Chapter 11,

Materials and Methods
Materials and Methods:

Materials

All chemicals were of analytical grade and obtained from Sigma Aldrich Chemicals (St Louis, MO, USA) except RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA), Sulphanilamide, Phenazine methosulphate (Sisco Research Laboratories, Mumbai, India), MTS or 3 - (4,5 -dimethylthiazol - 2 - yl) -5 - (3-carboxymethoxyphenyl) - 2 - (4-sulfophenyl) - 2H - tetrazolium, inner salt (Promega, Madison, WI, USA), 5-chloromethylfluorescein-diacetate (CMFDA), 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide, (JC-1), Fluoro 4AM were obtained from Invitrogen, Carlsbad, CA, USA), Z-Val-Ala-DLAsp (methoxy) -fluoromethylketone (Z-VAD-FMK), fluochrome conjugated antibodies were from BD Biosciences (San Jose, CA, USA) and Caspase-3/CPP32 Colorimetric assay kit (Biovision, Mountain View, CA, USA); protease inhibitor cocktail, in situ TUNEL assay kit (from Roche, Germany), N-1 napthyl ethylene diamine dihydrochloride (NED, Loba Chemie Pvt. Ltd., Mumbai, India), RNAnaqueous® Kit (Ambion, Austin, TX, USA), one Step RT-PCR kit (Qiagen, Hilden, Germany) and primers from Siga Genosys, India. Artemisinin (100 mM in DMSO), Miltefosine (2 mM in stock medium) were freshly prepared, and Sodium antimony gluconate (SAG) was a kind gift from Albert David (Kolkata, India).

Parasite culture

Promastigote strains representing several species included Leishmania donovani, L. infantum, L. tropica, L. braziliensis, L. mexicana and L. amazonensis. They were routinely cultured at 24°C in M-199 medium; supplemented with 10% heat inactivated fetal calf serum (HIFCS), penicillin G (50 IU/ml) and streptomycin (50 μg/ml). Cells were passaged every 72 h, 1 x10^5/ml being the inoculum.

Cell line culture

A mouse-monocyte macrophage cell line, RAW 264.7, human monocyte-lymphoma cell line, U937 and human monocyte cell line, THP1 were obtained from National Centre for Cell Science, Pune, India. Cells were maintained at 37°C, 5% CO₂ in RPMI medium, supplemented with 10% FCS, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). The cells were passaged every 72 h, 5 x 10^5 cells/ml being the initial inoculum.
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Animals

BALB/c mice (average wt, 25-30 g) were maintained at standard temperature (25 ± 5°C), in a 12 hr day/night cycle and were fed a standard pellet diet along with water provided *ad libitum*. All experimental protocols received prior approval from the Institutional Animal Ethical Committee.

Preparation of plant extracts

Preparation of Malabaricone plant extracts

The dry fruit rinds (20 g) of *Myristica malabarica* or rampatri were powdered with a grinder and extracted successively with ether and methanol (60 mL × 4 days with each solvent at room temperature). The supernatants were decanted and the entire process was repeated thrice and each of the combined supernatants was filtered through a nylon mesh and evaporated at <40°C in vacuum desiccators to yield the respective extracts. These were designated as rampatri ether (R1; 7.3 g, 36.5% w/w) and methanol (R2; 5.77 g, 28.9% w/w) extracts and stored in a vacuum desiccator, separated into two distinctly visible layers. The top oily layer was carefully separated and designated as R3, while the sticky residue that remained at the bottom of the flask yielded fraction R4.

Isolation of chemical constituents from R2

A portion of R2 was subjected to partial fractionation using silica gel (25 g) column and eluting with hexane to collect five 300 mL fractions, which on evaporation *in vacuo* gave the sub-fractions F1–F5. The F2 sub-fraction was subjected to rigorous column chromatography (silica gel, 0–20% EtOAc/hexane) to isolate five compounds 1–5, which were fully characterized using IR and 1H NMR spectroscopic data. The yields reported for compounds 1–5 are with respect to the weight of dry fruit rinds. Compounds 2–5 that were used for the present investigation were identified as malabaricones A–D.

Each of the test samples was dissolved in tissue culture grade DMSO (20 mg/mL) and stored at −20°C.

Preparation of extracts from *Artemisia indica*

Leaves of *A. indica* plant were collected locally and authenticated by the Botanical Survey of India, Shibpore, Howrah. The material was cut into small pieces, air dried and crushed into powder (stored in aliquots of 10 g); each aliquot was extracted with 100 ml of each solvent namely (a) Petroleum ether (Art 1), (b) CH₂Cl₂ (Art 2), (c) Methanol (Art 3) and
(d) Ethanol (Art 4). The solutions obtained were filtered thrice and the resultant liquid pooled and evaporated in a rotary evaporator. Stock solutions (100 mg/ml in DMSO) were prepared and stored at -20°C until used.

**Preparation of *Piper betle* extract**

Fresh leaves of *P. betle* were locally collected and authenticated by the Botanical Survey of India, Shibpore, Howrah. After being air dried and powdered in a hand crusher, the powder (300 g) was extracted twice with absolute ethanol (900 ml each time) and the resultant filtrate was 'solvent-recovered' by distillation in a Soxhlet apparatus. The concentrated extract was subsequently dried in a rotary evaporator and lyophilized, yield being 3.63 g and referred to as 'PB'. PB was dissolved in propylene glycol (100 mg/mL) and stored at 4°C.

