dependent topoisomerase I-mediated DNA cleavage, implying that diospyrin derivatives mediate a conformational change of topoisomerase I. It is noteworthy that in vitro splicing reactions revealed that diospyrin derivatives alter various steps of splicing. Some diospyrin derivatives inhibit either the first or the second catalytic step of splicing but not spliceosome assembly, whereas diospyrin itself prevents the formation of full spliceosome. Our data revealed for the first time that diospyrin derivatives are able to stall the dynamic assembly of the spliceosome and open the exciting possibility of using these derivatives to correct aberrant splicing in human genetic diseases.

Topoisomerase I is primarily known as a DNA nicking/closing enzyme capable of resolving topological constraints in DNA (Wang, 2002). Its ability to relax supercoiled DNA and its implication in chromatin (de)condensation have been thoroughly discussed (Wang, 2002). In addition to DNA, topoisomerase I interacts with different protein partners, among which are p53, nucleolin, and various other nuclear proteins (Tazi et al., 1997). One key feature of this enzyme is its capacity to perform reactions, via two distinct activity domains, on both DNA and a class of proteins called SR proteins implicated in RNA splicing, such as the SF2/ASF splicing factor (Rossi et al., 1996; Chen and Hwang, 1999; Andersen et al., 2002; Soret and Tazi, 2003). With DNA, topoisomerase I functions as a phosphodiesterase, introducing simple strand breaks and then as a ligase to reseal the break after the DNA has been conformationally manipulated (Champoux, 2001). With SR proteins, topoisomerase I acts as a kinase to phosphorylate their arginine-serine-rich domain, as has been shown for the SF2/ASF splicing factor (Rossi et al., 1996; Labourier et al., 1998). We have demonstrated previously that drugs that interfere with the kinase activity of topoisomerase I, and thereby with the phosphorylation status of SR proteins, prevent spliceosome assembly and modulate splicing profile of several genes (Labourier et al., 1999a; Pilch et al., 2001; Soret and Tazi, 2003). More importantly, topoI/kinase-mediated phosphorylation has been shown to be required for the exonic splicing element (ESE)-dependent splicing (Soret et al., 2003), implying that small molecules can achieve selective inhibition of splicing events.

Over the past 20 years, a large variety of topoisomerase I inhibitors targeting the topoisomerase I-DNA (cleavable) covalent complexes has been discovered (Meng et al., 2003).

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ABBREVIATIONS: topo, topoisomerase; ESE, exonic splicing element; DMSO, dimethyl sulfoxide; bp, base pair(s); snRNP, small nuclear ribonucleoprotein; CPT, camptothecin.

Two camptothecin derivatives, topotecan and irinotecan, are on the market for the treatment of ovarian and colon cancers, and several second- and third-generation camptothecins (e.g., exatecan and diflomotecan) are currently undergoing clinical trials (Bailly, 2003a). Noncamptothecin topoisomerase I "poisons" are also developed, including glycoside indolocarbazoles (Garcia-Carbonero and Supko, 2002; Bailly, 2003b), indenoisoquinolines (Strumberg et al., 1999), and lamellarins (Facompré et al., 2003), to cite a few. In sharp contrast, the kinase activity of topoisomerase I has not yet received much pharmacological attention, and this is the issue we addressed here via the use of the plant product diospyrin and six synthetic derivatives.

Diospyrin (compound D1 in Fig. 1) is a natural bisnaphthoquinoid extracted from the stem bark of the *Diospyros montana* Roxb. (Hazra et al., 1984), an indigenous medicinal plant of the Ebenaceae family, common in India. Plants of the genus *Diospyros* (from *dios*, divine, and *pyros*, heat; i.e., celestial food) are rich in secondary metabolites, and for this reason, they have frequently been used in folk medicine since ancient times to treat various diseases or symptoms (for review, see Mallavadhani et al., 1998). This compound and its derivatives have revealed interesting antitumor activities (Hazra et al., 1984, 1994; Pal et al., 1996; Chakrabarty et al., 2002), and it also has been investigated as an antibacterial (Adeniyi et al., 2000; Lall et al., 2003) and antiparasitic agent, active against *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi*, and *Trypanosoma brucei brucei* (Hazra et al., 1987, 1995; Yardley et al., 1996), as well as *Pneumocystis carinii* (Cushion et al., 2000; Kaneshiro et al., 2000). Diospyrin and synthetic derivatives have been shown to induce apoptosis of different human tumor cells (Chakrabarty et al., 2002), but otherwise the mechanism of action of this compound remains essentially unknown. Nevertheless, two previous independent studies point to a role of topoisomerase I in the pharmacological activity of diospyrin and derivatives. Selective inhibition of topoisomerase I from *L. donovani* has been reported with diospyrin itself (Ray et al., 1998), and very recently, the related compound isodiospyrin, extracted from *Diospyros morrisiana*, was shown to inhibit human topoisomerase I (Ting et al., 2003). These two studies suggested that the diospyrin-type drug interacts directly with topoisomerase I but not with its DNA partner as is frequently the case with other inhibitors. Most interestingly, it was reported that isodiospyrin antagonizes camptothecin-induced DNA cleavage mediated by topoisomerase I and strongly inhibits the kinase activity of the enzyme toward SF2/ASF (Ting et al., 2003). These considerations, together with our long-term interest in the discovery of topoisomerase I-targeted antitumor agents acting at the DNA relaxation (Bailly, 2000, 2003) and/or the kinase level (Labourier et al., 1999a; Pilch et al., 2001), prompted us to investigate the effects of selected diospyrins on the activity of topoisomerase I. We report here a study aimed at characterizing the inhibitory activity of diospyrin (D1), the dimethyl ether D2, the hydroquinonoid derivative D3, the diethyl ether D7, the aminoethanol derivative D16, the epoxide D17, and the thioethanol derivative D18 (Fig. 1). Their capacities to antagonize camptothecin-dependent topoisomerase I-mediated DNA cleavage and to inhibit the kinase activity of topoisomerase I were investigated. These results also prompted us to test whether these components can be used to selectively block splicing in vitro. Inasmuch as we have identified diospyrins as potent inhibitors of the RNA splicing machinery, this raises novel opportunities to design specific molecular tools targeting various steps of spliceosome assembly.

