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Aloe vera leaf exudate induces a caspase-independent cell death in *Leishmania donovani* promastigotes

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Leishmaniasis constitutes a complex of diseases with clinical and epidemiological diversity and includes visceral leishmaniasis, a disease that is fatal when left untreated. In earlier studies, the authors reported that *Aloe vera* leaf exudate (AVL) is a potent antileishmanial agent effective in promastigotes of *Leishmania braziliensis*, *Leishmania mexicana*, *Leishmania tropica*, *Leishmania major* and *Leishmania infantum* and also in axenic amastigotes of *Leishmania donovani*. In the present study, it has been demonstrated that, in promastigotes of *L. donovani* (IC_{50}=110 μg ml^{-1}), AVL mediates this leishmanicidal effect by triggering a programmed cell death. Incubation of promastigotes with AVL caused translocation of phosphatidylserine to the outer leaflet of the plasma membrane as measured by annexin V binding, which was accompanied by loss of mitochondrial membrane potential, release of cytochrome *c* into the cytosol and concomitant nuclear alterations that included chromatin condensation, deoxynucleotidyltransferase-mediated dUTP end labelling and DNA laddering. As this AVL-induced leishmanicidal effect could not be inhibited by protease inhibitors including Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone, a broad-spectrum caspase inhibitor, non-involvement of caspases and major proteases was suggested. Additionally, AVL treatment caused no increase in cytosolic Ca^{2+} or generation of reactive oxygen species, indicating that although promastigote death was induced by an apoptotic-like mechanism similar to metazoan apoptosis, the pathways of induction and/or execution differed at the molecular level.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are obligately intracellular, reside in mononuclear phagocytes and cause a wide spectrum of clinical manifestations resulting in substantial morbidity and mortality in an estimated 12 million people worldwide (Desjeux, 2004). The major clinical presentations depend upon the causative species and immunological state of the host. These range from a simple cutaneous lesion through to the disfiguring mucocutaneous leishmaniasis and finally to the visceralized form or kala-azar, which is fatal if left untreated (Murray *et al.*, 2005). In recent years, an unprecedented increase in unresponsiveness to sodium antimony gluconate, the first line of treatment, in visceral leishmaniasis patients has been observed in the Indian subcontinent, a major endemic area of visceral leishmaniasis (Sundar & Chatterjee, 2006).

In the ongoing search for better leishmanicidal compounds (Dupouy-Camet, 2004; Handman, 2001), plant-derived products (Kayser *et al.*, 2003; Akendengue *et al.*, 1999) are being evaluated. We have recently reported the efficacy of the *Aloe vera* leaf exudate (AVL) against promastigotes of *Leishmania braziliensis*, *Leishmania mexicana*, *Leishmania tropica*, *Leishmania major* and *Leishmania infantum* (Dutta *et al.*, 2007). Among various mechanisms for mediating parasiticidal activity, programmed cell death (PCD) appears to be the most preferred, as has been observed in

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Abbreviations: AVL, *Aloe vera* leaf exudate; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyltransferase mediated dUTP end labelling.

kinetoplastids (Szallies *et al.*, 2002; Arnoult *et al.*, 2002) in response to diverse stimuli, e.g. heat shock (Moreira *et al.*, 1996), chemotherapeutic agents such as pentostam, amphotericin B (Lee *et al.*, 2002), oxidants such as H_2O_2 (Das *et al.*, 2001) or even serum depletion (Zangger *et al.*, 2002). In this study, our objective was to evaluate the putative mechanism(s) mediating the leishmanicidal property of AVL. Herein, we report that AVL induces PCD in *L. donovani* promastigotes via a protease- and caspase-independent signalling pathway involving changes in the mitochondrial membrane potential and release of cytochrome *c* from hypopolarized mitochondria and culminating in DNA fragmentation.

METHODS

Materials. M-199 medium and fetal calf serum (FCS) were obtained from Gibco-BRL, DMSO from SRL, methanol from Merck, Apoptotic DNA ladder kit from Roche, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), Hoechst 33258, 1,4-diazabicyclo[2.2.2]octane, JC-1, propidium iodide (PI), mitotracker deep red and fluo-3/AM from Molecular Probes, and cytofix, cytoperm, murine anti-cytochrome *c* antibody, anti-murine IgG–FITC, annexin V–FITC and Apo-Direct kit from BD Biosciences. All other chemicals were from Sigma unless stated.

