

## ORIGINAL ARTICLE

# Isobenzofuranone derivatives exhibit antileishmanial effect by inhibiting type II DNA topoisomerase and inducing host response

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## Keywords

Antileishmanial agents, apoptosis, DNA topoisomerase II, isobenzofuranone, *Leishmania donovani*

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## Abstract

*Leishmania*, a protozoan parasite, causes a wide range of human diseases ranging from the localized self-healing cutaneous lesions to fatal visceral leishmaniasis. Toxicity of traditional first line drugs and emergence of drug-resistant strains have worsened the situation. DNA topoisomerase II in kinetoplastid protozoan parasites are of immense interest as drug target because they take part in replication of unusual kinetoplast DNA network. In this study, we have taken target-based therapeutic approaches to combat leishmaniasis. Two isobenzofuranone compounds, viz., (1) 3,5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH3) and (2) (4-bromo)-3'-hydroxy-5'-(4-bromophenyl)-benzophenone (JVPH4) were synthesized chemically and characterized by NMR and mass spectrometry analysis. Activity of type II DNA topoisomerase of leishmania (LdTOPII) was monitored by decatenation assay and plasmid cleavage assay. The antiparasitic activity of these compounds was checked in experimental BALB/c mice model of visceral leishmaniasis. Isobenzofuranone derivatives exhibited potent antileishmanial effect on both antimony (Sb) sensitive and resistant parasites. Treatment with isobenzofuranone derivatives on promastigotes caused induction of reactive oxygen species (ROS)-mediated apoptosis like cell death in leishmania. Both the compounds inhibited the decatenation activity of LdTOPII but have no effect on bi-subunit topoisomerase IB. Treatment of LdTOPII with isobenzofuranone derivatives did not stabilize cleavage complex formation both in vitro and in vivo. Moreover, treatment with isobenzofuranone derivatives on *Leishmania donovani*-infected mice resulted in clearance of parasites in liver and spleen by induction of Th1 cytokines. Taken together, our data suggest that these compounds can be exploited as potential antileishmanial agents targeted to DNA topoisomerase II of the parasite.

## Abbreviations

ATP, adenosine triphosphate; DHBA, dihydrobetulinic acid; BSA, bovine serum albumin; EtBr, ethidium bromide; FBS, fetal bovine serum; H<sub>2</sub>DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; hTOPII $\alpha$ , human topoisomerase II $\alpha$ ; kDNA, kinetoplast DNA; LDU, Leishman Donovan Units; M199, Medium199; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; Ni-NTA, Ni-nitrilo triacetic acid; NO, nitric oxide; PCD, programmed cell death; ROS, reactive oxygen species; SAG, sodium antimony gluconate; SLA, soluble leishmanial antigen; TGF- $\beta$ , transforming growth factor- $\beta$ .

## Introduction

Leishmaniasis is one of the major fatal parasitic diseases with significant morbidity and mortality worldwide and is caused by protozoan parasites belonging to the genus *Leishmania* (Herwaldt 1999). Depending on the species variation, the disease can have a wide range of clinical manifestation and categorized into three different types, namely, cutaneous, mucocutaneous, and visceral leishmaniasis. Cutaneous leishmaniasis is the most common form and visceral leishmaniasis is the most severe form (Coler and Reed 2005). Kala-azar or visceral leishmaniasis caused by *Leishmania donovani* is a major problem in the developing countries (Topno et al. 2010). Most of the drugs used for the therapy of this disease are antimonial preparations such as sodium stibogluconate (pentostam) and meglumine antimonate (glucantime) and are known as first-line drugs. The second-line drugs like amphotericin B, miltefosine, and paromomycin although clinically used are all severely toxic and as a consequence lead to patient noncompliance and emergence of drug-resistant strains (Ashutosh and Goyal 2007; Chappuis et al. 2007). Presently, there is no available vaccine against leishmaniasis and therefore chemotherapy remains the major medical mode for managing the diseases (Kedzierski 2010).

DNA topoisomerases are long known as targets for antibacterial and anticancer therapy (Pommier et al. 2010). The enzymes participate in all kind of DNA metabolic processes. Based on the number of strands they cleave, these enzymes are classified as type I or type II DNA topoisomerase (Champoux 2001). DNA topoisomerase II in kinetoplastid protozoan parasites are of immense interest because they take part in replication of unusual kinetoplast DNA network (kDNA) inside mitochondria (Shapiro 1994; Das et al. 2008). Type II DNA topoisomerases have emerged as principal therapeutic targets, with a group of targeting agents having a broad spectrum of antiparasitic activity (Bakshi and Shapiro 2003). DNA topoisomerase II of leishmania consists of an N-terminal ATPase, a central DNA-binding, and an unconserved C-terminal domain (Sengupta et al. 2003, 2005a,b). The inhibitors of type II topoisomerase can be divided into two classes: topoisomerase II poisons and catalytic inhibitors or class II inhibitors (Nitiss 2009). Poisons stabilize the covalent enzyme–DNA complex and block rejoining of the DNA break. These include bacterial DNA gyrase poisons such as quinolone antibiotics and eukaryotic topoisomerase II poisons doxorubicin, amsacrine, etoposide, and teniposide (Pommier et al. 2010). Class II inhibitors interfere with DNA topoisomerase II during different stages of the catalytic cycle without trapping the covalent enzyme–DNA complexes (Larsen et al.

2003). All class II drugs mediate their action by either binding to the enzyme, which prevents them to sit on the substrate DNA, (e.g., merbarone and acetyl boswellic acids) (Fortune and Osheroff 1998; Syrovets et al. 2000) or by interacting with the ATPase domain and thus interfering with the ATPase activity of the enzyme that produces “closed clamps”, for example, bisdioxopiperazines ICRF-187 and ICRF-193 (Jensen et al. 2000). This class II inhibitors are ‘catalytic inhibitors’ and they exert their antiproliferative effects by depleting the essential enzymatic function (Larsen et al. 2003; Nitiss 2009).

*Leishmania donovani* infection causes strong immunosuppressive effect in the host (Awasthi et al. 2004) by downregulating the production of reactive oxygen species (ROS) and nitric oxide (NO) within the host macrophages (Lima-Junior et al. 2013). *Leishmania* infection impairs the production of host protective (Th1) cytokines, for example, interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-12, and interleukin-2 and induces the production of the disease-promoting (Th2) cytokines, for example, transforming growth factor  $\beta$  (TGF- $\beta$ ), interleukin-10, and interleukin-4 (Kemp et al. 1993). Consequently, a potential therapy for leishmaniasis would not only be limited to the antileishmanial property of the molecule but also be to modulate immune responses mediated by the parasite-infected immune cells (Walker et al. 1999).

Phthalides or 1(3H)-Isobenzofuranone are a prominent class of natural products that possess significant biological properties (Lin et al. 2005). Phthalides also serve as valuable synthetic intermediates. An aryl-substituted isobenzofuranone-1(3H)-one was found to be the inhibitors of the lymphocyte pore-forming protein perforin (Spicer et al. 2012). Antiplatelet activity of 3-butyl-6-bromo-1(3H)-isobenzofuranone was reported both in vitro and ex vivo in rat platelets aggregation (Ma et al. 2012). Several compounds in the group of 1(3H)-Isobenzofuranone exhibited anticancer activity (Ye et al. 1998; Karna et al. 2010). In a recent communication, it was reported that different derivatives of 2,4-dihydroxybenzophenone exhibited leishmanicidal activity against *Leishmania amazonensis* (MacielRezende et al. 2013).

