Disuccinyl betulin triggers Metacaspase Dependent
Endonuclease G Mediated Cell death in Unicellular
Protozoan Parasite *Leishmania donovani*

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List of Non-Standard Abbreviations:
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. donovani topoisomerase I small subunit; DMSO, dimethyl sulfoxide; EtBr, Ethidium bromide; SAG, Sodium antimony gluconate; M199, Medium 199; FBS, Fetal Bovine Serum; EndoG, Endonuclease G; MC, Metacaspase; PCD, Programmed cell death; AIF, Apoptosis inducing factor.
Abstract

The unicellular organism *Leishmania* undergoes apoptosis-like cell death in response to external stress or exposure to antileishmanial agents. Here we showed that, triterpenoid disuccinyl betulin (DiSB), a potent topoisomerase IB inhibitor induced parasitic cell death by generating oxidative stress. The characteristic feature of the death process resembled programmed cell death (PCD) in higher eukaryotes. Generation of reactive oxygen species (ROS) followed by depolarization of mitochondrial membrane potential ($\Delta \Psi_m$) caused loss of ATP production in *Leishmania* parasites. This further gave positive feedback to produce high amount of ROS, which in turn caused oxidative DNA lesions and genomic DNA fragmentation. Treatment of promastigotes with DiSB induced high expression of metacaspase protein that led to cell death of this unicellular organism. The PCD was insensitive to zVAD-fmk, suggesting that the death process was not associated with activation of caspases. DiSB treatment translocated LdEndoG (*Leishmania donovani* Endonuclease G) from mitochondria to nucleus which was responsible for DNA degradation process. Conditional antisense knockdown of LdMC (*Leishmania donovani* Metacaspase) as well as EndoG subverted death of the parasite and rescued cell cycle arrest in G1 phase. The present study on effectors molecules associated with PCD pathway of the parasite should not only help to manifest the mechanisms of PCD but also could be exploited in antileishmanial chemotherapy.
**Introduction**

Cell death, particularly apoptosis, is one of the most widely-studied phenomena for cell biologists. Understanding apoptosis in disease conditions is very important as it not only gives insights into the pathogenesis of a disease but also leaves clues on how the disease can be treated. Type I PCD involves three main types of biochemical changes: 1) activation of caspases, 2) DNA and protein breakdown and 3) membrane changes and recognition by phagocytic cells (1). Early in apoptosis, there is expression of phosphatidylserine (PS) in the outer layers of the cell membrane, which has been "flipped out" from the inner layers. This allows early recognition of dead cells by macrophages, resulting in phagocytosis without the release of pro-inflammatory cellular components (2). In higher eukaryotes, activated caspase-3 activates caspase-activated DNases (CADs) (3). Endonuclease G (EndoG) (4) and Apoptosis Inducing Factor (AIF) comprise caspase-independent effector endonucleases. Cytotoxic agents induce oxidative stress and cause nuclear translocation of EndoG, which thereby induce DNA fragmentation, and PCD (5).

Leishmaniasis is the most serious form of parasitic diseases caused by the protozoan flagellates of the genus *Leishmania* and presents a spectrum of clinical manifestations (6). More than 350 million people are at risk of the infection and the disease causes 70,000 deaths each year (7). The control measures that are mainly based on early detection and chemotherapy are hampered by huge toxicity, side-effects of the drugs and emergence of drug-resistant parasites. For last six decades, organic pentavalent antimonials [Sb (V)] have been the first line drugs for treatment of this disease. However, emergence of clinically resistant isolates to these drugs poses a serious obstacle for disease control and treatment (8). Therefore identification of new molecular targets for improved therapy is clear and justified.
A better understanding of the cell death mechanism induced by drugs will be helpful in developing intervention strategies against the parasite.

In *Leishmania*, PCD helps in altruistic growth control and organizes them into clonal populations (9) by (i) selecting for the fitter cells within the population, (ii) optimally regulating the cell number to adapt to the environmental constraints and (iii) tightly controlling the cell cycle and cell differentiation. Topoisomerases are DNA manipulators that relieve the torsional strain in DNA, built up during vital cellular processes. The heterodimeric topoisomerase IB of *Leishmania* has been established as an attractive therapeutic target (10).

In higher eukaryotes, ‘DNA sensors’ recognize inhibitor trapped topoI-DNA cleavable complex and activate Bax to subtly permeabilize the mitochondrial outer membrane. This generates an oxidative stress and causes nascent cytochrome c release (11). Cytochrome c forms the ‘apoptosome’ and also binds to inositol triphosphate receptors and releases Ca\(^{2+}\) into the cytosol (12). Maintenance of proper mitochondrial transmembrane potential (\(\Delta\psi_{mt}\)) is essential for survival of the cell because it drives the synthesis of ATP and maintains oxidative phosphorylation (13). In caspase-independent PCD, the increase in intracellular calcium increases mitochondrial calcium and causes further mitochondrial membrane depolarization, generation of reactive oxygen species (ROS) and activation of endonucleases (3). In *Leishmania*, the potent topoisomerase IB inhibitor camptothecin (CPT) is known to induce DNA degradation and PCD (14).

Betulin (lup-20)-ene-3b, 28-diol), an abundant naturally occurring triterpene and its derivative betulinic acid exhibit anti-malarial (15), anti-HIV and anti-inflammatory (16) as well as cytotoxic activities on cancer cell lines (17). Betulin derivatives are chemically synthesized products, which affect DNA-topoisomerase activity (18). Betulin induces apoptotic cell death and inhibition of growth of human gynecologic and colon cancer cells.
Treatment with betulin also alters tumour cell's morphology, decreases their motility and induces apoptotic cell death. Betulin induces cell death more rapidly as compared to betulinic acid, but to achieve similar extents of cell death, a considerably higher concentration of betulin is needed. Although a few reports exist which showed antiprotozoal activity of betulin derivatives, there is no extensive study on cell death induced by betulin derivatives. We have shown that DiSB is a potent antileishmanial agent that binds to topoisomerase I and inhibits binding of the enzyme to DNA and thus affects relaxation activity of Leishmania topoisomerase. DiSB is also effective in reducing the parasite burden in cultured macrophages and is effective in case of SAG-resistant parasites.

In the present study, we have shown that DiSB induces caspase-independent PCD of the parasites. While studying nuclear, mitochondrial, and cytosolic changes associated with PCD, it was found that the compound causes depolarization of mitochondrial membrane. Loss of ΔΨm leads to release of cytochrome c into the cytosol and cell death is triggered by activation of metacaspases. This is evidenced by down regulation of DiSB-mediated cell death process after inhibition of metacaspase activity. Taken together, our results provide an insight into the mitochondria-dependent apoptotic-like death pathway induced by DiSB in Leishmania spp. The depletion of ATP level enhances apoptosis by creating a cellular oxidative stress followed by DNA fragmentation which is caused by nuclear translocated mitochondrial LdEndoG. Such information has great potential in determining the role of mitochondria in apoptosis-like death of leishmanial cells and in designing better drugs for leishmaniasis.
Materials and Methods

Chemicals

DiSB, DiGDHB, DiSDHB (18), CPT, NAC (20 mM concentration each), CCCP (100 mM), BAPTA-AM (50 mM) and H$_2$DCFDA (10 mM) were dissolved in 100% DMSO and stored at -20°C. Antipain (10 mM) and ATA (ammonium salt at 20 mM) were dissolved in water and stored at -20°C. All the above chemicals were purchased from Sigma Aldrich (St Louis, MO, USA). cis-parinaric acid was purchased from Life Technologies (Carlsbad, California, USA).

Parasite Culture and Maintenance.

The *Leishmania donovani* (MHOM/IN/AG/83) promastigotes were grown at 22°C in M199 media supplemented with 10% fetal calf serum as described previously (24). For knockdown studies, the *L. tarentolae* T7.TR strain (JenaBioscience) was used (25). To maintain the genome integrated T7 RNA polymerase and Tet repressor genes, the parasites were cultured in the presence of nourseothricin and hygromycin (100 μg/ml each).

Measurement of cell viability

The *L. donovani* AG83 promastigotes were treated with different concentrations (1, 2.5, 5, 10 and 25 μM) of DiSB, DiGDHB and DiSDGB for 12 h. In another set of experiment, promastigotes (3.0 × 10$^6$ cells/ml) were incubated with three different concentrations of DiSB (1, 5 and 10 μM) for indicated time periods (2, 4, 6, 8, 10, 12, 16 and 24 h). The viability of promastigotes were measured using Alamar Blue dye. The dye is a cell permeable nontoxic nonfluorescent active ingredient (blue) that uses the natural reducing power of viable cells to convert resazurin to the fluorescent molecule, resorufin (very bright red). Metabolically active cells convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity (10).
Measurement of oxygen consumption.