Parasite culture. Promastigotes of *L. donovani* (MHOM/IN/83/AG83) were routinely cultured at 22 °C in M-199 medium supplemented with 10 % heat-inactivated FCS and gentamicin (100 μg ml^{-1}) and subcultured every 72 h.

Preparation of AVL. *Aloe vera* syn. *Aloe berbadensis* Mill. was collected from Midnapore, West Bengal, and a voucher specimen was obtained from the Botanical Survey of India, Shibpore, Howrah. Initially, fresh succulent leaves of *Aloe vera* (*Aloe berbadensis* Mill.) were collected and crushed in an electric grinder and this was considered the crude extract. Subsequently, the inner gelatinous portion and the outer leafy coat were manually separated and similarly crushed. All three components, i.e. crude, gel and leaf, were individually tested for their antileishmanial activity. As the maximal activity was identified in the exudate of the leaf (unpublished), it was lyophilized and stored at 4 °C; in all experiments, the DMSO (Sigma) soluble fraction was used (3.5 mg ml^{-1}). This fraction is referred to as leafy exudate of *Aloe vera* (AVL).

***In vitro* cytotoxicity assay.** Exponential-phase promastigotes were resuspended in modified RPMI 1640 medium (phenol red free) supplemented with 10 % FCS and 100 μg gentamicin ml^{-1} (Medium A); parasites were seeded in 96-well tissue culture plates (5×10^4 in 250 μl per well) and exposed to increasing concentrations of AVL (0–300 μg ml^{-1}) for 72 h at 22 °C. Parasite viability was evaluated using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Dutta *et al.*, 2005) wherein the amount of formazan produced is directly proportional to the number of metabolically active cells. Accordingly, the A_{492} represented the number of live cells and the IC_{50}, i.e. the concentration that decreased cell growth by 50 %, was determined by graphical extrapolation.

Flow cytometric analysis of externalized phosphatidylserine in *L. donovani* promastigotes. Double staining with annexin V–FITC and PI was performed as previously described (Mehta & Shaha, 2004). Briefly, promastigotes were incubated with AVL (87.5 μg ml^{-1}, 24 h); cells were centrifuged (3500 r.p.m. × 10 min), washed twice in PBS (0.02 M, pH 7.2) and resuspended in annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$; pH 7.4). Annexin V–FITC and PI were then added, according to the manufacturer's instructions, and incubated for 30 min in the dark at 20–25 °C. Acquisition was done on a FACS Calibur flow cytometer (BD) and analysed with CellQuest software. Miltefosine, an established inducer of apoptosis in *Leishmania* parasites (20 μM, 24 h), served as the positive control (Paris *et al.*, 2004).

Measurement of mitochondrial membrane potential of *L. donovani* promastigotes. Mitochondrial membrane potential was measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), a cell-permeable dye that exists in a monomeric form that on entering the cytoplasm emits a green fluorescence. Subsequently, on entering the mitochondria it forms J-aggregates and emits a red fluorescence. The ratio between red and green fluorescence, i.e. 585/530 nm, determines the mitochondrial membrane potential, $\Delta\Psi$m (Reers *et al.*, 1995). After being treated with AVL (87.5 μg ml^{-1}) for 0–24 h, promastigotes were centrifuged (3500 r.p.m. × 10 min). The cells were resuspended in PBS containing JC-1 (10 μg; Molecular Probes) and incubated at 37 °C for 7 min. Analysis for mean green and red fluorescence intensity was done using FACS Calibur and CellQuest software.

Release of cytochrome *c* from mitochondria of *L. donovani* promastigotes. Promastigotes were treated with AVL (87.5 μg ml^{-1}, 0–16 h) and initially loaded with mitotracker deep red as per the manufacturer's instructions (Molecular Probes). The cells were then fixed and permeabilized with cytofix and cytoperm, respectively. After nonspecific blocking with 2 % PBS–BSA, cells were incubated with murine anti-cytochrome *c* followed by FITC-labelled anti-murine IgG and viewed under a TCS-SP confocal microscope (Leica). At least 20 microscopic fields were observed for each sample.

Analysis of the nuclear morphology of promastigotes. Oligonucleosomal fragmentation of AVL-treated parasites was identified microscopically using the fluorescent nuclear stain Hoechst 33258. Briefly, promastigotes were incubated with AVL (87.5 μg ml^{-1}) for 0–72 h, cells were then centrifuged at each time point and the resultant pellet was loaded with Hoechst (5 μg ml^{-1}) for 30 min at 20–25 °C, attached on poly-L-lysine-coated glass slides, mounted in glycerol containing 1,4-diazabicyclo[2.2.2]octane and examined under a confocal microscope (Leica). At least 20 microscopic fields were observed for each sample.