In this study, we have shown that the chemically synthesized 3,5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH3) and (4-bromo)-3'-hydroxy-5'-(4-bromophenyl)-benzophenone (JVPH4) exhibit potent antileishmanial property. Both the compounds are cytotoxic to antimony-sensitive and also to antimony-resistant parasites. JVPH3 and JVPH4 inhibit LdTOPII activity by interfering the initial topoisomerase II–DNA binary complex formation. Isobenzofuranone derivatives mediated inhibition of topoisomerase II hampers the basic metabolic process in cell and ultimately lead to apoptosis like cell death of the parasite. Moreover, these compounds

modulate host immune response by increasing the production of Th1 cytokines in experimental mouse model and reduced the parasite burden in liver and spleen of the infected animal. Our data demonstrate for the first time the novel antileishmanial property of isobenzofuranone derivatives that exert their action via targeting topoisomerase II in leishmania.

## Materials and Methods

### Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen Life Technologies. dimethyl sulfoxide (DMSO), pyruvate kinase, lactate dehydrogenase, camptothecin, and etoposide were purchased from Sigma chemicals (St. Louis, MO). Recombinant human topoisomerase II $\alpha$  (hTOPII $\alpha$ ) was purchased from Topogen Inc. (Port Orange, FL). Recombinant LdTOPIB was prepared as described previously (Das et al. 2006). All drugs were dissolved in 100% DMSO at a concentration of 20 mmol/L and stored at  $-20^{\circ}\text{C}$ .

### Synthesis of 3,5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH-3) and (4-bromo)-3'-hydroxy-5'-(4-bromophenyl)-benzophenone (JVPH-4)

To a 50 mL flask fitted with magnetic stirrer were added activated  $\text{I}_2$  (45 mg, 20 mol%),  $\text{K}_2\text{CO}_3$  (276 mg, 2 mmol/L), (E)-1,4-bis(4-chlorophenyl)but-2-ene-1,4-dione (234 mg, 1 mmol/L) or (E)-1,4-bis(4-bromophenyl)but-2-ene-1,4-dione (394 mg, 1 mmol/L) and methyl acetoacetate (128 mg, 1.1 mmol/L) in dry i-PrOH (10 mL). The reaction mixture was then stirred under reflux at  $110^{\circ}\text{C}$  for 0.5 h. After disappearance of the starting material (monitored by thin layer chromatography) the reaction mixture was allowed to cool at room temperature. Solvent was removed from the reaction mixture under reduced pressure in vacuum. The residue was then diluted with water (10 mL) followed by extraction with  $\text{CHCl}_3$  ( $3 \times 25$  mL). The organic layer was collected, washed with brine, and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Removal of solvent resulted in a solid mass which was subjected to column chromatography over neutral alumina using petroleum ether and increasing proportion of chloroform as eluent. Petroleum ether: chloroform (70:30) eluent gave a solid which was recrystallized from chloroform–petroleum ether as a white solid to afford 3,5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH-3) (235 mg, 60%), whereas elution of the corresponding reaction mixture (E)-1,4-bis(4-bromophenyl)but-2-ene-1,4-dione with petroleum ether: chloroform

(50:50) afforded a solid which was recrystallized to give a white solid (4-bromo)-3'-hydroxy-5'-(4-bromophenyl)-benzophenone (JVPH-4) (150 mg, 35%).

### Parasite maintenance and cultures

*Leishmania donovani*, sodium antimony gluconate (SAG)-sensitive ( $\text{Sb}^{\text{S}}$ ) MHOM/IN/1983/AG83 (AG83) and the one SAG-resistant ( $\text{Sb}^{\text{R}}$ ) strain, GE1 were used. Amastigotes obtained from the spleens of infected hamsters were cultured at  $22^{\circ}\text{C}$  in Medium199 (M199) media to obtain promastigotes. Promastigotes were further grown in 10% (v/v) heat-inactivated fetal bovine serum (FBS) as described (Saha et al. 2013).

### Measurement of cell viability

*Leishmania donovani* AG83 promastigotes and laboratory developed SAG-resistant ( $\text{Sb}^{\text{R}}$ ) GE1 parasites ( $3.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) were incubated with different concentrations of JVPH3 and JVPH4 for 12 h, following which the survival percentage was estimated by MTT assay. Parasites treated with 0.5% DMSO served as controls. MTT is reduced to purple formazan in the mitochondria of living cells. Formazan is then solubilized, and the concentration determined by optical density at 570 nm. Percentage of viable promastigotes in each treatment groups was determined with respect to untreated control cells (Roy et al. 2008).

### Measurement of ROS

Promastigotes ( $2.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) were treated with JVPH3 or JVPH4 for indicated time periods. Parasites treated with 0.5% DMSO served as controls. After different treatments, parasites were washed and loaded with a cell permeant dye 2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ ) for 1 h. Fluorometric measurements ( $\lambda_{\text{ex}} = 510$  nm and  $\lambda_{\text{em}} = 530$  nm) were performed in triplicate, and the results were expressed as in the mean fluorescence intensity per  $10^6$  cells (Roy et al. 2008).

### Measurement of reduced glutathione level

*Leishmania donovani* promastigotes ( $2.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) were treated with JVPH3 or JVPH4 at different times. Parasites treated with 0.5% DMSO served as controls. The cells were then lysed by cell lysis buffer according to the manufacturer's protocol (Apo Alert glutathione assay kit; Clontech, Mountain View, CA). Cell lysates were incubated with monochlorobimane for 3 h and glutathione levels were detected by fluorometer ( $\lambda_{\text{ex}} = 395$  nm and  $\lambda_{\text{em}} = 480$  nm) (Sen et al. 2004).

Spectrofluorometric data presented here are representative of three experiments.

### Measurement of total fluorescent lipid peroxidation product

Promastigotes ( $2.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) were treated with JVPH3 or JVPH4 for indicated time periods. Parasites treated with 0.5% DMSO served as controls. After different treatments, parasites were washed twice with 1X PBS and the pellet was resuspended in 2 mL of 15% SDS-PBS solution. The fluorescence intensities of the total fluorescent lipid peroxidation products were measured with excitation at 360 nm and emission at 430 nm and expressed as relative fluorescence units with respect to quinine sulfate (Sen et al. 2004). Spectrofluorometric data presented here are representative of three experiments.

### Double staining with annexinV and PI

Externalization of phosphatidyl serine on the outer membrane of untreated and JVPH3- and JVPH4-treated promastigotes was measured by the binding of FITC-annexinV and PI using an annexinV staining kit (Invitrogen BioServices India Pvt. Ltd, Bangalore, India). 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 (Chowdhury et al. 2012).

### DNA fragmentation assay

The extent of DNA fragmentation after drug treatments was estimated using the cell death detection ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN). Promastigote samples ( $5.0 \times 10^6$  cells  $\text{mL}^{-1}$ ) were collected 0, 2, 4, and 6 h post treatment with these compounds and the histone-associated DNA fragments (mononucleosome and oligonucleosome) were detected using the manufacturer's protocol (Chowdhury et al. 2012).

