BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 1143–1150
© 2010 The Authors
Journal compilation © 2010 The British Pharmacological Society All rights reserved
0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

16α-Hydroxycleroda-3,13 (14)Z-dien-15,16-olide from *Polyalthia longifolia*: a safe and orally active antileishmanial agent

Pragya Misra[1], Koneni V Sashidhara[2], Suriya Pratap Singh[2], Awanish Kumar[1], Reema Gupta[1], Shailendra S Chaudhaery[2], Souvik Sen Gupta[3], HK Majumder[3], Anil K Saxena[2] and Anuradha Dube[1]

[1]*Parasitology Division, CDRI, CSIR, Lucknow, India,* [2]*Medicinal and Process Chemistry Division, CDRI, CSIR, Lucknow, India, and* [3]*Molecular Parasitology, IICB, CSIR, Kolkata, India*

Background and purpose: New antileishmanials from natural products are urgently needed due to the emergence of drug resistance complicated by severe cytotoxic effects. 16α-Hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1) from *Polyalthia longifolia* was found to be a potential antileishmanial and non-cytotoxic, as evidenced by long-term survival (>6 months) of treated animals. This prompted us to determine its target and, using molecular modelling, identify the interactions responsible for its specific antileishmanial activity.
Experimental approach: *In vitro* activity of compound was assessed using intracellular transgenic green fluorescent protein-stably expressed *Leishmania donovani* parasites. *In vivo* activity and survival of animals post-treatment were evaluated in *L. donovani*-infected hamsters. Known property of clerodane diterpenes as potent human DNA topoisomerase inhibitors led us to evaluate the inhibition of recombinant *L. donovani* topoisomerase I using relaxation assay. Mode of cell death induced by Compound 1 was assessed by phosphotidylserine exposure post-treatment. Molecular modelling studies were conducted with DNA topoisomerase I to identify the binding interactions responsible for its activity.
Key results: Bioassay-guided fractionation led to isolation of Compound 1 as a non-cytotoxic, orally active antileishmanial. Compound 1 inhibited recombinant DNA topoisomerase I which, ultimately, induced apoptosis. Molecular docking studies indicated that five strong hydrogen-bonding interactions and hydrophobic interactions of Compound 1 with *L. donovani* DNA-topoisomerase are responsible for its antileishmanial activity.
Conclusions and implications: The data reveal Compound 1 is a potent and safe antileishmanial. The study further exploited the structural determinants responsible for its non-cytotoxic and potent activity, to raise the feasibility of specifically targeting the target enzyme responsible for its activity through rational drug design.
British Journal of Pharmacology (2010) **159,** 1143–1150; doi:10.1111/j.1476-5381.2009.00609.x; published online 5 February 2010

Keywords: *Leishmania donovani*; *Polyalthia longifolia*; antileishmanial activity; apoptosis; *L. donovani* topoisomerase I; Molecular docking

Abbreviations: CDRI, Central Drug Research Institute; CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals; FACS, fluorescent activated cell sorter; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; *L. donovani*, *Leishmania donovani*; Ld-topoI, *L. donovani* DNA-topoisomerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; *P. longifolia*, *Polyalthia longifolia*; PDB, Brookhaven Protein Data Bank; PI, propidium iodide; VL, visceral leishmaniasis

Introduction

Visceral leishmaniasis (VL or Kala-azar) is the most devastating form of leishmaniasis of all leishmaniases and is caused by the invasion of the reticuloendothelial system (spleen, liver and bone marrow) by the haemoflagellate protozoan parasite *Leishmania donovani*. The disease is widely distributed in the Indian subcontinent and South-West Asia (Desjeux *et al.*,

Correspondence: Anuradha Dube, Parasitology Division, Central Drug Research Institute, CSIR, Lucknow-226001, India. E-mail: anuradha_dube@hotmail.com; Koneni V Sashidhara, Medicinal and Process Chemistry Division, Central Drug Research Institute, CSIR, Lucknow-226001, India. E-mail: sashidhar123@gmail.com
Received 22 August 2009; revised 25 September 2009; accepted 13 October 2009

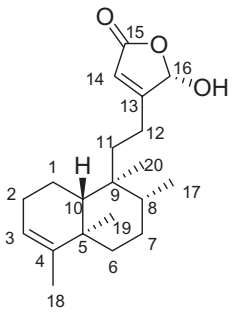

Figure 1 Structure of 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1).

2001; Sundar, 2001). Since the 1940s, pentavalent antimonial compounds constitute the first-line treatment for all forms of leishmaniasis and in case of therapeutic resistance to these compounds, amphotericin B desoxycholate, liposomal amphotericin B and miltefosine are also being used (Croft and Yardley, 2002). However, as most of these drugs are expensive, toxic and have unacceptable side effects and their administration is complicated by the fact that they are given parenterally, they are not entirely satisfactory (Murray, 2000). Moreover, cases of drug resistance are on the rise (Berman *et al*., 1982; Croft, 2001). This has caused a renewed interest in the study of medicinal plants as a source for new antiparasitic compounds with better activities and fewer side effects. Many people living in endemic areas of leishmaniasis rely on traditional medicines for treatment; these consist of oral administration of plant extracts that combat the systemic forms of the disease (Iwu *et al*., 1994).

