Molecular Mechanisms of *In vitro* Betulin-Induced Apoptosis of *Leishmania donovani*

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Abstract. Although leishmanial infections of humans occur globally, the major health impact lies in developing nations, thus, leishmaniasis remains “neglected” diseases for new drugs development. Multidrug resistance has been documented in most countries where leishmaniasis is endemic. Betulin is a widely available and affordable natural product exerting leishmanicidal activity at micromolar concentration. In this study, the molecular mechanisms of death that contribute to the anti-leishmanial activity of betulin are investigated. In promastigotes, betulin stimulated reactive oxygen species generation at micromolar concentrations in *Leishmania*. Apoptosis was observed in betulin-treated promastigotes using flow cytometric analysis of treated cells stained with annexin V-FITC and propidium iodide. Furthermore, betulin treatment of promastigotes led to mitochondrial membrane damage, activation of caspase-like proteases, and DNA fragmentation in *Leishmania donovani* promastigotes. Betulin treatment of amastigotes cultured within macrophages, resulted in a reduced number of amastigotes, with no substantive cytotoxic damage to the host macrophage cells at leishmanicidal drug concentrations.

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by intramacrophage obligate protozoan parasites of genus *Leishmania*. The disease is known for its diverse and complex characteristic infection. *Leishmania* parasite lives within mammalian macrophages, and is transmitted by sand flies. Various manifestations of leishmanial pathology are recognized, including cutaneous, mucocutaneous, and visceral¹; visceral leishmaniasis, also known as kala-azar in India, is caused by *Leishmania donovani*.¹ Kala-azar is the most common form of leishmaniasis in India. One of the major hurdles in eradicating the disease is the fact that the parasite has developed resistance to the most commonly used drugs. The search for a successful vaccine is still elusive.^{2,3} Current drugs of choice for leishmaniasis treatment have severe side effects, some potentially life-threatening. Existing drugs’ toxicity makes these drugs unsuited even for prophylactic treatment.^{4–8} Cost and availability of known anti-leishmanials is frequently problematic. There is an urgent need for discovery of new more effective, less toxic, and affordable anti-leishmanials.

An ideal drug target is the one that is parasite specific and is essential for the parasite survival. Such parasite-specific targets can be identified by exploring fundamental differences in metabolic and/or biochemical targets between host and parasite. A marked metabolic difference between *Leishmania* and its mammalian hosts lies in this trypanosomatids redox metabolism, which functions to reduce cellular oxidative stress.^{9,10} The key metabolite in the redox system of the *Leishmania* is trypanothione bis(glutathionyl)spermidine; (T[SH]₂), which maintains the cellular redox homeostasis.¹¹ Whereas, the functionally analogous redox metabolite in the mammalian host is glutathione (GSH). The biological synthesis of T[SH]₂ is carried out by trypanothione synthetase (TryS), one of the key enzymes of the parasite’s redox metabolism. In addition to maintaining redox homeostasis, T[SH]₂ is involved in various cellular processes, such as synthesis of deoxynucleotides, and parasite resistance to metal stress and chemical stress, including resistance to anti-leishmanials.^{11–14} The TryS has

been validated as a drug target by conducting knockout studies in *Trypanosoma brucei*.^{15,16} The exclusive nature of TryS in synthesizing T[SH]₂ in parasites and its absence in the host make it an attractive target.

In our earlier study, we have quantified the activity of several natural compounds that inhibit the TryS enzyme of *L. donovani* and also inhibited *L. donovani* promastigotes growth at micromolar concentration, but the molecular mechanism underlying parasite death was not studied.¹⁷ Betulin (Figure 1) was also reported as an inhibitor of the TryS with potent anti-leishmanial activity.¹⁷ Betulin is an abundant naturally occurring triterpene present in the bark of white birch trees. Other groups have reported betulin’s anti-inflammatory, anti-human immunodeficiency virus (HIV),¹⁸ anti-malarial,¹⁹ and natural medicine anticancer properties.^{20,21} Recently, betulin and its derivatives have also been reported to inhibit type IB DNA topoisomerase of *L. donovani*.²² Antiparasitics having more than one metabolic target are generally more effective and less likely to induce rapid parasite drug resistance, than drugs having only one mechanism of antiparasite action. The aim of this study was to investigate the precise molecular mechanism of parasite death.

