

# Racemoside A, an anti-leishmanial, water-soluble, natural steroidal saponin, induces programmed cell death in *Leishmania donovani*

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Leishmaniasis remains a major health problem of the tropical and subtropical world. The visceral form causes the most fatalities if left untreated. Dramatic increases in the rates of infection and drug resistance and the non-availability of safe vaccines have highlighted the need for identification of novel and inexpensive anti-leishmanial agents. This study reports that racemoside A, a water-soluble steroidal saponin purified from the fruits of *Asparagus racemosus*, is a potent anti-leishmanial molecule effective against antimonial-sensitive (strain AG83) and -unresponsive (strain GE1F8R) *Leishmania donovani* promastigotes, with IC<sub>50</sub> values of 1.15 and 1.31 µg ml<sup>-1</sup>, respectively. Incubation of promastigotes with racemoside A caused morphological alterations including cell shrinkage, an aflagellated ovoid shape and chromatin condensation. This compound exerts its leishmanicidal effect through the induction of programmed cell death mediated by the loss of plasma membrane integrity as detected by binding of annexin V and propidium iodide, loss of mitochondrial membrane potential culminating in cell-cycle arrest at the sub-G<sub>0</sub>/G<sub>1</sub> phase, and DNA nicking shown by deoxynucleotidyltransferase-mediated dUTP end labelling (TUNEL). Racemoside A also showed significant activity against intracellular amastigotes of AG83 and GE1F8R at a 7–8-fold lower dose, with IC<sub>50</sub> values of 0.17 and 0.16 µg ml<sup>-1</sup>, respectively, and was non-toxic to murine peritoneal macrophages up to a concentration of 10 µg ml<sup>-1</sup>. Hence, racemoside A is a potent anti-leishmanial agent that merits further pharmacological investigation.

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## INTRODUCTION

Leishmaniasis can occur in diverse clinical forms such as cutaneous, mucosal and visceral leishmaniasis (VL, the most severe) and remains a major health problem in the tropics and subtropics, threatening almost 350 million people in 88 countries (Chava *et al.*, 2005; Murray *et al.*, 2005). Approximately 50% of the world's cases of VL occur in the Indian subcontinent (Desjeux, 2004). To date, there are no vaccines against leishmaniasis and therefore treatment relies on chemotherapy. The use of chemotherapy, especially in India, is unsatisfactory due to increasing unresponsiveness to first-line treatment with pentavalent antimonials (Sundar & Chatterjee, 2006). These limitations also apply to amphotericin B and its lipid formulations due to toxicity, high cost and the need for parenteral administration (Guerin *et al.*, 2002).

**Abbreviations:** MFI, mean fluorescence intensity; PCD, programmed cell death; PI, propidium iodide; SAG, sodium antimony gluconate; TdT, terminal deoxynucleotidyltransferase; TUNEL, TdT-mediated dUTP end labelling.

Miltefosine exhibited promise as the first oral candidate, but a poor cost:benefit ratio and the possibility of the development of resistance have restricted its potential (Murray *et al.*, 2005; Perez-Victoria *et al.*, 2003), thus highlighting the urgent need for new drugs.

Programmed cell death (PCD) mechanisms are known to be operative in kinetoplastid parasites of the genus *Trypanosoma* (Szallies *et al.*, 2002) and *Leishmania* (Arnoult *et al.*, 2002) in response to various chemotherapeutic stimuli such as pentostam, amphotericin B (Lee *et al.*, 2002) and miltefosine (Paris *et al.*, 2004).

Plant-derived products have shown promise in the search for better therapeutics against leishmaniasis (Dupouy-Camet, 2004). In this regard, the leaf exudate of *Aloe vera* (Dutta *et al.*, 2007a, b) and triterpenoid saponin of *Careya arborea* (Mandal *et al.*, 2006a) have been reported to be leishmanicidal. Although water-insoluble saponins have shown anti-leishmanial properties (Delmas *et al.*, 2000; Maes *et al.*, 2004), to our knowledge, no leishmanicidal activity mediated by PCD has been demonstrated.

The main objectives of the present study were to evaluate the anti-leishmanial effect and putative mechanism(s) of a water-soluble steroidal saponin, racemoside A, purified from the fruits of *Asparagus racemosus*. Here, we report, for the first time, that racemoside A is a potent anti-leishmanial molecule against both promastigotes and amastigotes of *Leishmania donovani*, acting by inducing PCD.

## METHODS

**Materials.** M199 medium and fetal calf serum (FCS) were obtained from Gibco-BRL, DMSO from SRL, methanol from Merck and 5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and propidium iodide (PI) from Molecular Probes. Annexin V-FITC and the ApoDirect kit were from BD Biosciences. All other chemicals were from Sigma unless stated.

**Parasite culture.** Promastigotes were obtained from two Indian *L. donovani* strains, AG83 (MHOM/IN/83/AG83) and GE1F8R (a subclone of MHOM/IN/90/GE1) (Dutta *et al.*, 2005). Based on their clinical response to sodium antimony gluconate (SAG), they were classified as SAG sensitive (AG83) or SAG resistant (GE1F8R). They were routinely cultured at 22 °C in complete medium (M199 medium supplemented with 10% heat-inactivated FCS and 100 µg gentamicin ml<sup>-1</sup>).