Polyalthia longifolia var. *pendula* Linn, belonging to the family Annonaceae, is one of the most important medicinal plants of India (Lala *et al*., 2004). Activity-guided fractionation of the crude ethanolic extract of the leaves of *P. longifolia* var. *pendula* has led to the isolation of the clerodane diterpene 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1, Figure 1), which is a major secondary metabolite of this plant. Clerodane diterpenoids from *P. longifolia* are known to have antimicrobial activity (Marthanda Murthy *et al*., 2005), have cytotoxic effects on various cancer cell lines (Zhao *et al*., 1991; Chen *et al*., 2000; Chang *et al*., 2006) and have antimalarial activity (Ichino *et al*., 2006). Here, we report for the first time its bio-efficacy against *L. donovani* infection. Further studies were also performed to elucidate its mechanism of action at both the molecular and structural level.

In a bid to find new therapeutic targets, essential for parasite growth and survival, DNA topoisomerases have recently emerged as potential therapeutic targets possessing a broad spectrum of antiprotozoan activity (Chowdhury *et al*., 2003). Clerodane diterpenes, which have a wide spectrum of pharmacological properties, have been reported to be potent inhibitors of human DNA topoisomerases (Jamora *et al*., 2001). Herein we have investigated the potential antileishmanial activity of 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide, a clerodane diterpene from *P. longifolia* and found it to be an inhibitor of recombinant Leishmania DNA topoisomerase I. Molecular docking studies with the active site of *L. donovani* DNA-topoisomerase (Ld-topoI) [Brookhaven Protein Data Bank (PDB) ID: 2B9S] were also carried out to predict the binding mode of the compound, which would be useful in computer-aided/structure-based drug designing.

Methods

Plant material

The leaves of the plant were collected from Lucknow in April 2005. The identity of the plant was confirmed and a voucher specimen (No. 6381) has been deposited in the herbarium of the Botany Division, Central Drug Research Institute (CDRI), Lucknow, India.

Extraction and isolation

Air-dried and ground-up leaves (16 kg) of *P. longifolia* var. *pendula* were extracted with ethanol (50 L, three times) for three consecutive days at room temperature. Evaporation of the solvent under reduced pressure yielded an ethanolic extract (2 kg). This ethanolic extract was macerated with hexane (2 L, three times) and the remaining residue dissolved in water (2 L) and partitioned with chloroform (2 L, three times) and butanol (2 L, three times) to yield three fractions, which when concentrated under reduced pressure at a water bath temperature of 20–50°C yielded a hexane extract (1.2 kg), a chloroform extract (50.2 g) and a butanol extract (300 g).

The hexane extract (1.2 kg) was subjected to silica gel (60–120 mesh) column chromatography using EtOAc : hexane gradient (0:100 to 100:0) to give eight sub-fractions. The diterpene 1 was present in 20% EtOAc : hexane fraction (383 g). A portion of the 20% EtOAc : hexane fraction (30 g) was further taken up for the isolation of the diterpene. It was chromatographed over flash silica gel (230–400 mesh) with a hexane : EtOAc gradient (100:0 to 0:100) to furnish eight subfractions. Fraction IV (6% EtOAc : hexane fraction, 6.5 g) on purification gave 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1) (3.56 g). Compound 1 was identified by using 1D and 2D NMR coupled with other spectroscopic data (available as supporting data) and by comparing the observed values with those reported in the literature (Hara *et al*., 1995).

General experimental procedures

IR spectra were recorded on a Perkin Elmer 399B spectrometer. Optical rotations were obtained using a Perkin-Elmer 241 Polarimeter. All 1D and 2D NMR experiments were performed on Bruker Avance DPX 300MHz spectrometer. The NMR spectra were recorded in $CDCl_3$ solvent using TMS as an internal standard. Chemical shifts were given in δ (ppm), while ^1H-^1H coupling constant (J) values are given in Hz. ESI mass spectra were recorded on JEOL SX 102/DA-6000. Organic solvents were distilled prior to use. Silica gel was used as a stationary phase to isolate the compound. TLC was performed on silica gel $60F_{254}$ aluminium plates (Merck KGaA, Darmstadt, Germany). TLC plates were sprayed with 5%vanillin in 10% H_2SO_4 solution followed by heating at 110°C for 5 min. TLC spots were visualized by inspection of the plates under a UV lamp.

Parasite culture
The transgenic promastigotes tagged with stably expressing green fluorescent protein (GFP) of *L. donovani* (MHOM/80/Dd8) (Singh *et al*., 2009) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin at 26°C.

Animal host
Laboratory-bred male golden hamsters (*Mesocricetus auratus*) weighing 45–50 g were used as the experimental host. They were housed in climatically controlled rooms and fed with standard rodent food pellet (Lipton Ltd., Bombay, India) and water *ad libitum*. All animal care and experimental use of animals conformed to CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines for laboratory animal facility and was approved by the institutional animal ethics committee of the CDRI. Every effort was made to minimize both the number of animals used and their suffering.

Infection of animals
Hamsters were infected intracardially with late log phase promastigotes of *L. donovani*. Briefly, promastigotes were harvested by centrifugation at 2500× *g* for 15 min at 4°C, washed three times with phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of $1 \times 10^8 \cdot 0.1$ mL^{-1}. Hamsters were inoculated intracardially with promastigotes in 0.1 mL of PBS. Parasite burden was assessed on days 25–30 post-infection by performing splenic biopsies as described previously (Singh *et al*., 2008). Once the infection was established with promastigote form, further passages in hamsters were carried out with splenic amastigotes. For this, animals carrying 40–60-day-old infection were autopsied; their spleen removed aseptically, homogenized in PBS and centrifuged at 170× *g* for 5 min at 4°C to sieve out tissue debris. Supernatant was centrifuged at 1300× *g* for 10 min, pellet washed twice with PBS. Animals were infected intracardially with 1×10^7 amastigotes in 0.1 mL PBS. Animals carrying 25–30-day-old infection were employed for drug screening.

