

# Novel Betulin Derivatives as Antileishmanial Agents with Mode of Action Targeting Type IB DNA Topoisomerase

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**Running Title:** Betulin Derivatives, unique LdTOP1LS Inhibitors.

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- Number of text pages: 44
- Number of Tables: 2
- Number of Figures: 8
- Number of References: 40
- The number of words in Abstract: 194
- The Number of words in Introduction: 648
- The Number of words in Discussion: 951

- List of Non-Standard Abbreviations:

DHBA, dihydrobetulinic acid; DIM, 3,3'-diindolylmethane; CPT, camptothecin; DiSB, 3-O, 28-O disuccinyl betulin; DiGDHB, 3-O, 28-O diglutaryl dihydrobetulin; DiSDHB, 3-O, 28-O disuccinyl dihydrobetulin; LdTOP1L, *L. donovani* topoisomerase I large subunit; LdTOP1S, *L.*

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*donovani* topoisomerase I small subunit; DMSO, dimethyl sulfoxide; DMAP, 4 N, N – dimethylaminopyridine; DTT, Dithiothreitol; T.L.C, Thin Layer Chromatography; IPTG, isopropyl  $\beta$ -D-thiogalactoside; Ni-NTA, Ni<sup>2+</sup>-nitriloacetate-agarose; P11 cellulose, phosphocellulose; HAP, hydroxyapatite; EtBr, Ethidium bromide; SAG, Sodium antimony gluconate; M199, Medium 199; FBS, Fetal Bovine Serum; K<sub>d</sub>, dissociation constant; L<sub>k</sub>, Linking number; V<sub>max</sub>, maximal velocity.

## Abstract

Towards 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* plants exhibits several pharmacological properties. Three compounds viz. disuccinyl betulin, diglutaryl dihydrobetulin and disuccinyl dihydrobetulin inhibit growth of the parasite as well as relaxation activity of the enzyme type IB topoisomerase (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 dissociation constant of 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 affect the relaxation of supercoiled DNA. Interestingly, these compounds reduce the intracellular parasite burden in macrophages infected with wild type *Leishmania* 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 resistant parasites, against most commonly used pentavalent antimonials e.g. glucantime and pentostam (Ashutosh et al, 2007) or arsenite (Haimeur, 1999), together with lack of available vaccine (Kedzierski, 2010; Nylen and Gautam; 2010) pose a threat to cure the disease. This led to treatment with current drugs like expensive pentamidine (Mishra et al, 1992), amphotericin B (AmBisome) (Wortmann, 2010; Soloman, 2010) and the teratogenic drug miltefosine (Impavido) (Seifert et al, 2007; Sindermann and Engel, 2006). Therefore improvised drug therapy of *Leishmania* 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 catalyzes breakage and rejoining of DNA strands during several vital life processes like replication, repair, recombination, transcription, chromosome segregation and integration (Wang, 1996; Liu, 1989). These DNA manipulators modulate the dynamic nature of DNA secondary and higher order structure by transient nicking/closing of DNA strands (Champoux, 2001).

The topoisomerases can be classified into two types: type I and type II, both of which are of equal importance as chemotherapeutic targets (Fortune and Osheroff, 2000; Pommier, 2006; Teicher, 2008; Nitiss, 2009). Unlike eukaryotic type IB topoisomerases, kinetoplastid topoisomerases IB possess heterodimeric structure, consisting 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 small subunit (S), bearing 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* 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 coined 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 as class II inhibitors (Li et al, 1993; Chowdhury et al, 2003). The most well studied type IB topoisomerase poison CPT (camptothecin) promotes protein-DNA cleavable complex formation (Bodley and Shapiro, 1995) leading to apoptotic like cell death (Sen et al, 2004) in *Leishmania donovani*.

We have previously reported that dihydrobetulinic acid (DHBA), isolated from the leaves of *Bacopa monniera*, has profound effect on intracellular amastigotes with an IC<sub>50</sub> value of 4.1 μM). DHBA inhibits *Leishmania* 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-3b, 28-diol) is an abundant naturally occurring triterpene and constituent of the cork layer of the outer bark of *Betula alba*, *B. pendula*, *B. pubescent* and *B. platyphylla*. Betulin and the more active form betulinic acid exhibit anti-malarial (Steele et al, 1999), anti-HIV and anti-inflammatory (Reutrakul et al, 2010) as well as cytotoxic activities on cancer cell lines (Laszczyk et al, 2009).

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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. Interestingly these betulin derivatives can efficiently reduce parasite burden from macrophage cultures infected with antimony resistant and sensitive *Leishmania* parasites having less effect on host cells. Thus the need for new therapeutic agents against antimony resistant strains of *Leishmania* can be exploited using modification of plant-derived betulin.

## **Materials and Methods:**

### **Chemicals**

DMSO and Camptothecin were purchased from Sigma chemicals (St. Louis, MO, USA). 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. (Florida, USA).

### **Synthesis of several derivatives of betulin and dihydrobetulin**

Hydrogenation of betulin was carried out by dissolving 400 mg betulin in minimum volume of dry ethyl acetate (50 ml) and little dichloromethane was added to clear the solution. Then catalytic amount of 10 % palladised charcoal (Pd / C) was added and exposed to hydrogen gas atmosphere, the reaction mixture was stirred over night at room temperature, filtered and the hydrogenated product was dried over vacuum.

For general preparations of betulin or dihydrobetulin derivatives, a mixture of betulin (50 mg, 1 eqv) or dihydrobetulin (50 mg, 1 eqv) and 4 N, N – dimethylaminopyridine (DMAP) (2mg) was dissolved in pyridine (2 ml). Acid anhydride (10 eqv) was added and the reaction mixture was placed in 80<sup>0</sup>-90<sup>0</sup>C pre heated oil bath and stirred for 24 hours in anhydrous condition. After usual work up, the organic layer was collected and dried under reduced pressure.