*L. donovani* AG/83 promastigotes (5 × 10^6 cells/ml) were grown in M199 medium containing 5 and 10 μM DiSB or 0.2% DMSO. Aliquots of cells were taken at indicated time intervals, harvested and washed with respiration buffer (50 μM sucrose, 145 μM KCl, 5μM NaCl, 1 μM EDTA, 1 μM MgCl₂ and 10 μM sodium phosphate buffer, pH 7.4) and finally suspended in the same buffer. Oxygen uptake was determined with a Clarke-type oxy-electrode having a cell capacity of 2 ml (14). Calibration of the oxy-electrode was carried out using air-saturated water containing sodium metabisulfite.

Measurement of ROS

Intracellular ROS levels were measured in DiSB-treated and untreated parasites. Promastigotes (2 × 10^7 cells/ml) were treated with 5 and 10 μM DiSB for indicated times. Parasites treated with 0.2% DMSO served as controls. In another set, the parasites were incubated with N-acetyl cysteine (NAC) prior to treatment with DiSB. The parasites were washed and resuspended in 500 μl of M199 (without phenol red) and incubated with a cell permeant dye H₂DCFDA for 30 min (25). Fluorometric measurements (λ_ex = 510 nm and λ_em=525 nm) were performed in triplicate, and the results were expressed as mean fluorescence intensity per 10^6 cells.

Measurement of GSH and GSSG Levels.

GSH level was measured by monochlorobimane dye that gives a blue fluorescence when bound to glutathione (14). *L. donovani* promastigotes (approximately 3 × 10^6 cells) were treated with or without DiSB for different times. Cells were then harvested and lysed by cell lysis buffer according to the manufacturer’s protocol (ApoAlert glutathione assay kit; Clontech, Mountain View, CA). Cell lysates were incubated with monochlorobimane (2 μM)
for 3 h at 37°C. The decrease in glutathione levels in the extracts of nonapoptotic and
apoptotic cells were detected by fluorometer with 395-nm excitation and 480-nm emission
wavelengths and normalized to total cell numbers. Spectrofluorometric data presented here
are representative of three experiments.

For reduced glutathione detection, Glutathione Fluorometric Assay Kit (GSH, GSSG and
Total) from Biovision (Catalog #K264-100) was used. The preparation was done according to
manufacturer’s protocol. Briefly, 2 × 10⁶ cells were treated with DiSB (5 and 10 μM) for 8 h
or after pre-treatment with NAC. Cells were lysed in 100 μl of Glutathione Assay Buffer.
Sixty μl of each lysate was transferred to a prechilled tube containing PCA and vortexed for
several seconds to achieve uniform emulsion. Finally the emulsions were kept on ice for 5
min and centrifuged for 2 min at 13,000 x g at 4°C and supernatant (containing glutathione)
was collected. Total GSH and GSSG were measured according to manufacturer’s protocol.

Measurement of total Fluorescent Lipid Peroxidation Product.
DiSB-treated and untreated *L. donovani* cells were centrifuged and pellets were washed twice
with 1X PBS. The pellets were suspended in 500 μl of 1X PBS. The cell suspension was
further incubated with cis-parinaric acid (final concentration 5 μM) and incubated in dark at
room temperature for 30 min (26). The fluorescence intensities of the total fluorescent lipid
peroxidation products were measured with excitation at 320 nm and emission at 415 nm and
expressed as relative fluorescence units. Spectrofluorometric data (normalized with total
number of promastigotes) presented here are representative of three experiments.

Double staining with annexinV and PI
Externalization of phosphatidyl serine on the outer membrane of untreated and DiSB-treated
promastigotes was measured by binding of FITC-annexinV and PI using an annexinV
staining kit (Invitrogen Incorporation Ltd). Flow cytometry was carried out for treated and untreated parasites. The gating was done so that the FL-1 channel denotes the mean intensity of FITC-annexinV, whereas the FL-2 channel denotes the mean intensity of PI. The data represented here are one of three experiments.

**Intracellular Ca$^{2+}$ measurement.**

Intracellular Ca$^{2+}$ concentration was measured with the fluorescent probe Fura-2AM as described previously (27). Briefly, cells with differently treated groups were harvested and washed twice with wash buffer containing 116 μM NaCl, 5.4 μM KCl, 0.8 μM MgCl$_2$, 5.5 μM glucose, 1 μM CaCl$_2$, and 50 μM MOPS, pH 7.4. Cells were then suspended in the same buffer containing 15% sucrose and were incubated with Fura 2AM (6 μM) at 27°C for 1 h with mild shaking. Cells were pelleted down, and after two subsequent washing, cells were suspended in the same wash buffer. Fluorescence measurements were performed with excitation at 340 nm and emission at 510 nm. To convert fluorescent values into absolute calcium concentration, calibration was performed at the end of each experiment.

Cytosolic Ca$^{2+}$ concentration was calculated using the following equation:

$$[\text{Ca}^{2+}] = K_d (F - F_{\text{min}})/(F_{\text{max}} - F)$$

where $K_d$ is the dissociation constant of the calcium-bound Fura 2 complex (224 nM), $F_{\text{max}}$ corresponds to the maximum fluorescence obtained by treating cells with 10 μM calcium ionophore A23187 and $F_{\text{min}}$ represents the minimum fluorescence of the cells (obtained from ionophore treated cells in the presence of 4 μM EGTA). Data were normalized with mg of total cellular protein extracts.

**Measurement of $\Delta \Psi_m$.**
Mitochondrial transmembrane potential was investigated using JC-1 dye. This dye accumulates in the mitochondrial matrix under the influence of ΔΨ<sub>m</sub>, where it reversibly forms monomers (green) with characteristic absorption and emission spectra (14). In brief, leishmanial cells, after different treatment with DiSB (5 or 10 µM), or with 10 µM DiSB after 60 min pretreatment with NAC (20 µM), BAPTA-AM (25 µM), zVAD-fmk (25 µM), TLCK (1 µM) or antipain (2 µM) were harvested and washed with PBS (1X). Cells were then incubated at 28°C in 5% CO<sub>2</sub> incubator for 1 h with a final concentration of JC-1 dye at 5 µg/µl. Cells were then analyzed by fluorescence measurement through spectrofluorometer using 507 and 530 nm as excitation and emission wavelengths, respectively (for green fluorescence); and 507 and 590 nm as excitation and emission wavelengths (for red fluorescence), respectively, to analyze ΔΨ<sub>m</sub>. Spectrofluorometric data presented here are representative of three experiments. The ratio of the reading at 590 nm to the reading at 530 nm (590:530 ratios) was considered as the relative ΔΨ<sub>m</sub> value. In flow cytometer FL-1 channel denotes the mean fluorescence intensity.

Measurement of Cellular ATP Level.

ATP content was determined by the luciferin/luciferase method as described previously (14). The assay is based on the requirement of luciferase for ATP in producing light (emission maximum, 560 nm, at pH 7.8). In brief, promastigotes (3 × 10<sup>7</sup>) were treated with different concentrations of drugs for the indicated times and washed with PBS (1X) twice and suspended in PBS (1X). The mitochondrial and cytosolic extracts were prepared as described above. An aliquot of these fractions was assayed for ATP using the luciferase ATP assay kit (Invitrogen Inc. Ltd). The amount of ATP in the experimental samples was calculated from standard curve prepared with ATP. The data were normalized to mg of cellular protein extract.
Cell fractionation.

Nuclear/kinetoplast (N/K) fraction was prepared by lysing parasites with 140 μM NaCl, 1.5 μM MgCl₂, 10 μM Tris-HCl, pH 8.6, 0.5% NP-40 and protease inhibitors. After 10 min cell suspension was centrifuged at 6000 × g for 5 min at 4°C. The supernatant served as the cytosolic fraction, whereas the pellet was the N/K fraction (as both share more or less similar mass for kinetoplastids). For immunoblotting analysis, the nuclear extract was prepared by resuspending the N/K fraction in the same buffer with 0.25 mg/ml digitonin and 100 U of DNaseI. The suspension was kept for 15 min at room temperature. Thereafter it was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant served as the nuclear extract. The mitochondrial pellet was resuspended in the same buffer containing 1 mg/ml digitonin and 0.5% Triton X-100. After incubation for 10 min at room temperature, the suspension was centrifuged at 12,000 × g for 10 min at 4°C to obtain the mitochondrial fraction.

Preparation of Cytoplasmic Extract.

