Parasitology Research (2019) 118:335–345
https://doi.org/10.1007/s00436-018-6157-3

TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

# Berberine chloride mediates its antileishmanial activity by inhibiting *Leishmania* mitochondria

Sritama De Sarkar[1] · Deblina Sarkar[1] · Avijit Sarkar[1] · Aishwarya Dighal[1] · Katrin Staniek[2] · Lars Gille[2] · Mitali Chatterjee[1]

Received: 6 August 2018 / Accepted: 14 November 2018 / Published online: 23 November 2018


## Abstract

Berberine chloride, a plant-derived isoquinoline alkaloid, has been demonstrated to have leishmanicidal activity, which is mediated by generation of a redox imbalance and depolarization of the mitochondrial membrane, resulting in a caspase-independent apoptotic-like cell death. However, its impact on mitochondrial function remains to be delineated and is the focus of this study. In UR6 promastigotes, berberine chloride demonstrated a dose-dependent increase in generation of reactive oxygen species and mitochondrial superoxide, depolarization of the mitochondrial membrane potential, a dose-dependent inhibition of mitochondrial complexes I–III and II–III, along with a substantial depletion of ATP, collectively suggesting inhibition of parasite mitochondria. Accordingly, the oxidative stress induced by berberine chloride resulting in an apoptotic-like cell death in *Leishmania* can be exploited as a potent chemotherapeutic strategy, mitochondria being a prime contributor.

**Keywords** *Leishmania* · Promastigotes · Mitochondria · Reactive oxygen species (ROS) · Mitochondrial superoxide · Berberine chloride

**Abbreviations**

| | |
|---|---|
| ATP | Adenosine triphosphate |
| ETC | Electron transport chain |
| FDA | Food and Drug Administration |
| FBS | Fetal bovine serum |
| $H_2$DCFDA | Dichlorodihydrofluorescein diacetate |
| $IC_{50}$ | Inhibitory concentration$_{50}$ |
| MMP | Mitochondrial membrane potential |
| NADH | Nicotinamide adenine dinucleotide |
| NCC | NADH cytochrome c reductase |
| NTDs | Neglected tropical diseases |
| $O_2^{\bullet-}$ | Superoxide anion |
| ONOO• | Peroxynitrite |
| PKDL | Post kala-azar dermal leishmaniasis |
| PMS | Phenazine methosulfate |
| ROS | Reactive oxygen species |
| SCC | Succinate cytochrome c reductase |
| TTFA | Thenoyltrifluoroacetone |

Handling Editor: Julia Walochnik

Sritama De Sarkar and Deblina Sarkar should be considered as joint first authors

✉ Mitali Chatterjee
ilatim@vsnl.net; ilatimc@gmail.com

[1] Department of Pharmacology, Institute of Post Graduate Medical Education and Research, 244B Acharya JC Bose Road, Kolkata 700020, India

[2] Institute of Pharmacology and Toxicology, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria

## Introduction

Leishmaniasis includes a group of diseases whose manifestations range from a cutaneous self-healing variant, disseminating mucocutaneous lesions to a life-threatening visceral form, and is caused by more than 20 species of the protozoan parasite of the genus *Leishmania*. The disease is endemic in about 97 countries, 350 million people being at risk of acquiring the disease with an estimated 1.5 to 2 million new cases of leishmaniasis occurring per year, causing up to 70,000 deaths (World Health Organization 2018; Torres-Guerrero et al. 2017). Leishmaniasis is one of the neglected tropical diseases (NTDs) where research investments are disproportionately lower than their impact on human health worldwide. This could be mitigated by drug repurposing using target-based

screening and/or phenotypic screening (Andrade-Neto et al. 2018). In fact, drug repurposing accounted for 30% of the new drugs and vaccines approved by the Food and Drug administration (FDA). Drug repurposing can attenuate the risks which amplify throughout the various stages of development in view of the pharmacokinetics and safety profiles being already determined. This is particularly relevant in leishmaniasis where one of the presentations, post kala-azar dermal leishmaniasis (PKDL), has no animal model, and as many key features of the immunopathology associated with PKDL remain to be identified, opportunities for identifying novel therapeutics remain limited. In fact, the mainstay of present day antileishmanials is repurposed antimicrobial (e.g., amphotericin B, paromomycin) and anticancer (e.g., miltefosine) drugs. Antimalarials such as artemisinin, atovaquone, and chloroquine could well be repositioned based on their efficacy demonstrated in experimental models of leishmaniasis (Andrade-Neto et al. 2018 and references therein).

A common feature of all repurposed antileishmanial drugs is their ability to cause oxidative stress in *Leishmania* parasites, based on their relative weak anti-oxidant defense, as they lack catalase or classical selenocysteine containing glutathione peroxidase, rendering the parasites more vulnerable to free radical toxicity (Krauth-Siegel and Comini 2008; van Assche et al. 2011; Flohé et al. 1999). Several plant-derived compounds namely luteolin, quassin, *Aloe vera*, *Piper betle*, berberine chloride, and artemisinin (Dutta et al. 2008; Sarkar et al. 2008; Saha et al. 2011a; Sen and Chatterjee 2011; and references therein) have shown the proclivity to mediate their leishmanicidal activity by augmenting a redox imbalance.

Berberine, an active ingredient of many traditionally used medicinal plants, has been demonstrated to have a broad spectrum of pharmacological activities that include antimicrobial (Sahibzada et al. 2018), anticancer (Li et al. 2018), antidiabetic (Turner et al. 2008), and antiprotozoal (Bahar et al. 2011). Furthermore, berberine and its derivatives have shown efficacy in experimental models of leishmaniasis (Vennerstrom et al. 1990) and intralesional administration of berberine sulfate was effective in canine cutaneous leishmaniasis (Ahuja et al. 1993). The anticancer potential of berberine has been extensively delineated and, in human prostate carcinoma (PC-3) cell lines, has been attributed to increased generation of ROS, altered mitochondrial membrane potential, and increased release of cytochrome c that led to apoptosis (Meeran et al. 2008). Similarly, in breast cancer MCF-7 cells, berberine triggered the apoptotic process through formation of ROS, over-expression of p53 and p21 along with inhibition of Bcl-2 (Tillhon et al. 2012). In human hepatoma cells, berberine inhibited the ERK and PI3K-AKT pathways and reduced the expression of MMP-9 (Liu et al. 2011). The impact of berberine upon mitochondria included inhibition of mitochondrial respiration in cardiomyoblasts (Chang et al. 2017), inhibition of complex I (Pereira et al. 2007), and induction of mitochondrial fission (Yan et al. 2017). Importantly, its impact on macrophage-derived cell lines was not promising (Letasiová et al. 2006). This is important while considering berberine chloride for an intramacrophage pathogen like *Leishmania*. Berberine has been shown to mediate its leishmanicidal activity by inducing a redox imbalance following enhanced generation of ROS and concomitant depletion of non-protein thiols, which culminated in a caspase-independent apoptotic-like death (Saha et al. 2009). In trypanosomatids like *Leishmania*, the presence of a single mitochondrion as one of the major producers of ROS renders it an exploitable target, and as the ability of berberine to induce oxidative stress via mitochondrial dysfunction in cancers is well documented, this study aimed to establish its impact on parasite mitochondria.

## Materials and methods

### Reagents

All chemicals were of analytical grade and obtained from Sigma Aldrich Chemicals (St Louis, MO, USA), except MTS [3-(4, 5 dimethylthiazol-2-yl) 5- (3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium] from Promega (Madison, WI, USA), ATP determination kit and MitoSOX™ red [3, 8- phenanthridinediamine, 5-(6-triphenylphosphoniumhexyl)-5,6 dihydro-6-phenyl] from Molecular Probes (Carlsbad, CA, USA), protein assay dye reagent concentrate from Bio Rad (Haryana, India), and fetal bovine serum (FBS) from Gibco (Thermo Fischer Scientific, Waltham, MA, USA). A stock solution of berberine chloride (Sigma, cat no. B3251, purity > 98%, 100 mM in DMSO) was stored at − 20 °C until use.