***In situ* detection of DNA fragmentation by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP end labelling (TUNEL).** DNA fragmentation within the cell was analysed using the Apo-Direct kit (BD) following the manufacturer's instructions. Briefly, promastigotes were treated with AVL (87.5 μg ml^{-1} for 0, 6, 24, 48 and 72 h); cells were then washed twice in PBS and fixed in paraformaldehyde (1 %) for 30 min. Cells were again washed in PBS and kept in chilled ethanol (70 %) for 30 min on ice following which they were washed twice, reacted with TdT enzyme in reaction buffer and stained with PI and dUTP–FITC (Apo-Direct kit). Finally, cells were resuspended in PBS before acquisition on a FACS Calibur and analysed by CellQuest software.

Oligonucleosomal DNA fragmentation assay. Analysis of the presence of DNA fragments generated as a function of cell death was performed using the Apoptotic DNA Ladder kit (Roche) as per the manufacturer's instructions. Briefly, genomic DNA was isolated from promastigotes exposed to AVL (87.5 μg ml^{-1} for 0, 24, 48 and 72 h) or miltefosine (40 μM for 24 h). Extracted DNA was quantified spectrophotometrically by the absorbance ratio of 260/280 nm and DNA (2–3 μg per lane) was run on an agarose (1 %) gel containing ethidium bromide in TBE buffer (50 mM; pH 8.0) for 1.5 h at 75 V

and visualized under UV light. Miltefosine-treated promastigotes (40 μM, 24 h) were used as the positive control.

Generation of reactive oxygen species (ROS) in promastigotes. To measure AVL-mediated generation of ROS by promastigotes, H_2DCFDA, a non-fluorescent dye, was used based on the existing knowledge that it is converted by free radicals to a fluorescent dye, dichlorofluorescein (Wan *et al.*, 1993). Briefly, promastigotes were incubated with AVL (87.5 μg ml^{-1}) at 22 °C for different time points (0–72 h). At each time point, cells were fixed in paraformaldehyde (4 %), nonspecific binding sites were blocked with 2 % PBS–BSA and then probed with H_2DCFDA (10 μM) for 30 min at 20–25 °C. Cells were analysed for intracellular ROS by FACS Calibur using CellQuest software.

Flow cytometric assessment of intracellular Ca^{2+} in *L. donovani* promastigotes. Changes in intracellular Ca^{2+} were monitored using the fluorescent probe fluo-3/AM as previously described (Mukherjee *et al.*, 2002). Briefly, AVL-treated promastigotes (87.5 μg ml^{-1} for 0, 6, 12, 24, 48 and 72 h) were loaded for 30 min at 20–25 °C with fluo-3/AM (5 μM) containing pluronic acid F127 (1 μM) for proper dispersal and sulfinpyrazone (0.25 mM), an organic anion transport inhibitor, to inhibit leakage of the fluo-3 dye. Fluorescence intensity was expressed as the increase in fluorescence with respect to baseline fluorescence intensity before stimulation. For detection of fluo-3 staining in promastigotes, fluo-3-labelled cells were excited with a 488 nm laser and images were collected at 530 nm using a FACS Calibur. Promastigotes treated with Ca^{2+} ionophore or Ca^{2+} ionophore with EGTA served as positive and negative controls, respectively.

Determination of protease activity in AVL-treated promastigotes. To study the role of caspases and other proteases in AVL-induced death, exponential-phase promastigotes were harvested and resuspended in Medium A. Cells were seeded (1×10^5 in 250 μl per well) in 96-well tissue culture plates and pre-incubated for 2 h with one of the protease inhibitors, namely aprotinin (3 μg ml^{-1}), leupeptin (100 μM), phenylmethylsulfonylfluoride (PMSF, 1 mM), pepstatin (10 μM), trypsin inhibitor (1 mM), EDTA (10 mM), EGTA (10 mM) or Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK, 100 μM). Subsequently, AVL (200 μg ml^{-1}) was added and co-incubated for a further 72 h. The viability of promastigotes was evaluated using the colorimetric 'modified MTT assay' (Dutta *et al.*, 2005).

Statistical analysis. *In vitro* antileishmanial activity was expressed as the IC_{50} by linear regression analysis. Values are mean ± SD from at least three independent experiments in duplicate.