All the reaction products were purified by chromatography over silicagel (60-120 mesh) and were monitored through T.L.C (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<sub>600</sub>=0.6 with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) at 22°C for 12 h. Cells harvested from 1 litre of culture were



separately lysed by lysozyme/sonication, and the proteins were purified through Ni-NTA (Ni<sup>2+</sup>-nitriloacetate-agarose column (Qiagen) 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 Bio-Rad Protein Estimation Kit 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, pH7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM PMSF 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 pBS (SK<sup>+</sup>) [pBluescript (SK<sup>+</sup>)] DNA in agarose gel. The relaxation assay was carried out as described previously with LdTOP1LS (Das et al, 2005; Sen et al, 2004) and HTOP91, serially diluted in the relaxation buffer (25 mM Tris-HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 25 mM EDTA and 150 µg/ml BSA) and supercoiled plasmid pBS (SK<sup>+</sup>) DNA (85-95% were negatively supercoiled, with remainder being nicked circles). The reconstituted enzyme LdTOP1LS was assayed at 50 mM KCl concentration whereas HTOP91 was assayed at 150 mM KCl concentration as described before (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 µg/ml) and the amount

of supercoiled monomer DNA band fluorescence were quantified by integration using Gel Doc 2000 under UV illumination (Bio-Rad Quantity One Software), as described previously (Das et al, 2005). Initial velocities (nM of DNA base-pairs relaxed/min) were calculated using the following equation:

$$\text{Initial Velocity} = ([\text{Supercoiled DNA}]_0 - (\text{Int}_t \cdot [\text{supercoiled DNA}]_0 / \text{Int}_0)) / t$$

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  $t$  (Osheroff et al, 1983). The effect of DNA concentration on the kinetics of relaxation was examined over a range of 4-60 nM supercoiled pBS (SK<sup>+</sup>) DNA (0.16-2.4  $\mu\text{g}/25 \mu\text{l}$  of reaction mixture) at constant concentration of 10 mM  $\text{MgCl}_2$  and 0.94 nM enzyme (LdTOP1LS) at 37°C for 1 min. the data were analyzed by a Lineweaver-Burk plot. Intercept of the y-axis is  $1/V_{\text{max}}$ , and catalytic-center activity =  $V_{\text{max}}/\text{enzyme concentration}$  (plasmid molecules relaxed/min/molecule of enzyme).

### **Plasmid cleavage assay**

Cleavage assay was carried out as described (Ray et al, 1998). Briefly, 50 fmol of pHOT1 supercoiled DNA (containing topoisomerase I cleavage site) and 100 fmol of reconstituted LdTOP1LS were incubated in standard reaction mixture (50  $\mu\text{l}$ ) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM  $\text{MgCl}_2$ , 0.5 mM DTT, 0.5 mM EDTA and 30  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$  proteinase K and further incubated for 1 h at 37°C.

DNA samples were electrophoresed in 1% agarose gel containing 0.5  $\mu\text{g/ml}$  EtBr to resolve more slowly migrating nicked product (Form II) from the supercoiled molecules (Form I).

### **Suicide Cleavage Assay**

A 14-mer (5'-GAAAAAAGACTT↓AG-3') oligonucleotide (ML14) containing a topoisomerase IB-specific cleavage site was 5'-<sup>32</sup>P-end-labelled and annealed to 25-mer (3'-CTTTTTTCTGAATCTTTTTAAAAAT-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  $\mu\text{M}$  enzyme (LdTOP1LS) in 20  $\mu\text{l}$  reaction mixtures under standard assay conditions (10 mM Tris/HCl, pH 7.5, 10 mM  $\text{MgCl}_2$ , 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  $\mu\text{l}$  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 for 1.25  $\mu\text{M}$ . 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 PerkinElmer LS55 luminescence spectrometer.

### Spectrofluorimetric binding assay

LdTOP1LS (200 nM) was incubated in fluorescence buffer (20 mM Tris/HCl, 50 mM NaCl and 10 mM MgCl<sub>2</sub>) with varying concentrations of each of the inhibitors (0-22.5 μM) 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,  $F$  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 Figure 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;  $L_f = C - B[J]$ , 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 SAG-sensitive ( $Sb^s$ ) MHOM/IN/1983/AG83 (AG83) and the one SAG-resistant ( $Sb^r$ ) strains, 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 of penicillin and 100 mg/ml of 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 Jackson Laboratories, 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 hr post injection (intraperitoneal) with 2% (w/v) hydrolyzed starch by peritoneal lavage with ice-cold phosphate-buffered saline. Cells were washed and cultured for 18-24 hr (for adherence) in RPMI 1640 (supplemented with 100 IU/ml of penicillin and 100 µg/ml of streptomycin) containing 10% (v/v) heat-inactivated FBS (RPMI-FCS) at 37°C at 5% CO<sub>2</sub> in air on sterile cover glass (22 mm × 22 mm) placed in disposable plates 35 mm in diameter (Tarsons India Ltd.). The culture medium was washed off and fresh RPMI-FBS was added. About  $5 \times 10^5$  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 hours to ensure entry of parasite. Following incubation, unphagocytosed parasites were removed by washing with medium, and cells were resuspended in RPMI-FBS at 37°C, 5% CO<sub>2</sub> and then

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incubated for 6 hrs. Cultures were transferred to a CO<sub>2</sub> incubator at 37°C and incubated for another 10-12 hr. Respective inhibitors were added at different concentrations (ranging from 1 μM to 20 μM) to infected macrophages and left for another 24 hr 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 analogues of betulin (Figure 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**), 3-o, 28-o Dicrotonyl betulin (**6**) were prepared from betulin as mentioned in Materials and Methods. Dihydrobetulin (Figure 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 bi-subunit type IB topoisomerase of *L. donovani* (LdTOP1LS) was examined by plasmid relaxation assays as described in the Materials and Methods. The relaxation assays were carried out under standard assay conditions where 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 said 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  $\mu$ 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 $\mu$ M (Fig 2A, lanes 4, 9, 14 respectively) under this same conditions. The inhibition increases in a dose dependent fashion for all the three compounds (Figure 2A, lanes 5-8 for DiSB, lanes 10-13 for DiGDHB and lanes 15-18 for DiSDHB). At 100  $\mu$ M concentration of DiSB and DiGDHB almost 98% inhibition was achieved (Figure 2A, lane 7 for DiSB and lane 12 for DiGDHB), but DiSDHB inhibits only to the extent of 94% at 200  $\mu$ M concentration (Figure 2A, lane 18).

To investigate whether these compounds interact with the enzyme, LdTOP1LS was preincubated with these compounds at different concentrations for 5 min at 37°C before the addition of substrate DNA (Figure 2B). Inhibitory effect of these compounds in preincubation condition was compared with the inhibition by these compounds incubated simultaneously with the enzyme (LdTOP1LS) and supercoiled DNA in the relaxation reaction (Figure 2A). Ninety eight percent inhibition was achieved at 20  $\mu$ M concentration of DiSB (Figure 2B, lane 7) and DiGDHB (Figure 2B, lane 13); but for DiSDHB, 98% inhibition takes place only at 50  $\mu$ M concentration (Figure 2B, lane 20).

