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An insight into the mechanism of inhibition of unusual bi-subunit topoisomerase I from *Leishmania donovani* by 3,3′-di-indolylmethane, a novel DNA topoisomerase I poison with a strong binding affinity to the enzyme

Amit ROY*, Benu Brata DAS†, Agneyo GANGULY*, Somdeb BOSE DASGUPTA*, Neeta V. M. KHALKHO*, Churala PAL‡, Sumit DEY‡, Venkatachalam Sesha GIRI‡, Parasuraman JAISANKAR‡, Sanjit DEY§ and Hemanta K. MAJUMDER*[1]

*Department of Molecular Parasitology, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata-700032, India, †Laboratory of Molecular Pharmacology, National Cancer Institute, NIH (National Institutes of Health), 37 Convent Drive MSC 4255, Bethesda, MD 20892-4255, U.S.A., ‡Department of Medicinal Chemistry, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata-700032, India, and §Department of Physiology, University of Calcutta, 92, APC Road, Kolkata-700009, India

DIM (3,3′-di-indolylmethane), an abundant dietary component of cruciferous vegetables, exhibits a wide spectrum of pharmacological properties. In the present study, we show that DIM is a potent inhibitor of *Leishmania donovani* topoisomerase I with an IC_{50} of 1.2 μM. Equilibrium dialysis shows that DIM binds strongly to the free enzyme with a binding constant of 9.73×10^{-9} M. The binding affinity of DIM to the small subunit is 8.6-fold more than that of the large subunit of unusual LdTOP1LS (bi-subunit *L. donovani* topoisomerase I). DIM stabilizes topoisomerase I–DNA cleavage complexes *in vitro* and also *in vivo*. Like CPT (camptothecin), DIM inhibits the religation step when the drug was added to preformed topoisomerase I–DNA binary complex. Hence, DIM is similar to CPT with respect to its ability to form the topoisomerase I-mediated 'cleavable complexes' *in vitro* and *in vivo*. But unlike CPT, DIM interacts with both free enzyme and substrate DNA. Therefore DIM is a non-competitive class I inhibitor of topoisomerase I. DIM also inhibits the relaxation activity of the CPT-resistant mutant enzyme LdTOP1Δ39LS (N-terminal deletion of amino acids 1–39 of LdTOP1LS). The IC_{50} values of DIM in simultaneous and enzyme pre-incubation relaxation assays were 3.6 and 2.9 μM respectively, which are higher than that of wild-type topoisomerase I (LdTOP1LS), indicating that the affinity of DIM to LdTOP1Δ39LS is less than that for LdTOP1LS. This is the first report on DIM as an *L. donovani* topoisomerase I poison. Our study illuminates a new mode of action of enzyme inhibition by DIM that might be exploited for rational drug design in human leishmaniasis.

Key words: cleavable complex, 3,3′-di-indolylmethane (DIM), *Leishmania donovani*, reconstitution, topoisomerase I, topoisomerase poison.

INTRODUCTION

DNA topoisomerases are a family of DNA-processing enzymes that catalyse the breakage and rejoining of DNA strands to govern the topological changes in DNA molecules [1]. These ubiquitous enzymes play a pivotal role in modulating the dynamic nature of DNA secondary and higher-order structures, and carry out vital cellular processes, e.g. replication, repair, recombination, transcription, integration and chromosomal segregation [2,3]. The topoisomerases are broadly classified into two types: type I and type II, both of which have gained importance as chemotherapeutic targets. Topoisomerase II is the target of various antitumour agents, e.g. m-AMSA (amsacrine), etoposide, teniposide and doxorubicin, which stabilize the 'cleavable complex' between the enzyme and DNA [2]. Catalytic inhibitors that display high activity against topoisomerase II have been identified previously, e.g. merbarone [4] and aclarubicin [5]. In contrast, DNA topoisomerase I inhibitors are very rare, the most widely studied and characterized inhibitor being CPT (camptothecin), a topoisomerase I poison [6]. A few other topoisomerase I inhibitors have also been reported, such as β-lapachone [7], diospyrin [8], betulinic acid [9], DHBA (dihydrobetulinic acid) [10] and luteolin [11].

Topoisomerases I from all eukaryotes are type IB enzymes that catalyse the trans-esterification reaction by attaching to the 3′-end of cleaved DNA by forming a phosphotyrosine linkage. The catalytic cycle is completed by a single-strand rotation followed by a religation step. Most eukaryotic type IB topoisomerases, including human topoisomerase I, are monomeric enzymes comprising a conserved DNA-binding domain and a C-terminal domain. But interestingly, DNA topoisomerase I of the kinetoplastid protozoan parasite *Leishmania donovani* is an unusual bi-subunit enzyme, which is expressed from two open reading frames to form a heterodimeric enzyme consisting of LdTOP1L (*L. donovani* topoisomerase I large subunit; 635 amino acids, 73 kDa) and LdTOP1S (*L. donovani* topoisomerase I small subunit; 262 amino acids, 29 kDa) [12]. The two subunits are synthesized from two different genes and associate with each other through protein–protein interaction to form an active heterodimeric topoisomerase I within the parasite [12]. The first 39 amino acids of the N-terminal region of LdTOP1LS (bi-subunit *L. donovani* topoisomerase I) that resemble the cap region of the monomeric

Abbreviations used: CPT, camptothecin; CT DNA, calf thymus DNA; DHBA, dihydrobetulinic acid; DIM, 3,3′-di-indolylmethane; DTT, dithiothreitol; EtBr, ethidium bromide; 3HF, 3-hydroxyflavone; IFNγ, interferon γ; LdTOP1L, *Leishmania donovani* topoisomerase I large subunit; LdTOP1LS, bi-subunit *L. donovani* topoisomerase I; LdTOP1S, *L. donovani* topoisomerase I small subunit; LdTOP1Δ39LS, N-terminal deletion of amino acids 1–39 of LdTOP1LS; NF-κB, nuclear factor κB; pBS (SK$^+$), pBluescript (SK$^+$); TNF-α, tumour necrosis factor-α.

[1] To whom correspondence should be addressed (email hkmajumder@iicb.res.in).

enzymes have a modulating role in non-covalent interaction with DNA and sensitivity towards CPT. Thus the N-terminal of the bi-subunit enzyme has an important role in strand rotation and DNA relaxation, whereas amino acid residues 40–99 of LdTOP1L have an important role in the interaction with LdTOP1S [13].

All the topoisomerase inhibitors are broadly divided into two classes. The class I topoisomerase inhibitors, referred to as 'topoisomerase poisons', act by stabilizing the enzyme–DNA covalent complex (cleavable complex). A large number of topoisomerase inhibitors were developed that play a key role in cancer therapy. Some of them also act as antiparasitic agents, e.g. betulinic acid [9], luteolin [11,14], baicalein [15] and CPT [16]. Improved drug therapy of *Leishmania* infections is still desirable, and the need for new molecular targets for developing therapeutic agents is clear and justified. DNA topoisomerases have emerged as principal therapeutic targets, with a group of targeting agents having a broad spectrum of antiparasitic activity [2].

DIM (3,3′-di-indolylmethane) is a type of novel topoisomerase inhibitor. DIM is a major acid condensation product of I3C (indole-3-carbinol), a natural compound found in vegetables of the genus *Brassica*, including turnips, kale, broccoli, cabbage, Brussels sprouts and cauliflower. It has an anticarcinogenic effect in experimental animals and inhibits the growth of human cancer cells [17,18]. Several studies have demonstrated that dietary exposure to DIM has a pronounced effect against tumour development [19]. Thus there is considerable interest in the mode of action of DIM because of its pronounced antitumour activities in human breast cancer cell lines [20]. DIM is a novel immunomodulator [20] and induces G_1 arrest in breast cancer cells [18] and leads to apoptosis [21]. DIM significantly inhibits TNF-α (tumour necrosis factor-α)-induced translocation of NF-κB (nuclear factor κB) to the nucleus, and down-regulates NF-κB function and promotes apoptotic signalling while protecting cells from DNA-damaging agents, such as TNF-α [22]. DIM up-regulates the expression of IFNγ (interferon γ) in human MCF-7 breast cancer cells. This novel effect may contribute to the anticancer effects of DIM, because IFNγ plays an important role in preventing the development of primary and transplanted tumours [23]. Recently, Gong et al. [24] have demonstrated that DIM is a novel topoisomerase IIα catalytic inhibitor in human hepatoma HepG2 cells and also inhibits topoisomerase I at high concentrations.

The most widely studied inhibitor of type IB topoisomerase is CPT, an important class of antitumour agents; CPT is an uncompetitive topoisomerase I inhibitor that traps the enzyme–DNA covalent complex and slows the religation step of the nicking/closing cycle [7,25]. In *Trypanosoma brucei*, *Trypanosoma cruzi* and *L. donovani*, CPT promotes protein–DNA complex formation with nuclear as well as kinetoplast DNA [16]. CPT enhances the formation of 'cleavable complex' at low salt concentrations [12] and induces cellular dysfunction of *L. donovani* with features that are well characterized by several cytoplasmic and nuclear events of apoptosis [26].

In the present study, we demonstrate for the first time that DIM strongly inhibits *L. donovani* topoisomerase I and stabilizes topoisomerase I–DNA cleavable complex. This cleavable complex formation is inhibited when LdTOP1LS was pre-incubated with DHBA, a catalytic inhibitor of topoisomerase I [10], prior to the addition of DIM. In the present study, we have also shown that DIM binds to enzyme as well as DNA. The binding of DIM to enzyme is stronger than flavones or CPT. Our results suggest that DIM is a novel *L. donovani* topoisomerase I poison that acts as a non-competitive inhibitor. Thus this intriguing dietary component might be exploited for therapeutic development against leishmaniasis.

EXPERIMENTAL

Chemicals

The bioactive DIM ($C_{17}H_{14}N_2$; M_r 246; Figure 1A) was synthesized chemically from indole and urotropine by the addition of $InCl_3$ (10 mol%) and propan-2-ol [27]. DHBA and luteolin were synthesized and purified as described previously [11,12]. CPT was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). All drugs were dissolved in 100% DMSO at a concentration of 20 mM and stored at −20 °C.

