Free Radical Research, November 2009; 43(11): 1101–1110

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Berberine chloride causes a caspase-independent, apoptotic-like death in *Leishmania donovani* promastigotes

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(Received 15 June 2009; revised 14 July 2009)

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Abstract
Berberine chloride, a quarternary isoquinoline alkaloid, is a promising anti-leishmanial compound, IC_{50} being 7.1 μM in *L. donovani* promastigotes. This leishmanicidal activity was initiated by its pro-oxidant effect, evidenced by enhanced generation of reactive oxygen intermediates that was accompanied by depletion of thiols; pre-incubation in N-acetyl cysteine, attenuated its cell viability, corroborating that generation of free radicals triggered its parasiticidal activity. Externalization of phosphatidylserine and elevation of intracellular calcium preceded depolarization of the mitochondrial membrane potential, which translated into an increase in the sub G_0/G_1 population and was accompanied by DNA laddering, hallmarks of apoptosis. Berberine chloride failed to induce caspase activity and anti-leishmanial activity in the presence of a pan caspase inhibitor, Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone remained unchanged, which indicated that the apoptosis was caspase independent. Collectively, the data indicates that Berberine chloride triggers an apoptosis-like death following enhanced generation of reactive oxygen species, thus meriting further pharmacological investigations.

Keywords: *Anti-leishmanial, apoptosis, Berberine chloride, Leishmaniasis, oxidative stress, reactive oxygen species*

Introduction

The protozoan parasite *Leishmania*, a member of the order *Kinetoplastida*, is the causative agent of leishmaniasis, a disease affecting 12 million people worldwide; an estimated 350 million people are at risk and the global yearly incidence is 1–1.5 million for cutaneous and 500 000 for visceral leishmaniasis (VL) [1]. The flagellated, extracellular promastigote form of the parasite resides within the mid-gut of the vector, the female sandfly, where it undergoes differentiation from a proliferating, non-infectious procyclic form to an infectious metacyclic stage. Following a blood meal by the sand fly, the parasite infects mammalian host macrophages, differentiating into amastigotes who deviously survive and multiply within the macrophage phagolysosome [2].

The circumvention of oxidative stress is vital for survival of Trypanosomatids as, during a host-parasite interaction, the host creates a hostile environment by generating reactive oxygen and nitrogen intermediates [3]. The anti-oxidant pathway of *Leishmania* includes two iron containing superoxide dismutases which only marginally protect the parasite [4,5], as the absence of catalase and the glutathione peroxidase system renders the parasite more susceptible to free radical damage [6,7]. To minimize the deleterious effects of hydroperoxides, *Leishmania* depends on a less efficient, unique trypanothione dependent antioxidant system which includes the bis-glutathionyl spermidine

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2009 Informa UK Ltd.
DOI: 10.1080/10715760903186124

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conjugate trypanothione, trypanothione reductase, tryparedoxin and tryparedoxin peroxidase, accordingly, Trypanothione in parasites is considered as a pivotal intermediate in regulation of the redox homeostasis and a vital component for defense against xenobiotics and oxidative stress [8–10].

Exploiting the impaired antioxidant capacity of *Leishmania* parasites, triggering of oxidative stress seems a logical chemotherapeutic modality. Indeed, sodium antimony gluconate, the main stay of treatment for leishmaniasis [11], generates reactive oxygen species (ROS) both within macrophage phagolysosomes [12] and the parasite [13,14], thereby inducing apoptosis [15,16] by mitochondrial dysfunction in promastigotes following uncoupling of oxidative phosphorylation [13].

Several plant-derived anti-leishmanial compounds have shown a similar propensity to mediate their anti-leishmanial activity by inflicting an enhanced oxidative insult upon susceptible *Leishmania* parasites [17–19]. In the present study, we have characterized the apoptotic pathway induced by Berberine chloride in *L. donovani* promastigotes.

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Materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri) except N-1 napthyl ethylene diamine dihydrochloride (Loba Chemie Pvt. Ltd., Mumbai, India), phenazine methosulphate (PMS) and phenylmethylsulphonylfluoride (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, Wisconsin), JC-1, Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK) and Fluo 4AM (Invitrogen, California), Caspase-3/CPP32 Colorimetric assay kit (Biovision, California), Quick apoptotic DNA ladder detection kit, Invitrogen (California) (Catalog no. KH01021). Berberine chloride (50 mM in methanol) was stored at -20°C.

Parasite culture

Promastigotes of an Indian *Leishmania donovani* strain, NS2 previously isolated from a patient with VL were routinely cultured at 24°C in Medium 199 (M199) supplemented with 10% foetal bovine serum (FBS), penicillin G (50 IU/ml) and streptomycin (50 µg/ml). Log phase promastigotes were subcultured every 72–96 h, inoculum being 1×10^6/ml.

Cell culture

A human non-adherent leukaemic cell line (U937) was sub-cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin G (50 IU/ml) and streptomycin (50 µg/ml), at 37°C in a humidified atmosphere containing 5% CO_2; log phase cells were sub-cultured every 72 h, inoculum being 5×10^5/ml.

