

Artemisinin triggers induction of cell-cycle arrest and apoptosis in *Leishmania donovani* promastigotes

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A major impediment to effective anti-leishmanial chemotherapy is the emergence of drug resistance, especially to sodium antimony gluconate, the first-line treatment for leishmaniasis. Artemisinin, a sesquiterpene lactone isolated from *Artemisia annua*, is an established anti-malarial compound that showed anti-leishmanial activity in both promastigotes and amastigotes, with IC₅₀ values of 160 and 22 µM, respectively, and, importantly, was accompanied by a high safety index (>22-fold). The leishmanicidal activity of artemisinin was mediated via apoptosis as evidenced by externalization of phosphatidylserine, loss of mitochondrial membrane potential, *in situ* labelling of DNA fragments by terminal deoxyribonucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) and cell-cycle arrest at the sub-G₀/G₁ phase. Taken together, these data indicate that artemisinin has promising anti-leishmanial activity that is mediated by programmed cell death and, accordingly, merits consideration and further investigation as a therapeutic option for the treatment of leishmaniasis.

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INTRODUCTION

Leishmania, a unicellular trypanosomatid protozoan parasite, is the causative organism of leishmaniasis, which comprises a wide disease spectrum ranging from localized, self-healing, cutaneous lesions to disfiguring mucocutaneous leishmaniasis and the visceral form, which can be fatal if neglected (Murray *et al.*, 2005). In the past decade, an unprecedented increase in unresponsiveness to antimonials, the first line of treatment, in visceral leishmaniasis has been observed, importantly in the Indian subcontinent, a major endemic area of visceral leishmaniasis (Croft *et al.*, 2006). In the absence of an effective vaccine, chemotherapy remains the sole weapon in the arsenal against leishmaniasis (Murray *et al.*, 2005). Current treatment modalities are limited, have the potential to develop resistance and possess unacceptable toxicity (Sundar & Chatterjee, 2006).

In the ongoing search for better leishmanicidal compounds, plant-derived products are gaining ground (Dutta *et al.*, 2007a, b; Sen *et al.*, 2007). Artemisinin, a sesquiterpene endoperoxide isolated from *Artemisia annua*, is an estab-

lished anti-malarial drug (Bhakuni *et al.*, 2001; Meshnick, 2002). However, there has only been one study investigating its anti-leishmanial activity (Yang & Liew, 1993), where its efficacy against experimental cutaneous leishmaniasis was demonstrated. Studies of the efficacy of artemisinin against *Leishmania donovani*, the causative organism of visceral leishmaniasis, have been restricted to promastigotes (Avery *et al.*, 2003), and studies of its efficacy in amastigotes, the biologically relevant form, are notably absent. The anti-leishmanial activity of an ethanolic extract of the leaves of *Artemisia indica* was investigated in exponential-phase promastigotes from six strains responsible for cutaneous, mucocutaneous or visceral leishmaniasis and the IC₅₀ values ranged from 0.21 to 0.58 mg ml⁻¹ (Ganguly *et al.*, 2006a). Accordingly, the current study was undertaken to establish the leishmanicidal efficacy of artemisinin in *L. donovani*, and to demonstrate the high safety index of the drug and delineate the molecular mechanisms that contribute to the observed leishmanicidal activity.

METHODS

Chemicals. All chemicals used were of analytical grade. RPMI 1640 was obtained from Gibco-BRL, methanol and ethanol from Merck, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) from Molecular Probes, FITC-conjugated annexin V

Abbreviations: MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium inner salt; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labelling.

from BD Biosciences and a Cell Death Detection kit from Roche. All other chemicals were from Sigma. A 200 mM stock solution of artemisinin was prepared in tissue-culture-grade DMSO and stored at -20°C ; miltefosine and pentamidine were freshly prepared in M199 medium (serum-free).

