

BnSP-7 toxin, a basic phospholipase A₂ from *Bothrops pauloensis* snake venom, interferes with proliferation, ultrastructure and infectivity of *Leishmania (Leishmania) amazonensis*

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(Received 19 October 2012; revised 6 December 2012; accepted 1 January 2013; first published online 27 February 2013)

SUMMARY

This paper reports the effects of BnSP-7 toxin, a catalytically inactive phospholipase A₂ from *Bothrops pauloensis* snake venom, on *Leishmania (Leishmania) amazonensis*. BnSP-7 presented activity against promastigote parasite forms both in the MTT assay, with IC₅₀ of 58.7 µg mL⁻¹ of toxin, and a growth curve, inhibiting parasite proliferation 60–70% at concentrations of 50–200 µg mL⁻¹ of toxin 96 h after treatment. Also, the toxin presented effects on amastigotes, reducing parasite viability by 50% at 28.1 µg mL⁻¹ and delaying the amastigote–promastigote differentiation process. Ultrastructural studies showed that BnSP-7 caused severe morphological changes in promastigotes such as mitochondrial swelling, nuclear alteration, vacuolization, acidocalcisomes, multi-flagellar aspects and a blebbing effect in the plasma membrane. Finally, BnSP-7 interfered with the infective capacity of promastigotes in murine peritoneal macrophages, causing statistically significant infectivity-index reductions ($P < 0.05$) of 20–35%. These data suggest that the BnSP-7 toxin is an important tool for the discovery of new parasite targets that can be exploited to develop new drugs for treating leishmaniasis.

Key words: basic phospholipase A₂, *Bothrops pauloensis*, cytotoxicity, *Leishmania (Leishmania) amazonensis*.

INTRODUCTION

Leishmaniasis is a disease caused by protozoa of the *Leishmania* genus, which are transmitted by the bite of a female sandfly of the genus *Phlebotomus* (Mitropoulos *et al.* 2010). In the case of *Leishmania (Leishmania) amazonensis*, the parasite affects skin causing ulcers characteristic of cutaneous leishmaniasis (Grevelink and Lerner, 1996; Basano and Camargo, 2004; García-Almargo, 2005). The systemic pharmacological treatment of leishmaniasis includes pentavalent antimonials as first-choice drugs (Brasil, 2007). However, when the therapeutic response is unsatisfactory or in cases of inability to use them, second-choice drugs such as amphotericin B and pentamidins can be used for the treatment (Gontijo and Carvalho, 2003; Brasil, 2007). This current scenario of antileishmanial drugs – characterized by their high toxicity, high

cost and, in some cases, parasite resistance – has motivated the search for new therapeutic approaches. In this sense, natural products appear as sources of bioactive molecules, which are important tools for discovering new therapeutic targets.

Snake venom toxins are commonly used as tools for drug design, in view of the wide variety of pharmacological actions attributed to these substances (Tempone *et al.* 2001). Microbicidal activity described for snake venom has been associated with L-amino acid oxidase and/or the enzymes phospholipase A₂ (PLA₂) (Barbosa *et al.* 2005; Samy *et al.* 2007). Phospholipase A₂ (EC 3.1.1.4) is a class of enzymes defined by their ability to catalyse the hydrolysis of the *sn*-2-acyl bond of *sn*-3-phospholipids, generating free fatty acids and lysophospholipids as products (van Deenen and de Haas, 1963; Diaz and Arm, 2003). The action of PLA₂s on *Leishmania* sp. was assessed in cytotoxicity studies by several research groups (Stábeli *et al.* 2006; Costa *et al.* 2008; Passero *et al.* 2008; Torres *et al.* 2010; Peichoto *et al.* 2011). With the exception of one

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investigation by Passero *et al.* (2008), these studies evaluated the action of the toxin only by the viability of the parasite. Thus, there are few data on the effect of toxins on parasite–host interactions, as well as a shortage of studies seeking to elucidate the mechanism of cytotoxicity or the biochemical and physiological changes observed in the parasite. It should be emphasized that no study, so far, has shown morphological alterations due to snake toxin action on *Leishmania* sp. Studies on the effects of bioactive molecules against *Leishmania* sp. are important not only for ascertaining the parasite biology, but also for generating new drugs to treat leishmaniasis since they may serve as tools in the discovery of new therapeutic targets. The present work aimed to elucidate the cytotoxic effects of a PLA₂ isolated from the *Bothrops pauloensis* snake venom, denominated BnSP-7 (Soares *et al.* 2000), on *L. (L.) amazonensis*, by evaluating the effect of this toxin on proliferation, cell viability, morphology and amastigote–promastigote differentiation, as well as to determine the toxin's effect on the infective capacity of promastigotes in murine peritoneal macrophages.

MATERIALS AND METHODS

Chemicals

Dimethylsulfoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), RPMI 1640 medium, penicillin and streptomycin were purchased from Sigma Chemical Co. (St Louis, USA), heat-inactivated fetal bovine serum (FBS) from Cultilab (Brazil) and Schneider's insect medium from LGC Biotecnologia (Brazil). All other reagents were analytical grade or superior.

Promastigote culture

Leishmania (L.) amazonensis (IFLA/BR/67/PH8 strain) promastigotes were cultured in Schneider's insect medium, pH 7.0, supplemented with 10% FBS, penicillin (100 UI mL⁻¹) and streptomycin (100 µg mL⁻¹) – complete Schneider's insect medium – at 23 °C. Promastigotes used in all experiments were isolated from the stationary growth phase (metacyclic promastigotes, in 5- to 6-day-old culture).

Preparation of amastigotes

Leishmania (L.) amazonensis amastigotes were obtained by footpad infection of BALB/c mice (6–8 weeks old) with promastigote forms (1 × 10⁷ cells/footpad) for 5 to 6 weeks. Footpad lesions were surgically removed and the tissue homogenized in phosphate-buffered saline (PBS).