Materials and Methods

Drugs. D1 and its derivatives D2, D3, and D7 were synthesized and purified as described previously (Hazra et al., 1984; Sanyal et al., 2003). The syntheses of D16, D17, and D18 are to be described in the doctoral dissertation of M. Das Sarma at Jadavpur University (unpublished). Stock solutions (5 mM) of the compounds were prepared in DMSO, and solutions were diluted with water extemporaneously.

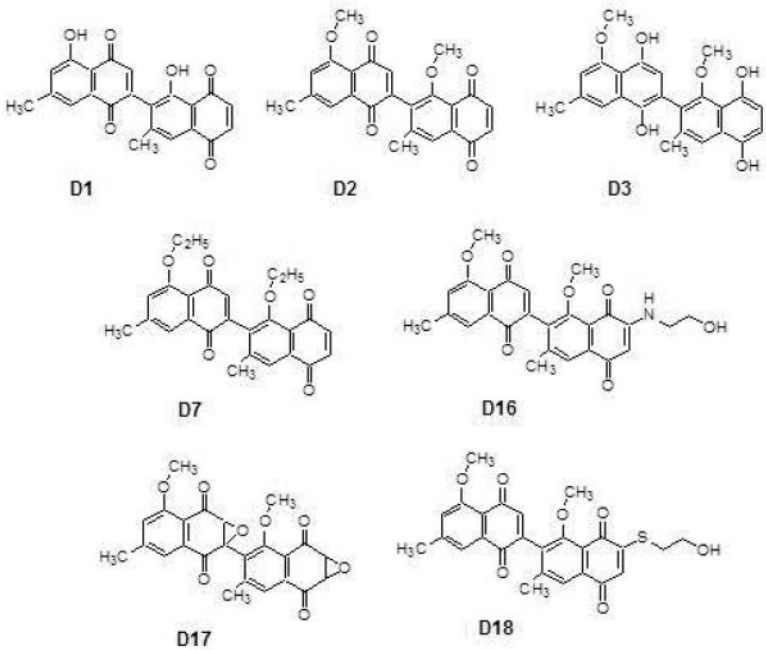

Fig. 1. Structure of the diospyrins used in this study.

Biophysical Measurements. DNA binding studies using melting temperature, UV-visible absorption, and circular dichroism measurements were performed as described previously (Goossens et al., 2001).

DNA Relaxation Experiments. Recombinant topoisomerase I protein was produced and purified from baculovirus-infected Sf9 cells (Rossi et al., 1996). Supercoiled pLAZ3 DNA (0.25 μg) was incubated with 3 units of human topoisomerase I at 37°C for 1 h in relaxation buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/ml. DNA samples were then added to the electrophoresis dye mixture (3 μl) and electrophoresed at room temperature for 2 h at 120 V in 1% agarose gels containing ethidium bromide (1 μg/ml). After electrophoresis, gels were washed and photographed under UV light (Bailly, 2001).

Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites. The 117-bp DNA fragment was prepared by 3′-^{32}P-end labeling of the EcoRI-PvuII double digest of the pBS plasmid (Stratagene, La Jolla, CA) using [α-^{32}P]dATP (3000 Ci/mmol; Amersham Biosciences, Piscataway, NJ) and avian myeloblastosis virus reverse transcriptase (Roche Diagnostics, Indianapolis, IN).

Each reaction mixture contained 2 μl of 3′-end ^{32}P-labeled DNA (~1 μM), 5 μl of water, 2 μl of 10 times concentrated topoisomerase I buffer, and 10 μl of drug solution at the desired concentration (50 μM final concentration). After 10-min incubation to ensure equilibration, the reaction was initiated by addition of 2 μl (20 units) of topoisomerase I. Samples were incubated for 45 min at 37°C before adding SDS to 0.25% and proteinase K to 250 μg/ml to dissociate the drug-DNA-topoisomerase I-cleavable complexes. The DNA was precipitated with ethanol and resuspended in 5 μl of formamide-TBE loading buffer, denatured at 90°C for 4 min, and then chilled in ice for 4 min before loading onto the sequencing gel. DNA-cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions.

Purification of Recombinant Topoisomerase I, SF2/ASF Proteins, and Kinase Assays. Recombinant topoisomerase I and hexahistidine-tagged SF2/ASF protein were produced and purified from baculovirus-infected Sf9 cells and *Escherichia coli*, respectively, as described previously (Ge et al., 1991; Rossi et al., 1996, 1998). The reaction mixtures for protein kinase assay contained 100 ng of recombinant topoI/kinase, 300 ng of recombinant SF2/ASF protein, 1 μM ATP, 3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 1 μl of diospyrin or DMSO in a final volume of 20 μl of buffer A (50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, and 0.5 mM dithiothreitol). The samples were incubated at 30°C for 30 min and then mixed with 6 μl of 4× loading buffer and applied on a 12% SDS-polyacrylamide gel. Radioactivity incorporated into SF2/ASF was