### Parasite culture

*Leishmania* promastigotes (MHOM/IN/78/UR6, Mukhopadhyay et al. 2000) were maintained at 24 °C in blood agar slants, containing brain heart infusion agar (3.25%) supplemented with glucose (1.3%) and rabbit blood (2%) along with penicillin G (50 IU/ml), streptomycin (50 μg/ml) or at 24 °C in M199 medium supplemented with 10% FBS, penicillin G (50 IU/ml), streptomycin (50 μg/ml), and hemin (3.25 mg/L); cells were sub-cultured every 48–72 h, inoculum being $1 \times 10^6$ cells/ml.

#### In vitro evaluation of antipromastigote activity of berberine chloride

The antileishmanial activity of berberine chloride in UR6 promastigotes was measured in terms of cell viability using

the modified MTS-PMS assay (Ganguly et al. 2006). Briefly, log phase promastigotes ($1 \times 10^5$ cells/200 μl medium/well) were incubated with berberine chloride (0–100 μM) for 48 h and parasite viability was measured. MTS [3-(4, 5 dimethylthiazol-2-yl) 5- (3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium] (2.0 mg/ml) and PMS (phenazine methosulfate, 0.92 mg/ml) were added in a ratio of 5:1 (20 μl/well) and plates were incubated for 3 h at 37 °C; resultant absorbances were measured at 490 nm in a spectrometer (Merilyzer EIAQuant, Meril Life Sciences, India). The mean percent viability was calculated as follows:

(Mean specific absorbance of treated parasites/Mean specific absorbance of untreated parasites) × 100

Results were expressed as the $IC_{50}$, i.e., the concentration that inhibited viability by 50%; both $IC_{50}$ and $IC_{90}$ were enumerated by graphical extrapolation using GraphPad Prism software (version 5).

## Measurement of free radical generation in *Leishmania* promastigotes

To study the effect of berberine chloride on generation of reactive oxygen species (ROS) in UR6, log phase promastigotes ($2 \times 10^4$ cells/500 μl) following incubation with berberine chloride (0–50 μM, 37 °C, 3–24 h) were washed with phosphate-buffered saline (0.02 M phosphate, pH 7.2, PBS) and then incubated with 2, 7 dichlorodihydrofluorescein diacetate ($H_2$DCFDA, 100 μM, 30 min, 37 °C). Fluorescence was acquired on a flow cytometer (FACS Verse, Becton Dickinson, CA, USA) using forward vs. side scatter to gate the parasite population and an FL1 histogram quantified the fluorescence of viable parasites. A time kinetic measurement was done to monitor the generation of ROS in UR6 at 3, 18, and 24 h. Treatment with hydrogen peroxide ($H_2O_2$, 1 mM, 1 h, 37 °C) served as the positive control.

## Evaluation of mitochondrial functions

### Measurement of mitochondrial superoxide in Leishmania promastigotes

To determine the generation of ROS within the mitochondrion of *Leishmania* promastigotes, a live-cell permeant dye MitoSOX™ red was used which rapidly and selectively targets mitochondria (Shadab et al. 2017). The entry of the probe is dependent on membrane potential, and once inside the mitochondria, the MitoSOX™ red reagent is rapidly oxidized by superoxide and exhibits a red fluorescence (excitation/emission being 510/580 nm) directly proportional to the amount of superoxide present in the mitochondria (Polster et al. 2014). Briefly, UR6 promastigotes ($2 \times 10^4$ cells in 250 μl PBS) were pre-stained with MitoSOX™ (2.5 μM, 1 h, 37 °C) followed by incubation with berberine chloride (0–50 μM, 37 °C) for 3, 18, and 24 h. Cells were then acquired in the FL3 channel of a flow cytometer; miltefosine (HePC, 1 μM, 3 h, 37 °C) served as the positive control.

### Isolation of mitochondria

Mitochondria from log phase promastigotes were isolated following hypotonic lysis and differential centrifugation as previously described (Roy et al. 2008) with some modifications. Briefly, *Leishmania* parasites (UR6, $1–2 \times 10^9$ cells) were washed twice with PBS and the cell pellet resuspended in Tris-HCl (5 mM, pH 7.4) at 25 °C for 10 min to osmotically lyse the cells. The suspension was then passed through a needle (26 gauge) followed by homogenization using a pre-chilled Dounce homogenizer (~ 10 cycles, each cycle for 5 min, 4 °C) and observed microscopically for optimum cell lysis. This was followed by immediate addition of a one-third volume of mitochondria stabilization buffer (1 M sucrose, 35 mM EDTA, 50 mM Tris). The homogenate was then centrifuged (1000*g*, 10 min, 4 °C); the resultant supernatant was further centrifuged (13,000*g*, 20 min, 4 °C). The resultant pellet containing mitochondria was then resuspended in phosphate buffer (50 mM, pH 7.4) containing a protease inhibitor cocktail (04693116001, Sigma), and protein concentrations were measured by using Bradford reagent (Bradford 1976). The isolated mitochondria were stored as aliquots at − 20 °C until use.

### Complex I–III (NADH cytochrome c reductase, NCC) coupled assay

The activity of complex I–III (NADH cytochrome c reductase) was measured in mitochondria, isolated from UR6 promastigotes by the NADH supported reduction of ferricytochrome c to ferrocytochrome c, absorbances being measured at 550 nm (Chen et al. 2001). Briefly, mitochondria (30–50 μg protein) in a final volume of 200 μl of phosphate buffer (50 mM, pH 7.4) containing NADH (250 μM, Sigma, cat no. N4505), cytochrome c (1.2 mg/ml, Sigma, cat no. C2506), sodium cyanide (2 mM), and EDTA (0.01 mM) were incubated with berberine chloride (Ber, 0–50 μM, 25 °C). The increase in absorbance was immediately measured at 550 nm, every 15 s for 5 min in a quartz cuvette in a spectrophotometer (Genesys 10S, UV-VIS, Thermo Fisher Scientific, Waltham,

MA, USA), with rotenone (inhibitor of complex I, 0.25 mM; Mehta and Shaha 2004; Upegui et al. 2014) serving as the positive control. The enzyme activity was calculated in terms of nanomoles of cytochrome c reduced/min/mg protein, the molar extinction co-efficient of cytochrome C being 29.5 $mM^{-1}$ $cm^{-1}$.

### Complex II–III (succinate cytochrome c reductase, SCC) coupled assay

The activity of complex II–III (succinate cytochrome c reductase) in parasite mitochondria isolated from UR6 promastigotes, treated with berberine chloride (0–50 μM, 25 °C), was assayed by monitoring the succinate-supported reduction of ferricytochrome c to ferrocytochrome c at 550 nm (Chen et al. 2001). Briefly, mitochondria, 30–50 μg in 200 μl of phosphate buffer (100 mM, pH 7.4), were incubated with succinate (2 mM), sodium cyanide (1.0 mM), EDTA (0.3 mM), and cytochrome c (1.2 mg/ml, Sigma, cat no. C2506); the reference cuvette contained all the reagents, except the sample and increase in absorbances at 550 nm was monitored every 15 s for 5 min in a spectrophotometer. Thenoyltrifluoroacetone (TTFA, 400 μM), an inhibitor of complex II, confirmed the assay specificity, and enzyme activity was calculated in terms of nanomoles of cytochrome c reduced/min/mg protein, molar extinction co-efficient of cytochrome C being 29.5 $mM^{-1}$ $cm^{1}$.

### Measurement of mitochondrial membrane potential

The mitochondrial transmembrane electrochemical gradient was measured using a cell permeable, cationic, lipophilic dye, JC-1 wherein the ratio of red to green fluorescence of JC-1 indicated the cellular mitochondrial transmembrane potential, and the impact of berberine chloride was measured in UR6 promastigotes as previously described (Sen et al. 2007). Briefly, parasites ($5 \times 10^5$/ml) were stained with 10 μM JC-1 at 24 °C for 10 min and fluorescence measured in a flow cytometer, hydrogen peroxide ($H_2O_2$, 40 mM, 30 min, 37 °C) being the positive control.