MATERIALS AND METHODS

Parasites, cell lines, and chemicals. The *L. donovani* (MHOM/IN/2010/BHU1081) promastigotes culture was obtained from Prof. Shyam Sundar, Banaras Hindu University. Macrophage cell line J774A.1 used in the study was obtained from the National Center for Cell Science (NCCS), Pune, India. The H2DCFDA dye was obtained from Invitrogen. The apoptosis detection kit and mitochondrial membrane potential detection kit were procured from Calbiochem. The Caspase 3/7 detection kit was purchased from Promega. Betulin (> 98% pure, Sigma-Aldrich St. Louis, MO) 5 mM stock was prepared in 100% dimethyl sulfoxide (DMSO) and subsequently diluted with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) for the experiments. For all experiments 0.5% DMSO served as a negative control. All the chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich or Merck.

Parasite cultures and maintenance of host cells. The promastigotes of *L. donovani* (MHOM/IN/2010/BHU1081)

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FIGURE 1. Chemical structure of betulin.

were cultivated in M199 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin. The macrophage cell line J774A.1 was cultured in RPMI 1640 media supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin.

Cytotoxicity of betulin on macrophage cells and *Leishmania* parasite. We have already reported the anti-leishmanial activity of betulin against *L. donovani* promastigotes with an IC₅₀ value of 11.71 ± 0.56 µM.¹⁷ However, the previous study reported on the MHOM/IN/1978/UR6 strain of the parasite. The experiment was performed on the strain used in the current study as well (MHOM/IN/2010/BHU1081) and the IC₅₀ value was found to be very close (11.91 ± 0.62 µM). The toxicity of betulin on macrophage cell line J774A.1 was investigated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously^{17,23} and 50% cytotoxic concentration (CC₅₀) was determined. To study the effect on the intracellular amastigote form of the parasite, macrophage cell line J774A.1 was cultured overnight on glass coverslips by maintaining ~5 × 10⁵ cells for proper distribution on the coverslip. Non-adherent cells were removed by washing with PBS and fresh media was added. Macrophages were infected with *L. donovani* promastigotes by maintaining a parasite/macrophage ratio of 10:1 and incubated at 37°C in 5% CO₂ for 6 h to ensure parasite phagocytized by macrophage cells. After incubation, unphagocytized parasites were removed by twice washing with PBS. Fresh media was added and the amastigote cell cultures were incubated for 6 h. The graded concentrations of betulin were added and further incubated for 48 h. The cells were then fixed in methanol and Giemsa stained. Anti-leishmanial efficacy of betulin for intracellular amastigotes was evaluated by microscopic counting of 200 infected macrophage cells and compared with untreated control (*Leishmania* parasite treated with 0.5% DMSO but not treated with betulin). Three independent experiments were performed. The therapeutic index (TI) for betulin was defined as the ratio of CC₅₀ on macrophage cells to IC₅₀ on *L. donovani* intramacrophage amastigotes.

Flow cytometric studies. The reactive oxygen species (ROS) and apoptosis studies with *L. donovani* promastigotes either betulin-treated/untreated or N-acetylcysteine (NAC) (20 mM) pre-treated were carried out using the BD FACS Calibur flow cytometer and analyzed using CellQuestPro software (BD Biosciences, San Jose, CA). The ROS was determined as previously reported^{24,25}; to determine ROS, *L. donovani* promastigotes, pretreated with an IC₅₀ dose of betulin for 3 h, were subsequently treated with the cell permeable probe, 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (H2DCFDA), with ROS fluorescent signal measured via flow cytometry. The apoptosis in *L. donovani* was studied

using an annexin V-FITC apoptosis detection kit (Calbiochem). In brief, the cells were treated with an IC₅₀ dose of betulin for 6 h and phosphatidylserine externalization was detected by staining with annexin V-FITC antibody and propidium iodide (PI) as per instructions given by the manufacturer. For all the flow cytometer studies, cells were washed twice in PBS before flow cytometry analysis.

