

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. Previously we have demonstrated that human DNA topoisomerase I, an extensively studied target for anticancer drugs, has also an intrinsic protein kinase activity which specifically phosphorylates proteins involved in splice sites selection. Accordingly, 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. While 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. Importantly, *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, while 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.

Introduction

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 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; Soret and Tazi, 2003, Chen and Hwang, 1999; Andersen et al., 2002). 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 (RS domain) as it has been shown for the SF2/ASF splicing factor (Rossi et al., 1996; Labourier et al., 1998). Over the past, we have demonstrated that drugs which 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, topo I/kinase mediated phosphorylation have 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 twenty years, a large variety of topoisomerase I inhibitors targeting the topoisomerase I-DNA (cleavable) covalent complexes has been discovered (Meng et al., 2003). Two camptothecin derivatives, topotecan and irinotecan are on the market for the treatment of ovarian and colon cancers and several 2nd and 3rd generation of camptothecins (e.g. exatecan, diflomotecan) are currently undergoing clinical trials (Bailly, 2003a). Non-

camptothecin 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 only a few of them. 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 D-1 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 genus, 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 are frequently used in folk medicine since olden times to treat various diseases, or symptoms (reviewed in Mallavadhani et al., 1998). This compound and its derivatives have revealed interesting antitumour activities (Hazra et al., 1984, 1994; Pal et al., 1996; chakrabarty et al., 2002), and it has been also investigated as an antibacterial (Adeniyi et al., 2000; Lall et al., 2003) and antiparasitic agent, active against *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi* and *T. brucei brucei* (Hazra et al., 1987, 1995; Yardley et al., 1996), as well as *Pneumocystis carinii* (Kaneshiro et al., 2000; Cushion et al., 2000). Diospyrin and synthetic derivatives have been shown to induce apoptosis of different human tumour 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 *Leishmania 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 it 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 (D-1), the dimethyl ether D-2, the hydroquinonoid derivative D-3, the diethyl ether D-7, the aminoethanol derivative D-16, epoxide D-17 and thioethanol derivative D-18 (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. Diospyrin (D1) and its derivatives D2, D3 and D7 were synthesized and purified as previously described (Hazra et al., 1984; Sanyal et al., 2003). The syntheses of D16, D17 and D18 are to be described in the Ph.D. dissertation of M. Das Sarma at Jadavpur University (unpublished data). Stock solutions (5 mM) of the compounds were prepared in DMSO and solutions were diluted with water extemporaneously.

Biophysical measurements. DNA binding studies using melting temperature, UV-visible absorption and circular dichroism measurements were performed as previously described (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 μ g) was incubated with 3 units human topoisomerase I at 37°C for 1 h in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 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 μ g/ml. DNA samples were then added to the electrophoresis dye mixture (3 μ l) and electrophoresed at room temperature for 2 h at 120V in 1 % agarose gels containing ethidium bromide (1 μ g/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'-[³²P]-end labelling of the *EcoRI-PvuII* double digest of the pBS plasmid (Stratagene) using α -[³²P]-dATP (Amersham, 3000 Ci/mmol) and AMV reverse transcriptase (Roche).

Each reaction mixture contained 2 μ l of 3'-end [³²P] labelled DNA (~1 μ M), 5 μ l of water, 2 μ l of 10 time concentrated topoisomerase I buffer, 10 μ l of drug solution at the desired concentration (50 μ M final concentration). After 10 min incubation to ensure equilibration, the reaction was initiated by addition of 2 μ l (20 units) topoisomerase I. Samples were incubated for 45 min at 37°C prior to adding SDS to 0.25% and proteinase K to 250 μ g/ml to dissociate the drug-DNA-topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 μ l of formamide-TBE loading buffer, denatured at 90°C for 4 min then chilled in ice for 4 min prior to loading on to 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 *E. coli*, respectively, as previously described (Rossi et al., 1996, 1998, Ge et al., 1991). The reaction mixtures for protein kinase assay contained 100 ng of recombinant topo I/kinase, 300 ng of recombinant SF2/ASF protein, 1 μ M ATP, 3 μ Ci [γ - 32 P]ATP (3000 Ci/mmmole) and 1 μ l of Diospyrin or DMSO in a final volume of 20 μ l of buffer A (50 mM Hepes pH 7.0, 10 mM MgCl₂, 3 mM MnCl₂, 50 mM KCl, 0.5 mM DTT). The samples were incubated at 30°C for 30 min, then mixed with 6 μ l of 4X loading buffer and applied on a 12% SDS-polyacrylamide gel. Radioactivity incorporated into SF2/ASF was detected by autoradiography of the dried gel and quantitated by a Molecular Dynamics imaging analyzer using ImageQuant software version 3.22.

