

Novel Betulin Derivatives as Antileishmanial Agents with Mode of Action Targeting Type IB DNA Topoisomerase^[S]

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ABSTRACT

Toward developing antileishmanial agents with mode of action targeted to DNA topoisomerases of *Leishmania donovani*, we have synthesized a large number of derivatives of betulin. The compound, a natural triterpene isolated from the cork layer of *Betula* spp. plants exhibits several pharmacological properties. Three compounds (disuccinyl betulin, diglutaryl dihydrobetulin, and disuccinyl dihydrobetulin) inhibit growth of the parasite as well as relaxation activity of the enzyme type IB topoisomerase [*Leishmania donovani* topoisomerase I (LdTOP1LS)] of the parasite. Mechanistic studies suggest that these compounds interact with the enzyme in a reversible manner. The stoichiometry of these compounds binding to LdTOP1LS is 1:1 (mole/mole) with a dis-

sociation constant on the order of $\sim 10^{-6}$ M. Unlike CPT, these compounds do not stabilize the cleavage complex; rather, they abrogate the covalent complex formation. In processive mode of relaxation assay condition, these compounds slow down the strand rotation event, which ultimately affects the relaxation of supercoiled DNA. It is noteworthy that these compounds reduce the intracellular parasite burden in macrophages infected with wild-type *L. donovani* as well as with sodium antimony gluconate resistant parasite (GE1). Taken together, our data suggest that these betulin derivatives can be exploited as potential drug candidates against threatening drug resistant leishmaniasis.

Introduction

Leishmaniasis is one of the major fatal parasitic diseases affecting millions of people around the world. The disease presents a variety of symptoms ranging from self-healing cutaneous lesions through the metastasizing mucocutaneous form to the often-fatal visceralizing form (Olivier et al., 2005). Development of parasites resistant to most commonly used pentavalent antimonials (e.g., glucantime and Pentostam) (Ashutosh et al., 2007) or arsenite (Haimeur et al., 1999), together with lack of available vaccine (Kedzierski, 2010; Nylén and Gautam 2010), pose a threat to cure the

disease. This led to treatment with current drugs, such as expensive pentamidine (Mishra et al., 1992), amphotericin B (AmBisome; Astellas Pharma, Deerfield, IL) (Wortmann et al., 2010; Solomon et al., 2011) and the teratogenic drug miltefosine (Impavido; Aeterna Zentaris, Quebec, QC, Canada) (Sindermann and Engel, 2006; Seifert et al., 2007). Therefore, improvised drug therapy of *Leishmania* spp. infection is still desirable, and the need for new molecular targets on which to base improved therapies is clear and justified.

DNA topoisomerases are a family of DNA-processing enzymes that release torsional stress in the DNA. The DNA-resolving enzymes catalyze breakage and rejoining of DNA strands during several vital life processes, such as replication, repair, recombination, transcription, chromosome segregation, and integration (Liu, 1989; Wang, 1996). These DNA manipulators modulate the dynamic nature of DNA secondary and higher order structure by transient nicking/closing of DNA strands (Champoux, 2001).

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ABBREVIATIONS: LdTOP1L, *Leishmania donovani* topoisomerase I large subunit; LdTOP1S, *Leishmania donovani* topoisomerase I small subunit; CPT, camptothecin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; pBS, pBluescript; BSA, bovine serum albumin; EtBr, ethidium bromide; FBS, fetal bovine serum; RPMI-FBS, RPMI 1640 medium (supplemented with 100 IU/ml penicillin and 100 μ g/ml streptomycin) containing 10% (v/v) heat-inactivated FBS; DiSB, 3-O,28-O-disuccinyl betulin (**4**); DiGDHB, 3-O,28-O-diglutaryl dihydrobetulin (**8**); DiSDHB, 3-O,28-O-disuccinyl dihydrobetulin (**10**); LdTOP1LS, *Leishmania donovani* type IB topoisomerase; Sb^S, antimony-sensitive; Sb^R, antimony-resistant; *L*_k, Linking number.

The topoisomerases can be classified into types I and II, both of which are of equal importance as chemotherapeutic targets (Fortune and Osherooff, 2006; Pommier, 2006; Teicher, 2008; Nitiss, 2009). Unlike eukaryotic type IB topoisomerases, kinetoplastid topoisomerases IB possess heterodimeric structure consisting of a large subunit of 635 amino acids (LdTOP1L) and a small subunit of 262 amino acids (LdTOP1S) (Das et al., 2004). Reconstitution of the large subunit (L) bearing DNA binding "VAILCNH" motif with the small subunit (S) bearing the active site SKXXY motif occurs through protein-protein interaction to form an active heterodimeric enzyme within the parasite (Das et al., 2004).

Improvised drug therapy of *Leishmania* spp. infections has become a need and use of topoisomerase inhibitors for antileishmanial therapy are of immense interest. These inhibitors can be broadly classified into two classes: the class I inhibitors stabilize the formation of topoisomerase-DNA covalent complex (cleavable complex) and are known as "topoisomerase poisons." Inhibitors with property to abrogate only the catalytic property of the enzyme and thus interfere with the formation of covalent complex formation are termed class II inhibitors (Li et al., 1993; Chowdhury et al., 2003). The most well studied type IB topoisomerase poison camptothecin (CPT) promotes protein-DNA cleavable complex formation (Bodley and Shapiro, 1995) leading to apoptosis-like cell death (Sen et al., 2004) in *Leishmania donovani*.

We have reported previously that dihydrobetulinic acid, isolated from the leaves of *Bacopa monnieri*, has profound effect on intracellular amastigotes with an IC_{50} value of 4.1 μ M. Dihydrobetulinic acid inhibits *Leishmania* spp. topoisomerases and induces apoptosis in *L. donovani* with an effective clearance of parasites from infected golden hamsters (Chowdhury et al., 2003).

Betulin [lup-20(29)-ene-3 β ,28-diol] is an abundant naturally occurring triterpene and constituent of the cork layer of the outer bark of *Betula alba*, *Betula pendula*, *Betula pubescens*, and *Betula platyphylla*. Betulin and the more active form betulinic acid exhibit antimalarial (Steele et al., 1999), anti-HIV, and anti-inflammatory (Reutrakul et al., 2010) as well as cytotoxic activities on cancer cell lines (Laszczyk, 2009).

In the present study, we report some novel derivatives of betulin, which interfere with the relaxation activity of *L. donovani* topoisomerase I. These derivatives act like class II inhibitors and abrogate topoisomerase I-DNA complex formation. These compounds prevent the strand rotation step by binding to enzyme in 1:1 ratio through a weak interaction. It is noteworthy that these betulin derivatives can efficiently reduce parasite burden from macrophage cultures infected with antimony resistant and sensitive *Leishmania* spp. parasites having less effect on host cells. Thus, the need for new therapeutic agents against antimony-resistant strains of *Leishmania* spp. can be exploited using modification of plant-derived betulin.

Materials and Methods

Chemicals. DMSO and camptothecin were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in 100% DMSO at a concentration of 20 mM and stored at -20°C . Recombinant human topoisomerase I was purchased from TopoGEN, Inc. (Port Orange, FL).

Synthesis of Several Derivatives of Betulin and Dihydrobetulin. Hydrogenation of betulin was carried out by dissolving 400 mg of betulin in a minimal volume of dry ethyl acetate (50 ml) and a little dichloromethane was added to clear the solution. Then catalytic amount of 10% palladized charcoal was added and exposed to a hydrogen gas atmosphere, the reaction mixture was stirred overnight at room temperature and filtered, and the hydrogenated product was dried over vacuum.