Measurement of mitochondrial membrane potential (ΔΨ_m). The mitochondrial transmembrane potential was investigated using a MitoCapture apoptosis detection kit (Calbiochem) according to the manufacturer's instructions. After a 6-h culture with an IC₅₀ betulin dose, *Leishmania* promastigotes (1 × 10⁷ cells mL⁻¹) were harvested by centrifugation (1,000 × g for 5 min at 4°C), then centrifugally (1,000 × g for 5 min at 4°C), and washed with cold PBS. The cells (1 × 10⁶ cells mL⁻¹) were suspended in 100 µL of incubation buffer containing MitoCapture reagent, which was diluted as per instructions given by the manufacturer, and followed by incubation at room temperature (25°C) for 30 min. Staining with MitoCapture reagent for < 30 min time is not sufficient for intense red fluorescence in control *Leishmania* cells. Moreover, some green fluorescence is also seen in control cells, using a shorter staining time. Thus, a longer incubation time (30 min) was used to insure maximum uptake of the dye by mitochondria. After staining with MitoCapture reagent, the cells were centrifugally washed (1,000 × g for 5 min at 4°C) twice with PBS, mounted on a glass slide and photographed using a fluorescent microscope, at ~570 nm (red) and ~500 nm (green) wavelength.

Determination of caspase-3/7-like protease activity. The caspase-like protease activity was measured fluorometrically using the Apo-1 homogenous caspase 3/7 activity assay kit (Promega). The assay was performed according to the instructions provided by the manufacturer with minor changes. In brief, *L. donovani* promastigotes, treated or untreated with an IC₅₀ dose of betulin for 6 h, were harvested by centrifugation (1,000 × g for 5 min at 4°C) and centrifugally washed (1,000 × g for 5 min at 4°C) with cold PBS. The cells were suspended in a 100 µL of reaction buffer containing caspase substrate Z-DEVD-R110 and incubated for 4 h in the dark at room temperature (25°C). After completion of the incubation period, the increase in fluorescence caused by cleavage of the Z-DEVD-R110 substrate was measured fluorometrically at excitation and emission wavelengths of 485 and 530 nm, respectively. In a parallel set of reactions, the caspase inhibitor Ac-DEVD-CHO was added to the reaction mixture before the addition of betulin-treated cells.

Determination of DNA fragmentation by agarose gel electrophoresis. To analyze the DNA fragmentation, standard genomic DNA isolation protocol was followed.²⁶ In brief, *L. donovani* promastigotes were cultured in 6-well plates either treated or untreated with an IC₅₀ dose of betulin for a 48-h time period. After incubation the time point samples were harvested by centrifugation (1,000 × g for 5 min). *Leishmania* promastigotes, lysed in 0.5 mL of extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 0.5% SDS) containing proteinase K (15.6 mg mL⁻¹), were vortexed and allowed to digest at 50°C for 1 h. The lysates were then extracted using phenol-chloroform-isoamylalcohol (25:24:1 v/v) and centrifuged for 10 min at 16,000 × g. The upper aqueous phase was carefully collected in fresh tubes and incubated overnight with 3 M sodium acetate and 100% ethanol at

-20°C . The samples were centrifuged ($16,000 \times g$ for 10 min at 4°C), supernatant was removed and the pellet was washed with 70% ethanol. The DNA was solubilized in nuclease-free water and quantified spectrophotometrically at 260/280 nm. On a 1.5% agarose gel containing $0.5 \mu\text{g mL}^{-1}$ ethidium bromide, $5 \mu\text{g}$ of DNA was loaded and electrophoresed. The gel was run for 1.5 h at 60 V using Tris-acetate-EDTA buffer and visualized in a UV illuminator.