Debris was eliminated by nylon Nitex filtration (pore size, 80 µm). The cell suspension was centrifuged at 1800 g for 10 min. To lyse erythrocytes, the pellet was re-suspended in ammonium chloride solution (8.29 g of NH₄Cl, 1 g of KHCO₃ and 37.3 mg of EDTA per litre) for 10 min, and the insoluble material containing infected macrophage cells was homogenized (20 strokes). The resulting suspension was centrifuged 4 times at 1800 g, and the final pellet was re-suspended in RPMI 1640 in the presence of penicillin/streptomycin. The suspension was shaken for 3 h and the amastigotes washed 4 times by centrifugation at 1800 g (Tanaka *et al.* 2007). All animal experimental procedures were approved by the Institutional Ethics Committee for Animal Use at the Federal University of Uberlândia (CEUA 052/09).

Peritoneal macrophage culture

Resident peritoneal macrophages were harvested from the peritoneal cavity of male BALB/c mice (6–8 weeks old) by washing with cold PBS. Collected cells were washed 3 times with cold PBS by centrifugation at 950 g and re-suspended in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, penicillin (100 U mL⁻¹) and streptomycin (100 mg mL⁻¹) – complete RPMI medium. Macrophages were placed on 96-well plates (2 × 10⁵ cells/well) for the viability assay or placed on sterile glass coverslips in 24-well plates (5 × 10⁵ cells/well) for the infectivity assay, in both cases for 1 h. Non-adherent cells were removed by several washings with RPMI and the plates were kept at 37 °C in a CO₂ incubator. All animal experimental procedures were approved by the Institutional Ethics Committee for Animal Use at the Federal University of Uberlândia (CEUA 052/09). BALB/c mice were supplied by the Centre for Animal Experimentation of the Federal University of Uberlândia. They were maintained in sterilized cages under a controlled environment (25 ± 5 °C, 12 h day/night cycle) and received water and food *ad libitum*.

Toxin

BnSP-7 toxin was isolated from *B. pauloensis* snake venom according to the description of Rodrigues *et al.* (1998) and Soares *et al.* (2000) with minor modifications. Briefly, approximately 200 mg of whole venom was dissolved in 2 mL of 50 mM ammonium bicarbonate ((NH₄)HCO₃) buffer (pH 7.8) and centrifuged at 3000 g for 10 min at 4 °C. The supernatant was recovered and injected on a CM-Sephacose column previously equilibrated and initially eluted with the same buffer. A convex gradient was then applied until obtaining 0.5 M (NH₄)HCO₃ buffer, pH 7.8. Fractions of 1 mL/tube were collected at a flow rate of 6.5 mL h⁻¹.

The CM5 fraction containing the PLA₂ BnSP-7 (1 mg) was dissolved in 500 µL of solvent A (0.1% trifluoroacetic acid) and applied to an HPLC system, using C2–C18 µRPC 4.6/100 (GE Health Care) column, previously equilibrated with solvent A. The elution of the protein was then conducted using a linear gradient of solvent B (80% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100% at a flow rate of 1 mL min⁻¹ at room temperature. To certify protein homogeneity, polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli (1970).

The BnSP-7 PLA₂, derived from some RP-HPLC, was lyophilized and solubilized in DMSO to obtain a stock solution (40 mg mL⁻¹), and maintained at -20 °C. In viability assays, the highest concentration tested in the parasite was 400 µg mL⁻¹, and thus the final DMSO concentration in culture medium never exceeded 1% (v/v). It is well established that DMSO at the concentration of 1% does not affect parasite proliferation (Granthon *et al.* 2007; Santos *et al.* 2008; Neto *et al.* 2011).

Proliferation and viability assays

In order to evaluate the BnSP-7 toxin effect on cellular proliferation of *L. (L.) amazonensis* promastigotes, parasites (5 × 10⁶ cells mL⁻¹) were cultured in 25 cm² cell culture flasks containing complete Schneider's insect medium with progressively higher concentrations of toxin (25.0–200.0 µg mL⁻¹) at 23 °C. At 24, 48, 72 and 96 h after incubation with toxin, the number of promastigotes was determined by counting in a Neubauer chamber.

Viability assays in the presence of BnSP-7 toxin were performed on parasites (promastigote and amastigote forms) and peritoneal macrophages by a colorimetric method based on mitochondrial oxidation of MTT reagent. First, parasites were re-suspended in complete Schneider's insect medium and placed on 96-well culture plates (5 × 10⁵ parasites/well) and incubated at 23 °C (promastigote) or at 37 °C (amastigote) with different toxin concentrations (2-fold serial dilution from 400.0 µg mL⁻¹ of toxin to promastigote and 200.0 µg mL⁻¹ of toxin to amastigote) for 72 h (promastigote) or 24 h (amastigote). To determinate the macrophage viability in the presence of toxin, resident peritoneal macrophages collected from BALB/c mice, as described above, were placed on a 96-well culture plate (2 × 10⁵ cells/well) and incubated with complete RPMI medium containing toxin (2-fold serial dilution from 400 µg mL⁻¹ of toxin) at 37 °C with 5% CO₂ for 24 h. After incubation, the parasite and macrophage viabilities were accessed by adding MTT reagent (5 mg mL⁻¹ in Schneider's insect medium, 100 µg/well) to the plates and incubation proceeded for about 4 h at

37 °C protected from light. The reaction was stopped by adding 100 µL of PBS containing 10% SDS and 0.01 M HCl. The absorbances were measured in an EL × 800 microplate reader (BioTek Instruments) at 595 nm. Each assay was carried out in triplicate and independent experiments were performed. The IC₅₀ values with 95% confidence limits were determined by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA).

Amastigote differentiation

The effect of toxin on amastigote differentiation in promastigotes was analysed by the addition of BnSP-7 toxin (100 µg mL⁻¹) in 1 × 10⁶ amastigotes mL⁻¹ cultured in 25 cm² cell culture flasks containing complete Schneider's insect medium at 23 °C. The normal differentiation profile from amastigotes to promastigotes was evaluated by amastigote culture in the absence of toxin. Aliquots of culture were collected daily for 6 days to determine the cell density of promastigotes and amastigotes.

Promastigote morphology

In order to evaluate morphological changes, promastigotes treated for 72 h with BnSP-7 toxin (100 µg mL⁻¹) were collected by centrifugation, washed 3 times in PBS and fixed with 2% formaldehyde in PBS. Fixed parasites were placed on glass slides, stained with the fast Panótico kit (LB Laborclin, Brazil) and analysed by optical microscopy.

