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ORIGINAL ARTICLE

Iron enhances generation of free radicals by Artemisinin causing a caspase-independent, apoptotic death in *Leishmania donovani* promastigotes

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Abstract

An increasing incidence of unresponsiveness to antimonials in Leishmaniasis has led to identification of plant-derived anti-leishmanial compounds like Artemisinin. Since iron-mediated generation of free radicals sustains the anti-malarial activity of Artemisinin, this study investigated whether similar mechanisms accounted for its activity in *Leishmania* promastigotes. Artemisinin effectively disrupted the redox potential via an increased generation of free radicals along with a decrease in levels of non-protein thiols. Attenuation of its IC_{50} by a free radical scavenger N-acetyl l-cysteine and an iron chelator desferoxamine established the pivotal role of free radicals and of the potentiating effect of iron. An enhanced Fluo-4 fluorescence reflected Artemisinin-induced mobilization of intracellular calcium, which triggered apoptosis. However, the absence of any detectable caspase activity indicated that the leishmanicidal activity of Artemisinin is mediated by an iron-dependent generation of reactive intermediates, terminating in a caspase-independent, apoptotic mode of cell death.

Keywords: *Artemisinin, anti-leishmanial, free radicals, iron, leishmaniasis*

Introduction

Leishmaniasis, caused by intracellular trypanosomatid parasites of the genus *Leishmania*, manifests primarily as three forms, namely cutaneous (CL), mucocutaneous (MCL) and visceral (VL), the last being fatal if left untreated. Faced with increasing resistance, traditional antimony-based drugs are being sidelined with a renewed emphasis on development of anti-leishmanial compounds targeting parasite-specific biochemical pathways [1].

In intracellular trypanosomatids, protection against oxidative stress is vital for survival as host phagocytic cells can generate copious amounts of reactive oxygen and reactive nitrogen intermediates, all directed against invading pathogens [2,3]. In *Leishmania* parasites, their anti-oxidant defences are relatively compromised owing to the absence of catalase and selenium-dependent glutathione peroxidases, thus making them overly reliant upon sulphur (SH) catalysis ([4] and references therein). Many organisms, including their mammalian hosts, maintain a number of selenium-dependent peroxidases, as these selenoenzymes have a far greater catalytic efficiency than SH-dependent peroxidases due to the greater nucleophilicity of selenium [5]. *Leishmania* instead possesses a unique but relatively weak trypanothione-dependent anti-oxidant system, which specifically targets the deleterious hydroperoxides, intermediates crucial to the regulation of redox homeostasis [4].

Understandably, a credible chemotherapeutic approach would be to exploit this impaired anti-oxidant capacity of *Leishmania* parasites by inducing oxidative stress [6,7]. This is indeed the case with sodium

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antimony gluconate, the therapeutic mainstay for leishmaniasis which generates reactive oxygen species (ROS) both within host macrophage phagolysosomes [3,8] and the parasite [9]. Indeed this also holds true for emerging experimental anti-leishmanial agents such as Curcumin [10], metalloids [11] and Berberine chloride [12].

Artemisinin, a sesquiterpene lactone endoperoxide, was isolated from the Chinese medicinal plant *Artemisia annua L* (qinghaosu) and is used worldwide for the treatment of malaria [13–16]. Studies have confirmed its anti-parasitic effect in *Leishmania* [17–19] and confirmed its therapeutic potential in experimental models of Leishmaniasis [20,21], *Trypanosoma* sp. [22], *Clonorchis* [23], Schistosomiasis [24] and cancer cell lines [15,25]. As its anti-malarial action has been attributed to an intra-parasitic iron- or heme-catalysed cleavage of the endoperoxide bridge leading to generation of toxic free radicals or intermediates [13,25], we provide evidence for the involvement of iron and free radicals in triggering apoptosis in Artemisinin-treated *L. donovani* promastigotes [17].