Parasite culture, maintenance and cytotoxicity assay

L. donovani strain AG83 promastigotes were grown at 22 °C in M199 liquid medium supplemented with 10% (v/v) fetal calf serum or in Ray's modified medium as described previously [10,14]. Cytotoxicity of DIM was estimated by microscopic counting of the viable parasites by the Trypan Blue exclusion method after treatment of *L. donovani* promastigotes with DIM.

Purification and reconstitution of recombinant proteins to examine *L. donovani* topoisomerase I activity

Escherichia coli BL21 (DE3) pLysS cells harbouring pET16bLdTOP1L, pET16bLdTOP1Δ39L and pET16bLdTOP1S, described previously [15], were separately induced at an attenuance (D_{600}) of 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 an Ni–NTA (Ni^{2+}–nitrilotriacetate)–agarose column (Qiagen) followed by a phosphocellulose column (P11 cellulose; Whatman) as described previously [16]. Finally, the purified proteins LdTOP1L, LdTOP1Δ39L and LdTOP1S were stored at −70 °C.

Purified LdTOP1L or LdTOP1Δ39L was mixed with purified LdTOP1S separately 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 (dithiothreitol), 1 mM EDTA, 0.1 mM PMSF and 10% (v/v) glycerol]. The mixtures were dialysed overnight at 4 °C and the dialysed fractions were used for the plasmid relaxation activity [12,13].

Plasmid relaxation assay

The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBS (SK^+) [pBluescript (SK^+)] DNA in an agarose gel. The relaxation assay was carried out as described previously [13,26] with LdTOP1LS and LdTOP1Δ39LS (N-terminal deletion of amino acids 1–39 of LdTOP1LS) serially diluted in the relaxation buffer (25 mM Tris/HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM $MgCl_2$, 2.5 mM EDTA and 150 μg/ml BSA), supercoiled pBS (SK^+) DNA (85–95% was negatively supercoiled, with the remainder being nicked circles) and 50 mM KCl. The amount of supercoiled monomer DNA band florescence after EtBr (ethidium bromide; 0.5 μg/ml) staining was quantified by integration using Gel Doc 2000 under UV illumination (Bio-Rad Quality One software), as described in [13]. Initial velocities (nM of DNA base-pairs relaxed/min) were calculated by the 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, Int_0 is the area under the supercoiled DNA

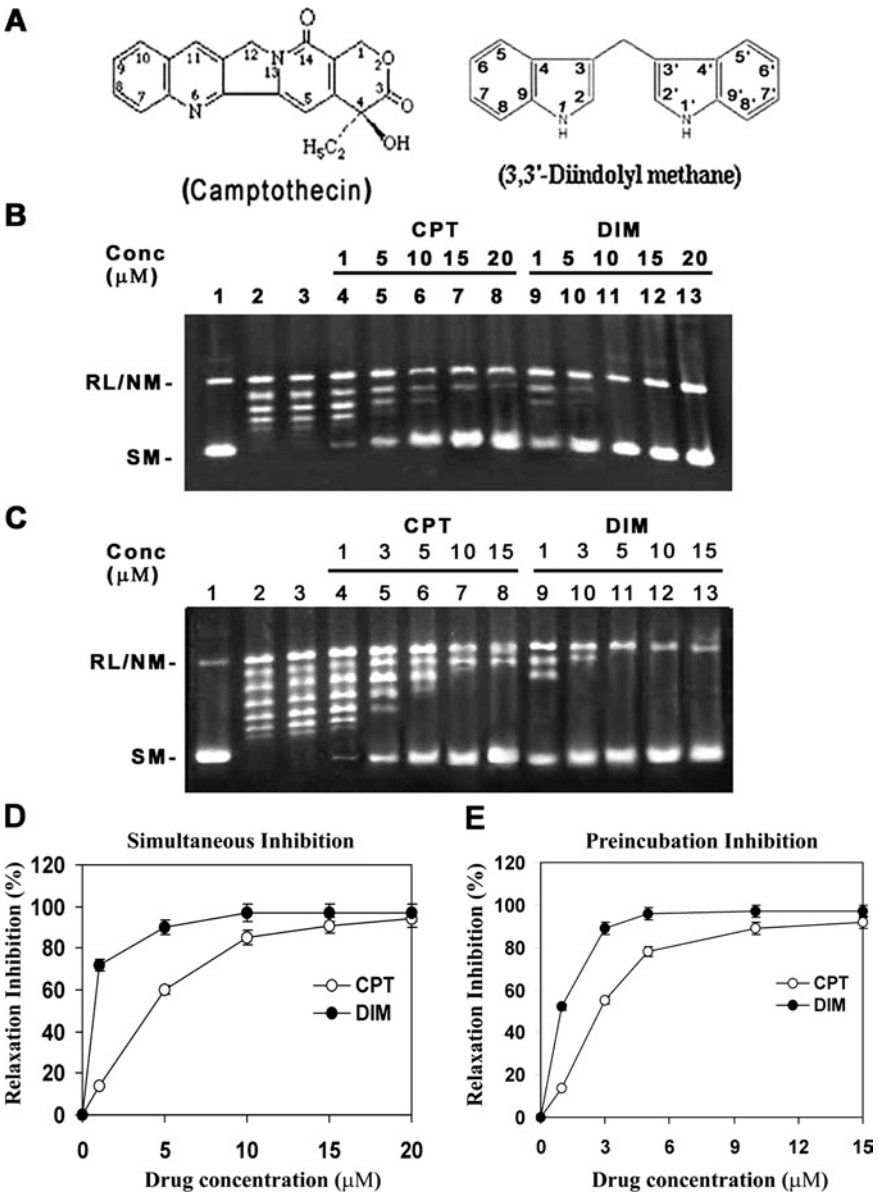

Figure 1 Inhibition of LdTOP1LS activity by DIM

(**A**) Structures of CPT and DIM. (**B**) 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 the presence of 4 % (v/v) DMSO; lanes 4–8, same as lane 2, but in the presence of 1, 5, 10, 15 and 20 μM CPT respectively; lanes 9–13, same as lane 2, but in the presence of 1, 5, 10, 15 and 20 μM DIM respectively. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. (**C**) Pre-incubation of LdTOP1LS with CPT and DIM followed by addition of DNA. Lane 1, 90 fmol of pBS (SK^+) DNA; lane 2, same as lane 1, but 30 fmol of LdTOP1LS was pre-incubated with reaction buffer for 5 min at 37 °C, followed by addition of 90 fmol of pBS (SK^+) DNA, and was further incubated for 15 min at 37 °C, lane 3, same as lane 2, but the enzyme was pre-incubated with 4 % (v/v) DMSO; lanes 4–8, same as lane 2, but the enzyme was pre-incubated with 1, 3, 5, 10 and 15 μM CPT respectively at 37 °C for the indicated time periods; lanes 9–13, same as lane 2, but the enzyme was pre-incubated with 1, 3, 5, 10 and 15 μM DIM respectively. Reactions were stopped by addition of SDS to a final concentration of 0.5 % and were electrophoresed in 1 % agarose gel. (**D**) Quantitative representation of enzyme inhibition in the presence of DIM in relaxation experiments. LdTOP1LS (100 fmol) was incubated simultaneously with 1, 5, 10, 15 and 20 μM CPT and DIM simultaneously with pBS (SK^+) DNA for 30 min at 37 °C in relaxation buffer. (**E**) The enzyme was pre-incubated separately with 1, 3, 5, 10 and 15 μM CPT and DIM respectively for 5 min at 37 °C in relaxation buffer, followed by addition of 50 fmol of pBS (SK^+) DNA, and was further incubated for 15 min at 37 °C. Reactions were stopped by the addition of SDS and electrophoresed as described above. The percentage of relaxation inhibition is plotted as a function of drug 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. Variations among different sets of experiments were <5 %.

band at zero time and Int_t is the area at the reaction time t [28]. The effect of DNA concentration on the kinetics of relaxation was examined over the range of 4–40 nM supercoiled pBS (SK^+) DNA (0.16–1.6 μg/25 μl of reaction mixture) at constant concentrations of 10 mM $MgCl_2$ and 0.9 nM enzyme (LdTOP1LS) at 37 °C for 1 min. The data were analysed by a Lineweaver–Burk plot. Intercept of the y-axis is $1/V_{max}$, and catalytic-centre activity $= V_{max}$/enzyme concentration (plasmid molecules relaxed/min per molecule of enzyme).

Plasmid cleavage assay

Cleavage assay was carried out as described in [8]. 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 μl) containing 50 mM Tris/HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA and 30 μg/ml BSA in the presence of various concentrations of drugs at 37 °C for 30 min. The reactions were terminated by adding 1 % SDS and 150 μg/ml proteinase K and further incubated for 1 h at 37 °C. DNA samples were electrophoresed in a 1 % agarose gel containing 0.5 μg/ml EtBr.

Duplex oligonucleotide cleavage assay

The 25-mer duplex of oligonucleotide 1 (5′-GAAAAAAGACTT↓AGAAAAATTTTTA-3′) and oligonucleotide 2 (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) containing a topoisomerase I-binding motif was labelled and annealed as described in [13]. Cleavage was carried out using 20-fold molar excess of the wild-type (LdTOP1LS) and mutant enzymes (LdTOP1Δ39LS) over duplex 25-mer DNA (enzymes, 0.2 μM; DNA, 10 nM). The reactions were carried out in a standard assay mixture containing 10 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EDTA and 50 mM KCl in the presence or absence of drugs at 37 °C for 30 min or for the indicated time periods. All the reactions were stopped by addition of SDS to a final concentration of 2 % (w/v). The samples were precipitated with ethanol, digested with 5 μl of 1 mg/ml trypsin and resolved on 12 % (w/v) denaturing polyacrylamide gel followed by autoradiography as described above. The amount of strand cleavage in the presence of drugs for the wild-type enzymes was determined by film densitometry as described previously [13].