In vitro *evaluation of anti-promastigote activity*

The anti-leishmanial activity of Berberine chloride was established in promastigotes and cell viability was measured using the modified MTS-PMS assay [20]. Briefly, log phase promastigotes (2×10^5 cells/200 µl of M199 medium/well) were incubated with Berberine chloride (0–50 µM) for 48 h and parasite viability measured. MTS [3-(4, 5 dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(-4-sulphonyl)-2H-tetrazolium, inner salt] (2.0 mg/ml) and PMS (Phenazine methosulphate) (0.92 mg/ml) were added in a ratio of 5:1 (20 µl per well) and plates incubated for 3 h at 37°C; resultant absorbances were measured at 490 nm in an ELISA reader. Accordingly, the specific absorbance that represented formazan production was calculated by subtraction of background absorbance from total absorbance. The mean percentage viability was calculated as follows:

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

Results were expressed as the $IC_{5,}$ i.e. the concentration that inhibited 50% cell growth which was enumerated by graphical extrapolation using Graph pad Prism software (version 4).

Generation of reactive oxygen species (ROS)

To study the effect of Berberine chloride on generation of ROS, log phase promastigotes (1×10^6/ml) following incubation with Berberine chloride (0–50 µM, 3 h) were washed with phosphate buffered saline (0.02 M, pH 7.2, PBS) and then incubated with 2, 7 dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 µM) for 45 min at 37°C and fluorescence 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 viable parasites [14]. The subsequent analyses were done using BD CellQuest Pro software.

Measurement of non-protein thiols

Non-protein thiols were measured as previously described [14]. Briefly, promastigotes (1×10^6 cells/ml) in serum-free M199 were incubated with Berberine chloride (0–50 µM) at 37°C for 3 h. Cells were then washed with ice cold PBS, resuspended in mercury orange (500 µM in acetone) and incubated precisely for 5 min on ice. Cells were immediately washed thoroughly with chilled PBS and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) using forward vs. side scatter to gate the parasite population and a FL3 histogram to quantify

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fluorescence of viable parasites and analysed using BD CellQuest Pro software.

Analysis of phosphatidylserine externalization

Double staining for annexin V–FITC and propidium iodide (PI) was performed as previously described [21]. Briefly, promastigotes incubated with an IC_{90} concentration of Berberine chloride (50 μM) for 0–6 h at 24°C were centrifuged (1000 g × 10 min), washed twice in PBS 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 was added according to the manufacturers' instructions and cells kept for 30 min in the dark at 24°C. Prior to acquisition, PI (0.1 μg/ml) was added and the percentage of positive cells were determined on a flow cytometer (FACSCalibur, BD Biosciences, USA) using Cell Quest Pro software.

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 [22]. Briefly, log phase promastigotes (2×10^6 cells) were equilibrated with loading medium (Fluo-4 AM, 2.5 μM, pluronic acid F127, 0.02% and sulphinpyrazone, 0.25 mM in M199 medium) at 24°C for 30 min. Cells were then washed with medium containing 0.25 mM sulphinpyrazone (Medium A) and incubated at 24°C for 30–60 min to allow for de-esterification of Fluo-4AM. Subsequently, cells were incubated with an IC_{90} concentration of Berberine chloride (50 μM, 0–3 h); in parallel, cells were incubated with a Ca^{2+} ionophore (Ionomycin 3 μM) and specificity confirmed by addition of a chelating agent, EGTA (5 mM). Cells were washed, resuspended in Medium A and fluorescence measured in a spectrofluorimeter (excitation, 485 nm and emission, 520 nm). To convert fluorescence values into absolute $[Ca^{2+}]_t$, calibration was performed at the end of each experiment and calculated as follows [23]:

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

where K_d is the dissociation constant of the Ca^{2+} Fluo 4 complex (345 nM), F represents fluorescence intensity of Berberine chloride treated cells, $F_{\max}$ is maximal fluorescence, i.e. cells treated with Ionomycin (3 μM), while $F_{\min}$ corresponded to minimum fluorescence, i.e. cells treated with Ionomycin (3 μM) and EGTA (5 mM).

Analysis of mitochondrial transmembrane potential

The mitochondrial transmembrane electrochemical gradient ($\triangle\psi_m$) was measured using JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide), a cell permeable, cationic and lipophilic dye as previously described [24]. Briefly, promastigotes were incubated with an IC_{90} concentration of Berberine chloride (50 μM, 0–6 h at 24°C) and stained with JC1 (2 μM in PBS) for 10 min at 37°C. Cells were acquired on a FACS Calibur on the basis of quadrant dot plot to determine monomers and J aggregates and analysed using Cell Quest Pro software. To set quadrants, promastigotes were treated with H_2O_2 (15 mM, 10 min), representing cells with depolarized mitochondrial membrane potential.

Cell cycle analysis

Promastigotes (1×10^6) were treated at 24°C with an IC_{90} concentration of Berberine chloride (50 μM, 0–12 h) and fixed in chilled ethanol (70%) and kept at 4°C until analysis. Prior to analysis, cells were washed in PBS and the resultant pellet resuspended in DNase-free RNase (200 μg/ml, 0.5 ml) for 1 h at 37°C; cells were then stained with PI (40 μg/ml), acquired on a Flow cytometer (FACS Calibur) and analysed using Cell Quest Pro software [24].