### Purification of recombinant LdTOPII

*Escherichia coli* BL21 (DE3) pLysS cells harboring pET16bLdTOPII, (Sengupta et al. 2005b) were induced at 0.6 OD<sub>600</sub> with 0.5 mmol/L isopropyl  $\beta$ -D-1-thiogalactopyranoside at 22°C for 4 h. The cells were harvested and resuspended in phosphate buffer (pH 7.8) containing 300 mmol/L NaCl, 200  $\mu\text{g mL}^{-1}$  lysozyme, 0.1% Triton X-100, 0.25% Sarkosyl and 1 mmol/L phenylmethylsulfonyl fluoride. The lysates obtained after sonication on ice was cleared by centrifugation. The cleared lysate was passed through Ni-nitrilo triacetic acid (Ni-NTA) agarose

column (Qiagen India Pvt. New Delhi, India). After washing with phosphate buffer (pH 7.8) containing 300 mmol/L NaCl and 30 mmol/L imidazole, elution was done using 250 mmol/L imidazole. For further purification, the fractions were pooled dialyzed and chromatographed on phosphocellulose column (P11 cellulose, Whatman) as described (Sengupta et al. 2005b).

### Decatenation assay

Decatenation assays were performed in total volumes of 25  $\mu\text{L}$  containing 25 mmol/L Tris-HCl pH 7.9, 10 mmol/L  $\text{MgCl}_2$ , 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L DTT, 50 mmol/L NaCl, 10% glycerol, 2 mmol/L adenosine triphosphate (ATP), 200 ng of kDNA from *L. donovani* strain UR6, and one unit of purified LdTOPII or hTOPII $\alpha$ . One unit of enzyme activity is defined as the amount of enzyme needed for 50% decatenation of 200 ng kDNA networks into minicircles. The assays were carried at 30°C for 30 min and the reaction products were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide (EtBr) (0.5  $\mu\text{g mL}^{-1}$ ), and photographed under UV illumination (Sengupta et al. 2003).

### Plasmid cleavage reaction

Cleavage reaction was performed as described previously (Sengupta et al. 2005b). A 20  $\mu\text{L}$  reaction mixture contained 200 ng negatively supercoiled pRYG DNA, 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L KCl, 0.1 mmol/L EDTA, 5 mmol/L  $\text{MgCl}_2$ , 0.5 mmol/L DTT, 2 mmol/L ATP, 30  $\mu\text{g mL}^{-1}$  bovine serum albumin (BSA), and 5 unit of enzymes. Reaction mixture was incubated at 37°C for 30 min and terminated by the addition of 0.5% SDS and 10 mmol/L EDTA. The mixture was further incubated with 100  $\mu\text{g mL}^{-1}$  proteinase K at 37°C for 30 min and analyzed by electrophoresis on a 1% agarose gel. EtBr at a final concentration of 0.5  $\mu\text{g/mL}$  was included in the gel to resolve the linear product (Form III) from the supercoiled molecule (Form I). Control assays always contained an amount of drug diluent (DMSO) equivalent to that present in drug containing reaction.

### DNA unwinding assay

Unwinding assay was performed with 50 fmol of supercoiled and relaxed pBluscript (SK+) DNA in the presence or absence of different concentrations of JVPH3 and JVPH4 in a 50  $\mu\text{L}$  reaction mixture as described (Pommier et al. 1987). Relaxed DNA was prepared by treatment of the supercoiled plasmid DNA with excess of topoisomerase I, followed by proteinase K digestion at 37°C, phenol/chloroform extraction, and ethanol

precipitation. After incubation at 37°C for 15 min, reactions were terminated by the addition of prewarmed stop solution (5% SDS, 15% Ficoll and 0.25% Bromophenol Blue) and electrophoresed on to 1% agarose gel. The DNA band was stained with 0.5  $\mu\text{g mL}^{-1}$  of EtBr and visualized by UV light.

### ATPase assay

ATPase measurements were carried out by the pyruvate kinase/lactate dehydrogenase assay described previously (Sengupta et al. 2005a). Reaction mixture contained 0.1 mmol/L NADH, 2 mmol/L phosphoenolpyruvate, 2 mmol/L ATP, three units of pyruvate kinase, and four units of lactate dehydrogenase. Reactions were initiated by mixing the reaction mixture and ATP, both preequilibrated to 30°C. Decrease in NADH concentration was monitored by measuring the absorbance at 340 nm in a UV-visible spectrophotometer.

### Electrophoretic mobility shift assay

The labeling of 36-mer oligonucleotide 1 (5'-ATGAAATCTAACAAATGCGCTCATCGT CATCCTCGGC3') containing high-affinity topoisomerase II binding site was done using polynucleotide kinase (Roche Biochemicals). The labeled oligonucleotide 1 was annealed to oligonucleotide 2 (3'-TACTTTAGATTGTTACGCGAGTAGCAGT AGGACCG5') in a buffer containing 40 mmol/L Tris-HCl (pH 7.5), 20 mmol/L  $\text{MgCl}_2$ , and 50 mmol/L NaCl at 70°C for 1 h and allowed to cool down slowly to room temperature. Briefly, the reaction was done in a 20  $\mu\text{L}$  binding buffer containing 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L DTT, 4 mmol/L  $\text{MgCl}_2$ , 50 mmol/L KCl, and 15  $\mu\text{g mL}^{-1}$  BSA and 5 pmol of 36 bp duplex oligonucleotide and LdTOPII enzyme and varying concentrations of JVP3 and JVP4. Reaction mixtures were incubated at 4°C for 30 min and electrophoresed through 7% nondenaturing polyacrylamide gel and autoradiographed (Sengupta et al. 2005b).

### Immunoband depletion assay

Promastigotes ( $2.0 \times 10^7$ ) were cultured for 12 h at 22°C with or without drugs. Nuclear fractions were isolated as previously described (Sen et al. 2004). The nuclear fractions after lysis with 1% SDS were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrophoretically transferred on to nitrocellulose membranes. Immunoblotting of immobilized proteins was carried out using a rabbit antibody raised against ATPase domain (43 kDa) of *L. donovani* topoisomerase II (Sengupta et al. 2005a).

## Bioethics

BALB/c mice, originally obtained from Jackson Laboratories, Bar Harbor, ME and reared in the Institute Animal facilities, were used for experimental purposes with prior approval of the Animal Ethics Committee. The studies and animal handling were approved by IICB Animal Ethical Committee (Registration no. 147/1999, CPCSEA), registered with Committee for the purpose of Control and Supervision on Experiments on Animals (CPCSEA), Govt. of India.

### Infection of mice and treatment regimen

For experimental visceral infections, female BALB/c mice (4–6 week old and 20–25 g each) were injected via intracardiac route with  $2 \times 10^7$  hamster spleen-transformed *L. donovani* promastigotes. Three weeks post infection, JVP3 and JVP4 were administered to infected animals via intraperitoneal routes at 5 and 10 mg  $\text{kg}^{-1}$  body weight separately twice a week for a period of 3 weeks (Chowdhury et al. 2012). Visceral infection was determined by Giemsa-stained impression smears of spleen and liver from 6-week infected mice and reported as Leishman Donovan Units (LDU), calculated as the number of parasites per 1000 nucleated cells  $\times$  organ weight (in mg) (Stauber 1956).