**Anti-promastigote activity of indigenous plant extracts**

The *in vitro* effect of the following compounds was tested using MTS-PMS assay [Ganguly et al., 2006b]:

(i) Malabaricones or Rampatri extracts (0 – 50 µg/ml)
(ii) Organic solvent extracts of *A. indica* (0–1.0 mg/ml)
(iii) Artemisinin (0 – 0.5 mM)
(iv) 19 analogs of Artemisinin (0 – 0.5 mM)
(v) *Piper betle* extract or PB (0 – 20 µg/ml)

Briefly, exponentially growing parasites were resuspended in 96-well tissue culture plates (2 x 10⁵ /200 µl/well). After incubation at 24°C for 2 h, one of the above mentioned compounds was added for an additional 48 h. At the end of 48-h of drug treatment, 20 µl of a solution comprising MTS and PMS in a ratio of 5:1 was added per well. The plates were then incubated further for 3 h at 37°C and absorbances of resultant formazan measured at 490 nm using a plate reader (BioRad, California, USA). MTS is converted to formazan by mitochondrial dehydrogenases of viable parasites in presence of an electron coupler PMS. Therefore, the amount of formazan produced was considered as a measure of cell viability. The mean percent viability was calculated as:

\[
\text{Mean percent viability} = \frac{(\text{Mean specific absorbance of drug treated parasites}) \times 100}{\text{Mean specific absorbance of untreated parasites}}
\]
where specific absorbances were determined by subtracting the background absorbance of medium. The results were expressed as the IC$_{50}$ i.e. the concentration that inhibited 50% cell growth, which was enumerated by graphical extrapolation using GraphPad Prism software (version 5). Accordingly, the IC$_{50}$ for each drug, i.e., the concentration of drug that decreased the percent viability by 50% was graphically extrapolated by plotting percent viability against the respective drug concentration.

**Evaluation of ex vivo anti-amastigote activity of Artemisinin and PB**

Exponential phase, non-adherent THP1 monocytes seeded in 16 chambered slides (5 x 10$^4$ cells/200 μl/well) were incubated with phorbol myristate acetate (PMA, 20 nM) at 37°C, 5% CO$_2$ for at least 12 h. Adherent macrophages were equilibrated in PMA-free medium for at least 24 h and then incubated with stationary-phase promastigotes for at least 3 h at 37°C, 5% CO$_2$. After removal of non-phagocytosed promastigotes, infected macrophages were incubated with either Artemisinin/ its analogs (0 - 50 μM)/ PB (0 - 20 μg/ml) for 48 h at 37°C, 5% CO$_2$. Cells were then fixed, stained with Giemsa and examined microscopically for intracellular amastigotes. At least 100 macrophages per well were counted to calculate the percentage of infected macrophages. The infection rate of treated macrophages was normalized to 100% for further analysis of results.

**Measurement of safety Index**

To evaluate the cytotoxic activity of the compounds in mammalian cells, log phase RAW 264.7 cells seeded in 96 well tissue culture plates (5 x 10$^4$/200 μl RPMI 1640/well) were incubated in presence or absence of the compounds (0 - 0.5 mM) for 48 h at 37°C, 5% CO$_2$ and cytotoxic effects enumerated by the MTS assay [Ganguly et al., 2006b]. In addition toxic effect of Artemisinin and PB was also determined in mouse peritoneal macrophages. The safety indexes were derived from IC$_{50}$ of compounds in macrophages/IC$_{50}$ of compounds in amastigotes.

**Characterization of the reaction products of Artemisinin in the presence of iron**

In a 1-cm quartz cuvette, the absorption spectra was recorded on a spectrophotometer (Systronics, India) of solutions containing Fe$_2$SO$_4$ (100 μM) or Artemisinin (100 μM) or both in de-ionised (pH. 7.2) water maintaining 25°C [Sibmooh et al., 2001].
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*Generation of reactive oxygen species (ROS) in promastigotes*

Generation of ROS in promastigotes were measured using H$_2$DCFDA, a dye which within the cell, is cleaved off by non-specific esterases into impermeable non-fluorescent H$_2$DCF that subsequently gets oxidized by ROS into a highly fluorescent compound DCF. Therefore, the intensity of fluorescence is directly proportional to the amount of ROS present. To study the effect of Artemisinin on generation of ROS, log phase promastigotes (1 x 10^6/ml) were incubated with Artemisinin (0 - 0.5 mM) for 3 h at 37°C. Cells were washed with phosphate buffered saline (0.02 M, pH 7.2), incubated with H$_2$DCFDA (50 μM) for 45 min at 37°C and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) using forward vs. side scatter to gate the parasite population and a FL1 histogram to quantify fluorescence of parasites [Mandal et al., 2007]. The subsequent analyses were done using BD CellQuest Pro software.