DNA fragmentation assay by agarose gel electrophoresis

To determine DNA fragmentation, total cellular DNA was isolated from *Leishmania donovani* promastigotes treated with Berberine chloride (50 μM, 48 h) according to manufacturer's instructions and analysed by 1.5% agarose gel electrophoresis containing ethidium bromide (0.5 μg/ml) in TBE buffer and visualized on a Molecular Imager Chemi Doc XRS System (Bio Rad, California) [25].

Determination of caspase activity in Berberine chloride treated promastigotes

Caspase activity was measured using a commercially available kit, as per the manufacturer's instructions. Briefly, Berberine chloride (50 μM, 12 h) treated promastigotes were washed with ice cold PBS, cell lysates were prepared and protein concentration estimated. To detect activity of CED3/CPP32 group of proteases, cell lysates (100 μg protein in 50 μl lysis buffer) was combined with 50 μl of 2× reaction buffer (containing 10 mM DTT), substrate DEVD-pNA (4 mM, 5 μl) and incubated at 37°C up to 4 h; the emission of pNA was quantified by measuring absorbances at 405 nm. In parallel, *Leishmania* parasites and U937 cells were treated with H_2O_2 (4 mM, 30 min) and Miltefosine (40 μM, 12 h).

To study the biological role of caspases in Berberine chloride-induced death, log phase promastigotes (2×10^5 in 200 μl/well) were seeded in 96 well tissue culture plates. Following a 48 h incubation at 24°C with Berberine chloride (0–50 μM) in the presence of a pan caspase inhibitor Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK, 100 μM),

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cell viability and IC_{50} values were evaluated by the MTS-PMS assay [20].

Statistical analysis

Results were expressed as mean $\pm$ SD/SEM as indicated. Statistical analysis was evaluated by Students *t*-test using Graph Pad Prism software (version 4), $p < 0.05$ was considered as statistically significant.

Results

Anti-promastigote activity of Berberine chloride

Berberine chloride (0–50 μM, 48 h) demonstrated a dose-dependent inhibition of parasite growth with a 50% inhibitory concentration (IC_{50}) achieved at 7.1 μM and IC_{90} at 50 μM. The effect of methanol (0.05%), representative of the highest concentration present in Berberine chloride (50 μM), was studied and showed no loss in parasite viability occurred.

Berberine chloride demonstrated pro-oxidant activity in promastigotes

To evaluate the effect of Berberine chloride on the oxidative status of promastigotes H_2DCFDA, a lipid soluble, membrane permeable compound was used based on evidence that following cleavage by cellular non-specific esterases, an impermeable H_2DCF is formed which is subsequently oxidized by intracellular reactive oxygen species (ROS) to produce a fluorescent compound DCF [26]; therefore, the resultant fluorescence is directly proportional to the quantum of ROS generated.

Initially log phase promastigotes (1×10^6/ml) were incubated with an IC_{90} concentration of Berberine chloride (50 μM) and demonstrated a time-dependent increase in the generation of ROS, maximum being at 3 h, with no change in cell viability as measured by PI exclusion (data not shown). Subsequently, the effect of Berberine chloride (0–50 μM) was studied; the mean $\pm$ SEM of GMFC representing baseline ROS was 27.56 ± 3.03 which progressively increased to 80.54 ± 18.00 ($p < 0.01$) with 50 μM (Figure 1A). To exclude any autofluorescence generated by Berberine chloride, if any, cells were incubated with only Berberine chloride (0–50 μM, 3 h) and GMFC was quantified. As no measurable increase in GMFC was observed, we concluded that Berberine chloride induced an oxidative burst, the observed fluorescence being specifically attributed to enhanced generation of ROS.

To examine the effect of Berberine chloride on levels of non-protein thiols, mercury orange (MO) was used as it reacts with all sulphhydryl (-SH) groups generating a non-permeable fluorescent product which is retained within cells. However, as the reaction rate of mercury orange with non-protein thiols is much faster than with protein thiols, incubation for 5 min on ice allowed MO to react selectively with non protein –SH groups; accordingly, the fluorescence represented the level of cellular non-protein thiols [27]. Berberine chloride (0–50 μM) caused a dose-dependent decrease in levels of non-protein thiols, mean $\pm$ SEM of GMFC in controls being 148.83 ± 6.45, which with the addition of Berberine chloride (10, 25 and 50 μM) decreased progressively to 49.56 ± 5.15 ($p < 0.0001$) (Figure 1B).

To confirm whether the oxidative burst induced by Berberine chloride was a major contributory factor towards its leishmanicidal activity, promastigotes were co-incubated with Berberine chloride and a non-toxic concentration of N-acetyl-L-cysteine (NAC, 5 mM), an established antioxidant. With the addition of NAC, the IC_{50} of Berberine chloride increased 6-fold from 7.1 μM to 43.55 μM, substantiating that induction of oxidative burst is a key factor triggering the parasiticidal activity of Berberine chloride (Figure 1C).

