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

Rupashree Sen,1 Samiran Bandyopadhyay,2 Avijit Dutta,1 Goutam Mandal,1 Sudipto Ganguly,1 Piu Saha1 and Mitali Chatterjee1

Correspondence
Mitali Chatterjee
ilatim@vsnl.net

1Department of Pharmacology, Institute of Postgraduate Medical Education & Research, 244 B Acharya J. C. Bose Road, Kolkata 700 020, India
2National Research Centre on Yak, Indian Council of Agricultural Research, Dirang, Arunachal Pradesh 790101, India

Received 26 April 2007
Accepted 22 May 2007

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 deoxynucleotidyltransferase-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.

**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 established 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'-tetracloro-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.
Analysis of mitochondrial transmembrane potential. To assess
the mitochondrial transmembrane electrochemical gradient (ΔΨm), 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
software. Heat-killed promastigotes, representative of cells with
depolarized mitochondria, were processed and analysed similarly.

In situ detection of DNA fragmentation by terminal deoxynu-
cleotidyl transferase (TdT)-mediated dUTP nick end labelling
(TUNEL). In situ detection of DNA fragments following treatment of
promastigotes with an IC50 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 parafomaldehyde (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 on to slides and air dried; they were washed with PBS and incubated with H2O2 (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-fluorescin 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 software.

Effect of artemisinin on the cell cycle. Parasites (1 × 10^6 cells)
were treated with an IC50 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 flow
cytometer (BD Biosciences) and analysed with CELLQUEST 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 amphoterin 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
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 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 haemazoin, 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 µM for 24 and 48 h, 24 °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 µM 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-G0/G1 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 fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G0/G1 cells, i.e. a sub-G0/G1 peak. In promastigotes incubated with artemisinin (160 µM for 24 h), the proportion of cells in the sub-G0/G1 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-G0/G1 phase increased further to 33.11 % compared with 3.03 % in control cells. This increase in sub-G0/G1 phase was accompanied by a decrease in the number of cells in the G2/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-G0/G1 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 IC50 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.

**ACKNOWLEDGEMENTS**

This work received financial assistance from the Life Sciences Research Board, DRDO, and the University Grants Commission, Government of India. S.G. and G.M. are recipients of a Senior Research fellowship from the University Grants Commission and the Council of Scientific and Industrial Research, Government of India, respectively.
REFERENCES