Splicing and spliceosome assembly assays. The single-intron human β -globin construct pSPH β m3S1 was previously described (Labourier et al., 1999b). Radiolabelled β 3S1 RNA was synthesized by *in vitro* transcription in presence of 20 units of SP6 RNA polymerase (Boehringer), 1 μ g of pSPH β m3S1 plasmid linearized with *Bam*HI and 5 μ M [α - 32 P] UTP (3000 Ci/mmmol) in 25 μ l reactions according to manufacturer conditions. The splicing reactions were performed under standard conditions for the indicated time in a total volume of 20 μ l containing 50 fmol labelled pre-mRNA and 10 μ l 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 were performed as described (Konarska and Sharp, 1987). Aliquots (5 μ l) from the various reactions treated with heparin (2 mg/ml) were

mixed with 1 μ l of 97% glycerol, 1% bromophenol blue and resolved directly on a 4% non-denaturing 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 μ M. The absorption spectra of the drugs was 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 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 references compounds camptothecin (for topoisomerase I) and etoposide (for topoisomerase II), which stimulate DNA single strand (Lane CPT in Fig. 2A) or double strand (Lane Etop. in Fig. 2B) 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 analogues. The amount of nicked DNA remained high in the presence of compounds D2, D16, D17, D18 whereas it was reduced by more than 50% in the presence of compounds D1, D3 or D7.

To investigate further this antagonism, DNA cleavage reactions were performed with a 117-bp radiolabelled DNA substrate which 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 favourable 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 compound 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. Interestingly, these observations are consistent with cytometry data showing that the compounds antagonize the pro-apoptotic activity of camptothecin to different extent (data not shown).

Selective inhibition of topo I-kinase activity by diospyrin derivatives. The data above suggested that diospyrin derivatives possibly bind topoisomerase I and thereby changes 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 alter 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 μ M of diospyrin (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 Topo I/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 (pSpH β m-3S1) and whose splicing strictly depends on a ASF/SF2 enhancer, demonstrated that compounds D1 and D3 (at 50 μ M) 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-exon2) were detected after 2 hours 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 D3 (compare lane 8 and lane 11) and no inhibition of splicing was observed with a concentration up to 50 μ M of compounds D2 and D18 (data not shown). In contrast, compound D7 and D17 induced a different kind of splicing inhibition, as they were able to induce accumulation of splicing intermediates (Fig. 6A, lanes 1-3 and 4-6, respectively). This

result indicates that the later 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 compounds were pre-incubated for 15 min with nuclear extract before adding the substrate. While this pre-incubation 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 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, since the splicing profile is similar to control if the drugs were added after 1h of pre-incubation (Fig. 6B, compare lanes 4 and 8 with lane 9). Altogether, these results further confirm that compound D3 inhibits splicing at both catalytic steps, while D7 affect only the second catalytic step.

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. ³²P-labelled 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 fashion at early time points and correspond to complex A and complex B, in addition to a fast migrating non specific complex H (lane 3). The complex A formation involves the stable binding of U2 snRNP to the pre-