Phosphatidyl serine externalization was altered by Berberine chloride treatment

Translocation of phosphatidylserine from the inner aspect to the outer leaflet of the plasma membrane occurs during apoptosis of metazoan and unicellular parasites [12,23,28,29]. The high binding affinity of Annexin V, a Ca^{2+}-dependent phospholipid binding protein towards phosphatidylserine helps ascertain whether parasite death is via apoptosis or necrosis, the latter being identified by PI, a non-permeable stain having affinity towards nucleic acids and selectively entering necrotic cells. Therefore, co-staining of Annexin V and PI helps discriminate between live parasites (PI and Annexin V negative), cells in early apoptosis (Annexin V positive, PI negative), cells undergoing late apoptosis (both Annexin V and PI positive) or necrotic cells (PI positive, Annexin V negative).

In healthy, log phase promastigotes, the basal binding of annexin V was 5.06%, whereas with Berberine chloride this increased to 36.50% at 6 h, the majority of the population undergoing early apoptosis (Figure 2, lower and upper right quadrant). The percentage of PI-positive cells (Figure 2, upper left quadrant) at baseline was minimal and remained comparable at 2, 4 and 6 h, being 2.29%, 3.86% and 3.61%, respectively. Taken together, Berberine chloride causes externalization of phosphatidyl serine, to a degree comparable with Miltefosine (20 μM, 12 h), an established inducer of apoptosis, whose percentage positivity was 37.28 (data not shown).

Berberine chloride caused an increase in cytosolic Ca^{2+}

Alterations in cytosolic Ca^{2+} in response to changes in the redox potential have been reported to induce mitochondrial dysfunction [23]. The addition of

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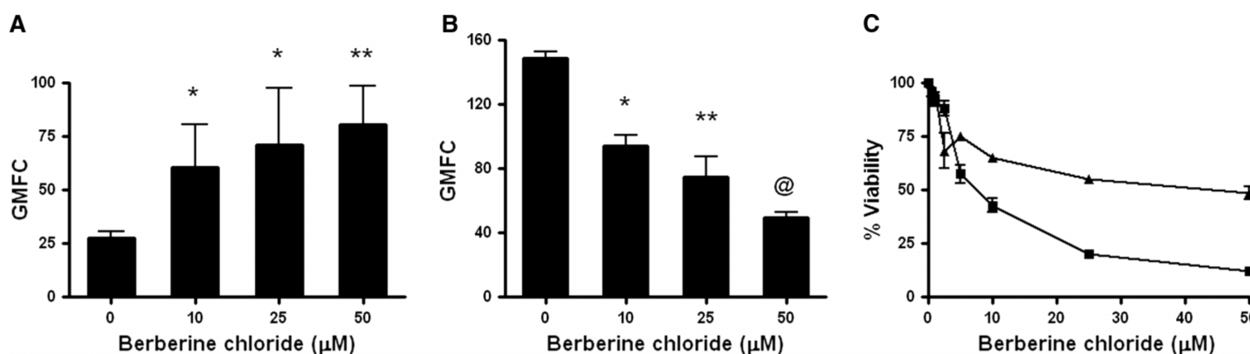

Figure 1. Modulation of oxidant status of *L. donovani* promastigotes by Berberine chloride. (A) Effect of Berberine chloride on ROS generation. Log phase promastigotes were incubated with Berberine chloride (0–50 μM) and labelled with 2, 7 dichlorodihydroflurescein diacetate (H_2DCFDA, 50 μM) as described in Materials and methods. Data are expressed as the mean GMFC ± SEM of at least three experiments in duplicate. $^*p < 0.05$ and $^{**}p < 0.01$ as compared to controls. (B) Effect of Berberine chloride on level of non-protein thiols. Log phase promastigotes incubated with Berberine chloride (0–50 μM) for 3 h were labelled with mercury orange (500 μM in acetone) and analysed for fluorescence as described in Materials and methods. Data are expressed as mean GMFC ± SEM of at least three experiments in duplicate. $^*p < 0.0005$, $^{**}p < 0.005$ and $^{@}p < 0.0001$ as compared to controls. (C) Effect of antioxidant on survival of promastigotes. Promastigotes (2×10^5/200 μl/well) were incubated with Berberine chloride (0–50 μM, ■) along with N-acetyl-L-cysteine (NAC, ▲) for 48 h and the MTS-PMS assay was performed as described in Materials and methods. Each point corresponds to the mean ± SD of at least three experiments in duplicate.

Ionomycin, a potent, Ca^{2+} ionophore, increased intracellular Ca^{2+}, which decreased when cells were treated with Ionomycin in the presence of EGTA, confirming assay specificity in *Leishmania* parasites. In control cells, a steady concentration of intracellular [Ca^{2+}] was maintained, whereas Berberine chloride caused a time-dependent increase in cytosolic [Ca^{2+}], maximal at 120 min (Figure 3). No increase in fluorescence occurred when cells were incubated with Berberine chloride alone or methanol (0.05%), the vehicle control.