RESULTS AND DISCUSSION

Determination of the IC_{50} of AVL-mediated death in *L. donovani* promastigotes

The viability of promastigotes was evaluated by the 'modified MTT assay' wherein the conversion of MTT to formazan by mitochondrial enzymes serves as an indicator of cell viability; accordingly, a decrease in formazan production indicates decreased cell viability and vice versa (Dutta *et al.*, 2005). Treatment of promastigotes with AVL demonstrated a dose-dependent inhibition of parasite growth and the IC_{50} in AG83 (MHOM/IN/83/AG83) promastigotes was 110 μg ml^{-1} (Fig. 1).

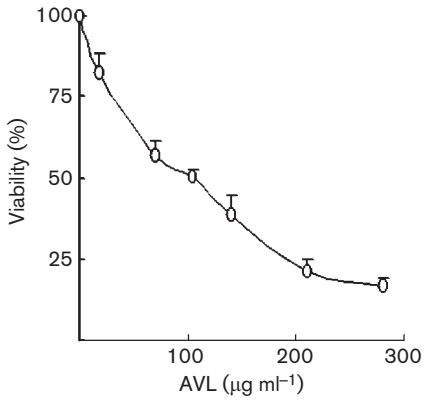

Fig. 1. Analysis of the antileishmanial activity of AVL. Promastigotes (MHOM/IN/83/AG83; 5×10^4 in 250 μl per well) were incubated with increasing concentrations of AVL (0–300 μg ml^{-1}) for 72 h and the MTT assay was performed as described in Methods. Each point corresponds to the mean ± SD of at least three experiments in duplicate.

AVL-treated promastigotes have externalization of phosphatidylserine

In metazoan and unicellular cells, translocation of phosphatidylserine from the inner side to the outer layer of the plasma membrane occurs during PCD (Mehta & Shaha, 2004; Sudhandiran & Shaha, 2003; Koonin & Aravind, 2002). Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with affinity for phosphatidylserine, is routinely used to label externalization of phosphatidylserine. Since annexin V can also label necrotic cells following the loss of membrane integrity, simultaneous addition of PI, which does not permeate cells with an intact plasma membrane, allows discrimination between apoptotic cells (annexin V-positive, PI-negative), necrotic cells (both annexin V- and PI-positive) and live cells (both annexin V- and PI-negative).

Accordingly, in order to study whether the mechanism of cell death triggered by AVL is via apoptosis or necrosis, AVL-treated promastigotes (87.5 μg ml^{-1} for 0, 6, 16 and 24 h) were double-stained with FITC-conjugated annexin V and PI. As shown in Fig. 2, a significant percentage (51.0 %) of promastigotes of AVL-treated cells (24 h) stained positive for annexin V (Fig. 2, lower and upper right quadrants) compared to only 2.0 % in untreated cells (Fig. 2). This was comparable with apoptosis triggered by miltefosine (64.5 %; Fig. 2, lower and upper right quadrants), which served as a positive control based on previous studies confirming that its antileishmanial activity is mediated via apoptosis (Paris *et al.*, 2004). The percentage of PI-stained (upper left quadrant) or double-stained (upper right quadrant) cells ranged from 2 to 11 % in all experimental sets, indicating that AVL exerts its leishmanicidal activity primarily via apoptosis.

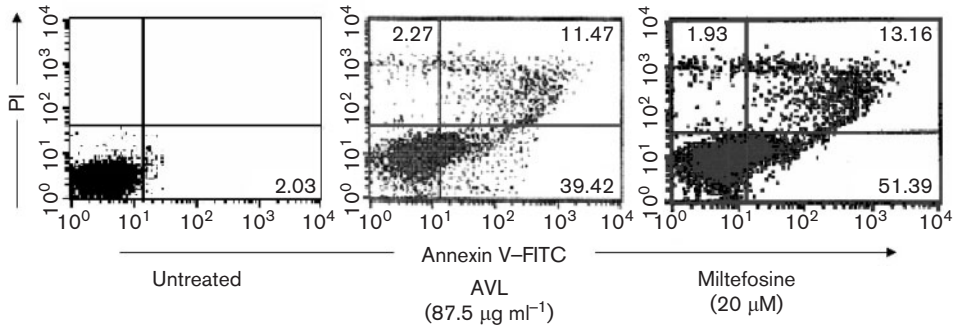

Fig. 2. Externalization of phosphatidylserine in AVL-treated promastigotes. Promastigotes (MHOM/IN/83/AG83) were incubated with AVL (87.5 μg ml^{-1}) or miltefosine (20 μM) for 24 h, co-stained with PI and annexin V–FITC and analysed by flow cytometry as described in Methods. This is a representative profile of at least three experiments.