The percentage of relaxation inhibition was plotted against the concentration of the compounds for both simultaneous and preincubation assay conditions (Fig 2C and 2D). The IC<sub>50</sub> values were determined under varying concentration of these compounds with the same amount of enzyme and substrate. The IC<sub>50</sub> values of DiSB, DiGDHB and DiSDHB under simultaneous relaxation assays were 12.6  $\mu$ M, 15.9  $\mu$ M and 23.1  $\mu$ M respectively; whereas under preincubation assay conditions, these compounds have IC<sub>50</sub> values of 7.35  $\mu$ M, 8.2  $\mu$ M and 10  $\mu$ M respectively. To rule out the binding of BSA present in the relaxation assay mixture, the inhibition kinetics was also studied in the absence of BSA (data not shown). The IC<sub>50</sub> values calculated for each of the



inhibitors in simultaneous relaxation assay in such condition were 11.8  $\mu\text{M}$  for DiSB, 15.3  $\mu\text{M}$  for DiGDHB and 24.2  $\mu\text{M}$  for DiSDHB respectively. All the  $\text{IC}_{50}$  values were calculated using variable slope model for finding  $\text{EC}_{50}$  in GraphPad Prism version 5.0 which fits the non-linear regression equation.

**Betulin and dihydrobetulin derivatives inhibit *L. donovani* topoisomerase IB mediated DNA cleavable complex formation by abrogating topoisomerase - DNA interaction.**

Topoisomerase I changes the linking number ( $L_k$ ) of DNA by transiently cleaving single strand and thus producing nick, about which the uncleaved strand swivels via a ‘controlled rotation’ mechanism followed by religation of the nicked phosphodiester bond. The putative intermediate of this reaction is a covalent enzyme-DNA complex (cleavable complex) which can be irreversibly converted to topoisomerase I linked DNA single strand breaks by addition of strong protein denaturing agents like NaOH or SDS. Topoisomerase inhibition can be achieved by prevention of enzyme-DNA binary complex formation or by stabilizing the enzyme-DNA cleavable complex. Camptothecin, the most established uncompetitive inhibitors of type IB topoisomerase, traps the protein-DNA cleavable complex. In the present study, the ability of these compounds to stabilize cleavable complex formation between LdTOP1LS and pHOT1 DNA (Figure 3A), containing a topoisomerase IB specific binding site as mentioned in the Materials and Methods section, has been investigated. The cleavage assay was performed at increasing concentrations of these compounds up to 200  $\mu\text{M}$  with CPT as a positive control under standard assay conditions. As shown in Figure 3A, both 25 and 50  $\mu\text{M}$  camptothecin convert closed circular DNA (form I) to nicked circular DNA (form II) by stabilization of the ‘cleavable complex’ (lanes 5 and 6). Lane 3 shows the formation of form II DNA due to

cleavage of pHOT1 DNA with 100 fmol of LdTOP1LS alone. Amount of form II DNA increases when the nicked products are trapped with SDS and proteinase K (lane 4). This serves as the background cleavage of LdTOP1LS. When the cleavage assay was performed at increasing concentration (50, 100 and 200  $\mu\text{M}$ ) of these compounds using 100 fmol of LdTOP1LS, no remarkable nicked products (Figure 3A, lanes 7-9 for DiSB; lanes 10-12 for DiGDHB and lanes 13-15 for DiSDHB) were observed; whereas camptothecin at 25  $\mu\text{M}$  (lane 5) and 50  $\mu\text{M}$  (lane 6) stabilizes almost 70% and 90% of the cleavable complex respectively. EtBr at a final concentration of 0.5%  $\mu\text{g/ml}$  was included in the gel to resolve the more slowly migrating nicked product (form II) from the relaxed molecules (form I').

The experiment was further supported by suicidal cleavage assay by reacting LdTOP1LS with ML14/ML25 duplex oligonucleotides in presence of 200  $\mu\text{M}$  of each inhibitor (Supplemental Figure S1). The result indicates that these inhibitors do not affect the LdTOP1LS mediated cleavage reaction i.e. second step of the reaction.

Moreover, when 100 fmol of topoisomerase I was preincubated with 25, 50 and 100  $\mu\text{M}$  of these derivatives prior to addition of 100  $\mu\text{M}$  of CPT (Figure 3B lanes 6-8 for DiSB; lanes 9-11 for DiGDHB and lanes 12-14 for DiSDHB), the CPT mediated cleavage was inhibited drastically with increasing concentration of these compounds and completely inhibited at 100  $\mu\text{M}$  concentrations of each derivatives (Figure 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 to 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 following 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 where 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, as topoisomerase I acts in a strictly processive manner at low salt concentrations (15 mM NaCl, coming from the enzyme preparation and 5 mM MgCl<sub>2</sub>). Under these conditions, the enzyme completes the relaxation of bound DNA substrate before it attacks another molecule of substrate DNA and thus provides condition independent of the association/dissociation rates (McConaughy et al, 1981). A molar excess of enzyme compared to substrate also limits the effect of association/dissociation on relaxation reaction. The relaxation assay was resolved in the presence of 3 µg/ml of 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 (Figure 4A, lane 2) and complete relaxation under

processive assay condition was achieved after 60 min of incubation (Figure 4A, lane 7). On the other hand, in presence of 100  $\mu$ M of DiSB, relaxed molecules started to appear from 60 min of incubation (Figure 4B, lane 7); whereas, incubation with same amount of DiGDHB (Figure 4C) and DiSDHB (Figure 4D), relaxed form appeared from 40 min (Figure 4C, lane 5) and 30 min (Figure 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 to LdTOP1LS alone can best be explained by a slower strand rotation event which ultimately effects on general relaxation reaction.

### **Betulin and dihydrobetulin derivatives act reversibly against the LdTOP1LS.**

The selected derivatives are potent inhibitors of LdTOP1LS. Preincubation experiment supports that these compounds interact with the enzyme almost in the same fashion, but it is not clear whether the interaction is strong enough so that they can act on the enzyme in an irreversible manner. This critical issue has been sorted out by doing dilution experiment. Reconstituted LdTOP1LS was preincubated with 20, 20 and 50  $\mu$ M of DiSB, DiGDHB and DiSDHB respectively (Figure 5, lanes 4-6); the concentration at which 95-99% inhibition of enzyme has been achieved. The reaction mixtures were subsequently diluted 5-fold so that the final concentrations become 4, 4 and 10  $\mu$ M respectively (lanes 7-9). The results show that partial (about 20%) relief of inhibition have been achieved. Further dilution to 10-fold (lanes 10-12) and 20-fold (lanes 13-15) show that almost 50% and complete relief of inhibition have been achieved

respectively. In the drug control reaction, i.e. inhibition study 1, 1, and 2.5  $\mu\text{M}$  of the respective compounds, 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 CY, 1982). In this specified plot, concentration of both LdTOP1LS and one of the derivatives were continuously varied keeping the total ligand-protein concentration fixed at 1.25  $\mu\text{M}$ . The excitation and emission slit widths were 2.5 nm and 5 nm respectively. Appropriate blanks corresponding to the buffer were subtracted to eliminate background fluorescence. The results are shown in Figure 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 Figures 6B and 6C. 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  $\mu\text{M}$ ). All the fluorescence readings were corrected for the inner filter effect. The dissociation constant ( $K_d$ ) of enzyme-inhibitor interaction can be determined and is given in a tabular format (Table 1). Detail calculation and formulation from the binding data have been summarized in the 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