For general preparations of betulin or dihydrobetulin derivatives, a mixture of betulin (50 mg, 1 Eq) or dihydrobetulin (50 mg, 1 Eq) and 4-*N,N*-dimethylaminopyridine (2 mg) was dissolved in pyridine (2 ml). Acid anhydride (10 Eq) was added, and the reaction mixture was placed in 80–90°C pre heated oil bath and stirred for 24 h in anhydrous condition. After the usual work-up, the organic layer was collected and dried under reduced pressure. All the reaction products were purified by chromatography over silica gel (60–120 mesh) and were monitored through thin layer chromatography (solvent system, 2% methanol in chloroform).

Purification and Reconstitution of Recombinant Proteins of Topoisomerase I Activity. *Escherichia coli* BL21 (DE3)pLysS cells harboring pET16bLdTOP1L and pET16bLdTOP1S, described previously (Das et al., 2006), were separately induced at $OD_{600} = 0.6$ with 0.5 mM isopropyl β -D-thiogalactoside at 22°C for 12 h. Cells harvested from 1 liter of culture were separately lysed by lysozyme/sonication, and the proteins were purified through Ni^{2+} -nitriloacetate-agarose column (QIAGEN, Valencia, CA) followed by a phosphocellulose column (P11 cellulose; Whatman) as described previously (Das et al., 2004). Finally, the purified proteins LdTOP1L and LdTOP1S were stored at -70°C . The concentrations of each protein were quantified by Bradford reaction using a Protein Estimation Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Purified LdTOP1L was mixed with purified LdTOP1S at a molar ratio of 1:1 at a total protein concentration of 0.5 mg/ml in reconstitution buffer [50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol]. The mixture was dialyzed overnight at 4°C, and dialyzed fractions were used for plasmid relaxation activity (Das et al., 2004, 2005).

Plasmid Relaxation Assay. The type I DNA topoisomerases were assayed by decreased mobility of the relaxed isomers of supercoiled pBlueScript (SK⁺) [pBS (SK⁺)] DNA in agarose gel. The relaxation assay was carried out as described previously with LdTOP1LS (Sen et al., 2004; Das et al., 2005), serially diluted in the relaxation buffer (25 mM Tris-HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM MgCl₂, 50 mM KCl, 25 mM EDTA, and 150 μ g/ml BSA) and supercoiled plasmid pBS (SK⁺) DNA (85–95% was negatively supercoiled, with remainder being nicked circles). The reconstituted enzyme LdTOP1LS was assayed at 50 mM KCl concentration, whereas human topoisomerase I was assayed at 150 mM KCl concentration as described previously (Ganguly et al., 2006). For all kinetic studies, the reaction mixtures containing the buffer and DNA were heated to 37°C before addition of the enzymes. The reactions were rapidly quenched using stop solution and kept on ice. The gels were stained with ethidium bromide (EtBr) (0.5 μ g/ml), and the amount of supercoiled monomer DNA band fluorescence was quantified by integration using Gel Doc 2000 under UV illumination (Bio-Rad Quantity One Software), as described previously (Das et al., 2005). Initial velocities (nanomoles of DNA base pairs relaxed per min) were calculated using the following equation: Initial Velocity = $([\text{Supercoiled DNA}]_0 - [\text{Int}_t \cdot [\text{supercoiled DNA}]_0 / [\text{Int}_0]] / t$, where $([\text{Supercoiled DNA}]_0)$ is the initial concentration of supercoiled DNA, $[\text{Int}_0]$ is the area under the supercoiled DNA band at zero time, and $[\text{Int}_t]$ is the area at the reaction time t (Osherooff et al., 1983). The effect of DNA concentration on the kinetics of relaxation was examined over a range of 4 to 60 nM supercoiled pBS (SK⁺) DNA (0.16–2.4 μ g/25 μ l of reaction mixture) at constant concentration of 10 mM MgCl₂ and 0.98 nM enzyme (LdTOP1LS) at 37°C for 1 min. The data were

analyzed by a Lineweaver-Burk plot. Intercept of the y -axis is $1/V_{\max}$, and catalytic-center activity = $V_{\max}/\text{enzyme concentration}$ (plasmid molecules relaxed per minute per molecule of enzyme).

Plasmid Cleavage Assay. Cleavage assay was carried out as described previously (Ray et al., 1998). In brief, 50 fmol of pHOT1 supercoiled DNA (containing topoisomerase I cleavage site) and 100 fmol of reconstituted LdTOP1LS were incubated in standard reaction mixture (50 μ l) containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, and 30 μ g/ml BSA in the presence of various concentrations of inhibitors at 37°C for 30 min. The reactions were terminated by adding 1% SDS and 150 μ g/ml proteinase K and further incubated for 1 h at 37°C. DNA samples were electrophoresed in 1% agarose gel containing 0.5 μ g/ml EtBr to resolve more slowly migrating nicked product (form II) from the supercoiled molecules (form I).

Suicide Cleavage Assay. A 14-mer (5'-GAAAAAGACTT \downarrow AG-3') oligonucleotide (ML14) containing a topoisomerase IB-specific cleavage site was 5'-³²P-end-labeled and annealed to 25-mer (3'-CTTTTCTGAATCTTTTAAAAAT-5') oligonucleotides (ML25) as described previously (Das et al., 2005). The suicidal cleavage reaction was carried out with 5 nM DNA substrate and 0.2 μ M enzyme (LdTOP1LS) in 20- μ l reaction mixtures under standard assay conditions (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA, and 50 mM KCl) at 23°C for 2 h in the presence or absence of betulin inhibitors. Finally, all the reactions were stopped by adding SDS, and DNAs were subsequently precipitated by ethanol. Samples were digested with 5 μ l of 1 mg/ml trypsin, electrophoresed in 12% (w/v) denaturing polyacrylamide gel, and autoradiographed.

Job Plot. The binding stoichiometry for each of the inhibitors with LdTOP1LS was determined using the method of continuous variation (Huang, 1982; Ward, 1985). Several mixtures of each of the three inhibitors and LdTOP1LS were prepared by continuously varying the concentrations of recombinant LdTOP1LS and one inhibitor in the mixtures, keeping the total concentration of inhibitor plus recombinant LdTOP1LS constant at 1.25 μ M. Reaction mixtures were incubated for 10 min at 25°C, and the quenching of tryptophan fluorescence was recorded at 350 nm upon excitation at 295 nm on a luminescence spectrometer (LS55; PerkinElmer Life and Analytical Sciences, Waltham, MA).

Spectrofluorimetric Binding Assay. LdTOP1LS (200 nM) was incubated in fluorescence buffer (20 mM Tris/HCl, 50 mM NaCl, and 10 mM MgCl₂) with varying concentrations of each of the inhibitors (0–22.5 μ M) at 25°C for 10 min. The fluorescence intensity was measured at 350 nm upon excitation at 295 nm. Excitation and emission slit widths were 2.5 and 5 nm, respectively. The measurements in the fluorescent values (performed in duplicate) in the presence of continuous increasing concentrations of inhibitors were corrected for the inner filter effect for excitation and emission wavelength. The fraction of binding sites (B) occupied by inhibitor was determined by the following equation: $B = (F_0 - F)/F_{\max}$, where F_0 is the fluorescence intensity of LdTOP1LS alone in the absence of any inhibitors, and F is the corrected fluorescence intensity of LdTOP1LS in the presence of inhibitor. F_{\max} is obtained from the plot of $1/(F_0 - F)$ versus $1/[X]$ and by extrapolating of $1/[X]$ to zero as shown in Fig. 6B, where $[X]$ is the concentration of one of the three inhibitors. The dissociation constant (K_d) was determined as described previously (Acharya et al., 2008) using the following equation: $F_{\max}/(F_0 - F) = 1 + K_d/L_f$ where L_f denotes the free concentration of inhibitor; $L_f = C - B \times [J]$, where C is the total concentration of inhibitor and $[J]$ is the molar concentration of ligand-binding sites using a stoichiometry from the Job plot.