*Measurement of non protein thiols using CMFDA*

The measurement of non protein thiols were done by CMFDA, a cell permeable, non-fluorescent dye that upon entering the cell, rapidly binds with non-protein thiols and becomes non-permeable; simultaneous cleavage of the diacetate moiety by cellular esterases yields a fluorescent thioether. In *Leishmania* parasites, the fluorescence represents primarily trypanothione methylfluorescein [TSMF, Sarkar et al., 2009]. Accordingly, the fluorescence is directly proportional to the amount of intracellular non protein thiols. To measure levels of non-protein thiols, Artemisinin-treated promastigotes were incubated with increasing concentrations of drug (0 - 0.5 mM) and incubated at 37°C for 3 h. After washing with PBS, cells were incubated with CMFDA (1.0 μM) at 37°C for 15 min in dark and cells acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) and subsequent analyses were done using BD CellQuest Pro software [Sarkar et al., 2009].

*Measurement of nitric oxide (NO)*

Nitrite level, a stable oxidized product of NO that accumulated in the culture medium and therefore an indicator of NO production, were measured using the Griess assay [Sarkar et al., 2008b]. The Griess Reagent System is based on the chemical reaction which uses sulfanilamide and NED under acidic (phosphoric acid) conditions in the presence of nitrite to yield an azo compound that can be measured spectrophotometrically. This system detects NO$_2$ in a variety of biological and experimental liquid matrices such as plasma, serum, urine
and tissue culture medium. Briefly, murine peritoneal macrophages (1 x 10^6/ml of complete RPMI-1640 medium) seeded in 6-well plates were infected with *L. donovani* promastigotes at a macrophage: parasite ratio of 1:10 for 5 h at 37°C, 5% CO_2_. Non-internalized parasites were removed and infected macrophages as well as uninfected macrophages were incubated with Artemisinin (10 and 25 μM) at 37°C; supernatants were collected at 24 to 72 h. NO was measured using Griess reagent and a standard curve was generated using NaN_2O (0 - 100 μM).

**Determination of role of free radicals in Artemisinin-induced leishmanicidal activity**

To evaluate the contribution of ROS in the leishmanicidal activity of Artemisinin, promastigotes were incubated with N-acetyl L-cysteine (NAC), a free radical scavenger. Briefly, log phase promastigotes (2 x 10^5 cells/200μl of M199/well) resuspended in 96 well tissue culture plates, were co-incubated with Artemisinin (0 - 0.5 mM) and NAC (2.5 mM) for 48 h at 24°C and cell viability measured using the MTS-PMS cell viability assay as described above.

**Determination of role of iron in Artemisinin-induced leishmanicidal activity**

As iron is proposed to play a synergistic role in generation of carbon centered free radicals from Artemisinin, generation of ROS in Artemisinin treated promastigotes in the presence of a ferrous compound was measured. Log phase promastigotes (1 x 10^6/ml) were initially incubated with Artemisinin (0 - 0.5 mM) for 1 h at 37°C followed by addition of a non toxic concentration of Fe_2SO_4 (0.2 mM) for 1 h. Cells were then washed with PBS incubated with H_2DCFDA (50 μM) for 45 min at 37°C and acquired as previously described. The influence of a non toxic conc. of Desferoxamine (DFO), an iron chelator on the leishmanicidal activity of Artemisinin was also studied by co-incubating log phase promastigotes with Artemisinin (0 - 0.5 mM) and DFO (0.25 mM) for 48 h at 24°C and cell viability measured using the MTS-PMS assay.

**Flow cytometric analysis of externalized phosphatidylserine (PS) in *L. donovani* promastigotes**

Cells upon undergoing apoptosis have flippage of PS which binds strongly with Annexin V and therefore it can be used as a probe to identify apoptotic cells. Annexin V-FITC (Annexin V conjugated with Fluorescein isothiocyanate or FITC) typically used in conjunction with a vital dye Propidium iodide (PI) to distinguish apoptotic cells (Annexin V-FITC...
FITC positive, PI negative) from necrotic cells (Annexin V-FITC negative, PI positive). Double staining with Annexin V–FITC and PI was performed. Briefly, promastigotes were incubated and Artemisinin (0.16 mM, 24 and 48 h); cells were centrifuged (5000 rpm x 5 min), washed twice in PBS (0.02 M, pH 7.2) and resuspended in annexin-V binding buffer (1x, BD Biosciences). Annexin V- FITC and PI were then added, according to the manufacturer’s instructions, and incubated in dark for 30 min at RT. Acquisition was done on a FACS Calibur flow cytometer (BD) and analysed with CellQuest software.

**Measurement of mitochondrial membrane potential of L. donovani promastigotes**

Mitochondrial membrane potential was measured using JC-1, a cell-permeable dye that exists in a monomeric form that on entering the cytoplasm emits green fluorescence. Subsequently, on entering the mitochondria it forms J-aggregates and emits red fluorescence. The ratio between red and green fluorescence, i.e. 585/530 nm, determines the mitochondrial trans-membrane potential. After being treated with Artemisinin (0.16 mM, 24 and 48 h), promastigotes were centrifuged (5000 rpm x 5 min). The cells were resuspended in PBS containing JC-1 (2 µM) and incubated at 37 °C for 15 min. Analysis for mean green and red fluorescence intensity was done using FACS Calibur and CellQuest software.