### Estimation of cytokine levels by enzyme-linked immunosorbent assay

Splenocytes were isolated from different groups of BALB/c mice by mechanical disruption of spleen and lysis of red blood corpuscles by 0.14 mol/L Tris-buffered  $\text{NH}_4\text{Cl}$ . Cells were plated in triplicate at  $5.0 \times 10^5$  cells  $\text{mL}^{-1}$  in 96-well plates (BD Biosciences, San Diego, CA) and allowed to proliferate for next 48 h at 37°C in 5%  $\text{CO}_2$  incubator in presence or absence of 25  $\mu\text{g mL}^{-1}$  soluble leishmanial antigen (SLA) (Mukherjee et al. 2013). Cytokines production by SLA pulsed splenocytes from different mice groups was determined by ELISA kit (BD Biosciences) as per manufacturer's instruction.

### Statistical analysis

Data are provided as means  $\pm$  SEM or means  $\pm$  SD, of the number of independent experiments. Data were tested for significance using the paired Student's *t*-test. Differences were considered statistically significant when  $P < 0.05$ . Statistical analysis and graphical representation were performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA).

## Results

### Synthesis and spectral analysis of isobenzofuranone derivatives

Two isobenzofuranone derivatives, namely, 3, 5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH-3) and (4-bromo)-3'-hydroxy-5'-(4-bromophenyl) benzophenone (JVPH-4) were synthesized chemically as described in "Materials and Methods" section. The chemical structure of JVPH3 and JVPH4 is presented in Figure 1A and B, respectively.

Spectral data of JVPH3 were given below.

IR  $\nu_{\max}$  (KBr,  $\text{cm}^{-1}$ ): 3210, 1728, 821  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 600 MHz):  $\delta$  7.79 (s, 1H), 7.55-7.57 (m, 4H), 7.40 (dd,  $J = 6.6, 1.8$  Hz, 2H), 7.20 (dd,  $J = 6.6, 1.8$  Hz, 2H), 7.15 (s, 1H), 6.40 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 150 MHz):  $\delta$  171.4, 156.4, 150.1, 149.9, 138.3, 134.8, 132.3, 132.2, 129.0 (2C), 128.8 (4C), 123.8, 123.4, 114.8, 112.8, 109.8, 83.1; EI MS  $m/z$ : 392.86  $[\text{M}+\text{Na}]^+$ ; Anal. Calcd. For  $\text{C}_{20}\text{H}_{12}\text{Cl}_2\text{O}_3$ : C, 64.71; H, 3.26; Found: C, 64.62; H, 3.22.

Spectral data of JVPH4 were given below

IR  $\nu_{\max}$  (KBr,  $\text{cm}^{-1}$ ): 3249, 1635, 822  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 600 MHz):  $\delta$  7.78 (d,  $J = 8.4$ , 2H), 7.49-7.43 (m, 6H), 7.40 (d,  $J = 8.4$ , 2H), 7.26 (s, 1H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 150 MHz):  $\delta$  195.1, 156.3, 141.9, 139.3, 139.2, 138.1, 135.5, 134.2, 132.1, 131.8, 131.5, 129.1 (2C), 128.8, 128.4 (2C), 121.1, 118.2, 115.5; EI MS  $m/z$ : 432.05  $[\text{M}]^+$ ; Anal. Calcd. For  $\text{C}_{19}\text{H}_{12}\text{Br}_2\text{O}_2$ : C, 52.81; H, 2.80; Found: 52.59, 2.72.

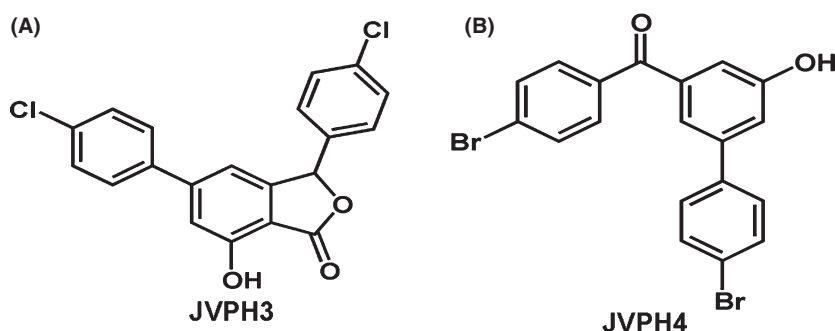
### Isobenzofuranone derivatives exhibited cytotoxic activity on *L. donovani* by induction of apoptosis like cell death

To determine the cytotoxic effect of isobenzofuranone derivatives (JVPH3 and JVPH4) on the growth of leishmania, the *L. donovani* AG83 promastigotes and on labo-

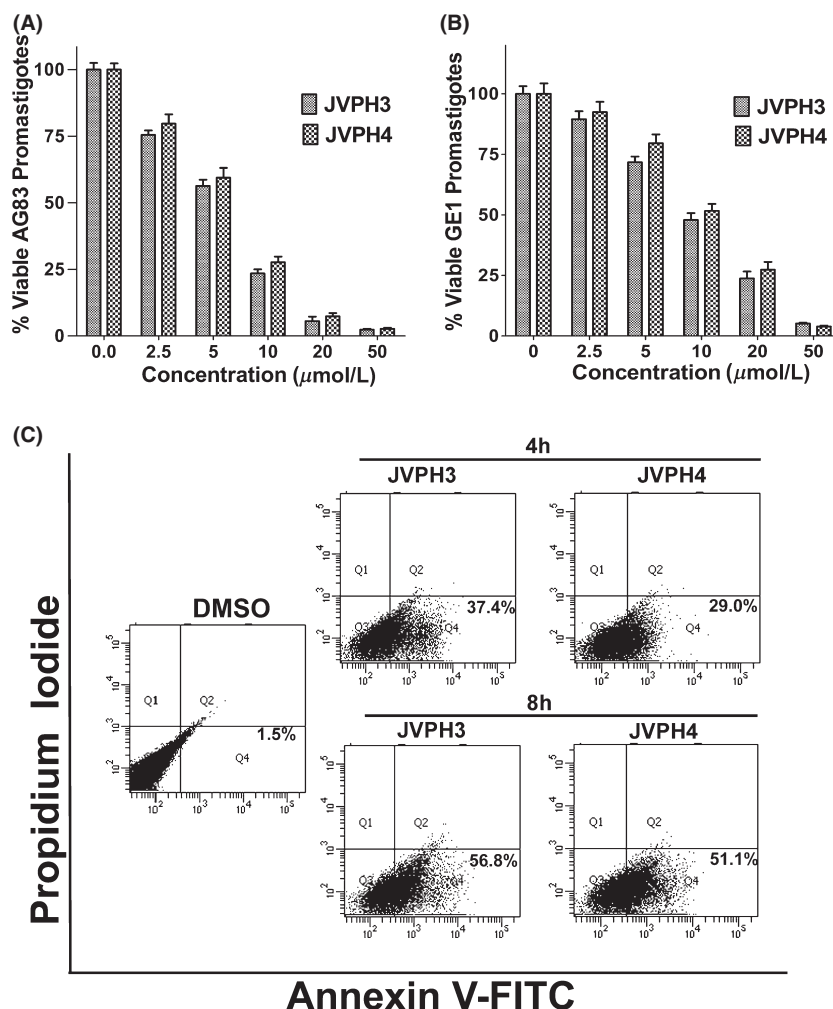
ratory grown antimony-resistant ( $\text{Sb}^{\text{R}}$ ) GE1 parasite were exposed to five different concentrations of JVPH3 and JVPH4 for 12 h, after which the cell viability was determined by the MTT assay as described under "Materials and Methods" (Fig. 2A and B). Cells treated with 0.2% DMSO served as control.  $\text{IC}_{50}$  values of the compounds on AG83 and GE1 promastigotes are given in Table 1. We checked the morphological changes in JVPH3- and JVPH4-treated parasite by microscopic study and no attributable phenotypic changes were observed (data not shown). Antileishmanial effect of both JVPH3 and JVPH4 was further checked on amastigote form of AG83 and GE1 parasite (Fig. S1A and B).