Macrophage cell line culture and maintenance
The adherent mouse macrophage cell line J774A.1 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin at 37°C in 5% CO_2 in a humidified atmosphere.

Antipromastigote activity
Log phase transgenic GFP-expressing promastigotes (1×10^6 cells·mL^{-1}) were put into 48-well tissue culture plates, and different concentrations of standard drugs as well as test samples were administered. Untreated cells served as control. Each assay was performed in triplicate. At different time intervals (48–72 h) after treatment, cells were removed, washed in PBS and analysed in a fluorescent activated cell sorter (FACS) Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15 mV 488 nm air-cooled argon laser with excitation at 488 nm and emission at 515 nm. Ten thousand cells were acquired for each analysis. Multiparametric data were analysed by Cell Quest software (Becton Dickinson). The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated parasites with those of untreated control parasites. Miltefosine was used as a reference drug.

Activity against intracellular amastigotes
J774A.1 macrophages were cultured in 24-well plates, to a cell density of 10^5 cells per well and infected with late log phase transgenic GFP-expressing promastigotes at a multiplicity of infection of 10:1 (parasite/macrophage) and incubated at 37°C in 5% CO_2 for 8–12 h; later on wells were washed three times with incomplete medium to remove non-phagocytosed parasites, and finally, the wells were supplemented with complete medium. At different time intervals (48–72 h) after treatment, cells were removed, washed in PBS and analysed in a FACS Caliber flow cytometry (Becton Dickinson, USA) equipped with a 15 mV 488 nm air-cooled argon laser with excitation at 488 nm and emission at 515 nm. Ten thousand cells were acquired for each analysis. Multi-parametric data were analysed by Cell Quest software (Becton Dickinson). The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated parasites with that of untreated control parasites.

Cytotoxicity assay
In vitro cytotoxicity of Compound 1 on J774A.1 macrophages was assessed by colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] reduction assay. Cells (10^5 mL^{-1}) were incubated with various concentrations of the test samples and incubated at 37°C in 5% CO_2 for 72 h. MTT to a final concentration of 400 μg·mL^{-1} was added and incubated further for 3 h at 24°C. Cells were centrifuged at 2800× *g*, and pellets were dissolved in DMSO before taking the absorbance at 540 nm. The mean percentage of post-treatment viable cells was calculated relative to control, and results are expressed as the concentration inhibiting cell growth by 50% (IC_{50}).

Efficacy evaluation of Compound 1 in hamsters infected with L. donovani
The antileishmanial activity of different oral doses of Compound 1 against established infection (25–30 days) of *L. donovani* was assessed. Infected animals were divided into groups with six animals in each. Treatment was initiated 3–4 days after spleen biopsies (pretreatment) at a dose level of 250 mg·kg^{-1} and continued for five consecutive days. One group of animals was treated orally with the reference drug – miltefosine. One group served as an untreated control. The effects of other doses of the compound were also investigated to obtain optimum efficacy. Three animals were killed on day 7 post-treatment and dab smears were prepared from spleen, liver and bone marrow. The % inhibition of the infection was assessed using the following formula:

$$PI = AT \times 100/IT \times TI$$

where propidium iodide (PI) – % inhibition; AT – actual number of amastigotes per 100 spleen cell nuclei in treated animals; IT – initial number of amastigotes per 100 spleen cell nuclei in treated animals; and TI – times increase in untreated control animals.

The three animals remaining in each group were observed for measuring their survival and their live/dead counts were made on different days post-treatment. Two to three replicates were obtained for each dose.

Plasmid relaxation assay

The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBluescript (SK^+) DNA in an agarose gel after treatment with enzyme. A relaxation assay was carried out as previously described with LdTOP1LS (Das *et al.*, 2004). The standard topoisomerase assay contained 25 mM Tris–HCl, pH 7.5, 5% glycerol, 50 mM KCl, 0.5 mM DTT, 10 mM $MgCl_2$, 150 mg·L^{-1} bovine serum albumin, 0.5 mg of pBS plasmid and 1 U of enzyme (1 U of topoisomerase I activity is the amount of enzyme that converts 0.5 mg of superhelical DNA to the relaxed state under the conditions of the assay). The reaction was carried out at 37°C for 30 min. Reactions were stopped by adding 1% SDS, 10 mM EDTA, 0.25 mg·L^{-1} bromophenol blue and 15% glycerol. Samples were applied to a horizontal 1% agarose gel and subjected to electrophoresis in Tris–acetate–EDTA buffer (0.04 M Tris–acetate, 0.002 M EDTA, pH 8.0) at 1.5 V·cm^{-1} for 14–16 h at room temperature. The gels were stained with ethidium bromide (5 mg·L^{-1}), de-stained in water and photographed under UV illumination. The percentage of relaxation was measured by microdensitometry of negative photographs of supercoiled monomer DNA band fluorescence after ethidium bromide staining with a microdensitometer (LKB BROMMA 2202 Ultrascan), and the area under the peak was calculated.