**Assessment of intracellular Ca²⁺ in L. donovani promastigotes in fluorimeter**

Changes in intracellular Ca²⁺ were monitored using the fluorescent probe Fluo-4 AM, a membrane-soluble dye. Upon entering the cells, the AM or acetoxymethyl ester is cleaved off by the cellular esterases and binds with intracellular Ca²⁺ and the fluorescence is a measure of intracellular Ca²⁺. Briefly, promastigotes (2 x 10⁶ cells/ml) were incubated with loading medium containing Fluo-4 AM (2.5 µM), pluronic acid FI27 (0.02%) and sulfipyrazone (0.25 mM) at 24°C for 30 min. After incubation with loading dye, cells were washed with medium containing 0.25 mM sulfipyrazone (Medium A), subsequently incubated up to 6 h with an IC₇₅ conc. of Artemisinin (0.5 mM); in parallel, cells were incubated with a Ca²⁺ ionophore (Ionomycin, 2.5 µM) in the absence and presence of a chelating agent, EGTA (5.0 mM) and these served as positive and negative controls, respectively. Cells were then washed and resuspended in Medium A and fluorescence measured on a fluorimeter at an excitation of 485 nm and emission of 520 nm. To convert fluorescence values into absolute [Ca²⁺], calibration was performed at the end of each experiment and was calculated using the following equation:
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\[ [\text{Ca}^{2+}]_r = K_d (F - F_{\text{min}})/(F_{\text{max}} - F) \]

where \( K_d \) is the dissociation constant of the Ca\(^{2+}\) Fluo 4 complex (345 nm), where \( F \) represented fluorescence intensity of cells. \( F_{\text{max}} \) represented maximal fluorescence i.e. cells treated with Ionomycin while \( F_{\text{min}} \) corresponded to minimum fluorescence i.e. cells treated with Ionomycin and EGTA.

**Determination of caspase activity in Artemisinin treated promastigotes**

Caspase activity was measured using a commercially available kit as per the manufacturer’s instructions. The assay is based on the spectrophotometric detection of the chromophore \( p \)-nitroanilide (\( p \)NA) following cleavage from the labeled CED3/CPP32 group substrate DEVD-\( p \)NA. The \( p \)NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Briefly, promastigotes were treated for 12 h with an IC\(_{75}\) conc. of Artemisinin (0.5 mM); promastigotes were pelleted, washed with ice cold PBS, cell lysates prepared and protein concentration was estimated [Lowry, 1958]. To detect activity of CED3/CPP32 group proteases, cell lysates (100 \( \mu \)g protein) in reaction buffer containing 10 mM DTT, substrate DEVD-\( p \)NA (4 mM, 5 \( \mu \)L) were incubated at 37\(^\circ\)C up to 4 h; the emission of \( p \)NA was quantified by measuring absorbances at 405 nm.

To study the biological role of caspases in Artemisinin induced death, log phase promastigotes (2 x 10\(^5\)/200 \( \mu \)L/well) were seeded in 96 well tissue culture plates. Following 48 h incubation at 24\(^\circ\)C with Artemisinin (0 - 0.5 mM) in the presence and absence of the pan-caspase inhibitor, Z-Val-Ala-DL-Asp (methoxy) - fluoromethylketone (Z-VAD-FMK, 100 \( \mu \)M), cell viability was evaluated by the MTS-PMS assay. The results were expressed as IC\(_{50}\) values i.e. the concentration that inhibited 50% of cell growth as enumerated by graphical extrapolation.

**In situ detection of DNA fragmentation by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL)**

DNA fragmentation within the cell was analysed following the manufacturer’s instructions. Briefly, following DNA nicking and exposure of the 3'-OH ends, the enzyme TdT was used along with FITC labeled nucleotide (dUTP). The enzyme TdT helps the dUTP-FITC to bind to the nick end. This binding is then detected using HRP labeled antifITC which was quantified microscopically and detected on a flow cytometer (where the last step of adding HRP-tagged anti FITC was omitted). Briefly, promastigotes were treated with Artemisinin (0.16 mM) for 24 and 48 h, after treatment, cells were washed, fixed with
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Paraformaldehyde and kept on ice for 1 h. Thereafter, the cells were centrifuged (5000 rpm x 
5 min) and resuspended in PBS. One set of cells were spotted on a grease free slide, air dried 
after which the slides were washed with PBS, placed on ice and permeabilized with freshly 
prepared, chilled Na-Citrate in Triton X-100 solution for 15 min. Cells were again washed 
twice with PBS following which 25 µl of reaction mixture containing enzyme (TdT) and 
nucleotide mixture was added. The cells were then incubated in a humidified chamber at 
37°C for 1 h, washed with PBS and convertor POD (Anti-fluorescein antibody conjugated 
with horse - radish peroxidase, 25 µl) was added and incubated for 30 min at 37°C. After 
incubation one set was acquired in FACS and for microscopic observation, the substrate 
Diaminobenzidine (DAB, 25 µl) was added; slides were kept at 4°C for 10 min, washed with 
deionised water and observed microscopically (under oil immersion objective, 100 x magnifications). At least 20 microscopic fields were observed for each sample. Images were 
taken using a digital compact camera with (7x zoom, Olympus, Singapore, CAMEDIA, C- 
7070) and modified using Adobe Photoshop 8.0 (Adobe Systems Inc., Mountain View, CA, 
USA).