Cytoplasmic extracts were prepared from both treated and untreated Leishmania cells as described previously (14). In brief, cells (3 × 10⁷) after different treatment were harvested, suspended in cell extraction buffer (20 μM HEPES-KOH, 10 μM KCl, 1.5 μM MgCl₂, 1 μM EDTA, 1 μM dithiothreitol, 200 μM PMSF, and 10 μg/ml leupeptin and pepstatin) and lysed by a process of freeze-thaw using nitrogen cavitations and incubating at 37°C for 5 min simultaneously. Lysate was centrifuged at 10,000 x g for 1 h, and supernatants were used as a source of cytoplasmic extract.

Detection of cytochrome c Release by Western Blotting.

DiSB-treated and untreated cells were harvested and washed twice with 1X PBS, suspended in cell fractionation buffer (Apo Alert cell fractionation kit), and homogenized. After separation of cytosolic and mitochondrial fraction, 50 μg of each of cytosolic fractions were
separated by electrophoresis on 12% SDS-polyacrylamide gel and immunoblotted with the rabbit polyclonal cytochrome c antibody. Horseradish peroxidase-conjugated secondary antibody was used, and protein band was visualized by enhanced chemiluminescence reaction.

**Metacaspase activity**

The fluorogenic substrates Boc-GRR-AMC were obtained from Bachem (Bubendorf, Switzerland), and Ac-DEVD-AMC, Ac-VEID-AMC, and Ac-LEHD-AFC were from Molecular Probes. The caspase inhibitor Z-VAD-fmk were obtained from Calbiochem. All the other protease inhibitors were obtained from Sigma (St. Louis, MO). *L. donovani* promastigotes (5 × 10^6) were treated with or without DiSB for different time periods. Different groups of cells were pretreated for 60 min with NAC, Antipain, TLCK or zVAD-fmk followed by DiSB treatment. Cells were then harvested and lysed by cell lysis buffer according to the manufacturer’s protocol (Apo Alert caspase assay kit, Clontech). Cell lysates were incubated with respective caspase or metacaspase buffers to detect CED3/CPP32 and ICE group of protease or metacaspase activities. Individual fluorogenic peptide substrates at 100 μM and 1X reaction buffer containing 100 μM DTT were added to corresponding cell lysates. AFC and AMC release were measured after incubating these samples at 37°C for 2 h by a fluorometer with 400 nm excitation and 500 nm emissions and 380 nm excitation and 460 nm emissions, respectively. Data were normalized with mg of total cellular protein extracts.

**Generation of antisense constructs.**

Antisense constructs of LdMC were generated by PCR amplification of the 1-177 nucleotide region using sense primer 5’-CGGGATCCATGATCGCCCGGCTGCTCGGC -3’ and antisense primer 5’-CCCAAGCTTTCACTGGCTGCTGTCGCT-3’ and cloned in the
antisense orientation in the HindIII/BamHI site of pLew82v4. It was electroporated into
Lt.T7.TR strain (JenaBioscience) and trasfectants were selected using phleomycin and termed
antiMC. For control, empty vector transfectant was also prepared and termed as pLew82.
Tetracycline (2 μg/ml) induction for 24 hrs was carried out in both the transfectants after
which DiSB treatment was done.

Parasite growth in LdEndoG and LdMC-downregulated parasites.
Wild-type LdEndoG and LdMC were downregulated separately using the conditional
antisense approach as described previously (25). DiSB treatment was carried out after 24 h of
tetracycline induction in the above said transfectants, whereas the pLew82 transfectants
served as the empty vector control. Live promastigotes were calculated at given time
intervals. Three sets of experiments were carried out and the values were averaged and
plotted.

DNA fragmentation Assay
An estimate of the extent of DNA fragmentation after drug treatments was carried out using
the cell death detection ELISA kit (Roche Biochemicals). Promastigote samples (5 × 10^6
cells/ml) were collected at different time intervals and the histone-associated DNA fragments
(mononucleosome and oligonucleosome) were detected using the manufacturer’s protocol.
DNA fragmentation was estimated by spectrophotometric measurement of microtiter plates in
a Thermo MULTISCAN EX plate reader at 405 nm. The results were normalized with
number of total parasites. The relative percentage (with respect to samples treated with
micrococcal nuclease and normalized to percentage values) was plotted versus time.
Genomic DNAs from exponentially growing L. donovani promastigotes (2 × 10^6) treated
with or without DiSB and H2O2 were isolated with an apoptotic DNA ladder kit (Roche
Biochemicals). In another set, L. donovani promastigotes were separately preincubated with 2
µM antipain for 1 h followed by treatment with 10 µM DiSB for different time points. DNAs were electrophoresed in 1% agarose gel at 75 V for 2 h, stained with ethidium bromide and photographed under UV-illumination.

**In situ labeling of DNA fragments by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL).**

Treated and untreated *L. donovani* promastigote cells were fixed with 2% paraformaldehyde and incubated with 0.5% Triton X-100 for 5 min for permeabilization and layered with TdT reaction mixture containing FITC labeled dUTP for 1 h at 37°C according to the manufacturer’s protocol (Apo AlertTM DNA fragmentation assay kit). Cells were stained with propidium iodide and acquired by BD FACS ARIA II flow cytometer through dual pass FITC/PI filter set. Data were analyzed by BD FACS SCAN software.

**Assay of PARP Cleavage.**

Cell lysates were prepared from DiSB-treated and untreated cells, separated on 12% SDS-polyacrylamide gel and subjected to western blot analysis as described previously (14). Anti-poly (ADP-ribose) polymerase (PARP) polyclonal antibody diluted to 1000-fold, and horseradish peroxidase-conjugated secondary antibody was used to visualize the reactive band by chemoluminescence reaction.

**Cell cycle Analysis**

Exponentially grown *L. donovani* promastigote cells (2.5 × 10⁶) were treated with DiSB (10 µM) for 2, 4, 6 and 8 h. Cells were then harvested, washed three times with PBS (1X), and fixed in 50% ethanol (diluted in 1× PBS) for 4 h. Fixed cells were washed thoroughly, treated with 100 µg/ml RNase A and then suspended in 1 ml of staining solution [1 mg/ml stock solution of PI diluted to 3 µM PI in staining buffer (100 µM Tris, pH 7.4, 150 µM NaCl, 1
μM CaCl₂, 0.5 μM MgCl₂, 0.1% Nonidet P-40). The samples were incubated for 30 min in the dark at room temperature and were analyzed by flow cytometry. The cells (20,000) were analyzed from each sample. The percentage of cells in G1, S, and G2/M phases of the cell cycle were determined in Becton Dickinson flow cytometer and data were processed by BD FACS SCAN Software (San Diego, CA).

**In vivo analysis of BrdU incorporation**

Promastigotes were treated with DiSB for different time periods and samples were labeled with BrdU for 2 h, fixed with ethanol and then incubated with a monoclonal antibody to BrdU. The antibody to BrdU supplied with the kit (Roche Biochemicals) contains an optimized mixture of nucleases. These nucleases generate single-stranded DNA fragments, which allow binding of the antibody to BrdU without destruction of the cellular morphology. The samples were incubated in the presence of anti-BrdU antibody raised in mouse. The samples were counterstained with propidium iodide (in presence of 10 μg/ml RNase). The samples were acquired in BD FACS ARIA II and the data were analysed using BD FACS SCAN software.

**Measurement of in vivo DNA Synthesis.**

Exponentially growing *L. tarentolae* promastigote cells (8 × 10⁶ cells/ml) were radiolabeled by adding *methyl-³H* thymidine to final concentration of 5 μCi/ml (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) into the medium M199 supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cultures were incubated for 4 to 24 h at 22°C in presence or absence of different concentrations of DiSB. After incubation, cells were lysed with 10% trichloroacetic acid and harvested in a Skatron Combi cell harvester. Readings were normalized with total number of parasites. The incorporation of radioactivity in acid precipitable DNA was measured in a liquid scintillation counter as described previously (10).
Results

Betulin derivatives inhibit growth of *Leishmania* sp promastigotes.