**Extraction, isolation and structure elucidation of racemoside A.** Racemoside A was purified from dried fruits of *Asparagus racemosus* (Mandal *et al.*, 2006b). The compound was characterized as (25S)-5β-spirostan-3β-ol-3-O-β-D-glucopyranosyl(1→6)-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside with the molecular formula C<sub>51</sub>H<sub>84</sub>O<sub>22</sub> (Fig. 1). Racemoside A was dissolved in RPMI 1640 at a concentration of 1 mg ml<sup>-1</sup> for evaluation of its anti-leishmanial activity.

**Anti-promastigote activity of racemoside A (in vitro).** Exponential-phase promastigotes of *L. donovani* AG83 and GE1F8R were resuspended in modified RPMI 1640 (without phenol red) supplemented with 10% FCS and 100 µg gentamicin ml<sup>-1</sup>,

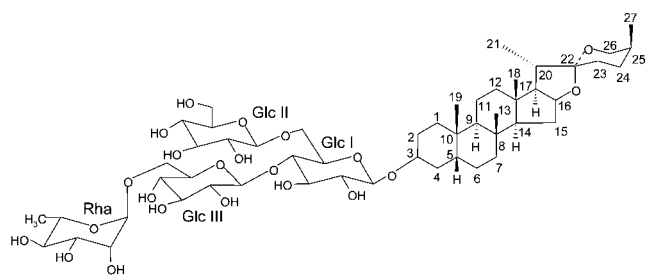
designated medium A. Parasites (2 × 10<sup>5</sup>) were seeded in 96-well tissue culture plates (0.25 ml per well) and exposed to increasing concentrations of racemoside A (0–10 µg ml<sup>-1</sup>) or amphotericin B (0–10 µg ml<sup>-1</sup>) for 72 h at 22 °C. Parasite viability was evaluated using a modified MTT assay, where the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan by mitochondrial enzymes served as an indicator of cell viability and the amount of formazan produced was directly proportional to the number of metabolically active cells (Dutta *et al.*, 2005). Accordingly, absorbance at 492 nm represented the number of live cells. The concentration that decreased cell growth by 50% (IC<sub>50</sub>) was determined by graphic extrapolation.

**Anti-leishmanial activity in a macrophage–amastigote model (ex vivo).** Murine peritoneal macrophages were lavaged using cold medium A from the peritoneum of BALB/c mice following starch induction (2% starch administered intraperitoneally, 2 ml per mouse) and harvested in cold medium A. Cells (5 × 10<sup>5</sup>) were then seeded on 16-well tissue culture glass slides (0.10 ml per well) and allowed to adhere overnight in an atmosphere of 5% CO<sub>2</sub> at 37 °C. After removal of non-adherent macrophages, stationary-phase promastigotes of strain AG83 were added to the macrophages at a ratio of 5:1 (parasite:macrophage) and incubated overnight in the same environment. Non-phagocytosed parasites were removed by gentle washing and infected macrophages were incubated with racemoside A (0–1 µg ml<sup>-1</sup>) or amphotericin B (0–1 µg ml<sup>-1</sup>) for an additional 72 h. The slides were fixed in methanol and Giemsa stained, and the number of amastigotes within macrophages was determined microscopically. The number of parasites per 100 macrophages per well was determined from quadruplicate cultures and the IC<sub>50</sub> was established from the number of amastigotes in 100 macrophages. The *ex vivo* effect of racemoside A on the antimonial-resistant strain GE1F8R was enumerated in a similar way.

In parallel, the toxic effect of racemoside A on murine peritoneal macrophages was determined by incubating cells for 72 h in the presence or absence of racemoside A (0–10 µg ml<sup>-1</sup>) in medium A and evaluating the viability of macrophages as above. The absorbance by macrophages in the absence of racemoside A was considered to be 100%.

**Analysis of promastigote cellular morphology.** Morphological changes in parasites as a result of racemoside A treatment were identified microscopically. Briefly, exponential-phase promastigotes were incubated in the absence (controls) or presence of racemoside A (10 µg ml<sup>-1</sup>) for 0–24 h in medium A. At various time points, cells were centrifuged, fixed in 4% paraformaldehyde, attached to poly-L-lysine-coated glass slides, mounted in glycerol and examined under a confocal microscope (Leica). At least 20 microscope fields were observed for each sample.

**Analysis of externalized phosphatidylserine in promastigotes by flow cytometry.** Exponential-phase promastigotes were incubated with racemoside A (10 µg ml<sup>-1</sup>) for 0–6 h. Cells were centrifuged (3000 g for 5 min), washed twice in PBS and resuspended in annexin V binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>]. Annexin V-FITC and PI were then added according to the manufacturers' instructions, and incubated for 30 min in the dark at 20–25 °C. Data acquisition was carried out on a FACSCalibur flow cytometer (BD) and analysed using CELLQUEST software. If there is an alteration of the membrane integrity (due to externalization of phosphatidylserine), annexin V detects both pro- and late-apoptotic cells. Therefore, the simultaneous addition of PI, which does not enter healthy cells with an intact plasma membrane, discriminates between pro-apoptotic (annexin V-positive and PI-negative), late-apoptotic (both annexin V- and PI-positive), necrotic (PI-positive) and live (both annexin V- and PI-negative) cells.



**Fig. 1.** Structure of racemoside A. Racemoside A was purified from a methanol extract of air-dried powdered fruits of *A. racemosus*. Elucidation of the structure was based on <sup>1</sup>H and <sup>13</sup>C NMR, distortionless enhancement by polarization transfer, correlation spectroscopy, total correlation spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear multiple-quantum coherence and heteronuclear multiple-bond correlation experiments. Glc, Glucose; Rha, rhamnose.