DNA fragmentation assay by agarose gel electrophoresis

To determine DNA fragmentation, total cellular DNA was isolated from L. donovani 
promastigotes treated with Artemisinin (0.16 mM, 48 h) according to manufacturer’s 
instructions and analysed by 1.5% agarose gel electrophoresis containing ethidium bromide 
(0.5 mg/ml) in TBE buffer and visualized on a Molecular Imager Chemi Doc XRS System 
(Bio Rad, California, USA).

Effect of plant compounds on the cell cycle

The cell cycle protocol is based on flow cytometric analysis to quantify the 
percentage of cells in different phases of the cell cycle, the amount of bound dye (Propidium 
iodide) representing DNA content. Briefly, parasites (1x 10^6 cells) were treated with an IC_{50} 
dose of Artemisinin (0.16 mM) for 24 and 48 h at 24 °C; at each time point, cells were fixed 
in chilled 70% ethanol and kept at freezer until use. After washing the cells in PBS, the 
resultant pellet was resuspended in 500 µl RNase (200 µg/ ml) and incubated for 1 h at 37 
° C. Cells were then stained with PI (40 µg/ ml) and incubated in the dark for 20 min at 20– 25 ° C. Data acquisition was carried out using a FACS Calibur and analysed using
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CELLQUEST PRO software. Similarly, Cell cycle analysis of Artemisinin and PB treated (48 h) PBMC were also studied, isolation of PBMC is as following:

*Isolation of peripheral blood mononuclear cells (PBMC):*

Peripheral blood was carefully layered over ficoll-hypaque and centrifuged (400 g for 30 min). The PBMC-rich interface was washed twice in PBS (0.01 mM; pH 7.2) and resuspended in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% heat-inactivated fetal calf serum. Cell viability was confirmed using trypan blue (>95% viability) and seeded (1 x 10^6 cells/mL) in multi-well tissue culture plates and incubated at 37°C in 5% CO2.

*Reverse transcriptase polymerase chain reaction (RT-PCR)*

RNA was isolated from uninfected and infected macrophages treated with Artemisinin (10 and 25 µM) for 18 h using the RNAqueous® Kit. Subsequently, RT-PCR was carried out using one-step RT-PCR kit with RNA (200 ng/reaction) and gene-specific primers for β-actin, iNOS and IL-10. For reverse transcription, all samples were subjected to an initial incubation at 50°C for 30 min followed by an initial PCR activation (95°C for 15 min) as per the manufacturer’s instructions. The amplification cycle comprised 35 cycles of denaturing (94°C for 30 s), annealing for 30 s (varying temperatures for each primer set), extension (72°C for 60 s) and a final extension at 72°C (10 min) [Sarkar et al., 2008b]. RT-PCR products were resolved on agarose gels (1.5%) containing ethidium bromide (0.5 µg/ml) and visualized with the Molecular Imager Chemi Doc XRS System (Bio Rad, California, USA). The extent of mRNA expression was quantified by densitometric analysis using Total lab Nonlinear Dynamic Image analysis software (Nonlinear USA, Inc., Durham, NC), values being normalized to β-actin.

**Primers and amplification conditions for RT-PCR**

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<th>Gene</th>
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<th>Anti sense</th>
<th>Annealing temperature (°C)</th>
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Experimental model of VL in BALB/c mouse

To assess the effectivity of any compound, one should screen its efficacy in an animal model. We developed an experimental model of VL using Th1 suppressed animal, BALB/c mice, to screen the effectivity of the compounds. Late log phase *L. donovani* promastigotes were injected intra-peritoneally in 4-6 weeks old BALB/c mice (1 x 10^7/animal) that were chosen since their infection pattern closely resembles human VL. For monitoring the anti-leishmanial antibody levels, every 20 days post infection, blood was collected from the retro-orbital plexus and ELISA was performed and serum IgM (for primary response) and IgG (for secondary response) levels for primary and secondary infection of Leishmaniasis was monitored.

Preparation of *Leishmania donovani* antigen (LDA)

Crude *Leishmania* antigen was prepared from a *L. donovani* strain. Briefly, *Leishmania* promastigotes were routinely cultured in M-199 medium supplemented with 10% FCS, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). Cells were bulked by adding fresh medium every 72 h at 24°C up to 20 ml and a cell density of approx. >2 x 10^7/ml; cells were centrifuged (5000 rpm for 5 min., 4°C) and washed twice with chilled PBS. The pellet was resuspended in 300 μL of lysis buffer. After vigorous vortexing, the cells were subjected to at least three rapid freeze - thaw cycles, for effective lysis, which was confirmed microscopically. Protein content of lysates was estimated using Lowry’s method [Lowry, 1958]. The lysates were aliquoted, kept at -20°C and diluted immediately before use.