*L. donovani* AG83 promastigotes (3.0 × 10^6 cells/ml) were incubated with different concentrations (1, 2.5, 5, 10, and 25 μM) of three different betulin derivatives (DiSB, DiGDHB and DiSDHB) up to 12 h, after which the number of live promastigotes were estimated with Alamar Blue reagent (Fig. 1A). EC_{50} values of the derivatives are presented in Table 1. Previous study has shown that, these betulin derivatives are potent topoisomerase inhibitors and kill intracellular parasites (18). All the three compounds are very effective against the *L. donovani* promastigotes and inhibit almost 90-98% of growth of the parasite. DiSB shows about 98% inhibition of growth of the parasites in culture medium (Fig. 1A). So we performed our study with DiSB thereafter. At 12 h, 90% growth was inhibited by 1 μM DiSB, which was comparable with the inhibition (92%) achieved by 10 μM DiSB at 8 h, and 98% growth was inhibited by 5 μM DiSB at 16 h, which was comparable with the inhibition achieved by 10 μM DiSB at 12 h (Fig. 1B). Consistent with these results, it was observed that after 6 h treatment with 20 μM DiSB, growth of *L. major*, *L. donovani*, and *L. tarentolae* promastigotes were inhibited to the extent of 90, 89, and 93%, respectively (Fig. 1C). IC_{50} values for these leishmanial strains are presented in Table 2. We further extended our study with the growth inhibition of parasites resistant to sodium antimony gluconate (GE1), miltefosine (Mil), camptothecin (CPT) and diindolylmethane (DIM) (Fig. 1D). All the parasites showed similar kind of growth inhibition in presence of different concentration of DiSB for 6 h while untreated controls proliferated at normal rate. Microscopic examination of all the samples indicated that the observed effect is leishmanicidal (as the promastigotes were lysed) rather than leishmanistatic. Microscopy also confirmed that. Therefore, our present
data demonstrate that under *in vitro* conditions DiSB can prove to be a highly cytotoxic
leishmanicidal compound.

**Treatment with DiSB hampers cellular oxygen consumption and causes induction of
reactive oxygen species generation.**

Oxygen uptake was measured in the DiSB-treated and untreated (0.2% DMSO) parasites with
time. *L. donovani* promastigotes consumed oxygen at the rate of 68±14 nmol/min/mg of
protein, which remains unchanged for 6 h. Treatment with 10 μM DiSB decreases oxygen
consumption rate to 31±9 nmol/min/mg of protein in 2 h and to 12±0.8 nmol/min/mg of
protein in 6 h.

Cytotoxic activity of betulin on many cancer cells is attributed to its pro-oxidant activity
through the generation of ROS (20). This prompted us to examine ROS production in *L.
donovani* promastigotes to determine if DiSB could trigger oxidative stress that could serve
as a possible pre-requisite for apoptosis-like cell death. During oxidative phosphorylation, the
release of ROS in the form of superoxide anion occurs to the extent of 3 to 5% of total
oxygen consumed (28). However, under certain conditions when drugs inhibit oxidative
phosphorylation or cellular processes, this rate of ROS production can be increased greatly
(29). To verify the effect of DiSB on intracellular ROS levels, we treated *Leishmania*
parasites separately with DiSB (10 μM) and antioxidant (NAC), and examined the ROS
production over time (Fig. 2B). Cellular ROS formation after treatment with DiSB in *L.
donovani* promastigotes was measured fluorometrically by conversion of CM-H₂DCFDA to
highly fluorescent 2, 7-dichlorofluorescein. The level of ROS increased in parasites
significantly within 2 h of treatment with DiSB and increases up to 4 h of incubation.
Treatment with DiSB generated a 3.7-fold higher amount of ROS (compared to DMSO
treatment) inside the parasites When cells were treated with NAC before treatment with DiSB
(10 μM), the level of ROS generation decreased (Fig. 2B) to almost 2.3 fold at 4h compared with DiSB at 10 μM treatment.

Oxidative stress causes depletion of GSH level and upregulation of lipid peroxidation.

One of the most important cellular defences against intracellular oxidative stress is GSH, which plays a critical role in mediating apoptosis in eukaryotes, including leishmanial cells. The actual mechanism by which GSH exerts its influence in leishmanial cells is yet to be explored. GSH is an important molecule for protecting kinetoplastids from ROS or toxic compounds (27), and increase in ROS may induce a loss of Δψm. DiSB caused 22% decrease in GSH level after 2 h (Fig. 3A), and the effect was more pronounced after 4 h of treatment with DiSB (52%). When cells were preincubated with NAC for 30 min, before treatment with DiSB, the decrease in GSH level was protected significantly, and the level tends to become normal. Generation of ROS in the parasites may have caused a concomitant decrease in the cellular trypanothione (GSH) levels (Fig. 3A). Decrease in cellular GSH level produces oxidised GSSG levels inside cells. As a result, GSH to GSSG decreases in time dependent manner (Fig. 3B). When cells were pre-treated with ROS scavenger NAC, it reverted back to the level of untreated controls.

Lipid peroxidation was assessed by measuring total fluorescent lipid peroxidation products in leishmanial cells after treatment with DiSB. The treatment led to an increase in lipid peroxides after 2.5 h (almost 2.6-fold) and reached to saturating level after 5 h (4.5-fold). When leishmanial cells were treated with BHT (20 μM), a specific inhibitor of lipid peroxidation, and subsequently washed and treated with DiSB for indicated times, the level of fluorescent products decreased as observed after treatment with NAC at 20 μM (Fig. 3C). The above mentioned results suggest that the effect of DiSB is similar to that of CPT with
respect to depletion of GSH level and it increases the level of lipid peroxidation in leishmanial cells.

DiSB triggers externalization of phosphatidyl serine which is reversed by metacaspase inhibitor.

Induction of apoptosis causes the externalization of phosphatidyl serine on the surface of apoptotic cells (14), which was detected by using annexin V that binds with exposed phosphatidyl serine of apoptotic cells in a Ca²⁺- dependent manner. To determine the percentage of apoptotic and necrotic cells in Leishmania spp. promastigotes, cells were incubated with DiSB (10 μM) for 2, 4, 8 and 12 h and then the percentage of cells undergoing apoptosis/necrosis were determined by flow cytometry after staining with annexin V-FITC and PI (Fig. 4A). Cells in the bottom right quadrant indicated apoptosis, whereas cells in the top right quadrant represented post-apoptotic/necrotic population. It is known that dead cells are reactive to PI and that externalized phosphatidylserine residues undergoing apoptosis are detected by annexin V-FITC. Flow cytometric analysis with annexin V/PI staining showed that when cells were exposed to DiSB (10 μM) for 2, 4, and 8 h, the percentage of apoptotic cells were increased with slight increase of necrotic cells. The proportion of apoptotic and necrotic cells increased from 6 and 1% in control cells to 38 and 3%, respectively after treatment with DiSB for 4 h. After 8 h of treatment, the proportion of apoptotic and necrotic cells were increased to 61 and 5%, respectively (Fig. 4A). The number of viable cells decreased from 98 to 13% after treatment with DiSB for 8 h. After treatment with 10 μM DiSB for 12 h, the percent of necrotic population is increased to 23%, whereas late apoptotic/early necrotic population increased to 62% and only a small population exhibits annexin V positive cells (8%). These results suggest that DiSB-induced apoptosis is the main cause of death. Treatment of promastigote cells with 10 μM protease inhibitor (VAD-fmk)
for 60 min causes no significant decrease in annexin V positivity which supports that the appearance of phosphatidyl serine on outer leaflet apparently requires no caspase-like protease activation in *Leishmania* spp. promastigote cells (Fig. 4B). But pretreatment with metacaspase inhibitors like antipain or TLCK (for 60 min) greatly reduced the apoptotic events induced by DiSB (Fig. 4B) supporting that metacaspase is an effector protease for DiSB-mediated cell death.

**DiSB triggers accumulation of intracellular Ca^{2+} pool followed by depolarization of mitochondrial membrane potential.**

The morphological changes in PCD result from several physiological imbalances that arise inside the parasites. To confirm that DiSB-induced oxidative stress disrupts the intracellular cation homeostasis of the parasites, intracellular Ca^{2+} level was estimated. Previous studies have shown that oxidative stress causes mitochondrial depolarization in *L. donovani* by increasing cytosolic Ca^{2+} levels (27). It was also shown that calcium flux is absolutely necessary not only for the activation of different proteases (14) but also for the appearance of phosphatidylserine on the outer leaflet of the plasma membrane during apoptosis. Considering the importance of cytosolic Ca^{2+} level in inducing apoptosis we measured the Ca^{2+} concentration in control and DiSB-treated promastigotes at different time intervals. In this study, we have shown that control leishmanial cells maintained intracellular [Ca^{2+}] at 52±7 nM while DiSB treated cells exhibit a time dependent increase in cytosolic [Ca^{2+}] (Fig. 5A). Pretreatment of cells with selective calcium channel blocker like verapamil (100 nM) and non-selective cation channel blocker FFA (250 μM) for 30 min before treatment with DiSB lowers the increase in cytosolic [Ca^{2+}] revealing that both these channels are affected by treatment with DiSB (Fig. 5A). There was a substantial decrease in the level of intracellular Ca^{2+} during treatment with NAC suggesting that ROS has an important role in the
elevation of intracellular \([\text{Ca}^{2+}]\). The \(\text{Ca}^{2+}\) ion is an important element in the progression of cell death as most endonucleases require the presence of \(\text{Ca}^{2+}\) to cleave DNA strands. Therefore, the molecular mechanism for DiSB induced apoptosis-like death involves ROS mediated damage to calcium channels leading to an increase in intracellular calcium pool.