RESULTS

Selective cytotoxicity of betulin toward *Leishmania* parasites. Previously, we reported anti-leishmanial activity of betulin on *Leishmania* promastigotes with an IC_{50} value of $11.71 \mu\text{M}$ ¹⁷; we have further evaluated the effect of betulin on intracellular *L. donovani* amastigote *in vitro* (Figure 2A). To investigate whether betulin selectively inhibits *L. donovani* promastigotes, the cytotoxicity of betulin to a macrophage cell line J774A.1 was investigated using an MTT assay. The betulin concentration 50% cytotoxic to host macrophages, or CC_{50} , was calculated to be $288.61 \pm 4.83 \mu\text{M}$ (Figure 2B). The betulin anti-leishmanial effect on amastigotes was dose-dependent. The *Leishmania* intracellular amastigotes decreased with increased betulin concentration in a dose-dependent manner up to $25 \mu\text{M}$; whereas the host macrophage cells did not show any significant toxicity even at a higher concentration of $100 \mu\text{M}$ betulin. The IC_{50} value of betulin for amastigotes was calculated to be $12.43 \pm 0.64 \mu\text{M}$ with a TI (ratio of host cell toxic drug concentration divided by the cytotoxic drug concentration for the amastigotes) of 23.2. Any TI, > 1.0 , indicates that the drug tested is more effective to eliminate the infection than harm the host cells.

Betulin induces generation of ROS and oxidative stress in parasite. The ROS generated in the form of superoxide anion radicals as a result of oxidative phosphorylation in aerobic organisms can extend up to 3% to 5% of the total oxygen used.²⁷ The results of our earlier studies show that betulin inhibits key redox enzyme, TryS, of *L. donovani*.¹⁷ Hence, it was desirable to investigate the ROS levels in betulin-treated *Leishmania* promastigotes. *Leishmania donovani* promastigotes treated with IC_{50} concentration of betulin for 3 h resulted in a significant ROS generation as evident by flow cytometric analysis (Figure 3). In NAC (20 mM) pretreated

FIGURE 3. Betulin-induced reactive oxygen species (ROS) is measured by DCF fluorescence as assayed by flow cytometer. NAC represents data after N-acetylcysteine (NAC) pre-treatment, whereas betulin represents data after betulin treatment. Data shown is a representative experiment of at least three experiments that gave similar results.

L. donovani promastigotes, the level of ROS produced decreased drastically and was almost the same as that of control cells. Thus, in summary, these results indicate that betulin treatment causes significant oxidative stress in *Leishmania* parasites.

Betulin-treated *Leishmania* promastigotes exhibited externalization of phosphatidylserine. Cells undergoing apoptosis exhibit the translocation of phosphatidylserine from the inner side of the cell membrane to the outer cell surface. Phosphatidylserine translocation can be detected using annexin V, which binds to exposed phosphatidylserine. Furthermore, during necrosis or in late apoptosis, there is membrane damage that permits PI to diffuse inside the cell and stain DNA. As evident from ROS studies, betulin induces ROS inside the *Leishmania* promastigotes. Because it is very well known that intracellular ROS is a key regulator for inducing

FIGURE 2. (A) Dose-dependent effect of betulin on intracellular *Leishmania donovani* amastigotes. The IC_{50} value of betulin was determined to be $12.43 \pm 0.64 \mu\text{M}$. (B) Effect of betulin on mouse macrophage cell line J774A.1. The betulin concentration 50% cytotoxic to host macrophages (CC_{50}) was calculated to be $288.61 \pm 4.83 \mu\text{M}$. Data represent the mean $\pm$ SD of three independent experiments.