Double staining with Annexin V and PI

Externalization of phosphatidylserine on the outer membrane of untreated and Compound 1 treated promastigotes was measured by the binding of Annexin V-FITC (fluorescein isothiocyanate) and PI as previously described (Mehta and Shaha, 2004). Briefly, promastigotes were incubated with Compound 1 (5.7 μg·mL^{-1}) for different time periods and then the cells were washed by centrifugation (2500× *g*, 10 min), in PBS (0.02 M, pH 7.2) and resuspended in Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$; pH 7.4). Annexin V-FITC and PI were then added, according to the manufacturer's instructions, and incubated for 15 min in dark at 20–25°C. Acquisition was done on a FACS Calibur flow cytometer (BD) and analysed with Cell Quest software. Miltefosine, an established inducer of apoptosis in *Leishmania* parasites (10 μM, 48 h), served as the positive control (Paris *et al.*, 2004).

Molecular modelling and molecular dynamics studies

The Genetic Optimization for Ligand Docking (GOLD) version 2.2 on windows-based PC was used for docking studies (Jones *et al.*, 1997). This method allows a partial flexibility of protein and full flexibility of ligand. The reported crystal structure of Ld-topoI (PDB ID: 2B9S) with vanadate ligand was downloaded from the PDB for the present docking study. Initially, the protein was considered without ligand, DNA and water molecules for the purpose of docking studies. It is also important to note that such a strategy has already been applied by another group for Ld-topoI (Chhabra *et al.*, 2007). The protein (PDB ID: 2B9S) was minimized up to a gradient of 0.01 kcal·(mol Å)$^{-1}$, and hydrogens were added using the CHARMm force field available in the software Discovery studio 2.0. The energy-minimized structure was used for further docking analysis. In the GOLD docking software/programme, the default parameters: population size (100); selection-pressure (1.1); number of operations (10 000); number of islands (1); niche size (2); and operator weights for migrate (0), mutate (100) and crossover (100) were applied. The active site was defined within 10 Å and the ligand binding sites were analysed. The docked poses were scored using scoring functions: Goldscore (GS) and ChemScore (CS) to find the required docking pose. The inhibitor, Compound 1, was built using the ISIS draw and exported to Discovery Studio 2.0 software where CHARMm force field was applied and energy minimization was performed using the steepest descent algorithm with a convergence gradient value of 0.001 kcal·mol^{-1}.

Statistical analysis

The data are presented as mean ± SD.

Enzyme and chemicals

Recombinant type I DNA topoisomerase was prepared as described previously (Das *et al.*, 2004). Camptothecin, obtained from Sigma (St. Louis, MO, USA), was dissolved in DMSO at 20 mM concentrations and kept frozen at –20°C. RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, DMEM, penicillin and streptomycin were all bought from Sigma, and the tissue culture plates were from CellStar.

Results

Spectral data

$[\alpha]_D$: -68.92^0 (MeOH, c 3.04), IR ν_{max}(neat)cm^{-1}: 3379, 2935, 1752, 1647, 1457, 1382, 1131, 953, 757, ESIMS: m/z(rel. int.) 318[M^+] (13), ^{1}H NMR($CDCl_3$, 300 MHz): δ 1.52(2H, m, H-1), 2.04(2H, m, H-2), 5.18(1H, brs, H-3), 1.75(1H, m, H-6a), 1.18(1H, m, H-6b), 1.44(2H, m, H-7), 1.45(1H, m, H-8), 1.34(1H, m, H-10), 1.70(1H, m, H-11a), 1.52(1H, m, H-11b), 2.26(2H, m, H-12), 5.85(1H, s, H-14), 6.07(1H, s, H-16), 0.81(3H, d, J = 6.4 Hz, H-17), 1.58(3H, s, H-18), 1.00(3H, s, H-19), 0.77(3H, s, H-20), ^{13}C NMR($CDCl_3$, 75 MHz): δ 18.4(C-1), 26.9(C-2), 120.5(C-3), 144.5(C-4), 38.3(C-5), 36.9(C-6), 27.5(C-7), 36.5(C-8), 38.8(C-9), 46.6(C-10), 35.0(C-11), 21.5(C-12), 170.4(C-13), 117.6(C-14), 172.0(C-15), 101.8(C-16), 16.1(C-17), 18.1(C-18), 20.0(C-19), 18.3(C-20).

Table 1 Antileishmanial activity of Compound 1 in *Leishmania donovani* infected hamsters

Treated groups	*Dose, mg·kg⁻¹ × 5 p.o.*	*Total no. of animals*	*(% inhibition)*		
			Bone marrow	*Liver*	*Spleen*
Compound 1	250	6	89.07 ± 2.1	87.5 ± 1.8	91.0 ± 2.0
	100	6	84.23 ± 2.2	83.7 ± 1.45	86.88 ± 1.27
	50	6	85.5 ± 1.55	77.9 ± 3.00	80.2 ± 2.30
	25	6	40.75 ± 3.2	35.5 ± 2.4	43.9 ± 1.45
Miltefosine	40	6	94.7 ± 3.8	93.2 ± 2.8	95.5 ± 1.22

Inhibitory effect of 1 on L. donovani *promastigotes*
The concentration of Compound 1 at which nearly 50% death of *L. donovani* promastigotes would occur was calculated using log phase transgenic GFP-expressing promastigotes by flow cytometry. The % cell death, as measured by decrease in mean fluorescence intensity values on treatment with drug, was initially negligible at a concentration of 10 μg·mL^{-1}. However, a very rapid and dose-dependent death occurred with Compound 1 at concentrations between 2 and 50 μg·mL^{-1}. The IC_{50} was calculated to be 8.04 ± 0.40 μg·mL^{-1}. The reference drug, miltefosine, exhibited nearly 50% inhibition of parasite multiplication at a concentration of 10 μg·mL^{-1}.