The loss of mitochondrial membrane potential is a characteristic feature of metazoan apoptosis associated with the calcium ion imbalance and plays a key role in drug induced death in *Leishmania* (14). Previous reports on betulin suggests that it induces apoptosis in cancer cells through mitochondrial pathways (30). This prompted us to determine the changes in mitochondrial membrane potential using the mito-sensor dye JC-1. JC-1, a cationic lipophilic dye becomes concentrated in the mitochondria of healthy cells. This aggregate fluoresce red at higher potential but at lower potential this reagent cannot accumulate in the mitochondria and remains as monomers in the cytoplasm that fluoresces green. Fall in the mitochondrial membrane potential increases the green fluorescence intensity. Experiment with JC-1 dye shows accumulation of red signal in the mitochondria of control parasites (Fig. 5B, untreated panel), and the entire cytoplasm coloured light green. But when the cells were treated with DiSB the red signal completely disappears from mitochondria (Fig. 5B, lower panel) suggesting that there is a loss of mitochondrial membrane potential. Flow cytometry shows that DiSB-treated parasites exhibit a right shift in the FL-1 channel (Fig. 5C). The mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydrazone; 1 \(\mu\text{M}\)) causes a greater right shift and served as the positive control. Hence a loss in \(\Delta\psi_m\) was clearly evident. Parasites pretreated with antioxidant, N-acetyl cysteine (NAC) for 60 min or intracellular \(\text{Ca}^{2+}\) chelator BAPTA-AM \((1,2\text{-bis(o-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid})\) for 30 min, Antipain or TLCK for 60 min prior to DiSB treatment prevent rapid loss of \(\Delta\psi_m\). Hence, DiSB-induced cellular stress alters the calcium homeostasis, which
thereby induces loss of $\Delta \psi_m$. Mitochondrial dysfunction may result through a change in
cation homeostasis brought about by high ROS levels (31). The ratio of 590/530 was
measured through spectrofluorometric analysis. As shown in Fig. 5D, following treatment
with 10 $\mu$M DiSB, there was a significant fall (61%) in $\Delta \psi_m$ within first 4 h of treatment with
DiSB as compared with relative $\Delta \psi_m$ observed at 0 h. This is concomitant with the ROS
production suggesting ROS generation precedes loss of $\Delta \psi_m$. From the above data it can be
inferred that the $\Delta \psi_m$ in promastigotes is sensitive to increasing oxidative stress induced by
DiSB treatment. The early loss of potential is indicative of considerable cellular changes
taking place soon after the application of oxidative stress. This state of $\Delta \psi_m$ is continued till 4
h of treatment with DiSB. At 8 h of treatment with DiSB there is a further loss in $\Delta \psi_m$. When
cells were treated with antioxidant NAC, before treatment with DiSB, the loss of $\Delta \psi_m$ was
only 18% after 8 h of DiSB treatment (Fig. 5D). Similar results were achieved when
promastigotes were pretreated with TLCK, antipain or BAPTA-AM. Since NAC could
greatly abolish the $\Delta \psi_m$ collapse, these results indicate that the overproduced ROS was
responsible for triggering the collapse in mitochondrial potential.

To confirm that the single mitochondrion of *Leishmania* adopts a low energy state where $\Delta \psi_m$
is lowered and minimum ATP level is maintained to ensure the entry of cell into apoptotic
pathway (27), we measured intracellular ATP content in normal and DiSB-treated parasites.
The parasites maintain a higher level of ATP in DMSO treated conditions (~170.2 nmole/10^8
cells). The parasites when treated with 5 $\mu$M of DiSB for 2 h, the ATP level remains almost
same (162.2 nmole/10^8 cells). The dramatic decrease in ATP level has been observed after
treatment with DiSB for 4h and the level of cellular ATP becomes about 102.1 nmole/10^8
cells. Treatment of cells for 6 h further decreases the level of ATP to about 68.9 nmole/10^8
cells and at 8 h of treatment the ATP level decreases to 21.6 nmole/10^8 cells (Fig. 5E). This
ensures that generation of ROS followed by depolarization of mitochondrial $\Delta \psi_m$ finally hampers cellular ATP generation which is followed by apoptosis.

**DiSB treatment triggers release of cytochrome c into the cytosol.**

Cytochrome c is a component of mitochondrial electron transport chain and it is present in the inner membrane. A small amount of cytochrome c is present in the cytoplasmic fraction of the control promastigotes. Permeabilization of mitochondrial outer membrane causes increased release of cytochrome c into the cytoplasm with time (Fig. 6A). In case of untreated promastigotes the punctate cytochrome c resides in mitochondria. Treatment with DiSB rapidly translocates cytochrome c into cytosol (Fig. 6A, lower panel). This cytosolic efflux of cytochrome c is concomitant with a reciprocal decrease in mitochondrial cytochrome c (Fig. 6B). Pretreatment of the parasites with NAC, BAPTA-AM, antipain or TLCK decreases the rate of cytochrome c release. But pretreatment of the parasites with the caspase inhibitor, zVAD-fmk for 60 min could not prevent rapid cytochrome c release (Fig. 6C). Disruption of the outer mitochondrial membrane by apoptotic stimuli results in the release of the cytochrome c into the cytoplasm which activates a cascade of proteases involved in apoptosis.

**DiSB causes activation of non-canonical metacaspase proteases.**

Metacaspases are caspase-related cysteine-proteases that are present in organisms without caspases such as plants, yeast, and protozoan parasites. Since caspases are important effector molecules in mammalian apoptosis, the possible role of metacaspases in PCD was evaluated in the *L. donovani* promastigotes after treatment with DiSB. Our data suggests that the programmed cell death cannot be reversed by pretreatment with caspase inhibitors but is inhibited by metacaspase specific inhibitors. In order to compare the level of LdMC (metacaspase) activity in the drug-treated promastigotes, enzyme assays were done with
lysates of promastigotes harvested in mid-log growth phase and using Boc-GRR-AMC as the substrate. Levels of expression of metacaspase detected in 4 and 8 hours post treatment with DiSB (Fig. 7A). Untreated cells expressed metacaspase gene steadily in several time points. Our results showed an increase in the activity of LdMC in parasites treated with DiSB compared to untreated controls (Fig. 7A).

To further support the potential role of LdMCs in DiSB-triggered apoptosis, parasites were transfected with conditional antisense construct of LdMC, where antisense mRNA is produced under the control of Tet repressor gene. DiSB was added to parasites after 24 h of Tet-induced or uninduced pLew82 (empty vector) and antiMC transfectants and the percentage of live promastigotes was plotted against time after DiSB treatment (Fig. 7B). Treatment of LdMC-downregulated parasites (antiMC) with DiSB induced a slow death, while DiSB treatment of other parasites induced LdMC-mediated PCD progression. DNA fragmentations were considerably reduced in DiSB-treated LdMC-downregulated parasites (Fig. 7C). Therefore, the absence of effector metacaspases in antiMC parasites generates a negative feedback response, which suppresses the signalling cascades in the parasites.

DiSB-induced DNA fragmentation is associated with LdEndoG activation.

The internucleosomal DNA digestion by an endogenous nuclease (genomic DNA fragmentation) is considered as a hallmark of apoptotic cell death (32). To establish the DiSB-induced DNA fragmentation, we performed the DNA fragmentation assay by DNA laddering experiment. DMSO-treated parasites exhibited a single genomic DNA band, whereas DiSB-treated parasites showed a laddering pattern indicative of DNA fragmentation. H$_2$O$_2$-treated parasites exhibit more prominent ladders (Fig. 8A). DNA fragmentation assay using ELISA showed that DiSB treatment of parasites along with antipain caused 42% reduction in DNA fragmentation in 4 h. It was observed that there was 45 and 76%
fragmentation of DNA by treatment with DiSB (10 μM) at 4 and 8 h, respectively (Fig. 8B).