### Measurement of ATP

ATP was measured using an ATP determination kit, wherein the luminescence generated is proportional to the amount of ATP present, based on the utilization of ATP by luciferase ($Em_{max}$ 560 nm, pH 7.8, Roy et al. 2008). Promastigotes (UR6, $1 \times 10^6$ cells) treated with berberine chloride (0–50 μM, 3 h, 37 °C) were lysed using triton X-100 (0.25%), and the lysed cells (10 μl) were added to a reaction mix of 190 μl containing luciferin (0.5 mM), luciferase (1.25 μg/ml), and reaction buffer in deionized $H_2O$. The amount of intracellular ATP was measured in a total volume of 200 μl in a 96-well black, clear bottom microtest plate (Optilux, BD Falcon, CA, USA) in terms of chemiluminescence (Spectramax M2e, Molecular Devices, San Jose, CA, USA). The amount of ATP was calculated from a standard curve (0–1000 nM) and expressed as nanomoles of ATP per $10^6$ cells. Oligomycin (10 μM) served as the positive control.

### Pyruvate kinase/lactate dehydrogenase-coupled ATP hydrolysis activity

The mitochondrial $F_0F_1$-ATPase activity was measured spectrophotometrically at 340 nm by coupling the hydrolysis of ATP to the oxidation of NADH via the pyruvate kinase and lactate dehydrogenase reaction (Roy et al. 2008). Briefly, mitochondria from UR6 promastigotes were initially permeabilized by incubating in a digitonin (1%) containing solubilizing buffer (30 min, 4 °C) comprising Tris-HCl (50 mM, pH 7.4), NaCl (120 mM), KCl (5 mM), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM), and glycerol (10%). The ATPase activity of solubilized mitochondria was measured in terms of the oxidation of NADH via pyruvate kinase and lactate dehydrogenase, molar extinction co-efficient of NADH being 6.22 $mM^{-1}$ $cm^{-1}$ (Roy et al. 2008). Accordingly, the reaction mixture (200 μl) contained mitochondrial protein (30–50 μg) in Tris-HCl (50 mM, pH 8.0) along with ATP (1.0 mM), $MgCl_2$ (1.0 mM), KCl (25 mM), EDTA (0.10 mM), NADH (0.10 mM, Sigma, cat no. N4505), phosphoenolpyruvate (0.5 mM), pyruvate kinase (2.5 U), lactate dehydrogenase (4 U), cyanide (5 μM), rotenone (5 μM), and antimycin A (5 μM). These assay conditions minimized the contribution of other transport ATPases, such as $Na^+$, $K^+$-ATPase. Berberine chloride (0–50 μM, 25 °C) or oligomycin (10 μM, 25 °C, a complex V inhibitor) was added and decrease in absorbance was measured at 340 nm every 15 s for 5 min in a spectrophotometer. Data was expressed as nanomoles of NADH oxidized/min/mg protein.

## Flow cytometry

Promastigotes ($2 \times 10^4$ cells/500 μl) from different experimental groups were monitored for their intracellular fluorescence on a flow cytometer. The parasites were gated based on their forward and side scatter and fluorescence was then measured in the log mode using BD FACS Suite™ software (BD Biosciences, CA, USA). Acquisition was performed on 8000 gated events and data expressed as geometrical mean fluorescence channel (GMFC), i.e., average or central tendency of fluorescence of analyzed particles. Data was analyzed using BD FACS Suite™ (BD Biosciences, CA, USA).

### Statistical analysis

Each experiment was performed at least thrice and results expressed as mean ± standard error of the mean (SEM). Statistical analysis was evaluated by Kruskal Wallis multiple comparison test followed by Dunnett's multiple comparison test for non-parametric data using GraphPad Prism software, version 5 (La Jolla, CA, USA); $p < 0.05$ was considered as statistically significant.

## Results

### Antipromastigote activity of berberine chloride in UR6 promastigotes

Berberine chloride (Ber, 0–25 μM, 48 h) demonstrated a dose-dependent inhibition of cell growth in *Leishmania* promastigotes (UR6); the 50% inhibitory concentration ($IC_{50}$) being 4.80 μM (Fig. 1) and the $IC_{90}$ being achieved at 50 μM (data not shown). DMSO (0.25%) representative of the amount present in the highest concentration of berberine chloride (50 μM) showed no effect on cell viability, confirming its inertness (data not shown).

### Berberine chloride stimulated generation of reactive oxygen species in *Leishmania* promastigotes

In cancer cell lines, berberine exerted its cytotoxicity via enhanced generation of free radicals. Accordingly, the ability of berberine chloride to generate ROS in *Leishmania* was evaluated using the membrane permeable probe $H_2$DCFDA that

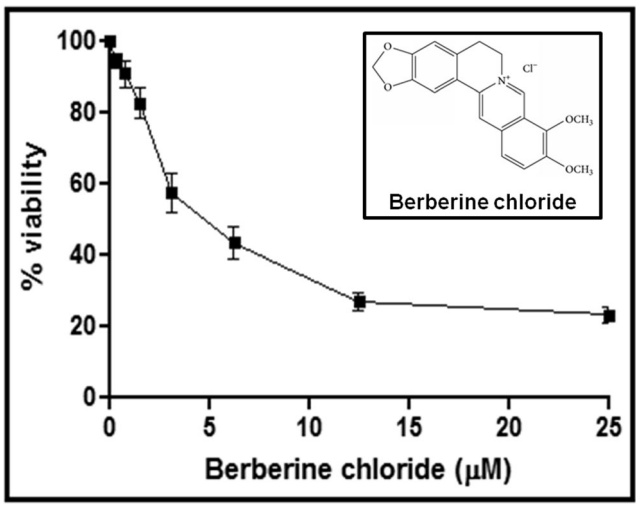


**Fig. 1** Antipromastigote activity of berberine chloride. Log phase promastigotes (UR6, $2 \times 10^5$/200 μl/well, ■) were incubated with berberine chloride (0–25 μM, 48 h), a quaternary ammonium salt of an isoquinoline alkaloid (inset). Cell viability was measured by the MTS-PMS assay as described in "Materials and methods." Data are expressed as the mean ± SEM of at least three experiments in duplicate

generates a fluorescent compound DCF, following its oxidation by intracellular free radicals (Wan et al. 1993); the resultant fluorescence being representative of the quantum of ROS generated. In UR6, berberine chloride (Ber, 0–50 μM, 37 °C, 3 h) increased the GMFC by 2.35-, 2.42-, 2.66-, and 2.49-fold respectively (Fig. 2a and d). Upon extending the incubation period to 18 h, berberine chloride caused a similar dose-dependent enhanced fluorescence from 12.5 μM onwards, the fold increase being 1.30, 1.96, and 2.56 (Fig. 2b**)**, and the trend remained at 24 h (Fig. 2c); $H_2O_2$ (1 mM, 1 h, 37 °C) served as the positive control, wherein a 41.6-fold increase in fluorescence was observed (4309.00 ± 10.68, Fig. 2d).

### Berberine chloride enhanced generation of mitochondrial superoxide in *Leishmania* promastigotes

Mitochondria generate superoxide ($O_2^{\bullet-}$), with complexes I and III of the electron transport chain (ETC) being the major sites of generation (Brookes 2005). Since berberine chloride generated ROS in *Leishmania* promastigotes, generation of mitochondrial superoxide was evaluated. Berberine chloride (Ber, 0–50 μM, 37 °C) showed a dose-dependent increase in MitoSOX™ fluorescence (GMFC) in *Leishmania* promastigotes (UR6; Fig. 3). At 3 h, berberine chloride (6.25–50 μM), the fluorescence increased by 1.39-, 1.50-, 1.58-, and 1.75-fold (Fig. 3a and d). At longer time points (18 h), a similar dose-dependent increase in the levels of mitochondrial superoxide was evident, fold increase being 1.27, 1.41, 1.59, and 2.06 respectively (Fig. 3b) and was similar at 24 h **(**Fig. 3c**)**. Miltefosine (1 μM, 3 h) served as a positive control (Getachew and Gedamu 2012) and demonstrated a 4.14-fold increase (2457.00 ± 91.80) in the generation of mitochondrial superoxide (Fig. 3d).