Inhibitory effect of Compound 1 on intra-macrophage amastigotes
The leishmanicidal effect of Compound 1 was assessed on the intracellular transgenic amastigote forms of *L. donovani* expressing GFP by flow cytometry. The IC_{50} was found to be 5.79 ± 0.31 μg·mL^{-1}. Miltefosine, which was used as a reference drug, has an IC_{50} of 5 μg·mL^{-1}.

Antileishmanial activity of Compound 1 in L. donovani *infected hamsters*
The *in vivo* efficacy of Compound 1 administered by the oral route at four dose schedules (25, 50, 100 and 250 mg·kg^{-1} body weight for 5 days) was assessed against established *L. donovani* infection in hamsters. At 250 mg·kg^{-1} a higher order of efficacy of the compound was observed in spleen (91 ± 2%) liver (87.5 ± 1.8%) and bone marrow (89.1 ± 2.1%). At 100 mg·kg^{-1} it exhibited 86.88 ± 1.3%, 83.7 ± 1.5% and 84.2 ± 2.2% inhibition of parasites in spleen, liver and bone marrow respectively. A lower 50 mg oral treatment regimen of Compound 1 reduced parasitic burden by 85.5 ± 1.6% in spleen and 77.9 ± 3% and 80.2 ± 2.3% in liver and bone marrow respectively. No activity was observed at 25 mg·kg^{-1} × 5 days. The reference drug miltefosine resulted in 95.5 ± 1.2% inhibition of *Leishmania* parasite at a dose of 40 mg·kg^{-1} × 5 days. (Table 1) It was inferred from survival studies conducted with each dose schedule that Compound 1 is completely safe, as all the animals in all groups survived beyond 6 months of treatment as compared with untreated infected control where none survived beyond 2 months post-infection.

Cytotoxicity of Compound 1
Treatment of J774A.1 macrophages with Compound 1 was carried out at various concentrations to assess the safety of this pure compound for mammalian cells. After 48 h the viability of macrophages was checked by MTT assay. The compound was found to be devoid of any cytotoxic effect to macrophages even at the concentration of 200 μg·mL^{-1}, which was many times higher than the IC_{50} of the compound (data not shown).

Compound 1 inhibits DNA topoisomerases I of L. donovani
While studying the *in vitro* effect of Compound 1 on *L. donovani* topoisomerase I, we found that, when added together with the DNA and enzyme, the compound inhibited relaxation activity (Figure 2, lanes 15–20). Inhibition of enzyme activity is more significant when the enzyme is pre-incubated with the compound for 5 min at 37°C in the relaxation assay mixture before addition of the DNA substrate at same concentration. Figure 2 (lanes 5–10) shows the inhibition of catalytic activity by Compound 1 in the above reaction condition. Densitometric analysis of the agarose gel shows that the compound exerts 82% inhibition only at 5 μM concentration (lane 5). The inhibition by the compound occurs in a highly dose-dependent manner at increasing concentrations of 10 (87.6%), 20 (87.9%), 50 (92%), 100 (94%) and 200 (99.5%) μM. Lane 12 shows the relaxation of super coiled pBS by 1 unit of purified topoisomerase I of *L. donovani* under simultaneous assay conditions. It was found that at this condition, Compound 1 at 5 μM inhibited only 20% relaxation (lane 15).

Molecular docking and binding studies
The binding interactions of 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1) within the *L. donovani* DNA-topoisomerase (Ld-topoI) binding sites are illustrated in Figure 3. The most stable ligand–receptor complex with Compound 1 shows that the butenolide ring of clerodane is present at the active site Tyr222, Arg314, Lys352 and Arg410 amino acid residues, which are present in the conserved core domain of the leishmanial enzyme, which characterize the active site. The following interactions of Compound 1 with the active site were observed:

1. The keto oxygen at C-15 of the butenolide ring participates in one hydrogen bonding interaction with the NH of Arg314 (distance = 3.09 Å).
2. The oxygen atom of butenolide ring participates in one hydrogen bonding interaction with the NH of Arg314 (distance = 3.2 Å), which is considered essential for inhibitory activity.