Parasite culture. Promastigotes of *L. donovani* strain MHOM/IN/83/AG83 were routinely cultured at 24°C in M199 medium supplemented with 10% fetal calf serum, 50 U penicillin ml^{-1} and 50 μg streptomycin ml^{-1} , and subcultured every 72 h using an inoculum of 1×10^6 cells ml^{-1} .

Cell line culture. A non-adherent human monocyte cell line, THP1, obtained from the National Centre for Cell Sciences (Pune, India), was maintained in RPMI 1640 supplemented with 10% fetal calf serum, 50 U penicillin ml^{-1} and 50 μg streptomycin ml^{-1} at 37°C , 5% CO_2 . The cells were subcultured every 72 h using an inoculum of 2×10^5 cells ml^{-1} .

In vitro evaluation of anti-promastigote activity. The anti-leishmanial activity of artemisinin was initially established in promastigotes, with cell viability being measured using a modified MTS assay (Ganguly *et al.*, 2006b). Briefly, exponential-phase promastigotes (2×10^5 cells in 200 μl M199 medium per well) were incubated with artemisinin (0–0.5 mM) for 48 h. Pentamidine (0–10 μM) and miltefosine (0–50 μM) served as reference drugs, as well as 0.25% DMSO, which represented the highest concentration of DMSO present in the artemisinin (0.5 mM). After treatment, parasite viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethylthioxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium inner salt (MTS). The results were expressed as IC_{50} values, i.e. the concentration that inhibited 50% of cell growth, enumerated by graphic extrapolation using GRAPHPAD PRISM software (version 4).

Evaluation of ex vivo anti-amastigote activity. Exponential-phase, non-adherent THP1 monocytes seeded in eight-well slides (BD Biosciences) (5×10^4 cells in 200 μl per well) were incubated with 20 nM phorbol myristate acetate at 37°C , 5% CO_2 for at least 12 h. Adherent macrophages were equilibrated in phorbol myristate acetate-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 artemisinin (0–250 μM) 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 (Dube *et al.*, 2005).

To evaluate the safety index of artemisinin, murine peritoneal macrophages were lavaged from pre-primed BALB/c mice (2% starch, 2 ml per mouse) and seeded in 96-well plates (2×10^5 in 200 μl per well) at 37°C , 5% CO_2 . After removal of non-adherent cells, macrophages were incubated with artemisinin (0–0.5 mM) for 48 h at 37°C , 5% CO_2 , and its effect on macrophage viability was enumerated using an MTS assay as described above.

Analysis of phosphatidylserine externalization. Double staining for annexin V–FITC and propidium iodide (PI) was performed as described previously (Dutta *et al.*, 2007a). Briefly, promastigotes were incubated with an IC_{50} dose of artemisinin (160 μM) for 24 and 48 h at 24°C and cells were centrifuged (1000 g for 10 min), washed twice in 0.02 M PBS (pH 7.2) and resuspended in annexin V binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2]. Annexin V–FITC and PI (1 μg ml^{-1}) were then added according to the manufacturers' instructions and incubated for 30 min in the dark at 20 – 25°C . Data acquisition was carried out using a FACSCalibur flow cytometer (BD Biosciences) and analysed with CELLQUEST PRO software.

Analysis of mitochondrial transmembrane potential. To assess the mitochondrial transmembrane electrochemical gradient ($\Delta\psi_m$), a cell-permeable, cationic and lipophilic dye, JC-1, was used. This probe aggregates within mitochondria and fluoresces red at higher transmembrane potentials. However, at lower transmembrane potentials, JC-1 cannot accumulate within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green. Therefore, the ratio of red to green fluorescence gives a measure of the transmembrane electrochemical gradient (Verma *et al.*, 2007).

Accordingly, to evaluate the effect of artemisinin on mitochondrial membrane potential, promastigotes were incubated with 160 μM artemisinin for 24 and 48 h at 24°C . Drug-treated and control cells were stained at corresponding time points with JC-1 (3 μM in PBS) and analysed by flow cytometry as described previously (Dutta *et al.*, 2007b). Briefly, after drug treatment, cells were washed in PBS and incubated with JC-1 for 15 min at 20 – 25°C . Data acquisition was carried out using a FACSCalibur and analysed using CELLQUEST PRO software. Heat-killed promastigotes, representative of cells with depolarized mitochondria, were processed and analysed similarly.