In order to evaluate ultrastructural changes, promastigotes treated for 72 h with BnSP-7 toxin (100 µg mL⁻¹) were analysed by electron transmission microscopy. After treatment with toxin, parasites were fixed for 2 h at 4 °C in a solution containing 2.5% glutaraldehyde in PBS. Fixed parasites were washed in PBS and post-fixed for 1 h in a solution containing 1% osmium tetroxide (OsO₄) and 0.8% potassium ferrocyanide in PBS. The cells were washed in PBS, dehydrated in a graded acetone series and embedded in resin. Ultrathin sections were stained with uranyl acetate and lead citrate then observed under a Zeiss EM 109 transmission electron microscope (Zeiss, Oberkochen, Germany). Three independent experiments were performed in triplicate.

Macrophage infectivity by L. (L.) amazonensis promastigotes

Promastigote forms previously incubated for 72 h in the presence of BnSP-7 toxin (25, 100 and 200 µg mL⁻¹) or absence of the toxin (control parasite) were harvested by centrifugation and washed 3 times in PBS. Then, parasites were added onto macrophages placed on glass coverslips in a 24-well plate, obtained

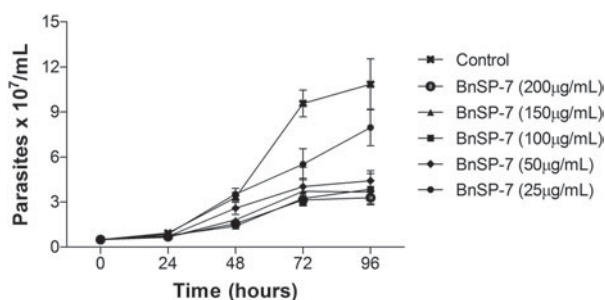


Fig. 1. Effect of BnSP-7 toxin on the proliferation of *Leishmania (L.) amazonensis* promastigotes: The growth pattern of *L. (L.) amazonensis* promastigotes was monitored in parasites cultivated in Schneider's insect medium at 23 °C in the absence (control) or presence of increasing doses of BnSP-7 toxin (25–200 µg mL⁻¹) up to 96 h. The toxin was added to the culture at hour 0. The parasite concentration was determined daily. Data are expressed as the mean ± s.d. of 3 independent experiments performed in triplicate.

as previously described, at a 10 : 1 ratio (parasites : macrophage), and kept at 25 °C for 1 h. Plates were washed with RPMI 1640 medium to remove non-internalized parasites. Complete RPMI medium was added and the plates were incubated at 37 °C in a CO₂ incubator for 24 h. Coverslips containing macrophages infected with parasites were fixed with 2% formaldehyde in PBS, stained by Giemsa and analysed through optical microscopy. The infectivity index was determined by multiplying the percentage of macrophages that had at least 1 intracellular parasite by the average number of intracellular parasites per infected macrophage. These data were determined by randomly counting at least 100 cells in each of the triplicate coverslips. Three independent experiments were performed.

Statistical analysis

All experiments were performed at least 3 times. Representative results of experiments developed in triplicate are shown throughout the article. The data were analysed statistically using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Data are expressed as mean ± s.d. of 3 independent experiments performed in triplicate. Comparisons of data for each group were analysed by one-way ANOVA and post-test, when necessary. Differences were considered statistically significant for a *P* value < 0.05.

RESULTS

Effect of BnSP-7 toxin on proliferation and cellular viability

The PLA₂ BnSP-7 was shown to be homogeneous by SDS-PAGE after chromatographic isolation (results

Table 1. Effects of BnSP-7 toxin on *Leishmania (L.) amazonensis* viability

Cell type	*IC ₅₀ value (95% confidence intervals) µg/ml
Promastigote ^a	58.7 (43.4–79.3)
Amastigote ^b	28.1 (23.8–33.1)
Mammalian cell ^b	5.6 (4.6–6.9)

* IC₅₀: concentration that produces 50% inhibitory effect on cell viability.

^a The viability assay was tested 72 h after BnSP-7 treatment.

^b The viability assay was tested 24 h after BnSP-7 treatment.

not shown). Thus, in order to evaluate the effect of toxin on the proliferation of *L. (L.) amazonensis* promastigotes, BnSP-7 toxin (25–200 µg mL⁻¹) was added to promastigotes from stationary growth phase and monitored for 96 h. The toxin caused a dose-dependent inhibition on promastigote proliferation that was evident 72 h after incubation with toxin. Proliferation inhibitions of between 60 and 70% for toxin concentrations of 50–200 µg mL⁻¹ were observed 96 h after incubation (Fig. 1).

Viability assays on parasites and macrophage were performed by MTT assay, based on the ability of viable cells to reduce MTT reagent to an insoluble formazan product. The toxin provoked dose-dependent cytotoxicity in both amastigotes and promastigotes. The data were analysed statistically to determine BnSP-7 toxin IC₅₀ values. The IC₅₀ values were 58.7 µg mL⁻¹ 72 h after incubation and 28.1 µg mL⁻¹ 24 h after incubation for promastigotes and amastigotes, respectively (Table 1). These results indicate that amastigotes were more susceptible than promastigotes to BnSP-7 toxin. Despite the lower IC₅₀ value for amastigotes in relation to promastigotes, 100 µg mL⁻¹ of toxin caused similar cytotoxicity regardless of the parasite form (85 and 88% for amastigote and promastigote forms, respectively). Also, the cytotoxic effect of BnSP-7 toxin on peritoneal murine macrophages was analysed by MTT assay and showed that in the presence of 5.6 µg mL⁻¹ of toxin only 50% of cells remained viable after 24 h when compared with the experimental control.