**Measurement of the mitochondrial membrane potential of promastigotes.** Mitochondrial damage was assessed using a cell-permeable dye, JC-1, to analyse the change in mitochondrial transmembrane potential ( $\Delta\psi_m$ ). The carbocyanine dye JC-1 exists as a monomer or as aggregates, depending on concentration and membrane potential. In the mitochondria of healthy cells, with high  $\Delta\psi_m$ , the dye spontaneously forms complexes, known as J-aggregates, that emit red fluorescence at 585 nm. Following the onset of apoptosis, as the mitochondrial potential decreases, JC-1 remains in the monomeric form, emitting green fluorescence at 530 nm. Thus, the ratio of red (585 nm) to green (530 nm) fluorescence represents  $\Delta\psi_m$ , which is dependent only on the membrane potential (Reers *et al.*, 1995). After exposure to racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 0–6 h, exponential-phase promastigotes were centrifuged, resuspended in PBS containing JC-1 ( $10 \mu\text{g}$  in 0.1 ml per well) and incubated at  $37^\circ\text{C}$  for 10 min. Analysis of the mean green and red fluorescence intensities was carried out using a FACSCalibur and CELLQUEST software. The mitochondrial transmembrane potential was calculated from the ratio of the mean fluorescence intensity (MFI) of J-aggregates and monomers.

**Analysis of the cell cycle.** Exponential-phase promastigotes were incubated for 0–24 h in the presence of racemoside A ( $10 \mu\text{g ml}^{-1}$ ) and then washed twice with PBS (pH 7.2). Cells were fixed in chilled methanol by incubating for 3 min on ice and then resuspended in 0.5 ml PBS containing  $0.5 \mu\text{g}$  PI and  $50 \mu\text{g}$  RNase A and incubated for 1 h in the dark at room temperature. Data acquisition was carried out using a FACSCalibur and analysed using CELLQUEST software.

**In situ detection of DNA fragmentation of promastigotes.** DNA fragmentation within the cell was analysed by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP end labelling (TUNEL) using an Apo-Direct kit according to the manufacturer's instructions. Briefly, promastigotes were incubated with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 0, 18 or 24 h, washed twice in PBS (pH 7.2) and fixed in 1% paraformaldehyde for 30 min. Cells were washed again in PBS and incubated in chilled 70% ethanol for 30 min on ice. After washing, these cells were allowed to react with TdT enzyme in reaction buffer and stained with PI and dUTP-FITC. Finally, cells were resuspended in PBS before data acquisition using a FACSCalibur and CELLQUEST software.

**Statistical analysis.** *In vitro* anti-leishmanial activity was expressed as  $\text{IC}_{50}$  values by linear regression analysis. Values were determined as means  $\pm$  SD from at least three independent experiments in duplicate. *P* values were estimated using Student's *t*-test.

## RESULTS AND DISCUSSION

### Racemoside A-mediated death in *L. donovani* promastigotes

The viability of promastigotes after treatment with racemoside A was evaluated using a modified MTT assay. MTT is converted to formazan by active mitochondrial enzymes. Therefore, a decrease in formazan production indicates a reduction in the number of metabolically active cells, i.e. a decrease in cell viability. Treatment of promastigotes with racemoside A demonstrated a dose-dependent inhibition of parasite growth irrespective of SAG sensitivity.  $\text{IC}_{50}$  values in the SAG-sensitive strain AG83 and SAG-resistant strain GE1F8R promastigotes were 1.15 and  $1.31 \mu\text{g ml}^{-1}$ , respectively (Fig. 2a). The established

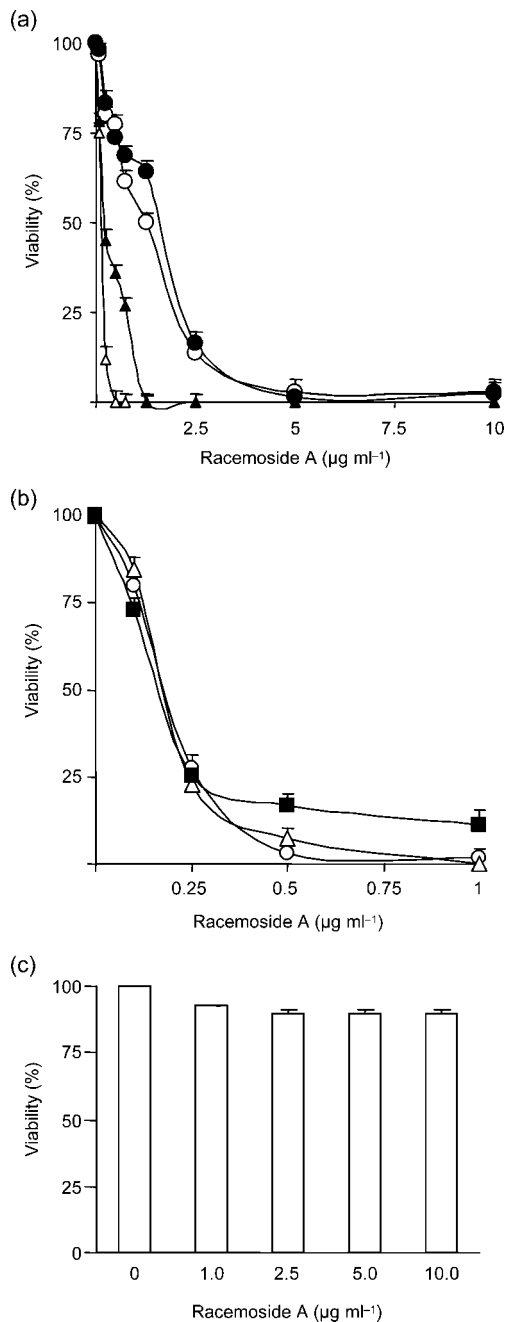
anti-leishmanial drug amphotericin B, used as a positive control, showed a similar trend in dose-dependent parasite killing, with  $\text{IC}_{50}$  values of 0.17 and  $0.22 \mu\text{g ml}^{-1}$  for AG83 and GE1F8R, respectively.

