CHAPTER-3

EFFECT OF BERBERINE CHLORIDE ON

*L. DONOVANI* PROMASTIGOTES


**Introduction**

The *Leishmania* parasites after infecting mammalian host macrophages differentiate into amastigotes. They deviously survive and multiply within the macrophage phagolysosome (Naderer and McConville, 2008). For survival, circumvention of oxidative stress is vital as during a host-parasite interaction, the host creates a hostile environment by generating reactive oxygen and nitrogen intermediates (Iyer et al., 2008). The anti-oxidant pathway of *Leishmania* includes two iron containing superoxide dismutases which only marginally protect the parasite (Paramchuk et al., 1997; Ghosh et al., 2003), as the absence of catalase and the classical selenium dependent glutathione peroxidase system renders them more susceptible to free radical damage (Augustyns et al., 2001; Jaeger and Flohe, 2006). To minimize the deleterious effects of hydroperoxides, *Leishmania* depend on a less efficient, unique trypanothione dependent antioxidant system which includes the bis-glutathionyl spermidine 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 (Wyllie et al., 2008; Fairlamb and Cerami, 1992; Krauth-Siegel, 2003). Exploiting the impaired antioxidant capacity of *Leishmania* parasites, triggering of oxidative stress seems to be a logical chemotherapeutic modality. Indeed, sodium antimony gluconate, the main stay of treatment for leishmaniasis (Sundar and Chatterjee, 2006) generates reactive oxygen species (ROS) both within macrophage phagolysosomes (Sudhandiran and Shaha, 2003) and the parasite (Mehta and Shaha, 2006; Mandal et al., 2007) thereby inducing apoptosis (Das et al., 2001; Mukherjee et al., 2002) by mitochondrial dysfunction in promastigotes following uncoupling of oxidative phosphorylation (Mehta and Shaha, 2006). 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 (Sen et al., 2007; Das et al., 2008; Mittra et al., 2000). In the present study, we have demonstrated the apoptotic pathway mediated by Berberine chloride in *L. donovani* parasites.
Berberine chloride:  
Berberine chloride [1, 8,13α- tetra-hydro-9, 10- demethoxy-2, 3- (methyl-ene-dioxy) – berberium chloride], a benzodioxoloquinolizine is widely distributed in nine plant families. Berberine is found in such plant as Berberis, Hydrastis canadensis, and Coptis chinensis, Berberis aristata, Berberis vulgaris and Berberis aquifolium, usually in the roots, rhizomes, stems, and bark. It is a medically important quaternary isoquinoline alkaloid that has been used for its medicinal properties in virtually all traditional medicinal systems dating back at least 3000 years in Ayurvedic and Chinese medicine. It has a broad pharmacological spectrum that includes anti-microbial activity (Schmeller et al., 1997) and its effectiveness in leishmaniasis has been amply demonstrated (Ghosh et al., 1983; Ghosh et al., 1985; Vennerstrom et al., 1990). Berberine has drawn attention for its antineoplastic effects (Tang et al., 2009). It seems to suppress the growth of a wide variety of tumor cells including breast cancer (Kim et al., 2008; Kim et al., 2010), leukemia (Li et al., 2008), epidermoid carcinoma (Ho et al., 2009), oral carcinoma (Lin et al., 2007), prostate carcinoma (Muralimanoharan et al., 2009) and gastric carcinoma (Lin et al., 2006). Besides that Berberine seems to act as an herbal antidepressant and a neuroprotector (Ye et al., 2009). New experimental results show that Berberine may have the potential in prevention of Alzheimer’s disease (Ye et al., 2009). Recently Berberine has reported to reduce blood glucose levels in diabetes (Zhang et al., 2010). In the present study, we have characterized the apoptotic pathway induced by Berberine chloride in L. donovani promastigotes.
Materials and methods

Parasite culture
Promastigotes of Indian *Leishmania donovani* strains, previously isolated from patients with VL were routinely cultured at 24°C in Medium 199 (M199) supplemented with 10% fetal 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 x 10⁶/ml.

Cell culture
A human non adherent leukemic monocyte lymphoma cell line (U937) was subcultured 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% CO₂, log phase cells were subcultured every 72 h, inoculum being 5 x 10⁵/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 as described in Materials and methods (Ganguly et al., 2006).