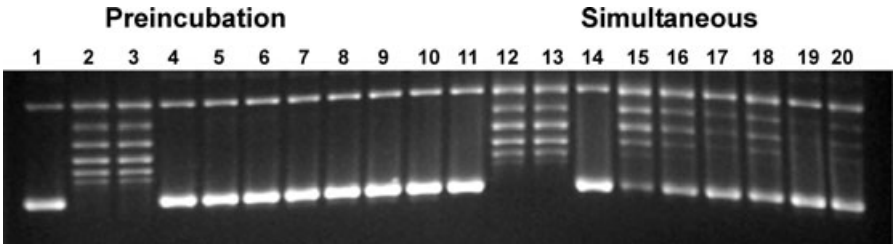

Figure 2 Inhibition of catalytic activity of *Leishmania donovani* DNA topoisomerase I. Lanes 1–10: pre-incubation of enzyme and Compound 1; lanes 11–20, simultaneous addition of topoisomerase I, Compound 1 and DNA. Lane 1, supercoiled pBS DNA; lane 2, DNA with 1 U of purified *L. donovani* topoisomerase I, lane 3: DNA with 1 U of purified *L. donovani* topoisomerase I and DMSO; lane 4, inhibition of catalytic activity with camptothecin (50 mM); lanes 5–10, inhibition of catalytic activity with increasing concentration of 1 (5, 10, 20, 50, 100 and 200 μM respectively); lane 11, DNA control; lane 12, DNA with 1 U of purified *L. donovani* topoisomerase I; lane 13, DNA with 1 U of purified *L. donovani* topoisomerase I and DMSO; lane 14, inhibition of catalytic activity with camptothecin (50 mM); lanes 15–20: inhibition of catalytic activity with increasing concentrations of Compound 1 (5, 10, 20, 50, 100 and 200 μM respectively).

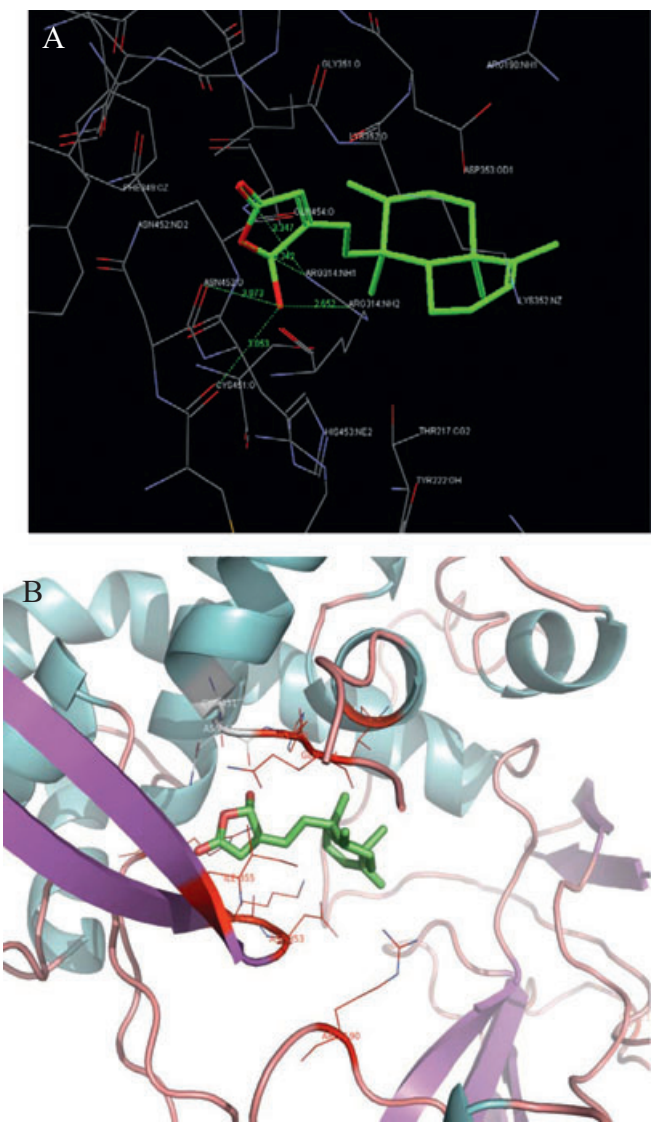

Figure 3 (A) Docking of 16α-hydroxycleroda-3,13 (14)Z-dien-15,16-olide (Compound 1) into the active site of *Leishmania donovani* DNA-topoisomerase (Ld-topoI) (green dotted line represents hydrogen bonding). (B) Superimposition of K9 in to the active site display as Ribbon form.

3. The 16α-hydroxyl group of the butenolide ring participates in three hydrogen bonding interactions with the NH group of Arg314 (distance = 2.61 Å), oxygen of Asn452 (distance = 3.11 Å) and oxygen of Cys451 (distance = 3.12 Å) amino acid residues.
4. The trans-decalin ring of clerodane skeleton is oriented in the hydrophobic pocket consisting of Asp 353, Lys352 and Gln454 amino acid residues. The 18-methyl of the octahydronaphthalene ring undergoes hydrophobic interaction with Gln454 (distance = 2.59 Å).
5. The methylene group at C-12 undergoes hydrophobic interaction with Ile 355(distance = 3.198 Å) amino acid residue.

The finding that Compound 1 undergoes five hydrogen bonding interactions, hydrophobic interactions and its binding energy (–88 kcal·mol^{-1}) suggest that this compound will have high antileishmanial activity.

Externalization of phosphatidylserine on treatment with Compound 1

During apoptosis in metazoan and unicellular cells, phosphatidylserine is translocated from the inner side to the outer layer of the plasma membrane (Mehta and Shaha, 2004). Annexin V, a Ca^{2+}-dependent phospholipid-binding protein having an affinity for phosphatidylserine, is routinely used to label externalization of phosphatidylserine. Because Annexin V can also label necrotic cells, PI stain was used to differentiate the apoptotic cells (Annexin V-positive, PI-negative), necrosis cells (both Annexin V- and PI-positive) and normal cells (both Annexin V- and PI-negative). Promastigotes treated with Compound 1 (8 μg·mL^{-1} for 8 h) were double-stained with FITC-conjugated Annexin V and PI, and analysed by flow cytometry. In untreated control promastigotes only 5.1% stained positive for Annexin V (Figure 4A, lower right quadrant) whereas a significant percentage of cells (30%) stained positive after 8 h of treatment with Compound 1 (Figure 4B, lower right quadrant).