In situ detection of DNA fragmentation by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL).

In situ detection of DNA fragments following treatment of promastigotes with an IC_{50} concentration of artemisinin (160 μM) for 24 and 48 h at 24°C was measured by TUNEL using a Cell Death Detection kit (Roche) according to the manufacturer's instructions. Cells were then washed, fixed with paraformaldehyde (2% in PBS, pH 7.4) and kept on ice for 1 h. Thereafter, 2×10^5 cells were centrifuged (1800 g for 5 min), resuspended in 10 μl PBS, spotted on to slides and air dried; they were washed with PBS and incubated with H_2O_2 (3% in methanol) for 10 min at 4°C . The slides were washed again with PBS, placed on ice and permeabilized with freshly prepared, chilled 0.1% sodium citrate in 0.1% Triton X-100 solution for 15 min. Cells were washed twice with PBS, after which a 25 μl reaction mixture containing TdT and dUTP–FITC (Cell Death Detection kit; Roche) was added. The slides were then incubated in a humidified chamber at 37°C for 1 h, washed with PBS and 25 μl converter POD (anti-fluorescein antibody conjugated to horseradish peroxidase, Cell Death Detection kit; Roche) was added and incubated for 30 min at 37°C . Finally, 25 μl diaminobenzidine (Cell Death Detection kit; Roche) was added and slides were incubated at 4°C for 10 min, washed with deionized water and observed microscopically under an oil-immersion objective; at least 20 microscope fields were observed for each sample. In parallel, after addition of the reaction mixture containing TdT and dUTP–FITC, cells were incubated at 37°C for 1 h; cells were then washed and resuspended in PBS before data acquisition on a FACSCalibur and analysis using CELLQUEST PRO software.

Effect of artemisinin on the cell cycle. Parasites (1×10^6 cells) were treated with an IC_{50} dose of artemisinin (160 μM) for 24 and 48 h at 24°C ; at each time point, cells were fixed in chilled 70% ethanol and kept at -20°C until analysis. After washing the cells in PBS, the resultant pellet was resuspended in 500 μl DNase-free RNase (200 μg ml^{-1}) and incubated for 1 h at 37°C . Cells were then stained with PI (40 μg ml^{-1}) and incubated in the dark for 20 min at 20 – 25°C . Data acquisition was carried out using a FACSCalibur and analysed using CELLQUEST PRO software.

RESULTS AND DISCUSSION

Anti-promastigote activity of artemisinin

The leafy portion of *Artemisia* sp. (Chinese name qinghao) was used in traditional Chinese medicine as early as 168 BC,

but it was not until 1972 that scientists purified and determined the chemical structure of this sesquiterpene lactone and designated it artemisinin (Meshnick, 2002). The effect of artemisinin on the viability of promastigotes was evaluated using an MTS assay wherein the conversion of MTS to formazan by mitochondrial enzymes in the presence of the electron coupler phenazine methosulfate served as an indicator of cell viability; thus, a decrease in formazan production indicated decreased cell viability and vice versa (Ganguly *et al.*, 2006b). Treatment of promastigotes with artemisinin (0–0.5 mM for 48 h) demonstrated a dose-dependent inhibition of parasite growth and an IC₅₀ value in promastigotes of 160 µM. In parallel, the IC₅₀ values of the reference drugs miltefosine and pentamidine were evaluated as 21.0 and 1.25 µM, respectively, which correlated with previous reports (Ganguly *et al.*, 2006b). The addition of 0.25 % DMSO caused no change in parasite viability (data not shown).