Materials and methods

Materials

All chemicals were of analytical grade and obtained from Sigma Aldrich Chemicals (St Louis, MO) except Phenazine methosulphate (PMS, Sisco Research Laboratories, Mumbai, India), MTS or 3-(4,5-dimethylthiazol–2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI), 5-chloromethylfluorescein-diacetate (CMFDA), fLUO-4am were obtained from (Invitrogen, Carlsbad, CA), Z-Val-Ala-DLAsp (methoxy)-fluoromethylketone (Z-VAD-FMK) were from BD Biosciences (San Jose, CA) and Caspase-3/CPP32 Colorimetric assay kit (Biovision, Mountain View, CA); Artemisinin (100 mM in DMSO) was stored at 4°C until used.

Parasite culture

Promastigotes of *L. donovani* were routinely cultured at 24°C in M-199 medium, supplemented with 10% foetal calf serum (FCS), penicillin G (50 IU/ml) and streptomycin (50 μg/ml); cells were passaged every 72 h, 1×10^6/ml being the inoculum.

Cell culture

U937, a human leukaemia cell line, was obtained from the National Centre for Cell Science, Pune, India, maintained at 37°C, 5% CO_2 in RPMI (PR^+) medium, supplemented with 10% FCS, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). The cells were passaged every 72 h, 2×10^5 cells/ml being the initial inoculum.

Measurement of reactive oxygen species (ROS)

To study the effect of Artemisinin on generation of ROS, log phase promastigotes (1×10^6/ml) were incubated with Artemisinin (0–0.5 mM) for 3 h at 37°C. Cells were washed with phosphate buffered saline (PBS, 0.02 M, pH 7.2), incubated with 2, 7-dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 μM) for 45 min at 37°C and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA) using forward vs side scatter to gate the parasite population and a FL1 histogram to quantify fluorescence [9]. Subsequent analyses were done using BD CellQuest Pro software.

Measurement of non-protein thiols using CMFDA

To measure levels of non-protein thiols, Artemisinin-treated promastigotes were incubated with increasing concentrations of drug (0–0.5 mM) 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 the dark and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA) with subsequent analyses done using BD CellQuest Pro software [26].

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 (2×10^5 cells/200 μl of M199 + 10% FCS/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 MTS-PMS cell viability assay [27]; after drug treatment, cells were examined microscopically and then 20 μl of a solution comprising MTS (2.0 mg/ml) and PMS (0.92 mg/ml) in a ratio of 5:1 were added per well. The plates were then further incubated for 3 h at 37°C and absorbances of resultant formazan measured at 490 nm using a plate reader (BioRad, CA). MTS is converted to formazan by mitochondrial dehydrogenases of viable parasites in the presence of an electron coupler PMS. Therefore, the amount of formazan produced was considered as a measure of cell viability. The mean percentage viability was calculated as:

$$\frac{\text{Mean specific absorbance of drug treated parasites}}{\text{Mean specific absorbance of untreated parasites}} \times 100$$

where specific absorbances were determined by subtracting the background absorbance of medium which contained Artemisinin ± NAC. 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).

Determination of role of iron in Artemisinin-induced leishmanicidal activity

As iron is proposed to play a synergistic role in generation of carbon-centred free radicals from Artemisinin, generation of ROS by Artemisinin treated promastigotes in the presence of a ferrous compound was measured. Log phase promastigotes (1×10^6/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 ($7H_2O$, 0.2 mM) for an additional 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 concentration of desferoxamine (DFO, 0.25 mM), an iron chelator on the leishmanicidal activity of Artemisinin, was similarly studied by co-incubating log phase promastigotes with Artemisinin (0–0.5 mM) for 48 h at 24°C and cell viability measured using the MTS-PMS assay [27].