Indirect ELISA to detect presence of antileishmanial antibodies

The enzyme linked immunosorbent assay (ELISA) is a serological technique that can detect antigen-antibody reactions by the use of enzyme linked antibodies. ELISA is based on the use of labeled antibodies so that the resulting conjugates have both immunological and enzymatic activity. Indirect ELISA is the most commonly used method for antibody detection. Briefly; it involves the coating of the ELISA plate with the antigen against the specific antibodies that may be present in the serum. To detect the presence of antileishmanial antibodies, crude LDA (preparation, as described above) was used to coat 96 well flat bottoms ELISA plates (1 μg/ 100 μL/ well in phosphate buffer or PB, 0.02 M, pH. 7.8) and incubated overnight at 4°C. After an overnight incubation, wells were washed thrice with wash buffer. To prevent non-specific binding, 200 μL/ well blocking buffer was added.
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and incubated for at least 2 hrs at room temperature. Serum samples of normal and infected mice diluted in blocking buffer (diluted 1: 250) were added to respective wells (100 μL/well). Following an overnight incubation, wells were rinsed thrice with washing buffer. Binding was detected using horseradish peroxidase conjugated anti-mouse IgM (diluted 1:5,000, 100 μL/well) and anti-mouse IgG (diluted 1:1000, 100 μL/well) and incubated for 30 min at 37° C. The wells were rinsed with washing buffer five times, leaving buffer for five min. in wells during each wash and incubated with 2,2'- azino-bis (3 ethylbenzthiazoline-6-sulfonic acid) or ABTS (100 μL/well) for 15 to 30 min. till the colour develops, after which absorbance were measured at 405 nm in an ELISA reader. The data were expressed either using fold increase with respect to control uninfected mice or the absorbance measured.

In vivo evaluation of anti amastigote activity of Artemisinin

To measure the therapeutic efficacy of Artemisinin, BALB/c mice were infected with *L. donovani* promastigotes (1 x 10⁶/animal, IP). Approximately, after 12 weeks of infection, parasitemia was confirmed by parasite transformation from splenocytes of two arbitrarily selected animals. Mice were then randomly distributed into five groups (n = 5 per group) as follows: (a) Inf: *Leishmania* infected mice (0.9 % NaCl, p.o.) (b) SAG: sodium antimony gluconate (SAG, 20 mg/kg b.w., s.c.) (c) A10: Artemisinin (10 mg/kg b.w, p.o) (d) A25: Artemisinin (25 mg/kg b.w, p.o) and (e) Veh: Infected mice (1% DMSO, p.o, vehicle control), Artemisinin or SAG was administered orally or s.c. respectively for five alternate days. In addition, one group of uninfected mice (N, 0.9% NaCl, p.o., n = 5) were included. Two weeks after completion of treatment, all animals were sacrificed, splenic weight measured and parasitic burden estimated in terms of Leishman-Donovan units (LDU) based on the Stauber's formula [Stauber, 1958]:

Leishman Donovan Unit = (No. of amastigotes/1000 host cell nuclei) X weight of spleen (mg).

Measurement of Gr1+ve surface staining in peripheral blood

Peripheral blood (100 μL) was collected from infected and Artemisinin treated mice via the retro-orbital plexus, surface tagged with rat anti mouse Gr1 for 15-20 min; thereafter cells were stained with goat anti-rabbit FITC labeled secondary antibody, along with appropriate isotype controls. After incubation for 15-20 min on ice, cells were centrifuged (400 g for 5 min), and the resultant pellet washed with PBS and analyzed on a FACS Calibur.
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Flow cytometer (BD Biosciences). Cells were gated on the basis of characteristic linear forward and side scatter features of neutrophils with fluorescence measured on a logarithmic scale, using CellQuest Pro software.

Intracellular cytokine analysis:

Flow cytometric analysis of the intracellular cytokine stained cells allows characterization of a heterogeneous cell population. Briefly, splenocytes were obtained by macerating the spleen through a wire mesh (diameter 230 μM); cells were washed with PBS and resuspended in RPMI-1640 medium containing 10% FCS and seeded (1 x 10^6 / ml/ well) in 6 well plates. Following an overnight incubation at 37°C, 5% CO2 they were treated with Phorbol Myristate Acetate (PMA, 5 ng/ml), Ionomycin (1 μM) and Brefeldin A (10 μg/ml) for 4 h. Cells were then harvested in BD Pharmingen stain buffer (100 μl) and incubated with anti mouse CD3- fluorescein isothiocyanate (FITC) for 15 min at 25°C in dark. Cells were fixed and permeabilized in BD Cytofix/Cytoperm buffer (100 μl/10^6 cells) at room temperature for 20 min., following which were incubated with either phycoerythrin (PE)-conjugated isotype or antibodies to IFN-γ, IL-2, IL-4 or IL-10 for 15 min. Cells were acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) with lymphocytes being gated on the basis of a characteristic forward scatter vs. side scatter dot plot and percentages of cytokine-positive T lymphocytes (based on CD3+ve cells) quantified on a FL1 vs. FL2 dot plot. Quadrant markers were set on the basis of isotype fluorescence and final analysis done using BD CellQuest Pro Software.

Similarly, tested analogs of Artemisinin, GC003 (5 and 10 mg/kg b.w, p.o.), GC012 (1 and 5 mg/kg b.w, p.o.) and ML86-1(5 and 10 mg/kg b.w, p.o.) were also screened for their antileishmanial activity in in vivo model of VL.