The IC₅₀ value of artemisinin agreed with previous reports using *L. donovani* promastigotes (Avery *et al.*, 2003) in which artemisinin and over 70 of its derivatives were studied. Some derivatives had an IC₅₀ value as low as 1.4 µM, suggesting that, with minor modifications, the anti-leishmanial potency of artemisinin could be enhanced; however, these need to be tested in the classical amastigote–macrophage model and their safety profile evaluated. With regard to *Leishmania* strains responsible for cutaneous leishmaniasis, artemisinin was more potent, with an IC₅₀ value of 750 nM for promastigotes and 3–30 µM for intracellular amastigotes (Yang & Liew, 1993).

Anti-amastigote activity of artemisinin

During *Leishmania* infection, promastigotes transform into amastigotes within phagolysosomal vacuoles of macrophages. Accordingly, the anti-leishmanial activity of artemisinin (0–250 µM for 48 h) was tested against intracellular amastigotes in *L. donovani*-infected THP1 cells. The infection rate of artemisinin-treated macrophages was normalized to 100 % for further analysis of the results. Artemisinin showed a dose-dependent reduction in parasitic load and an IC₅₀ value of 22 µM, which was 7-fold lower than the IC₅₀ value obtained in promastigotes.

To evaluate the safety index of artemisinin, its effect (0–0.5 mM for 48 h) on viability of murine peritoneal macrophages was evaluated using an MTS assay. Macrophage viability remained unaffected up to 0.25 mM; at the highest concentration of 0.5 mM, cell viability was 60 % and therefore the IC₅₀ value of artemisinin in macrophages was at least 22-fold higher than in amastigotes.

The IC₅₀ value of artemisinin as an anti-malarial drug is in the nanomolar range (4.2–16.2 nM) whereas, for anti-leishmanial activity, micromolar concentrations are required. However, the safety index of the drug as an anti-leishmanial is high and, as oral, parenteral and rectal dosages can achieve micromolar concentrations (Räth *et al.*,

2004), artemisinin could well be considered a potential anti-leishmanial drug. Artemisinin compounds have also been shown to inhibit *in vitro* growth of *Trypanosoma cruzi* and *Trypanosoma brucei* subsp. *rhodesiense* at micromolar concentrations, providing evidence of its expanding therapeutic potential (Mishina *et al.*, 2007).

Artemisinin induces phosphatidylserine externalization in *L. donovani* promastigotes

Artesunate, an analogue of artemisinin, has been shown to trigger apoptosis in tumour cells by both p53-dependent and -independent pathways (Efferth, 2007). In parasites, apoptosis, or programmed cell death, appears to be the predominant form of cell death, as has been observed in kinetoplastids (Arnoult *et al.*, 2002) in response to diverse stimuli, e.g. heat shock, chemotherapeutic agents such as pentostam and amphotericin B, oxidants such as H₂O₂, serum depletion and plant extracts such as *Aloe vera* leaf exudate (Dutta *et al.*, 2007b).

Following an apoptotic stimulus, phosphatidylserine present in the inner leaflet of the plasma membrane flips out to the outer leaflet of the plasmalemma; thus, externalization of phosphatidylserine is considered to be a marker of apoptosis (Debrabant & Nakhasi, 2003). The binding of annexin V, a Ca²⁺-dependent phospholipid-binding protein known to have a strong affinity towards phosphatidylserine, is a proven measure of apoptosis. To distinguish apoptotic cell death from necrotic cell death, cells were counterstained with PI, a non-permeable stain with an affinity for nucleic acids, as it selectively enters necrotic cells. Therefore, co-staining of annexin V and PI can differentiate between cells undergoing early apoptosis (annexin V-positive, PI-negative) or necrosis (PI-positive, annexin V-negative) and live cells (PI- and annexin V-negative).

In untreated promastigotes, the degree of binding of annexin V at 24 and 48 h was 2.31 and 2.35 %, respectively. Following treatment of promastigotes with artemisinin at its IC₅₀ value of 160 µM for 24 and 48 h, the percentage of annexin V-positive cells increased to 7.62 % at 24 h and 38.97 % at 48 h (Fig. 1, lower-right quadrant). The percentage of PI-stained cells (Fig. 1, upper-left quadrant) ranged from 0.19 to 0.55 %, indicating that artemisinin exerts its anti-leishmanial activity primarily via apoptosis.