Measurement of intracellular Ca^{2+} in L. donovani promastigotes

Changes in intracellular Ca^{2+} were monitored using the fluorescent probe Fluo-4 AM as previously described [12]. Briefly, promastigotes (2×10^6 cells/ml) were pre-loaded with Fluo-4 (2.5 μM) in medium containing pluronic acid F127 (0.02%) and sulphinpyrazone (0.25 mM) at 24°C for 30 min. The cells were then washed with medium containing 0.25 mM sulphinpyrazone (Medium A) and incubated up to 6 h with an IC_{90} concentration of Artemisinin (0.5 mM). In parallel, cells were incubated with a Ca^{2+} ionophore Ionomycin (2.5 μM) in the absence (positive control) and presence of a chelating agent, ethylene glycol tetraacetic acid (EGTA, 5 mM, negative control). 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 $[Ca^{2+}]_t$, calibration was performed at the end of each experiment and calculated using the following equation [11]:

$$[Ca^{2+}]_t = K_d[(F - F_{min})/(F_{max} - F)],$$

where K_d =dissociation constant of the Ca^{2+} Fluo-4 complex (345 nM); F= fluorescence intensity of Artemisinin-treated cells; F_{max} = maximal fluorescence, i.e. cells treated with Ionomycin, and F_{min} = minimum fluorescence, i.e. cells treated with Ionomycin (2.5 μM) and EGTA (5 mM).

Determination of caspase activity in Artemisinin treated promastigotes

Caspase activity was measured using a commercially available kit (Biovision, Mountain View, CA), as per manufacturer's instructions. Briefly, promastigotes were treated for 12 h with an IC_{90} concetnration of Artemisinin (0.5 mM); promastigotes were pelleted, washed with ice-cold PBS, cell lysates prepared and protein concentration estimated. To detect activity of CED3/CPP32 group proteases, cell lysates (100 μg protein) in reaction buffer containing 10 mM DTT, substrate DEVD-pNA (4 mM, 5 μL) were incubated at 37°C up to 4 h; the absorbance of pNA was quantified at 405 nm [12]. In parallel, assay specificity was confirmed using U937 cells that were treated with a similar concentration of hydrogen peroxide (H_2O_2, 4 mM, 30 min) as also parasites were similarly treated.

To study the biological role of caspases in Artemisinin-induced death, log phase promastigotes (2×10^5/200 μL/well) were seeded in 96-well tissue culture plates. Following a 48 h incubation at 24°C with Artemisinin (0–0.5 mM) in the presence and absence of the pan-caspase inhibitor, Z-VAD-FMK (100 μM), 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, enumerated by graphic extrapolation using GraphPad Prism software (version 5).

Statistical analysis

The data are presented as mean ± SEM using GraphPad Prism software (version 5.0) to assess significance of differences; $p < 0.05$ was considered as statistically significant.

Results

Artemisinin increased generation of ROS along with a concomitant decrease in levels of non-protein thiols

To establish whether Artemisinin has a pro-oxidant effect on promastigotes, we measured the generation of ROS using H_2DCFDA, a lipid-soluble, membrane-permeable primarily non-fluorescent compound which, following enzymatic cleavage by non-specific esterases, forms H_2DCF which is then oxidized by intracellular ROS to produce a fluorescent compound DCF; therefore, the resultant fluorescence is a direct measure of the amount of ROS generated.

Log phase promastigotes (1×10^6/ml) incubated with increasing doses of Artemisinin (0–0.5 mM, 3 h) at 37°C showed a dose-dependent increase in generation of ROS, the basal mean ± SEM of GMFC (Geometric mean fluorescence channel) of healthy cells being 33.80 ± 0.37, which in the presence of Artemisinin remained unchanged at 0.05 mM (33.75 ± 0.25) and marginally increased with 0.1 mM (52.26 ± 5.61). With higher concentrations of 0.25 and 0.5 mM, the GMFC increased significantly to 95.40 ± 8.17 ($p < 0.05$) and 155.90 ± 3.80, ($p < 0.01$), respectively (Figure 1A).