Parasite Maintenance and Cultures. Two strains of *L. donovani*, one sodium antimony gluconate-sensitive (Sb^s) MHOM/IN/1983/AG83 (AG83) and one sodium antimony gluconate-resistant (Sb^r) GE1 (raised in hamsters) (Basu et al., 2005), were used. Amastigotes obtained from the spleens of infected hamsters were cultured at 22°C to obtain promastigotes and cultured in M199 containing 20% (v/v) heat-inactivated FBS supplemented with 100 IU/ml peni-

cillin and 100 μ g/ml streptomycin at 22°C to obtain promastigotes. Promastigotes were further grown in 10% (v/v) heat-inactivated FBS for 3 to 5 days at 22°C before use.

In Vitro Macrophage Infection. BALB/c mice, originally obtained from The Jackson Laboratory (Bar Harbor, ME) and reared in the institute animal facilities, were used for experimental purposes with prior approval of the animal ethics committee. Macrophages were isolated from mice 36 to 48 h after injection (intraperitoneal) with 2% (w/v) hydrolyzed starch by peritoneal lavage with ice-cold phosphate-buffered saline. Cells were washed and cultured for 18 to 24 h (for adherence) in RPMI 1640 medium (supplemented with 100 IU/ml penicillin and 100 μ g/ml streptomycin) containing 10% (v/v) heat-inactivated FBS (RPMI-FBS) at 37°C at 5% CO₂ in air on sterile cover glass (22 \times 22 mm) placed in disposable plates 35 mm in diameter (Tarsons India Ltd., Kolkata, India). The culture medium was washed off, and fresh RPMI-FBS was added. Approximately 5×10^5 macrophages were maintained for proper distribution on cover glass. During the course of this study, macrophages were infected with promastigotes at a macrophage-to-parasite ratio of 1:10 in RPMI-FBS for 6 h to ensure entry of parasite. After incubation, unphagocytosed parasites were removed by washing with medium, and cells were resuspended in RPMI-FBS at 37°C, 5% CO₂ and then incubated for 6 h. Cultures were transferred to a CO₂ incubator at 37°C and incubated for another 10 to 12 h. Respective inhibitors were added at different concentrations (ranging from 1 to 20 μ M) to infected macrophages and left for another 24-h period. Cells were then fixed in methanol and stained with 2% Giemsa. Percentages of infected cells and total number of intracellular parasites were determined by manual counting in at least 200 cells using light microscope.

Results

Derivatives of Betulin and Dihydrobetulin Inhibit the Catalytic Activity of *L. donovani* Topoisomerase IB. A series of analogs of betulin (Fig. 1A, 1) [e.g., 3-*O*,28-*O*-diglutaryl betulin (2), 3-*O*,28-*O*-diphthaloyl betulin (3), 3-*O*,28-*O*-disuccinyl betulin (4), 3-*O*,28-*O*-diacetyl betulin (5), and 3-*O*,28-*O*-dicrotonyl betulin (6)] was prepared from betulin as mentioned under *Materials and Methods*. Dihydrobetulin (Fig. 1B, 7) was synthesized by hydrogenation of betulin, and similar reactions were carried out to generate 3-*O*,28-*O*-diglutaryl dihydrobetulin (8), 3-*O*,28-*O*-diphthaloyl dihydrobetulin (9), 3-*O*,28-*O*-disuccinyl dihydrobetulin (10), 3-*O*,28-*O*-diacetyl dihydrobetulin (11), and 3-*O*,28-*O*-dicrotonyl dihydrobetulin (12).

The effect of these chemically synthesized derivatives of betulin and dihydrobetulin on the unusual bisubunit type IB topoisomerase of *L. donovani* (LdTOP1LS) was examined by plasmid relaxation assays as described under *Materials and Methods*. The relaxation assays were carried out under standard assay conditions in which the plasmid DNA and the enzyme were present at a molar ratio of 3:1. Under this reaction condition, in the absence of any inhibitor, LdTOP1LS relaxes supercoiled plasmid DNA completely after 30 min of incubation (Roy et al., 2008). Screening of all the above compounds on relaxation activity of LdTOP1LS was carried out using 2% (v/v) DMSO. Betulin and dihydrobetulin have no inhibitory effect on LdTOP1LS but 3-*O*,28-*O*-disuccinyl betulin (DiSB) (4) and 3-*O*,28-*O*-diglutaryl dihydrobetulin (DiGDHB) (8) completely inhibit the enzyme activity at 200 μ M, while 3-*O*,28-*O*-disuccinyl dihydrobetulin (DiSDHB) (10) achieves approximately 93% inhibition at the same condition (data not shown). DiSB, DiGDHB, and DiSDHB show little inhibition at 10 μ M (Fig. 2A, lanes 4, 9, 14, respectively)