Saponins comprise a large group of compounds known for their ability to permeate cells. However, the concentration required ( $1 \text{ mg ml}^{-1}$ ) is  $\sim$ 100-fold higher than that required for racemoside A ( $10 \mu\text{g ml}^{-1}$ ). Anti-leishmanial activity of a water-insoluble triterpenoid saponin isolated from *C. arborea* has been reported against *L. donovani* promastigotes, with an  $\text{IC}_{50}$  of  $15 \mu\text{g ml}^{-1}$  (Mandal *et al.*, 2006a), but its effect against the intracellular form of the parasite (amastigotes) has yet to be elucidated. Similarly, water-insoluble sapogenin from *Asparagus africanus* was parasitocidal ( $\text{IC}_{50}$   $31 \pm 4 \mu\text{g ml}^{-1}$ ) against *Leishmania major* promastigotes (Oketch-Rabah *et al.*, 1997). However, this sapogenin has not been tested against VL-causing forms of *Leishmania* such as *L. donovani*, which cause the most fatalities. Racemoside A has sugar moieties attached to the core structure and this core structure is similar to the structure of sapogenin. Our data demonstrated that racemoside A, a unique water-soluble steroidal saponin, is much more active against *L. donovani* promastigotes ( $\text{IC}_{50}$  1.15– $1.31 \mu\text{g ml}^{-1}$ ; Fig. 2a) than is the water-insoluble sapogenin ( $\text{IC}_{50}$   $31 \pm 4 \mu\text{g ml}^{-1}$ ). Therefore, it can be envisaged that these additional sugar molecules not only increase the compound's hydrophilicity, but also enhance its leishmanicidal efficacy. Triterpenoid saponins isolated from *Maesa balansae* showed anti-leishmanial efficacy against *L. donovani* and *Leishmania infantum* at very low doses, with  $\text{IC}_{50}$  values of 7–40  $\text{ng ml}^{-1}$  (Germonprez *et al.*, 2005; Maes *et al.*, 2004). Unfortunately, unlike racemoside A, these triterpenoid saponins are water-insoluble. To our knowledge, this is the first report of the leishmanicidal efficacy of a naturally occurring, water-soluble, steroidal saponin.

### Racemoside A-mediated death of *L. donovani* amastigotes

Treatment with racemoside A demonstrated a dose-dependent removal of phagocytosed amastigotes (Fig. 2b), as shown by microscopic observation of Giemsa-stained cells. The  $\text{IC}_{50}$  of racemoside A in amastigotes was  $0.17 \mu\text{g ml}^{-1}$  in strain AG83 and  $0.16 \mu\text{g ml}^{-1}$  in strain GE1F8R, which is comparable to the  $\text{IC}_{50}$  of amphotericin B, used as a reference compound, with an  $\text{IC}_{50}$  of  $0.16 \mu\text{g ml}^{-1}$  against AG83. The cytotoxic effect of racemoside A against murine peritoneal macrophages was enumerated biochemically using an MTT assay. The viability of murine peritoneal macrophages was  $>92\%$  after treatment with racemoside A ( $1 \mu\text{g ml}^{-1}$ ; Fig. 2c). A similar degree of macrophage viability ( $>89\%$ ) was observed up to a concentration of  $10 \mu\text{g}$  racemoside A  $\text{ml}^{-1}$ .

Racemoside A is thus highly potent at very low doses, even against the intracellular form of the parasite, i.e. approximately 7-fold more effective ( $\text{IC}_{50}$  0.16– $0.17 \mu\text{g ml}^{-1}$ )



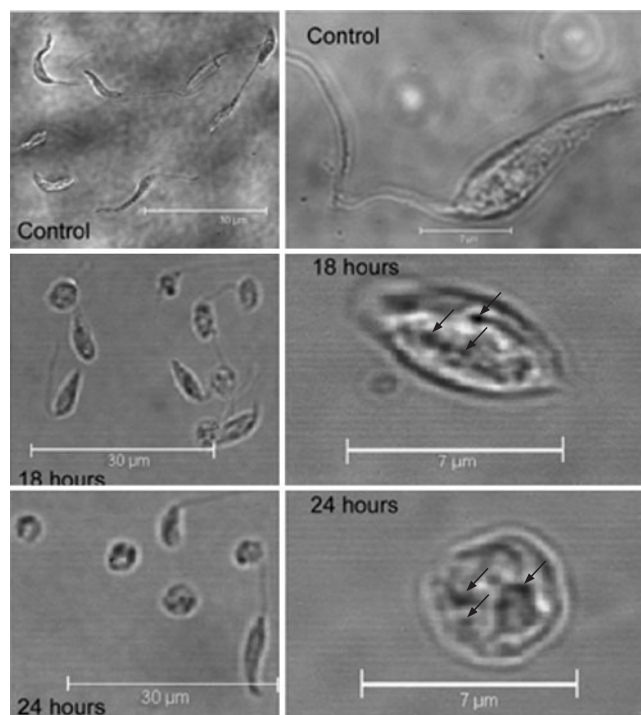
against amastigotes than against promastigotes, which was comparable with the effect of amphotericin B ( $IC_{50}=0.16 \mu\text{g ml}^{-1}$ ; Fig. 2b). More importantly, the absence of shared toxicity towards murine peritoneal macrophages, even up to a concentration of  $10 \mu\text{g ml}^{-1}$  (Fig. 2c), increases its therapeutic ratio (the ratio between leishmanicidal concentration and the concentration that is toxic to host cells). The efficacy of racemoside A against intra-macrophage amastigotes ( $IC_{50} 0.17 \mu\text{g ml}^{-1}$ ) was found to be higher than that of known anti-leishmanial compounds such as Pentostam ( $IC_{50} 4.9\text{--}50 \mu\text{g ml}^{-1}$ ), miltefosine ( $IC_{50} 13.6 \mu\text{M}$ ; Paris *et al.*, 2004), SAG ( $IC_{50} 154 \mu\text{g ml}^{-1}$ ; Roberts & Rainey, 1993) and lipid formulations of amphotericin B ( $IC_{50}$