A concomitant decrease in non-protein thiols was evidenced as the mean ± SEM of GMFC in promastigotes was 612.00 ± 28.50; with the addition of 0.05, 0.1, 0.25 and 0.5 mM of Artemisinin, the GMFC significantly decreased in a dose-dependent fashion to 331.50 ± 1.48 ($p < 0.01$), 287.50 ± 7.50 ($p < 0.01$), 228.00 ± 2.90 and 177.80 ± 2.80 ($p < 0.01$), respectively (Figure 1A).

Iron enhanced the leishmanicidal effect of Artemisinin in promastigotes

To establish whether iron executed a major role in generation of carbon-centred free radicals induced by Artemisinin and thereby contributed to parasite death, log phase parasites were incubated with iron (Fe^{2+}) in the presence of Artemisinin. In untreated cells, the baseline GMFC remained unaffected by Fe^{2+}, 0.2 mM (Figure 1B). However, Artemisinin (0.5 mM) increased the GMFC to 109.80 ± 17.25 and iron had an additive effect, as the GMFC of Artemisinin and treated cells increased significantly to 193.70 ± 39.64 ($p < 0.05$) as compared to Artemisinin alone (Figure 1B).

NAC and DFO diminished the leishmanicidal activity of Artemisinin

To establish whether generation of free radicals by Artemisinin was pivotal towards its leishmanicidal activity, promastigotes were incubated with Artemisinin in the presence or absence of a non-toxic concentration of NAC, an established anti-oxidant. Artemisinin, in the absence of NAC, demonstrated a dose-dependent inhibition of parasite growth, the 50% inhibitory concentration (IC_{50}) in promastigotes being 0.16 mM (Figure 1C), which, following co-incubation with NAC, was sharply attenuated, indicating that the altered redox status of parasites is vital for Artemisinin to mediate its parasiticidal activity (Figure 1C). Additionally, the presence of an iron chelator, DFO (0.25 mM), decreased the leishmanicidal activity of Artemisinin, as at its highest concentration of 0.5 mM only 60% of cells could be eliminated, corroborating the additive role of iron (Figure 1C).