Figure 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 JJ, 2001). It stabilizes the enzyme-DNA covalent complex and slows down the religation step which is essential to decrease the  $L_k$  of supercoil structure. On the other hand 3,3' di-indolylmethane (DIM), interacts with both the free enzyme as well as DNA bound enzyme and acts like a non-competitive inhibitor to LdTOP1LS (Roy et al, 2008). Preincubation relaxation assay and cleavage experiment suggest that betulin and dihydrobetulin derivatives do not interact with the DNA-bound enzyme, rather they interact with the free enzyme and thus affecting 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 37°C, where the concentration of supercoiled substrate pBS (SK<sup>+</sup>) DNA was varied over a range of 8-60 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 Figure S3). The maximal velocity ( $V_{max}$ ) for the LdTOP1LS was  $6.67 \times 10^{-8}$  M base pairs of supercoiled DNA relaxed/min/0.98nM of enzyme which corresponds to a turnover number of about 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 towards 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* topoisomerase I, the effect of these compounds was next assessed on human topoisomerase I (hTopI). The relaxation experiment was performed under standard assay conditions (Ganguly et al, 2006). Interestingly all the three compounds showed partial inhibition at 200  $\mu\text{M}$  concentration (Figure 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  $\mu\text{M}$  concentrations are not sufficient to inhibit hTopI completely (Figure 7B, lane 8 for DiSB; lane 13 for DiGDHB and lane 18 for DiSDHB).

### **Selective inhibitors can reduce intracellular $\text{Sb}^{\text{S}}$ and $\text{Sb}^{\text{R}}$ 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 ( $\text{Sb}^{\text{S}}$ ) and laboratory developed  $\text{Sb}^{\text{R}}$  (GE1) parasites *in vitro*. Infected macrophages, after subsequent washing, were incubated with different concentrations (1, 2.5, 5, 7.5, 10, 15, and 20  $\mu\text{M}$ ) of each of these inhibitors for 24 h (Figure 8A). Macrophages were fixed and intracellular amastigotes were counted by Giemsa staining. The  $\text{EC}_{50}$  of each inhibitor against  $\text{Sb}^{\text{S}}$  and  $\text{Sb}^{\text{R}}$  were given in Table 2. 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 on the availability and stability of these derivatives in cell culture medium. Also the percentage of infected macrophages has been reduced after subsequent treatment with these compounds (Supplemental Figure S4). The parasite burden is reduced to almost 98% in case of intracellular Sb<sup>S</sup> AG83 amastigotes which is very much comparable to that of intracellular Sb<sup>R</sup> GE1 parasites when treated with 20μM of DiSB (Figure 8B). In case of DiGDHB and DiSDHB the efficiency of amastigote killing is up to 96% for DiGDHB and 78% for DiSDHB when infected with Sb<sup>S</sup> parasites respectively (Figure 8A).



## Discussion:

In the present study, we have shown for the first time that the derivatives of betulin and dihydrobetulin inhibit *L. donovani* topoisomerase I (LdTOP1LS). The mechanism of inhibition by these compounds differs from that of CPT. These derivatives bind with enzyme, but do not stabilize 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, i.e. (i) binding of the enzyme to the substrate DNA, (ii) cleavage of one strand by trans-esterification reaction followed by strand rotation leading to the change of linking number by one and (iii) strand religation and turnover of the enzyme. Suicidal cleavage experiment proves that these derivatives do not affect the cleavage of DNA (Supplemental Figure S1). But unlike camptothecin (CPT), these derivatives are not only unable to stabilize the 'cleavable complex' (Figure 3A), but also inhibit camptothecin mediated 'cleavable complex' formation (Figure 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 competitive class of inhibitors unlike CPT. The increase in  $K_M$  suggests that the affinity of the enzyme towards DNA decreases when enzyme is bound to inhibitor (Supplemental Figure 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). The rotation of the free 5'-hydroxyl DNA end around the intact strand takes place in a manner which is

restricted by the surrounding protein (Stewart et al, 1998; Redinbo 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 ~ 2:1, low salt buffer). In this condition, the relaxation rate depends mainly on the speed of strand rotation which acts as 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 as there is lesser degree of interaction between LdTOP1LS and DNA. As a result of which, 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 be best 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 DiSB-enzyme of  $1.31 \pm 0.27 \mu\text{M}$ , DiGDHB-enzyme of  $0.59 \pm 0.20 \mu\text{M}$  and DiSDHB-enzyme of  $0.49 \pm 0.19 \mu\text{M}$ . Job Plot analysis also reveals that there is a 1:1 interaction of each of these inhibitors with LdTOP1LS.

In the subsequent study we have shown that all these inhibitors have profound effects on the clearance of antimony sensitive ( $\text{Sb}^{\text{S}}$ ) and resistant ( $\text{Sb}^{\text{R}}$ ) intracellular parasites in cultured macrophages. Upon infection with the wild type *L. donovani* ( $\text{Sb}^{\text{S}}$ ) on the isolated murine peritoneal macrophages followed by treatment with the inhibitors individually at  $20 \mu\text{M}$  for 24 hours, there is significant reduction of the parasite burden within macrophages (upto 95% for DiSB, 96% for DiGDHB and 78% for DiSDHB). In case of  $\text{Sb}^{\text{R}}$  parasites, the extent of clearance of intracellular amastigotes is similar when treated with DiSB (Figure 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 to LdTOP1LS (Figure 7). Cell viability test with isolated murine macrophages also suggests that these compounds exert no remarkable cytotoxicity up to 50  $\mu\text{M}$  concentrations (data not shown); however these compounds showed some toxicity (23% for DiSB, 18% for DiGDHB and 15% for DiSDHB) when incubated at 100  $\mu\text{M}$  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. Recently Alakurtii et al. showed that some chemically synthesized heterocycloadducts of betulin with acetyl and methyl substitution displayed about 98% growth inhibition of amastogote with a  $\text{GI}_{50}$  of 8.9  $\mu\text{M}$ . Substitution with more bulky groups decreases the antileishmanial activity which is also observed in our derivatives. This investigation specially demonstrates that simple aliphatic-acid group variations of parent compound like 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

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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 against wild type as well as resistant parasites can be well exploited in developing rational approaches to chemotherapy against the dreaded disease.