Statistical analysis

For non-parametric data, Kruskal-Wallis ANOVA followed by Dunn's multiple comparison tests was used to analyze data for individual groups. All analyses were performed on GraphPad Prism software, version 5 (GraphPad Software Inc, San Diego, CA, USA).
Materials and Methods

Preparation of solutions:

Unless mentioned, reagents were prepared in deionised water or tissue culture grade dimethyl sulfoxide (DMSO).

Artemisinin (MW= 282.34, 100 mM): 2.83 mg Artemisinin was dissolved in 100 µl DMSO.

Miltefosine (MW= 407.57, 2 mM): 0.8 mg Miltefosine was dissolved in 1 mL stock medium.

Phosphate buffered saline (PBS, 0.2 M or 10x):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weigh/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄ (MW= 178)</td>
<td>35.6 g</td>
</tr>
<tr>
<td>NaH₂PO₄ (MW= 156, 0.2 M)</td>
<td>31.2 g in 1L</td>
</tr>
<tr>
<td>NaCl (8.5 %)</td>
<td>85 g</td>
</tr>
</tbody>
</table>

Preparation:

Na₂HPO₄ was dissolved in approx. 500 ml of water, NaCl was added to it and the pH was adjusted with NaH₂PO₄ (0.2 M) to pH 6.8; volume was then made up to 1L (we have observed that addition of NaCl changes the pH slightly, so it was added before); check by diluting 1:10 and pH should be 7.2-7.4, stored at 4°C.

MTS (2 mg/ml): 2.0 mg MTS was dissolved in 1 ml PBS (0.02M, kept in dark), stored at -20°C.

PMS (9.2 mg/ml, 10x): 9.2 mg was dissolved in 1 ml PBS (0.02M, kept in dark); then diluted ten times in PBS (1x, 0.92 mg/ml), aliquoted and stored at -20°C.

N-acetyl L-cysteine (NAC, MW= 163.19, 50 mM): 4.07 mg NAC dissolved in 0.5 ml medium.

Desferoxamine (DFO, MW= 657, 10 mM): 6.57 mg DFO was dissolved in 1 ml water.

Determination of H₂O₂ conc.:

Using Beer's law, the conc. of H₂O₂ was determined; H₂O₂ was initially diluted in 50 mM PBS (1:5) and then further diluted (1:20) and absorbances measured at 240 nm keeping PBS as blank.

Conc. of H₂O₂ in mM = Sp. ab of H₂O₂ at 240 nm/ 0.0436 (where 0.0436 = ε[{M}⁻¹ cm⁻¹], molar extinction coefficient of H₂O₂).

H₂DCFDA stock solution (MW= 487.29, 2 mM in PBS containing 3% ethanol)

- 20.0 mg H₂DCFDA was weighed in a beaker (50 ml) placed on ice; dissolved in 600 µl absolute ethanol (kept protected from light).
- Gradually 19.4 ml cold PBS was added, stirred for at least 30 min. Aliquoted in small volumes, stored at -20°C and freeze thawing avoided.

Fe₂SO₄, 7H₂O (MW= 278.02, 10 mM): 2.78 mg Fe₂SO₄ was mixed in 1 ml water. Freshly prepared.

CMFDA stock (MW= 464.86, 1 mM):

CMFDA (50 µg, 1 vial) was dissolved in 107.5 µl DMSO, vortexed and stored in small aliquots at -20°C. Repeated freeze thawing was avoided and kept protected from light.

Griess reagent preparation:

- NED (0.1%): 0.01 g NED was dissolved in 10 ml water.
- H₃PO₄ (5%): Add 588 µl H₃PO₄ (88%) to 9.412 ml water.
- Sulphanilamide (1%): 0.1 g sulphanilamide was dissolved in 5 ml 5% H₃PO₄ solution, vortexed vigorously and make up volume to 10 ml. To dissolve bigger particles, the solution was kept at 37°C for a short period (kept on checking).

Griess reagent was always freshly prepared using 1:1 ratio of NED (0.1%) and Sulphanilamide (1%).
Materials and Methods

**NaNO₂ (MW = 69, 10 mM):** 0.69 mg NaNO₂ was dissolved in 1 ml de-ionised water. A working stock of 100 μM was freshly prepared.

**Solutions for measurement of intracellular Calcium**

Fluo4-AM stock (MW = 1096.94, 5 mM): 1 mg Fluo4-AM was dissolved in 182.3 μl DMSO in dark. Stored as small aliquots at -20°C, freeze-thawing was avoided.

Pluronic acid F127 (20% w/v): 2 mg of pluronic acid was dissolved in 10 ml water.

Sulfinpyrazone (MW= 404.48, 250 mM): 101 mg of sulfinpyrazone was dissolved in 1 ml DMSO.