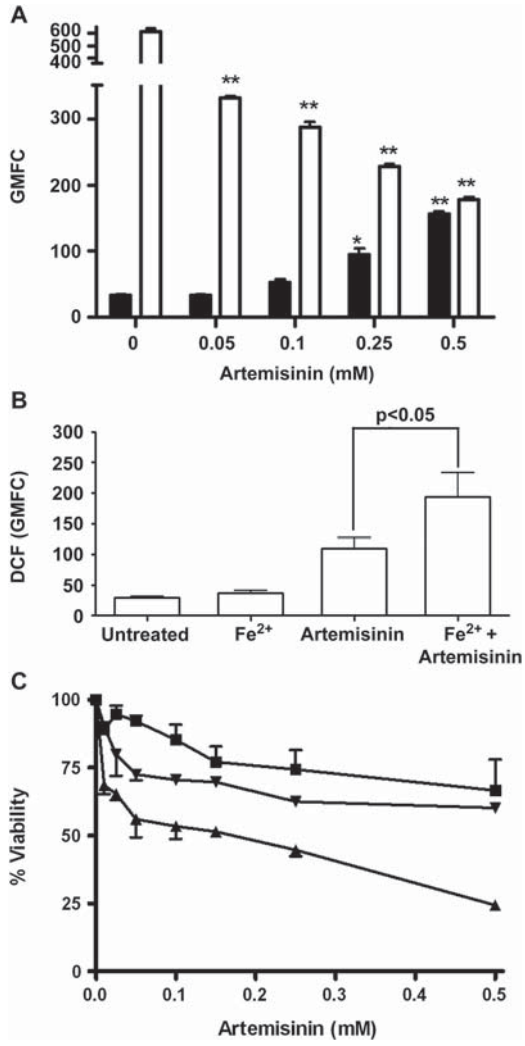

Figure 1. Generation of oxidative stress by Artemisinin in promastigotes. (A) Effect of Artemisinin upon generation of ROS and levels of non-protein thiols. Log phase promastigotes (*L. donovani*, 1×10^6 cells/ml) were incubated at 37°C for 3 h in the presence of Artemisinin (0–0.5 mM) as described in Materials and methods. Generation of ROS (-■-) was measured using 2'7'-dichloro-dihydrofluorescein diacetate (H_2DCFDA) while non-protein thiols (-□-) with 5-chloromethylfluorescein diacetate (CMFDA) as described in Materials and methods. Each data bar represents the mean ± SEM of GMFC of at least three experiments in duplicate. $^*p < 0.05$ and $^{**}p < 0.01$ as compared to untreated cells. (B) Role of iron in production of ROS by Artemisinin in *L. donovani* promastigotes. Log phase promastigotes (*L. donovani*, 1×10^6 cells/ml) were incubated at 37°C for 1 h in the presence of Artemisinin (0.5 mM) and further incubated with Fe^{2+} (0.2 mM) for 1 h as described in Materials and methods. Generation of ROS (-□-) was measured using H_2DCFDA as described in Materials and methods. Each data bar represents the mean ± SEM of GMFC of at least three experiments in duplicate. (C) Role of free radicals and iron in viability of Artemisinin treated *L. donovani* promastigotes. Log phase *L. donovani* promastigotes (2×10^5 cells in 200 µl/well) were incubated with Artemisinin (0–0.5 mM, -▲-) along with N-acetyl l-cysteine (2.5 mM, -■-) or iron chelator, desferoxamine (0.25 mM, -▼-) for 48 h. Cell viability was measured by the MTS-PMS assay as described in Materials and methods. Each data point represents the mean ± SEM value of at least three experiments in duplicate.

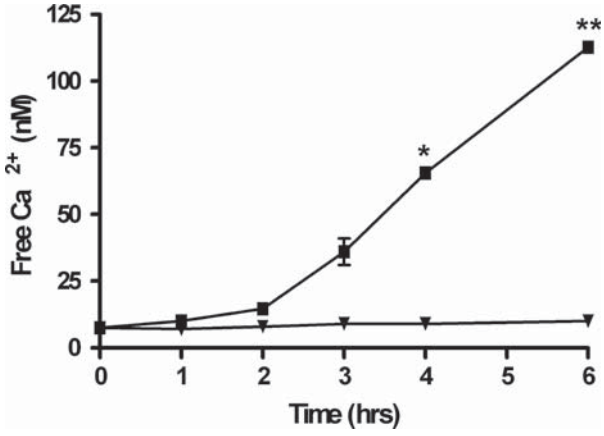

Figure 2. Effect of Artemisinin upon intracellular Ca^{2+} in promastigotes. Changes in Ca^{2+} levels of *L. donovani* promastigotes following treatment with Artemisinin (0.5 mM, -■-) was measured fluorimetrically as described in Materials and methods; vehicle control, 0.5% DMSO (-▼-) showed no changes in cytosolic Ca^{2+}. Data are expressed as mean ± SEM of at least three experiments in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to DMSO (0.5 %)-treated parasites.

Artemisinin caused an increase in cytosolic Ca^{2+} in promastigotes

Promastigotes when treated with Ionomycin, a potent Ca^{2+} ionophore, showed an increase in fluorescence, which following the addition of EGTA, a Ca^{2+} chelator translated into a pronounced decrease in fluorescence, confirming assay specificity in *Leishmania* parasites. With the addition of an IC_{90} concentration of Artemisinin (0.5 mM), a gradual increase in cytosolic [Ca^{2+}] up to 6 h was evident, whereas cytosolic [Ca^{2+}] remained close to basal levels in parasites incubated with DMSO (0.5%) that represented the concentration of DMSO present (Figure 2).