The drug concentration (10 μM) used has been optimized on the basis of percentage of DNA fragmentation induced at 8 h of drug treatment. Further treatment with the drug for 12 h did not increase DNA fragmentation (data not shown), and it remains almost same as before (79%). Treatment with protease inhibitor VAD-fmk (25 μM) had no effect on the DNA fragmentation but antioxidant NAC, metacaspase inhibitor antipain and Endo G inhibitor ATA reduced the percentage of DNA fragmentation. So, these results indicate that caspase-like protease has no role on DiSB-mediated PCD, but metacaspases as well as LdEndoG and ROS are also responsible for DNA fragmentation in DiSB-induced cell death. *Leishmania* parasites exhibit stress-induced activation of metacaspases, which thereby induce DNA fragmentation.

PARP is an enzyme involved in DNA repair when DNA damage is sensed (33). PARP cleavage was studied after treatment of *L. donovani* promastigotes with 10 μM DiSB for various time periods. The expression of PARP could be detected in cells at 2, 4 and 8 hours post-treatment (Fig. 8C). PARP protein (78 kDa) generates a 63-kDa cleaved fragment upon treatment of cells with DiSB for 4 and 8 h and the extent of PARP cleavage is more in 8 h. However, the cleavage of this PARP protein did not occur when cells were pre-treated with antipain or endonuclease inhibitor ATA.

Immunoblotting with anti-LdEndoG for mitochondrial and nuclear extracts prepared from parasites showed gradual translocation of LdEndoG to the nucleus with time upon treatment with DiSB (Fig. 8D). The equal band intensities corresponding to LdCOXIV for the mitochondrial extracts and LdHistone H1 for the nuclear extracts served as loading controls.
Terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling (TUNEL) assays helped to visualize the efficacy of these inhibitors in preventing DiSB-induced DNA fragmentation. Figure 8E shows time course increase (24% in 2 h and 77% in 8 h) in DNA fragmentation upon DiSB treatment as evident from the flow cytometry analysis. zVAD-fmk could not prevent DiSB-induced DNA fragmentation, hence parasites were TUNEL positive (72%). Parasites pretreated with ATA (30 min) or antipain (60 min) prior to DiSB treatment showed reduced (15-18%) TUNEL-positive parasites as they inhibit the DiSB-activated endonucleases. Microscopic analysis also revealed that the number of TUNEL positive nuclei is reduced following antipain pre-treatment (Fig. 8F). Thus, DiSB-induced oxidative stress activates endonucleases and is independent of caspase-like proteins in these parasites. The concomitant increase in metacaspase activity and percentage of TUNEL-positive cells in parasites treated with DiSB suggest a possible involvement of LdMCs in DiSB-induced Leishmania PCD.

Conditional knock-down of LdMC and LdEndoG bypasses Leishmania cell cycle arrest and DNA synthesis followed by DiSB treatment.

The cell cycle distribution was analyzed by flow cytometry after treatment with DiSB for different times (Fig. 9A). In control cells the G1, S, and G2/M population remain almost same. DiSB caused *L. tarentolae* promastigotes to remain at resting G0/G1 phase and inhibited their entry into the S phase. The flow cytometric experiment showed that 90% of cells were in G1 phase, whereas 4% and 6% were in S phase and G2/M phase, respectively after treatment with DiSB (10 μM) for 8 h. Treatment with NAC, antipain or ATA could overcome the cell cycle arrest whereas caspase inhibitor had less effect on the reversal. AntiMC and antiEg transfectants could easily bypass the arresting step. Arrest of cell cycle was reversed almost to untreated controls when we used endonuclease G inhibitor ATA on
antiMC transfectants and vice versa proving that both metcaspase and endonuclease G are key effector molecules to trigger DiSB-mediated cell death.

The effect of DiSB on DNA synthesis was studied by incorporation of BrdU (Fig. 9B) and [3H] thymidine into DNA (Fig. 9C) of *L. tarentolae* promastigotes in the presence and absence of different concentrations of DiSB. Treatment with DiSB at 10 μM for 2, 4 and 8 h decreased the BrdU incorporation as well as [3H] thymidine incorporation in promastigotes. After 24 h of incubation with 5 and 10 μM of DiSB, the [3H] thymidine incorporation decreased by 52 and 85%, respectively. The decrease in the rate of thymidine incorporation with time at a particular concentration indicates a decrease in DNA replication activity within the parasites. When antisense constructs of MC were transfected to *L. tarentolae* parasites, it could revert the DNA synthesis inhibition by almost 82% (Fig 9C). This was concomitant with the BrdU incorporation. Transfectants of anti-metcaspase as well as EndoG could relieve the DiSB-mediated cell cycle arrest. The reversal was almost similar to that of untreated cells when endonuclease G inhibitor ATA was used on antiMC transfectants and vice versa.
Discussion

Although the PCD of unicellular organisms has been established (34, 35) the pathway in protozoan parasites has not been well characterized. A search in the *Leishmania* genome database failed to identify most of the effector molecules associated with the caspase-dependent PCD pathway. A recent report identified Endo G of kinetoplastids as an effector molecule of kinetoplast PCD pathway (36), but the initiation of PCD has not been clearly understood. Recently metacaspases, have come up as effector molecules in these parasites (37). We have shown previously that DiSB directly interacts with topoisomerase I and thus inhibits all the life processes associated with dsDNA transactions in leishmanial cells and acts as a catalytic inhibitor of topoisomerase I (18). CPT, a potent antitumor agent, can trap topoisomerase 1-mediated cleavable complex in the mitochondria (38), and increases the cellular ROS generation in leishmanial cells (14).

In the present study, we found inhibition of growth of *L. donovani* promastigotes by DiSB in a dose dependent manner. In accordance with previous studies on betulin, a significant population of leishmanial cells in G2/M phase were decreased when parasites were exposed to 10 μM DiSB. The present study indicates that DiSB was an effective inducer of apoptosis in *L. donovani* and that is confirmed by phosphatidylserine externalization and DNA fragmentation. An established event in most apoptotic cells is the generation of ROS in the cytosol that directs the cell towards the path of death. Because DiSB interacts with topoisomerase I, it might generate ROS inside mitochondria. Therefore, we measured the endogenous ROS in leishmanial cells after treatment with DiSB. It was found that there was consistent increase in cytosolic ROS. But pre-treatment with NAC could not reverted the ROS level upto normal state. This may be due to the topoisomerase lesions that DiSB generates inside parasites which becomes irreversible.
DiSB-induced oxidative stress increases intracellular calcium ion concentration, which concomitantly accumulates in mitochondria. Cation imbalance inside the parasite mitochondria causes its membrane to depolarize. When calcium reaches critical threshold, the mitochondrial outer membrane is permeabilized (mediated by VDAC channels) (29). Increased ROS production depletes cellular GSH levels in *Leishmania* and thereby accelerates growth arrest. There is concomitant inner membrane permeabilization, promoted by the permeability transition pore. Resultant generation of ROS boosts up the stress pathway and also causes oxidative DNA lesions (39), which is generated by abrogating topoisomerase DNA interactions. Lipid peroxidation causes release of cytochrome c into the cytosol along with the effector endonuclease, LdEndoG (14). ATA inhibits the effector endonuclease and generates a negative feedback response to arrest the interlinked signaling cascade and helps to maintain cation homeostasis (40).

There was depletion of mitochondrial ATP level in DiSB-treated cells, and the extent of depletion within 30 min was almost 20%, which increased to almost 60% in 4 h. Almost similar type of mitochondrial ATP depletion was observed in oligomycin-treated cells (data not shown). The above-mentioned results suggest that mitochondrial ATP depletion after treatment with DiSB is due to inhibition of mitochondrial oxidative phosphorylation. In the absence of proper functional mitochondria, cells would cease to synthesize ATP from their mitochondrial source and cause a rapid decrease in cellular ATP levels to the extent of 53% by 120 min. Our result is consistent with the study reported previously that the ATP level gradually decreases after the loss of Δψm during treatment with H2O2 (27). But, ATP is a key molecule for chromatin condensation, nuclear fragmentation and regulation and the maintenance of ion homeostasis during apoptosis. So, we can assume that the ATP levels...
generated before the loss of $\Delta \psi_{\text{m}}$ and ATP supplied by glycolysis are sufficient to carry out these cellular activities and to propagate PCD in leishmanial cells.

In higher eukaryotes, release of cytochrome c results in ‘apoptosome’ formation that activates the caspase cascade and thereby caspase-activated DNases (CAD) (41). The negative assays for caspase-like proteins (data not shown), ineffectiveness of zVAD-fmk and activity of Boc-GRR-AMC indicated the activation of a metacaspase-dependent PCD mechanism by DiSB.