FIGURE 4. Betulin elicits apoptosis in *Leishmania* promastigotes. (A) The *Leishmania* promastigotes treated with 0.2% dimethyl sulfoxide (DMSO) for 6 h were double stained with propidium iodide (PI) and annexin V-FITC and used as control. (B) N-acetylcysteine (NAC) pre-treated *Leishmania* promastigotes, incubated with an IC_{50} dose of betulin for 6 h, were double stained with both PI and annexin V-FITC. (C) *Leishmania donovani* promastigotes treated with an IC_{50} dose of betulin for 6 h were double stained with PI and annexin V-FITC. All data shown are representative experiments of at least three experiments which gave similar results.

apoptosis,^{28,29} we investigated if betulin causes apoptosis in *Leishmania* promastigotes. *Leishmania donovani* promastigotes treated with an IC_{50} concentration of betulin for 6 h were stained with annexin V-FITC and PI. A significant percent of *Leishmania* promastigotes stained positive for annexin V-FITC. As shown in the Figure 4C, 27.78% of betulin-treated cells are in the early apoptotic stage (lower right quadrant) compared with control parasites, where only 0.11% are positively stained (Figure 4A). A clear cut difference in cells that are in the late apoptotic stage (upper right quadrant) was observed in betulin treated and control cells. In betulin-treated *Leishmania* promastigotes 55.59% of cells are in late apoptotic stage (Figure 4C), whereas only 0.31% of cells were present in control (Figure 4A). When the cells were treated with NAC (20 mM) before treatment with betulin, phosphatidylserine externalization was suppressed and cells behaved

normally as that of control (Figure 4B). From this data it may be concluded that betulin induced ROS apoptotic processes in *Leishmania* promastigotes.

Betulin induces depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in *Leishmania* promastigotes. Depolarization of mitochondrial membrane potential is the characteristic feature observed in cells that are undergoing programmed cell death. Betulin-treated *Leishmania* promastigotes were investigated for change in $\Delta\Psi_m$. The detection of $\Delta\Psi_m$ in betulin treated/untreated *Leishmania* promastigotes was done using the MitoCapture apoptosis detection kit (Calbiochem). MitoCapture is a cationic mitochondrial specific dye that gets aggregated in normal mitochondria, whose $\Delta\Psi_m$ is retained, giving red fluorescence. Whereas in cells with altered $\Delta\Psi_m$, MitoCapture fails to aggregate in mitochondria and this stain accumulates in the parasite cytosol in its monomeric form,

FIGURE 5. Betulin induces apoptosis as measured by mitochondrial membrane injury. *Leishmania* promastigotes were treated with an IC_{50} dose of betulin for 6 h, stained with mitochondria-specific cation dye, MitoCapture (Calbiochem). *Leishmania* promastigotes, not treated with betulin, were used as a control. Both untreated control and betulin-treated *Leishmania* promastigotes were photographed using excitation wavelengths of ~ 570 nm (red) and ~ 500 nm (green), respectively, using fluorescence microscope (60 $\times$). Normal mitochondrial membrane potential is indicated by the red fluorescence, whereas green fluorescence indicates depolarization mitochondrial membrane. Data shown are a representative experiment of at least three experiments that gave similar results.

resulting in green fluorescence. Thus, the fluorescence of MitoCapture reagent can be regarded as an indicator of $\Delta\Psi_m$ energy state. The promastigotes were treated with an IC_{50} dose of betulin for 6 h, and then analyzed using a fluorescent microscope. A green fluorescence was observed using the fluorescent microscope in 6-h betulin-treated cells caused by complete loss in the mitochondria $\Delta\Psi_m$ as detected by MitoCapture reagent. Although, in control untreated cells MitoCapture reagent gave red fluorescence indicating retention of membrane potential (Figure 5).