AVL-induced modification in mitochondrial membrane potential

Previous studies have suggested that nuclear features of apoptosis in metazoan cells such as condensation and fragmentation of DNA are preceded by alterations in the mitochondrial membrane potential or $\Delta\psi$m (Green & Kroemer, 2004). These changes in $\Delta\psi$m were determined by flow cytometry using JC-1, a cell-permeable dye that exists within the cytosol in a monomeric form emitting a green fluorescence; subsequently, on entering the mitochondria, it forms J-aggregates and emits a red fluorescence. The ratio of red/green fluorescence represents $\Delta\Psi$m (Sen *et al.*, 2004; Reers *et al.*, 1995). Accordingly, the 585/530 nm ratio, i.e. J-aggregates, within the mitochondria vs monomers in the cytosol represents the $\Delta\psi$m.

As shown in Fig. 3, a 3 h treatment with AVL (87.5 μg ml^{-1}) caused hyperpolarization evidenced by a 33.3 % increase in the 585/530 nm ratio of AVL-treated versus untreated cells, being 6.37 ± 0.19 versus 4.78 ± 0.23, respectively. This was sustained up to 6 h, being 6.22 ± 0.15 versus 4.71 ± 0.21. However, at 10 h, the degree of hyperpolarization decreased, the 585/530 nm ratio of treated versus non-treated cells being 3.81 ± 0.27 versus 3.21 ± 0.31, respectively. At 14 h, AVL induced hypopolarization of $\Delta\psi$m as the 585/530 nm ratio of treated versus non-treated cells was 2.89 ± 0.38 versus 3.66 ± 0.31, respectively. At 24 h, a further decrease in the $\Delta\psi$m was observed, the 585/530 nm ratio in treated versus non-treated cells being 2.08 ± 0.23 versus 3.98 ± 0.17, respectively (Fig. 3). Taken together, the data indicate that AVL up to the 10th hour caused mitochondrial membrane hyperpolarization, which was followed by a sustained hypopolarization thereafter (Fig. 3).

AVL treatment triggered release of cytochrome *c* into the cytosol

Following AVL treatment, disruption of the outer mitochondrial membrane by apoptotic stimuli resulted in the release of cytochrome *c* into the cytosol as evident by confocal microscopy (Fig. 4). On receiving an apoptotic stimulus, cytochrome *c* present in the inter-membrane space of mitochondria leaches into the cytoplasm, where it exists in a reduced monomeric form (Liu *et al.*, 1996). In AVL-treated cells (16 h), the presence of this reduced monomeric cytochrome *c* (approx. 15 kDa) was detected in the cytoplasm using an FITC-labelled secondary antibody. In control promastigotes, reduced monomeric cytochrome *c* was absent in the cytoplasm, evident by an absence of green fluorescence (Fig. 4), whereas in AVL-treated cells, presence of reduced monomeric cytochrome *c* was detected in the cytosol from the 16th hour (Fig. 4). The irreversible hypopolarization is known to commit cells to release cytochrome *c* into the cytosol (Sen *et al.*, 2004) and a

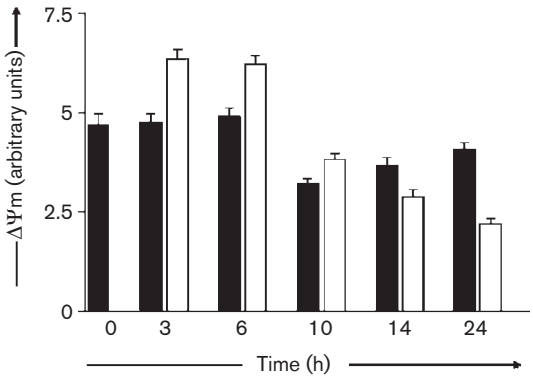

Fig. 3. Changes in mitochondrial membrane potential following treatment with AVL. Promastigotes (MHOM/IN/83/AG83) were incubated at 22 °C in the absence (filled bars) or presence (87.5 μg ml^{-1}; open bars) of AVL for different time points, washed in PBS and probed with JC-1 (10 μg in 100 μl per well) and analysed by flow cytometry as described in Methods. The 585/530 nm ratio, i.e. J-aggregates, in the mitochondria versus monomers in the cytosol represents the $\Delta\psi$m.