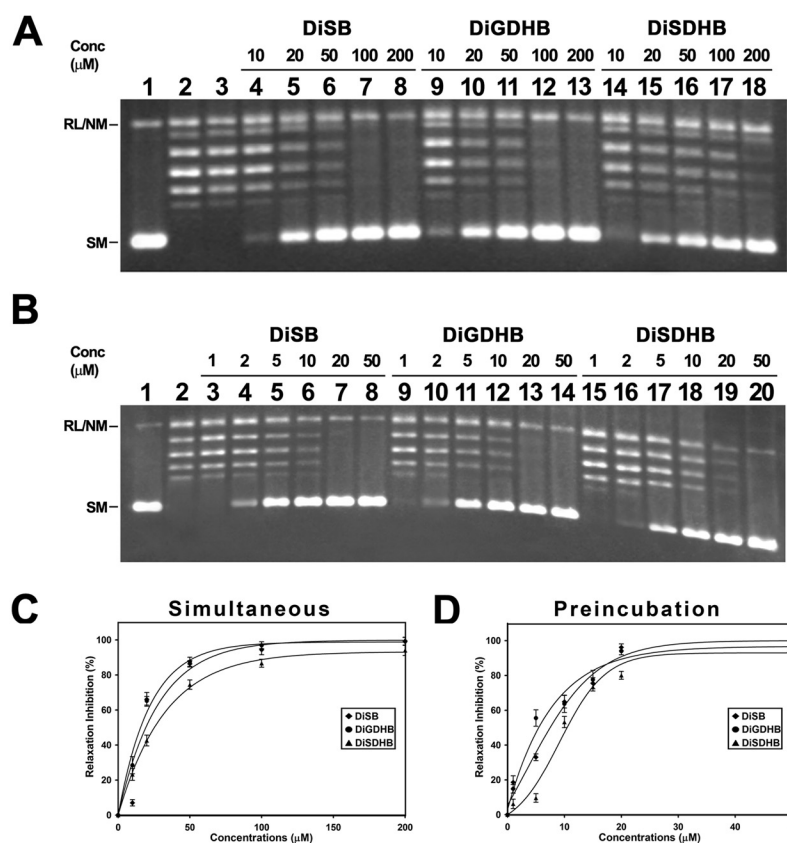


Fig. 2. Inhibition of catalytic activity of LdTOP1LS by derivatives of betulin and dihydrobetulin. A, relaxation of supercoiled pBS (SK⁺) DNA with reconstituted LdTOP1LS at a molar ratio of 3:1. Lane 1, 90 fmol of pBS (SK⁺) DNA; lane 2, same as lane 1, but simultaneously incubated with 30 fmol of LdTOP1LS for 30 min at 37°C; lane 3, same as lane 2, but in presence of 2% (v/v) DMSO; lanes 4–8, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μM DiSB, respectively; lanes 9 to 13, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μM DiGDHB, respectively; lanes 14 to 18, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μM DiSDHB, respectively. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. B, preincubation of LdTOP1LS with respective inhibitors followed by addition of DNA. Lane 1, 90 fmol of pBS (SK⁺) DNA; lane 2, same as lane 1, but the enzyme was preincubated with 2% (v/v) DMSO; lanes 3 to 8, same as lane 2, but the enzyme was preincubated with 1, 2, 5, 10, 20, and 50 μM DiSB, respectively; lanes 9 to 14, same as lane 2, but the enzyme was preincubated with 1, 2, 5, 10, 20, and 50 μM DiGDHB, respectively; lanes 15 to 20, same as lane 2, but the enzyme was preincubated with 1, 2, 5, 10, 20, and 50 μM DiSDHB, respectively. Reactions were stopped by the addition of SDS to a final concentration of 0.5% and electrophoresed in 1% agarose gel. C and D, quantitative representation of enzyme inhibition in presence of DiSB, DiGDHB, and DiSDHB in relaxation experiments. The percentage of relaxation inhibition is plotted as a function of inhibitor concentrations as indicated. The results depicted are the means for three independent experiments, and the representative results from one set of these experiments are expressed as means ± S.D. The fitted lines (sigmoidal) from these data points have the *R*² values of 0.9828, 0.9793, and 0.9820, respectively.

cally with increasing concentration of these compounds and was completely inhibited at a 100 μM concentration of each derivative (Fig. 3B, lane 8 for DiSB, lane 11 for DiGDHB, and lane 14 for DiSDHB). These results not only depict the inability of betulin and dihydrobetulin derivatives to trap the topoisomerase I-mediated cleavable complex but also highlight the antagonistic nature of these derivatives compared with camptothecin-mediated cleavage. This is a clear indication of the fact that these derivatives inhibit the binding of enzyme to substrate DNA and inhibit cleavable complex formation.

Betulin and Dihydrobetulin Derivatives Interfere with the Controlled Strand Rotation after the Single Strand Cleavage of Topoisomerase IB. In the general mechanism of a topoisomerase-mediated DNA relaxation reaction, strand rotation about the nicked topoisomerase I-DNA cleavage complex occurs, leading to change of a linking number. The speed of enzyme-associated relaxation may depend on several factors, including DNA association, cleavage, strand rotation, ligation, and dissociation. The actual aim of this experiment was to investigate the speed of the catalytic steps occurring in the relaxation reaction (i.e., cleavage, strand rotation, and ligation). Here, we performed the relaxation assays under conditions in which the DNA association/dissociation rates are not rate-limiting. This was achieved by varying the salt concentrations in the reaction mixtures using a plasmid/enzyme ratio of 1:2, because topoisomerase I acts in a strictly processive manner at low salt concentrations (15 mM NaCl, coming from the enzyme preparation and 5 mM MgCl₂). Under these conditions, the enzyme completes the relaxation of bound DNA substrate before it attacks another molecule of substrate DNA and thus provides a

condition independent of the association/dissociation rates (McConaughy et al., 1981). A molar excess of enzyme compared with substrate also limits the effect of association/dissociation on relaxation reaction. The relaxation assay was resolved in the presence of 3 μg/ml chloroquine to better resolve the topoisomers and to improve the visualization of the relaxed band.

It was found that in the absence of any inhibitor, relaxed plasmid monomers start appearing from 10 min of incubation in low salt buffer (Fig. 4A, lane 2), and complete relaxation under processive assay condition was achieved after 60 min of incubation (Fig. 4A, lane 7). On the other hand, in presence of 100 μM DiSB, relaxed molecules started to appear from 60 min of incubation (Fig. 4B, lane 7), whereas after incubation with same amount of DiGDHB (Fig. 4C) and DiSDHB (Fig. 4D), the relaxed form appeared from 40 min (Fig. 4C, lane 5) and 30 min (Fig. 4D, lane 4) of incubation, respectively. These results suggest a slower rate of completion of catalytic cycle of LdTOP1LS in presence of the respective inhibitors with respect to LdTOP1LS alone. Considering the fact that multiple strand rotations can occur for every cleavage event, as suggested by the controlled strand rotation model (Stewart et al., 1998), the speed of the strand rotation rather than cleavage/ligation rates are likely to be the rate-limiting step under these conditions. Taken together, the slower rate of LdTOP1LS in presence of each inhibitor compared with LdTOP1LS alone can best be explained by a slower strand rotation event that ultimately affects the general relaxation reaction.

Betulin and Dihydrobetulin Derivatives Act Reversibly against the LdTOP1LS. The selected derivatives are potent inhibitors of LdTOP1LS. Preincubation experiments