In vivo cleavable complex formation and measurement of DNA synthesis

DNA–protein complexes can be trapped within cells and quantified by the KCl–SDS co-precipitation assay [7,15]. Exponentially growing *L. donovani* promastigotes (4×10^6 cells/ml) were radiolabelled by adding [Me-^{3}H]thymidine (Amersham) to the medium to a final concentration of 5 μCi/ml for 24 h at 22 °C. Cells were then pelleted by centrifugation at 800 ***g*** for 5 min at room tempeature (25 °C), washed twice with PBS and resuspended in fresh M199 liquid medium supplemented with 10 % (v/v) fetal calf serum for 3 h. Cells were then exposed to various concentrations of DIM, CPT and DHBA at 22 °C for indicated time periods. Finally, the cells were lysed in 200 μl of SDS-containing solution [1.25 % (w/v) SDS, 5 mM EDTA, pH 8.0, and 0.4 mg/ml CT DNA (calf thymus DNA)] prewarmed at 65 °C. The lysates were transferred to 1.5 ml Microfuge tubes containing 250 μl of 325 mM KCl. After vigorous mixing, the samples were cooled on ice for 10 min and centrifuged at 8000 ***g*** for 30 min at 4 °C. The pellets were resuspended in 500 μl of wash solution (10 mM Tris/HCl, pH 8.0, 100 mM KCl, 1 mM EDTA and 0.1 mg/ml CT DNA) and warmed at 65 °C for 10 min with occasional shaking. The suspensions were cooled on ice for 10 min and re-centrifuged. The pellets were washed as above and mixed with 4 ml of scintillation liquifluor (Spectrochem) and the radioactivity was determined with a liquid–scintillation counter [15].

Immunoband depletion assay

Leishmania cells (2×10^7) were cultured for 12 h at 22 °C with or without drugs. Nuclear fractions were isolated as described previously [29]. Briefly, cells were suspended in hypo-osmotic buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine hydrochloride and 5 mM DTT) and homogenized. The homogenate was centrifuged at 10000 ***g*** for 10 min at 4 °C. The pellet was washed and used as the source of nuclear fraction. Then the nuclear fractions after lysis with 1 % SDS were subjected to SDS/10 % PAGE, and the proteins were electrophoretically transferred on to nitrocellulose membranes. Immunoblotting of immobilized proteins was carried out using a rabbit antibody raised against LdTOP1S [12] and ATPase domain (43 kDa) of *L. donovani* topoisomerase II [30].

Single turnover cleavage and religation assay

A 14-mer (5′-GAAAAAAGACTT↓AG-3′) oligonucleotide containing an topoisomerase IB-specific cleavage site was 5′-^{32}P-end-labelled and annealed to 25-mer (3′-CTTTTTTCTGAATC-TTTTTAAAAAT*P*-5′) oligonucleotides as described previously [13]. The suicidal cleavage reaction was carried out with 5 nM DNA substrate and 0.15 μM enzyme (LdTOP1LS) in 20 μl reaction mixtures under standard assay conditions (10 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA and 50 mM KCl) at 23 °C for 4 h in the presence or absence of drugs as described previously [13]. For religation experiments, covalent complexes generated by incubating suicide DNA substrate with LdTOP1LS in the presence or absence of drugs were transferred to 30 °C and pre-incubated for 2 min. The religation reaction was initiated by the addition of 300-fold molar excess of the 11-mer religation acceptor oligonucleotides (5′-OH-AGAAAAATTTT-3′) in the same reaction mixture and incubated for the indicated time periods. Finally, all the reactions were stopped by adding SDS, and DNAs were subsequently precipitated by ethanol. Samples were digested with 5 μl of 1 mg/ml trypsin, electrophoresed in 12 % (w/v) denaturing polyacrylamide gel and autoradiographed [13].

Equilibrium dialysis

The equilibrium dialysis experiment was performed as described previously [31]. The binding solution as well as the buffer used for equilibrium dialysis was 20 mM Tris/HCl (pH 7.5), 50 mM NaCl and 10 mM $MgCl_2$. Four sets of reactions were performed in 500 μl of binding solution containing 10 nM enzyme (LdTOP1LS) in one set, 60 μM BSA in other set as control, 10 μM LdTOP1L and 0.1 μM LdTOP1S and increasing concentrations of DIM (1, 5, 10, 15 and 20 μM) at 37 °C for 30 min. The reaction mixture was loaded into the dialysis bag with 12–14 kDa cut-off and placed in 5 ml of buffer solution. Corresponding 'blanks' containing only buffer solution and only buffer/enzyme solution without drug were run. The solutions were equilibrated for 24 h at 25 °C with shaking at 100 rev./min for the entire period. At the end of the equilibration, the concentration of DIM in the outside solutions was estimated by measuring the absorbance of the solution and using a molar absorption coefficient of 6.6×10^4 $M^{-1} \cdot cm^{-1}$ for DIM. Inside solutions could not be used, since protein interfered in the estimation. All the absorbance measurements were made at 25 °C using a Bio-Rad SmartSpec™3000 UV spectrophotometer in a 10 mm pathlength cell.

Spectrofluorimetric binding assay

Fluorescence titration was measured using a PerkinElmer LS55 luminescence spectrometer. The intrinsic binding of DIM to DNA was performed separately by fluorescence measurements at a λ_{ex} of 280 nm and λ_{em} in the range of 300–500 nm. Excitation and emission slit widths were 5 and 5 nm respectively. Background emission (<2 %) was corrected by subtraction of spectra of blank buffer, DNA plus sample buffer and DIM plus sample buffer respectively. Spectral titration was performed with DIM at 25 °C in fluorescence buffer (20 mM Tris/HCl, pH 7.5, 50 mM NaCl and 10 mM $MgCl_2$). CT DNA (double-stranded DNA) was added

in increasing concentrations (20, 30, 50, 80, 100, 120, 150 and 200 μM), and thermally denatured DNA (single-stranded) was added at 20, 50, 100, 150 and 200 μM, as indicated in the legend of Figure 6. All the assays were performed in duplicate, titration points were corrected as described above and binding constants for DIM–DNA interaction were determined according to the following equation [32]:

$$1/\Delta F = 1/\Delta F_{max} + (1/K_a \cdot S_t)(1/\Delta F_{max}) \quad (1)$$

where $\Delta F = F_x - F_0$, F_x and F_0 represent the fluorescence intensity of DIM in the presence or absence of the added total DNA (S_t) respectively. ΔF_{max} is the maximum change in the fluorescence intensity. The intercept of the plot on the $1/F$ axis corresponding to $1/S_t = 0$ measures the $1/F_{max}$, whereas the slope gives the estimation of the affinity constant (K_a). The dissociation constant $K_D = 1/K_a$.

RESULTS

DIM inhibits the catalytic activity of *L. donovani* topoisomerase I

The effect of DIM on *L. donovani* topoisomerase I was examined by plasmid relaxation assays as described in the Experimental section. The recombinant LdTOP1LS was purified as described in [12]. The relaxation experiments were performed under standard assay conditions where the plasmid DNA and the enzyme (LdTOP1LS) were mixed at a molar ratio of 3:1. Under this condition in the absence of DIM, LdTOP1LS completes its relaxation within 20 min (Figure 1B, lane 2). When added together with pBS (SK^+) DNA and enzymes, DIM partially inhibited relaxation of supercoiled DNA at 1 and 5 μM (Figure 1B, lanes 9 and 10). Lane 10 shows that 87 % inhibition was achieved by DIM at 5 μM. Under the same assay condition, 95 % of DNA relaxation activity of LdTOP1LS was inhibited by simultaneous addition of 10 μM DIM (lane 11). Similar inhibition (90 %) was achieved by CPT at 20 μM (lane 8). It can be inferred from the above results that DIM is also a good inhibitor of topoisomerase I, like CPT.

To investigate the interaction of DIM with the enzyme in the relaxation experiment, LdTOP1LS was pre-incubated separately with DIM at different concentrations for 5 min at 37 °C before the addition of DNA (Figure 1C). The inhibition of the enzyme by pre-incubation with DIM was compared with the inhibitory effects of DIM incubated simultaneously with enzyme (LdTOP1LS) and supercoiled DNA in the relaxation assay. Interestingly, 85 % inhibition was achieved at 3 μM concentration of DIM (lane 10), and 95 % inhibition at 5 μM concentration (lane 11).

The IC_{50} values were determined under different pre-incubation conditions. DIM was pre-incubated with enzyme for different time periods before the addition of substrate DNA and further incubated at the same temperature for 30 min. Results showed that IC_{50} decreased with increasing pre-incubation time and it was minimal for 5 min pre-incubation.

Further pre-incubation had no effect on IC_{50} (results not shown). The IC_{50} values of DIM and CPT are shown in Figures 1(D) and 1(E). The IC_{50} values of DIM in simultaneous and pre-incubation DNA relaxation assay were 1.2 μM (Figure 1D) and 0.8 μM (Figure 1E) respectively. But CPT inhibits the reaction at much higher concentrations (4.2 and 2.8 μM respectively) under both simultaneous and pre-incubation conditions (Figures 1B and 1C, lanes 4–8).