**Fig. 2.** (a) Analysis of the anti-promastigote activity of racemoside A. Exponential-phase promastigotes ( $5 \times 10^4$ ) of strains AG83 (○) and GE1F8R (●) in 0.25 ml per well were incubated with increasing concentrations of racemoside A ( $0\text{--}10 \mu\text{g ml}^{-1}$ ) for 72 h and a modified MTT assay was performed as described in Methods. Promastigotes of AG83 (△) and GE1F8R (▲) were treated with amphotericin B ( $0\text{--}10 \mu\text{g ml}^{-1}$ ) as an experimental control and evaluated similarly. Each point corresponds to the mean  $\pm$  SD of at least three experiments in duplicate. (b) Evaluation of the anti-amastigote activity of racemoside A. Murine peritoneal macrophages were infected overnight with stationary-phase promastigotes (at a ratio of 1:5) of AG83 (○) and GE1F8R (■). After removal of non-phagocytosed parasites, infected macrophages were incubated with increasing concentrations of racemoside A ( $0\text{--}1 \mu\text{g ml}^{-1}$ ) for 72 h and the degree of parasite removal was analysed microscopically from Giemsa-stained slides as described in Methods. *Leishmania*-infected macrophages (△) were treated with amphotericin B ( $0\text{--}1 \mu\text{g ml}^{-1}$ ) as an experimental control and evaluated similarly. Each point corresponds to the mean  $\pm$  SD of at least three experiments in duplicate. (c) Evaluation of the macrophage toxicity of racemoside A. Murine peritoneal macrophages were isolated and adhered overnight to 16-well glass slides in an atmosphere of 5%  $\text{CO}_2$  at 37 °C. After removal of non-adherent cells, racemoside A ( $0\text{--}10 \mu\text{g ml}^{-1}$ ) was added and the cells were incubated under similar conditions for 72 h. Cell viability was evaluated using a modified MTT assay as described in Methods. Each point corresponds to the mean  $\pm$  SD of at least three experiments in duplicate.

$0.2\text{--}2.6 \mu\text{g ml}^{-1}$ ; Yardley & Croft, 2000), but comparable to amphotericin B ( $IC_{50} 0.013\text{--}0.18 \mu\text{g ml}^{-1}$ ; Yardley & Croft, 2000). Higher leishmanicidal efficacy (low  $IC_{50}$ ), solubility in water and less shared toxicity towards mammalian cells (even at  $10 \mu\text{g ml}^{-1}$ ) make this readily available plant-derived compound a potent anti-leishmanial molecule for SAG-sensitive and -resistant strains and their amastigote forms, and thus it merits further pharmacological investigation.

### Racemoside A causes changes in promastigote morphology

During PCD, the promastigotes alter to an ovoid shape with nuclear condensation, which is accompanied by the formation of fragmented nuclei (Sen *et al.*, 2004). Phase-contrast microscopic studies on the morphology of racemoside A-treated *L. donovani* promastigotes demonstrated the induction of certain morphological changes that were similar to the signs of PCD. The flagellated promastigotes shrank and became aflagellated and oval or round with an increase in vacuoles as a result of racemoside A exposure. The nuclei of control promastigotes displayed a prominent central or slightly eccentric localized nucleolus, whilst chromatin was usually distributed peripherally beneath the nuclear membrane (Fig. 3). However, in most of the treated cells, condensed, irregular, bead-like, marginated chromatin and a fragmented nucleus with condensed cytoplasm were observed (Fig. 3).



**Fig. 3.** Analysis of the cellular morphology of racemoside A-treated promastigotes. Exponential-phase promastigotes ( $5 \times 10^4$  in 0.25 ml per well) of strain AG83 were incubated with increasing concentrations of racemoside A ( $0$ – $10 \mu\text{g ml}^{-1}$ ) for 18 and 24 h, fixed in 4% paraformaldehyde, adhered to poly-L-lysine-coated glass slides and analysed by confocal microscopy as described in Methods. Changes in chromatin following racemoside A treatment are indicated by arrows. Bars,  $30 \mu\text{m}$  (left panels),  $7 \mu\text{m}$  (right panels).

### Racemoside A-treated promastigotes show both annexin V and PI binding

Translocation of phosphatidylserine from the inner side to the outer layer of the plasma membrane is a common alteration during PCD (Koonin & Aravind, 2002; Mehta & Shaha, 2004). Annexin V, a  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein with a special affinity for phosphatidylserine, is a general reagent used to detect the externalization of phosphatidylserine, thus labelling cells that have lost their membrane integrity.