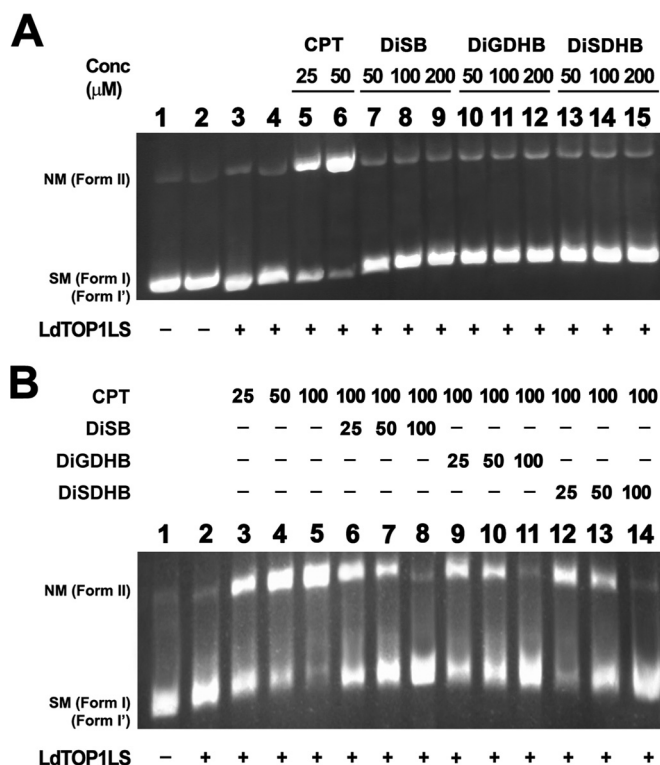


Fig. 3. Derivatives of betulin and dihydrobetulin do not induce LdTOP1LS mediated DNA cleavage. A, cleavage reaction and agarose gel electrophoresis were performed as described under *Materials and Methods*. Lane 1, 50 fmol of supercoiled pHOT1 DNA; lane 2, same as lane 1, but treated with SDS-proteinase K; lane 3, same as lane 1, but incubated with 100 fmol LdTOP1LS; lane 4, same as lane 3, but with SDS-proteinase K treatment; lanes 5 and 6, same as lane 4, but in presence of 25 and 50 μM CPT, respectively, as control; lanes 7 to 9, same as lane 4, but in the presence of 50, 100, and 200 μM DiSB; lanes 10 to 12, same as lane 4, but in the presence of 50, 100, and 200 μM DiGDHB; lanes 13 to 15, same as lane 4, but in the presence of 50, 100, and 200 μM DiSDHB. Positions of supercoiled monomer (SM; form I) and nicked monomer (NM; form II) are indicated. Form I', relaxed molecules. The figure represents set from three independent experiments. B, betulin and dihydrobetulin derivatives abrogate CPT-mediated cleavage. Reactions were described under *Materials and Methods*. Lane 1, 50 fmol of supercoiled pHOT1 DNA; lane 2, with 100 fmol of LdTOP1LS followed by treatment with SDS-proteinase K; lanes 3 to 5, same as lane 2, but incubated with 25, 50, and 100 μM CPT, respectively; lanes 6 to 8, same as lane 2, but the enzyme was preincubated with 25, 50, and 100 μM DiSB before addition of CPT (100 μM); lanes 9 to 11, same as lane 2, but the enzyme was preincubated with 25, 50, and 100 μM DiGDHB before addition of CPT (100 μM); lanes 12 to 14, same as lane 2, but the enzyme was preincubated with 25, 50, and 100 μM DiSDHB before addition of CPT (100 μM). The figure shown here is representative of three independent experiments.

support that these compounds interact with the enzyme in almost the same fashion, but it is not clear whether the interaction is strong enough that they can act on the enzyme in an irreversible manner. This critical issue has been sorted out by doing dilution experiments. Reconstituted LdTOP1LS was preincubated with 20, 20, and 50 μM DiSB, DiGDHB, and DiSDHB, respectively (Fig. 5, lanes 4–6), the concentration at which 95 to 99% inhibition of enzyme has been achieved. The reaction mixtures were subsequently diluted 5-fold so that the final concentrations became 4, 4, and 10 μM , respectively (lanes 7–9). The results show that partial (approximately 20%) relief of inhibition has been achieved. Further dilution to 10-fold (lanes 10–12) and 20-fold (lanes 13–15) show that almost 50% and complete relief of inhibi-

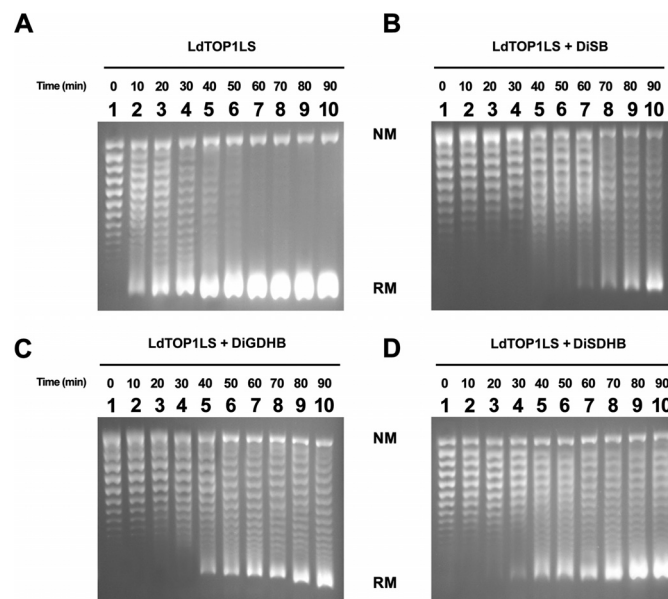


Fig. 4. Relaxation assay to assess the effect on strand rotation event under processive conditions. The relaxation of negatively supercoiled pBS (SK⁺) DNA at processive conditions were performed as described under *Materials and Methods*. Effect on of relaxation of LdTOP1LS activity under processive condition in absence of any inhibitor (A), and in presence of DiSB (100 μM) (B), DiGDHB (100 μM) (C), and DiSDHB (100 μM) (D) has been assayed by incubating 2 μg of negatively supercoiled plasmid pBS (SK⁺) (lane 1) with enzyme alone (A) or with respective inhibitors at 37°C at molar enzyme: plasmid ratio of 2:1. Reactions were stopped by addition of 2% (v/v) SDS after indicated times. After proteinase K digestion, samples were analyzed in 1.5% agarose gel containing 3 $\mu\text{g}/\text{ml}$ chloroquine followed by staining with ethidium bromide. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. The representative results are from one set of three independent experiments performed with or without any inhibitor.

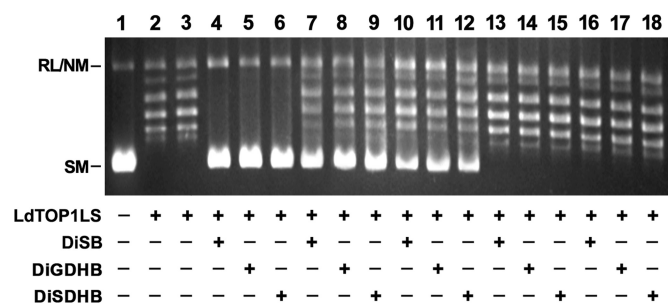


Fig. 5. Reversible interaction between betulin and dihydrobetulin derivatives with LdTOP1LS. Lane 1, 50 fmol of pBS (SK⁺) DNA. Lane 2, LdTOP1LS (100 fmol) incubated at 37°C for 5 min in the reaction mixture before addition of pBS (SK⁺) DNA. Lane 3, same as lane 2, but in presence of 2% (v/v) DMSO. Lanes 4 to 6, same as lane 2, but in presence of 20, 20, and 50 μM DiSB, DiGDHB, and DiSDHB, respectively, preincubated with LdTOP1LS for 5 min at 37°C in relaxation buffer followed by addition of 50 fmol of pBS (SK⁺) DNA and further incubated for 10 min at 37°C. Lanes 7 to 9, same as lanes 4 to 6, but diluted 5-fold so that the final inhibitor concentrations became 4, 4 and 10 μM DiSB, DiGDHB, and DiSDHB, respectively. These were followed by addition of pBS (SK⁺) DNA and further incubated for 10 min at 37°C. Lanes 10 to 12, same as lanes 4 to 6, but diluted 10-fold so that the final inhibitor concentrations became 2, 2, and 5 μM DiSB, DiGDHB, and DiSDHB, respectively. Reactions were followed by addition of pBS (SK⁺) DNA and further incubated for 10 min at 37°C. Lanes 13 to 15, same as lanes 4 to 6, but diluted 20-fold so that the final inhibitor concentrations became 1, 1, and 2.5 μM DiSB, DiGDHB, and DiSDHB, respectively. Reactions were followed by addition of pBS (SK⁺) DNA and further incubated for 10 min at 37°C. Lanes 16 to 18, same as lane 2, but in presence of 1, 1, and 2.5 μM DiSB, DiGDHB, and DiSDHB, respectively before addition of DNA. The experiments were performed three times and representative results are from one set of experiments.

tion have been achieved, respectively. In the drug control reaction (i.e., inhibition study), 1, 1, and 2.5 μM of DiSB, DiGDHB, and DiSDHB, respectively, the results showed the expected pattern of inhibition (lanes 16–18). Thus, in this dilution experiment, the relief of inhibition upon dilution suggests that the effective derivatives are acting in reversible fashion against LdTOP1LS.