Generation of reactive oxygen species (ROS)
To study the effect of Berberine chloride on generation of ROS, promastigotes (1 x 10⁶/ml) following incubation with Berberine chloride (0 - 50 μM, 3 h) were processed and incubated with H₂DCFDA (50 μM) for 45 min at 37°C and fluorescence acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) as described in Materials and methods (Mandal et al., 2007; Saha et al., 2009).

Measurement of non protein thiols
In *Leishmania* promastigotes after treatment with Berberine chloride, non protein thiols levels were measured with mercury orange as described in Materials and methods (Saha et al., 2009).

Analysis of phosphatidylserine externalization
In *Leishmania* promastigotes after treatment with Berberine chloride, phosphatidyl serine exposure was measured by Annexin -V positivity as described in Materials and methods (Dutta et al., 2007a; Saha et al., 2009).

Measurement of intracellular Ca²⁺ in L. donovani promastigotes
In *Leishmania* promastigotes after treatment wth Berberine chloride, changes in intracellular Ca²⁺ were monitored using the fluorescent probe Fluo-4 AM as described in Materials and methods (Nagamune et al., 2007; Saha et al., 2009).
Berberine chloride as an antileishmanial drug

Analysis of mitochondrial transmembrane potential
In *Leishmania* promastigotes after treatment with Berberine chloride, the mitochondrial transmembrane electrochemical gradient (Δψm) was measured using JC-1 (5, 5', 6, 6'-tetrachloro-1', 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide), a cell permeable, cationic and lipophilic dye as described in Materials and methods (Sen et al., 2007; Saha et al., 2009).

Cell cycle analysis
In *Leishmania* promastigotes after treatment with Berberine chloride, cells were processed for cell cycle analysis by flow cytometry as described in Materials and methods (Sen et al., 2007; Saha et al., 2009).

DNA fragmentation assay by agarose gel electrophoresis
To determine DNA fragmentation, total cellular DNA was isolated from *Leishmania donovani* promastigotes treated with Berberine chloride according to the manufacturer's instructions (Quick apoptotic DNA ladder detection kit, Invitrogen, California, USA) and analysed by 1.5% agarose gel electrophoresis as described in Materials and methods (Verma and Dey, 2004).

Determination of caspase activity in Berberine chloride treated promastigotes
Caspase activity was measured in *Leishmania* promastigotes using a commercially available kit, as per the manufacturer's instructions as described in Materials and methods. To study the biological role of caspases in Berberine chloride induced death, *Leishmania* promastigotes were treated 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) and then cell viability and IC50 values were evaluated by the MTS-PMS assay as described in Materials and methods (Ganguly et al., 2006).

Statistical analysis:
Results were expressed as mean ± 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 \( \mu M \), 48 h) demonstrated a dose dependent inhibition of parasite growth (Isolate: NS2) with a 50% inhibitory concentration (IC\(_{50}\)) achieved at 7.1 \( \mu M \) and IC\(_{90}\) at 50 \( \mu M \). In other *L. donovani* isolates the IC\(_{50}\) varied from 3.0 \( \mu M \) to 15.0 \( \mu M \) (Table 1). The effect of methanol (0.05%), representative of the highest concentration present in Berberine chloride (50 \( \mu M \)) was studied and showed no effect in parasite viability. As NS2 was a virulent isolate, it was used for *ex vivo* experiments and other subsequent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Designation</th>
<th>IC(_{50}) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS2</td>
<td><em>L. donovani</em></td>
<td>7.1</td>
</tr>
<tr>
<td>GE1</td>
<td><em>L. donovani</em> MHOM/IN/90/GE1F8R</td>
<td>3.0</td>
</tr>
<tr>
<td>41</td>
<td><em>L. donovani</em></td>
<td>12.2</td>
</tr>
<tr>
<td>AG83</td>
<td><em>L. donovani</em> MHOM/IN/83/AG83</td>
<td>8.33</td>
</tr>
<tr>
<td>39</td>
<td><em>L. donovani</em></td>
<td>14.8</td>
</tr>
<tr>
<td>2001</td>
<td><em>L. donovani</em></td>
<td>13.6</td>
</tr>
<tr>
<td>MC9</td>
<td><em>L. donovani</em></td>
<td>9.0</td>
</tr>
</tbody>
</table>

Table 1: Anti promastigote activity of Berberine chloride against different *Leishmania donovani* strains* (*Ganguly et al., 2007)*