Discussion and conclusions

The present treatment regimens for VL have severe limitations including cost and safety; hence new drugs are urgently

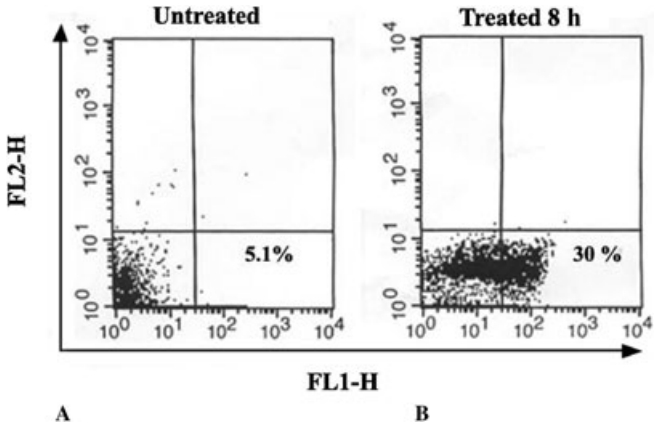

Figure 4 Detection of apoptosis in promastigotes by Annexin V and propidium iodide double staining in *Leishmania donovani* promastigotes. (A) Untreated and (B) treated with Compound 1.

required. In this regard, natural products have made and are continuing to make important contributions to this area of therapeutics. As part of a systematic search programme for antileishmanial agents of natural products-origin, activity-guided fractionation of the crude ethanolic extract of the leaves of *P. longifolia* var. *pendula* (Annonaceae) led to the isolation of the Compound 1, which exhibited considerably high antileishmanial activity *in vitro* and *in vivo* without any cytotoxicity.

To improve the drug therapy of leishmanial infections and for new drug development, there is a need to identify the molecular targets of antileishmanials that have the potential to be safe and effective treatments. Clerodane diterpenes are established human topoisomerase inhibitors (Jamora *et al*., 2001) and it has been suggested that these compounds bind to the enzyme prior to the formation of cleavable complexes with DNA. *Leishmania* DNA topoisomerases have recently emerged as principal therapeutic targets, and a number of agents that target these topoisomerases having a broad spectrum of antiprotozoan activity. This along with the fact that Compound 1 is also a clerodane diterpene prompted us to evaluate whether this compound has the potential to inhibit the catalytic activity of recombinant DNA topoisomerase I of the *Leishmania* parasite. A computational approach was utilized to study the relative binding modes of Compound 1 with the protein crystal structure of *L. donovani's* topoisomerase I. Since, Compound 1 has been found to have excellent *in vitro* and *in vivo* activity with no evident cytotoxicity, molecular modelling studies of Compound 1 with *Leishmania* topoisomerase will provide an understanding of the structural basis of ligand binding to the topoisomerase receptor, which may be used for the structure-based design of potent and novel ligands for antileishmanial therapy. The molecular dynamic studies showed that Compound 1 forms a stable complex with Ld-topoI enzyme (binding energy, –88 kcal·mol^{-1}) with five hydrogen bonding interactions and hydrophobic interactions. While studying the *in vitro* effect of Compound 1 on *L. donovani* topoisomerase I, by determining its relaxation of recombinant DNA topoisomerase I of *L. donovani*, we found that the compound, when added together with DNA and enzyme, marginally inhibited relaxation. Significant inhibition of enzyme activity was observed when the enzyme was pre-incubated with the compound for 5 min at 37°C in the relaxation assay mixture before addition of the DNA substrate. These results along with those from the docking studies show that Compound 1 inhibits DNA topoisomerase by directly interacting with the enzyme; inhibition of the enzyme was only observed in the pre-incubation reactions. This confines Compound 1 to a rare class of topoisomerase inhibitors that does not interact with substrate DNA and, therefore, is unable to stabilize the ternary cleavable complex. Instead it prevents the interaction between DNA and enzyme by interacting with the active site of the enzyme (Chowdhury *et al*., 2003). Earlier studies have suggested a direct correlation between DNA topoisomerase inhibition and apoptosis (Chowdhury *et al*., 2003). This led us to evaluate the mode of cell death induced by Compound 1 in *Leishmania* promastigotes; by use of Annexin V staining to determine the number of apoptotic cells after treatment with Compound 1, it was confirmed to be apoptosis.

The observation that Compound 1 has potent *in vitro* activity and an excellent therapeutic effect along with no cytotoxicity in macrophages, as well as the survival of animals of all of the groups after treatment for more than 6 months, indicates that it has the potential to be a potent antileishmanial drug. Our studies have also shown it to be a topoisomerase inhibitor that induces apoptosis, a safe mode of cell death. Taking all these merits into account, we aimed not only to explore the molecular mechanism of Compound 1 but also to explore and exploit its correct binding mode with *L. donovani* topoI using docking programmes for structure-based designing of new antileishmanials (Pommier *et al*., 2003). The results suggest that five hydrogen bonding interactions and hydrophobic interactions of Compound 1 with the active site of Ld-topoI are responsible for its potent antileishmanial activity. These results can be exploited for structure-based computer-aided drug designing of similar new and selective leishmanial-topoisomerase inhibitors, analogues of Compound 1, to optimize their antileishmanial efficacy.