Betulin and Dihydrobetulin Derivatives Bind Weakly to *L. donovani* Topoisomerase IB. To find out the nature of enzyme-inhibitor interaction, the binding of betulin and dihydrobetulin derivatives to LdTOP1LS was carried out by measuring the quenching of intrinsic tryptophan fluorescence of LdTOP1LS. The stoichiometry of the ligand-protein interaction was measured using the Job plot (Huang, 1982). In this specified plot, concentrations of both LdTOP1LS and one of the derivatives were continuously varied, keeping the total ligand-protein concentration fixed at 1.25 μM . The excitation and emission slit widths were 2.5 and 5 nm, respectively. Appropriate blanks corresponding to the buffer were subtracted to eliminate background fluorescence. The results are shown in Fig. 6A. The stoichiometry of binding, calculated using this method of continuous variation, was found to be 1:1.

The dissociation constant has been calculated from Fig. 6, B and C. Figure 6B emphasizes on the quenching profile of a fixed amount of LdTOP1LS (200 nM) with various concentrations of one of the derivatives (represented as X) (0–22.5 μM). All the fluorescence readings were corrected for the inner filter effect. The dissociation constants (K_d) of enzyme-

inhibitor interaction were as follows: DiSB, $1.321 \pm 0.281 \mu\text{M}$; DiGDHB, $0.981 \pm 0.372 \mu\text{M}$, and DiSDHB, $0.527 \pm 0.188 \mu\text{M}$. Detail calculation and formulation from the binding data have been summarized under *Materials and Methods* section, and the data clearly indicate that all the derivatives possess weak interaction with the LdTOP1LS ($K_d \sim 10^{-6}$ M). Incubation with CPT does not show any significant quenching (Supplemental Fig. S2), suggesting that these inhibitors specifically bind with LdTOP1LS. Dilution experiments with these compounds also suggest that the compounds bind reversibly with the enzyme LdTOP1LS.

Active Derivatives Are Competitive Inhibitors of *L. donovani* Topoisomerase IB. Camptothecin, the well established inhibitor of LdTOP1LS, acts as an uncompetitive inhibitor of topoisomerase IB (Champoux, 2001). It stabilizes the enzyme-DNA covalent complex and slows down the relaxation step, which is essential to decrease the L_k of supercoil structure. On the other hand, 3,3'-diindolylmethane interacts with both the free enzyme as well as DNA-bound enzyme and acts like a noncompetitive inhibitor to LdTOP1LS (Roy et al., 2008). Preincubation relaxation assay and cleavage experiments suggest that betulin and dihydrobetulin derivatives do not interact with the DNA-bound enzyme; rather, they interact with the free enzyme and thus affect their binding to the substrate DNA.

To investigate the characteristic of LdTOP1LS inhibition by these compounds, a time-course relaxation experiment was performed under standard relaxation assay conditions at

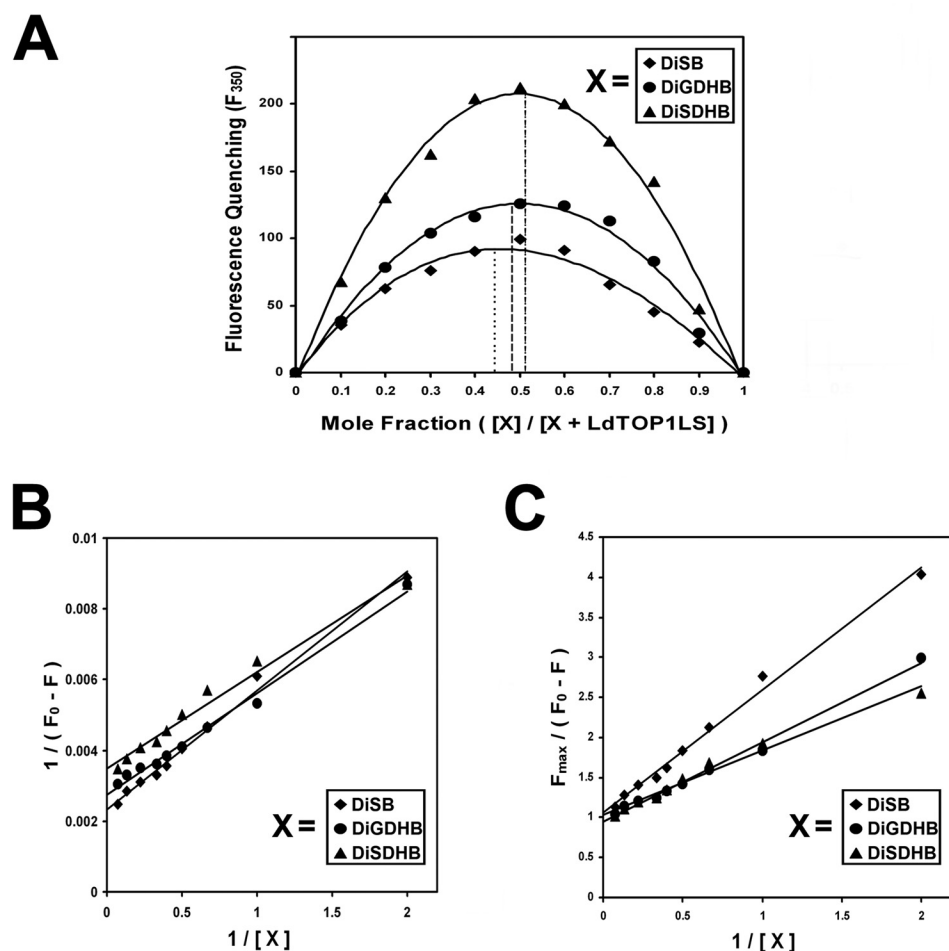


Fig. 6. Binding of betulin and dihydrobetulin derivatives with LdTOP1LS. A, Job's Plot of DiSB (◆), DiGDHB (●), and DiSDHB (▲) (as indicated by X) binding to LdTOP1LS. The concentrations of LdTOP1LS and each of the inhibitors were varied continuously (for each experiment), keeping the total concentration of LdTOP1LS plus inhibitor constant at 1.25 μM . The corrected fluorescence intensities at 350 nm were plotted against molar fractions of each inhibitor. B, double reciprocal plot of inhibitor binding to LdTOP1LS. F_{max} has been determined from the $1/(F_0 - F)$ versus $1/[X]$ plot, where $[X]$ represents concentration of each inhibitor in individual experiments. C, the linear plot of binding of each inhibitor to LdTOP1LS.

37°C, where the concentration of supercoiled substrate pBS (SK⁺) DNA was varied over a range of 4 to 60 nM and the enzyme/DNA ratio was kept within the steady-state assumption. The velocity of the enzyme remains linear for the first 5 min of reaction. All the subsequent velocities for this kinetic study were measured for the time point up to 1 min, which falls within the linear range for the velocity examined. The initial velocities for each substrate concentration were plotted on a Lineweaver-Burk plot (Supplemental Fig. S3). The maximal velocity (V_{\max}) for the LdTOP1LS was 6.67×10^{-8} M base pairs of supercoiled DNA relaxed/min/0.98 nM enzyme, which corresponds to a turnover number of approximately 70 plasmid molecules relaxed/min/molecules of enzyme and remains unaffected upon the incubation of each of the potent inhibitors with LdTOP1LS, whereas increase in K_M suggests that inhibitor-bound LdTOP1LS has low affinity toward the enzyme, and when DNA binds in this condition, strand rotation gets affected.