**Acknowledgements:**

We thank Prof. S. Roy, the Director of our Institute, for his interest in this work. We thank Miss Rupkatha Mukhopadhyay for her help in animal handling. SC is supported by a Senior Research Fellowship from Council of Scientific and Industrial Research, Government of India.

**Authorship Contributions:**

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

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*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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## Footnotes

This research work was supported by the Network Project from Council of Scientific and Industrial Research (CSIR), Government of India [Grant NWP-0038].

## Legends for Figures

**Figure 1: Chemical structure of betulin and its derivatives (A) and dihydrobetulin and its derivatives (B).**

**Figure 2: Inhibition of catalytic activity of LdTOP1LS by derivatives of betulin and dihydrobetulin.**

(A) Relaxation of supercoiled pBS (SK<sup>+</sup>) DNA with reconstituted LdTOP1LS at a molar ratio of 3:1. Lane 1, 90 fmol of pBS (SK<sup>+</sup>) 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 of DiSB respectively; lanes 9-13, same as lane 2, but in presence of 10, 20, 50, 100 and 200 μM of DiGDHB respectively; lanes 14-18, same as lane 2, but in presence of 10, 20, 50, 100 and 200 μM of 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<sup>+</sup>) DNA; lane 2, same as lane 1, but the enzyme was pre-incubated with 2% (v/v) DMSO; lanes 3-8, same as lane 2, but the enzyme was pre-incubated with 1, 2, 5, 10, 20 and 50 μM DiSB respectively; lanes 9-14, same as lane 2, but the enzyme was pre-incubated with 1, 2, 5, 10, 20 and 50 μM DiGDHB respectively; lanes 15-20, same as lane 2, but the enzyme was pre-incubated 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) 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  $\pm$  S.D. The fitted lines (sigmoidal) from these data points have the  $R^2$  values of 0.9828, 0.9793 and 0.9820 respectively.

**Figure 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 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  $\mu$ M of CPT respectively as control; lanes 7-9, same as lane 4, but in the presence of 50, 100 and 200  $\mu$ M DiSB; lanes 10-12, same as lane 4, but in the presence of 50, 100 and 200  $\mu$ M DiGDHB; lanes 13-15, same as lane 4, but in the presence of 50, 100 and 200  $\mu$ M 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 in 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 3-5, same as lane 2, but incubated with 25, 50 and 100  $\mu$ M of CPT respectively; lanes 6-8, same as lane 2, but the enzyme was pre-incubated with 25, 50 and 100  $\mu$ M of DiSB before addition of CPT (100  $\mu$ M); lanes 9-11, same as lane 2, but the enzyme was

pre-incubated with 25, 50 and 100  $\mu\text{M}$  of DiGDHB before addition of CPT (100  $\mu\text{M}$ ); lanes 12-14, same as lane 2, but the enzyme was pre-incubated with 25, 50 and 100  $\mu\text{M}$  of DiSDHB before addition of CPT (100  $\mu\text{M}$ ). The figure shown here is representative of three independent experiments.

**Figure 4: Relaxation assay to assess the effect on strand rotation event under processive conditions.**

The relaxation of negatively supercoiled pBS (SK<sup>+</sup>) DNA at processive conditions were performed as described in 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  $\mu\text{M}$ ) (**B**), DiGDHB (100  $\mu\text{M}$ ) (**C**) and DiSDHB (100  $\mu\text{M}$ ) (**D**) has been assayed by incubating 2  $\mu\text{g}$  negatively supercoiled plasmid pBS (SK<sup>+</sup>) (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 periods of time. Following 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.

**Figure 5: Reversible interaction between betulin and dihydrobetulin derivatives with LdTOP1LS.**

(**A**) Lane 1, 50 fmol of pBS (SK<sup>+</sup>) DNA. Lane 2, LdTOP1LS (100 fmol) incubated at 37°C for 5 min in the reaction mixture before addition of pBS (SK<sup>+</sup>) DNA. Lane 3, same as lane 2, but in presence of 2% (v/v) DMSO. Lanes 4-6, same as lane 2, but in presence of 20, 20 and 50  $\mu\text{M}$  of

DiSB, DiGDHB and DiSDHB, respectively, pre-incubated with LdTOP1LS for 5 min at 37°C in relaxation buffer followed by addition of 50 fmol of pBS (SK<sup>+</sup>) DNA and further incubated for 10 min at 37°C. Lanes 7-9, same as lane 4-6, but diluted 5-fold so that the final inhibitor concentrations became 4, 4 and 10 μM of DiSB, DiGDHB and DiSDHB, respectively. These were followed by addition of pBS (SK<sup>+</sup>) DNA and further incubated for 10 min at 37°C. Lanes 10-12, same as lane 4-6, but diluted 10-fold so that the final inhibitor concentrations became 2, 2 and 5 μM of DiSB, DiGDHB and DiSDHB, respectively. Reactions were followed by addition of pBS (SK<sup>+</sup>) DNA and further incubated for 10 min at 37°C. Lanes 13-15, same as lane 4-6, but diluted 20-fold so that the final inhibitor concentrations became 1, 1 and 2.5 μM of DiSB, DiGDHB and DiSDHB, respectively. Reactions were followed by addition of pBS (SK<sup>+</sup>) DNA and further incubated for 10 min at 37°C. Lanes 16-18, same as lane 2, but in presence of 1, 1 and 2.5 μM of DiSB, DiGDHB and DiSDHB, respectively before addition of DNA. The experiments were performed three times and representative result is from one set of experiments.

**Figure 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 mole 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.



**Figure 7: Inhibition of catalytic activity of human topoisomerase I by betulin and dihydrobetulin derivatives.**

Relaxation of negatively supercoiled pBS (SK<sup>+</sup>) 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<sup>+</sup>) 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 4-8, same as lane 2, but in presence of 10, 20, 50, 100 and 200 μM of DiSB respectively; lanes 9-13, same as lane 2, but in presence of 10, 20, 50, 100 and 200 μM of DiGDHB respectively; lanes 14-18, same as lane 2, but in presence of 10, 20, 50, 100 and 200 μM of 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<sup>+</sup>) DNA; lane 2, same as lane 1, but the enzyme was pre-incubated in relaxation buffer for 5 min followed by addition of pBS (SK<sup>+</sup>) DNA; lane 3, same as lane 2 but the enzyme was preincubated with 2% (v/v) DMSO; lanes 4-8, same as lane 2, but the enzyme was pre-incubated with 2, 5, 10, 20 and 50 μM DiSB respectively; lanes 9-13, same as lane 2, but the enzyme was pre-incubated with 2, 5, 10, 20 and 50 μM DiGDHB respectively; lanes 14-18, same as lane 2, but the enzyme was pre-incubated with 2, 5, 10, 20 and 50 μM 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.