Accordingly, in order to determine whether racemoside A triggered cell death following a similar phenomenon, promastigotes treated with racemoside A ( $10 \mu\text{g ml}^{-1}$  for 0–6 h) were double-stained with annexin V–FITC and PI. The percentage of racemoside A-treated promastigotes that were positive only for annexin V gradually decreased from 11.52% after 30 min to  $<1.0\%$  after 1 h (Fig. 4). However, the number of cells that were both annexin V- and PI-positive (Fig. 4, upper-right quadrant) gradually increased, to  $2.54 \pm 1.05$ ,  $47.01 \pm 2.07$  and  $76.13 \pm 0.89\%$

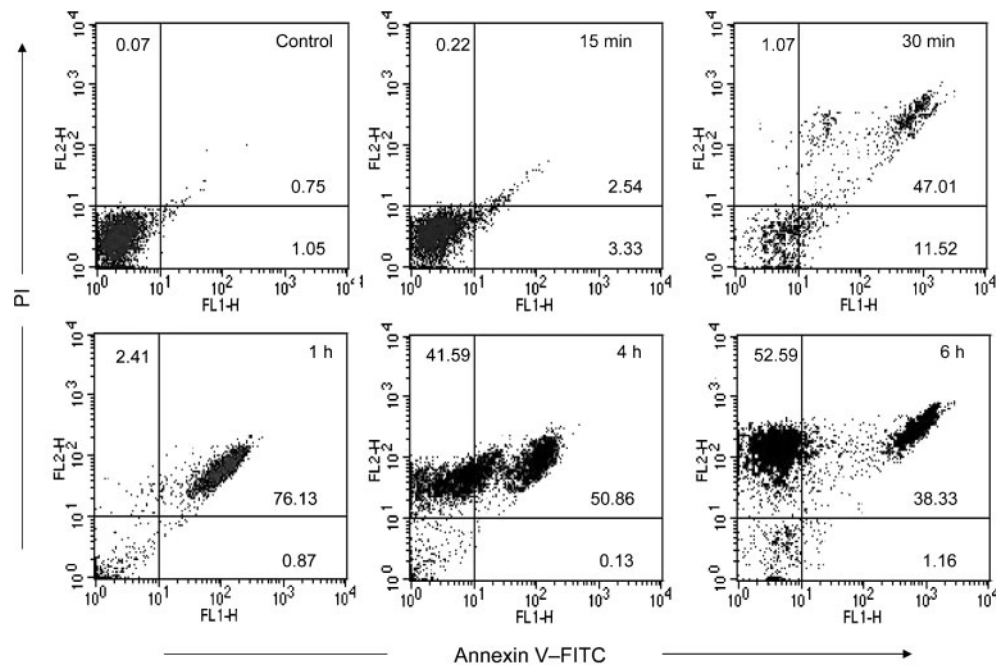
at 15 min, 30 min and 1 h, respectively, indicating late-apoptotic phase. A gradual decrease in these annexin V- and PI-positive cells was observed at 4 and 6 h of treatment, to  $50.86 \pm 3.28$  and  $38.33 \pm 4.28\%$ , respectively, with a simultaneous increase in the percentage of necrotic cells (annexin V-negative and PI-positive; Fig. 4, upper-left quadrant), to  $41.59 \pm 2.45\%$  and  $52.59 \pm 2.08\%$ , respectively. This was due to degradation of the parasites. These observations suggested that racemoside A induced cell death by loss of membrane integrity, as shown by the increased PI incorporation and annexin V binding, indicating late-apoptotic phase. In contrast, only  $0.75 \pm 0.08\%$  of untreated cells were annexin V- and PI-positive, and the number of cells positive for PI only was negligible at all time points ( $0.07 \pm 0.02\%$ ).

To analyse the pro-apoptotic stage, promastigotes were also incubated with a dose near the  $\text{IC}_{50}$  value ( $1.3 \mu\text{g ml}^{-1}$ ) of racemoside A for 30 min, after which  $8.26 \pm 2.02\%$  cells were positive for annexin V. At lower doses and with a shorter duration (30 min) of exposure, a dose-dependent increase in the number of cells positive for both annexin V and PI was observed in all experimental sets, being 2, 7.77 and 16.88% at concentrations of 1.3, 2.5 and  $5.0 \mu\text{g ml}^{-1}$ , respectively. This indicated that this steroidal saponin is a membrane-attacking molecule for *L. donovani* promastigotes, suggesting that racemoside A acts directly on the plasma membrane, making it leaky and thus driving PCD signals.

### Racemoside A induces sustained depolarization of mitochondrial membrane potential

The mitochondrial membrane potential ( $\Delta\psi_m$ ) is commonly determined by the ratio of red : green fluorescence at 585 and 530 nm, i.e. J-aggregates in mitochondria versus monomers in the cytosol, using JC-1 staining (Reers *et al.*, 1995; Sen *et al.*, 2004). Incubation of promastigotes with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 3 h caused depolarization, shown by a 16.49% decrease in the 585 : 530 ratio in cells incubated in the presence of racemoside A compared with unexposed cells (Fig. 5;  $11.34 \pm 0.35$  vs  $13.58 \pm 0.39$ ,  $P < 0.05$ ). By 4 h, the decrease was 18.25% (Fig. 5;  $11.11 \pm 0.51$  vs  $13.59 \pm 0.59$ ) and this was sustained up to 5 h. A maximum decrease of 32.64% was observed at 6 h (Fig. 5;  $9.12 \pm 0.37$  vs  $13.54 \pm 0.53$ ,  $P < 0.02$ ). Taken together, these results indicated that exposure to racemoside A caused sustained mitochondrial membrane depolarization for up to 6 h, which may be due to imperfect mitochondrial function.