**Berberine chloride demonstrated pro-oxidant activity in promastigotes:**

To evaluate the effect of Berberine chloride on the oxidative status of promastigotes H\(_2\)DCFDA, a lipid soluble, membrane permeable compound was used, based on evidence that following cleavage by cellular non-specific esterases, an impermeable H\(_2\)DCF is formed which is subsequently oxidized by intracellular reactive oxygen species (ROS) to produce a fluorescent compound DCF; therefore, the resultant fluorescence is directly proportional to the quantum of ROS generated.
Berberine chloride as an anti-leishmanial drug

Initially log phase promastigotes (1 x 10^6/ml) were incubated with an IC_{50} conc. 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 ± 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 auto fluorescence generated by Berberine chloride, treated (Berberine chloride, 0 - 50 μM, 3 h) unstained cells were analysed in flow cytometry. 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 sulfhydryl (-SH) groups generating a nonpermeable 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. Berberine chloride (0 - 50 μM) caused a dose dependent decrease in levels of non protein thiols, mean ± 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, 2.5 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).

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Figure 1: Modulation of oxidant status of *L. donovani* promastigotes by Berberine chloride

A: Effect of Berberine chloride on generation of ROS. *L. donovani* promastigotes were incubated with Berberine chloride (0 - 50 μM) and labeled with 2', 7' dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA, 50 μM) as described in Materials and methods. Data are expressed as the mean GMFC± SEM of at least 3 experiments in duplicate. *p <0.05 and **p <0.01 as compared to controls.

B: Effect of Berberine chloride on level of non protein thols. *L. donovani* promastigotes incubated with Berberine chloride (0 - 50 μM) for 3 h were labeled with mercury orange (500 μM in acetone) and analyzed for fluorescence as described in Materials and methods. Data are expressed as mean GMFC ± SEM of at least 3 experiments in duplicate. *p <0.0005, **p <0.005 and ***p <0.0001 as compared to controls.

C: Effect of an antioxidant NAC on survival of promastigotes. *L. donovani* promastigotes (2 x 10<sup>5</sup>/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.

Phosphatidyl serine externalization was altered by Berberine chloride treatment

The high binding affinity of Annexin V, a Ca<sup>2+</sup> 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 both 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).
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In healthy, log phase promastigotes, the basal binding of annexin V was 5.06% (3.44 + 1.62% = 5.06%) whereas with Berberine chloride, this increased to 36.50% (32.22 + 4.28% = 36.50%) at 6 h (Figure 2, lower and upper right quadrant). The percentage of PI-positive cells (Figure 2, upper left quadrant) at baseline was minimal and remained as being 2.29%, 3.86% and 3.61% at 2, 4 and 6 h 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 in Leishmania promastigotes, whose % positivity was 37.28 (data not shown).

Figure 2: Externalization of phosphatidyl serine in Berberine chloride treated promastigotes: *L. donovani* promastigotes (A) were incubated with an IC_{90} conc. 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 analyzed by flow cytometry as described in Materials and methods. The figure is a representative profile of at least three experiments.

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 (Mehta and Shaha, 2004). The addition of lonomycin, a potent, Ca^{2+} ionophore increased intracellular Ca^{2+} which decreased 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
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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 cysteine (NAC, 5 mM, 1 h, 37\(^{\circ}\)C), an established antioxidant followed by Berberine chloride. Pretreatment with NAC significantly reduced Berberine chloride induced cytosolic Ca\(^{2+}\) when compared to only Berberine chloride treated Leishmania promastigotes at 120 min and 180 min (Figure 3).

![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 \(\mu\)M, ■) and presence of NAC (▲) as described in Materials and methods. Vehicle control, methanol (▼) showed no alterations in cytosolic calcium level. Data are expressed mean ± SEM of atleast three experiments in duplicates. *p <0.05 as compared to Berberine chloride treated Leishmania promastigotes alone.](image)

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 \(\mu\)M, ■) and presence of NAC (▲) as described in Materials and methods. Vehicle control, methanol (▼) showed no alterations in cytosolic calcium level. Data are expressed mean ± SEM of atleast three experiments in duplicates. *p <0.05 as compared to Berberine chloride treated Leishmania promastigotes alone.

**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 (Sen et al., 2007). To measure the mitochondrial membrane potential, JC-1 (5, 5', 6, 6'-tetachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) was used as it is a lipophilic fluorescent cation that freely permeates the mitochondrial membrane and forms J aggregates that fluoresce
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red; accordingly, viable cells stained with JC-1 exhibit a pronounced red fluorescence. An apoptotic stimulus induces a decrease in the mitochondrial membrane potential; due to which JC-1 fails to enter the mitochondria and remains as cytosolic monomers giving 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 (Verma et al., 2007). In control promastigotes, the red/green fluorescence ratio was 14.96 which following the addition of an IC_{50} conc. 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 2.33 at 2 h, 1.31 at 4 h and 0.61 at 6 h.