**Fluo-4 AM loading medium (2.5 μM):**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Addition (final conc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-4 AM (5 mM)</td>
<td>2.5 μl (2.5 μM)</td>
</tr>
<tr>
<td>Pluronic acid F127 (20%)</td>
<td>5 μl (0.02%)</td>
</tr>
<tr>
<td>Sulfinpyrazone (250 mM)</td>
<td>5 μl (0.25 mM)</td>
</tr>
<tr>
<td>RPMI PR⁺ medium</td>
<td>4987.5 μl</td>
</tr>
</tbody>
</table>

**Medium A:** Washing medium containing 50 μl of sulfinpyrazone (250 mM) in 50 ml RPMI PR⁺ medium.

**EGTA (MW= 380.35, 250 mM):** 95 mg EGTA was dissolved in 1 ml water.

**JC-1 (MW= 652, 10 mM):**

- 3.26 mg JC-1 was weighed and dissolved in 0.5 ml of DMSO, in dark. Stored in small aliquots at -20°C, freeze-thawing was avoided.
- A working stock of JC-1 (2 μM) was freshly prepared, in the dark, initially diluted 1:10 in DMSO, and further diluted 1:500 in cold PBS; vortexed (till the dye was properly dissolved, solution was light pink in colour) and kept at 4°C.

**Paraformaldehyde fixative solution (PFA, 4%):** 0.4 g of paraformaldehyde was added to 10 ml of PBS (0.02 M), 5-10 μl NaOH (1N) was added to ensure complete solubility of paraformaldehyde; if necessary the solution was heated (65-70°C) for about 10 mins, stored at 4°C.

**Permeabilising solution:**

- Sodium citrate (0.1%) 0.01 g
- Triton X-100 (0.1%) 10 μl

**Preparation:**

Sodium citrate was dissolved in water and triton X-100 was added subsequently. Always used chilled and freshly prepared solution.

**DNase free RNase solution (10 mg/ml):**

5 mg of RNase was dissolved in 0.5 ml de-ionized water, heated to 65 -70°C for 15 min and allowed to cool and stored at -20°C in small aliquots; avoid freeze thawing.

**EDTA-Na₂ (MW= 372.24, 0.5 M, pH = 8.0):**

18.6 g of EDTA was added to 80 ml of ddH₂O and after pH was adjusted to 8.0 with NaOH (MW = 40, 1M), the final volume was made up to 100 ml.

**Tris-Borate-EDTA Buffer (TBE, 1x, 1L):**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weigh/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base (MW = 121.14)</td>
<td>5.4 g</td>
</tr>
<tr>
<td>Boric acid (MW = 61.83)</td>
<td>2.8 g</td>
</tr>
</tbody>
</table>
Materials and Methods

EDTA-Na₂-salt (0.5 mM) 2.0 ml
Preparation:
Tris-base and boric acid were added to 800 ml of ddH₂O, stirred, and after EDTA (0.5 M) was added to adjust the pH to 8.2-8.4, make up to 1L.

Gel loading dye (Specific for DNA laddering):

<table>
<thead>
<tr>
<th>Ingredient (final conc.)</th>
<th>Weigh/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS (0.1%)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bromophenol blue (0.25%)</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Glycerol (30%)</td>
<td>3 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to make up 10 ml</td>
</tr>
</tbody>
</table>

Preparation:
SDS, bromophenol blue were mixed in 7 ml of ddH₂O, thereafter 3 ml of glycerol was added.

DEPC (0.1%, v/v): 500 μl of DEPC was added to 500 ml of autoclaved water.

Tris-HCl (Tris MW = 121.14, pH 7.4, 200 mM): 2.42 g Tris was dissolved in 80 ml of water, thereafter pH was adjusted with HCl (MW = 36.5, 1N) to 7.4, volume made up to 100 ml.

NaCl (MW = 58.44, 400 mM): 233.76 mg was dissolved in 10 ml water.

Preparation of lysis buffer (1 ml):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 7.4, 0.2 M)</td>
<td>100 μl</td>
</tr>
<tr>
<td>NaCl (0.4M)</td>
<td>100 μl</td>
</tr>
<tr>
<td>EDTA (0.5M, pH 8.0)</td>
<td>10 μl</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail (20x)</td>
<td>20 μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>680 μl</td>
</tr>
</tbody>
</table>

Phosphate buffer (PB, 0.1 M, 200 ml):

<table>
<thead>
<tr>
<th>Ingredient (final conc.)</th>
<th>Weigh/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄ (MW = 178)</td>
<td>3.56 g</td>
</tr>
<tr>
<td>NaH₂PO₄ (MW = 156, 0.1M)</td>
<td>3.12 g in 200 ml</td>
</tr>
</tbody>
</table>

Preparation:
Na₂HPO₄ was dissolved in 50 ml; to this solution was added NaH₂PO₄ (0.1M) to make the pH 7.2; volume made up to 200 ml, then the working stock was 0.01 M and its pH should increase to 7.8.

Wash buffer: PBS (pH 7.2) containing 0.1% v/v Tween 20.

Blocking/dilution buffer (2% v/v): 1 ml FCS was added to 50 ml PBS (0.02 M).

Phorbol Myristate Acetate (PMA, 1 mg/ml): 1 mg PMA was dissolved in 1 ml of DMSO, stored in small aliquots at -20°C. Repeated freeze thawing was avoided and kept protected from light.

Ionomycin (MW = 747.07, 1 mM): 1 mg ionomycin was dissolved in 1338.6 μl DMSO, stored in small aliquots at -20°C. Repeated freeze thawing was avoided and kept protected from light.

Brefeldin A (10 mg/ml): 10 mg Brefeldin A was dissolved in 1 ml DMSO, stored as small aliquots at -20°C.