mRNA sequences near to the 3' splice site (pre-spliceosome) and subsequent binding of a tri-snRNP particle U4/U5/U6, rather than the individual snRNPs give rise to the (B) complex (spliceosome). Therefore, at 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 appears to be a critical step in the initiation of splicing reactions by the assembled B complex (Pilch et al., 2001). Surprisingly, with the exception of D1 (lanes 4-6) which markedly inhibited the complex B formation, all other derivatives did not alter neither complex A nor complex B formation. The complexes assembled in the presence of the various derivatives were similar to the complexes formed in the control reaction following the same kinetics (lanes 7-9, 10-12 and 13-15). Thus, diospyrin derivatives highlight a novel mechanism of splicing inhibition which is different from that mediated by indolocarbazoles. The later compounds inhibit the earliest step of spliceosome assembly that precedes binding of U2 snRNP, while the former act at later stages. Compounds 3, 7 and 17 inhibit splicing at either a pre-catalytic step (D3) or the second catalytic step (D7, 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 *Leishmania donovani* parasite (Ray et al., 1998) or 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 it 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 since 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 topoisomerase 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 tumour cells. The vast 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 for examples. Diospyrin does not belong to this group of poison. 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 of diospyrin (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 μM and 5 μM for D1 and D3, respectively. These two compounds were found to potently inhibit the splicing of a reporter pre-mRNA. Interestingly, the splicing reaction can be dissected with the diospyrins. Compound D3 inhibits splicing at a pre-catalytic step of the splicing reactions, whereas compounds D7 and D17 affect the second catalytic step. Diospyrin D1 is different as 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. Since two of the diospyrin derivatives tested here are able to target topo I/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 analogues that can be crosslinked 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). Targetting either the mutated sequences or the factors that bind to them may prove to be an important strategy to correct aberrant splicing. Thus, successful correction have been accomplished by RNase H inactive 2'-O-methylated phosphorothioated antisense-oligonucleotides (2MeAO), which hybridize to regulatory sequences and prevent their use by the splicing machinery (Sazani and Kole, 2003a; Sazani and Kole, 2003b). 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. Also, while 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 (ALS), 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. Recently, valproic acid, a drug used in long-term epilepsy therapy, has been effective to restore 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, since aclarubicin and sodium butylate were found as an anti-cancer 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/Sty SR protein kinase family (Muraki et al., 2004) and topo I/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, topo I/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 topo I and spliceosome assembly. Diospyrin derivatives represent a novel class of drugs 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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Figure legends

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

Fig. 2. Effect of the diospyrin derivatives on the stabilisation of covalent DNA-topoisomerase complexes. Native supercoiled DNA (lane DNA, 0.15 μ g) 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 μ M each). Diospyrin and its derivatives were tested at 10 and 50 μ M. DNA samples were separated by electrophoresis on agarose gels containing ethidium bromide (1 μ g/ml). Gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled.

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

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

Fig. 5. Inhibition of topoisomerase I kinase activity by diospyrin derivatives. Phosphorylation of SF2/ASF by topo I/kinase was performed as described (Materials and Methods) with increasing concentrations (1 μ M, 5 μ M, 25 μ M and 100 μ M 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 phosphor imaging. The position of SF2/ASF was localized by staining the gel with coomassie blue. The panels show 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 μ M for D7 and D17, but only 1 μ M and 5 μ M for D1 and D3, respectively.

Fig. 6. Inhibition of pre-mRNA splicing by diospyrin derivatives *in vitro*. (A) 32 P-labelled β -3S1 pre-mRNA (50 pmol) was incubated in 10 μ l of HeLa cell nuclear extract under splicing conditions without (lane 13) or with 25 μ M (lanes 1, 4, 7 and 10), 50 μ M (lanes 2,5,8 and 11) and 100 μ M (lanes 3,6,9 and 12) of the indicated diospyrin derivative. (B) Splicing reactions incubated for 60 min without (lane 9) or with 50 μ M of 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-labelled 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* of each panel. The open box indicate the first exon, the black box in the second exon correspond 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-labelled β -3S1 pre-mRNA at the indicated times in HeLa extracts alone (lanes 1-3) or containing 100 μ M of diospyrin derivative D1 (lanes 4-6), D3 (lanes 7-9), D7 (10-12)

or D17 (13-15) were separated on a 4% non denaturing polyacrylamide gel as described in 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 D-1, 85% for D-3, 95% for D-7 and 78% for D-17.