Betulin triggers activation of caspase-like protease and DNA fragmentation in *Leishmania* promastigotes. The altered $\Delta\Psi_m$ may result in release of cytochrome *c* into the cytosol leading to the activation of caspases, which in turn, trigger the downstream events leading to apoptosis.^{30,31} *Leishmania donovani* promastigotes treated with an IC_{50} dose of betulin for 6 h were studied by the spectrofluorometric method using Apo-ONE Homogeneous Caspase 3/7 assay kit (Promega). An increased caspase-like activity was observed in betulin-treated *Leishmania* promastigotes (Figure 6A). Caspase activity in the cell lysate of *Leishmania* promastigotes treated with an IC_{50} dose of betulin in the presence of caspase inhibitor (DEVD-CHO inhibitor) was very much similar to that of control *Leishmania* cells lysate (Figure 6A). The control betulin-untreated *Leishmania* promastigotes lysate did not show significant caspase-like protease activity compared with betulin-treated promastigotes lysate (Figure 6A). In this control, *L. donovani* promastigotes were treated with 0.2% DMSO to compensate the effect of DMSO that was used for dissolving caspase inhibitor or substrate solution.

Fragmentation of genomic DNA is considered as the hallmark of apoptotic cell death. To establish betulin-induced genomic DNA fragmentation, we used agarose gel electropho-

resis of genomic DNA isolated from betulin-treated/untreated *Leishmania* promastigotes as described in the Materials and Methods section. It was observed that *L. donovani* promastigotes treated with an IC_{50} dose of betulin for a 48-h time point showed fragmentation of DNA (Figure 6B). We hypothesize that the action of both ROS and caspase-like proteases may be responsible for DNA fragmentation in betulin-treated *Leishmania* promastigotes.

DISCUSSION

Multidrug-resistant leishmaniasis are increasing worldwide making the discovery of new more effective anti-leishmanials essential. Anti-leishmanial drugs that originate from natural sources often have the advantages including low toxicity, and fewer or no side effects. In our previous report, we have identified betulin as an inhibitor of TryS of *L. donovani* and as a potent anti-leishmanial compound¹⁷; in this study, we attempted to understand the molecular mechanisms underlying the anti-leishmanial property of betulin.

Historically, betulin was known as an anti-cancerous agent, inducing apoptosis in human cancer cells through the intrinsic apoptotic pathway²¹; its derivatives have been reported to inhibit type IB DNA topoisomerase of *L. donovani*.²² Because betulin inhibits redox enzyme of *Leishmania* parasites,¹⁷ betulin treatment of *L. donovani* promastigotes resulted in a significant ROS generation. Upon treatment with betulin, NAC pretreated *L. donovani* did not accumulate ROS, indicating the effective removal of ROS by NAC. Inhibition of TryS by betulin decreases the T(SH)₂ levels drastically in the parasites, thereby increasing the ROS accumulation caused by a lack of the required amount of T(SH)₂ for scavenging ROS, resulting in destabilization of cellular redox homeostasis. Furthermore, T(SH)₂ is known to participate in many metabolic processes, such as reduction of glutathione disulphide (GSSG), ovithiol disulphide,^{32,33} and direct reduction of dehydroascorbate (DHA), by at least two times faster than GSH. The T(SH)₂ is also known to interact with nitric oxide,^{34,35} involved in the detoxification of ketoaldehydes; display defense against toxic xenobiotics; reducing equivalent transfer to proteins such as ribonucleotide reductase¹²; and many other functions. Therefore, the several metabolic processes that are T(SH)₂-dependent are also affected as a result of the decreased levels of T(SH)₂ leading to enormous oxidative- and other stress.

The accumulation of ROS is a primary factor to elicit programmed cell death or apoptosis. Betulin-induced ROS, eliciting apoptosis in *Leishmania* promastigotes, was studied by double staining *Leishmania* promastigotes with annexin V-FITC and PI. Annexin V is a Ca²⁺-dependent phospholipid binding protein having an affinity for phosphatidylserine. As annexin V is also known to label necrotic cells, another stain, PI, was used to distinguish between apoptotic cells, which stain annexin V-positive and PI-negative; necrotic cells, staining annexin V-negative and PI-positive; from normal cells, staining both annexin V and PI negative. Our results indicated that betulin induces apoptotic in *Leishmania* promastigotes as confirmed by FACS analysis of externalization of phosphatidylserine in betulin-treated cells. It appears that betulin induced ROS generation resulting in parasite death by apoptosis.