### Berberine chloride induced depolarization of mitochondrial transmembrane potential in UR6 promastigotes

The loss of mitochondrial membrane potential is a characteristic feature of apoptosis and plays a key role in death of *L. donovani* promastigotes (Sen et al. 2007, 2010). The effect of berberine chloride on transmembrane potential was evaluated in UR6 promastigotes, wherein a concentration-dependent progressive increase in monomers was observed using 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1). JC-1 fluorescence was measured in the percentage gated population within two gates, namely P2 and P3, where P2 represented the mitochondrial resting membrane potential, while P3 represented cells whose mitochondrial membrane potential had been depolarized, and were prone to apoptosis (Fig. 4a and b). In healthy cells, percentage JC-1 monomers was 5.88 ± 0.77

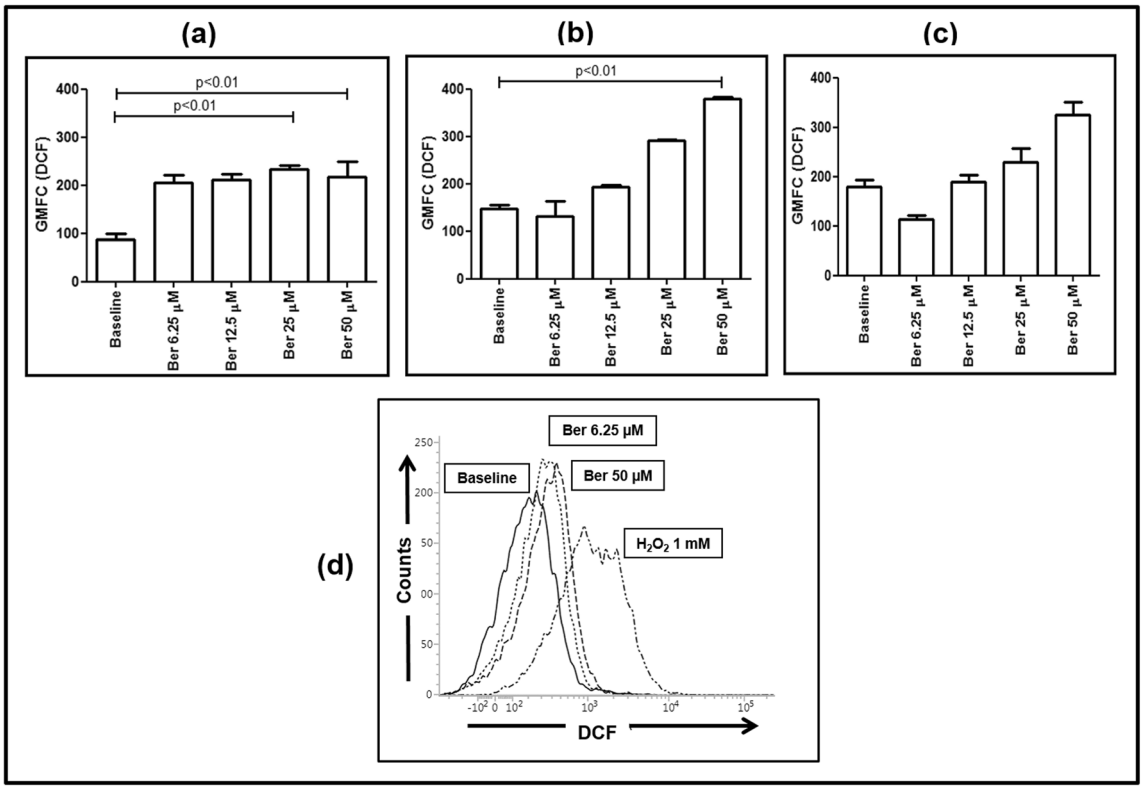


**Fig. 2** Effect of berberine chloride on generation of free radicals in *Leishmania* promastigotes. (**a**–**c**) Log phase promastigotes (UR6, 2 × $10^4$ cells/500 μl) were incubated with berberine chloride (Ber, 0–50 μM, 37 °C) for 3 h (**a**), 18 h (**b**), and 24 h (**c**), and after being labeled with $H_2$DCFDA (100 μM, 30 min, 37 °C), fluorescence was acquired and analyzed as described in "Materials and methods." Data are expressed as the mean ± SEM of GMFC of at least three experiments in duplicate; $p < 0.05$, $p < 0.01$ as compared to respective control. **d** Representative histogram profile of UR6 promastigotes (2 × $10^4$ cells/500 μl, Baseline) treated with berberine chloride (Ber 6.25 μM and 50 μM, 37 °C, 3 h) or $H_2O_2$ (1 mM, 37 °C, 3 h) and stained with $H_2$DCFDA as described in "Materials and methods"

and, with the addition of berberine chloride (6.25–50 μM), increased progressively to 11.87 ± 0.77, 21.37 ± 1.80, 42.96 ± 1.96, and 69.33 ± 3.58% respectively (Fig. 4c). This translated into a decline in the red/green fluorescence ratio from 17.21 ± 2.35 in healthy cells to 7.54 ± 0.63, 3.74 ± 0.43, 1.32 ± 0.10, and 0.45 ± 0.07 with berberine (6.25, 12.5, 25.0, and 50 μM respectively).

## Berberine chloride inhibited complex I–III (NADH cytochrome c reductase, NCC) and complex II–III (succinate cytochrome c reductase, SCC) activities in UR6 promastigotes

In view of berberine chloride being able to trigger superoxide generation in *Leishmania* promastigotes, its impact on the mitochondrial ETC was assessed. Berberine chloride demonstrated a dose-dependent inhibition of the complex I–III coupled activity (NCC) as the baseline cytochrome c reduction decreased from 49.30 ± 1.37 to 46.32 ± 2.52 (6.25 μM), 37.17 ± 4.74 (12.50 μM), 24.22 ± 2.34 ($p < 0.05$, 25.00 μM), and 24.09 ± 3.22 ($p < 0.05$, 50.00 μM) nmoles/min/mg respectively. This translated into a dose-dependent inhibition of 6.00, 24.60, 51.00, and 51.13% respectively (Fig. 5a). Rotenone (250 μM) served as the positive control, wherein a 3.2-fold decrease in cytochrome c reduction was observed (15.39 ± 3.02 vs. 49.30 ± 1.37, Fig. 5a).

With regard to the complex II–III (SCC) activity, the scenario was similar, as berberine chloride (0–50 μM) mediated a dose-dependent inhibition with nmoles of cytochrome c reduction/min/mg protein being decreased from 26.09 ± 2.34 to 22.46 ± 0.55 (6.25 μM), 17.90 ± 0.44 (12.50 μM), 15.36 ± 0.31 ($p < 0.05$, 25.00 μM), and 12.05 ± 0.78 ($p < 0.01$, 50.00 μM (Fig. 5b), respectively, which translated into a progressive inhibition of 14.00, 31.40, 41.12, and 57.00% respectively. Thenoyltrifluoroacetone (TTFA, 400 μM) served as the positive control, wherein there was a 5.8-fold decrease in cytochrome c reduction (4.46 ± 0.80 vs. 26.09 ± 2.34, Fig. 5b).