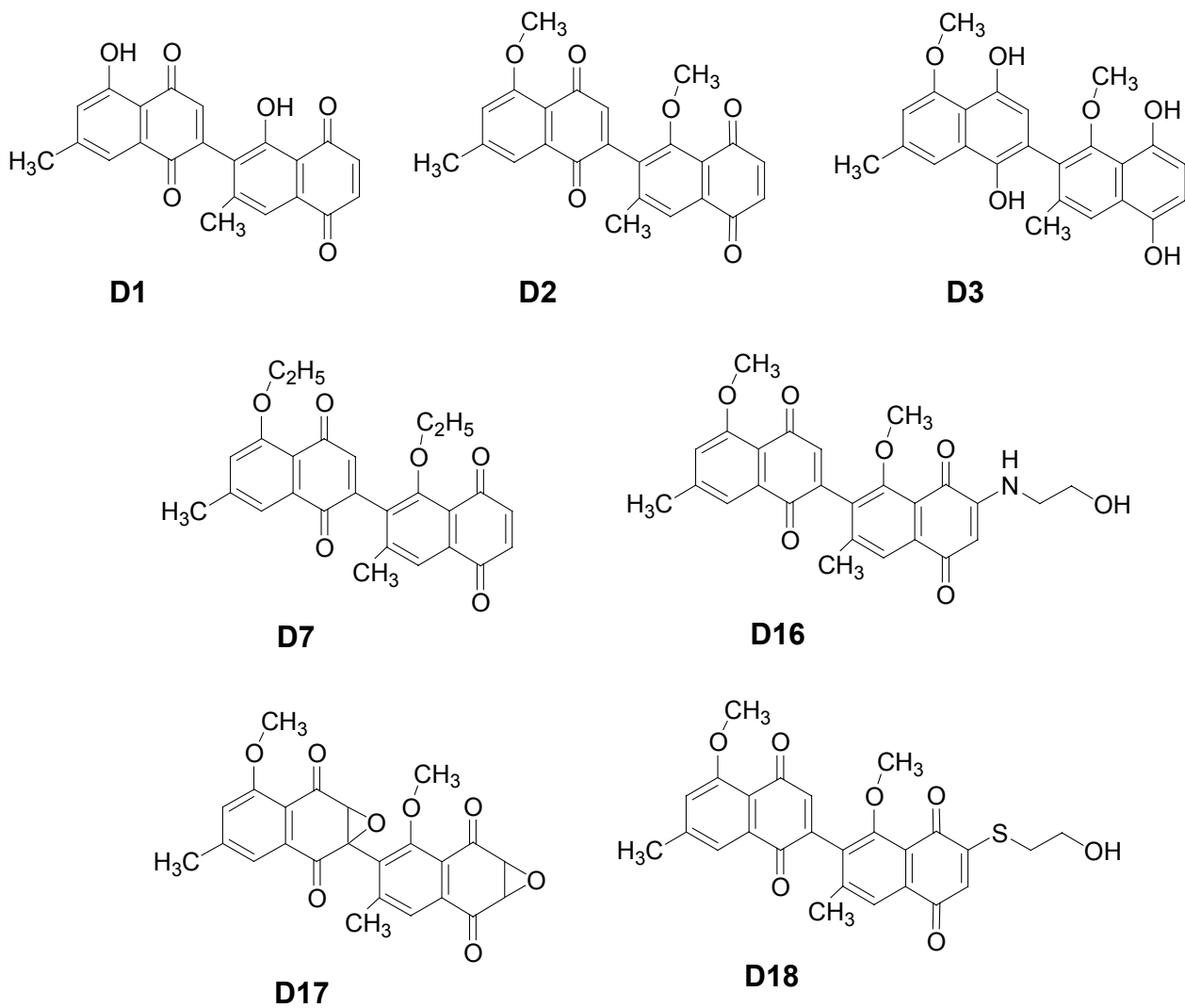


Figure 1
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