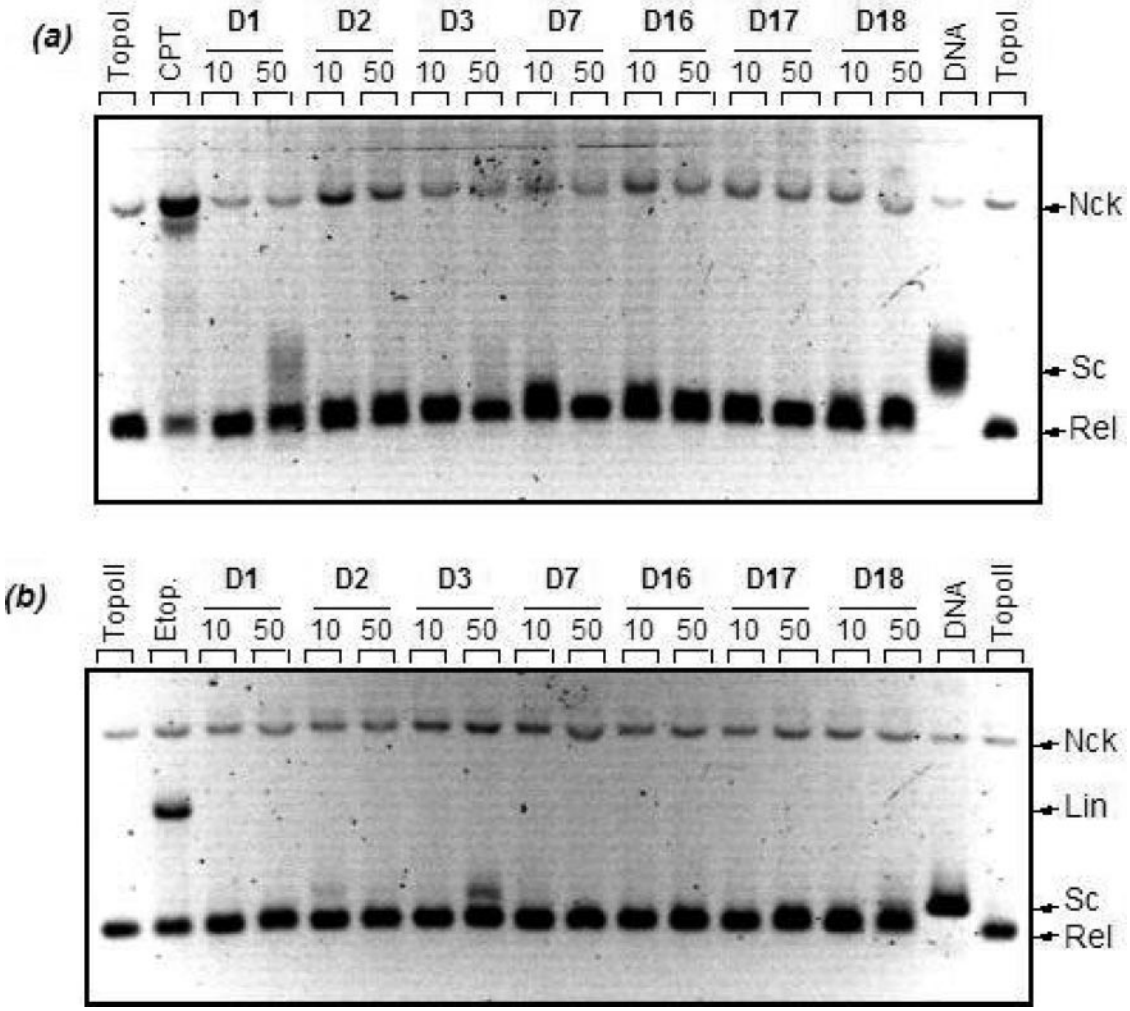

Fig. 2. Effect of the diospyrin derivatives on the stabilization of covalent DNA-topoisomerase complexes. Native supercoiled DNA (lane DNA, 0.15 μg) was incubated with topoisomerase I (a) or topoisomerase II (b) in the absence (lane TopoI or TopoII) or presence of the indicated compound. Lanes marked CPT and Etop. refer to camptothecin and etoposide (20 μM each), respectively. Diospyrin and its derivatives were tested at 10 and 50 μM. DNA samples were separated by electrophoresis on agarose gels containing 1 μg/ml ethidium bromide. Gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled.

detected by autoradiography of the dried gel and quantitated by an Amersham Biosciences imaging analyzer using ImageQuant software version 3.22.

Splicing and Spliceosome Assembly Assays. The single-intron human *β-globin* construct pSPHβm3S1 was described previously (Labourier et al., 1999b). Radiolabeled β3S1 RNA was synthesized by in vitro transcription in presence of 20 units of SP6 RNA polymerase (Roche Diagnostics), 1 μg of pSPHβm3S1 plasmid linearized with BamHI, and 5 μM [α-^{32}P]UTP (3000 Ci/mmol) in 25-μl reactions according to manufacturer's conditions. The splicing reactions were performed under standard conditions for the indicated time in a total volume of 20 μl containing 50 fmol of labeled pre-mRNA and 10 μl of HeLa nuclear extract complemented with buffer D (Tazi et al., 1986). Splicing products were analyzed by electrophoresis on denaturing 7% polyacrylamide gels and revealed by autoradiography.

Kinetics of appearance of splicing complexes was performed as described previously (Konarska and Sharp, 1987). Aliquots (5 μl) from the various reactions treated with 2 mg/ml heparin were mixed with 1 μl of 97% glycerol, 1% bromphenol blue, and resolved directly on a 4% nondenaturing polyacrylamide gel (acrylamide/bis-acrylamide weight ratio of 80:1) in 50 mM Tris-glycine, pH 8.3.

Results

Diospyrin Derivatives Neither Bind to DNA nor Stabilize Topoisomerase I-DNA Covalent Complexes. The interaction of the diospyrin derivatives with purified DNA and polynucleotides was investigated by spectroscopic and biochemical methods, but in all cases, no significant interaction was observed. Melting temperature experiments and circular dichroism measurements showed no stabilization of duplex DNA against heat denaturation in the presence of the diospyrin, even at a concentration as high as 50 μM. The absorption spectra of the drugs were practically not affected by the titration with DNA. Only a weak hypochromism (about 12% in the 310-nm absorption band) was detected with compounds D2 and D3 with a massive excess of DNA and at a low ionic strength (data not shown). DNase I footprinting experiments also failed to reveal any significant interaction of the drugs with DNA. In parallel, we tested the effects of the compounds on DNA topoisomerases I and II by using conventional topoisomerase assays. In the relaxation assay using supercoiled plasmid and electrophoresis on agarose gels to resolve the topological forms of DNA, none of the diospyrins promoted DNA cleavage by topoisomerases I or II (Fig. 2). A potent inhibition of the enzymes was observed with the reference compounds camptothecin (for topoisomerase I) and etoposide (for topoisomerase II), which stimulate DNA single-strand (Fig. 2a, lane CPT) or double-strand (Fig. 2b, lane Etop.) breaks by the topoisomerase. No such effects were seen with the diospyrins; they do not stabilize topoisomerase-DNA covalent complexes.