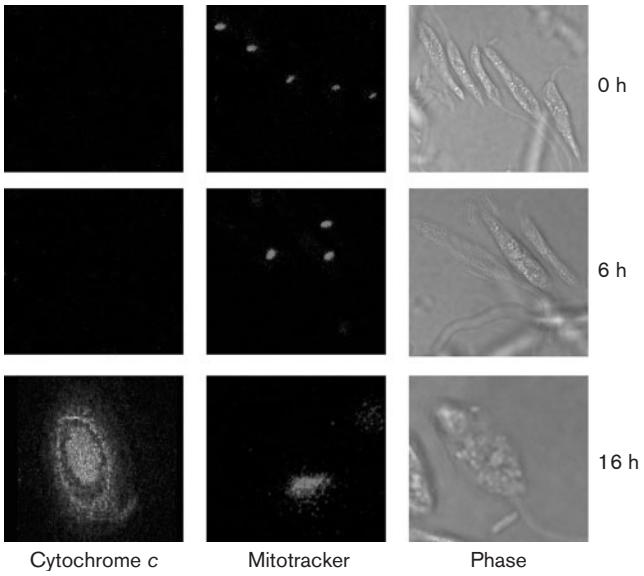

Fig. 4. Presence of cytosolic cytochrome *c* in promastigotes following AVL treatment. Promastigotes (MHOM/IN/83/AG83) were incubated with AVL (87.5 μg ml^{-1}) for different time points and the binding of FITC-labelled cytochrome *c* was analysed by confocal microscopy as described in Methods. Mitotracker was used to identify the mitochondria.

similar scenario was observed with AVL, where hypopolarization of the mitochondria caused cytochrome *c* to leach into the cytosol (Fig. 4).

AVL caused blebbing of promastigote nuclei

During PCD, the cleavage patterns of genomic DNA are typical of internucleosomal DNA digestion by endonucleases and are considered as a hallmark of apoptosis that is preceded by chromatin condensation and nuclear blebbing. In untreated promastigotes (0 h) stained with Hoechst 33258, the nuclei appeared as discrete blue spots, whereas in AVL-treated promastigotes (24, 48 and 72 h), the nuclei appeared hazy and after 72 h treatment >90 % of cells showed blebbed nuclei (Fig. 5; 48 h and 72 h). However, the mitochondrial DNA remained unchanged following AVL treatment.

Oligonucleosomal DNA fragmentation in AVL-treated promastigotes

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. The occurrence of DNA nicking by AVL was detected by the TUNEL assay wherein the proportion of DNA nicks was quantified by measuring the binding of FITC-labelled dUTP that gets attached to the nicked ends via TdT. Accordingly, the proportion of DNA nicks is directly proportional to the fluorescence obtained. The treatment of promastigotes with AVL (87.5 μg ml^{-1}) caused a time-dependent increase in nuclear DNA fragmentation as evidenced from dUTP–FITC binding. After AVL treatment for 24 h, the degree of DNA nicking was comparable with that of control cells, the mean fluorescence intensity (MFI) being 3.7 versus 3.0, respectively. However, at 48 h, the MFI in AVL-treated cells increased to 7.8 and increased further at 72 h treatment to 26.7. As positive and negative controls, ethanol-fixed