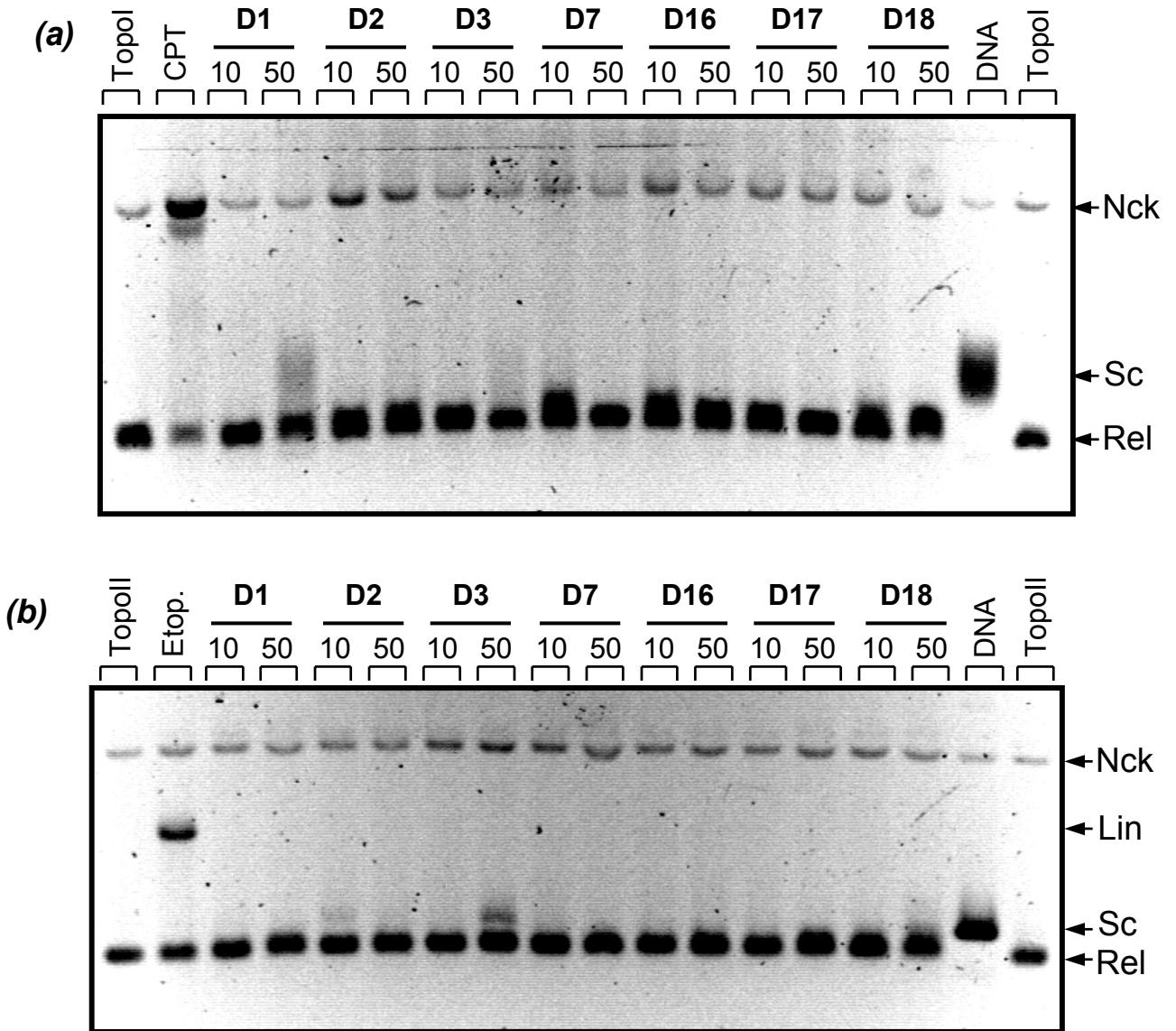


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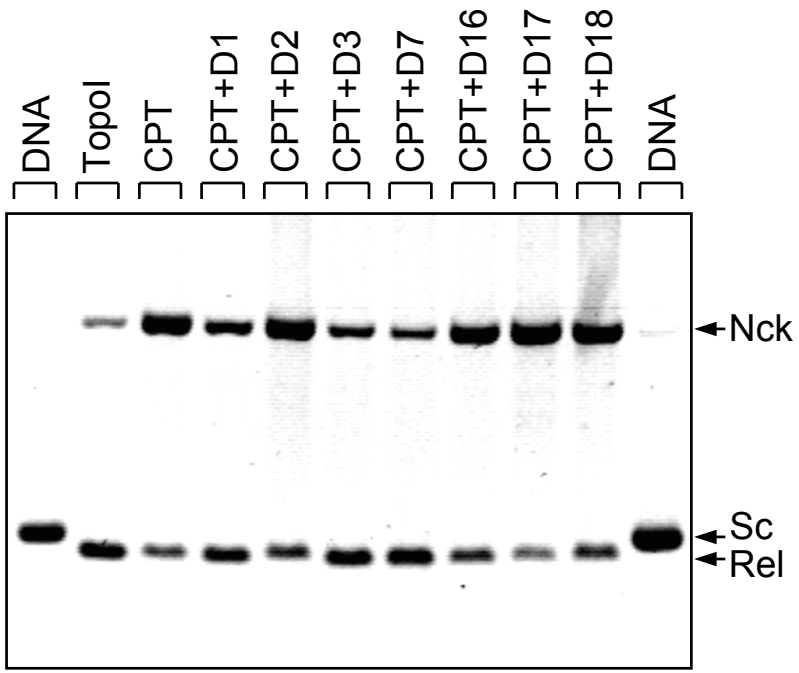