DIM stabilizes *L. donovani* topoisomerase I–DNA cleavable complexes formation

The topoisomerase reaction has three general mechanistic steps: (i) binding of enzyme to the substrate DNA, (ii) single strand breakage and subsequent strand rotation through the break, leading to a change in linking number, and (iii) strand religation. The second step of reaction is the introduction of a single-stranded nick in the phosphodiester bond of the DNA, through which an intact strand is allowed to pass. During this process, a covalent bond is formed between the 3′-phosphoryl group of the DNA backbone and the tyrosine residue at the active site of topoisomerase I. Topoisomerase inhibition can be achieved by prevention of enzyme–DNA binary complex formation or by stabilization of enzyme–DNA cleavable complex. CPT, the most established topoisomerase IB inhibitor, has been shown to stabilize the topoisomerase I–DNA cleavable complex. In order to investigate the mechanism of inhibition of LdTOP1LS, trans-esterification was examined under equilibrium conditions by reacting LdTOP1LS with pHOT1 DNA in the presence of DIM and CPT. In the present study, we investigated the ability of DIM to stabilize cleavable complex formation between LdTOP1LS and pHOT1 DNA (Figure 2A) containing a topoisomerase IB-specific binding motif as described in the Experimental section. This experiment was performed at increasing DIM concentrations with CPT under standard assay conditions. It has been shown that closed circular DNA (form I) was converted into nicked circular DNA (form II) in the presence of 100 fmol of LdTOP1LS with increasing drug concentrations. Approx. 50 % of form I DNA was converted into form II at 1 μM DIM (Figure 2A, lane 6). The amount of form II DNA increased with increasing concentrations of DIM (Figure 2A, lanes 6–11). Approx. 95 % cleavable complex was stabilized at 5 μM DIM, and complete cleavage (100 %) was induced by 10 μM DIM (Figure 2B), whereas 60 μM CPT fully stabilizes the cleavable complex (lane 5). The background cleavage, i.e. the formation of form II DNA in the presence of topoisomerase I only, is shown in lane 2. Lane 3 shows the formation of nicked product when the covalent complex was trapped with SDS and proteinase K. EtBr at a final concentration of 0.5 μg/ml was included in the gel to resolve the more slowly migrating nicked product (form II) from the relaxed molecules (form I′). This result shows that DIM does not inhibit step II of a topoisomerase I-catalysed reaction. Instead, DIM stabilizes the topoisomerase I-mediated cleavable complex and acts as a topoisomerase poison.

This conclusion was further justified by the experiment with DHBA as a negative control. DHBA prevents the stabilization of cleavable complex formation as described previously [10]. This experiment was performed at increasing DIM concentrations prior to the addition of 10 μM DHBA and at increasing DHBA concentrations prior to the addition of 10 μM DIM, as mentioned in Figure legends in standard assay conditions (Figure 2C). Moreover, when LdTOP1LS was pre-incubated with increasing concentrations of DIM (1–10 μM) prior to the addition of 10 μM DHBA, the DIM-mediated cleavage was not inhibited by DHBA (lanes 4–7). But when LdTOP1LS was pre-incubated with increasing concentrations of DHBA (1–20 μM) prior to the addition of 10 μM DIM, the DIM-mediated cleavage was inhibited at that concentration of DHBA (lanes 8–11). So DHBA inhibits the DIM-mediated cleavage only when the enzyme is pre-incubated with DHBA prior to the addition of DIM.

The above experiment was further supported by duplex oligonucleotide cleavage under equilibrium conditions by reacting LdTOP1LS with 25-mer duplex oligonucleotides in the presence of 5, 10, 15 and 20 μM DIM. The cleavage experiment was carried out with 5′-^{32}P-end-labelled 25-mer duplex oligonucleotides containing a topoisomerase IB-specific binding motif as described in the Experimental section. DIM enhanced cleavage with increasing concentration of the drug, and the change is approx. 80 % at 20 μM with respect to the extent of cleavable complex

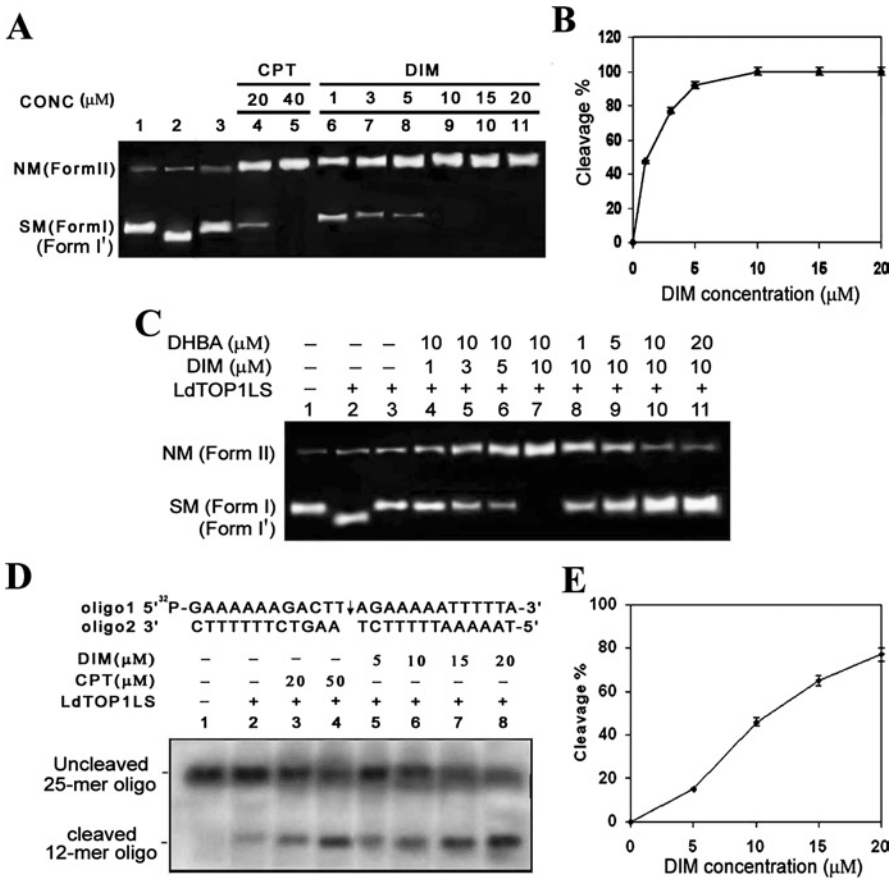

Figure 2 DIM stabilizes LdTOP1LS-mediated DNA cleavage

(**A**) Cleavage reaction and agarose gel electrophoresis were performed as described in the Experimental section. Lane 1, 50 fmol of pHOT1 DNA; lane 2, with 100 fmol LdTOP1LS; lane 3, same as lane 2, but with SDS–proteinase K treatment; lanes 4 and 5, same as lane 3, but in the presence of 20 and 40 μM CPT respectively as a control; lanes 6–11, same as lane 3, but in the presence of 1, 3, 5, 10, 15 and 20 μM DIM. Positions of supercoiled monomer (SM;form I) and nicked monomer (NM;form II) are indicated. Form I′, relaxed molecules. (**B**) Graphical representation of the extent of covalent complex formation plotted as increasing DIM concentrations (1, 3, 5, 10, 15 and 20 μM respectively). Cleavage %=Form II DNA/(Form I+Form II) DNA $\times$ 100 was determined by densitometry. Results are presented as the means $\pm$ S.D. ($n = 3$). (**C**) DHBA antagonizes DIM-mediated DNA cleavage. Reactions were as described in the Experimental section. Lane 1, 50 fmol of supercoiled pHOT1 DNA; lane 2, with 100 fmol of LdTOP1LS; lane 3, same as lane 2, but with SDS–proteinase K treatment; lanes 4–7, same as lane 2, but in the presence of 1, 3, 5 and 10 μM DIM pre-incubated with enzyme prior to the addition of 10 μM DHBA; lane 8–11, same as lane 2, but the enzyme was pre-incubated with 1, 5, 10 and 20 μM DHBA before addition of DIM (10 μM) and DNA. (**D**) Duplex oligonucleotide cleavage. The cleavage reactions and electrophoresis in a denaturing polyacrylamide gel were performed as described in the Experimental section. Lane 1, 10 nM 5′-P-end-labelled 25-mer duplex oligonucleotides as indicated above. Lane 2, same as lane 1, but incubated with 0.2 μM LdTOP1LS in the absence of inhibitors. Lanes 3 and 4, same as lane 2, but incubated with 20 and 50 μM CPT respectively for 60 min at 23 °C. Lanes 5–8, same as lane 2, but incubated with 5, 10, 15 and 20 μM DIM respectively for 60 min at 23 °C. 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 resolved on 12 % (w/v) denaturing polyacrylamide gel as described in the Experimental section. (**E**) Quantitative representation of percentage of cleavage in the presence of DIM in duplex oligonucleotide cleavage experiment. The percentage of cleaved DNA substrate (12-mer) was plotted as a concentration of DIM. The results depicted were performed three times and representative results from one set of experiments are expressed as means $\pm$ S.D. Variations among different sets of experiments were <6 %.

formed without the drug (Figure 2D, lane 8). In contrast, at 50 μM CPT, the extent of cleavage reached approx. 45 % (lane 4). The graphical representation of the extent of cleavage is shown in Figure 2(E). These results indicate that the efficiency of DIM stabilization of the covalent complex formed between 25-mer duplex DNA and LdTOP1LS was 2-fold compared with that of CPT and correlates with the reduction in relaxation activity of the enzyme in the presence of DIM.