Inhibition of Camptothecin-Dependent Topoisomerase I-Mediated DNA Cleavage. Camptothecin is very potent at stabilizing topoisomerase I-DNA covalent complexes and promoting DNA cleavage by the enzyme. This activity is antagonized by the diospyrins, at least some of them. As shown in Fig. 3, the formation of nicked DNA, which arises from single-stranded cleavage of supercoiled DNA by camptothecin-trapped topoisomerase I, is reduced when the reaction was performed in the presence of the diospyrins D1, D3, or D7 but not the other analogs. The amount of nicked DNA remained high in the presence of compounds D2, D16, D17, and D18, whereas it was reduced by more than 50% in the presence of compounds D1, D3, and D7.

To investigate further this antagonism, DNA cleavage reactions were performed with a 117-bp radiolabeled DNA substrate that contains three well spaced major cleavage sites for topoisomerase I at nucleotide positions 26, 48, and 81 (Fig. 4). In the presence of camptothecin, the enzyme is trapped at these three sites (all three corresponding to a TG dinucleotide step known as the most favorable sites for the alkaloid), and cleavage bands can be easily identified on the polyacrylamide gel under denaturing conditions. This sensitive assay is useful to study the effects of the diospyrins, and, as shown in Fig. 4, a complete inhibition of DNA cleavage was detected with compounds D1 and D7. The inhibition was only partial with D3 and a residual inhibitory activity was noted for D2. Compounds D16, D17, and D18 had absolutely no effect, in agreement with the results of the relaxation assay. It is interesting that these observations are consistent with cytometry data showing that the compounds antagonize the proapoptotic activity of camptothecin to different extents (data not shown).

Selective Inhibition of TopoI-Kinase Activity by Diospyrin Derivatives. The data mentioned above suggested that diospyrin derivatives possibly bind topoisomerase I and thereby change its sensitivity to camptothecin. Topoisomerase I has been shown to exhibit at least two incompatible conformations. One conformation is in the form of a complex with one of the substrates of the kinase reaction (ATP or the SR splicing factor ASF/SF2), which inhibits DNA relaxation activity, and the other, a topoisomerase I-DNA complex, which inhibits protein kinase activity (Chen and Hwang, 1999; Andersen et al., 2002). We were therefore interested to know whether the binding of diospyrin derivatives to topoisomerase I introduces conformational changes that may al-

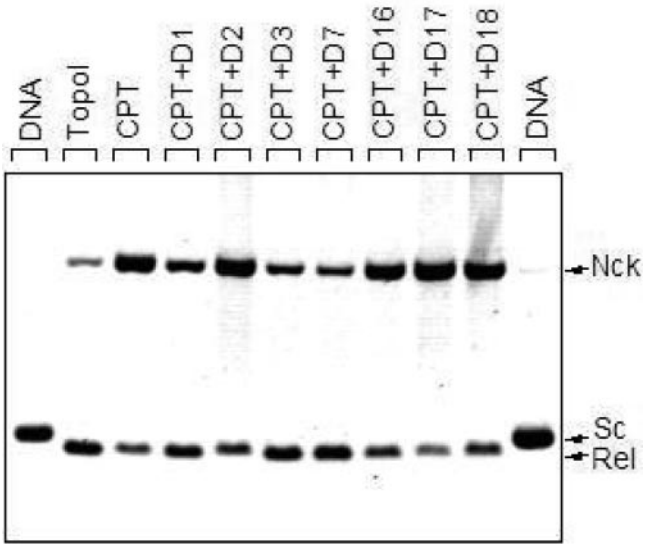

Fig. 3. Antagonistic effects of diospyrins on camptothecin-induced DNA cleavage mediated by topoisomerase I. Native supercoiled DNA (0.15 μg) (lanes DNA) was incubated with topoisomerase I in the absence (lane TopoI) or presence of camptothecin alone (10 μM) or camptothecin (10 μM) and each diospyrin derivative (50 μM). Reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on agarose gels containing 1 μg/ml ethidium bromide. The gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled DNA.

ter the kinase activity of topoisomerase I. Standard kinase assays performed with bacterially expressed recombinant SF2/ASF and [γ-^{32}P]ATP and various diospyrin derivatives showed that the phosphorylation of SF2/ASF by topoisomerase I is markedly inhibited in the presence of 50 μM D1 and the D3 derivative, whereas compounds D2, D7, D16, D17, and D18 were less effective (Fig. 5).

Diospyrin Derivatives Affect Different Steps of Splicing Reaction. Given that TopoI/kinase is required for ESE-dependent splicing but not constitutive splicing (Soret et al., 2003), we decided to test the ability of each compound to inhibit in vitro splicing of reporter pre-mRNAs whose splicing depends on such ESE sequences in the second exon. Splicing reactions performed with a substrate derived from the β-globin pre-mRNA (pSpHβm-3S1) and whose splicing strictly depends on a ASF/SF2 enhancer, demonstrated that compounds D1 and D3 (at 50 μM) have a strong inhibitory effect on splicing (Fig. 6A, lanes 7–9 and 10–12). Neither intermediates (exon 1 and lariat-exon 2) nor products (lariat intron and exon1-exon 2) were detected after 2 h of incubation of the pre-mRNA substrate under splicing conditions. Consistent with the kinase assays, however, the inhibitory effect of D1 was much stronger than that of D3 (compare lane 8 and lane 11), and no inhibition of splicing was observed with a concentration up to 50 μM D2 and D18 (data not shown). In contrast, compounds D7 and D17 induced a different kind of splicing inhibition, because they were able to induce accumulation of splicing intermediates (Fig. 6A, lanes 1–3 and 4–6, respectively). This result indicates that the latter compounds, unlike compounds D1 and D3, affect the second catalytic step of splicing.