JC-1 fluorescence was also measured by estimating the % gated population in two gates, namely R2 and R3, wherein R2 represented the healthy, non apoptotic population and R3 represented the apoptotic cell population. These gatings were set following addition of H_2O_2 (15 mM, 10 min) wherein the % gated in R2 and R3 was 17.67% and 79.73% respectively (data not shown). In healthy cells, the R2 and R3 % positivity was 92.8% and 6.2 % respectively. With Berberine chloride (50 μM), the population in R3 gate increased progressively with time, being 61.0 % at 6 h (Figure 4) indicating that Berberine chloride induces depolarization of mitochondrial membrane potential leading to apoptotic cell death.

Figure 4: Effect of Berberine chloride on mitochondrial transmembrane potential in L. donovani promastigotes: L. donovani 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.
Berberine chloride increased the sub G₀/G₁ population in promastigotes:

In promastigotes treated with an IC₉₀ conc. of Berberine chloride, proportion of cells in the sub G₀/G₁ phase at 8 and 12 h progressively increased to 12.95% and 51.84% respectively whereas in controls, the sub G₀/G₁ population was 3.62% (Figure 5, Table 2). Taken together, this progressive increase in the proportion of cells in the sub G₀/G₁ phase corroborated that Berberine chloride induced apoptosis in promastigotes, culminating in DNA degradation and arrest of cell cycle progression.

Table 2: Effect of Berberine chloride on cell cycle progression of L. donovani promastigotes

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>Sub G₀/G₁ (M1)</th>
<th>G₀/G₁ (M2)</th>
<th>S+ G₂/M (M3)</th>
</tr>
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<tbody>
<tr>
<td>-</td>
<td>3.62</td>
<td>78.76</td>
<td>18.87</td>
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</tr>
<tr>
<td>12</td>
<td>51.84</td>
<td>19.63</td>
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</tr>
</tbody>
</table>

Figure 5: Effect of Berberine chloride on cell cycle progression of L. donovani promastigotes. L. donovani promastigotes (1 x 10⁶/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. M1, M2 and M3 represent the cells present in Sub G₀/G₁, G₀/G₁, and S+ G₂/M stage of the cell cycle.

Table 2: Effect of Berberine chloride on cell cycle progression of L. donovani promastigotes.
Berberine chloride as an antileishmanial drug

Berberine chloride induced oligonucleosomal DNA fragmentation in promastigotes:

One of the hallmarks of apoptotic cell death is inter-nucleosomal 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 6, lane 3). A similar type of smearing was also observed with H$_2$O$_2$ (4 mM, 6 h) (Figure 6, lane 4) corroborating with previous studies (Das et al., 2001).

Berberine chloride mediated apoptosis via a caspase-independent pathway:

As caspases are considered as 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$_2$O$_2$ or Miltefosine showed minimal caspase activity (Figure 7A); however, caspase activity was evident in U937 cells treated with a similar conc. of H$_2$O$_2$ (4 mM) and Miltefosine (40 μM) (Figure 7A). To confirm assay specificity, U937 cells were treated 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
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co-incubated for 48 h with Z-VAD-FMK (pan caspase inhibitor) and Berberine chloride (0 - 50 μM), % parasite viability being the end point. The IC₅₀ 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 IC₅₀ remained at 7.19 μM, (Figure 7B) validating that Berberine chloride induces apoptosis via a caspase-independent pathway.

Figure 7A: Caspase activity in L. donovani promastigotes

Cell lysates from L. donovani promastigotes (○) were treated with H₂O₂ (△) or Berberine chloride (□) as described in Materials and methods. In parallel, U937 cells (●) were treated with H₂O₂ (4 mM, ■) or Miltefosine (40 μM, ▲). The data is a representative profile of at least three experiments.