Artemisinin induces depolarization of mitochondrial transmembrane potential in promastigotes

Maintenance of the mitochondrial transmembrane potential is essential for parasite survival, as *Leishmania* has a single mitochondrion. JC-1, a cationic lipophilic dye, becomes concentrated in the mitochondria in proportion to the membrane potential wherein it forms J-aggregates, which emit red fluorescence at 590 nm, whereas, during depolarization, the dye remains as a monomer, emitting

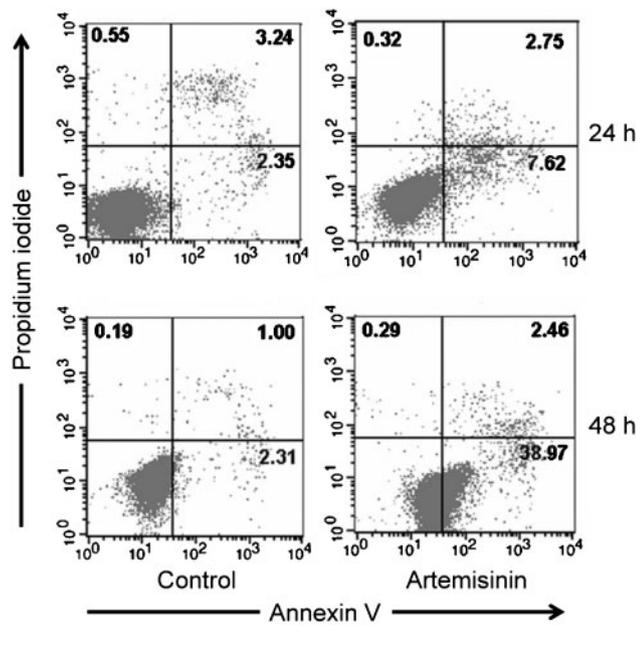


Fig. 1. Externalization of phosphatidylserine in artemisinin-treated promastigotes. Promastigotes (strain MHOM/IN/83/AG83; 1×10^6 cells ml^{-1}) were incubated with an IC_{50} dose of artemisinin (160 μM) for 24 and 48 h, co-stained with PI and annexin V-FITC and analysed by flow cytometry as described in Methods. The lower-left quadrant indicates the percentage of unstained cells, the upper-left shows PI-positive cells, the lower-right shows annexin V-stained cells and the upper-right shows PI- and annexin V-positive cells. The figure shows a representative result of at least three experiments.

green fluorescence at 530 nm. Therefore, the ratio of red:green fluorescence serves as an indicator of the mitochondrial energy state of the parasite.

The loss of mitochondrial membrane potential is a characteristic feature of metazoan apoptosis and has been observed to play a key role in drug-induced death in protists such as *Leishmania* (Sen *et al.*, 2004). In untreated cells, the red:green fluorescence ratio at 24 and 48 h was 0.978 and 1.95, respectively. However, the addition of 160 μM artemisinin caused a loss of mitochondrial membrane potential, blocking JC-1 entry to the mitochondria, leaving the JC-1 monomers to fluoresce green within the cytoplasm. This was reflected in the red:green fluorescence ratio, which decreased to 0.521 and 0.55 following drug treatment for 24 and 48 h, respectively (Fig. 2).