## Berberine chloride decreased levels of ATP in *Leishmania* promastigotes

As berberine chloride inhibited I–III and II–III activities (Fig. 5), its effect on levels of ATP was measured. Berberine chloride (0–50 μM) depleted ATP substantially from 122.00 ± 11.50 nmoles to 44.17 ± 5.66, 38.95 ± 3.36, 19.09 ± 1.45 ($p < 0.01$), and 11.65 ± 1.66 ($p < 0.001$) nmoles, respectively, translating into a

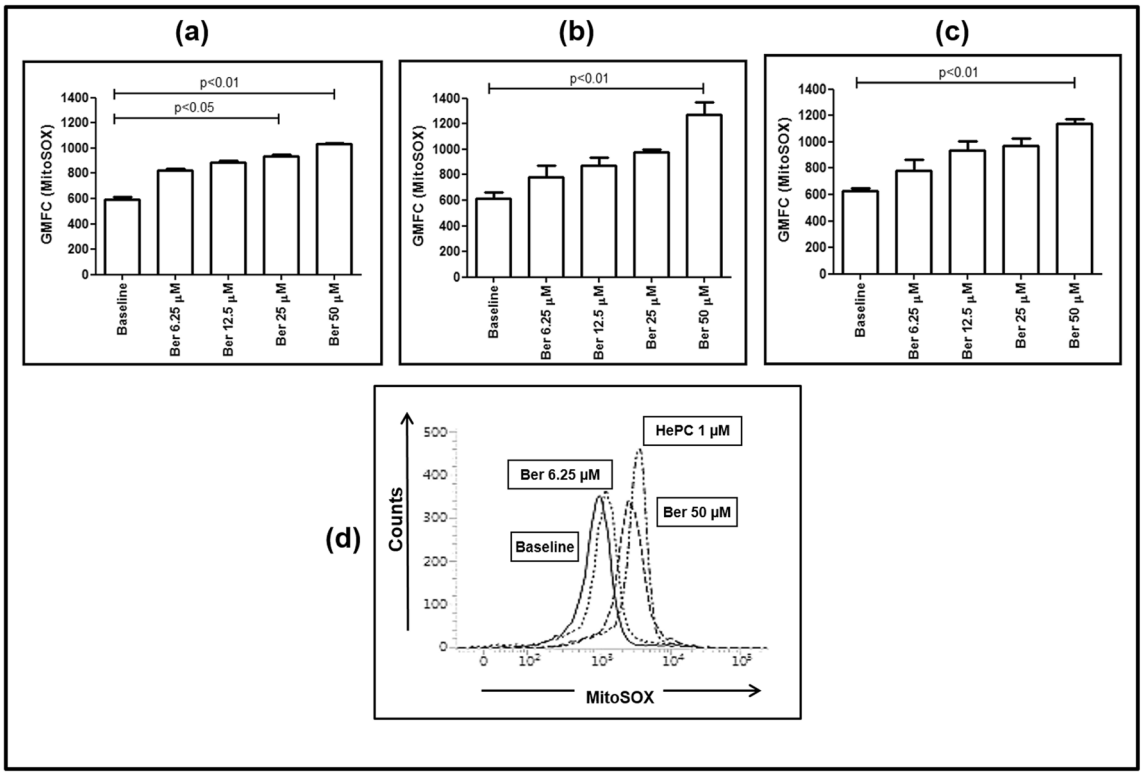


**Fig. 3** Effect of berberine chloride on generation of mitochondrial superoxide in *Leishmania* promastigotes. (**a**–**c**) Log phase promastigotes (UR6, $2 \times 10^4$ cells/500 μl, Baseline) were pre-stained with MitoSOX™ (2.5 μM, 37 °C, 1 h) followed by incubation with berberine chloride (Ber, 0–50 μM, 37 °C) for 3 h (**a**), 18 h (**b**), and 24 h (**c**) followed by acquisition and analysis as described in "Materials and methods." Data are expressed as the mean ± SEM of GMFC of at least three experiments in duplicate; $p < 0.05$, $p < 0.01$ as compared to respective controls. **d** Representative histogram profile of UR6 promastigotes ($2 \times 10^4$/500 μl, baseline) treated with berberine chloride (Ber 6.25 μM and 50 μM, 37 °C, 3 h) or miltefosine (HePC, 1 μM, 37 °C, 3 h) and stained with MitoSOX (2.5 μM, 37 °C) as described in "Materials and methods"

depletion of 63.80, 68.07, 84.35, and 90.45% respectively (Fig. 6a). Oligomycin, an inhibitor of the $F_0F_1$ complex, significantly decreased levels of ATP by 82.00% to 22.16 ± 5.10 nmoles, $p < 0.001$ (Fig. 6a).

### Effect of berberine chloride upon ATP hydrolysis in UR6 promastigotes

The $F_0F_1$-ATP synthase (complex V) is responsible for the last step of the electron transport chain during oxidative phosphorylation, and depending on the available proton gradient, mitochondrial $F_0F_1$-ATP synthase ($F_0F_1$-ATPase) catalyzes synthesis or hydrolysis of ATP (Faccenda and Campanella 2012). Berberine chloride up to 12.50 μM did not affect the $F_0F_1$-ATP synthase activity in UR6 promastigotes (Fig. 6b). However, at the higher concentrations (25 and 50 μM), ATP hydrolysis was inhibited as baseline levels of 274.50 ± 15.62 decreased to 220.00 ± 19.27 and 157.40 ± 8.57 nmoles of NADH oxidized/min/mg protein respectively (Fig. 6b). Oligomycin, a potent inhibitor of complex V, significantly inhibited the $F_0F_1$-ATP synthase activity by 80% to 53.09 ± 17.08 ($p < 0.001$) NADH oxidized/min/mg protein (Fig. 6b).

## Discussion

The generation of oxidative stress by berberine in cancer cell lines has been established as the underlying factor mediating its cytotoxicity, and a similar scenario was demonstrated in virulent *L. donovani* promastigotes (NS2, Saha et al. 2009) and amastigotes (Saha et al. 2011b). Importantly, as the impact of berberine on macrophages sourced from peritoneal (Saha et al. 2011b) or monocyte-macrophage cell lines was limited, further studies on delineating its target site of action were undertaken. As studies with mitochondria require high cell densities, this is only possible in non-pathogenic rapidly growing strains. Accordingly, this work was performed in a non-pathogenic strain of *Leishmania* promastigotes, namely UR6. However, its $IC_{50}$ was comparable (Fig. 1) with virulent strains (Saha et al. 2009) and, importantly, was substantially lower than its $IC_{50}$ in murine macrophages (Saha et al. 2011b). This redox imbalance induced by berberine can be secondary to enhanced generation of ROS (Fig. 2) and/or depletion of thiols, the former owing to mitochondrial dysfunction. The antioxidant defense of *Leishmania* is relatively compromised owing to their inability to express catalase or classical

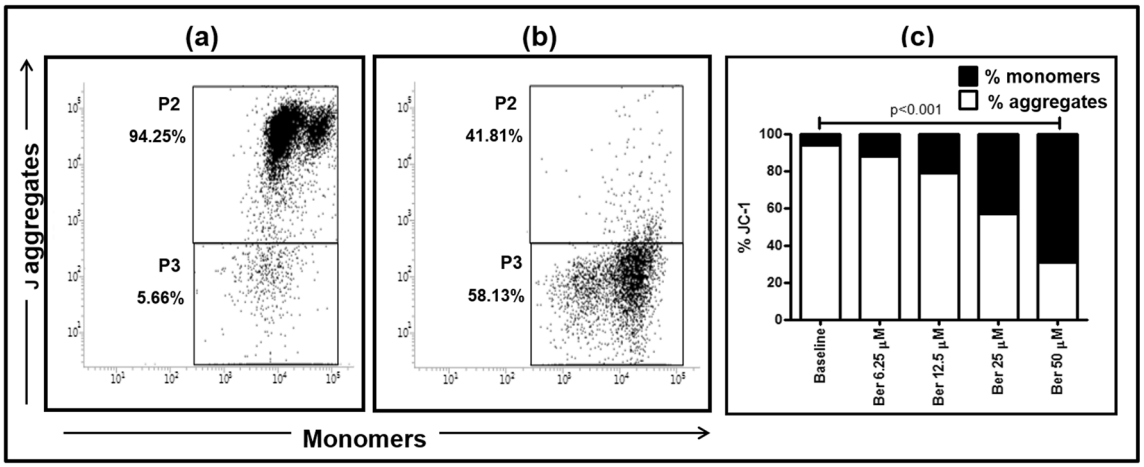


**Fig. 4** Effect of berberine chloride on generation of mitochondrial membrane potential in *Leishmania* promastigotes. (**a**) and (**b**) Representative profiles of JC-1 mediated red: green fluorescence in UR6 promastigotes ($1 \times 10^6$/ml, **a**) treated with berberine chloride (50 μM, **b**) as described in "Materials and methods." **c** Log phase UR6 promastigotes ($1 \times 10^6$/ml) incubated with berberine chloride (6.25–50 μM) were stained with JC-1 and analyzed as described in "Materials and methods." Data are expressed as mean percentage JC-1 monomers/aggregates of at least three experiments in duplicate

selenocysteine containing glutathione peroxidase, the two major $H_2O_2$-metabolizing enzymes usually present in eukaryotes. This Achilles heel of parasites renders them more vulnerable to free radical toxicity and is a key target for antileishmanial drugs (Krauth-Siegel and Comini 2008). Furthermore, the cytotoxicity of berberine could also be mediated secondary to its ability to deplete thiols (Saha et al. 2009), which may occur following disruption of thiol synthesis or its enhanced utilization following higher generation of superoxide; studies are underway.