Effect of BnSP-7 toxin on amastigote differentiation

Amastigote forms isolated from BALB/c mice footpad lesions were incubated with BnSP-7 toxin (100 µg mL⁻¹) and maintained at 23 °C for 6 days, in order to evaluate the effect of the toxin on the differentiation of amastigotes into promastigotes, as shown in Fig. 2. After 3 days, the number of promastigotes in culture without BnSP-7 toxin was

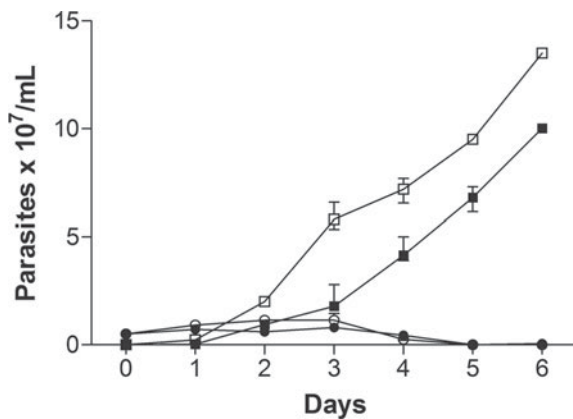


Fig. 2. Effect of BnSP-7 toxin on *Leishmania (L.) amazonensis* amastigote–promastigote differentiation. Amastigotes (1×10^6) isolated from BALB/c mice lesions were cultured in Schneider's insect medium at 23 °C in the absence (open circles and squares) or presence of $100 \mu\text{g mL}^{-1}$ of BnSP-7 toxin (filled circles and squares) up to 7 days. Aliquots were collected daily to determine the numbers of amastigotes (circles) and promastigotes (squares).

6.0×10^7 parasites mL^{-1} whereas in the presence of toxin the number of promastigotes was 65% less, suggesting a toxic effect on amastigote–promastigote differentiation. After the fourth day of cultivation, the effect of the toxin on differentiation was no longer detectable while the number of promastigotes had also increased in the culture containing the toxin. On the sixth day, the number of promastigotes in control cultures (absence of BnSP-7 toxin) reached approximately 1.35×10^8 parasites mL^{-1} whereas in the presence of toxin the number of promastigotes was 26% lower. These results indicate that the toxin prevented the differentiation process only during the first 3 days, after which the differentiation process occurred, a delay probably attributable to the initial action exerted by the toxin during the first days.

Effect of BnSP-7 toxin on promastigote morphology

Based on toxic and antiproliferative effects provoked by BnSP-7 toxin on *L. (L.) amazonensis*, the next step was to investigate the action exerted by the toxin on parasite morphology. Initial studies by optical microscopy showed that parasites cultured with BnSP-7 toxin present relevant alterations in their typical morphologies including an altered shape of the promastigote body and either 2 flagella or a single short flagellum (results not shown). To ascertain the effect of the toxin on parasite ultrastructure, promastigotes cultured with BnSP-7 toxin ($100 \mu\text{g mL}^{-1}$) for 72 h were analysed by transmission electron microscopy (TEM) (Fig. 3) and compared morphologically with control promastigotes (cultured in the absence of toxin). Analysis by TEM confirmed the presence of parasites containing 2 flagella (Fig. 3I,

J, D) as indicated by optical microscopy analysis (results not shown), and revealed additional details. Results showed blebs detaching from the entire cell surface (Fig. 3E–G, B), the presence of a crenate nucleus and chromatin condensation (Fig. 3K–N, n), a higher number of vacuoles (Fig. 3H, *), acidocalcisomes (Fig. 3L–N, white arrows), mitochondrial swelling (Fig. 3K, L, m) and shrinkage of the cytoplasm (Fig. 3G, K, d). These results corroborate the deleterious effects of BnSP-7 toxin on promastigote morphology.

Effect of BnSP-7 toxin on the infective capacity of promastigote forms

Promastigote forms of *L. (L.) amazonensis* pre-treated with BnSP-7 toxin (25, 100 and $200 \mu\text{g mL}^{-1}$) for 72 h were added to a culture of macrophages, and the infectivity indices were calculated 24 h after infection. The results showed that BnSP-7 toxin reduced the infective capacity of promastigotes in murine peritoneal macrophages, causing statistically significant reductions of approximately 20–35% (Fig. 4) at all toxin concentrations tested after 24 h, in relation to the control ($P < 0.05$). There was no statistically significant difference between groups ($P < 0.05$). Approximately 60% of macrophages were infected with control parasites after 24 h. Interestingly, the parasites pre-treated with toxin showed percentages very similar to control parasites, regardless of BnSP-7 toxin concentration. However, the number of parasites per infected macrophage by pre-treated parasites was significantly lower than among macrophages infected with control parasites, in a dose-dependent manner (data not shown).

DISCUSSION

As mentioned earlier, the effect of the whole snake venoms on *Leishmania* promastigotes has been well documented by several research groups (Fernandez-Gomez *et al.* 1994; Tempone *et al.* 2001; Gonçalves *et al.* 2002; Passero *et al.* 2007), since they are recognized as useful sources of bioactive substances showing a wide range of pharmacological activities. The PLA₂ action on *Leishmania* sp. promastigote growth and/or viability was previously demonstrated by other research groups. MjTX-II, a Lys49 PLA₂ isolated from *Bothrops moojeni*, reduced *Leishmania* sp. viability by around 50% in the presence of $75 \mu\text{g mL}^{-1}$ of toxin (Stábeli *et al.* 2006). Costa *et al.* (2008) assayed 2 PLA₂s (MTX-I and II) from *Bothrops brazili* and their respective C-terminal region-derived synthetic peptides (pepMTX-I and pepMTX-II). Both MTX-I, an Asp49 PLA₂, and MTX-II, a Lys49 PLA₂, caused an inhibition of approximately 90% in parasite viability at