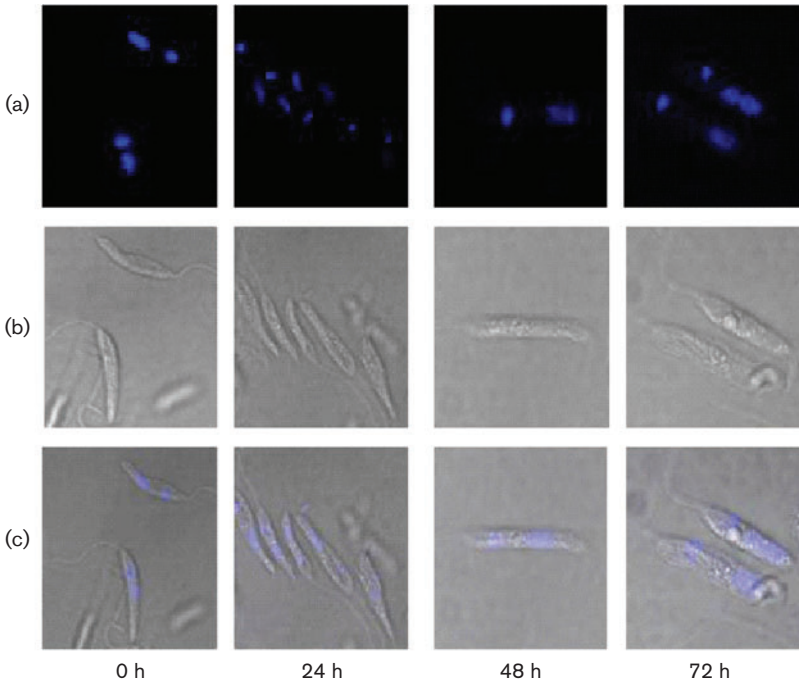

Fig. 5. Analysis of nuclear blebbing of AVL-treated promastigotes. Promastigotes (MHOM/IN/83/AG83) were incubated with AVL (87.5 μg ml^{-1}) for different time points (0, 24, 48 and 72 h), probed with Hoechst 33258, and analysed by confocal microscopy as described in Methods. (a) Fluorescence panel; (b) phase-contrast panel; (c) overlay of the previous two.

mammalian cell populations supplied in the kit were tested (data not shown).

This *in situ* finding was corroborated by oligonucleosomal DNA fragmentation analysis of promastigotes treated with AVL (87.5 μg ml^{-1}). A characteristic ladder was observed and a time-dependent increase in fragmentation of nuclear DNA was evident as detectable by gel electrophoresis. Promastigotes treated for 72 h with AVL showed a similar degree of fragmentation to that observed in miltefosine-treated cells (40 μM, 24 h).

Late-stage events in the process of apoptosis in *Leishmania* include nuclear condensation and DNA nicking (Verma & Dey, 2004). As this was evident in AVL-treated promastigotes, it strongly suggested that AVL triggers an apoptosis-like death in *Leishmania* parasites. Formation of a DNA ladder (~200 bp) is the hallmark of apoptosis, and as AVL-treated promastigotes showed a similar profile, this provided corroboratory evidence that AVL promoted apoptosis in *Leishmania* promastigotes.

AVL failed to generate ROS in promastigotes

To investigate whether AVL caused ROS generation within promastigotes, a fluorescent probe, H_2DCFDA, was used. This probe primarily detects H_2O_2 and hydroxyl radicals, O_2H^-, and fluoresces after forming dichlorofluorescein; therefore, an increase in signal indicates augmented generation of H_2O_2 and hydroxyl radicals (Wan *et al.*, 1993). Although an inherent basal level of ROS production in promastigotes was detectable (MFI=10.87), the addition of AVL (6, 12, 16, 24, 48 and 72 h) failed to induce any changes in the MFI, being 10.91, 11.43, 11.06, 9.11, 11.29 and 9.59, respectively, indicating that AVL did not trigger ROS generation in promastigotes. Although kinetoplastid parasites share features reminiscent of mammalian nucleated cell apoptosis, the pathways (induction/execution) may be different at the molecular level. An established event in most apoptotic cells is generation of ROS in the cytosol, which directs the cell and its neighbouring cells towards the path of death (Chipuk & Green, 2005). However, with AVL treatment, no ROS generation was evident.

Intracellular Ca^{2+} was unaltered by AVL treatment

An elevation in intracellular Ca^{2+} for endonuclease activity is generally considered a requirement for PCD. In promastigotes, calcium ionomycin increased intracellular Ca^{2+} levels from baseline levels as evidenced by the given fluorescence of fluo 3/AM, the MFI being 88.35 versus 21.93, respectively. The addition of EGTA, a Ca^{2+}-specific metalloprotease, decreased the MFI to 1.56. However, AVL (87.5 μg ml^{-1}; 6, 12, 24, 48 and 72 h) failed to change the Ca^{2+} levels in promastigotes, which remained close to baseline levels, the MFI being 21.30, 26.09, 19.76, 16.41 and 17.29, respectively.

Calcium is an important element in the progression towards cell death, as most endonucleases require the presence of Ca^{2+} to cleave DNA strands, and therefore in apoptosis, elevation of Ca^{2+} is consistently increased. However, with regard to AVL, the absence of a measurable alteration in Ca^{2+} despite the presence of DNA cleavage as confirmed by the TUNEL assay and DNA laddering, suggests that either the existing cellular Ca^{2+} is sufficient for endonuclease activity or possibly these endonucleases are Ca^{2+}-independent (Kawabata *et al.*, 1996).