To test the specificity and selectivity of action of the tested drugs, D3 or D7 was preincubated for 15 min with nuclear extract before adding the substrate. Although this preincubation reduces the level of splicing intermediate produced in the presence of D7 (Fig. 6A, compare lanes 1 and 2), a clear difference in splicing inhibition is still visible between D7 and D3 (Fig. 6B, compare lanes 2 and 6). No splicing intermediates are detected in the presence of D3. However, both compounds were able to alter the second catalytic step when

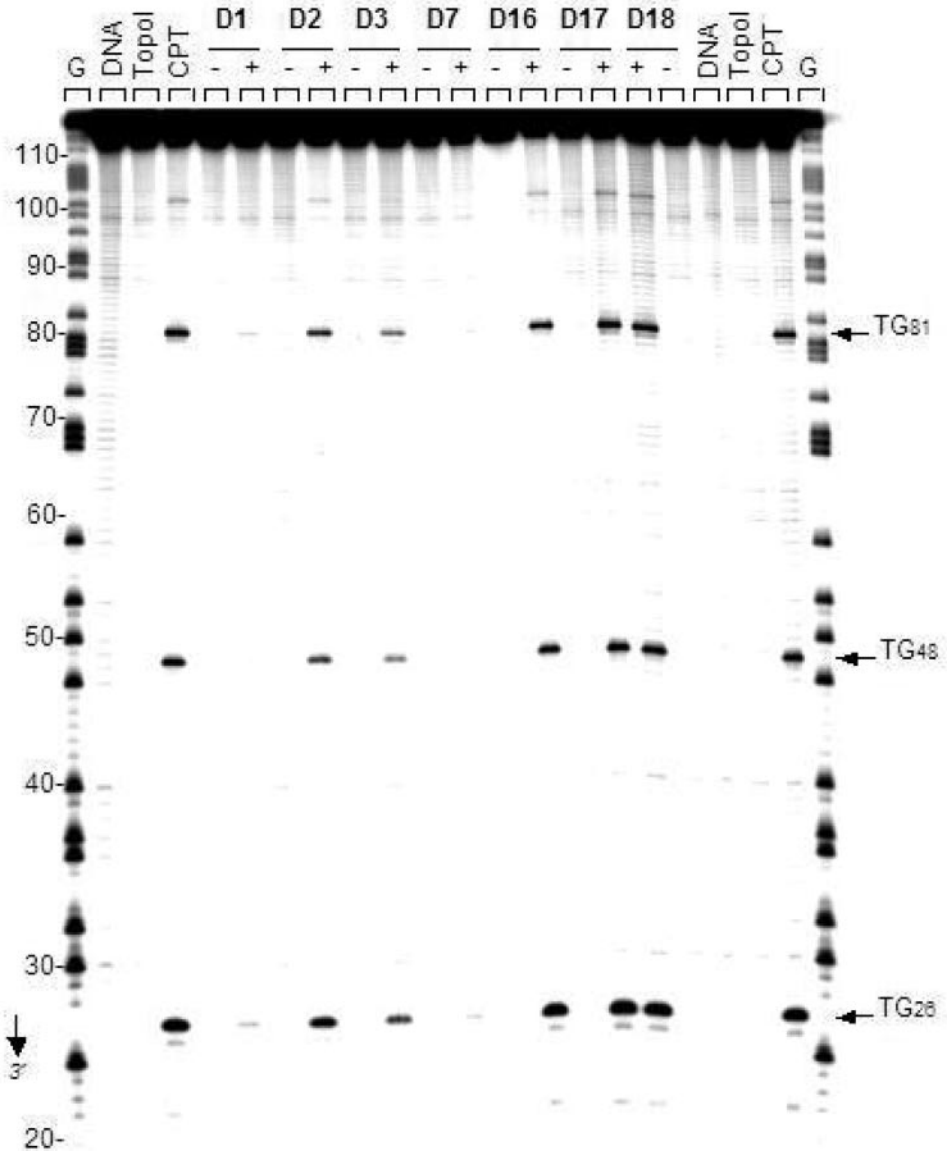

Fig. 4. Cleavage of the 117-bp DNA fragment by topoisomerase I in the presence of the diospyrin derivatives. The 3′-end-labeled fragment was incubated in the absence (lane TopoI) or presence of the test drug at 50 μM, in the presence (+) or absence (−) of 20 μM CPT. Topoisomerase I cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel. Numbers at the right side of the gels show the nucleotide positions, determined with reference to the guanine tracks labeled G. Arrows point to the three TG cleavage sites.

they were added after 30 min of incubation of the splicing reaction (Fig. 6B, compare lanes 3 and 7 with lane 9). The possibility of a selective degradation of splicing products mediated by compounds D3 or D7 can be ruled out, because the splicing profile is similar to control if the drugs were added after 1 h of preincubation (Fig. 6B, compare lanes 4 and 8 with lane 9). Together, these results further confirm that compound D3 inhibits splicing at both catalytic steps, whereas D7 affects only the second catalytic step.