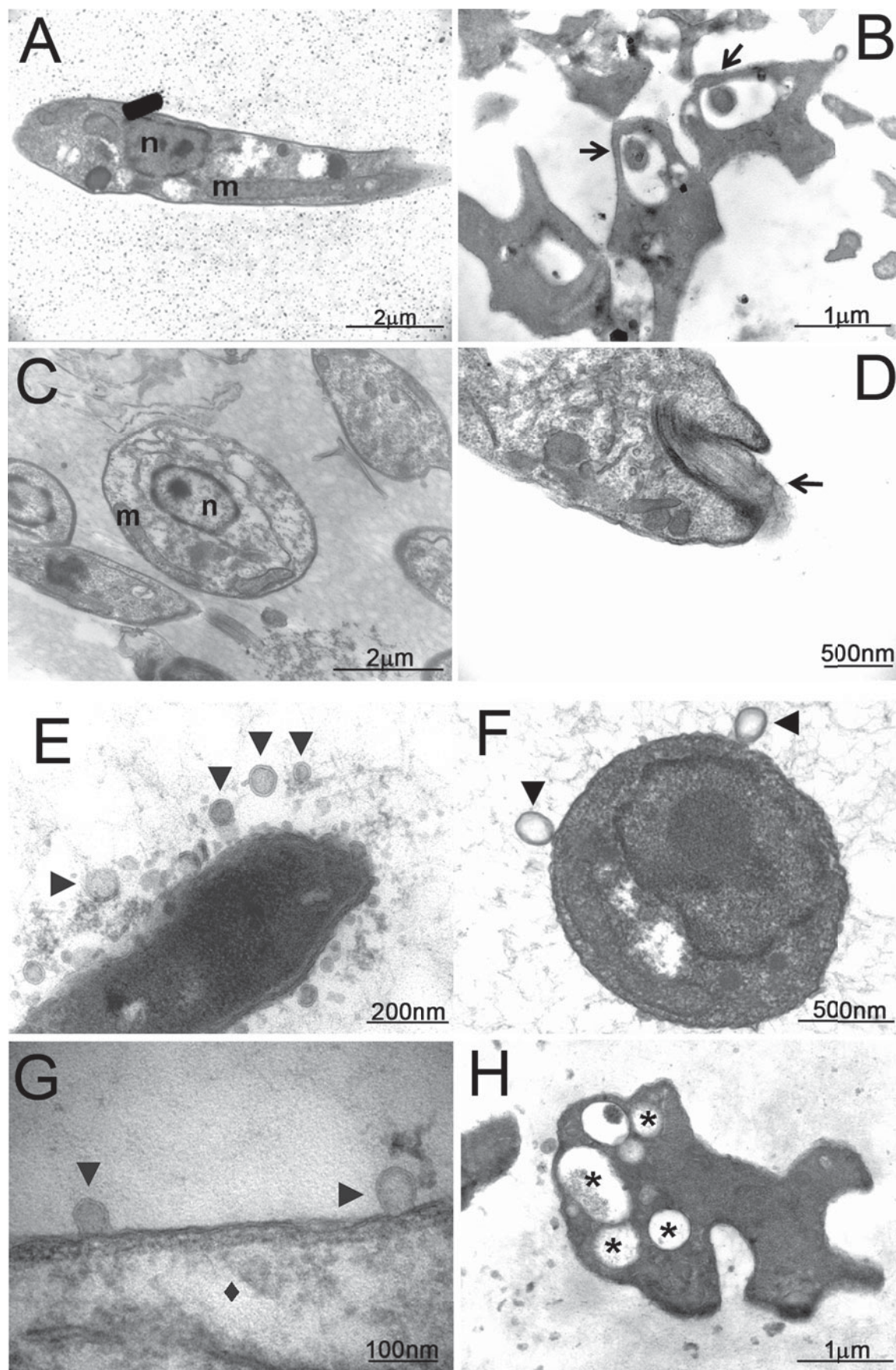


Fig. 3. See the following page for legend.