DIM stabilizes *in vivo* cleavable complex formation in *L. donovani* promastigotes

CPT promotes protein–DNA complex formation with nuclear as well as kinetoplast DNA in *T. brucei*, *T. cruzi* and *L. donovani* [19]. The ability of DIM to induce covalent complexes of topoisomerases and DNA in the *L. donovani* promastigotes was quantified by KCl–SDS precipitation assay [9]. The experiments were performed with [^{3}H]thymidine-labelled promastigotes, treated with different concentrations of DIM, CPT and DHBA. The treatment of cells with 5, 10, 20, 50, 100, 150 and 200 μM of DIM for 6 h significantly increased the SDS–K^+ precipitable complex compared with the untreated control cells (Figure 3A). The extent of SDS–K^+ precipitable complex formed with DIM was similar to that obtained by treatment with different concentrations of CPT for 6 h. It was shown earlier that DHBA antagonizes CPT-induced cleavage and it did not induce the formation of SDS–K^+ precipitable complex [10]. When *L. donovani* cells were pretreated with 150 μM DHBA for 15 min before incubation with DIM for 3 h, the formation of SDS–K^+ precipitable complex induced by DIM is inhibited (Figure 3A). These results revealed that the SDS–K^+ precipitable complex is due to the formation of covalent complexes between topoisomerases and DNA, and not to any other protein cross-linked to DNA. Thus we can summarize that

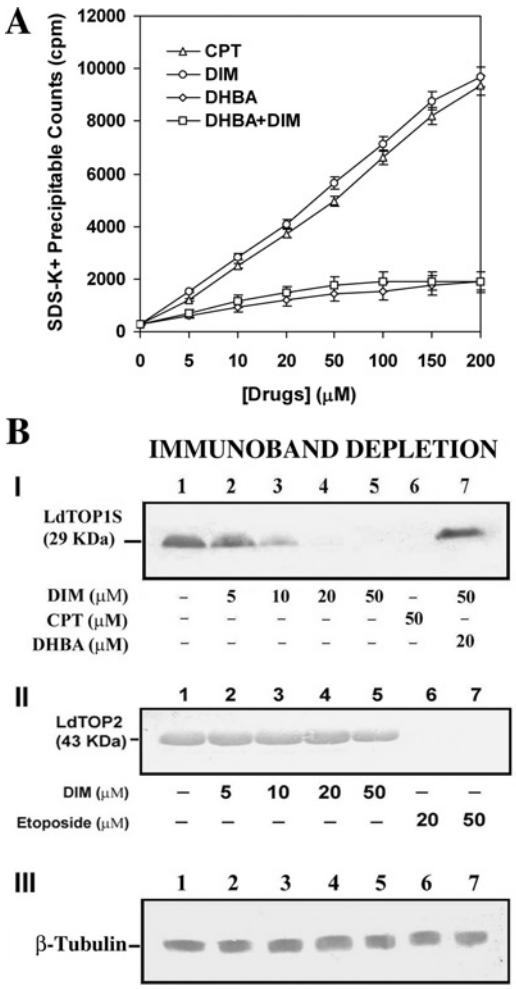

Figure 3 DIM induces the *in vivo* formation of topoisomerase–DNA cleavable complex within the cells

(**A**) Analysis of drug-induced covalent topoisomerase I–DNA complex formation in *L. donovani* promastigotes by the KCl–SDS precipitation assay. Exponentially growing *L. donovani* promastigotes (4×10^6 cells/ml) were labelled with [^{3}H]thymidine at 22 °C for 24 h and then treated with different concentrations of drugs as indicated. A fraction of the total population of labelled cells were treated with DHBA (100 μM) for 15 min before the addition of different concentrations of DIM as indicated. SDS–K$^+$ precipitable complexes were measured as described in the Experimental section. Experiments were performed three times and representative data from one set of experiments are presented as means $\pm$ S.D. Variations among different sets of experiments were <5 %. (**B**) Stabilization of topoisomerase-mediated cleavable complex was determined by the immunoband depletion assay. Panel I: immunoband depletion of *L. donovani* topoisomerase I using an antibody raised against LdTOP1S. Leishmanial cells were treated with 0.2 % DMSO alone (lane 1), 5, 10, 20 and 50 μM DIM (lanes 2–5), 50 μM CPT (lane 6) and 20 μM DHBA before treatment with DIM (lane 7). Panel II: immunoband depletion of *L. donovani* topoisomerase II using an antibody raised against ATPase domain of *L. donovani* topoisomerase II. The cells were treated with 0.2 % DMSO alone (lane 1), 5, 10, 20 and 50 μM DIM (lanes 2–5) and 20 and 50 μM etoposide (lanes 6 and 7). Panel III: loading control.

stabilization of topoisomerase-mediated duplex oligonucleotide DNA cleavage with DIM correlates with the protein–DNA breaks in *L. donovani* promastigotes and drug-induced cytotoxicity. But the SDS–K$^+$ precipitation assay does not differentiate between topoisomerase I–DNA cleavable complexes and topoisomerase II–DNA cleavable complexes.

In order to sort out whether DIM can be responsible for stabilization of covalent complex formation of topoisomerase I and DNA in intact cells, we carried out immunoband depletion experiments with *L. donovani* promastigotes. Nuclear fractions were prepared from untreated as well as drug-treated promastigotes and subjected to SDS/PAGE. If topoisomerase I can form a covalent complex with genomic DNA inside the cells, then topoisomerase I–DNA cleavable complex cannot enter the gel. On the other hand, if topoisomerase I does not form a complex with DNA and remains free, it will enter the gel. The presence of topoisomerase I was detected by immunoblotting as described in the Experimental section. The immunoband depletion data are summarized in Figure 3(B). It was observed that the immunoband of topoisomerase I gradually disappeared with increasing DIM concentration (from 5 to 50 μM) during a 6 h incubation (Figure 3B, panel I, lanes 2–5). The immunoband of topoisomerase I also disappeared after incubation of nuclear extract with 50 μM CPT for 6 h (lane 6). Pre-incubation with 20 μM DHBA before treatment with DIM (50 μM) causes the reappearance of the immunoband of *L. donovani* topoisomerase I, as cleavable complex formation is prevented (lane 7). The same experiment was performed with LdTOP2 to find out if DIM could enhance the *in vivo* cleavable complex formation by topoisomerase II. The topoisomerase II immunoband depletion was not found by increasing the DIM concentration (from 5 to 50 μM) at the same incubation time period (Figure 3B, panel II, lanes 2–5). But the extent of topoisomerase II immunoband depletion was observed by treatment with 20 and 50 μM etoposide, a topoisomerase II poison (Figure 3B, panel II, lanes 6 and 7). The above results suggest that DIM is responsible for the stabilization of topoisomerase I–DNA cleavable complex inside the cells. It should be mentioned here that topoisomerase I of *Leishmania* is a heterodimer and the catalytic site (SKXXY) is present in LdTOP1S, which is involved in the formation of the topoisomerase I–DNA covalent complex. So we have used the antibody raised against LdTOP1S and ATPase domain of LdTOP2 (43 kDa N-terminal recombinant enzyme) to study the immunoband depletion assay.

DIM inhibits single turnover cleavage and religation activity

Catalytic assays do not allow a precise chronological dissection of the inhibitory mechanism in relation to the catalytic cycle. Thus the step at which an inhibitor needs to enter the catalytic cycle and the step at which it becomes effective in trapping or inhibiting the enzyme cannot be differentiated. In order to overcome this problem, we have made the oligonucleotide suicide substrate of topoisomerase IB, which restricts the enzyme to a single round of cleavage and religation and allows one to address the two half-reactions separately as described previously [15].

The substrate consisted of 5′-^{32}P-labelled 14-base-pairs duplex with 11-base 3′ tail [15]. Upon cleavage and formation of topoisomerase–DNA complex, the AG dinucleotide at the 3′-end of the scissile strand is released. Suicidal cleavage assay was performed in the presence and absence of CPT and DIM at 23 °C for indicated time periods as described in the Experimental section. Religation was studied under single turnover conditions by assaying the ability of the covalent intermediate to attach a 5′-hydroxyl-terminated 11-mer to the covalently cleaved 12-mer to form a 23-mer product [15]. CPT stabilizes the suicide cleavage complex with topoisomerase I of *L. donovani* and inhibits the religation step (Figure 4, lane 3), which is in keeping with that of monomeric type IB topoisomerase [9]. Similarly, DIM also inhibits the religation step when added simultaneously with the enzyme and the DNA in the suicidal cleavage assay (lane 4), like CPT.

On the contrary, DHBA, a catalytic inhibitor of topoisomerase I, inhibits the suicidal cleavage reaction (Figure 4, lane 5). However, when DHBA was pre-incubated for 5 min with the enzyme, followed by simultaneous addition of DIM and suicidal substrate,

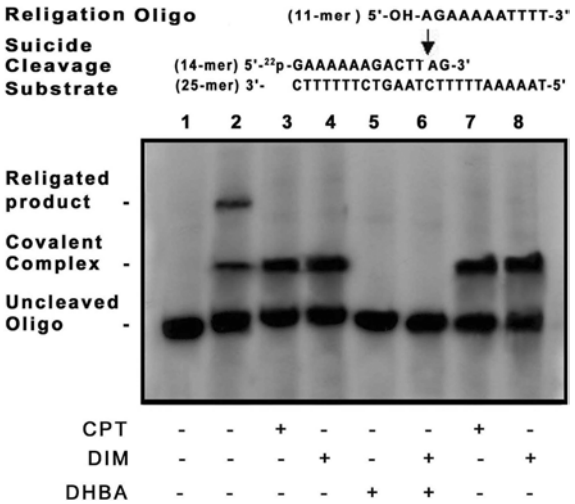

Figure 4 Effect of DIM on single turnover cleavage and religation

Suicidal cleavage assay was performed with the 5′-^{32}P-end-labelled suicide DNA substrate (14-mer/25-mer) as indicated. The DNA substrate was incubated with LdTOP1LS in the presence and in the absence of inhibitors at 23 °C as described in the Experimental section. Active cleavage complexes containing LdTOP1LS attached to the covalently cleaved 12-mer of the suicide substrate were made to react with 5′-hydroxyl-terminated 11-mer to form a 23-mer product for 10 min at 37 °C, and the products were analysed using a denaturing polyacrylamide gel. Lane 1, suicide DNA substrate only; lane 2, same as lane 1, but in the presence of LdTOP1LS and 5′-hydroxyl-terminated 11-mer religation oligonucleotides; lanes 3 and 4, same as lane 2, but with 60 μM CPT and 20 μM DIM respectively added simultaneously with the enzyme and DNA in the suicidal cleavage assay; lane 5, same as lane 2, but with 20 μM DHBA pre-incubated for 2 min at 23 °C with the enzyme before the addition of DNA in the suicidal cleavage assay; lane 6, same as lane 5, but followed by addition of 20 μM DIM simultaneously in the cleavage assay; lanes 7 and 8, same as lane 2, but 60 μM CPT and 20 μM DIM were added after suicidal cleavage reaction (on enzyme–substrate complex) together with 11-mer religation oligonucleotide. All the reactions were stopped by addition of 2 % (w/v) SDS. The samples were precipitated with ethanol, digested with trypsin and analysed by denaturing polyacrylamide sequencing gel electrophoresis. The uncleaved suicidal oligonucleotide, covalent complex and the religation products are indicated.

the suicide-complex formation was completely inhibited (lane 6). On the other hand, DIM also inhibits the religation reaction, like CPT, when added to the enzyme–substrate covalent complex that had been previously formed in the absence of the drug, i.e. a condition where the drugs and 11-mer religation oligonucleotides were added together (Figure 4, lanes 7 and 8) after the suicidal cleavage reaction. Taken together, these results suggest that interaction of DIM with enzyme during the trans-esterification reaction with DNA is a prerequisite for the stabilization of the topoisomerase I cleavable complex and subsequently inhibiting the religation step.