Next, we checked that whether this cytotoxic effect of isobenzofuranones was due to the occurrence of cell death in leishmania. Promastigotes were treated with 10  $\mu\text{mol/L}$  of each JVPH3 and JVPH4 for 4 and 8 h, and then the annexinV-positive cell population was determined by flow cytometry (Fig. 2C). Flow cytometry analysis revealed that JVPH3 treatment caused 37.4% and 56.8% annexinV-positive parasite for 4 and 8 h treatment, respectively. Similarly JVPH4 treatment for 4 h caused 29% annexinV-positive and for 8 h caused 51.1% annexinV-positive parasites, respectively.

To understand whether JVPH3 and JVPH4 induce ROS inside cells, promastigotes were exposed to 10  $\mu\text{mol/L}$  JVPH3 and JVPH4 for different time points. The time course experiment suggested that both the compounds induce ROS inside the parasite (Fig. S2A). Pretreatment of the parasite with *N*-acetyl cysteine (NAC), an antioxidant followed by treatment with JVPH3 did not show any induction of ROS in the experimental condition. We have also monitored other downstream events of ROS generation inside the cells such as peroxidation of membrane lipid molecules and reduction of endogenous antioxidant such as reduced glutathione level inside the cell after drug treatment. Treatment of the parasite with JVPH3 and JVPH4 increased the level of lipid peroxidation in a



**Figure 1.** Chemical structure of isobenzofuranone derivatives. 3,5-bis(4-chlorophenyl)-7-hydroxyisobenzofuran-1(3H)-one (JVPH3) (A) and (4-bromo)-3'-hydroxy-5'-(4-bromophenyl)-benzophenone (B).



**Figure 2.** Cytotoxic effect of isobenzofuranone derivatives on *Leishmania donovani* and cell death. Percentage of viable AG83 and GE1 promastigotes after treatment with different concentrations of JVPH3 and JVPH4 were plotted in (A) and (B), respectively. The results are shown as mean  $\pm$  SD of three independent experiments. Flow cytometric analysis of *L. donovani* promastigote death through PCD/necrotic processes using annexin V-FITC and PI in FL-1 versus FL-2 channels. The cells in the bottom right quadrant in each of the panels indicated apoptosis (C).

**Table 1.** IC<sub>50</sub> value (μmol/L) of isobenzofuranone compounds on antimony sensitive and resistant promastigotes.

Compounds	Parasite name	IC <sub>50</sub> (μmol/L)
JVPH3	AG83 (Sb <sup>S</sup> )	5.40 $\pm$ 0.0014
	GE1(Sb <sup>R</sup> )	9.81 $\pm$ 0.0017
JVPH4	AG83 (Sb <sup>S</sup> )	6.01 $\pm$ 0.0011
	GE1(Sb <sup>R</sup> )	11.54 $\pm$ 0.0012

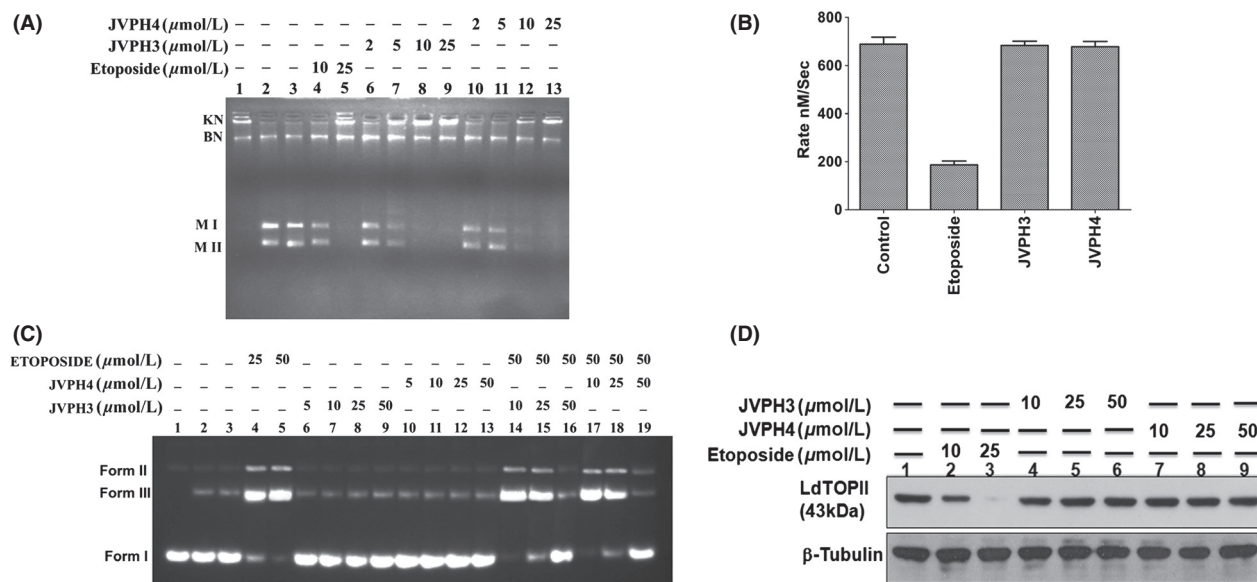
The results shown are the means of three independent experiments and represent mean  $\pm$  SD from three independent experiments.

time-dependent way, (Fig. S2B) on the other hand, level of reduced glutathione decreased drastically with increasing time of incubation increased (Fig. S2C).

We also checked the level of DNA fragmentation in JVPH3- and JVPH4-treated parasite. For these purposes, promastigotes were exposed to 20 μmol/L of JVPH3 or JVPH4 for different time points. The extent of DNA fragmentation in treated parasites was estimated using an enzyme-linked immunosorbent assay (ELISA)-based assay and time-dependent increase of DNA fragmentations were observed in both JVPH3- and JVPH4-treated parasites (Fig. S2D).