In a dot-plot analysis, JC-1 fluorescence was seen in both the FL-2 (R2-gated population) and FL-1 (R3-gated population) channels. The R2 gate was predominantly red fluorescence and reflected the non-apoptotic (healthy) population, whereas the R3 gate was prominently green fluorescence and reflected the apoptotic cell population. In heat-killed promastigotes, 10.44% of cells were within the

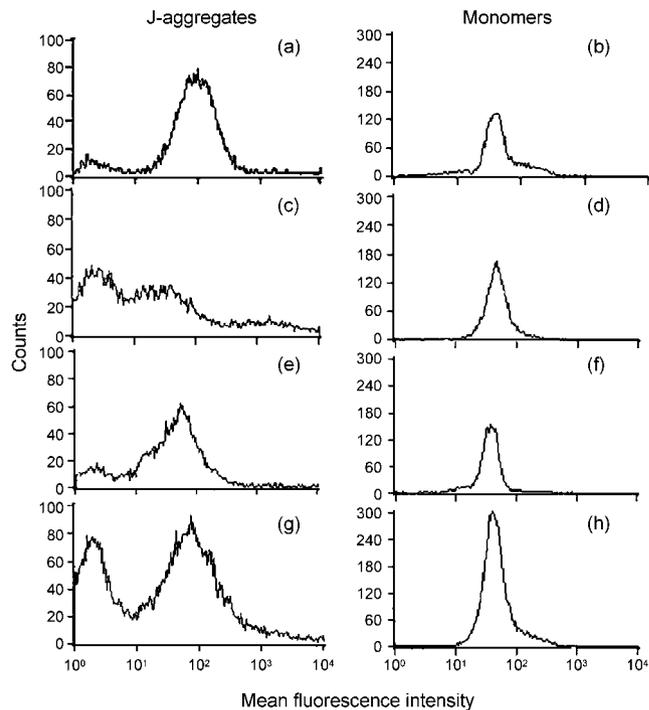


Fig. 2. Effect of artemisinin on mitochondrial function. Promastigotes (strain MHOM/IN/83/AG83, 1×10^6 cells ml^{-1}) were treated with an IC_{50} dose of artemisinin (160 μM) for 24 (a–d) and 48 h (e–h) and stained with JC-1 as described in Methods. The presence of J-aggregates (a, c, e, g) (red fluorescence, 590 nm) and monomers (b, d, f, h) (green fluorescence, 530 nm) for cells left untreated (a, b, e, f) or treated with artemisinin (c, d, g, h) is shown.

R2 gate and 82.89% within the R3 gate, confirming detection of depolarized membrane potential. In untreated cells at 24 h, the R2 gate contained 88.16% of the population, whereas the R3 gate contained only 7.27% of the population. With the addition of artemisinin (160 μM for 24 h) the percentage positivity in the R2 gate decreased to 30.63% and the R3 population increased dramatically to 49.44%. At 48 h, untreated cells had 75.35% in the R2 gate and 21.18% in the R3 gate. The addition of artemisinin (160 μM for 48 h) caused a decrease in the R2-gated population to 53.65% whilst, in the R3 gate, the percentage positivity increased to 34.55%. Taken together, these data indicated that artemisinin caused induction of apoptosis associated with depolarization of the mitochondria.

The nature of the trigger for artemisinin-mediated loss of mitochondrial membrane potential remains unclear. The anti-malarial action of artemisinin and its derivatives is proposed to be via its reaction with iron (ferrous compounds) (Golenser *et al.*, 2006). The haemoglobin within infected erythrocytes is digested by the parasite, and haem is released and neutralized by polymerization into haemozoin, with a high Fe^{2+} content. When artemisinin comes into contact with high iron concentrations, a chemical reaction is triggered that generates free radicals,

which are primarily responsible for the anti-malarial action (Golenser *et al.*, 2006). It remains to be seen whether a similar scenario occurs within the *Leishmania* parasite.

Artemisinin induces *in situ* DNA nicking in *L. donovani* promastigotes

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. To evaluate the endonuclease activity of artemisinin (160 μ M for 24 and 48 h, 24 $^{\circ}$ C), *in situ* TUNEL staining was performed. In parasites treated with artemisinin for 24 h, brown deposits representing incorporated TdT-labelled nuclei were observed, which increased further when parasites were incubated with artemisinin for 48 h, indicating nicking of the DNA (data not shown).