Acknowledgements

We are thankful to Mr AL Vishwakarma, Technical Officer, SAIF division for flow cytometry analysis and spectral data. Financial support was provided by the Department of Biotechnology, New Delhi, and Council of Scientific Research for fellowships of authors. This is CDRI communication No. 7384.

Conflict of interest

The authors declare no conflict of interest.

References

Berman JD, Chulay JD, Hendricks LD, Oster CN (1982). Susceptibility of clinically sensitive and resistant Leishmania to pentavalent antimony *in vitro*. *Am J Trop Med Hyg* **31**: 459–465.

Chang FR, Hwang TL, Yang YL, Li CE, Wu CC, Issa HH *et al*. (2006). Anti-inflammatory and cytotoxic diterpenes from formosan *Polyalthia longifolia* var. pendula. *Planta Med* **72**: 1344–1347.

Chen CY, Chang FR, Shih YC, Hsieh TJ, Chia YC, Tseng HY *et al*. (2000). Cytotoxic constituents of *Polyalthia longifolia* var. pendula. *J Nat Prod* **63**: 1475–1478.

Chhabra S, Sharma P, Ghoshal N (2007). A computational docking study for prediction of binding mode of diospyrin and derivatives: inhibitors of human and leishmanial DNA topoisomerase-I. *Bioorg Med Chem Lett* **17**: 4604–4612.

Chowdhury AR, Mandal S, Goswami A, Ghosh M, Mandal L, Chakraborty D *et al*. (2003). Dihydrobetulinic acid induces apoptosis in *Leishmania donovani* by targeting DNA topoisomerase I and II: implications in antileishmanial therapy. *Mol Med* **9**: 26–36.

Croft SL (2001). Monitoring drug resistance in leishmaniasis. *Trop Med Int Health* **6**: 899–905.

Croft SL, Yardley V (2002). Chemotherapy of leishmaniasis. *Curr Pharm Des* **8**: 319–342.

Das BB, Sen N, Ganguly A, Majumder HK (2004). Reconstitution and functional characterization of the unusual bi-subunit type I DNA topoisomerase from *Leishmania donovani*. *FEBS Lett* **565**: 81–88.

Desjeux P, Piot B, O'Neill K, Meert JP (2001). Co-infections of leishmania/HIV in south Europe. *Med Trop (Mars)* **61**: 187–193.

Hara N, Asaki H, Fujimoto Y, Gupta YK, Singh AK, Sahai M (1995). Clerodane and ent-halimane diterpenes from *Polyalthia longifolia*. *Phytochemistry* **38**: 189–194.

Ichino C, Soonthornchareonnon N, Chuakul W, Kiyohara H, Ishiyama A, Sekiguchi H *et al*. (2006). Screening of Thai medicinal plant extracts and their active constituents for *in vitro* antimalarial activity. *Phytother Res* **20**: 307–309.

Iwu MM, Jackson JE, Schuster BG (1994). Medicinal plants in the fight against leishmaniasis. *Parasitol Today* **10**: 65–68.

Jamora C, Theodoraki MA, Malhotra V, Theodorakis EA (2001). Investigation of the biological mode of action of clerocidin using whole cell assays. *Bioorg Med Chem* **9**: 1365–1370.

Jones G, Willett P, Glen RC, Leach AR, Taylor R (1997). Development and validation of a genetic algorithm for flexible docking. *J Mol Biol* **267**: 727–748.

Lala S, Pramanick S, Mukhopadhyay S, Bandyopadhyay S, Basu MK (2004). Harmine: evaluation of its antileishmanial properties in various vesicular delivery systems. *J Drug Target* **12**: 165–175.

Marthanda Murthy M, Subramanyam M, Hima Bindu M, Annapurna J (2005). Antimicrobial activity of clerodane diterpenoids from *Polyalthia longifolia* seeds. *Fitoterapia* **76**: 336–339.

Mehta A, Shaha C (2004). Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. *J Biol Chem* **279**: 11798–11813.

Murray HW (2000). Treatment of visceral leishmaniasis (kala-azar): a decade of progress and future approaches. *Int J Infect Dis* **4**: 158–177.

Paris C, Loiseau PM, Bories C, Breard J (2004). Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* **48**: 852–859.

Pommier Y, Redon C, Rao VA, Seiler JA, Sordet O, Takemura H *et al*. (2003). Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat Res* **532**: 173–203.

Singh N, Kumar A, Gupta P, Chand K, Samant M, Maurya R *et al*. (2008). Evaluation of antileishmanial potential of Tinospora sinensis against experimental visceral leishmaniasis. *Parasitol Res* **102**: 561–565.

Singh N, Gupta R, Jaiswal AK, Sundar S, Dube A (2009). Transgenic *Leishmania donovani* clinical isolates expressing green fluorescent protein constitutively for rapid and reliable ex vivo drug screening. *J Antimicrob Chemother* **64**: 370–374.

Sundar S (2001). Drug resistance in Indian visceral leishmaniasis. *Trop Med Int Health* **6**: 849–854.

Zhao GX, Jung JH, Smith DL, Wood KV, McLaughlin JL (1991). Cytotoxic clerodane diterpenes from *Polyalthia longifolia*. *Planta Med* **57**: 380–383.