Mitochondria are established producers of ROS, wherein inhibition of the mitochondrial ETC along with membrane depolarization leads to cell death (Fidalgo and Gille 2011; Nohl et al. 2003). In *Leishmania* species, functioning of the single mitochondrion is critical *vis-a-vis* other organisms who by virtue of their multiple mitochondria ensure compensation of injured mitochondria. Indeed, this disruption accounts for the parasiticidal activity of conventional antileishmanial drugs, namely, amphotericin B, miltefosine, and pentamidine (Lee et al. 2002). Additionally, many plant-derived compounds including the essential oil components from *Chenopodium* and *Cajucara* leaves, ethanolic extracts from *Withania* leaves, and xanthohumol target parasite mitochondria inhibit the electron transport chain and affect mitochondrial respiration (Monzote and Gille 2010; Monzote et al. 2014, 2015, 2017, 2018; Rodrigues et al. 2013; Chandrasekaran et al. 2013). Similarly, berberine demonstrated an enhanced generation of mitochondrial superoxide (Fig. 3), possibly secondary to reduction of $O_2$ to superoxide anion ($O_2^{\bullet-}$), the precursor of most reactive oxygen species and mediator of oxidative chain reactions (Brookes 2005). These superoxide radicals ($O_2^{-\bullet}$) upon reaction with $NO^{\bullet}$ can produce reactive nitrogen species (RNS) namely peroxynitrite ($ONOO^{\bullet}$). Dismutation of $O_2^{-\bullet}$, either spontaneously or

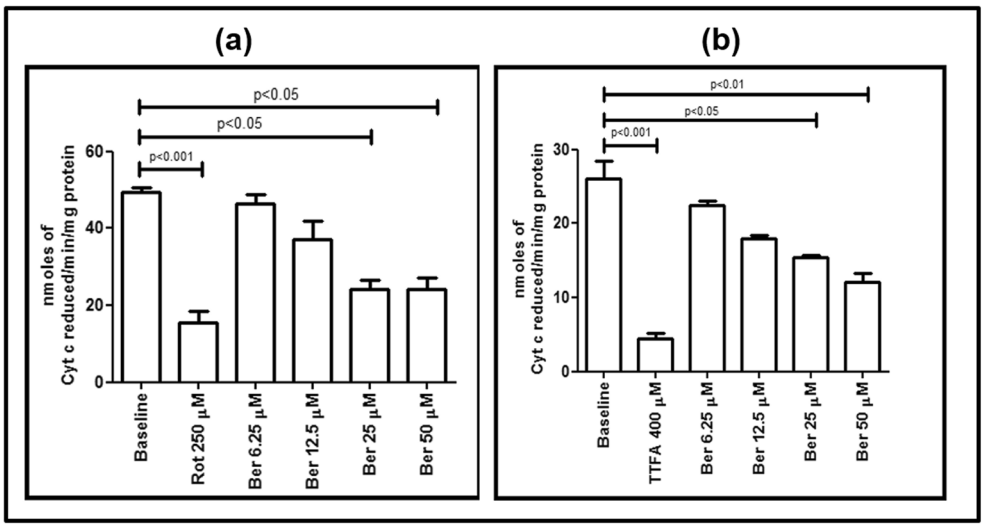


**Fig. 5** Effect of berberine chloride on the mitochondrial respiratory chain in *Leishmania*. (**a**) and (**b**) Mitochondria sourced from UR6 promastigotes were incubated with berberine chloride (Ber, 0–50 μM) and complex I–III (**a**) and complex II–III (**b**) assays performed as described in "Materials and methods." Data are expressed as mean ± SEM of nmoles of cytochrome c reduced/min/mg protein of at least three experiments in duplicate

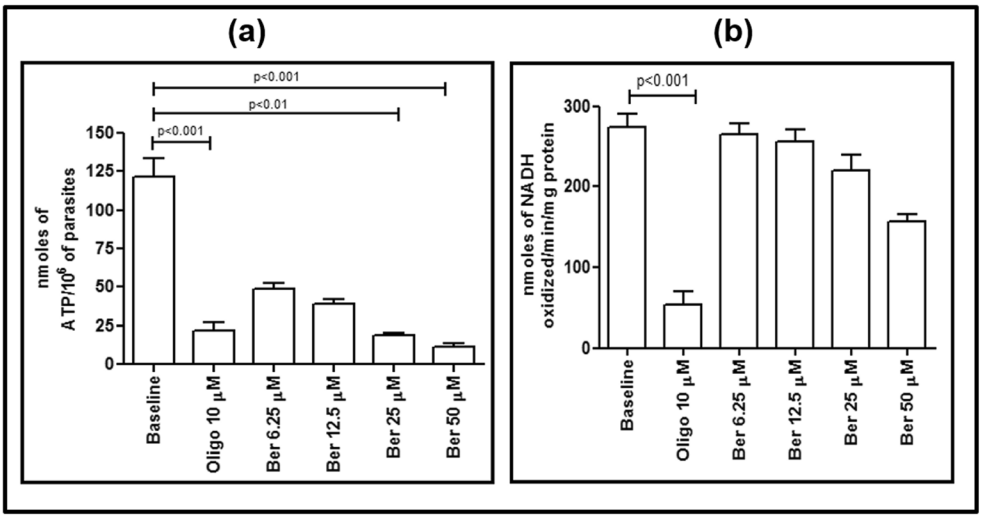


**Fig. 6** Effect of berberine chloride on levels of ATP and ATP hydrolysis in *Leishmania* promastigotes. (**a**) The levels of ATP were determined in UR6 promastigotes ($1 \times 10^6$) following treatment with berberine chloride (Ber, 0–50 μM) or oligomycin (Oligo, 10 μM). The ATP content was determined by the luciferin/luciferase reaction as described in "Materials and methods." The results are expressed as the mean ± SEM of nmoles of ATP per $10^6$ promastigotes of at least three experiments in duplicate. (**b**) Mitochondria from UR6 promastigotes treated with berberine chloride (Ber, 0–50 μM) were assayed for the $F_0F_1$-ATPase activity. The pyruvate kinase/lactate dehydrogenase-coupled assay was performed as described in "Materials and methods," with oligomycin (Oligo, 10 μM) as the positive control. Values represent the mean ± SEM of nanomoles of NADH oxidized/min/mg protein of at least three experiments in duplicate

through a reaction catalyzed by superoxide dismutases (SODs), produces hydrogen peroxide ($H_2O_2$) which can act as a signaling molecule and impacts on the cell cycle, induces stress responses, and influences energy metabolism among others (Brookes 2005).

In the respiratory chain, the proton pumps are critical to maintain the transmembrane proton gradient and the membrane potential ($\Delta\psi_m$). Therefore, alterations in the membrane potential can induce formation of ROS (Lee et al. 2001). Conventional as well as plant-derived antileishmanials namely miltefosine and artemisinin respectively induced loss in mitochondrial membrane potential in *L. donovani* resulting in mitochondrial dysfunction, which translated into apoptosis (Getachew and Gedamu 2012; Sen et al. 2007; De Sarkar et al. 2018). Berberine has been reported to induce mitochondrial dysfunction via mitochondrial membrane depolarization in cancer cells (Tillhon et al. 2012). Likewise, berberine chloride demonstrated loss of transmembrane potential by increasing the proportion of JC-1 monomers with concomitant decrease in the JC-1 aggregates (Fig. 4).