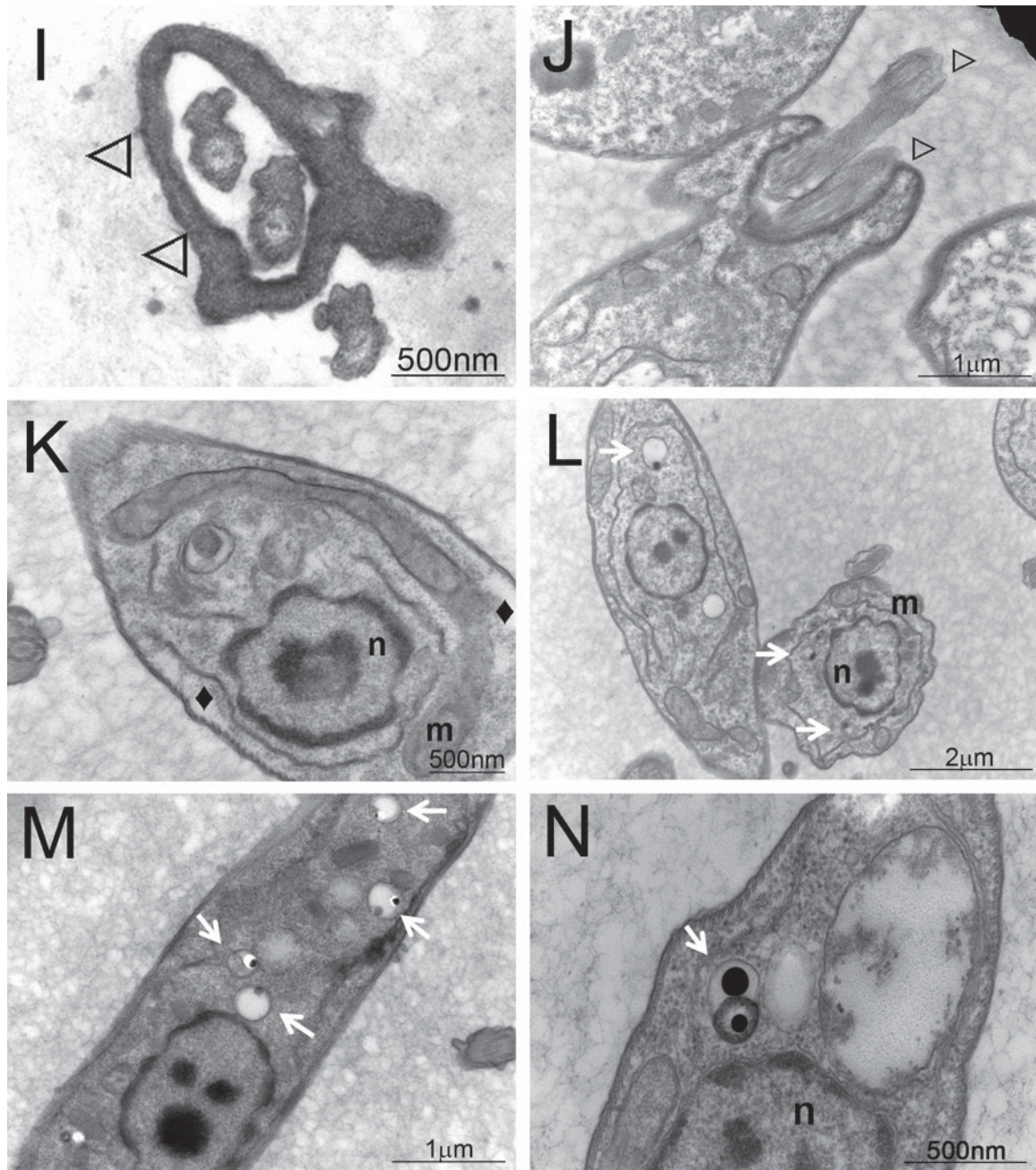


Fig. 3. Ultrastructural changes in *Leishmania (L.) amazonensis* promastigotes treated with BnSP-7 toxin. Promastigotes were cultured in Schneider's insect medium at 23 °C in the absence (panels A–D) or presence of 100 µg mL⁻¹ of BnSP-7 for 72 h (panels E–N). Subsequently, the parasites were processed for electron transmission microscopy. Single flagellum (panels B, D, black arrows), membrane blebbing (panels E–G, ►), membrane shrinkage (panels G, K, ◆), vacuoles (panel H, *), presence of 2 flagella in a flagellar pocket (panels I, J, ▷), acidocalcisomes (panel L–N, white arrows). Lower-case letters indicate: m, mitochondria; n, nucleus.

100 µg mL⁻¹ concentration, while the peptides killed about 70% of *Leishmania* sp. at 120 µg mL⁻¹.

In contrast, another study demonstrated that an enzymatically active PLA₂ from *Crotalus durissus collilineatus* venom stimulates the growth of *L. (L.) amazonensis* promastigotes and amastigotes (Passero *et al.* 2008). In a more recent study, Peichoto *et al.* (2011) found that Trimorphin, an Asp49 PLA₂ isolated from *Trimorphodon biscutatus lambda*, exhibited a dose-dependent biphasic effect on *L. (L.)*

major growth, with potent cytotoxicity at higher concentrations, and stimulation of proliferation at very low concentrations. According to the authors, this finding could be explained by the adaptive response of cells to a moderate stress. Since PLA₂s are important mediators of complex intracellular signalling pathways, low concentrations of these enzymes would lead to a stimulus of cell growth, whereas high concentrations of the enzyme would result in direct damage to the cell.

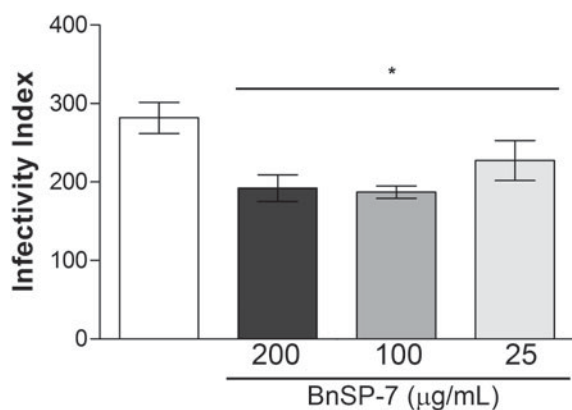


Fig. 4. Effect of BnSP-7 toxin on the infective capacity of *Leishmania (L.) amazonensis* promastigotes in murine peritoneal macrophages. Peritoneal macrophages were infected with promastigotes cultured in the absence or presence of BnSP-7 toxin (200, 100 and 25 $\mu\text{g mL}^{-1}$) for 72 h. Infected macrophages were incubated at 37 °C in a CO₂ incubator for 24 h. Infectivity indices were calculated by multiplying the percentage of macrophages that had at least 1 intracellular parasite by the average number of intracellular parasites per infected macrophage, counting 100 macrophages/cover slip ($n = 3$). *Statistically significant difference ($P < 0.05$) compared with the control. There is no statistically significant difference between groups ($P < 0.05$).

Our results showed that BnSP-7 toxin, a Lys49 PLA₂, provoked a leishmanicidal effect on *L. (L.) amazonensis* promastigotes, causing inhibition of both proliferation and mitochondrial dehydrogenase activity of the parasite, as determined by the growth curve and MTT assay, respectively. BnSP-7 toxin showed a dose-dependent inhibitory effect on the proliferation of promastigotes. Parasite growth inhibition started 48 h after culturing and became evident after 72 h. A 60% inhibition of promastigote growth was observed 96 h after incubation with 50 $\mu\text{g mL}^{-1}$ of BnSP-7 toxin *vs* a maximum inhibition of about 70% on growth produced by both 150 and 200 $\mu\text{g mL}^{-1}$ of toxin. These data indicate that 150 $\mu\text{g mL}^{-1}$ of toxin presents the maximum anti-proliferative effect against *L. (L.) amazonensis* promastigotes.

The study of the cytotoxic action was supplemented by viability assays showing that 58.7 $\mu\text{g mL}^{-1}$ of BnSP-7 toxin provoked the death of 50% of promastigotes whereas 28.1 $\mu\text{g mL}^{-1}$ killed 50% of amastigotes, suggesting that the amastigote is more susceptible to BnSP-7 toxin than the promastigote form. Tempone *et al.* (2001) observed that *L. (L.) amazonensis* amastigotes were not affected by L-amino acid oxidase (LAAO) from *B. moojeni*, even in the presence of 300 $\mu\text{g mL}^{-1}$ of toxin, whereas 1.44 $\mu\text{g mL}^{-1}$ of LAAO killed 50% of promastigote forms. Considering that the cytotoxic mechanism for LAAOs is related to hydrogen

peroxide production, the result obtained by incubating toxin with amastigotes is justified because this parasite form provides more efficient protection against free radicals and hydrogen peroxide when compared with promastigotes. In contrast, our results suggest that PLA₂s are more effective against free amastigotes. The greater effectiveness of the toxin BnSP-7 against amastigotes is very interesting because it is responsible for the maintenance of the infection in the vertebrate host. In addition to the cytotoxic action on amastigotes, the toxin caused a delay in the differentiation process to the promastigote form.