To establish whether reactive oxygen species (ROS) triggered elevation of Ca^{2+}, cells were pre-treated with N-Acetyl L-cysteine (NAC, 5 mM, 1 h, 37°C), an established antioxidant followed by Berberine chloride. Pre-treatment with NAC caused a significant reduction in Berberine chloride-induced cytosolic Ca^{2+} level when compared to Berberine

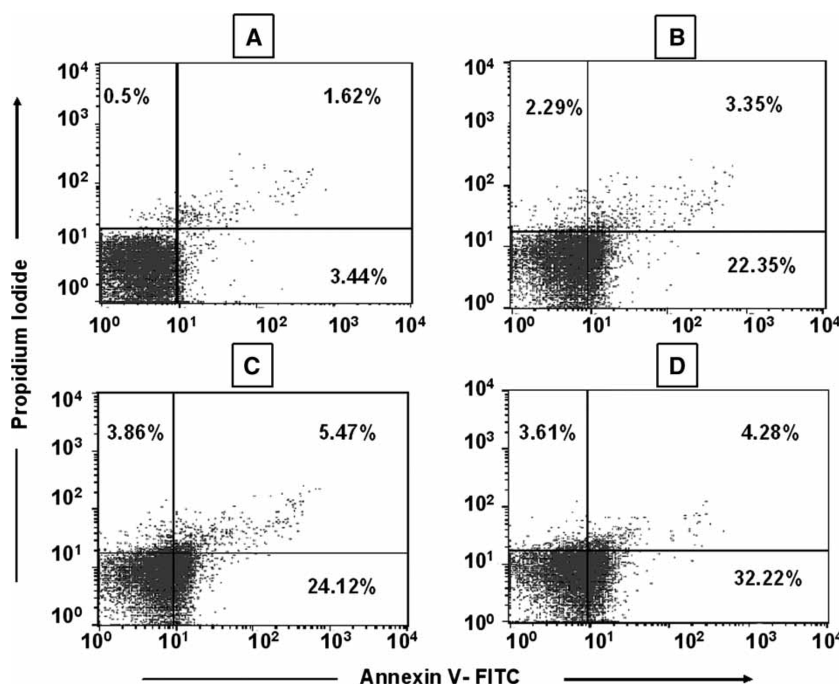

Figure 2. Externalization of phosphatidyl serine in Berberine chloride-treated promastigotes: Promastigotes (A) were incubated with an IC_{90} concentration of Berberine chloride (50 μM) for 2 h (B), 4 h (C) or 6 h (D), co-stained with PI and annexin V–FITC and analysed by flow cytometry as described in Materials and methods. The figure is a representative profile of at least three experiments.

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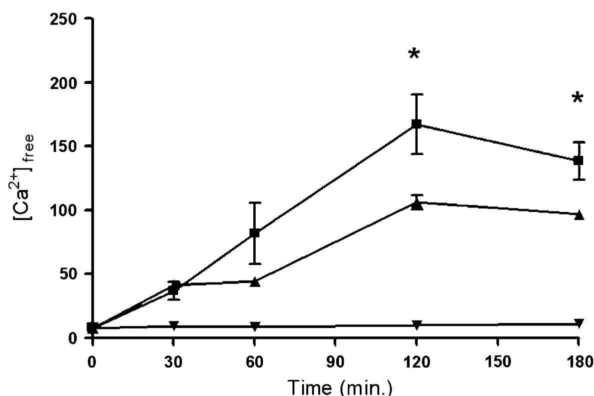

Figure 3. Effect of Berberine chloride upon intracellular Ca^{2+}: *L. donovani* promastigotes showing changes in Ca^{2+} levels following treatment with Berberine chloride in the absence (50 µM, ■) and presence of NAC (▲) as described in Materials and methods. Vehicle control, methanol (▼), showed no alterations in cytosolic calcium level. Data are expressed as mean ± SEM of at least three experiments in duplicate. $^{*}p < 0.05$ as compared to Berberine chloride treated *Leishmania* promastigotes alone.

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chloride treated *Leishmania* promastigotes at 120 min and 180 min (Figure 3).

Berberine chloride-induced depolarization of mitochondrial transmembrane potential in promastigotes

The loss of mitochondrial membrane potential is a characteristic feature of metazoan apoptosis, also evident in protozoans [24]. To measure the mitochondrial membrane potential, JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide) was used as it is a lipophilic fluorescent cation that freely permeates the mitochondrial membrane and forms J aggregates that fluoresce red; accordingly, viable cells stained with JC-1 exhibit a pronounced red fluorescence. An apoptotic stimulus induces a decrease in the mitochondrial membrane potential, JC-1 fails to enter the mitochondria, remaining as cytosolic monomers and emits a green fluorescence. Therefore the ratio of J-aggregates/monomers serves as an effective indicator of the mitochondrial energy state of the parasite, allowing apoptotic cells to be easily distinguished from their non-apoptotic counterparts [30]. In control promastigotes, the red/green fluorescence ratio was 14.92, which, following the addition of an IC_{90} concentration of Berberine chloride (50 µM, 2–6 h), induced a dramatic decrease in mitochondrial membrane potential, resulting in predominance of JC-1 monomers fluorescing green, which translated into a decrease in the red/green fluorescence ratio to 2.27 at 2 h, 1.76 at 4 h and 0.61 at 6 h.

JC-1 fluorescence was also measured by estimating the percentage gated population in two gates, namely R2 and R3, wherein R2 represented the healthy, non-apoptotic population, while R3 represented the apoptotic cell population. These gatings were set following addition of H_2O_2 (15 mM, 10 min) wherein the percentage gated in R2 and R3 was 17.67% and 79.73%, respectively (data not shown). In healthy cells, the R2 and R3 percentage positivity was 92.97% and 6.23%, respectively. With Berberine chloride (50 µM), the population in R3 gate increased progressively with time, being 61.28% at 6 h (Figure 4) indicating that Berberine chloride induces depolarization of mitochondrial membrane potential leading to apoptotic cell death.