### Isobenzofuranone derivatives inhibit the activity of LdTOPII but not LdTOPIB

We have checked the inhibitory effect of isobenzofuranone derivatives on the activity of LdTOPII. Decatenation



**Figure 3.** Inhibitory activity of isobenzofuranone derivatives on LdTOPII. Purified LdTOPII was incubated with 200 ng kDNA in presence of different concentrations of JVPH3 and JVPH4. Lane 1, kDNA only; lane 2, kDNA treated with LdTOPII; lane 3, same as lane 2 but in presence of DMSO. KN and BN indicate kDNA network and broken network, respectively. M II and M I indicate the released minicircles super coiled and nicked form (A). ATPase activity of LdTOPII was measured by the pyruvate kinase/lactate dehydrogenase assay. Rate of LdTOPII-mediated ATP hydrolysis in presence of (50  $\mu\text{mol/L}$ ) etoposide and 50  $\mu\text{mol/L}$  of JVPH3/JVPH4 were measured (B). The results are shown as mean  $\pm$  SD of three independent experiments. Inhibition of etoposide-induced cleavage complex formation by isobenzofuranone derivatives was analyzed by cleavage reaction and agarose gel electrophoresis. Lane 1, negatively supercoiled pRYG DNA; lane 2, pRYG DNA with LdTOPII alone; lane 3 same as lane 2 but in presence of proteinase K treatment (C). In vivo LdTOPII-mediated cleavage complex stabilization by isobenzofuranone derivatives was analyzed by immunoblotting depletion assay. Equal amounts of protein from different treatments were subjected to SDS-PAGE followed by immunoblotting with antibody raised against ATPase domain of LdTOPII.  $\beta$ -tubulin served as loading control (D).

assay was performed with the purified fraction of recombinant DNA topoisomerase (LdTOPII) and the release of minicircle DNA from kDNA network was checked by agarose gel electrophoresis (Fig. 3A). LdTOPII was incubated with different concentrations of JVPH3 and JVPH4 along with the substrate kDNA. Dose–response study revealed that both JVPH3 and JVPH4 completely inhibited decatenation activity of LdTOPII at 25  $\mu\text{mol/L}$  concentration. We also checked the inhibitory effect of isobenzofuranone derivatives on the activity of hTOPII $\alpha$ . Both JVPH3 and JVPH4 inhibited decatenation activity of hTOPII $\alpha$  in much more higher concentration (200  $\mu\text{mol/L}$ ) as compared to LdTOPII (Fig. S3).

To check the effect of JVPH3 and JVPH4 on the ATPase activity of LdTOPII, ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay as described in “Materials and Methods” section. It was observed that both JVPH3 and JVPH4 did not interfere with LdTOPII-mediated ATP hydrolysis (Fig. 3B). Etoposide inhibits the rate of enzyme-catalyzed ATP hydrolysis used as positive control in the experiments.

To check the effect of JVPH3 and JVPH4 on LdTOPII activity, plasmid DNA relaxation assay was performed.

None of these compounds exhibited any inhibitory effect on LdTOPIB (Fig. S4). To check whether JVPH3 and JVPH4 intercalate into DNA, DNA unwinding assay was performed with supercoiled and relaxed plasmid DNA as described in “Materials and Methods” section. Supercoiled and relaxed plasmid DNAs were separately treated with LdTOPIB in the presence of different concentrations of JVPH3 and JVPH4 (Fig. S5A and B). A net negative supercoiling of the relaxed substrate DNA was introduced in the presence of strong intercalative agent EtBr (positive control), but JVPH3 and JVPH4 had no effect on the topological state of the DNA up to 200  $\mu\text{mol/L}$  concentration.

### Isobenzofuranone derivatives inhibit cleavable complex formation by abrogating topoisomerase–DNA interaction

To explore the mechanism of inhibition of LdTOPII, electrophoretic mobility shift assays were performed to study the effect of JVPH3 and JVPH4 on the initial topoisomerase II–DNA binary complex formation.  $^{32}\text{P}$  labeled 36 bp duplex linear DNA containing high-affinity binding site



of topoisomerase II was used as substrate (Sengupta et al. 2005b). LdTOPII binds with this DNA substrate and caused the mobility shift of the labeled DNA. When LdTOPII was preincubated with increasing concentrations of JVPH3 and JVPH4, prior to the addition of 36 bp duplex DNA, no shift of mobility was observed. These results suggest that JVPH3 and JVPH4 prevented the primary interaction between LdTOPII and the substrate DNA (Fig. S6).

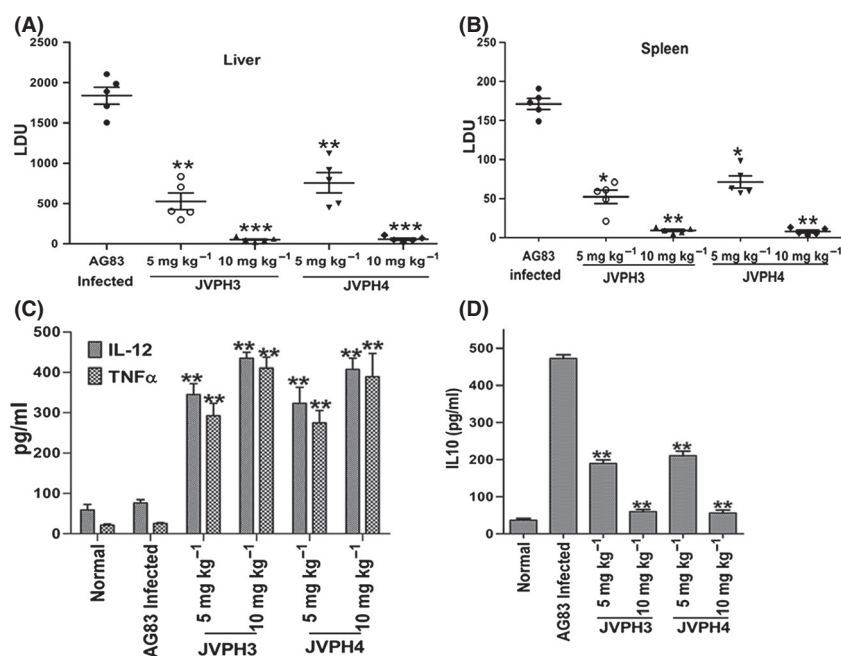
Since the binding of TOPII and DNA precedes the enzyme–DNA cleavable complex formation, LdTOPII-mediated DNA cleavage was assayed with different concentrations of JVPH3 and JVPH4. For this purpose, plasmid cleavage reaction was performed under equilibrium condition by reacting LdTOPII with pRYG DNA. Etoposide was used as positive control which stabilizes the cleavage complex formation. As shown in Figure. 3C, both 25 and 50  $\mu\text{M}$  etoposide convert closed circular DNA (Form I) to linear DNA (Form III) by stabilization of the “cleavable complex” (lanes 4 and 5). Lane 2 shows the formation of nicked circular DNA (Form II) and linear DNA (Form III) as a result of cleavage of pRYG DNA with LdTOPII alone. This serves as the background cleavage of LdTOPII. When the cleavage assay was performed with increasing concentrations of these compounds with LdTOPII, no remarkable linear products (lanes 6–9 for JVPH3, lanes 10–13 for JVPH4) were observed, whereas etoposide at 25 and 50  $\mu\text{mol/L}$  stabilized the cleavable complex. Moreover, when topoisomerase II was preincubated with 10, 25, and 50  $\mu\text{mol/L}$  concentrations of these derivatives before the addition of 50  $\mu\text{mol/L}$  etoposide (lanes 14–16 for JVPH3 and lanes 17–19 for JVPH4), the etoposide-mediated cleavage was inhibited drastically with increasing concentrations of these compounds and was completely inhibited at 50  $\mu\text{mol/L}$  concentration of each derivative. These results suggest that JVPH3 and JVPH4 derivatives inhibit the binding of enzyme to substrate DNA and thus inhibit cleavable complex formation. To understand whether such in vitro observations were comparable in vivo JVPH3- and JVPH4-treated *L. donovani* AG83 promastigotes were assayed for immunoband depletion experiments as described in “Materials and Methods”. If topoisomerase II can form a covalent complex with genomic DNA inside the cells, then topoisomerase II–DNA cleavable complex cannot enter into the gel. It was observed that etoposide treatment for 6 h on promastigotes caused the depletion of the immunoband of topoisomerase II. On the other hand, when the promastigotes were treated with increasing concentrations of JVPH3 and JVPH4, no remarkable depletion of the respective bands was observed (Fig. 3D).