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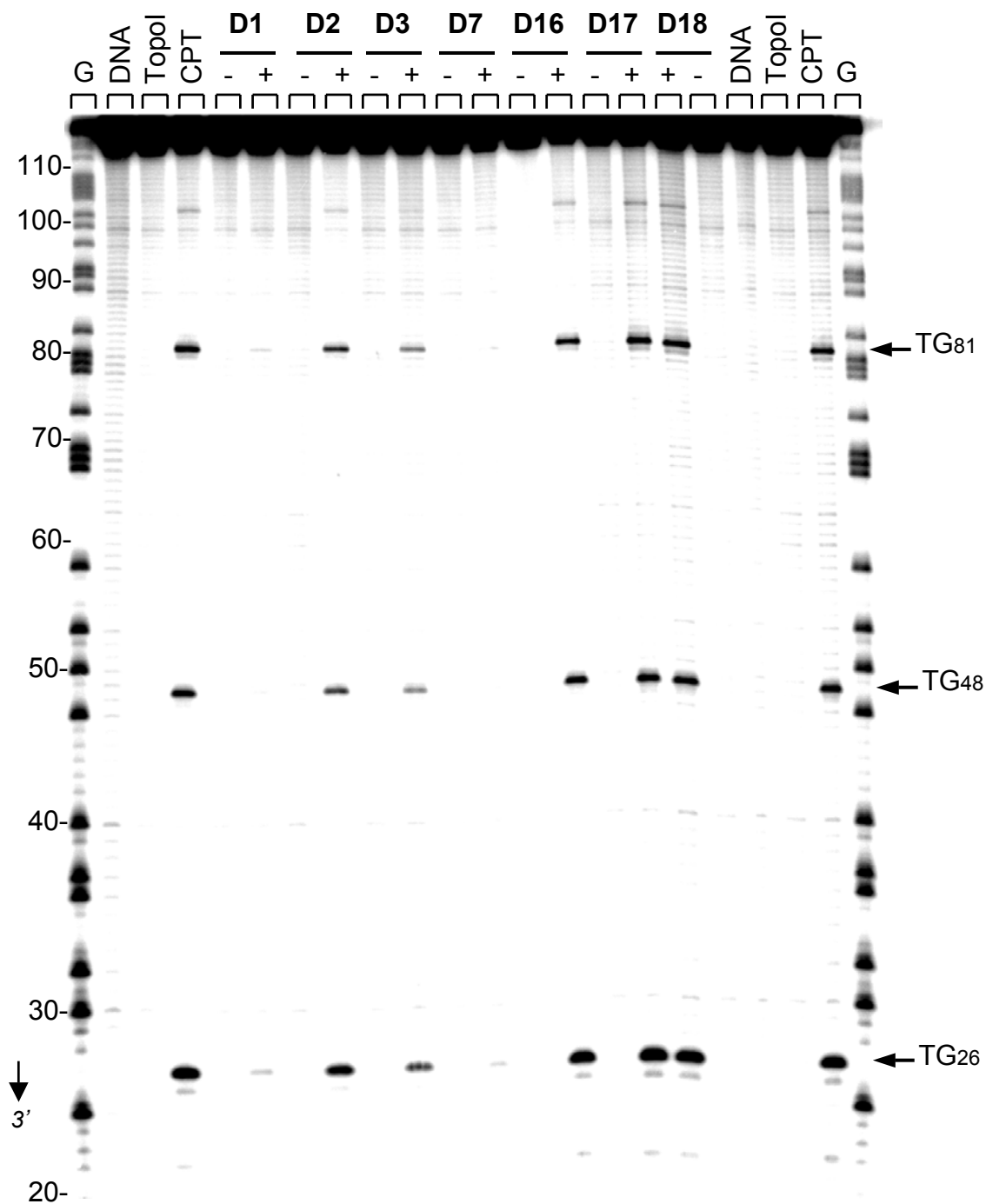