Berberine chloride increased the sub G_0/G_1 population in promastigotes

Flow cytometric analysis helped to quantify the percentage of cells in different phases of the cell cycle, the amount of bound dye representing DNA content. Accordingly, DNA fragmentation that occurs in apoptotic cells is known to translate into fluorescence intensity lower than that of G_0/G_1 cells translating into an increased sub G_0/G_1 peak. In promastigotes treated with an IC_{90} concentration of Berberine chloride, the proportion of cells in the sub G_0/G_1 phase at 8 and 12 h progressively increased to 12.95% and 51.84%, respectively, whereas in controls the sub G_0/G1 population remained at 3.62% (Table I). Taken together, this progressive increase in the proportion of cells in the sub G_0/G_1 phase corroborated that Berberine chloride induced apoptosis in promastigotes culminating in DNA degradation.

Berberine chloride-induced oligonucleosomal DNA fragmentation in promastigotes

One of the hallmarks of apoptotic cell death is internucleosomal DNA digestion by endogenous nucleases that yields a characteristic laddering pattern. Accordingly, oligonucleosomal DNA fragmentation following treatment of promastigotes with Berberine chloride (50 µM, 48 h) was studied; a degree of smearing together with distinct oligonucleosomal bands, similar to other protozoans was evident (Figure 5, lane 3) [15,31]. A similar degree of smearing was also observed with H_2O_2 (4 mM, 6 h) (Figure 5, lane 4) corroborating with previous studies [15].

Berberine chloride mediated apoptosis via a caspase-independent pathway

As caspases are considered key enzymes in the apoptotic pathways, their presence was examined in Berberine chloride-treated promastigotes. Promastigotes treated with Berberine chloride failed to show any caspase activity, while promastigotes treated with H_2O_2 or Miltefosine showed minimal caspase activity (Figure 6); however, caspase activity was evident in U937 cells treated with a similar concentration of H_2O_2 (4 mM) and Miltefosine (40 µM) (Figure 6). To confirm assay specificity, U937 cells were treated

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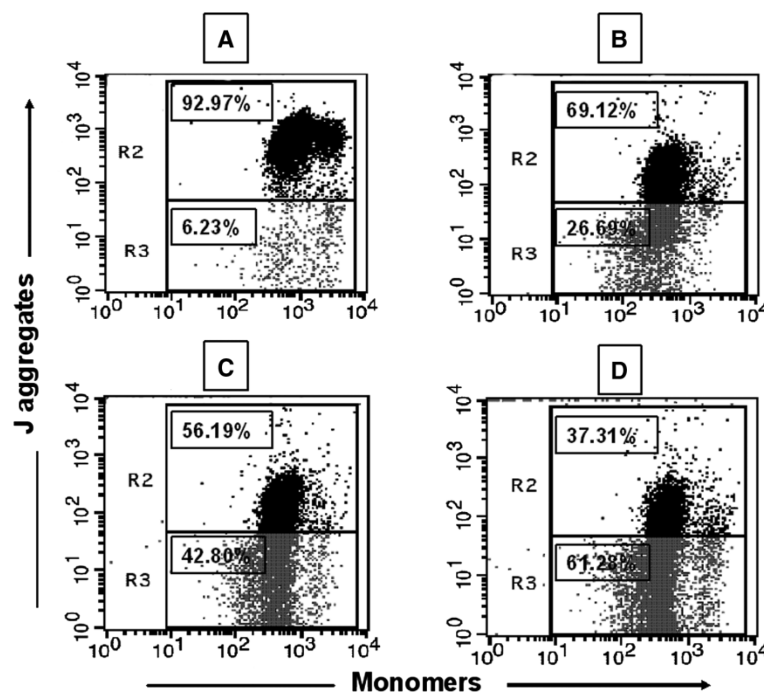

Figure 4. Effect of Berberine chloride on mitochondrial transmembrane potential in *L. donovani* promastigotes. Log phase promastigotes (A) were incubated with Berberine chloride (50 μM) for 2 h (B), 4 h (C) or 6 h (D) and probed with JC-1 as described in Materials and methods. The encompassed population of R2 represents viable cells whereas R3 represents apoptotic cells. The data is a representative profile of at least three experiments.

with Z-VAD-FMK, a pan caspase inhibitor, which resulted in decreased absorbances (data not shown).

To confirm the negligible role of caspases in Berberine chloride-induced apoptosis, promastigotes were co-incubated for 48 h with Z-VAD-FMK, a pan caspase and Berberine chloride (0–50 μM), percentage parasite viability being the end point. The IC_{50} of promastigotes treated with Berberine chloride was 7.1 μM and the addition of Z-VAD-FMK failed to attenuate Berberine chloride-induced parasiticidal activity as the IC_{50} remained at 7.19 μM, validating that Berberine chloride induces apoptosis via a caspase-independent pathway.