**Figure 8: Effectiveness of clearance of internalized Sb<sup>S</sup> *L. donovani* (AG83) and Sb<sup>R</sup> *L. donovani* (GE1) infection from *in vitro* infected mouse M $\phi$ .**

Macrophages from peritoneal extrudate of Balb/c mouse were infected with parasites. Cultures were treated with DiSB (■), DiGDHB (■) and DiSDHB (■) separately as indicated in Materials and Methods. Incubations were carried out for 24 hours. 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 were 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  $\mu$ M of inhibitor treatment. **(B)** Comparative analysis of effectiveness of DiSB on AG83 and GE1 amastigotes. The numbers of internalized Sb<sup>R</sup> (GE1) parasite (●) and Sb<sup>S</sup> (AG83) parasites (▲) were counted after 24 hour 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 Standard Error. The fitted lines were generated with GraphPad Prism version 5 software using Sigmoidal dose-Response (variable slope model) equation. The R<sup>2</sup> values for fitted non-linear curves are 0.9915 and 0.9941 respectively.

## Tables

**Table 1: Dissociation constant of betulin inhibitors with LdTOP1LS**

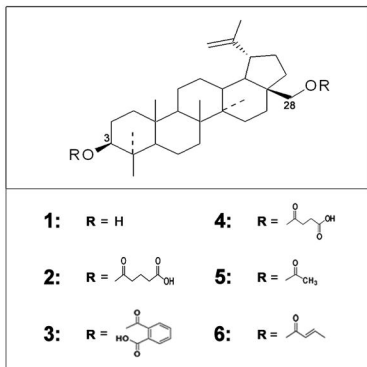
<b>Compound Name</b>	<b>K<sub>D</sub> (μM)</b>
DiSB	1.321 ± 0.281
DiGDHB	0.981 ± 0.372
DiSDHB	0.527 ± 0.188

**Table 2: Effect of betulin inhibitors on *Leishmania* amastigotes in macrophages**

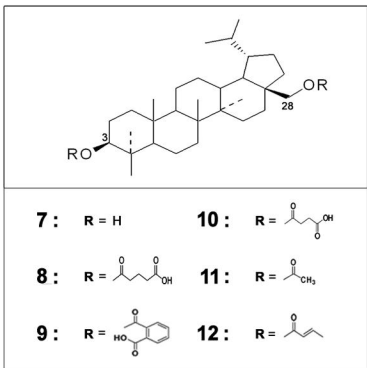
<b>Compound Name</b>	<b>EC<sub>50</sub> (μM)</b>
DiSB	6.05
DiGDHB	7.94
DiSDHB	10.54

# Figure 1

## A

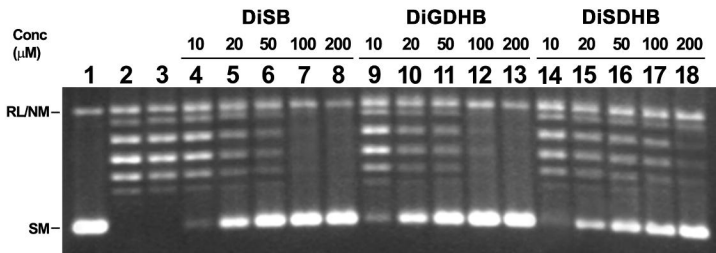


## B

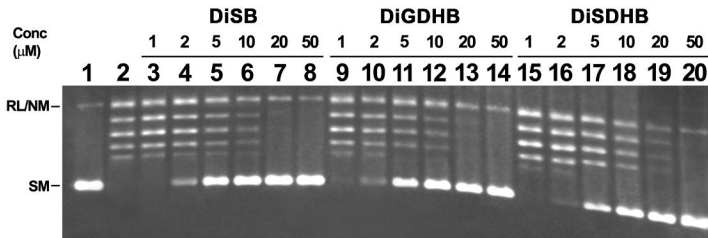


# Figure 2

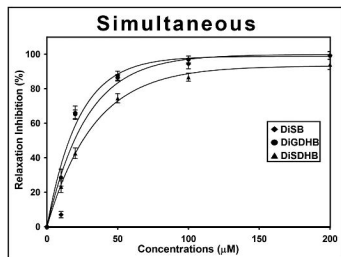
## A



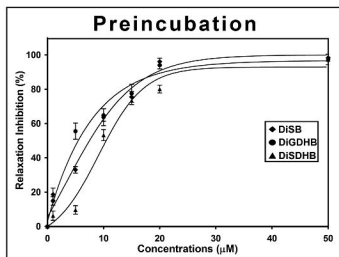
## B



## C

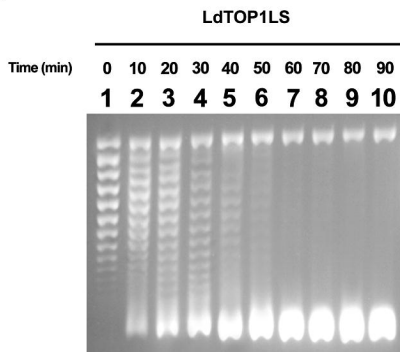
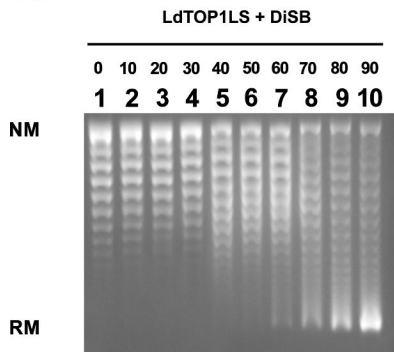
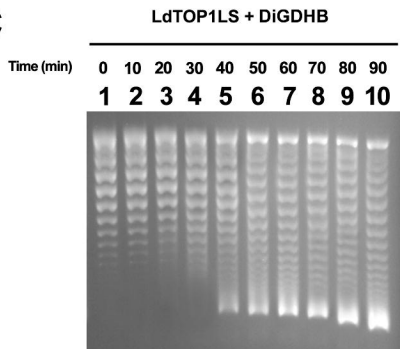
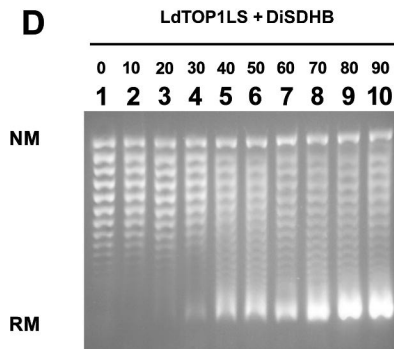