DiSB treatment translocates LdEndoG into the nucleus, which along with ROS produce nicked chromatin. Besides, DiSB generates single and double strand breaks by trapping DNA topoisomerases (18). These nicked chromatin forms the substrate of LdEndoG-assembled DNA ‘degradesome’ complex (25). DiSB treated Leishmania parasites activates metacaspase and downregulation to this metacaspase subverts cell death. Most mammalian caspases are synthesized as catalytically inactive zymogens that are processed into active proteases upon activation of the cell death pathway. L. donovani metacaspases do not appear to be processed under normal growth conditions or upon treatment with inducers of PCD (42). This suggests that LdMCs could be synthesized in active forms and that the parasites must have mechanism(s) to control/isolate metacaspase activity inside the cell.

In conclusion, DiSB-induced apoptosis appears to be primarily associated with the early activation of metacaspase through the mitochondrial pathway, as demonstrated by the simultaneous loss of mitochondrial membrane potential, and consequent release of cytochrome c (Fig. 10). Being a specific target to topoisomerase IB of Leishmania promastigotes, DiSB has the potency to be developed for antileishmanial drug (18). Exploiting the mechanistic understanding of the selective cytotoxic effect of DiSB on Leishmania respiting host cells may aid in the development of novel and potent chemotherapeutic agents accompanied by diminished side effects.
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Authorship Contributions:

- Participated in research design: Chowdhury, Sengupta and Majumder.
- Conducted experiments: Chowdhury, Roy Chowdhury and Mukherjee.
- Contributed new reagents or analytic tools: Mukherjee and Jaisankar.
- Performed data analysis: Chowdhury and Majumder.
- Wrote or contributed to the writing of the manuscript: Chowdhury and Majumder.
References:


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Figure Legends

Figure 1:

Betulin derivatives kill *Leishmania* parasites in dose and time dependent manner. (A) Promastigotes were cultured in presence of 0.2% DMSO or different concentrations of DiSB, DiGDHB and DiSDHB respectively up to 12 h. After 12 h and percentage of viable promastigotes was calculated using Alamar Blue dye. Values were taken in triplicate and averaged. Percentage of live promastigotes were calculated and plotted against concentration. (B) Analysis of antileishmanial activity in presence of 1 \( \mu \)M, 5 \( \mu \)M or 10 \( \mu \)M of DiSB for indicated times. Percentage of viable promastigotes was measured as above. (C) *L. major*, *L. donovani*, and *L. tarentolae* promastigotes were also cultured in presence of increasing concentrations of DiSB for 6 h. The percentage of viable promastigotes was measured by Alamar Blue assay. All data are expressed as percentage of live promastigotes and represent mean ± S.D. from three independent experiments. (D) Wild type AG83, laboratory grown sodium antimony gluconate resistant cells (GE1), miltefosine resistant *Leishmania* (Mil-R), camptothecin resistant cells (CPT-R) and diindolylmethane resistant strains (DIM-R) were cultured separately in FBS supplemented M199 medium. Early log phase promastigote cells \( (3 \times 10^6) \) were treated with 1, 5, 10, 20, and 50 \( \mu \)M DiSB for 6 h. Percentages of live promastigotes were calculated as above and plotted. Data represent means ± S.D. (n=3).

Figure 2:

Inhibition of oxygen consumption causes the formation of ROS inside cells. (A) Percentage of inhibition of \( O_2 \) consumption was determined after treating the cells in presence of 0.2% DMSO, 5 \( \mu \)M or 10 \( \mu \)M of DiSB for the period of 30 min to 10 h, respectively as described in Materials and Methods. Data are normalized with mg of protein and expressed as mean ± S.D. of three independent experiments. (B) Measurement of ROS
Figure 3:

**Determination of intracellular GSH level and level of lipid per oxidation during treatment with DiSB.** (A) Level of intracellular GSH in *L. donovani* promastigotes in treated and untreated cells. The intracellular GSH level was measured after treatment with DiSB (5 μM and 10 μM), 0.2% DMSO alone and with NAC prior (30 min) to treatment with DiSB. Intracellular GSH level has been corrected and normalized according to the number of viable parasites. (B) Ratio of GSH to GSSG of DiSB treated (5 and 10 μM respectively for 8h) *L. donovani* promastigotes. One set is pretreated with NAC for 60 min followed by DiSB treatment. (C) The level of fluorescent products of lipid peroxidation was measured after treatment of leishmanial cells with 0.2% DMSO alone, 5 μM and 10 μM of DiSB and with NAC separately prior to treatment with DiSB. Fluorescence readings were normalized with total numbers of parasites. Results are mean ± S.D. from three independent experiments. * < 0.5 (student's t-test), ** < 0.05 and *** < 0.01 compared to DMSO treated parasites. NS denotes non significant.

Figure 4:

**Flow cytometric analysis of promastigote death through PCD/necrotic processes.** (A) Externalization of phosphatidyl serine was detected in *L. donovani* promastigotes after annexin V labeling of *L. donovani* promastigotes. Cells were treated with 0.2% DMSO alone, with DiSB (10 μM) for 2, 4, 8 and 12 h, with CED3/CPP32 group of protease inhibitor.
(VAD-fmk), metacaspase inhibitor TLCK and with antipain (42) for 60 min prior to treatment with DiSB as described in Materials and Methods. Dot plots are representative of one of three similar results. Apoptotic cells were detected by flow cytometric analysis using annexin V and PI in FL-1 versus FL-2 channels. Cells in the bottom right quadrant indicated apoptosis, whereas cells in the top right quadrant represented post-apoptotic/early-necrotic population. (B) Bar diagram showing the early apoptotic cells, early-necrotic and necrotic cells as determined by fluorescence-activated cell sorting analysis. The results depicted were performed three times, and representative data from one set of experiments are expressed as means ± S.D. Variations among different set of experiments were <10%. * < 0.05 (student's t-test) and ** < 0.01, *** < 0.001 compared to DMSO treated parasites. NS denotes non significant.

Figure 5:

**Measurement of DiSB-induced changes in intracellular Ca\textsuperscript{2+} level and mitochondrial membrane depolarization.** (A) Increase in Ca\textsuperscript{2+} level was measured after treatment with 10 μM DiSB compared to treatment with 0.2% DMSO alone and with NAC, FFA and verapamil for 30 min prior to treatment with DiSB. Data were normalized to mg of cellular protein extract. Results are mean ± SD from three independent experiments. * < 0.05 (student's t-test),** < 0.01 and *** < 0.001 compared to DMSO treated parasites. NS denotes non significant. (B) Confocal microscopic analysis of JC-1 stain in Drug treated or untreated L. donovani cells. In normal cells, the cytoplasm stains as light green and mitochondria as red. In case of DiSB treated cells, the red colour disappears and the total cell appears green. For each set DIC, Red/Green merged channel and DIC merged pictures were provided. (C) Flow cytometric analysis of mitochondrial membrane potential. Parasites were treated with 0.2% DMSO, DiSB (5 or 10 μM), CCCP (1 μM) or with 10 μM DiSB after pretreatment with
NAC, BAPTA-AM, zVAD-fmk, Antipain or TLCK (60 min) mentioned in Materials and Methods. Thereafter promastigotes were incubated with BD mitosensor reagent and the green fluorescence intensity was monitored using flow cytometry. (D) L. donovani promastigotes were exposed to 0.2% DMSO, 10 μM DiSB for 2 h, 4 h and 8 h respectively and pretreated for 60 min with 20 μM NAC, 240 μM FFA, 100 nM Verapamil, zVAD-fmk, Antipain, TLCK and BAPTA-AM separately before treatment with DiSB for 8 h and subsequently stained with the potentiometric probe JC-1 (10 μM). Relative ΔΨm values are expressed as the ratio of the reading at 590 nm (aggregate) to the reading at 530 nm (monomer). Data are means ± SD from three independent experiments. * < 0.5 (student's t-test) and ** < 0.01 compared to DMSO treated parasites. NS denotes non significant. (E) L. donovani promastigotes were exposed to 0.2% DMSO, 5 μM DiSB for 2 h, 4 h, 6h and 8 h respectively and total cytosolic ATP was measured as describes in Materials and methods. Data are means ± SD from three independent experiments. ** < 0.5 (student's t-test) and *** < 0.01 compared to DMSO treated parasites.