Antipromastigote activity of AVL was via a caspase-independent pathway

To evaluate the role of caspases and other proteases in AVL-induced apoptosis, promastigotes were preincubated (2 h) with or without protease inhibitors, namely aprotinin, leupeptin, PMSF, pepstatin, trypsin inhibitor, EDTA and EGTA as well Z-VAD-FMK, followed by the addition of AVL (200 μg ml^{-1}, 72 h). The viability was measured using the modified MTT assay.

The percentage of AVL-induced promastigote killing was 77.35 ± 6.07 %. This remained unchanged in the presence of serine protease inhibitors (aprotinin, 83.84 ± 5.31 %; PMSF, 76.46 ± 6.23 %), a serine and cysteine protease inhibitor (leupeptin, 77.14 ± 4.28 %), an aspartic protease inhibitor (pepstatin, 74.70 ± 3.89 %), metalloprotease inhibitors (EDTA, 70.74 ± 4.12 %; EGTA, 75.91 ± 3.54 %) or trypsin inhibitor (82.10 ± 2.83 %). Even the broad-spectrum caspase 3 inhibitor Z-VAD-FMK failed to attenuate AVL-induced promastigote killing (80.93 ± 3.59 %), indicating that AVL-induced apoptosis is caspase-independent. It has been reported that caspase-mediated cell death causes formation of apoptotic bodies and shrinkage of the cell surface, which are absent in caspase-independent PCD (Chipuk & Green, 2005).

Studies pertaining to the signalling pathway, downstream of the formation of a cleavable complex, that ultimately leads to an increase in the number of apoptotic cells is still not clearly established. Generally, reduced cytochrome *c* attaches with apoptosis protease activating factor to activate caspase 3 downstream in metazoan cells (Green & Kroemer, 2004). However, caspase activation is not mandatory as studies with murine embryonic fibroblasts on exposure to UV irradiation showed release of cytochrome *c* into the cytosol in the absence of caspase activation (Carmen *et al.*, 2006). In *Leishmania*, the role of caspases in apoptosis remains controversial as stress promoted death is caspase-independent (Zangger *et al.*, 2002) whereas camptothecin causes a caspase-dependent death (Sen *et al.*, 2004). In our experiments, AVL-induced reduction in cell viability of promastigotes remained unchanged in the presence of a broad-spectrum caspase inhibitor (Z-VAD-FMK), implying that AVL-mediated parasite death is via a caspase-independent pathway. Alternative pathways resulting in caspase-independent apoptotic cell death have been reported in promastigotes

and amastigotes of *L. major* and *L. mexicana* upon serum depletion (Debrabant *et al.*, 2003). Following treatment with AVL, the decrease in cell viability remained unaltered in the presence of a number of cell-permeable protease inhibitors, virtually eliminating the possibility of proteases being involved in the apoptotic pathway. Therefore, our results suggest that AVL caused a protease-independent PCD in *L. donovani* promastigotes.

Apoptosis-like changes have been reported for mediating the leishmanicidal action of miltefosine (Verma & Dey, 2004), amphotericin B (Lee *et al.*, 2002) and camptothecin (Sen *et al.*, 2004). The efficacy of AVL against promastigotes of *L. braziliensis*, *L. mexicana*, *L. tropica*, *L. major* and *L. infantum* has recently been reported (Dutta *et al.*, 2007). In this study, we have shown that AVL has a similar leishmanicidal effect against promastigotes of *L. donovani* (IC_{50}=110 μg ml^{-1}; Fig. 1). To clarify the mode of action of AVL against *L. donovani*, using biochemical and morphological approaches we have demonstrated that AVL-induced cell death shared several phenotypic features observed with metazoan apoptosis (Debrabant *et al.*, 2003), which included phosphatidylserine exposure (Fig. 2), hypopolarization of mitochondrial potential (Fig. 3), release of cytochrome *c* into the cytosol (Fig. 4), nuclear blebbing by Hoechst staining (Fig. 5), *in situ* TUNEL staining of nicked DNA and oligonucleosomal DNA fragmentation. Taken together with the combined use of several techniques, including annexin V staining, changes in mitochondrial potential, release of cytochrome *c* into the cytosol and DNA fragmentation, we have conclusively proven that AVL induced a caspase-independent PCD in promastigotes that notably shares some, but not all, the classical features of apoptosis observed in higher eukaryotes. It is envisaged that study of the major pathways involved in *Leishmania* apoptosis-like death would help provide insight for future design of newer chemotherapeutic strategies.

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