## D



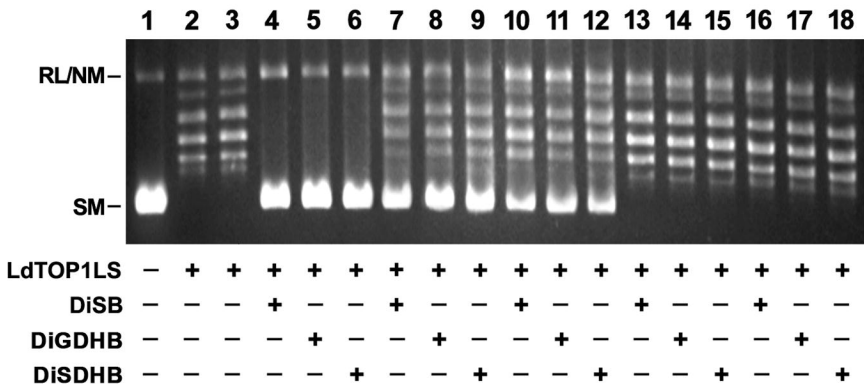


# Figure 4

**A****B****C****D**

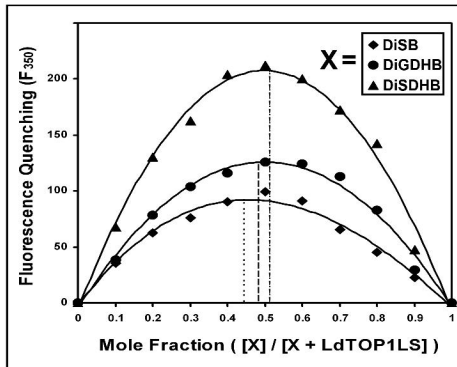


# Figure 5

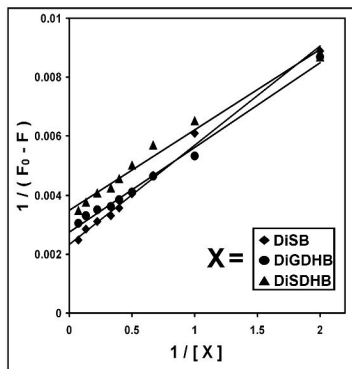


# Figure 6

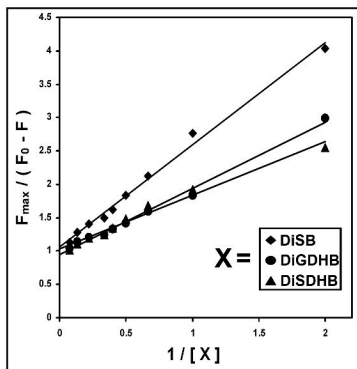
## A



## B

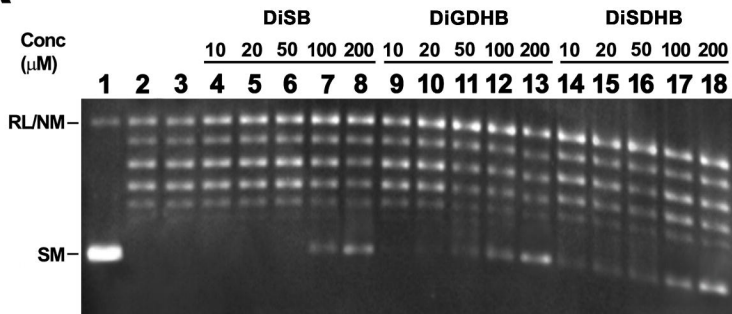


## C

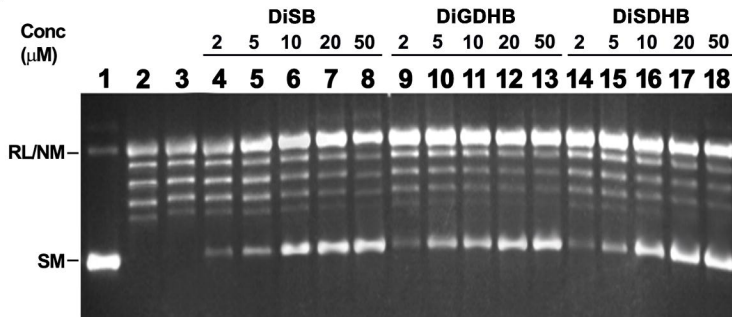


# Figure 7

## A

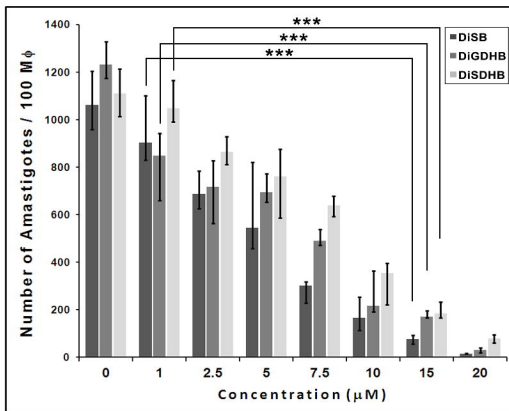


## B

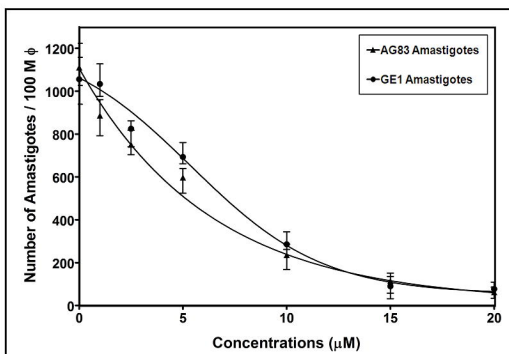


# Figure 8

## A



## B



## Supplemental Data

**Article Title:** Novel Betulin Derivatives as Antileishmanial Agents with Mode of Action Targeting Type IB DNA Topoisomerase.

**Authors:** Sayan Chowdhury, Tulika Mukherjee, Souvik Sengupta, Somenath Roy Chowdhury, Sibabrata Mukhopadhyay and Hemanta K. Majumder.

**Journal:** Molecular Pharmacology.

## **Supplemental Figure Legends**

### **Supplemental Figure S1:**

(A) Suicidal cleavage reactions and electrophoresis in a denaturing polyacrylamide gel were performed as described in Materials and Methods. Lane 1, 5 nM of 5'- <sup>32</sup>P- end labeled ML14/ML25 mer duplex oligonucleotides as indicated above. Lane 2, same as lane 1, but incubated with 0.2 μM of LdTOP1LS in the absence of inhibitors. Lanes 3–5, same as lane 2, but incubated with 200 μM of DiSB, DiGDHB and DiSDHB respectively for 2h. Positions of uncleaved oligonucleotide (14mer) and the cleavage product (12mer oligonucleotide complexed with residual topo I) are indicated. All the reactions were stopped by addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with ethanol, digested with trypsin and analyzed by 12% denaturing polyacrylamide gel as described in Materials and Methods.