### Treatment with isobenzofuranone derivatives reduced splenic and liver parasite burden and polarized Th1 immune response

To understand whether these compounds can be exploited as antileishmanial agents, the compounds were administered in an experimental visceral leishmaniasis mouse model as described in “Materials and Methods” section. Treatment with JVPH3 and JVPH4 (5 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) caused ~71.30% and ~58.80% reduction of liver parasite burden (Fig. 4A) and ~69.50% and ~58.40% reduction of splenic parasitic burden (Fig. 4B), respectively, compared to the parasitic load in infected animal liver and spleen tissues. The effect was more pronounced with 10 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$  of JVPH3 and JVPH4 administration, as there was huge reduction of hepatic and splenic parasite burden compared to infected control tissues. Treatment of both the compounds induced ROS and NO in splenocytes compared to drug untreated infected control (Fig. S7). For further investigation of antileishmanial mechanism of JVPH3 and JVPH4, we measured Th1 and Th2 cytokines level by ELISA analysis. Both JVPH3 and JVPH4 caused the increased production of Th1 cytokine (IL-12 and TNF- $\alpha$ ) and reduced Th2 cytokine (IL-10) production compared to drug untreated infected mice. The results demonstrated in Figure 4C suggest that treatment of JVPH3 in two different doses (5 and 10 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) caused the induction of IL-12 level of ~ 4.50 and ~5.68 fold and of TNF- $\alpha$  level of ~11.40 and ~16.02-fold, respectively, compared to drug untreated infected mice. Similarly, JVPH4 in two different doses (5 and 10 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) caused the induction of IL-12 level of ~ 4.21 and ~5.32-fold and TNF- $\alpha$  level of ~10.70 and ~15.17-fold, respectively, compared to drug untreated infected mice. However, Th2 cytokine IL-10 decreased after JVPH3 and JVPH4 treatment, compared to infected controls. Treatment of JVPH3 in two different doses (5 and 10 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) caused the reduction of IL-10 level of ~2.48 and ~7.90-fold, respectively, compared to drug untreated infected mice (Fig. 4D). Similarly, JVPH4 in two different doses (5 and 10 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) caused the reduction of IL-10 level of ~2.24 and ~8.42-fold compared to drug untreated infected mice. Collectively, these data suggest that isobenzofuranone derivatives confer immunobalance in *L. donovani* infected mice by switching to Th1 response.

### Discussion

Because of the emergence of drug unresponsive strains of leishmania and toxic side effects of available drugs, it is necessary to identify newer targets and also to develop



**Figure 4.** In vivo antileishmanial effects of isobenzofuranone derivatives on *Leishmania donovani* infected BALB/c mouse model of visceral leishmaniasis. Animals were sacrificed and liver (A) and splenic (B) parasite load was determined by stamp-smear method and expressed as LDU for all groups. Untreated, infected mice were used as controls. Data are presented as mean  $\pm$  SEM ( $n = 5$  animal per group). Splenocytes isolated from different group of mice were plated aseptically and incubated with  $25 \mu\text{g mL}^{-1}$  SLA for 48 h. IL-12 and TNF- $\alpha$  in supernatants of splenocyte cultures were assayed by ELISA (C). IL-10 in supernatants of splenocyte cultures were assayed by ELISA (D). Values represent the mean  $\pm$  SD. (3–5 mice/group) \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  (Student's  $t$ -test as compared to different JVPH3 and JVPH4 treatment groups with infection control). ns indicates that differences are not significant.

newer therapeutic agents to combat this infection. In this study, we have identified two novel isobenzofuranone derivatives, namely, JVPH3 and JVPH4 that exhibited potent antileishmanial effect on both antimony sensitive and resistant parasites via targeting DNA topoisomerase II. This study also illustrates the molecular mechanism of inhibition of LdTOPII and the mechanism of cell death. Earlier studies suggested several natural products and synthetic molecules that exhibited antileishmanial effect by targeting bi-subunit topoisomerase IB of *L. donovani* (Ray et al. 1998; Sen et al. 2004; Das et al. 2008; Roy et al. 2008). Flavonoids such as quercetin, luteolin, and baicalin have been shown to possess antileishmanial property by targeting topoisomerase IB and inducing DNA damage in leishmania (Das et al. 2006, 2008; BoseDasgupta et al. 2008). The pentacyclic triterpenoid, dihydrobetulinic acid (DHBA) also manifests antileishmanial activity by catalytically inhibiting the bi-subunit topoisomerase IB (Chowdhury et al. 2003, 2011). Very few compounds have recently been identified that exhibit antileishmanial effect by targeting leishmanial topoisomerase II (Mittra et al. 2000). The compounds JVPH3 and JVPH4 inhibited the decatenation activity of LdTOPII but have no inhibitory effect on bi-subunit topoisomerase IB activity. A topo-

isomerase reaction has three general mechanistic steps: binding of the enzyme to the substrate DNA, strand breakage and subsequent strand passage through the break and strand religation (Stewart et al. 1998; Koster et al. 2005). It is evident from EMSA analysis that both JVPH3 and JVPH4 inhibit the enzyme DNA complex formation and suggest that these compounds act on DNA-binding step. Since the binary complex formation precedes the enzyme–DNA cleavable complex formation, it was expected that isobenzofuranone would not stabilize the cleavable complex. Unlike etoposide, a known eukaryotic DNA topoisomerase II class I inhibitor that stabilizes cleavage complex, JVPH3 and JVPH4 do not form any cleavage complex in vitro, suggesting that these compounds act as catalytic inhibitors. Interestingly, when topoisomerase II was incubated with JVPH3 and JVPH4 prior to treatment with etoposide, the compounds abrogated the cleavage complex formation. The plausible explanation of this finding might be that the isobenzofuranone derivatives interfere with the DNA-binding activity of the enzymes, thus it cannot bind with the DNA and became unable to stabilize cleavage complex formation. This mechanism was verified by in vivo immunoband depletion assay.

DNA topoisomerase II exists both in the nucleus and in the mitochondria of leishmania (Shapiro 1994). The mtDNA or kDNA network of leishmania contains two types of DNA molecules; 'maxicircles and minicircles' (Simpson 1986). TOPII plays a vital role in kDNA metabolism (Shapiro 1994). Inhibition of LdTOPII by isobenzofuranone derivatives may interfere mitochondrial function. Increased population of annexinV-positive cells and DNA fragmentation in isobenzofuranones treated parasites indicate that isobenzofuranones promoted the apoptosis like cell death in leishmania. JVPH3 and JVPH4 induced ROS inside the parasite, cause the depletion of endogenous antioxidant level like reduced glutathione, and increase the production of lipid peroxidation in leishmania. Isobenzofuranone derivatives do not cause DNA fragmentation by the stabilization of LdTOPII–cleavage complex, rather it may occur due to ROS-mediated oxidative DNA lesions and frequent DNA modifications.