Figure 7B: Effect of Z-VAD-FMK on viability of Berberine chloride treated promastigotes

L. donovani promastigotes were co-incubated for 48 h with (●) and without (▲) Z-VAD-FMK (pan caspase inhibitor) and Berberine chloride (0 - 50 μM), % parasite viability was measured and IC₅₀ values were calculated as described in Materials and methods. Data are expressed mean ± SEM of at least three experiments in duplicates.
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 program, classically defined by unique morphological alterations that include membrane blebbing, cytoplasmic and nuclear condensation accompanied with DNA breakage (Vaux and Strasser, 1996). In *Leishmania* parasites, apoptosis appears to be the predominant form of cell death, in response to variable stimuli that include heat shock, H2O2 and antileishmanial drugs such as pentostam and Miltefosine (Verma and Dey, 2004). Additionally, several plant-derived anti leishmanial compounds including *Aloe vera* (Dutta et al., 2007), Artemisinin (Sen et al., 2007), *Piper betle* Linn (Sarkar et al., 2008), Luteolin (Mittra et al., 2000) and Curcumin (Das et al., 2008) 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 (Mignotte and Vayssiere, 1998). 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 (Sen et al., 2007). Similarly, another leishmanicidal agent, curcumin also enhanced generation of hydroxyl radicals (Das et al., 2008). 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 (Figures 1A and B). This enhanced oxidative insult was vital for the observed leishmanicidal activity, as attenuation of oxidative stress by N-acetyl cysteine caused a 6 fold increase in the IC50 of Berberine chloride (Figure 1C). This leishmanicidal activity has been confirmed in experimental models of VL using an Indian *L. donovani* strain (Ghosh et al., 1985) where >90% inhibition of parasite burden was reported. However, in a Sudanese strain of *L. donovani* (Mignotte and Vayssiere, 1998), 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 (Sen et al., 2007). 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 cytosolic Ca^{2+}, necessary for endonucleases generally to initiate DNA cleavage (Das et al., 2001; Mukherjee et al., 2002). The leishmanicidal activity of Curcumin was accompanied by an increase in Ca^{2+} (Das et al., 2008) whereas a leafy exudate from Aloe vera that also induced an apoptotic-like death in promastigotes minus alterations in Ca^{2+} levels (Dutta et al., 2007). 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, which by virtue of their multiple mitochondria ensure compensation of injured mitochondria. The loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) is generally an early change associated with apoptosis as dissipation of $\Delta\Psi_m$ following permeabilization of the inner mitochondrial membrane triggers release of several apoptotic factors. In Leishmaniasis, two metalloids, arsenic and antimony cause mitochondrial dysfunction in parasites, which are accompanied by DNA fragmentation (Mehta and Shaha, 2006). Artemisinin, a sesquiterpene lactone having established anti-malarial activity also demonstrated anti-leishmanial activity that was associated with a loss in mitochondrial membrane potential (Sen et al., 2007). 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 (Dutta et al., 2007). Berberine chloride too caused mitochondrial membrane depolarization, which was evident from treated with 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.
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proximal to the G₀-G₁ peak i.e. a sub G₀-G₁ peak. In promastigotes incubated with other plant derived anti-leishmanial compounds, a substantial proportion of cells have been identified in the sub G₀-G₁ phase (Sen et al., 2007). Our study is in agreement with previous observations as Berberine chloride also significantly increased the sub G₀-G₁ cell population (Table 2, Figure 5).

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 6, lane 3). DNA smearing has also been observed during apoptosis in yeast (Madeo et al., 1999) and some metazoan cell types (Oberhammer et al., 1993) similarly observed in promastigotes treated with curcumin (Das et al., 2008), Novobiocin (Singh et al., 2005). This non classical type DNA fragmentation could be attributed to the shorter H1 histone molecules present in *Leishmania* (Espinoza et al., 1996) 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 (Chipuk and Green, 2005). Interestingly, in *Leishmania*, apoptotic death has been reported to occur via a caspase independent pathway (Zangger et al., 2002). In fact, the absolute requirement for caspase activation in apoptosis, even in mammalian cells is no longer considered mandatory (Chipuk and Green, 2005). 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 (Zangger et al., 2002). Additionally, as no caspase genes have been identified in the *Leishmania* genome, a role of metacaspases in programmed cell death has been proposed (Gonzalez et al., 2007). Indeed, two metacaspase genes (LdMC1 and LdMC2) have been characterized in *L. donovani* (Lee et al., 2007). We did not observe any induction of caspase activity (Figure 7A) and failure to alter the leishmanicidal activity of Berberine chloride in the presence of a broad spectrum caspase inhibitor Z-VAD-FMK (Figure 7B) indicated that Berberine chloride mediates its antileishmanial 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, till date, is limited.