Similarly, treatment of promastigotes with racemoside A disturbed normal mitochondrial function as shown by the reduction in formazan formation in an MTT assay due to reduced mitochondrial dehydrogenases (Fig. 2a). The reduction in formazan formation served as a measure of the decrease in the number of viable cells and was also associated with a decrease in mitochondrial membrane potential, corroborated by the decreased red fluorescence



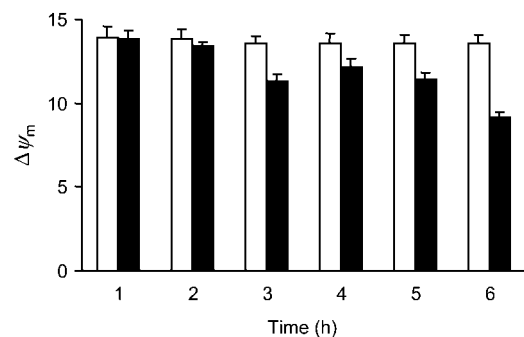
**Fig. 4.** Externalization of phosphatidylserine in racemoside A-treated promastigotes. Exponential-phase promastigotes of strain AG83 were incubated with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 0 (Control), 15 and 30 min and 1, 4 and 6 h, co-stained with PI and annexin V-FITC and analysed by flow cytometry as described in Methods. The results show a representative profile of at least three experiments.

of racemoside A-treated promastigotes following JC-1 staining (Fig. 5). This change in mitochondrial membrane potential appeared to occur more slowly than the PI/annexin V double staining at 3 h, when the majority of cells were already in the late-apoptotic phase. The cells are driven into the late-apoptotic phase by changes in the morphology of the cell membrane, which thus initiates the cascade of events involving the cell organelles (such as mitochondria) leading to PCD. Most probably, racemoside A directly targets the plasma membrane making it leaky and this in turn switches on the mitochondria-mediated death signal cascades. The depolarization is known to commit cells to DNA fragmentation (Sen *et al.*, 2004).

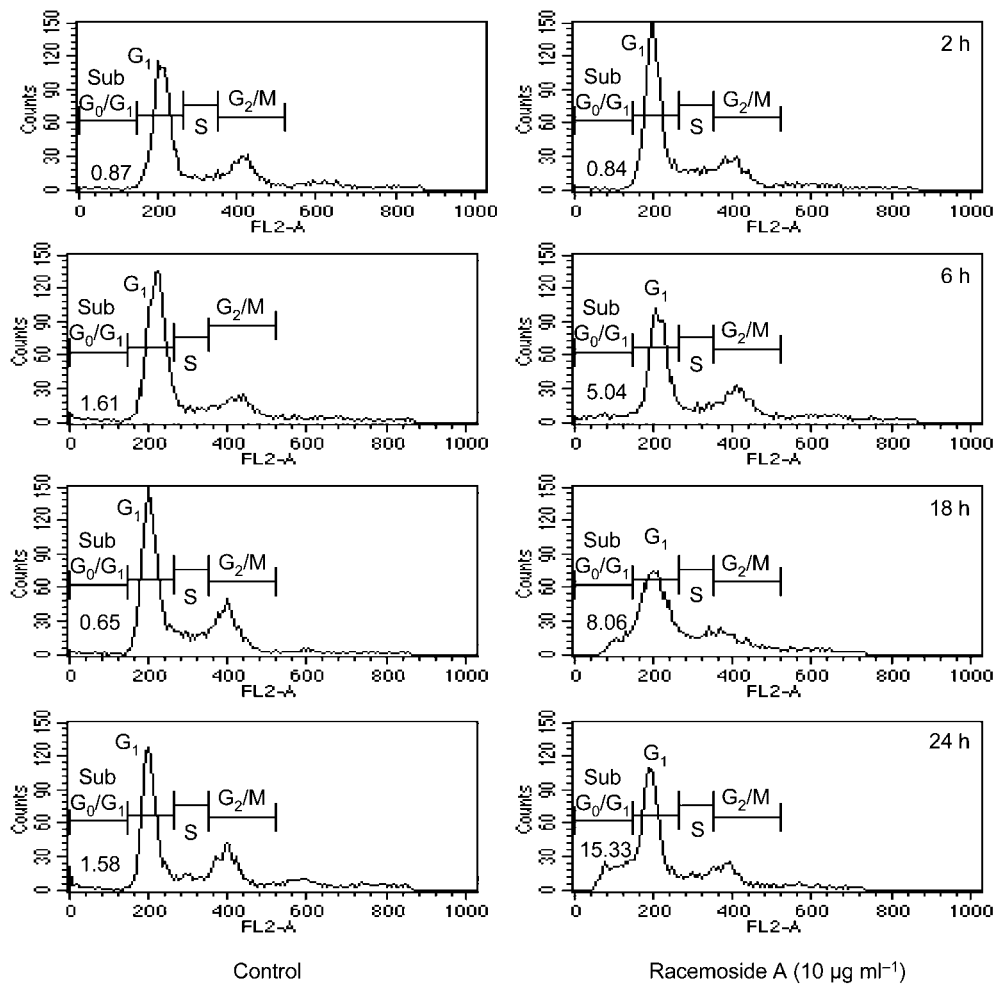
### Racemoside A causes cell-cycle arrest in *L. donovani* promastigotes