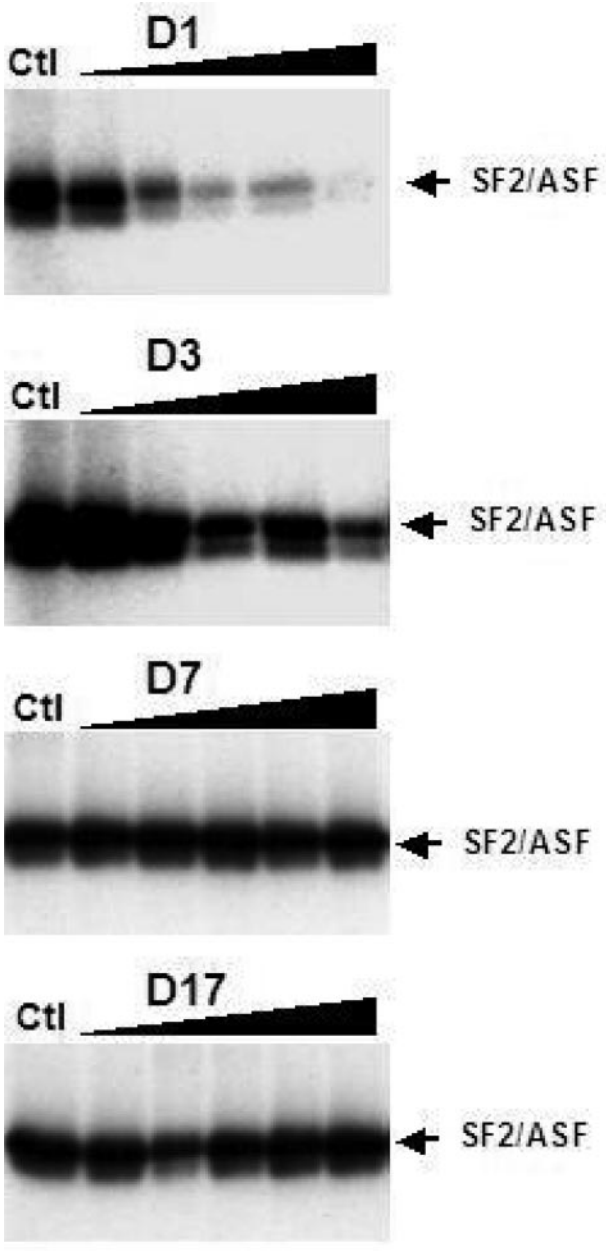

Fig. 5. Inhibition of topoisomerase I kinase activity by diospyrin derivatives. Phosphorylation of SF2/ASF by topoI/kinase was performed as described under *Materials and Methods* with increasing concentrations (1, 5, 25, and 100 μM of D1, D3, D7, and D17) of diospyrin derivatives. Ctl refers to the control reaction with DMSO alone instead of diospyrin derivatives. The phosphorylated SF2/ASF was analyzed on a 12% SDS-polyacrylamide gel and revealed either by autoradiography or quantitated by PhosphorImaging. The position of SF2/ASF was localized by staining the gel with Coomassie Blue. Shown are representative autoradiographs of kinase reactions with compounds D1, D3, D7, and D17. Duplicate assays revealed that the concentration required to inhibit 50% of the kinase activity is >50 μM for D7 and D17, but only 1 and 5 μM for D1 and D3, respectively.

D1 but Not Other Diospyrin Derivatives Inhibits Efficiently Spliceosome Assembly. The differential behavior of diospyrin derivatives in splicing reactions prompted us to test the effect of these compounds on spliceosome assembly. ^{32}P-Labeled β-3S1 was incubated with HeLa nuclear extracts treated with the highest concentration of diospyrin derivatives that demonstrated splicing alteration, and assembled ribonucleoprotein complexes were analyzed by native gel electrophoresis (Pilch et al., 2001). As shown in Fig. 7, the untreated control extract (lanes 1–3) shows the characteristic pattern of spliceosome assembly pathway. Two heparin-resistant complexes are formed in ATP-dependent manner at early time points and correspond to complex A and complex B, in addition to a fast-migrating nonspecific complex H (lane 3). Complex A formation involves the stable binding of U2 snRNP to the pre-mRNA sequences near to the 3′ splice site (prespliceosome) and subsequent binding of a tri-snRNP particle U4/U5/U6, rather than the individual snRNPs giving rise to the B complex (spliceosome). Therefore, at a later time point (30 min), the level of complex A decreased with parallel increase in the amount of complex B (lanes 1–3). A conformational change, probably involving disruption of base-paired U4 and U6 snRNAs, seems to be a critical step in the initiation of splicing reactions by the assembled B complex (Pilch et al., 2001). It was surprising that, except for D1 (lanes 4–6), which markedly inhibited complex B formation, no other derivatives altered formation of either complex A or B. The complexes assembled in the presence of the various derivatives were similar to the complexes formed in the control reaction after the same kinetics (lanes 7–9, 10–12, and 13–15). Thus, diospyrin derivatives highlight a novel mechanism of splicing inhibition that is different from that mediated by indolocarbazoles. The latter compounds inhibit the earliest step of spliceosome assembly that precedes binding of U2 snRNP, whereas the former compounds act at later stages. Compounds 3, 7, and 17 inhibit splicing at either a precatalytic step (D3) or the second catalytic step (D7 and D17), whereas D1 prevents the recruitment of U4/U5/U6 tri-snRNP and thereby the assembly of a full spliceosome.

Discussion

This study validates topoisomerase I as a target for diospyrin and provides for the first time structure-activity relationships for topoisomerase I inhibition by this family of bisnaphthoquinoids. Inhibitory effects of diospyrin on topoisomerase I from the *L. donovani* parasite (Ray et al., 1998) or from isodiospyrin on human topoisomerase I (Ting et al., 2003) had been previously mentioned in the literature. The present study not only confirms that topoisomerase I can effectively be considered as an essential target for this family of bisnaphthoquinoids but also indicates more precisely with which step of the various enzymatic reactions involving topoisomerase I the different compounds interfere. For a long time, the DNA cutting activity of topoisomerase I has attracted the attention of chemists and pharmacologists because the characterization in the early 1980s of the plant alkaloid camptothecin (CPT) as a potent and specific inhibitor of topoisomerase I. CPT promotes DNA cleavage by topo-

isomerase I, through the stabilization of topoisomerase I phosphotyrosine-DNA intermediates. The accumulation of DNA breaks activates specific molecular circuits through the nucleus, cytoplasm, and mitochondria, leading to apoptotic cell death, mostly in rapidly proliferating tumor cells. The majority of the topoisomerase I inhibitors reported thus far intervene at the level of DNA-topoisomerase I complex, through stabilization of the covalent intermediate whereby the enzyme is covalently attached to the DNA via a phosphotyrosyl linkage. This intermediate complex is selectively trapped by camptothecin or other drugs referred to as poisons, such as certain indolocarbazoles and indenoisoquinolines. Diospyrin does not belong to this group of poisons. Its action certainly results from a direct interaction with the enzyme, as suggested for isodiospyrin (Ting et al., 2003).