Figure 4
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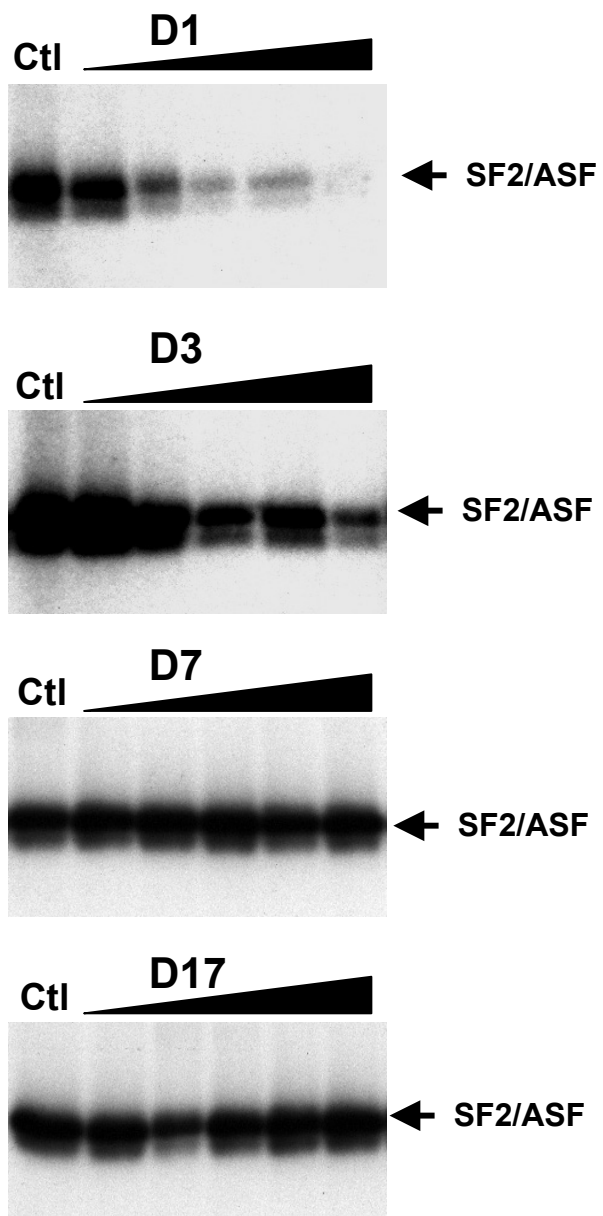


Figure 5
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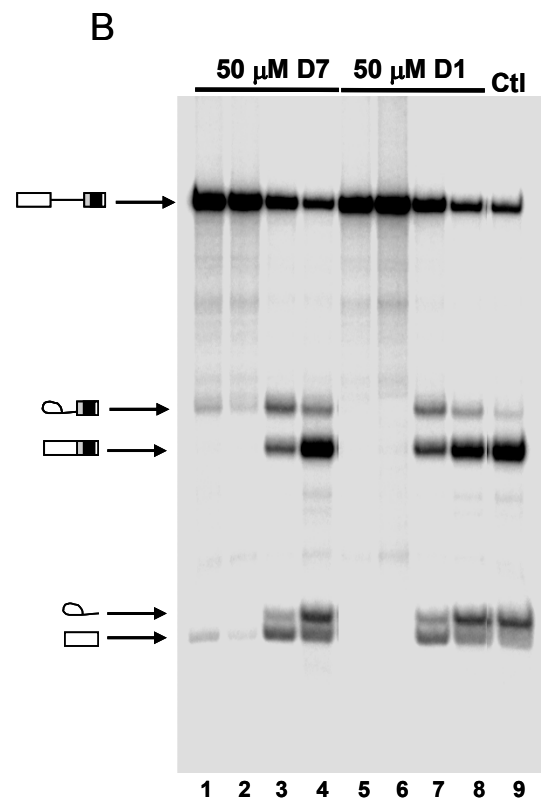
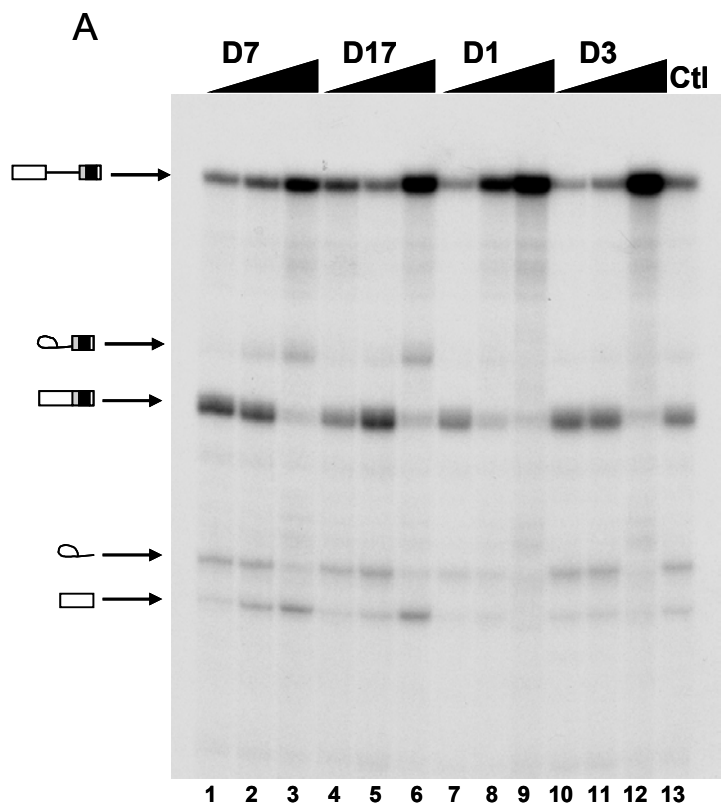