The occurrence of DNA nicking was also detected by quantifying the binding of FITC-labelled dUTP to the nicked ends via TdT, as the proportion of DNA nicks is directly proportional to the fluorescence obtained. Promastigotes treated with artemisinin (160 μ M for 24 h) caused an increase in nuclear DNA fragmentation, as the dUTP-FITC binding following artemisinin treatment for 24 h increased from a baseline mean fluorescence intensity of 35.96 in untreated cells to 110.27, thus confirming that the leishmanicidal activity of artemisinin occurs via apoptosis (Fig. 3).

Artemisinin induces sub- G_0/G_1 phase cell-division arrest

Flow cytometric analysis after cell permeabilization and labelling with PI was used to quantify the percentage of pseudohypodiploid cells. In a given cell, the amount of bound dye correlates with the DNA content and thus DNA

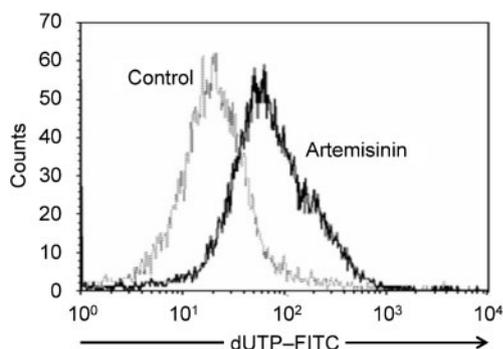


Fig. 3. Analysis of TUNEL positivity in artemisinin-treated promastigotes. Promastigotes (strain MHOM/IN/83/AG83; 1×10^6 cells ml^{-1}) incubated with an IC_{50} dose of artemisinin (160 μ M for 24 h) were stained with dUTP-FITC as described in Methods. The grey trace shows a control without artemisinin. The figure shows a representative profile of at least three experiments.

Table 1. Effect of artemisinin on the cell cycle of *L. donovani* promastigotes

L. donovani promastigotes (1×10^6 cells ml^{-1}) were treated with 160 μ M artemisinin for 24 or 48 h and processed for cell-cycle analysis as described in Methods. Values are percentages.

Group	Sub- G_0/G_1 (M1)	G_0/G_1 (M2)	S and G_2/M (M3)
24 h			
Control	3.62	55.92	41.03
Artemisinin	10.86	62.28	27.45
48 h			
Control	3.03	67.78	29.65
Artemisinin	33.11	57.42	12.03

fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G_0/G_1 cells, i.e. a sub- G_0/G_1 peak. In promastigotes incubated with artemisinin (160 μ M for 24 h), the proportion of cells in the sub- G_0/G_1 phase increased to 10.86% compared with 3.62% of controls (Table 1). Following 48 h of drug treatment, the proportion of cells in the sub- G_0/G_1 phase increased further to 33.11% compared with 3.03% in control cells. This increase in sub- G_0/G_1 phase was accompanied by a decrease in the number of cells in the G_2/M phase compared with untreated cells, with values of 27.45 vs 41.03% at 24 h and 12.03 vs 29.65% at 48 h (Table 1). Taken together, the increased proportion of cells in the sub- G_0/G_1 phase confirmed that artemisinin-induced apoptosis in promastigotes resulted in DNA degradation.

Apoptotic-like changes have been shown to mediate the action of several anti-leishmanial drugs including miltefosine (Verma *et al.*, 2007), amphotericin B (Moreira *et al.*, 1996) and *Aloe vera* leaf exudate (Dutta *et al.*, 2007a, b). In this study, we have shown that artemisinin has a leishmanicidal effect against both promastigotes and amastigotes of *L. donovani*, with cell death being via apoptosis. The 7-fold lower IC_{50} in amastigotes compared with promastigotes suggests that its leishmanicidal activity is enhanced by immunomodulation of macrophages. As the pharmacokinetics of artemisinin have been evaluated extensively and its oral bioavailability proven, the stage is set to study the potential of artemisinin as an anti-leishmanial compound in animal models, with the potential to strengthen the therapeutic control of leishmaniasis.

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