Figure 6:

Determination of cytochrome c release in DiSB treated L. donovani promastigotes. (A) Localization of cytochrome c in treated and untreated cells through confocal microscopy. Leishmania cells were treated with 0.2% DMSO and DiSB (10 μM). After 8 h cells were smeared and fixed with 3.7% paraformaldehyde followed by permeabilization with 0.1% Triton-X 100. The cells were incubated with anti cytochrome c antibody for overnight and counter stained with FITC-conjugated secondary antibody and confocal microscopic pictures were taken. Pictures are representative of one of three similar results. (B) Western blot analysis to detect the levels of cytochrome c for cytoplasmic (panel I) and mitochondrial (panel II) fractions from parasites taken at given times of differently treated cells. Lane 1,
cytosolic level of cytochrome c in DMSO-treated leishmanial cells; lane 2, CPT-treated (6 h)
leishmanial cells; lanes 3-5, cells treated with DiSB (10 μM) for 2, 4 and 8 h, respectively.
Immunoblots of Ldβ-tubulin and LdCOXIV served as loading controls for the cytosolic and
mitochondrial fractions, respectively. (C) immunoblot for cytochrome c in total cell lysate
(lane 1), cytosolic extracts from 0.2% DMSO-treated parasites (lane 2), DiSB (10 μM)
treated for 4 h (lane 3) and 8 h (lane 4), DiSB-treated parasites after pretreatment with 20 μM
NAC (lane 5), 25 μM zVAD-fmk (lane 6), 2 μM antipain (lane 7), 10 μM TLCK (lane 8) or
25 μM BAPTA-AM (lane 9). Immunoblot using anti-β-tubulin antibody served as the loading
control.

**Figure 7:**

**Determination of metacaspase activity and its downstream effects in the presence and in
the absence of protease inhibitor.** (A) Activation of metacaspases in the cytosol of
leishmanial cells was measured after treatment with 0.2% DMSO, 5 μM CPT alone, DiSB
(10 μM) alone for 4 and 8 h respectively and with NAC, antipain, TLCK and CED3/CPP32
group of inhibitor zVAD-fmk pre-treated (for 60 min) cells. Metacaspase activity was
measured using Boc-GRR-AMC as the substrate and expressed in relative fluorescence units
(RFU) and normalized to mg of protein of cellular extracts. The bar represents the averages
of three independent assays. Standard deviations (error bars) are shown. (B) Viability of
LdMC knocked down parasites upon DiSB treatment. Transfectants, pLew82 and antiMC
were induced by Tet (+T) for 24 h and thereafter treated with or without 10 μM DiSB for 24
h. Percentage of viable promastigotes was calculated using alamar blue reagent. Values taken
in triplicate were averaged and plotted against time. (C) Extent of DNA fragmentation upon
DiSB treatment for 24 h in cells transfected with pLew82 or with antiMC. The transfectants
were induced with or without Tet for 24 h prior to treatment with DiSB. Values were
obtained from the MULTISCAN EX readings at 405 nm. The percentage was plotted versus time. Data represent means ± SD (n=3). * < 0.05 (student's t-test) and ** < 0.01 and *** < 0.001 compared to DMSO treated parasites. NS denotes non significant.

Figure 8:

**DiSB-induced DNA fragmentation assay with activation of cellular Endonuclease. (A)**

Genomic DNAs were isolated from *L. donovani* promastigotes after treatment with 0.2% DMSO, 5 μM H₂O₂ for 4 h, 10 μM DiSB for 8 h and 10 μM DiSB along with 2 μM antipain. (B) Extent of genomic DNA fragmentation upon DiSB treatment. Parasites were treated with 5 μM or 10 μM DiSB alone or after pre-incubation with NAC, 25 μM zVAD-fmk, 2 μM antipain. As negative control, parasites were also treated with 0.2% DMSO. Values were obtained from the MULTISCAN EX readings at 405 nm and normalized to total number of cells. The relative percentage (with respect to samples treated with micrococcal nuclease and normalized to percentage values) was plotted as units of time. Data represent means±S.D. (n=3). * < 0.5 (student's t-test) and ** < 0.05 and *** < 0.01 compared to DMSO treated parasites. NS denotes non significant. (C) Western blot analysis to detect the cleavage of full-length PARP protein after treatment with 0.2% DMSO (lane 1), with DiSB (10 μM) for 2 h, 4 h and 8 h (lanes 2, 3 and 4), and with metacaspase group of protease inhibitor (antipain) before treatment with DiSB (lane 5) or with endonuclease G inhibitor ATA (lane 6). (D) Nuclear translocation of LdEndoG. Western blot analysis of the mitochondrial and nuclear fractions obtained from DiSB-treated parasites at different intervals or pretreated with antipain (for 60 min) and ATA (for 30 min); baicalein treatment served as positive control. Immunoblotting with anti-LdEndoG showed gradual decrease in LdEndoG from the mitochondrial fraction with concomitant increase in the nuclear fraction, Anti-COXIV and anti-Histone H1 served as the loading controls for the mitochondrial and nuclear fractions,
respectively. (E) DiSB-induced DNA fragmentation was analyzed by TUNEL assay using flow cytometry. Parasites were treated with 0.2% DMSO, 10 μM DiSB for 4 and 8 h respectively. DiSB treatment was also carried out after pre-incubation with NAC (60 min), 25 μM zVAD-fmk (60 min), 2 μM antipain (60 min) or 50 μM ATA (30 min). Treatment with CPT (10 μM) alone or after pre-incubation with 25 μM zVAD-fmk served as a positive control. Graphical representation of the percentage of TUNEL-positive nuclei is plotted. Mean values for three sets of 10,000 parasites were plotted. * < 0.05 (student's t-test) and ** < 0.01 compared to DMSO treated parasites. NS denotes non significant. (F) DiSB-induced DNA cleavage was characterized in L. donovani promastigote cells by TUNEL assay. Non-apoptotic cells (untreated), cells after treatment with 10 μM DiSB for 4 h and 8 h and with metacaspase inhibitor (antipain) were visualized. PI was added as counter stain.

Figure 9:

Subversion of cell cycle arrest in LdMC knocked down parasites upon DiSB treatment and analysis of DNA synthesis in DiSB-treated parasites. (A) DiSB-mediated cell cycle arrest in L. tarentolae promastigote cells. Histograms of distribution of DNA content with flow cytometry in Leishmania cells were shown. Cell cycle arrest was analysed after treatment with 0.2% DMSO as control, and with DiSB (10 μM) for 2, 4, 6 and 8 h or pre-treated with different inhibitors like NAC, zVAD-fmk, Antipain or ATA. Cells were then fixed and stained with propidium iodide, and nuclei were analysed for DNA content by flow cytometry. In another set, antisense construct of EndoG and Metacaspase were transfected individually or with antipain and ATA and were analysed. In total, 20,000 nuclei were counted from each sample. Percentages of cells within different cell stages were determined as described in Materials and Methods. (B) Flow cytometric analysis of DNA synthesis by BrDU incorporation as per manufacturer’s protocol (Roche Bioscience). After different
treatments (as shown in figure) cells were ethanol fixed and anti-BrDU antibody was used followed by FITC conjugated secondary antibody. Finally the cells were incubated with Propidium Iodide (with RNAse A). All the experiments were performed thrice and one such experiment was given. (C) Incorporation of [3H]thymidine (5 μCi/ml) into DNA of L. tarentolae cells. Incorporation was monitored at different times after addition of [3H]thymidine and 0.2% DMSO followed by the addition of DiSB (5 μM or 10 μM, 24 h) in Tet uninduced (-T) antisense construct of Metacaspase (aMC) transfected promastigotes, or with Tet induced (+T) antisense constructs. Aliquots of 200 μl each were withdrawn from the cultures at the indicated time intervals and processed to determine the incorporation of label into acid-precipitable DNA and normalized to total number of promastigotes. The experiments were performed three times, and representative data from one set of these experiments are presented as mean ± S.D. Variations among different set of experiments were <8%. * < 0.5 (student's t-test), ** < 0.05 and *** < .01 respectively compared to DMSO treated controls. NS denotes no significance.

Figure 10:
Schematic pathway of DiSB-induced metacaspase-dependent programmed cell death in Leishmania involving a LdEndoG.
### Table 1: EC<sub>50</sub> values of three betulin derivatives on Leishmania donovani promastigotes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>DiSB</th>
<th>DiGDHB</th>
<th>DiSDHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>1.43 ± 0.24</td>
<td>1.51 ± 0.35</td>
<td>3.32 ± 0.81</td>
</tr>
</tbody>
</table>

### Table 2: IC<sub>50</sub> values of different strains of parasites cultured for 6 h with DiSB.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>L. donovani</th>
<th>L. major</th>
<th>L. tarentolae</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>7.21 ± 2.11</td>
<td>5.84 ± 1.06</td>
<td>8.32 ± 2.03</td>
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