Artemisinin induced a caspase-independent apoptotic-like cell death in L. donovani promastigotes

Treatment with Artemisinin solicited minimal or no caspase activity in *L. donovani* promastigotes, as did H_2O_2 (4 mM); however, U937 cells treated with a similar concentration of H_2O_2 demonstrated pronounced caspase activity (Figure 3). To further confirm the absence of caspase activity in Artemisinin-treated parasites, promastigotes were incubated in the presence or absence of Z-VAD-FMK, a pan-caspase inhibitor followed by addition of Artemisinin (0–0.5 mM) and cell viability was measured after 48 h. As the IC_{50} of Artemisinin-treated promastigotes (0.16 mM) was comparable to the IC_{50} obtained when co-incubated with Z-VAD-FMK (0.15 mM), it validated that effector caspases were notably not involved in executing Artemisinin-induced apoptosis (data not shown).

Discussion

Artemisinin, a sesquiterpene lactone, is an established anti-malarial by virtue of its ability to generate carbon-centred radicals following the iron-mediated cleavage of its endoperoxide bridge [13,15] which then bind extensively to the thiol groups of cysteine residues [28]. In *Leishmania* spp. the absence of free radical mopping mechanisms such as catalase and selenium-dependent glutathione peroxidases renders these parasites more susceptible to free radical damage [4], as reported in response to chemotherapeutic agents such as SAG, Curcumin and Berberine chloride [3,10,12]. Therefore, it could be envisaged that Artemisinin, which generates reactive radicals in the malaria parasite, could in a similar manner also mediate its anti-leishmanial activity. Indeed, Artemisinin enhanced generation of ROS or reactive intermediates in promastigotes in a dose-dependent manner and was accompanied with concomitant depletion of cellular non-protein thiols (Figure 1A) resulting in alterations in the redox potential. Importantly, this oxidative imbalance was vital for the observed leishmanicidal activity of Artemisinin, as attenuation of oxidative stress by NAC translated into 66% cell death, even at the highest drug concentration of 0.5 mM (Figure 1C).

Iron is established as a vital component for the anti-malarial action of Artemisinin as in erythrocytes, iron bound in excess to haemoglobin activates Artemisinin by aiding the formation of intra-parasitic heme-iron. This subsequently catalyses cleavage of the endoperoxide ring, enabling transfer of an oxygen atom from the peroxide group to a chelated iron ion, generating a Fe (IV)=O species; the resultant free radical intermediate or iron-Artemisinin adduct then effectively kill parasites by alkylation, thus proving to be a lethal poison for the malaria parasite [13,14,25]. Therefore, iron acts as a double edged sword, as in one way it is essential for several biochemical pathways including replication, anti-oxidant defense of pathogens and hosts, while on the other hand it catalyses formation of deleterious free radicals [29,30]. Therefore, the central role of iron in both parasite metabolism and the host response makes it an attractive option for therapeutic intervention [31].

Leishmania are known to forage iron from its host macrophages [30] and it is therefore conceivable that the observed 7-fold decrease in IC_{50} of Artemisinin in amastigotes vs promastigotes (0.022 mM vs 0.16 mM) is due to the increased presence of iron [17,21]. Interestingly, following *Leishmania* infection, the anticipated decrease in production of nitric oxide (NO) was evident, but, importantly, addition of Artemisinin (0.025 mM) did translate into an increased production of NO, but only to levels comparable with uninfected macrophages, implying that Artemisinin exerts its parasiticidal effect directly rather than via a NO-dependent pathway [21].