### **Supplemental Figure S2: Fluorescence quenching analysis of Camptothecin-LdTOP1LS interaction.**

Fluorescence emission spectra ( $\lambda_{\text{excitation}} = 295 \text{ nm}$ ,  $\lambda_{\text{emission}} = 350 \text{ nm}$ ) of 200 nM LdTOP1LS were determined as a function of CPT concentration in the range of 0–22.5 μM. Control fluorescence spectrum of LdTOP1LS is shown. Arrow indicates the insignificant change in the emission spectra of LdTOP1LS on addition of CPT in increasing concentration.

**Supplemental Figure S3: Lineweaver-Burk representation of the kinetics of relaxation of negatively supercoiled pBS (SK<sup>+</sup>) DNA by LdTOP1LS alone and with each of the inhibitors respectively.**

The relaxation of negatively supercoiled pBS (SK<sup>+</sup>) DNA were performed for 1 min with increasing concentration of pBS (SK<sup>+</sup>) as described in Materials and Methods. DNA concentrations ranged from 8-60 nM and 0.98 nM enzyme has been added simultaneously without (■) or with each inhibitors of DiSB (◆), DiGDHB (●) or DiSDHB (▲) separately and analyzed by electrophoresis in analyzed in 1% agarose gel. Initial velocities were calculated as described in Materials and Methods and double reciprocal plot has been generated.

**Supplemental Figure S4: Mean of infected macrophages after treatment with betulin and dihydrobetulin derivatives.**

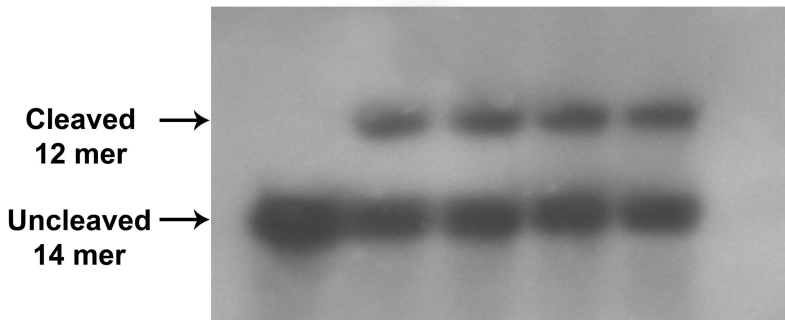
The percentage of macrophages infected with *L. donovani* and effect of each inhibitor of DiSB (◆), DiGDHB (■) or DiSDHB (▲) was plotted against their applied concentrations with standard error bars.

# Supplemental Figure S1

ML 14 : 5' <sup>32</sup>P- GAAAAAAGACTT↓AG 3'

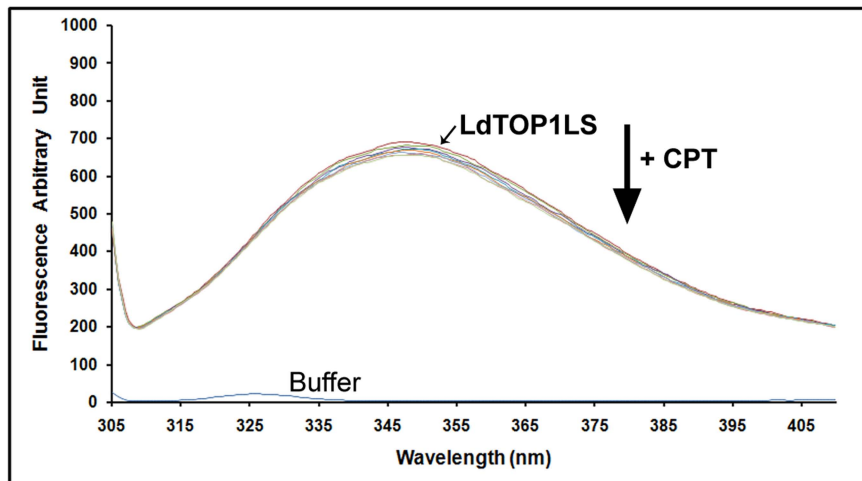
ML 25 : 3' CTTTTTCTGAA TCTTTTTAAAAAT 5'

DiSDHB (μM)	—	—	—	—	200
DiGDHB (μM)	—	—	—	200	—
DiSB (μM)	—	—	200	—	—
LdTOP1LS	—	+	+	+	+
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>

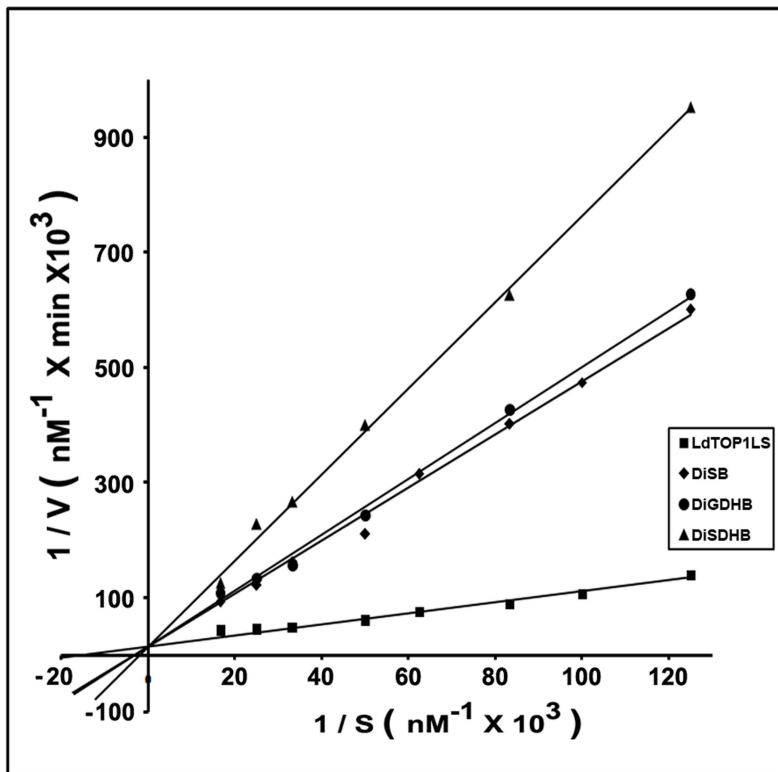




## Supplemental Figure S2



# Supplemental Figure S3



# Supplemental Figure S4