Discussion

Programmed cell death or apoptosis is considered as a genetically regulated, physiological process of cell suicide, pivotal for perpetuation and sustenance of most organisms. Apoptosis is generally triggered through a controlled programme, classically defined by unique morphological alterations that include membrane blebbing, cytoplasmic and nuclear

Table I. Effect of Berberine chloride on cell cycle progression of *L. donovani* promastigotes.

Time, h	*Sub G_0/G_1 (M1)	*G_0/G_1 (M2)	*S+ G_2/M (M3)
—	3.62	78.76	18.87
8	12.95	46.63	38.73
12	51.84	19.63	14.80

**L. donovani* promastigotes (1×10^6/ml/well), treated with Berberine chloride (50 μm) for 8 and 12 h were processed for cell cycle analysis as described in Materials and methods. Values are expressed as percentages and the data is a representative profile of at least three experiments.

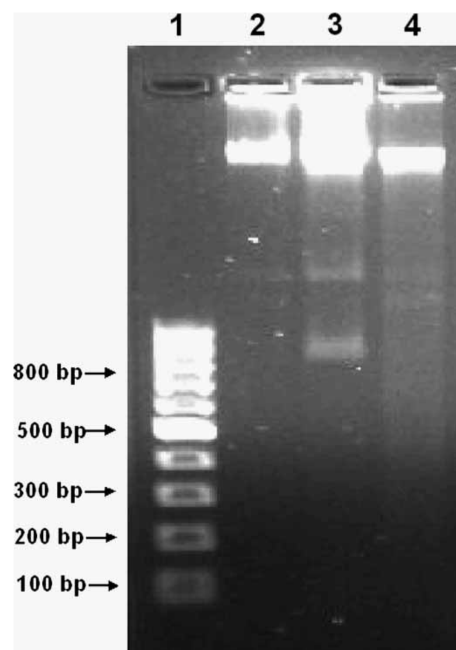

Figure 5. Berberine chloride induced DNA fragmentation in *L. donovani* promastigotes. Oligonucleosomal DNA fragmentation analysis of *L. donovani* promastigotes (lane 2) treated with Berberine chloride (50 μM, 48 h, lane 3) as described in Materials and methods; promastigotes treated with H_2O_2 served as positive control (lane 4) The figure is a representative profile of at least three experiments. Lane 1: 100 bp DNA ladder.

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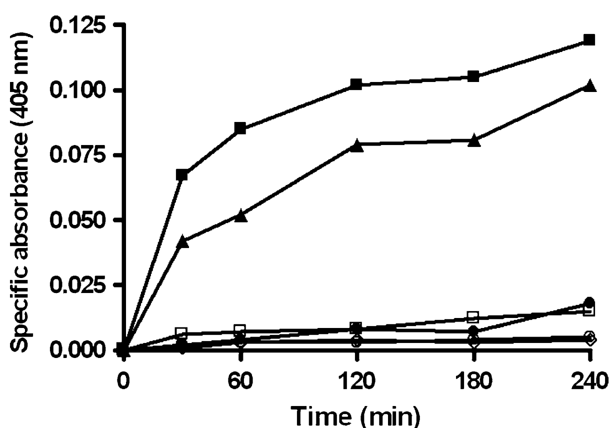

Figure 6. Caspase activity in *L.donovani* promastigotes. Cell lysates from *L. donovani* promastigotes (○) were treated with H_2O_2 (□) or Berberine chloride (◇) as described in Materials and methods. In parallel, U937 cells (●) were treated with H_2O_2 (4 mM, ■) or Miltefosine (40 μM, ▲). The data is a representative profile of at least three experiments.

condensation accompanied with DNA breakage [32]. In *Leishmania* parasites, apoptosis appears to be the predominant form of cell death, in response to variable stimuli that include heat shock, H_2O_2 and anti-leishmanial drugs such as pentostam and Miltefosine [25]. Additionally, several plant-derived anti-leishmanial compounds including *Aloe vera* [33], Artemisinin [24], *Piper betle* Linn [34], Luteolin [19] and Curcumin [18] amongst others also induce apoptosis.

In multicellular and unicellular organisms, the mitochondrion serves as an important cellular source for generation of reactive oxygen species (ROS), critical for induction of apoptosis [35]. The production of ROS during the early phase of apoptosis usually follows an imbalance in cellular redox homeostasis and is accompanied by depletion of cellular thiols. Withaferin A, an anti-leishmanial compound, generates ROS, inhibits protein kinase C and induces apoptosis [17]. Similarly, another leishmanicidal agent, curcumin, also enhanced generation of hydroxyl radicals [18]. In our study, Berberine chloride induced generation of ROS within promastigotes in a dose-dependent manner concomitant with depletion of cellular thiols, causing alterations in the redox potential (Figure 1A and B). This enhanced oxidative insult was vital for the observed leishmanicial activity, as attenuation of oxidative stress by NAC caused a 6-fold increase in the IC_{50} of Berberine chloride (Figure 1C). This leishmanicidal activity has been confirmed in experimental models of VL using an Indian *L. donovani* strain [36] where > 90% inhibition of parasite burden was reported. However, in a Sudanese strain of *L. donovani* [37], minimal reduction in parasite burden suggests that the sensitivity of Berberine chloride appears to be species-dependent.