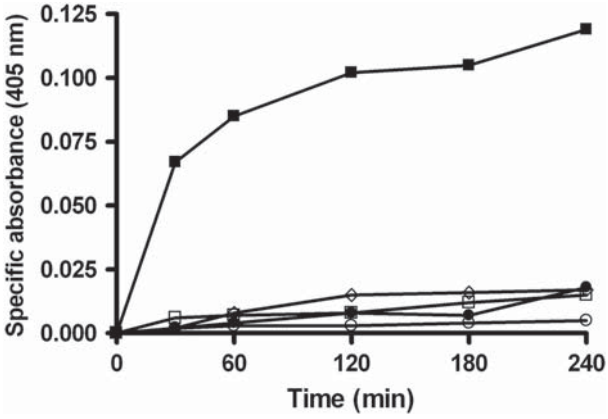

Figure 3. Effect of Artemisinin upon caspase activity in *L. donovani* promastigotes. *L. donovani* promastigotes (-○-) were treated with H_2O_2 (-□-) or Artemisinin (-◇-) and in parallel, U937 cells (-•-) were treated with H_2O_2 (-■-); cell lysates were prepared and the caspase activity was measured in terms of absorbances of pNA as described in Materials and methods. The data is a representative profile of at least three experiments.

Furthermore, the increase in production of reactive species following incubation of Artemisinin-treated cells with Fe^{2+} (Figure 1B) strengthened the role of iron, which was further corroborated when addition of an iron chelator, DFO, abrogated the action of Artemisinin (Figure 1C).

In *Leishmania* parasites, apoptosis or programmed cell death (PCD) appears to be the predominant form of cell death [32] in response to chemotherapeutic agents, for example Pentostam and Miltefosine, oxidants like H_2O_2 and also Artemisinin [21]. In both unicellular and multicellular organisms, the mitochondrion is an important source of reactive oxygen species, critical for induction of apoptosis [32,33]. Generation of ROS during early phase of apoptosis usually follows an imbalance in cellular redox homeostasis and leishmanicidal agents such as Withaferin A and Berberine chloride have been associated with enhanced production of oxide radicals [12,34]. Intracellular Ca^{2+} mobilization is an essential requirement for PCD as most endonucleases utilize Ca^{2+} to cleave DNA strands and elevation of Ca^{2+} is considered a feature of classical apoptosis, usually in tandem with early apoptotic changes including loss of mitochondrial membrane potential ($\Delta\psi$ m) [8]. In *Leishmania* parasites, antimony enhanced cellular levels of ROS that included superoxide, NO and H_2O_2 and, importantly, was associated with downstream events that included elevation of intracellular Ca^{2+} [8,12]. In our study, the surge in intracellular Ca^{2+} measured over 6 h with an IC_{90} concentration of Artemisinin (0.5 mM) indicated that Artemisinin-mediated generation of free radicals triggered mobilization of intracellular Ca^{2+} (Figure 2).

Degradation of nuclear DNA into nucleosomal units is another hallmark of apoptotic cell death [32]. In *Leishmania* parasites, a caspase-dependent mechanism of apoptotic cell death has been reported [6,34]. In *Leishmania*, knowledge about the total number of caspase-like proteases is limited, although the activation of the CED3/CPP32 group of proteases has been documented [6]. Lee et al. [35] have cloned and characterized two metacaspase genes in *L. donovani* (LdMC1 and LdMC2) and it has been established that caspase activation is not mandatory for triggering apoptosis [12,36,37]. As our study also demonstrated apoptosis by Artemisinin in the absence of detectable caspase activity (Figure 3), it corroborated previous studies that, in *Leishmania* parasites, the implementation of apoptosis can occur via a caspase-independent pathway, possibly via metacaspases; however, this aspect still needs to be validated.

Taken together, our results indicate that the antileishmanial activity of Artemisinin is enhanced by the presence of iron via an increased generation of free radicals or intermediates concomitant with depletion of non-protein thiols, culminating in an apoptotic mode of death. Further investigations aimed at dissecting the entry of iron into amastigotes and triggering of apoptosis could help generate useful inputs for novel drug targets.

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