Effect of the Active Derivatives on Human Topoisomerase I. As the three inhibitors potentially inhibit bi-subunit *Leishmania* spp. topoisomerase I, the effect of these compounds was next assessed on human topoisomerase I. The relaxation experiment was performed under standard assay conditions (Ganguly et al., 2006). It is noteworthy that all the three compounds showed partial inhibition at 200 μ M (Fig. 7A, lane 8 for DiSB, lane 13 for DiGDHB, and lane 18 for DiSDHB) when incubated simultaneously. Also in preincubation relaxation assay, inhibitors at 50 μ M concentrations are not sufficient to inhibit human topoisomerase I completely (Fig. 7B, lane 8 for DiSB, lane 13 for DiGDHB, and lane 18 for DiSDHB).

Selective Inhibitors Can Reduce Intracellular Sb^S and Sb^R Amastigotes from Cultured Murine Peritoneal Macrophage Cells. Primary macrophage cells were obtained from BALB/c mice peritoneal extrudates. After adherence and inactivation, these macrophage cells were infected with early-passaged *L. donovani* AG83 promastigotes (Sb^S) and laboratory developed Sb^R (GE1) parasites in vitro. Infected macrophages, after subsequent washing, were incubated with different concentrations (1, 2.5, 5, 7.5, 10, 15, and 20 μ M) of each of these inhibitors for 24 h (Fig. 8A). Macrophages were fixed, and intracellular amastigotes were counted by Giemsa staining. The EC₅₀ values of each inhibitor against Sb^S and Sb^R are as follows: DiSB, 6.05 μ M; DiGDHB, 7.94 μ M; and DiSDHB, 10.54 μ M. Stability of the compounds have been assessed by treating the infected macrophages with betulin and dihydrobetulin separately to check whether active esterases present in FBS or in macrophages can cleave the ester bond of the derivatives to produce parent compounds of active derivatives. Betulin and dihydrobetulin are inactive and cannot reduce the parasite burden (data not shown), which emphasizes the availability and stability of these derivatives in cell culture medium. In addition, the percentage of infected macrophages has been reduced after subsequent treatment with these compounds (Supplemental Fig. S4). The parasite burden is reduced to almost 98% in case of intracellular Sb^S AG83 amastigotes, which is very comparable with that of intracellular Sb^R GE1 parasites when treated with 20 μ M DiSB (Fig. 8B). The efficiency of amastigote killing is up to 96% for DiGDHB and 78% for DiSDHB when infected with Sb^S parasites (Fig. 8A).

Discussion

In the present study, we have shown for the first time that the derivatives of betulin and dihydrobetulin inhibit LdTOP1LS. The mechanism of inhibition by these compounds differs from that of CPT. These derivatives bind with enzyme but do stabilize neither the topoisomerase-mediated cleavable complex, like CPT, nor the cleavage step of topoisomerase reaction. On the other hand, these derivatives prevent the binary complex formed between the drug and the enzyme to interact with the substrate DNA.

A topoisomerase reaction has three general mechanistic steps: 1) binding of the enzyme to the substrate DNA, 2) cleavage of one strand by trans-esterification reaction followed by strand rotation leading to the change of linking number by one, and 3) strand religation and turnover of the enzyme. Suicidal cleavage experimentd prove that these derivatives do not affect the cleavage of DNA (Supplemental Fig. S1). However, unlike CPT, these derivatives are not only unable to stabilize the “cleavable complex” (Fig. 3A), but also

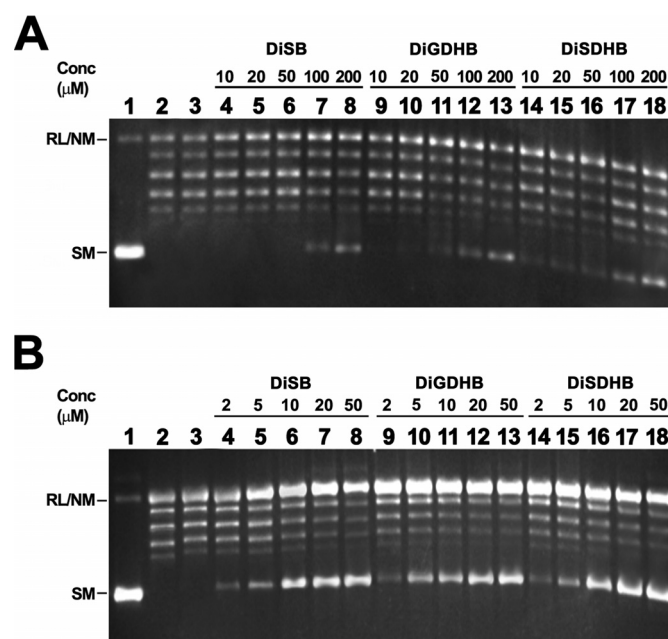


Fig. 7. Inhibition of catalytic activity of human topoisomerase I by betulin and dihydrobetulin derivatives. Relaxation of negatively supercoiled pBS (SK⁺) DNA with purified hTop I at a molar ratio of 3:1 in both simultaneous (A) and preincubation (B) conditions. A, lane 1, 90 fmol of pBS (SK⁺) DNA; lane 2, same as lane 1, but simultaneously incubated with 30 fmol of hTop I for 30 min at 37°C; lane 3, same as lane 2, but in presence of 2% (v/v) DMSO; lanes 4 to 8, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μ M DiSB, respectively; lanes 9 to 13, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μ M DiGDHB, respectively; lanes 14 to 18, same as lane 2, but in presence of 10, 20, 50, 100, and 200 μ M DiSDHB, respectively. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. B, preincubation of hTop I with respective inhibitors followed by addition of DNA. Lane 1, 90 fmol of pBS (SK⁺) DNA; lane 2, same as lane 1, but the enzyme was preincubated in relaxation buffer for 5 min followed by addition of pBS (SK⁺) DNA; lane 3, same as lane 2 but the enzyme was preincubated with 2% (v/v) DMSO; lanes 4 to 8, same as lane 2, but the enzyme was preincubated with 2, 5, 10, 20, and 50 μ M DiSB, respectively; lanes 9 to 13, same as lane 2, but the enzyme was preincubated with 2, 5, 10, 20, and 50 μ M DiGDHB, respectively; lanes 14 to 18, same as lane 2, but the enzyme was preincubated with 2, 5, 10, 20, and 50 μ M DiSDHB, respectively. Reactions were stopped by addition of SDS to a final concentration of 0.5% and electrophoresed in 1% agarose gel. The representative results were from one set of three experiments.

inhibit camptothecin-mediated cleavable complex formation (Fig. 3B). Preincubation study with these inhibitors suggests that these derivatives abrogate topoisomerase I-DNA interaction (i.e., the first step of topoisomerization). Enzyme kinetics study reveals that these derivatives act like a competitive class of inhibitors, unlike CPT. The increase in K_M suggests that the affinity of the enzyme toward DNA decreases when enzyme is bound to inhibitor (Supplemental Fig. S3).

Dynamic detailing of type IB topoisomerase-catalyzing DNA relaxation demonstrates that the relaxation proceeds in a stepwise, torque-dependent manner (Koster et al., 2005).