FIGURE 6. (A) Activation of Caspase-3/7-like proteases inside *Leishmania* promastigotes after treatment with an IC_{50} dose of betulin and in the absence or presence of a protease inhibitor (DEVD-CHO inhibitor) for a 6-h time point. *Leishmania* promastigotes not treated with betulin are used as control. Data are the mean $\pm$ SD of three independent experiments. (B) *Leishmania donovani* promastigotes genomic DNA fragmentation after treatment of betulin compared with control (untreated promastigotes). Lane M represents DNA molecular mass marker (1 kb DNA ladder), lane 1 represents control, whereas lane 2 shows cellular DNA fragmentation after treatment with an IC_{50} dose of betulin for 48 h, respectively.

Apoptosis is a cascade process involving physical and biochemical changes in the cell. Mitochondria are the power house of the cell where the cellular adenosine triphosphate is generated by oxidative phosphorylation and, therefore, are easy targets for induction of apoptosis under stress conditions. The mitochondrion is an important organelle for cell survival; any damage or dysfunction to it leads to cell death. Thus, this makes mitochondria a prime target to achieve programmed cell death or necrosis. Betulin treatment led to the accumulation of ROS, thereby increased cell stress resulting in depolarization of mitochondrial membrane potential in *Leishmania* promastigotes, as observed in a classical programmed cell death process. The depolarization of mitochondrial membrane potential acts as a switch to activate proteases, which play an important role in the apoptosis process.

We have found that betulin treatment in *Leishmania* promastigotes resulted in apoptotic death of the parasite. The parasite death is mediated through caspase-like proteases activity as cell lysates of apoptotic cells cleaved caspase substrate. There are a few other reports as well indicating caspase-like activity in *Leishmania*.^{36–38} Caspase that has an essential role in apoptosis in higher eukaryotic organism is absent in the genome of *Leishmania*.³⁹ Metacaspases, cysteine proteases distinctly related to caspase are found in *Leishmania* (but absent in mammal) were initially thought to be responsible for this parasite's caspase-like activity. However, recently metacaspases have been reported to have trypsin-like activity.⁴⁰ Thus, it will be crucial to rigorously further analyze the enzyme responsible for caspase-like activity. This may provide fundamental insights into apoptotic pathways in *Leishmania*.

The damaged mitochondrial membrane releases apoptotic eliciting signals that switch on the cascade for caspase-like proteases, which causes genomic DNA fragmentation leading to apoptotic death of the cell. Betulin-induced DNA fragmentation was observed in *Leishmania* promastigotes. Betulin inhibits TryS, a redox enzyme of the parasite that is involved in trypanothione [T(SH)₂] synthesis mitigating oxidative damage. Furthermore, the reduced T(SH)₂ facilitates deoxyribonucleotides (dNTPs) synthesis by transferring reducing equivalents to ribonuclease reductase during dNTPs synthesis.¹¹ Thus, inhibition of TryS may hinder the DNA synthesis leading to the formation of DNA suicidal complexes that are necessary events of apoptotic DNA fragmentation.³⁸ Because mammalian host cells lack trypanothione, betulin showed no apparent toxicity against macrophages even at high betulin concentrations. Betulin treatment of the amastigote stage of the parasite proved effective to reduce the intracellular parasite burden. We have shown that betulin can effectively clear parasites *in vitro*. Betulin cytotoxic activity was related to anti-leishmanial activity on amastigotes by determining the therapeutic index (TI = CC₅₀/IC₅₀), which was found to be 23.2, indicating the high selectivity against the *Leishmania* parasites than against the host macrophage cells.

Therefore, our study provides new data elucidating the molecular mechanism underlying the anti-leishmanial activities of betulin. Betulin generates oxidative stress in the *Leishmania* parasite resulting in apoptotic death. The current report provides, for the first time, data showing the molecular mechanism of betulin activity against both *Leishmania* promastigotes and amastigotes *in vitro*.

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