DIM strongly binds to the enzyme as evidenced by equilibrium dialysis

A equilibrium dialysis experiment was performed to check directly the binding of DIM to *L. donovani* topoisomerase I. The number of DIM molecules bound to protein is plotted against increasing concentrations of DIM (see Supplementary Figure 1A at http://www.BiochemJ.org/bj/409/bj4090611add.htm). LdTOP1LS (10 nM) was saturated at 10 μM DIM. The dissociation constant (K_D) calculated from the equilibrium dialysis experiment was 9.73×10^{-9} M, which is lower than that of flavones such as luteolin (4.6×10^{-5} M), quercetin (5.2×10^{-5} M) and baicalein (6.5×10^{-5} M) [17], indicating 10000-fold more affinity of DIM to enzyme than the flavones. Thus the equilibrium dialysis experiment results reveal that DIM strongly interacts with free enzyme (LdTOP1LS) and perhaps explains the stronger inhibition in the enzyme–drug pre-incubation experiments. 3HFs (3-hydroxyflavones) interact with BSA, and two binding sites are involved in this interaction, with a similar binding constant of $(1.1–1.3) \times 10^{-5}$ M [32]. Here, we have performed the DIM–BSA interaction by equilibrium dialysis (Supplementary Figure 1B) as a control experiment, and K_D was determined to be 7.14×10^{-5} M, which is comparable with the value reported for 3HF–BSA interaction.

To find out the differential affinity of DIM towards LdTOP1L and LdTOP1S, we have also performed the binding of individual subunits to DIM by equilibrium dialysis experiment. The number of DIM molecules bound by LdTOP1L and LdTOP1S are plotted against increasing concentration of DIM respectively (Supplementary Figures 1C and 1D). LdTOP1L (10 μM) was saturated with 15 μM DIM, and 0.1 μM LdTOP1S was saturated with 10 μM DIM. The binding constant of LdTOP1L–DIM and LdTOP1S–DIM was 4.28×10^{-7} and 5×10^{-8} M respectively, indicating that DIM has 8.6-fold higher affinities for the small subunit than for the large subunit. The results also suggest that DIM has a significantly higher affinity to the holoenzyme of *L. donovani* topoisomerase I than to either the small subunit or the large subunit alone.

DIM–enzyme interaction is much stronger than that with CPT or flavones

The time course relaxation experiment was performed to compare the binding affinities of DIM and CPT. The rate of relaxation is reduced in the presence of 60 μM CPT in standard assay conditions, and the time required to complete relaxation for LdTOP1LS is increased 15-fold. The inhibitory effect of CPT on enzyme is abolished within 15 min under simultaneous assay conditions [13]. Here, we found that the plasmid DNA is fully relaxed at 5 min (Figure 5A, lane 4), but when 10 μM DIM was added simultaneously under the same assay condition, the rate of relaxation is reduced and complete inhibition of relaxation was achieved within 10 min (lane 12). Upon further incubation up to 40 min, there is no appearance of relaxed DNA bands (lane 14), suggesting a strong interaction between the enzyme and DIM. We have also performed the dilution experiment (Figure 5B) to compare the enzyme-binding affinity of DIM with that of flavones. Reconstituted LdTOP1LS was pre-incubated with 5 μM DIM, a concentration at which almost 95 % inhibition is achieved before the addition of DNA. The reaction was subsequently diluted 2- and 5-fold so that the final DIM concentration becomes 2.5 μM (lane 13) and 1 μM (lane 14) respectively. Lane 2 serves as an enzyme control. Drug-control reactions, i.e. inhibition study with 1, 2.5 and 5 μM luteolin, showed the expected pattern of inhibition (lanes 3–5) followed by 2- and 5-fold dilution (lanes 7–8) as described previously [14]. Dilution from 5 μM to 2.5 and 1 μM DIM did not reverse the inhibition (lanes 12–14). This inhibition on dilution suggests that DIM is interacting strongly with the enzyme. Equilibrium dialysis experiments with a high affinity constant also support this strong interaction between DIM and enzyme.

DIM is a class I non-competitive inhibitor of *L. donovani* topoisomerase I

CPT is an uncompetitive class I inhibitor of topoisomerase I that traps the enzyme–DNA covalent complex and slows the religation step of the nicking/closing cycle [16] without interacting with enzyme. DIM interacts with DNA and is a weak DNA intercalator [24]. DIM also interacts with free enzyme as evidenced by the equilibrium dialysis experiment (Supplementary Figure 1).

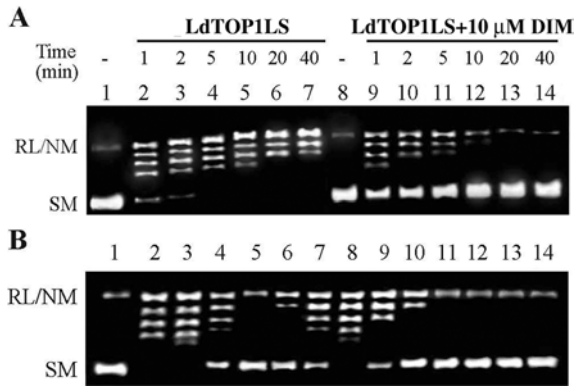

Figure 5 Enzyme–DIM interaction is stronger than that with CPT or flavones

(**A**) Relaxation of supercoiled pBS (SK^+) DNA with reconstituted LdTOP1LS at a molar ratio of 3:1, assayed in the absence or in the presence of DIM. Lanes 1 and 8, 90 fmol of pBS (SK^+) DNA; lanes 2–7, same as lane 1, but incubated with 30 fmol of LdTOP1LS in the absence of DIM at 37 °C for the indicated time periods as described in the Figure; lanes 9–14, same as lanes 2–7, but in the presence of 10 μM DIM. All the reactions were stopped by addition of SDS to a final concentration of 0.5 % and were electrophoresed in 1 % agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. (**B**) Pre-incubation and dilution with 5 μM luteolin and DIM respectively. 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. Lanes 3–5, same as lane 2, but in the presence of 1, 2.5 and 5 μM luteolin respectively. LdTOP1LS was pre-incubated with 5 μM luteolin for 5 min at 37 °C, then diluted 0-, 2- and 5-fold while maintaining or diluting the final drug concentrations to 5 μM (lane 6), 2.5 μM (lane 7) and 1 μM (lane 8) respectively. Lanes 9–11, same as lane 2, but in the presence of 1, 2.5 and 5 μM DIM respectively. LdTOP1LS was pre-incubated with 5 μM DIM for 5 min at 37 °C and then diluted 0-, 2- and 5-fold while maintaining or diluting the final drug concentrations to 5 μM (lane 12), 2.5 μM (lane 13) and 1 μM (lane 14) respectively. Reactions were performed at 37 °C for 15 min after addition of 50 fmol of pBS (SK^+) DNA.

DIM stabilizes *L. donovani* topoisomerase I-mediated cleavable complexes (Figure 2), suggesting that DIM acts as a non-competitive inhibitor of *L. donovani* topoisomerase I.

To investigate the non-competitive inhibition of LdTOP1LS by DIM, the time course relaxation experiments were performed in a standard assay mixture containing 10 mM Mg^{2+} and 50 mM KCl at 37 °C, where the plasmid DNA and the enzymes (LdTOP1LS) were mixed at a molar ratio of 3:1. The velocity of the enzyme is linear for the first 5 min of reaction. All the subsequent initial velocities during kinetic studies were calculated for the time point up to 1 min, within the linear range for the velocity examined. The kinetics of relaxation by LdTOP1LS was examined over a range of supercoiled pBS (SK^+) DNA (4–40 nM), and the initial velocity was plotted in a Lineweaver–Burk plot (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/409/bj4090611add.htm). The maximal velocity (V_{max}) for LdTOP1LS was 3.85×10^{-8} M base-pairs of supercoiled DNA relaxed/min per 0.5 nM enzyme. The maximal velocities (V_{max}) for LdTOP1LS in the presence of 5 and 10 μM DIM calculated by the same experiments were 2.44×10^{-8} and 1.57×10^{-8} M base-pairs of supercoiled DNA relaxed/min per 0.5 nM enzyme respectively.