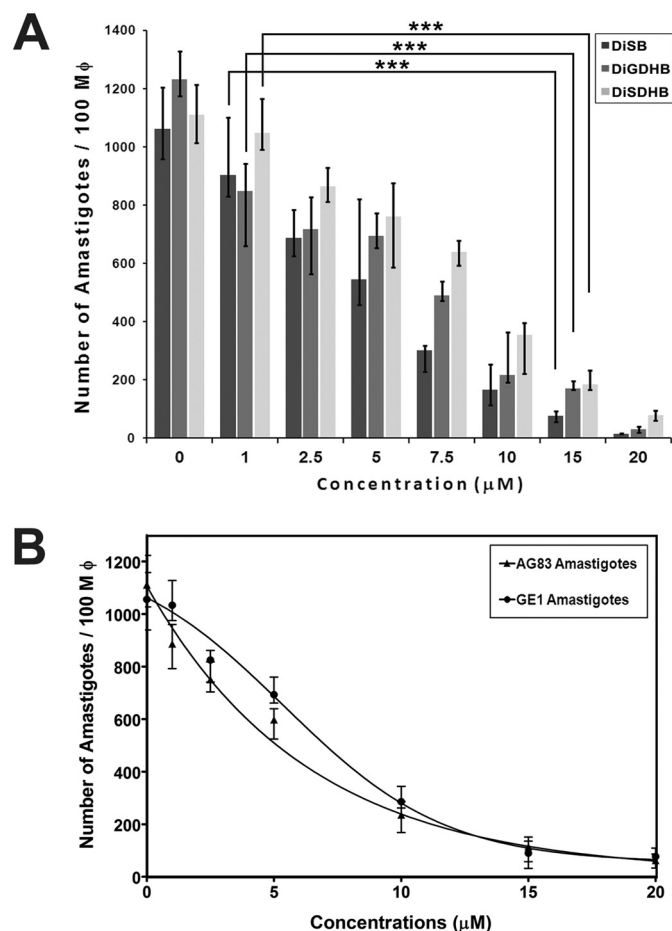


Fig. 8. Effectiveness of clearance of internalized Sb^S *L. donovani* (AG83) and Sb^R *L. donovani* (GE1) infection from in vitro infected mouse *Mφ*. Macrophages from peritoneal exudate of BALB/c mouse were infected with parasites. Cultures were treated with DiSB (■), DiGDHB (■), and DiSDHB (■) separately as indicated under *Materials and Methods*. Incubations were carried out for 24 h. Cells were fixed, stained with Giemsa, and counted under bright-field microscope. A, dose-dependent amastigote clearance by derivatives of betulin and dihydrobetulin from infected macrophages. The number of internalized AG83 amastigotes within each infected macrophages was counted under bright-field microscope. The results shown are the means of three independent experiments and plotted as mean \pm S.E. ***, $p < 0.001$ compared with 1 μ M inhibitor treatment. B, comparative analysis of effectiveness of DiSB on AG83 and GE1 amastigotes. The numbers of internalized Sb^R (GE1) parasite (●) and Sb^S (AG83) parasites (▲) were counted after 24 h treatment with or without any inhibitor. For each DiSB concentration, at least 200 macrophages were counted. The results shown are the means of three independent experiments with S.E. The fitted lines were generated with GraphPad Prism using Sigmoidal dose-Response (variable slope model) equation. The R^2 values for fitted nonlinear curves were 0.9915 and 0.9941, respectively.

The rotation of the free 5'-hydroxyl DNA end around the intact strand takes place in a manner that is restricted by the surrounding protein (Stewart et al., 1998). To assess the mode of enzyme inhibition, we analyzed the rate-limiting relaxation assays for each of the active derivatives under processive condition (molar enzyme:plasmid ratio of $\sim 2:1$; low salt buffer). In this condition, the relaxation rate depends mainly on the speed of strand rotation; which acts as the rate limiting step of topoisomerization (McConaughy et al., 1981). Strand rotation is affected maximally when the above relaxation assay is carried out with DiSB in simultaneous assay condition, because there is a lesser degree of interaction between LdTOP1LS and DNA. Consequently, time needed to relax the total supercoiled plasmid in presence of DiSB is highest with respect to enzyme alone. Considering the controlled strand rotation model, these results can best be explained by a drug-induced stalling of the enzyme in a conformation that restricts strand rotation imposed by flexible LdTOP1LS enzyme.

Direct measurement of enzyme-inhibitor interaction made by intrinsic tryptophan quenching of LdTOP1LS with each of these inhibitors reveals K_d values of 1.31 ± 0.27 , 0.59 ± 0.20 , and 0.49 ± 0.19 μ M for DiSB-enzyme, DiGDHB-enzyme, and DiSDHB-enzyme, respectively. Job Plot analysis also reveals that there is a 1:1 interaction of each of these inhibitors with LdTOP1LS.

In the subsequent study of in vitro parasite clearance, we have shown that all these inhibitors have profound effects on the clearance of antimony sensitive (Sb^S) and resistant (Sb^R) intracellular parasites in cultured macrophages. Upon infection with the wild-type *L. donovani* (Sb^S) on the isolated murine peritoneal macrophages followed by treatment with the inhibitors individually at 20 μ M for 24 h, there was significant reduction of the parasite burden within macrophages (up to 95% for DiSB, 96% for DiGDHB, and 78% for DiSDHB). In case of Sb^R parasites, the extent of clearance of intracellular amastigotes is similar when treated with DiSB (Fig. 8B). With the establishment of betulin derivatives as potent antileishmanial agents targeting LdTOP1LS, it was important to study the effect of these compounds on host cell topoisomerase (i.e., human topoisomerase I) or directly on host cell viability. Our results show that these inhibitors have less effect on human topoisomerase I compared with LdTOP1LS (Fig. 7). Cell viability test with isolated murine macrophages also suggests that these compounds exert no remarkable cytotoxicity up to 50 μ M concentrations (data not shown); however, these compounds showed some toxicity (23% for DiSB, 18% for DiGDHB, and 15% for DiSDHB) when incubated at 100 μ M concentration (data not shown).

A close inspection of all the synthesized derivatives (DiSB, DiGDHB and DiSDHB) that inhibit LdTOP1LS indicates that variation of carbon chain length at C-3 and C-28 positions in betulin and dihydrobetulin are important for inhibitory effect. The parent compounds betulin and dihydrobetulin do not inhibit enzymatic activity (data not shown), whereas substitution with succinyl group at C-3 and C-28 position of betulin and glutaryl or succinyl at the same positions of dihydrobetulin turns out to be very effective inhibitor of LdTOP1LS. However, substitution with phthaloyl group or crotonyl or acetyl group fails to convert the compound as inhibitors. Alakurtti et al. (2010) showed that some chemically synthesized heterocycloadducts of betulin with acetyl

and methyl substitution displayed approximately 98% growth inhibition of amastigote with a GI_{50} of 8.9 μ M. Substitution with more bulky groups decreases the antileishmanial activity, which is also observed in our derivatives. This investigation demonstrates in particular that simple aliphatic-acid group variations of parent compounds such as betulin or dihydrobetulin can produce potential inhibitors of *L. donovani* topoisomerase with less effect on host topoisomerase.

In conclusion, our results indicate that simple substitution on the parent skeleton of betulin and dihydrobetulin results in synthesis of potent *L. donovani* topoisomerase IB catalytic inhibitors. Structure-function analysis of these inhibitors and topoisomerase IB interaction along with modeling studies can be employed for developing more effective and potent leishmanicidal properties with lesser cytotoxicity for the host. The therapeutic importance of these compounds to treat wild-type as well as resistant parasites can be exploited in developing rational approaches to chemotherapy against the dreaded disease.

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Authorship Contributions

Participated in research design: Chowdhury, Sengupta, Mukhopadhyay, and Majumder.

Conducted experiments: Chowdhury, Roy Chowdhury and Mukherjee.

Contributed new reagents or analytic tools: Mukherjee and Mukhopadhyay.

Performed data analysis: Chowdhury and Mukherjee.

Wrote or contributed to the writing of the manuscript: Chowdhury and Majumder.

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