The actions of PLA₂s on *Leishmania* promastigote viability were described by other research groups and showed variations among IC₅₀ values. For example, the Lys49 PLA₂s (catalytically inactive) produced IC₅₀ values of 75 $\mu\text{g mL}^{-1}$ of MjTX-II toxin to *L. (L.) amazonensis*, *L. (L.) donovani* and *L. (L.) major* (Stábeli *et al.* 2006); approximately 40 and 60 $\mu\text{g mL}^{-1}$ of MTX-II toxin to *L. (L.) amazonensis* and *L. (V.) braziliensis*, respectively (Costa *et al.* 2008). These results are very close to those found in the present study, considering the wide variation in susceptibility observed among different species and strains of the parasite. On the other hand, no cytotoxic effect on *L. (L.) amazonensis* or *L. (L.) chagasi* was observed even at 200 $\mu\text{g mL}^{-1}$ of BmarPLA₂, which is also a Lys49 PLA₂ (Torres *et al.* 2010). For Asp49 PLA₂s (catalytically active) the IC₅₀s were approximately 40 and 20 $\mu\text{g mL}^{-1}$ of MTX-I to *L. (L.) amazonensis* and *L. (V.) braziliensis*, respectively (Costa *et al.* 2008); and more recently it was demonstrated that Trimorphin toxin presented an IC₅₀ of 3.6 $\mu\text{g mL}^{-1}$ to *L. (L.) major*.

Taken together, these findings indicate the possibility that catalytic activity is not necessary to produce anti-*Leishmania* action. Furthermore, studies have demonstrated that the C-terminal region, comprising amino acids from 115 to 129, is related to the cytotoxic and bactericidal effects of phospholipase A₂, independently of catalytic activity (Páramo *et al.* 1998; Stábeli *et al.* 2006; Nevalainen *et al.* 2008).

Besides the cytotoxic and anti-proliferative effects provoked by BnSP-7 toxin, the induction of several morphological changes was verified in the promastigote ultrastructure. Such alterations ranged from discrete to total destruction of the parasite. Control parasites presented normal ultrastructure, showing a typically elongated cell body, anterior flagellum, single mitochondrion containing the kinetoplast and a normal nucleus.

The rounded shape of the promastigote body, the blebbing effect, mitochondrial swelling and cytoplasm shrinking observed in BnSP-7-treated promastigotes indicate an important effect of the toxin on promastigote morphology. Other indications of morphological changes resulting from the toxin

action were localized in the nuclear region. These changes appeared as a nuclear crenate aspect (pyknotic effect) and altered chromatin organization, strongly resembling apoptotic cells. Apoptosis, a type of programmed cell death that plays a central role in normal tissue development and homeostasis (Paris *et al.* 2004; Deolindo *et al.* 2010; Paiva *et al.* 2011), involves phosphatidylserine externalization, release of cytochrome *c* with consequent activation of cellular proteases and DNA cleavage into oligonucleosomal fragments. These biochemical events lead to characteristic changes in cell morphology and subsequent death. In *Leishmania* sp., cellular alterations during this process include rounding up of the cell, cytoplasmic retraction (pyknosis), chromatin condensation, nuclear fragmentation, plasma membrane blebbing and mitochondrial swelling (Arnoult *et al.* 2002; Paris *et al.* 2004; Menna-Barreto *et al.* 2009; Jiménez-Ruiz *et al.* 2010; Paiva *et al.* 2011). Moreover, nuclear and mitochondrial alterations are probably among the main factors underlying the potent anti-proliferative effect and the loss of viability in *Leishmania* species (Ledezma *et al.* 2002).

The presence of multiple flagella was also observed by Nakamura *et al.* (2006), when *L. (L.) amazonensis* promastigotes were treated with Eugenol-rich essential oil from *Ocimum gratissimum*, and by Havens *et al.* (2000), in *L. (L.) donovani* exposed to tubulin inhibitors. The emergence of these multiflagellar forms suggests an interference with the process of cell division. In trypanosomatids, microtubules participate in 4 crucial processes: (i) subpellicular microtubules are involved in the protozoan structural maintenance; (ii) the flagella are associated with motility; (iii) the basal body and mitotic spindles are implicated in cell division; and (iv) the cytosome is involved in endocytosis (Menna-Barreto *et al.* 2009).

Also, photomicrographs obtained by electron microscopy of parasites cultured in the presence of BnSP-7 toxin revealed vacuolization and augmentation of the number of acidocalcisomes, which are membrane-bounded organelles with an electron-dense content, characterized by their acidic nature, high electron density and high content of polyphosphates (polyPs), calcium, magnesium and other elements. They appear to be involved in phosphorus (Pi, PPi and poly P) and cation storage, intracellular pH homeostasis and osmoregulation (Docampo and Moreno, 1999, 2001; Moreno and Docampo, 2009).

Drug-induced ultrastructural damage enables the following interpretations as to the mode of action and cell death pathway in pathogenic protozoa: such changes in myelin figures and autophagosome-like formations are indicative of autophagy; the membrane blebbing effect and chromatin alterations resemble apoptosis; and plasma membrane rupture suggests necrosis (Menna-Barreto *et al.* 2009). In the light of these interpretations and in accordance

with observations presented herein, it can be suggested that the cytotoxic mechanism of BnSP-7 toxin involves the activation of apoptosis in *L. (L.) amazonensis*. However, studies including time-dependent morphology and biochemical assays need to be performed to prove this hypothesis. Moreover, other morphological changes may also suggest an effect of the toxin on the promastigote cell cycle. In any case, the regulation and modulation of death pathways in *Leishmania* sp. are poorly understood (Jiménez-Ruiz *et al.* 2010) and need to be studied further to enable the development of new therapeutic agents and the elucidation of mechanisms involved in the cytotoxic action of different drugs.