DIM–DNA interaction by fluorescence quenching studies

To find out whether DIM interacts with DNA, fluorescence titration experiment with CT DNA was performed at different concentrations of DNA (20–200 μM). Free DIM has fluorescence emission maximum at a wavelength of 377 nm with a λ_{ex} of 280 nm. The excitation and emission slit widths were set at 5 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence. The intensity of fluorescence was decreased by increasing the concentration of DNA due to

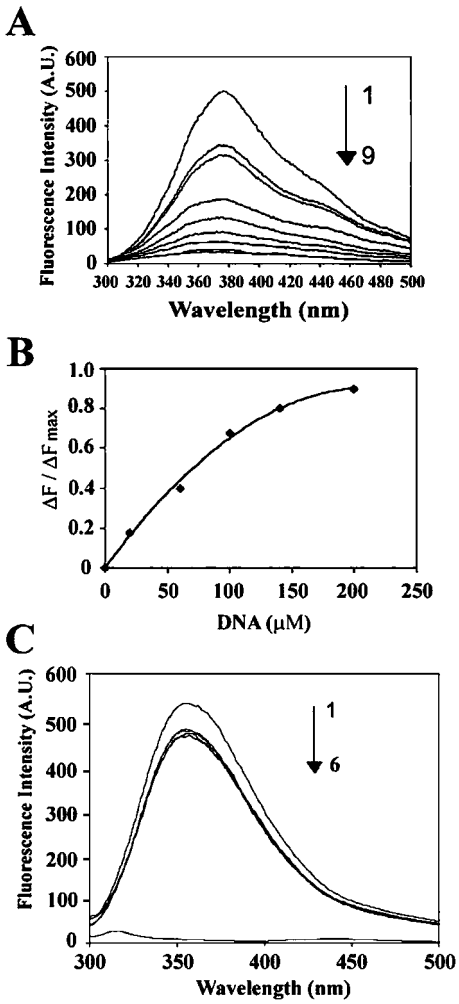

Figure 6 Fluorescence study of DIM–DNA interaction

(**A**) CT DNA (double-stranded DNA) concentration-dependently quenched DIM fluorescence at 377 nm. Fluorescence emission spectra ($\lambda_{ex} = 280$ nm) of 30 μM DIM were determined as a function of CT DNA concentration in the range of 20–200 μM. The arrow indicates the change in the emission spectra of DIM on addition of CT DNA. (**B**) A plot of $\Delta F/\Delta F_{max}$ against increasing concentration of CT DNA. (**C**) CT DNA (thermally denatured single-stranded DNA) concentration-dependently quenched DIM fluorescence at 377 nm. Fluorescence emission spectra ($\lambda_{ex} = 280$ nm) of 30 μM DIM were determined as a function of CT DNA concentration in the range of 20–200 μM. The arrow indicates the change in the emission spectra of DIM on addition of CT DNA.

quenching reaction. Addition of CT DNA causes a slight blue shift of the maximum peak (λ shift) from 377 to 373 nm with a concomitant decrease in fluorescence intensity (Figure 6A). A progressive change in the fluorescence spectra of DIM on addition of different concentrations of DNA indicated an association between them. The dissociation constant (K_D) calculated from the values using Scatchard analysis [33] for DIM was 2.2×10^{-5} M, which is much weaker than EtBr binding to DNA (4.0×10^{-7} M) [34]. A plot of $\Delta F/\Delta F_{max}$ against concentration of DNA was found to be hyperbolic, indicating the formation of a saturable complex (Figure 6B). We also performed the binding of DIM to heat-denatured calf thymus single-stranded DNA to find out the affinity of DIM to the single-stranded DNA (Figure 6C). The dissociation constant (K_D) value for single-stranded DNA binding to DIM was 11.6×10^{-4} M. The result suggests that single-stranded DNA has 53-fold less affinity for DNA compared with double-stranded DNA.

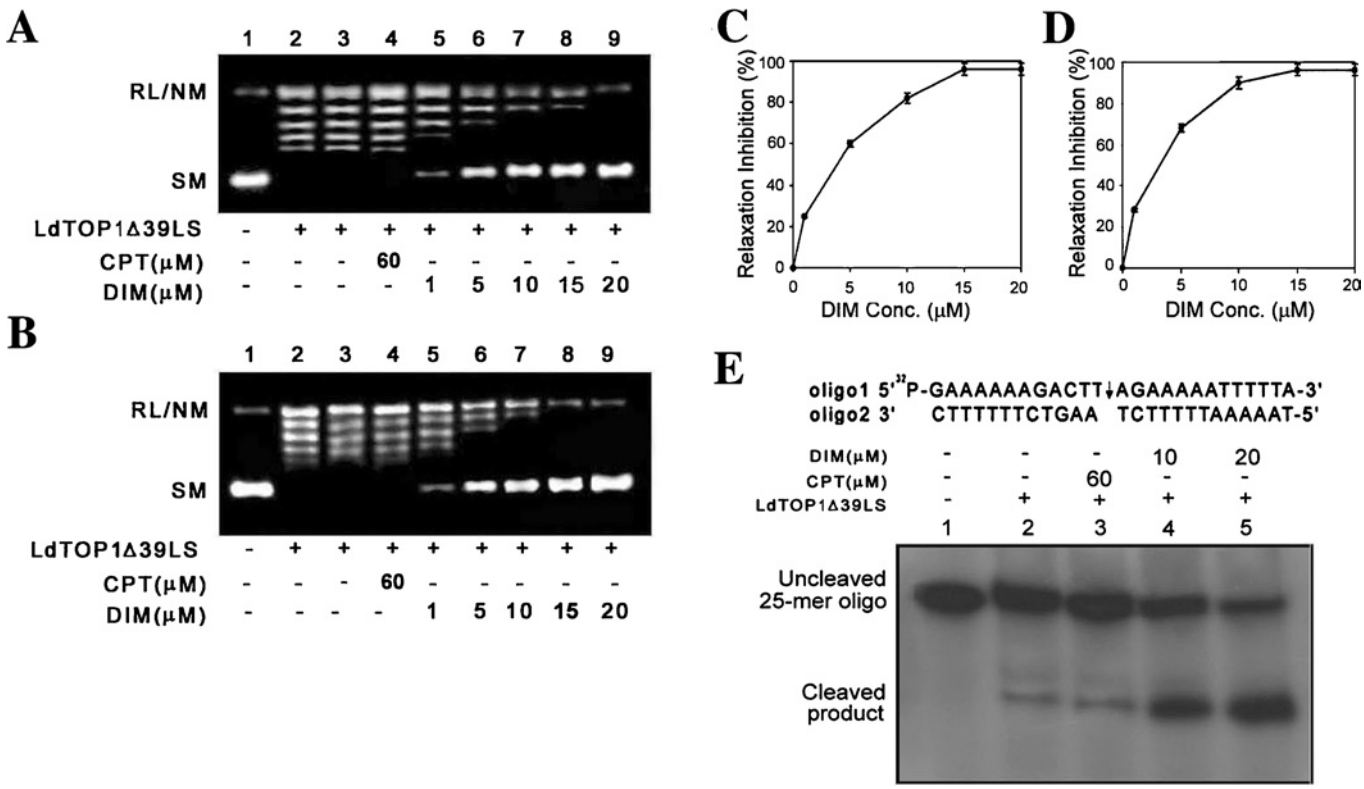

Figure 7 Effect of DIM on CPT-resistant *L. donovani* topoisomerase I

(**A**) Plasmid DNA relaxation experiments were performed with LdTOP1Δ39LS separately in the presence of DIM and CPT as indicated. pBS (SK$^+$) DNA and reconstituted mutant enzyme LdTOP1Δ39LS were mixed at a molar ratio of 1:2. Lane 1, 60 fmol of pBS (SK$^+$) DNA; lane 2, same as lane 1, but incubated with 120 fmol of LdTOP1Δ39LS in the absence of inhibitors; lane 3, same as lane 2, but in the presence of 4% (v/v) DMSO; lane 4, same as lane 2, but incubated simultaneously with 60 μM CPT at 37°C for 20 min; lanes 5–9, same as lane 2, but incubated simultaneously with 1, 5, 10, 15 and 20 μM DIM respectively at 37°C for 20 min. (**B**) Pre-incubation of LdTOP1Δ39LS with DIM and CPT followed by addition of DNA. Lane 1, 60 fmol of pBS (SK$^+$) DNA; lane 2, same as lane 1, but 120 fmol of LdTOP1Δ39LS was pre-incubated with reaction buffer for 5 min at 37°C, followed by addition of 60 fmol of pBS (SK$^+$) DNA and was further incubated for 15 min at 37°C; lane 3, same as lane 2, but the enzyme was pre-incubated with 4% (v/v) DMSO; lane 4, same as lane 2, but the enzyme was pre-incubated with 60 μM CPT at 37°C for indicated time periods; lanes 5–9, same as lane 2, but the enzyme was pre-incubated with 1, 5, 10, 15 and 20 μM DIM respectively. Reactions were stopped by addition of SDS to a final concentration of 0.5% and were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. (**C**) Quantitative representation of enzyme inhibition in the presence of DIM in relaxation experiments. LdTOP1Δ39LS (180 fmol) was incubated simultaneously with 1, 5, 10, 15 and 20 μM DIM for 20 min at 37°C in relaxation buffer. (**D**) The enzyme was pre-incubated separately with 1, 5, 10, 15 and 20 μM DIM for 5 min at 37°C in relaxation buffer, followed by addition of 60 fmol of pBS (SK$^+$) DNA, and was further incubated for 15 min at 37°C. Reactions were stopped by the addition of SDS and electrophoresed as described above. The percentage of relaxation inhibition is plotted as a function of drug 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. Variations among different sets of experiments were <5%. (**E**) Duplex oligonucleotide cleavage by LdTOP1Δ39LS. The cleavage reactions and electrophoresis in a denaturing polyacrylamide gel were performed as described in the Experimental section. Lane 1, 10 nM 5′-P-end-labelled 25-mer duplex oligonucleotides as indicated above. Lane 2, same as lane 1, but incubated with 0.2 μM LdTOP1Δ39LS in the absence of drugs. Lane 3, same as lane 2, but incubated with 60 μM CPT for 60 min at 23°C. Lanes 4 and 5, same as lane 2, but incubated with 10 and 20 μM DIM respectively for 60 min at 23°C. 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 analysed on 12% (w/v) denaturing gel as described in the Experimental section.