Figure 6
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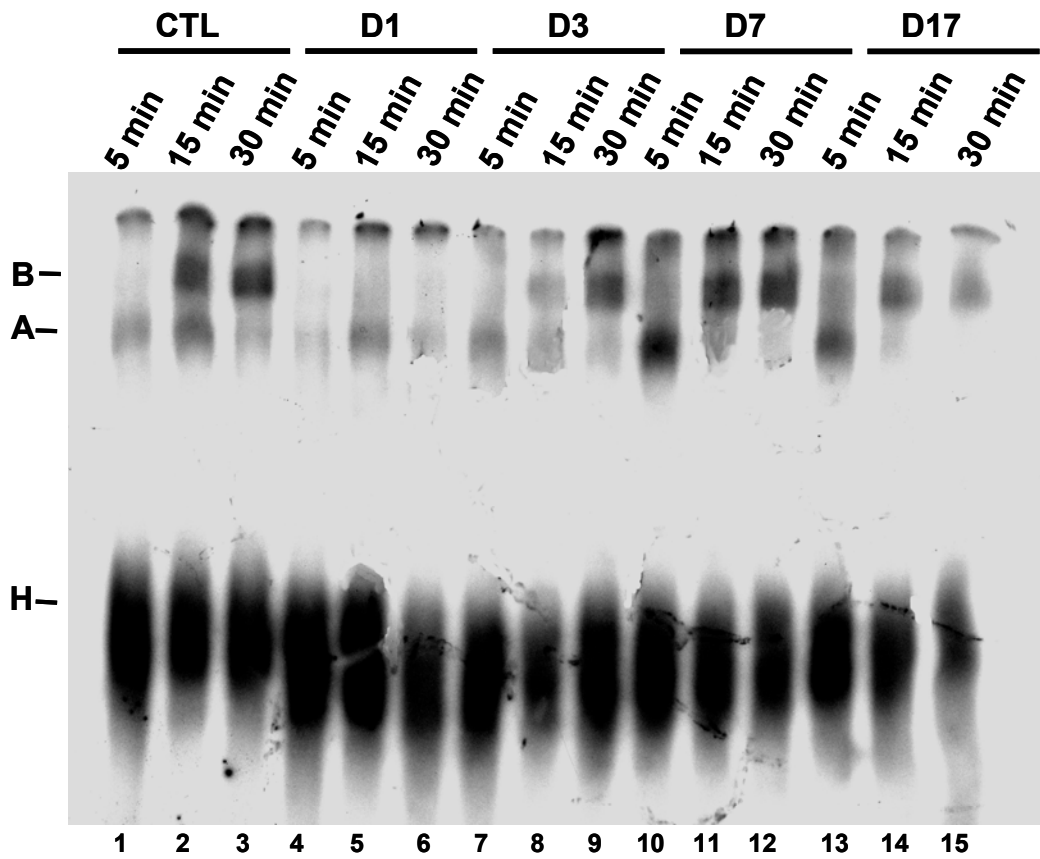


Figure 7
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