As berberine chloride induced membrane depolarization in promastigotes, this would impact on parasite mitochondrial functions as the primary source of $O_2^{-\bullet}$ is complex III generated through the Q-cycle (Dröse and Brandt 2008; Nohl et al. 2003). Complex I generates superoxide on the matrix side, while complex III accumulates superoxide on both the inner membrane and the matrix sides of the mitochondrial inner membrane (Mailloux and Harper 2011). In mouse melanoma cells, berberine chloride targeted complex I (Pereira et al. 2007). However, as complex I in protozoa is different from the mammalian counterpart with respect to structure, inhibitor sensitivity, and function (Chen et al. 2001), we limited our study to examining the impact of berberine on coupled I–II and II–III complex activities (NCC and SCC). Berberine mediated a pronounced impairment of NCC and SCC activities (Fig. 5a and b), which we propose accounted for the enhanced generation of superoxide (Fig. 3).

Mitochondria are the pivots of cellular energy metabolism generating ATP through oxidative phosphorylation, an essential requirement being maintenance of the MMP (Fidalgo and Gille 2011). In view of berberine chloride causing alteration of the MMP as also mitochondrial dysfunction, the decreased

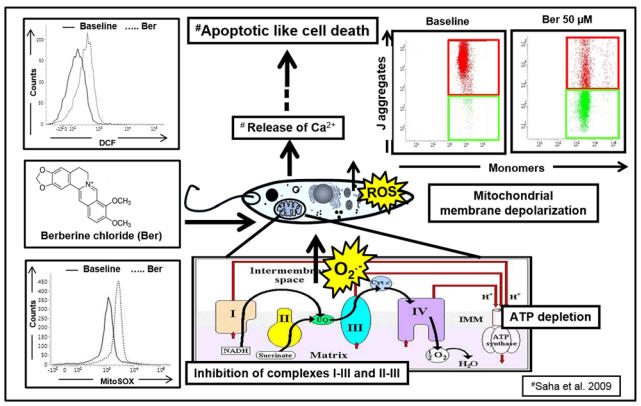


**Fig. 7** A proposed model demonstrating the effect of berberine chloride upon *Leishmania* parasites. In *Leishmania* promastigotes, Berberine chloride enhanced the generation of ROS, and mitochondrial superoxide caused membrane depolarization, inhibited the electron transport chain, and depleted ATP leading to apoptosis

levels of ATP in *Leishmania* promastigotes (Fig. 6a) corroborated that berberine mediated its action by mitochondrial inhibition. This depletion of ATP can result from multiple sources, namely (i) inhibition of complex V ($F_0F_1$-ATP synthase), (ii) disruption of the MMP, and/or (iii) increased ATP hydrolysis. The synthesis of ATP requires involvement of both $F_0$ and $F_1$ components and a chemiosmotic gradient (Faccenda and Campanella 2012). Accordingly, the inhibition of complex V (Fo-$F_1$ synthase, Fig. 6b) along with disruption of the MMP (Fig. 4), collectively contributed towards the depletion of ATP. This has been demonstrated by plant-derived antileishmanial compounds camptothecin and 3, 3′-diindolylmethane (Sen et al. 2004; Roy et al. 2008). Furthermore, as berberine did not alter the ATP hydrolysis activity up to 12.5 μM (Fig. 6b), it endorsed that berberine at its $IC_{50}$ dose promoted ATP hydrolysis which contributed towards the decreased levels of ATP.

Examination of the structure of berberine has revealed an extremely flat configuration, which may limit its absorption across the intestinal epithelia. This resulted in the need for relatively higher doses of the drug in a rodent model for diabetes and led to the derivation of dihydroberberine (Turner et al. 2008). Accordingly, studies with dihydroberberine should be undertaken in animal models of leishmaniasis. Taken together, berberine effectively mediated significant inhibition on the mitochondrial ETC along with mitochondrial depolarization that resulted in enhanced levels of mitochondrial superoxide and depletion of ATP, culminating in an apoptotic-like death (Fig. 7).

**Funding information** The work was supported by the International Bilateral Cooperation Division, Dept. of Science & Technology (DST), Govt. of India [INT/AUSTRIA/BMWF/P-06/2017] & Austrian Exchange Office (OEAD) in the Scientific & Technological Cooperation project with India IN 04/2017, Austrian Science Fund (FWF), grant P 27814-B22; Fund for Improvement of S&T infrastructure in Universities and Higher Educational Institutions (FIST) Program, DST, Govt. of India, [SR/FST/LS1-049/2010] and [SR/FST/LS1-663/2016]; and Dept. of Health Research, Govt. of India, "Establishment of Multidisciplinary Research Unit" no: [V.25011/103/2016-HR].

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Ahuja A, Purohit SK, Yadav JS, Netra PR (1993) Cutaneous leishmaniasis in domestic dogs. Indian J Public Health 37:29–31

Andrade-Neto VV, Cunha-Junior EF, Dos Santos Faioes V, Pereira TM, Silva RL, Leon LL, Torres-Santos EC (2018) Leishmaniasis treatment: update of possibilities for drug repurposing. Front Biosci (Landmark Ed) 23:967–996

Bahar M, Deng Y, Zhu X, He S, Pandharkar T, Drew ME, Navarro-Vázquez A, Anklin C, Gil RR, Doskotch RW, Werbovetz KA, Kinghorn AD (2011) Potent antiprotozoal activity of a novel semisynthetic berberine derivative. Bioorg Med Chem Lett 21:2606–2610

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brookes PS (2005) Mitochondrial H(+) leak and ROS generation: an odd couple. Free Radic Biol Med 38:12–23

Chandrasekaran S, Dayakar A, Veronica J, Sundar S, Maurya R (2013) An *in vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by withanolides. Parasitol Int 62:253–261

Chang W, Zhang M, Chen L, Hatch GM (2017) Berberine inhibits oxygen consumption rate independent of alteration in cardiolipin levels in H9c2 cells. Lipids 52:961–967

Chen M, Zhai L, Christensen SB, Theander TG, Kharazmi A (2001) Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. Antimicrob Agents Chemother 45:2023–2029

De Sarkar S, Sarkar D, Sarkar A, Dighal A, Chakrabarti S, Staniek K, Gille L, Chatterjee M (2018) The leishmanicidal activity of artemisinin is mediated by cleavage of the endoperoxide bridge and mitochondrial dysfunction. Parasitology 5:1–10

Dröse S, Brandt U (2008) The mechanism of mitochondrial superoxide production by the cytochrome bc1 complex. J Biol Chem 283:21649–21654

Dutta A, Sarkar D, Gurib-Fakim A, Mandal C, Chatterjee M (2008) In vitro and in vivo activity of *Aloe vera* leaf exudate in experimental visceral leishmaniasis. Parasitol Res 102:1235–1242

Faccenda D, Campanella M (2012) Molecular regulation of the mitochondrial F(1)F(o)-ATPsynthase: physiological and pathological significance of the inhibitory factor 1 (IF(1)). Int J Cell Biol 2012:367934

Fidalgo LM, Gille L (2011) Mitochondria and trypanosomatids: targets and drugs. Pharm Res 28:2758–2770

Flohé L, Hecht HJ, Steinert P (1999) Glutathione and trypanothione in parasitic hydroperoxide metabolism. Free Radic Biol Med 27:966–984

Ganguly S, Bandyopadhyay S, Sarkar A, Chatterjee M (2006) Development of a semi- automated colorimetric assay for screening antileishmanial agents. J Microbiol Methods 66:79–86

Getachew F, Gedamu L (2012) *Leishmania donovani* mitochondrial iron superoxide dismutase A is released into the cytosol during miltefosine induced programmed cell death. Mol Biochem Parasitol 183:42–51

Krauth-Siegel RL, Comini MA (2008) Redox control in trypanosomatids, parasitic protozoa with trypanothione-based thiol metabolism. Biochim Biophys Acta 1780:1236–1248

Lee I, Bender E, Arnold S, Kadenbach B (2001) New control of mitochondrial membrane potential and ROS formation–a hypothesis. Biol Chem 382:1629–1636

Lee N, Bertholet S, Debrabant A, Muller J, Duncan R, Nakhasi HL (2002) Programmed cell death in the unicellular protozoan parasite *Leishmania*. Cell Death Differ 9:53–64