An apoptotic stimulus causes externalization of phosphatidyl serine and is detected by the binding of annexin V, a Ca^{2+} dependent phospholipid binding protein owing to its strong affinity towards phosphatidyl serine [24]. In our study, Berberine chloride effected externalization of phosphatidyl serine (Figure 2), indicating that Berberine chloride exerts its anti-parasitic activity primarily via apoptosis.

An important component in the progression towards cell death is elevation of $[Ca^{2+}]c$, necessary for endonucleases generally to initiate DNA cleavage [15,16]. The leishmanicidal activity of Curcumin was accompanied by an increase in Ca^{2+} [18], whereas a leafy exudate from *Aloe vera* also induced an apoptotic-like death in promastigotes minus alterations in Ca^{2+} levels [33]. Berberine chloride caused a time-dependent increase in cytosolic $[Ca^{2+}]$ and additionally the presence of NAC, an established antioxidant, caused a substantial decrease in elevation of intracellular Ca^{2+} in Berberine chloride-treated cells, suggesting that generation of ROS triggered the increase in intracellular $[Ca^{2+}]$, an important element in the progression towards cell death (Figure 3).

Functioning of the single mitochondrion in *Leishmania* species is more vital as compared to other organisms that by virtue of their multiple mitochondria ensure compensation of injured mitochondria. The loss of mitochondrial transmembrane potential ($\triangle\psi_m$) is generally an early change associated with apoptosis as dissipation of $\triangle\psi_m$ following permeabilization of the inner mitochondrial membrane triggers release of several apoptotic factors. Two major metabolic alterations have been implicated in the loss of $\triangle\psi_m$ and include depletion of reduced thiols. In Leishmaniasis, two metalloids, arsenic and antimony, cause mitochondrial dysfunction in parasites, which are accompanied by DNA fragmentation [13]. Artemisinin, a sesquiterpene lactone having established anti-malarial activity, also demonstrated anti-leishmanial activity that was associated with a loss in mitochondrial membrane potential [24]. Similarly, a dramatic loss in mitochondrial membrane potential was demonstrated in promastigotes treated with an ethanolic extract of *Piper betle* (PB) and Racemoside A, a water-soluble steroidal saponin [33,34]. Berberine chloride too caused mitochondrial membrane depolarization, which was evident from 2 h onwards (Figure 4).

Apoptotic cells generally feature active endonucleases that preferentially cleave DNA, translating into an increased cell population located on a DNA frequency histogram proximal to the G_0–G_1 peak, i.e. a sub G_0–G_1 peak. In promastigotes incubated with other plant-derived anti-leishmanial compounds, a substantial proportion of cells have been identified in the sub G_0–G_1 phase [24]. Our study is in agreement with previous observations, as Berberine chloride also significantly increased the sub G_0–G_1 cell population (Table I).

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During apoptosis, cleavage patterns of genomic DNA following internucleosomal DNA digestion by endonucleases are a hallmark of apoptotic death. Electrophoretic analysis of DNA from promastigotes treated with Berberine chloride revealed a characteristic ladder pattern along with some amount of smearing (Figure 5, lane 3). DNA smearing has also been observed during apoptosis in yeast [38] and some metazoan cell types [39] similarly observed in promastigotes treated with curcumin [18], Novobiocin [40]. This non-classical type DNA fragmentation could be attributed to the shorter H1 histone molecules present in *Leishmania* [41] that render its nucleosomes more susceptible to enzymatic cleavage.

Executioner caspases are considered critical for the apoptotic cascade after induction by different stimuli that include growth-factor deprivation or various environmental stresses [42]. Interestingly, in *Leishmania*, apoptotic death has been reported to occur via a caspase-independent pathway [43]. In fact, the absolute requirement for caspase activation in apoptosis, even in mammalian cells, is no longer considered mandatory [42]. The absence of Caspase 3 and 7 activities in both stationary phase promastigotes and 5 day axenic cultures of *Leishmania* indicated that nucleosomal DNA degradation was caspase-independent [43]. Additionally, as no caspase genes have been identified in the *Leishmania* genome, a role for metacaspases in programmed cell death has been proposed [44]. Indeed, two metacaspase genes with trypsin like activity (LdMC1 and LdMC2) have been characterized in *L. donovani* [45]. Our inability to observe any induction of caspase activity (Figure 6) and failure to alter the leishmanicidal activity of Berberine chloride in the presence of a broad spectrum caspase inhibitor Z-VAD-FMK indicated that Berberine chloride mediates its anti-leishmanial activity via a caspase-independent pathway, possibly via metacaspases; studies are underway.

Taken together, our findings have indicated that Berberine chloride triggers cell-death machinery capable of executing several, but importantly, not all features of apoptosis ascribed to mammalian cells. It is envisaged that the study of the major pathways involved in apoptosis-like death in *Leishmania* would provide better insight for design of newer chemotherapeutic approaches critically needed for a disease whose therapeutic armamentarium, to date, is limited.

Acknowledgements

This work received financial assistance from Life Sciences Research Board, DRDO, Governmet of India and Department of Science & Technology, Government of India. PS and RS are recipients of Senior Research Fellowships from Indian Council of Medical Research, Government of India. We thank Professor Sasanka Chakaraborti, Department of Biochemistry, IPGMER, Kolkata for extending his laboratory facilities.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 6 August 2009.

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