Treatment of promastigotes with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 6, 18 and 24 h caused cell-cycle arrest at the sub- $G_0/G_1$  phase, as analysed by flow cytometry. The proportions of cells in this phase at these time points were 5.04, 8.06 and 15.33% compared with 1.61, 0.65 and 1.58%, respectively, for cells that were not exposed to racemoside A (Fig. 6). This suggests that a check in the cell cycle could initiate late events of PCD such as nuclear condensation and DNA nicking. However, at 2 h, the proportion of cells in different phases of the cell cycle was comparable in both sets. This finding indicated that

nuclear changes are switched on by alterations in the cell membrane and mitochondria and therefore can be considered as a relatively late phase of PCD.



**Fig. 5.** Changes in mitochondrial membrane potential following treatment with racemoside A. Exponential-phase promastigotes of strain AG83 were incubated with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 0–6 h at  $22^\circ\text{C}$  in complete medium (filled bars). Cells without racemoside treatment were used as a negative control (open bars). Cells were washed in PBS and probed with JC-1 ( $10 \mu\text{g}$  in 0.1 ml per well) and analysed by flow cytometry as described in Methods. The ratio of fluorescence at 585 and 530 nm [i.e. J-aggregates (red) in the mitochondria vs monomers (green) in the cytosol] represents  $\Delta\psi_m$ . Each bar corresponds to the mean  $\pm$  SD of at least three experiments in duplicate.

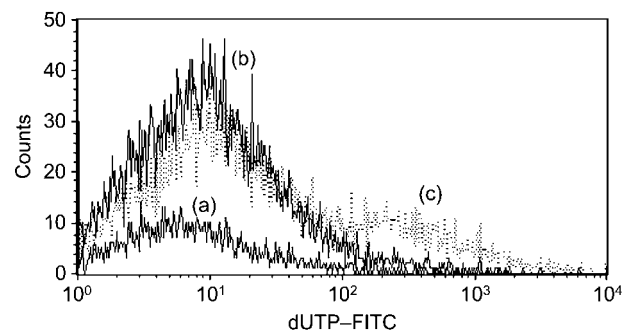


**Fig. 6.** Analysis of the cell-cycle status of racemoside A-treated promastigotes. Exponential-phase promastigotes of strain AG83 were incubated with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 2, 6, 18 and 24 h, fixed in chilled methanol, probed with PI and analysed using a FACSCalibur and CELLQUEST software as described in Methods.

### Oligonucleosomal DNA fragmentation in racemoside A-treated promastigotes

One of the hallmarks of apoptotic cell death is the degradation of nuclear DNA into nucleosomal units. DNA nicking resulting from exposure to racemoside A was detected using a TUNEL assay in which the proportion of DNA nicks was quantified by measuring the binding of dUTP-FITC to the nicked ends via TdT. Thus, the proportion of DNA nicks was directly proportional to the fluorescence obtained from dUTP-FITC.

The treatment of promastigotes with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) demonstrated a time-dependent increase in nuclear DNA fragmentation as shown by dUTP-FITC binding (Fig. 7). After 18 h of treatment, the degree of DNA nicking (MFI=9.05) was marginally increased compared with untreated promastigotes (MFI=6.91), which served as a control. However, after 24 h, the degree of DNA nicking was amplified (MFI=19.41) and the percentage of



**Fig. 7.** Analysis of TUNEL positivity in racemoside A-treated promastigotes. Exponential-phase promastigotes of strain AG83 were incubated with racemoside A ( $10 \mu\text{g ml}^{-1}$ ) for 0 h (a), 18 h (b) and 24 h (c). Cells were fixed, stained with PI and dUTP-FITC in the presence of TdT and RNase enzyme and analysed by flow cytometry as described in Methods.

dUTP-FITC-positive cells increased to 23.07% compared with 4.28% of untreated cells (ethanol-fixed mammalian cell populations, supplied in the kit, served as positive and negative controls; data not shown).

DNA nicking in promastigotes strongly suggests that the leishmanicidal effect is mediated by PCD. Cells were positive for both annexin V and PI at early time points, indicating that racemoside A directly targets the plasma membrane, making it more fragile and subsequently switching on mitochondria-mediated death signal cascades (Fig. 4). This signal was orchestrated by a substantial depolarization (~32.64%) of the mitochondrial membrane after 6 h (Fig. 5), chromatin condensation (Fig. 3), cell-cycle arrest at the sub-G<sub>0</sub>/G<sub>1</sub> phase after 24 h (Fig. 6) and TUNEL positivity after 24 h (Fig. 7).

In this study, we have shown for the first time that a newly identified, water-soluble, natural steroidal saponin, racemoside A (Fig. 1), had a leishmanicidal effect against promastigotes of both an SAG-sensitive and an SAG-unresponsive strain (Fig. 2a). This anti-leishmanial activity was almost seven times higher against intracellular amastigotes (Fig. 2b), with a high therapeutic ratio (Fig. 2c), making it a potent candidate for future chemotherapy.

Thus, we have conclusively demonstrated the leishmanicidal effect of racemoside A-induced PCD as shown by a number of biochemical and morphological techniques including annexin V and PI co-staining, changes in mitochondrial membrane potential, cell-cycle arrest and *in situ* DNA nicking. This study widens the scope for the design of new chemotherapeutic agents for better management of this disease.

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