The antileishmanial activity of isobenzofuranone derivatives was validated in *L. donovani*-infected BALB/c mice. At 10 mg kg<sup>-1</sup> body weight, there was almost complete clearance of parasites from the liver and spleen of the infected mice. Healing in visceral leishmaniasis is well associated with the development of strong cell-mediated immunological responses, such as T-cell response and NO production, which help in activating macrophages to kill the intracellular parasites (Kemp et al. 1993; Diefenbach et al. 1999). Both ROS and reactive nitrogen intermediates in activated macrophages play important role in parasite killing. Increased level of ROS and NO in JVPH3/JVPH4-treated mouse splenocytes strongly supports that these isobenzofuranone derivatives reduced the parasite burden in infected mice via activation of macrophages. An effective leishmanicidal response against *L. donovani* infection is dependent on the balance between Th1 and Th2 cytokines (Kemp et al. 1993). Therapy with isobenzofuranone derivatives mounts polarized Th1 responses with enhanced IL-12, TNF- $\alpha$  and NO production and reduced Th2-associated cytokine IL-10, production in all four groups of mice compared with the infected control groups. Collectively, it is evident that isobenzofuranones treatment on infected mice shifted the balance from Th2 to Th1 response.

In conclusion, our results documented that the novel isobenzofuranone derivatives exhibited potent antileishmanial effect on both antimony sensitive and resistant parasites targeting type II DNA topoisomerase and polarizing Th1 response during the healing of leishmaniasis in mouse model. As the target-based chemotherapy remains the only choice for the treatment of leishmaniasis, isobenzofuranone derivatives are the promising candidates for the development of antileishmanial drugs.

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## Disclosures

None declared.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Clearance of internalized Sb<sup>S</sup> (AG83) and Sb<sup>R</sup> (GE1) *Leishmania donovani* parasite from in vitro infected mouse macrophage in culture. Macrophages (M $\phi$ ) were isolated from BALB/c mice 36–48 h post injection (intra-peritoneal) with 2% (w/v) hydrolyzed starch by peritoneal lavage with ice-cold phosphate-buffered saline and infected with early-passaged. *L donovani* AG83 promastig-

otes and laboratory developed Sb<sup>R</sup> (GE1) parasites in vitro. Infected macrophages, after subsequent washing, were incubated with different concentrations of JVP3 and JVP4 and left for another 24-h period. Macrophages were fixed, and intracellular amastigotes were counted by Giemsa staining. The number of internalized AG83 and GE1 amastigotes within each infected macrophages was counted under bright field microscope in (A) and (B), respectively. The results are shown mean  $\pm$  SD of three independent experiments.

**Figure S2.** Isobenzofuranone derivatives induced the generation of ROS and fragmentation of genomic DNA in *Leishmania donovani*. ROS generation for the promastigotes treated with JVP3/JVP4 for different times was measured by fluometric analysis of H<sub>2</sub>DCFDA at 530 nm (A). The level of fluorescent products of lipid peroxidation was measured at 430 nm (B) and the intracellular GSH level was measured at 480 nm (C). The percentage of DNA fragmentation after treatment with JVP3 and JVP4 was plotted against time of incubation (D). The results are shown mean  $\pm$  SD of three independent experiments.

**Figure S3.** Effect of isobenzofuranone derivatives on hTOPII $\alpha$  activity. hLdTOPII $\alpha$  was incubated with 200 ng kDNA in presence of different concentrations of JVP3 and JVP4. Lane 1, kDNA only; lane 2, kDNA treated with LdTOPII; lane 3, same as lane 2 but in presence of DMSO. KN indicates kDNA network. M II and M I indicate the released minicircles super coiled and nicked form.

**Figure S4.** Effect of isobenzofuranone derivatives on LdTOPIB activity. The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBS (SK+) [pBluescript (SK+)] DNA in agarose gel. Different concentrations (2, 5, 10, 20, and 50  $\mu$ mol/L) of JVP3 and JVP4 were incubated with LdTOPIB followed by electrophoresis in 1% agarose gel. Camptothecin (CPT), a known topoisomerase I inhibitor, was used as positive control. The gels were stained EtBr (0.5  $\mu$ g mL<sup>-1</sup>) and photographed under UV illumination. Lane 1, pBS (SK+) DNA; lane 2, pBS (SK+) DNA with LdTOPIB; lane 3, same as lane 2 but in presence of 2% DMSO; lane 4, same as lane 2, but the enzyme was incubated with 25  $\mu$ mol/L CPT. NM, nicked monomer; RM, relaxed monomer; SM, supercoiled monomer.

**Figure S5.** Effect of isobenzofuranone derivatives on DNA unwinding. Unwinding assay was performed with supercoiled and relaxed plasmid DNA. Negatively supercoiled pBS (SK+) DNA was treated with LdTOPIB in presence of different concentrations of JVP3 and JVP4 (A). Relaxed pBS(SK+) DNA was treated with LdTOPIB in presence of different concentrations of JVP3 and JVP4 (B). EtBr (0.1 and 1  $\mu$ g mL<sup>-1</sup>) is used as positive

control in the experiments. Lane 1, pBS (SK+) DNA; lane 2, pBS(SK+) DNA with LdTOPIB; lanes 3–4, same as lane 2 but in presence of 0.1 and 1  $\mu\text{g mL}^{-1}$  EtBr; lanes 5–9, same as lane 2, but in presence of increasing concentration of JVPH3. Lanes 10–14, same as lane 2, but in presence of increasing concentration of JVPH4. NM, nicked monomer; RM, relaxed monomer; SM, supercoiled monomer.

**Figure S6.** Effect of isobenzofuranone derivatives on LdTOPII–DNA binary complex formation. Effect of isobenzofuranone derivatives on DNA-binding activity of LdTOPII proteins was analyzed by EMSA analysis. Lane 1, labeled 36-mer duplex oligonucleotide; lane 2, 36-mer oligonucleotide incubated with of LdTOPII; lanes 3 and 4

same as lane 2, but incubated in presence of JVPH3 (10 and 25  $\mu\text{mol/L}$ ); and lanes 5 and 6 same as lane 2 but incubated in presence of JVPH4 (10 and 25  $\mu\text{mol/L}$ ).

**Figure S7.** In vivo generation of ROS and NO from infected and isobenzofuranone derivatives treated BALB/c mice. Splenocytes ( $2.0 \times 10^6$ ) from different experimental mice groups were pulsed with SLA for 48 h in 5%  $\text{CO}_2$  incubator at 37°C. Level of ROS in splenocytes was measured by  $\text{H}_2\text{DCFDA}$  probe (A) and NO content in the culture supernatant was measured by Griess reagent (B). Data are presented as mean  $\pm$  SEM ( $n = 5$  animal per group). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.005$  (Student's  $t$ -test), as compared to different JVPH3 and JVPH4 treatment groups with infection control.