DIM also inhibits the relaxation activity of mutant *L. donovani* topoisomerase I and stabilizes the CPT-resistant topoisomerase I-mediated DNA cleavable complex

The N-terminal 1–39 amino acid residues of the large subunit of LdTOP1LS have a significant role in modulating CPT sensitivity [13]. So to understand the molecular interaction between the DIM and the enzyme, we investigated the inhibitory effect on the mutant enzyme (LdTOP1Δ39LS) in plasmid DNA relaxation assay. Interestingly, we observed that DIM shows considerable inhibition of relaxation activity up to 95% at 20 μM concentration when incubated simultaneously with enzyme and DNA (Figure 7A, lane 9). However, CPT at 60 μM concentration did not show any inhibition of relaxation activity (Figure 7A, lane 4). When LdTOP1Δ39LS was pre-incubated separately with DIM, at different concentrations, for 5 min at 37°C before the addition of DNA, inhibition to the same extent (approx. 95%) was achieved at a concentration of 15 μM (Figure 7B, lane 8). The IC_{50} values of DIM with the mutant enzyme (LdTOP1Δ39LS) in simultaneous and pre-incubation DNA relaxation assays were 3.6 and 2.9 μM, which are higher than those of wild-type topoisomerase I (LdTOP1LS) (1.2 and 0.8 μM respectively). The results indicate that the affinity of DIM to LdTOP1Δ39LS is less than that to LdTOP1LS (Figures 7C and 7D).

We investigated the interaction of DIM with CPT-resistant mutant enzyme (LdTOP1Δ39LS) in the equilibrium cleavage assay. CPT-induced stabilization of cleavable complex with LdTOP1Δ39LS was reduced to 6-fold compared with the wild-type enzyme [13]. The CPT (60 μM)-induced cleavage efficiency of LdTOP1Δ39LS is almost the same as that in the absence of CPT (Figure 7E, lane 3), whereas with 20 μM DIM, cleavage efficiency of the mutant enzyme increased to 62% (lane 5) compared with the control (lane 2). This result indicates that the amino acid residues (N-terminal 1–39) of the large subunit,

although important in modulating bi-subunit topoisomerase I activity *in vitro*, are not critical for DIM-induced topoisomerase I-mediated DNA cleavage.

DISCUSSION

In the present study, we have shown for the first time that DIM inhibits *L. donovani* topoisomerase I, and we have dissected the molecular mechanism of inhibition. DIM is found to be a potent inhibitor of LdTOP1S and stabilizes the cleavable complex like CPT. Plasmid cleavage experiment as well as oligonucleotide cleavage experiment (Figures 2A and 2D) show that DIM stabilizes the enzyme–DNA cleavable complex like CPT, i.e. mechanistic step (ii) of topoisomerase I-catalysed reaction (cleavage by trans-esterification). This is supported by further experiment with DHBA (Figure 2C). DHBA prevents the interaction between the enzyme and substrate DNA and inhibits the formation of enzyme–DNA cleavable complex [10]. When LdTOP1LS was pre-incubated with increasing concentrations of DIM (1–10 μM) prior to the addition of 10 μM DHBA, the DIM-mediated cleavage was not inhibited by DHBA (Figure 2C, lanes 4–7). But when LdTOP1LS was pre-incubated with increasing concentrations of DHBA (1–20 μM) prior to the addition of 10 μM DIM, the DIM-mediated cleavage was inhibited at that concentration of DHBA (lanes 8–11). So DHBA inhibits the DIM-mediated cleavage only when the enzyme is pre-incubated with DHBA prior to the addition of DIM. Subsequent cellular studies (SDS–K^+ precipitation assay) revealed that DIM also induced stabilization of *in vivo* covalent topoisomerase I–DNA cleavable complex in *L. donovani* promastigote cells (Figure 3A). To sort out whether DIM can be responsible for stabilization of covalent complex formation of topoisomerase I and DNA in intact cells, we carried out the immunoband depletion experiments with nuclear fractions of *L. donovani* promastigotes using specific antibody raised against *L. donovani* topoisomerase I. CPT, a topoisomerase I poison, induces the formation of cleavable complexes which did not enter the gel (Figure 3B, lane 6). When the cells were pre-incubated with DHBA prior to the treatment with DIM, DIM-mediated cleavage was inhibited. So from this result, it may be concluded that the *in vivo* cleavable complex induced by DIM is a substrate for topoisomerase I. Since in both SDS–K^+ precipitation assay and immunoband depletion assay, DIM shows CPT-like action, it may be concluded that DIM induces *in vivo* topoisomerase I–DNA cleavable complex formation also.

So from both *in vitro* and *in vivo* experiments, we can summarize that DIM is a class I inhibitor of topoisomerase I that acts as a topoisomerase poison similar to CPT. Thus, with stabilization of cleavable complex, DIM inhibits the subsequent religation reaction [step (iii)] like CPT, as observed in single turnover conditions (Figure 4, lanes 3 and 4). Flavones failed to inhibit the religation reaction when the drugs are added to the preformed enzyme–substrate cleavable complex [15]. We have shown here that, like CPT, DIM also inhibits the religation step under this condition (Figure 4, lanes 7 and 8 respectively). But unlike CPT, DIM is a non-competitive inhibitor, as it binds both the enzyme (LdTOP1LS) and the enzyme–substrate complex (LdTOP1LS–DNA complex). The interaction of DIM with enzyme during trans-esterification reaction with DNA is a prerequisite for stabilization of cleavable complex.

DIM inhibits the catalytic activity of enzyme (LdTOP1LS) in both simultaneous and pre-incubation relaxation assays (Figures 1B and 1C). Equilibrium dialysis experiment was performed to check the binding of DIM to *L. donovani* topoisomerase I (Supplementary Figure 1). The experiment revealed that the K_D value of DIM–enzyme interaction was 9.73×10^{-9} M, which is far less than that of flavones [15], which shows high affinity and strong binding of DIM to the enzyme. DIM also exhibits the differential affinity towards LdTOP1L and LdTOP1S with a binding constant of 4.28×10^{-7} and 5.0×10^{-8} M for LdTOP1L–DIM and LdTOP1S–DIM respectively. The results suggest that LdTOP1S has 8.6-fold higher binding affinity for DIM compared with LdTOP1L. But the binding affinity of DIM increased 5-fold for wild-type enzyme compared with the small subunit, and 44-fold compared with the large subunit. So from this result, it can be concluded that DIM has low affinity for the large subunit and the small subunit compared with LdTOP1LS.

The time course relaxation experiment was performed to compare the binding affinity of CPT and DIM towards the enzyme (Figure 5A). It was reported that the inhibition of catalytic activity of enzyme by CPT ceases within 15 min under simultaneous relaxation assay conditions [13]. In contrast, the inhibition of catalytic activity of the enzyme by DIM persists even up to 40 min in this relaxation reaction. This is because of the strong binding of the enzyme with the inhibitor (Figure 5A). This result is supported by the dilution experiment (Figure 5B). We have also shown previously that luteolin binds to topoisomerase I and the binding is reversible [11]. But binding of DIM to enzyme is not released by dilution of the drug concentration up to 5-fold. DIM can bind to DNA by intercalation, but it is a weak DNA intercalator [24]. Direct measurement of this interaction by fluorescence spectroscopy (Figure 6A) reveals the K_D value of DIM–DNA interaction to be 2.2×10^{-5} M. This DIM–DNA interaction is much weaker than EtBr binding to DNA (4.0×10^{-7} M) [34].

Recently, Gong et al. [24] reported DIM as a catalytic inhibitor of human topoisomerase IIα. It cannot stabilize cleavable complexes with human topoisomerase I and topoisomerase II in human hepatoma (HepG2) cells [24]. Although their study does not agree with our findings with unusual heterodimeric *Leishmania* topoisomerase I, this anomaly can be of potential interest in exploring the differential affinity of the drug for different enzymes. Being a potent inhibitor of ATPases, DIM is expected to inhibit topoisomerase II catalytically by directly affecting the ATP hydrolysis step of the enzyme. Moreover, the possibility that DIM exhibits differential affinity for human and *Leishmania* topoisomerase I cannot be ruled out, since the two enzymes vary considerably in their architecture. As evident from our results, DIM has a very high affinity for *Leishmania* topoisomerase I and exhibits a mixed type of inhibition. It is possible that apart from binding to the substrate-binding pocket, the drug binds strongly to a region on LdTOP1S. This may lead to a conformational change favourable for stabilization of the cleavable complex and hence exhibiting a mixed type of inhibition.

In our previous studies [35], we have shown that the replacement of the small subunit, to which DIM binds, with the C-terminal fragment of human topoisomerase I altered its sensitivity to CPT. Thus it is possible that the subtle differences around the active-site tyrosine residue between *L. donovani* and human topoisomerase I might affect their interactions with DIM in the way that DIM affects their catalytic cycles to poison *L. donovani* topoisomerase, but not human topoisomerase I.

This differential mechanism led us to investigate the effect of DIM on CPT-resistant mutant enzyme LdTOP1Δ39LS lacking 1–39 amino acids of the N-terminus of LdTOP1L [13]. LdTOP1Δ39LS showed an approx. 15-fold decrease in catalytic-centre activity compared with LdTOP1LS. Moreover, the mutant enzyme shows decreased sensitivity towards CPT in plasmid DNA relaxation experiments, whereas LdTOP1LS is 15-fold more

sensitive to that drug [13]. In duplex oligonucleotide cleavage assay with LdTOP1Δ39LS, the DNA cleavage efficiency of CPT decreased by 7.5-fold, whereas that of DIM was reduced by only 1.2-fold. These results indicate that the N-terminal 39 residues of LdTOP1L, although important in modulating topoisomerase I activity and CPT-sensitivity, are not critical for inhibition of relaxation activity by DIM and for DIM-induced topoisomerase–DNA cleavable complex formation.

In conclusion, the results of our study indicate that DIM is an *L. donovani* topoisomerase I poison which is a class I non-competitive inhibitor. DIM is more potent than CPT and it strongly binds to enzyme. The therapeutic importance of DIM as a lead compound for anti-leishmanial therapy is greatly enhanced by the present study. The emission properties of DIM provide a critical assessment of the nature of the microenvironments and the stoichiometry of binding in the topoisomerases. Structure–function analysis of DIM–topoisomerase I interaction along with modelling studies may be exploited in developing rational approaches to chemotherapy of leishmaniasis and also can facilitate the study of future drug resistance.

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