The phosphorylation of SF2/ASF by topoisomerase I is markedly inhibited in the presence of 50 μM D1 and the D3 derivative, whereas compounds D2, D7, D16, D17, and D18 are less effective. The concentration required to inhibit 50% of the kinase activity is $>$50 μM for D7 and D17, but only 1 and 5 μM for D1 and D3, respectively. These two compounds were found to potently inhibit the splicing of a reporter pre-mRNA. It is interesting that the splicing reaction can be dissected with the diospyrins. Compound D3 inhibits splicing at a precatalytic step of the splicing reactions, whereas compounds D7 and D17 affect the second catalytic step. Diospyrin D1 is different because it prevents the recruitment of U4/U5/U6 tri-snRNP and therefore the assembly of a full spliceosome. The two phenolic OH groups of D1 are likely to be essential for this activity. These diospyrin molecules represent the first molecular tools to decouple the splicing mechanism.

The various steps of spliceosome assembly are triggered by dynamic rearrangements of proteins associated with the different snRNPs (Makarov et al., 2002). The mechanisms by which such rearrangements are mediated are presently unknown. The discovery of drugs, such as diospyrin, that interfere with different stages of the spliceosome assembly is critical to uncover factors that contribute to the dynamics of the assembly process. Because two of the diospyrin derivatives tested here are able to target topoI/kinase, it is tempting to speculate that such related compounds may inhibit other key kinases involved in the spliceosome assembly. Identification of such kinases could be facilitated by the use of analogs that can be cross-linked to proteins in the purified spliceosome.

Diospyrin derivatives will also be a valuable tool to dissect the regulatory mechanisms involving splicing factor phosphorylation in vivo and may be applicable for the therapeutic manipulation of abnormal splicing. Indeed, many mutations at the origin of human diseases affect the splice sites as well as regulatory sequences to produce defective proteins (Cartegni et al., 2002; Faustino and Cooper, 2003; Garcia-Blanco et al., 2004). Targeting either the mutated sequences or the factors that bind to them may prove to be an important strategy to correct aberrant splicing. Thus, successful correc-

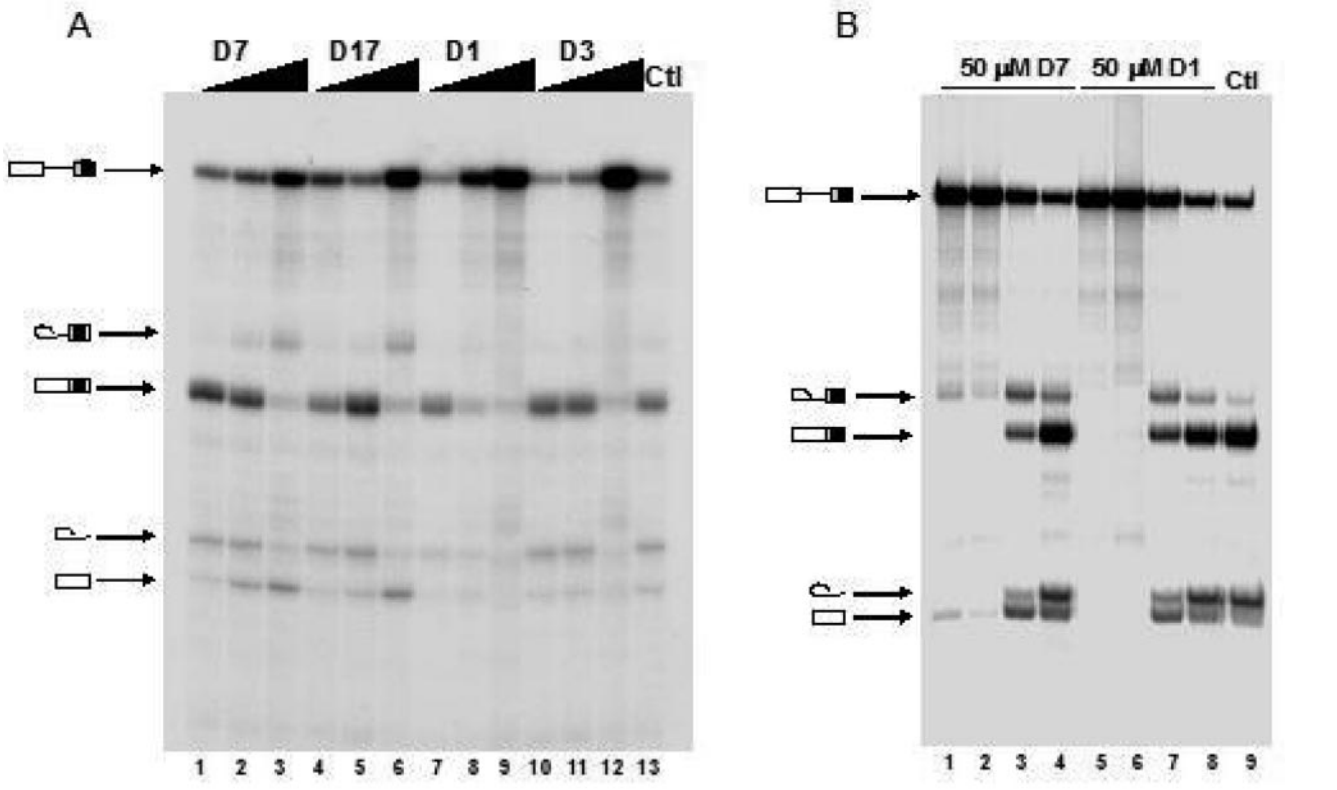

Fig. 6. Inhibition of pre-mRNA splicing by diospyrin derivatives in vitro. A, ^{32}P-labeled β-3S1 pre-mRNA (50 pmol) was incubated in 10 μl of HeLa cell nuclear extract under splicing conditions without (lane 13) or with 25 μM (lanes 1, 4, 7, and 10), 50 μM (lanes 2, 5, 8, and 11), and 100 μM (lanes 3, 6, 9, and 12) of the indicated diospyrin derivative. B, splicing reactions incubated for 60 min without (lane 9) or with 50 μM D7 (lanes 1–4) or D1 (lanes 5–8). The drugs were included at the same time (lanes 1 and 5), before 15 min (lanes 2 and 6), after 30 min (lanes 3 and 7), or after 1 h (lanes 4 and 8) of adding ^{32}P-labeled β-3S1 pre-mRNA. Splicing products were analyzed on a 7% denaturing polyacrylamide gel and revealed by autoradiography. The structure of splicing products and intermediates are depicted on the left. The open box indicates the first exon, the black box in the second exon corresponds to SF2/ASF high-affinity binding site, and linear or lariat lines represent the intron.