Letasiová S, Jantová S, Cipák L, Múcková M (2006) Berberine-antiproliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells. Cancer Lett 239:254–262

Li J, Liu F, Jiang S, Liu J, Chen X, Zhang S, Zhao H (2018) Berberine hydrochloride inhibits cell proliferation and promotes apoptosis of non-small cell lung cancer via the suppression of the MMP2 and Bcl-2/Bax signaling pathways. Oncol Lett 15:7409–7414

Liu B, Wang G, Yang J, Pan X, Yang Z, Zang L (2011) Berberine inhibits human hepatoma cell invasion without cytotoxicity in healthy hepatocytes. PLoS One 6:e21416

Mailloux RJ, Harper ME (2011) Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free Radic Biol Med 51:1106–1115

Meeran SM, Katiyar S, Katiyar SK (2008) Berberine-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen species generation. Toxicol Appl Pharmacol 229:33–43

Mehta A, Shaha C (2004) Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. J Biol Chem 279:11798–11813

Monzote L, García M, Pastor J, Gil L, Scull R, Maes L, Cos P, Gille L (2014) Essential oil from *Chenopodium ambrosioides* and main components: activity against *Leishmania*, their mitochondria and other microorganisms. Exp Parasitol 136:20–26

Monzote L, Geroldinger G, Tonner M, Scull R, De Sarkar S, Bergmann S, Bacher M, Staniek K, Chatterjee M, Rosenau T, Gille L (2018) Interaction of ascaridole, carvacrol, and caryophyllene oxide from essential oil of Chenopodium ambrosioides L. with mitochondria in Leishmania and other eukaryotes. Phytother Res 32:1729–1740

Monzote L, Lackova A, Staniek K, Cuesta-Rubio O, Gille L (2015) Role of mitochondria in the leishmanicidal effects and toxicity of acyl phloroglucinol derivatives: nemorosone and guttiferone A. Parasitology 142:1239–1248

Monzote L, Gille L (2010) Mitochondria as a promising antiparasitic target. Curr Clin Pharmacol 5:55–60

Monzote L, Lackova A, Staniek K, Steinbauer S, Pichle G, Jäger W, Gille L (2017) The antileishmanial activity of xanthohumol is mediated by mitochondrial inhibition. Parasitology 144:747–759

Mukhopadhyay S, Bhattacharyya S, Majhi R, De T, Naskar K, Majumdar S, Roy S (2000) Use of an attenuated leishmanial parasite as an immunoprophylactic and immunotherapeutic agent against murine visceral leishmaniasis. Clin Diagn Lab Immunol 7:233–240

Nohl H, Gille L, Kozlov A (2003) Are mitochondria a spontaneous source of reactive oxygen species? Redox Rep 8:135–141

Pereira GC, Branco AF, Matos JA, Pereira SL, Parke D, Perkins EL, Serafim TL, Sardão VA, Santos MS, Moreno AJ, Holy J, Oliveira PJ (2007) Mitochondrially targeted effects of berberine [natural yellow 18, 5, 6-dihydro-9, 10-dimethoxybenzo(g)-1,3-benzodioxolo(5, 6-a) quinolizinium] on K1735-M2 mouse melanoma cells: comparison with direct effects on isolated mitochondrial fractions. J Pharmacol Exp Ther 323:636–649

Polster BM, Nicholls DG, Ge SX, Roelofs BA (2014) Use of potentiometricfluorophores in the measurement of mitochondrial reactive oxygen species. Methods Enzymol 547:225–250

Rodrigues IA, Azevedo MM, Chaves FC, Bizzo HR, Corte-Real S, Alviano DS, Alviano CS, Rosa MS, Vermelho AB (2013) In vitro cytocidal effects of the essential oil from Croton cajucara (red sacaca) and its major constituent 7-hydroxycalamenene against *Leishmania chagasi*. BMC Complement Altern Med 13:249

Roy A, Ganguly A, Bose Dasgupta S, Das BB, Pal C, Jaisankar P, Majumder HK (2008) Mitochondria-dependent reactive oxygen species-mediated programmed cell death induced by 3, 3′-diindolylmethane through inhibition of $F_0F_1$-ATP synthase in unicellular protozoan parasite *Leishmania donovani*. Mol Pharmacol 74:1292–1307

Saha P, Mukhopadhyay D, Chatterjee M (2011a) Immunomodulation by chemotherapeutic agents against Leishmaniasis. Int Immunopharmacol 11:1668–1679

Saha P, Bhattacharjee S, Sarkar A, Manna A, Majumder S, Chatterjee M (2011b) Berberine chloride mediates its antileishmanial activity via differential regulation of the mitogen activated protein kinase pathway in macrophages. PLoS One 6:e18467

Saha P, Sen R, Hariharan C (2009) Berberine chloride causes a caspase-independent, apoptotic-like death in Leishmania donovani promastigotes. Free Radic Res 43:1101–1110

Sahibzada MUK, Sadiq A, Faidah HS, Khurram M, Amin MU, Haseeb A, Kaka M (2018) Berberine nanoparticles with enhanced in vitro bioavailability: characterization and antimicrobial activity. Drug Des Devel Ther 12:303–312

Sarkar A, Sen R, Saha P, Ganguly S, Mandal G, Chatterjee M (2008) An ethanolic extract of leaves of *Piper betle* (Paan) Linn mediates its antileishmanial activity via apoptosis. Parasitol Res 102:1249–1255

Sen N, Das BB, Ganguly A, Mukherjee T, Tripathi G, Bandyopadhyay S, Rakshit S, Sen T, Majumder HK (2004) Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. Cell Death Differ 11: 924–936

Sen R, Chatterjee M (2011) Plant derived therapeutics for the treatment of Leishmaniasis. Phytomedicine 18:1056–1069

Sen R, Bandyopadhyay S, Dutta A, Mandal G, Ganguly S, Saha P, Chatterjee M (2007) Artemisinin triggers induction of cell-cycle arrest and apoptosis in *Leishmania donovani* promastigotes. J Med Microbiol 56:1213–1218

Sen R, Ganguly S, Saha P, Chatterjee M (2010) Efficacy of artemisinin in experimental visceral leishmaniasis. Int J Antimicrob Agents 36:43–49

Shadab M, Jha B, Asad M, Deepthi M, Kamran M, Ali N (2017) Apoptosis-like cell death in *Leishmania donovani* treated with KalsomeTM10, a new liposomal amphotericin B. PLoS One 12: e0171306

Tillhon M, Guamán Ortiz LM, Lombardi P, Scovassi AI (2012) Berberine: new perspectives for old remedies. Biochem Pharmacol 84:1260–1267

Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R (2017) Leishmaniasis: a review. F1000Res 6:750 **eCollection 2017**

Turner N, Li JY, Gosby A, To SW, Cheng Z, Miyoshi H, Taketo MM, Cooney, GJ, Kraegen EW, James DE, Hu LH, Li J, Ye JM (2008) Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. Diabetes 57:1414–1418

Upegui Y, Gil JF, Quiñones W, Torres F, Escobar G, Robledo SM, Echeverri F (2014) Preparation of rotenone derivatives and *in vitro* analysis of their antimalarial, antileishmanial and selective cytotoxic activities. Molecules 19:18911–18922

van Assche T, Deschacht M, da Luz RA, Maes L, Cos P (2011) *Leishmania*-macrophage interactions: insights into the redox biology. Free Radic Biol Med 51:337–351

Vennerstrom JL, Lovelace JK, Waits VB, Hanson WL, Klayman DL (1990) Berberine derivatives as antileishmanial drugs. Antimicrob Agents Chemother 34:918–921

Wan CP, Myung E, Lau BH (1993) An automated micro-fluorometric assay for monitoring oxidative burst activity of phagocytes. J Immunol Methods 159:131–138

World Health Organization. Leishmaniasis. http://www.who.int/gho/neglected_diseases/leishmaniasis/en/; (last accessed on 11th October, 2018)

Yan XJ, Yu X, Wang XP, Jiang JF, Yuan ZY, Lu X, Lei F, Xing DM (2017) Mitochondria play an important role in the cell proliferation suppressing activity of berberine. Sci Rep 7:41712