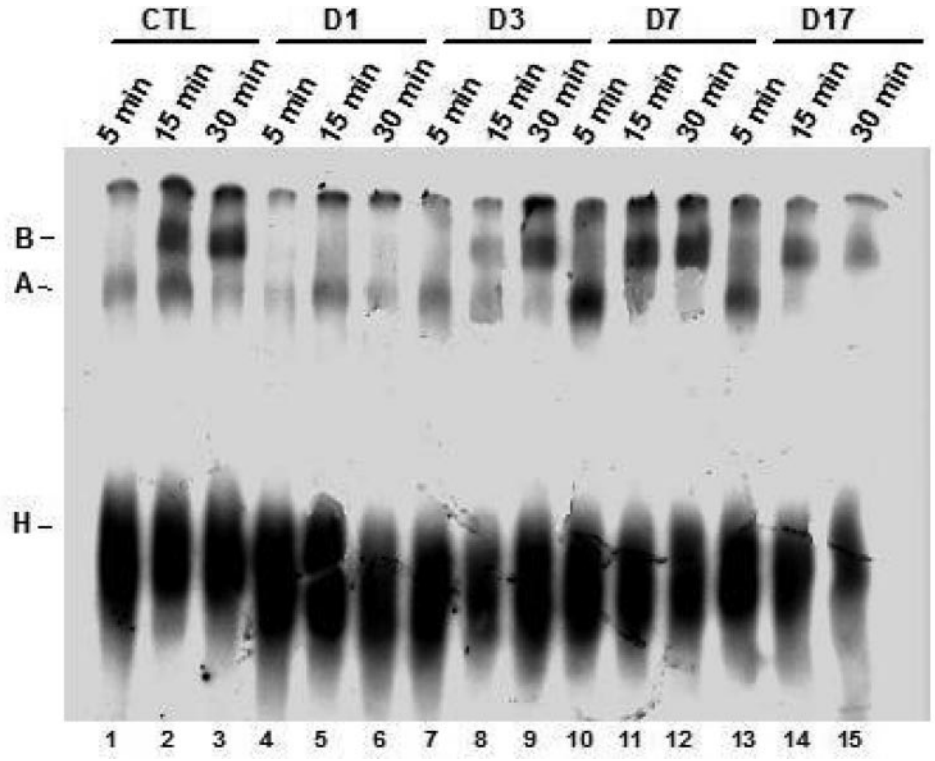

Fig. 7. Inhibition of spliceosome assembly by diospyrin derivatives. Splicing complexes formed on ^{32}P-labeled β-3S1 pre-mRNA at the indicated times in HeLa extracts alone (lanes 1–3) or containing 100 μM diospyrin derivative D1 (lanes 4–6), D3 (lanes 7–9), D7 (10–12), or D17 (13–15) were separated on a 4% nondenaturing polyacrylamide gel as described under *Materials and Methods* and revealed by autoradiography. Positions of splicing complexes H, A, and B are indicated. The potency of each drug to inhibit the spliceosome assembly was determined by quantitation of the signal of complex B relative to complex H assembled at 30 min of incubation. This signal is set 100% for the splicing reaction without drug and corresponds to 10% for D1, 85% for D3, 95% for D7, and 78% for D17.

tion has been accomplished by RNase H-inactive 2′-*O*-methylated phosphorothioated antisense oligonucleotides, which hybridize to regulatory sequences and prevent their use by the splicing machinery (Sazani and Kole, 2003a,b). Recent approaches related to ESE mechanisms in particular include antisense-induced exon skipping to suppress nonsense mutations and exon-skipping suppression by synthetic exon-specific effectors (bifunctional antisense peptide molecules or tailed antisense oligonucleotides) (Cartegni and Krainer, 2003; Skordis et al., 2003). However, as therapeutic agents, antisense oligonucleotides have the problem of limited duration and thus have to be administrated at regular intervals. In addition, although such approaches could be useful for manipulating a specific splice site selection of a known target sequence like β-globin, they will be less potent at correcting aberrant splicing, found in the patients of breast cancer, Wilm's tumor, and amyotrophic lateral sclerosis, which are not always accompanied with mutations around splice sites. As an alternative approach, small chemicals that target directly or indirectly splicing regulators can be used to inhibit and/or correct splicing. Valproic acid, a drug used in long-term epilepsy therapy, has been shown to be effective in restoring the correct splicing profile of one defective copy of the survival motor neuron SMN2 gene through transcriptional activation of the SR protein htra2 gene (Brichta et al., 2003). Some other small molecules such as aclarubicin (Andreassi et al., 2001) and sodium butyrate (Chang et al., 2001) have potency to increase an exon inclusion of the *SMN2* gene. However, the mechanisms of these effects remain unknown. Moreover, because aclarubicin and sodium butylate were found as an anticancer reagent and a histone deacetylase inhibitor affecting transcription, respectively, these compounds have obvious pleiotropic effects other than splicing.

More specific inhibitors include newly developed inhibitors of Clk/StySR protein kinase family (Muraki et al., 2004) and topoI/kinase (Labourier et al., 1999a; Pilch et al., 2001). Drugs that interfere with the kinase activity of topoisomerase I and thereby the phosphorylation status of SR proteins prevent spliceosome assembly and modulate splicing profile of several genes (Soret and Tazi, 2003). More importantly, topoI/kinase-mediated phosphorylation has been shown to be required for the ESE-dependent splicing (Soret et al., 2003), implying that small molecules can achieve selective inhibition of splicing events. These encouraging results prompted us to screen for drugs that can selectively block the kinase activity of topoI and spliceosome assembly. Diospyrin derivatives represent a novel class of drug-altering steps that occur after the spliceosome assembly. Identification of target proteins within purified spliceosome will not only shed light on the role of topoisomerase I in splicing but also on the mechanisms responsible for catalytic activation of the spliceosome.

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