Murine infection is one of the best-characterized experimental models for studying *Leishmania*-mammalian host cell interactions (Pereira and Alves, 2008). As to infectivity, it was observed that BnSP-7 toxin interfered with the infective capacity of promastigotes in murine peritoneal macrophages, causing statistically significant reductions at all toxin concentrations tested. Moreover, macrophages infected with BnSP-7-pre-treated promastigotes showed a decreased parasite load, suggesting parasite changes that hampered the intracellular survival in macrophages. In the last 20 years several research groups have studied the mechanism whereby the parasite subverts the host cell response to create an environment favourable for their differentiation into an amastigote form able to persist and divide within acid compartments. In this scope, many molecules are considered to be virulence factors on account of their involvement in the signalling pathways of the macrophage which contribute to influencing macrophage functions and consequently to establishing the infection (Olivier *et al.* 2012). Perhaps the BnSP-7 toxin acts on or prevents synthesis of molecules considered to be virulence factors essential for the infectious process.

The present study showed that BnSP-7 toxin, a phospholipase A₂ isolated from *B. pauloensis* snake venom, presents anti-*Leishmania* activity against promastigote and amastigote forms of *L. (L.) amazonensis*. The toxin is capable of inhibiting promastigote proliferation and promoting drastic changes in parasite morphology. It can also delay the process of differentiating amastigotes into promastigotes. The high toxicity observed for the toxin in mammalian cells prevented assays for testing the action of the toxin in intracellular amastigotes. Thus the action of the toxin in the infectivity process was only evaluated like pre-treatment of the parasite. This study revealed that the toxin also interferes in the infective capacity of the parasite in host cells during the establishment of infection.

In this sense, the findings reported herein are not intended to propose the use of BnSP-7 toxin as a disease treatment, but rather to show that this toxin is an important tool for studies focused on searching for

new targets in the parasite, as well as studies to improve the understanding of *Leishmania* biology. Additional detailed studies must be conducted to better understand the biological and molecular effects of BnSP-7 toxin on *L. (L.) amazonensis*, such as investigation of pathways involved in the death of *L. (L.) amazonensis* promastigotes and proteomic studies aimed at the discovery of new therapeutic targets. Furthermore, future perspectives should include studies of synthetic peptides derived from this toxin in order to ascertain not only targets in the parasite, but also to reveal a possible drug that utilizes the peptide itself or another drug designed from a cytotoxic peptide.

ACKNOWLEDGEMENTS

The authors thank Professors Amélia Hamaguchi and Maria Inês Homs-Brandeburgo for their valuable instruction, encouragement and confidence, and the laboratory technician Marcelo Arantes Levenhagen for his extensive help with electron microscopy. We declare that there is no potential conflict of interest.

REFERENCES

- Arnoult, D., Akarid, K., Grodet, A., Petit, P. X., Estaquier, J. and Ameisen, J. C. (2002). On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrial permeabilization. *Nature Cell Death and Differentiation* **9**, 65–81.
- Barbosa, P. S. F., Martins, A. M. C., Hayt, A., Toyama, O., Evangelista, J. S. M., Ferreira, D. P. P., Joazeiro, P. P., Beriam, L. O. S., Toyama, M. H., Fonteles, M. C. and Monteiro, H. S. A. (2005). Renal and antibacterial effects induced by myotoxin I and II isolated from *Bothrops jararacussu* venom. *Toxicon* **46**, 376–386.
- Basano, S. A. and Camargo, L. M. A. (2004). Leishmaniose tegumentar americana: histórico, epidemiologia e perspectivas de controle. *Revista Brasileira de Epidemiologia* **7**, 328–337.
- BRASIL (2007). Ministério da Saúde. Secretaria de Vigilância em Saúde. *Manual de Vigilância da Leishmaniose Tegumentar Americana*. 2nd Edn. atual. Editora do Ministério da Saúde, Brasília.
- Costa, T. R., Menaldo, D. L., Oliveira, C. Z., Santos-Filho, N. A., Teixeira, S. S., Nomizo, A., Fuly, A. L., Monteiro, M. C., de Souza, B. M., Palma, M. S., Stábeli, R. G., Sampaio, S. V. and Soares, A. M. (2008). Myotoxic phospholipases A₂ isolated from *Bothrops brazili* snake venom and synthetic peptides derived from their C-terminal region: cytotoxic effect on microorganism and tumor cells. *Peptides* **29**, 1645–1656.
- Deolindo, P., Teixeira-Ferreira, A. S., Damatta, R. A. and Alves, E. W. (2010). L-amino acid oxidase activity present in fractions of *Bothrops jararaca* venom is responsible for the induction of programmed cell death in *Trypanosoma cruzi*. *Toxicon* **56**, 944–955.
- Diaz, B. L. and Arm, J. P. (2003). Phospholipase A₂. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **69**, 87–97.
- Docampo, R. and Moreno, S. N. J. (1999). Acidocalcisome: a novel Ca²⁺ storage compartment in trypanosomatids and apicomplexan parasites. *Parasitology Today* **15**, 443–448.
- Docampo, R. and Moreno, S. N. J. (2001). The acidocalcisome. *Molecular and Biochemical Parasitology* **33**, 151–159.
- Fernandez-Gomes, R., Zerrouk, H., Sebt, F., Loyens, M., Benslimane, A. and Ouassii, M. A. (1994). Growth inhibition of *Trypanosoma cruzi* and *Leishmania donovani infantum* by different snake venoms: preliminary identification of proteins from *Cerastes cetrastes* venom which interact with the parasites. *Toxicon* **32**, 875–882.
- García-Almagro, D. (2005). Leishmaniasis cutánea. *Actas Dermosifiliográficas* **96**, 1–24.
- Gonçalves, A. R., Soares, M. J., Souza, W., Damatta, R. A. and Alves, E. W. (2002). Ultra structural alterations and growth inhibition of *Trypanosoma cruzi* and *Leishmania major* induced by *Bothrops jararaca* venom. *Parasitology Research* **88**, 598–602.
- Gontijo, B. and Carvalho, M. L. R. (2003). Leishmaniose tegumentar americana. *Revista da Sociedade Brasileira de Medicina Tropical* **36**, 71–80.
- Granthon, A. C., Braga, M. V., Rodrigues, J. C. F., Cammerer, S., Lorente, S. O., Gilbert, I. H., Urbina, J. A. and Souza, W. (2007). Alterations on the growth and ultrastructure of *Leishmania chagasi* induced by squalene synthase inhibitors. *Veterinary Parasitology* **146**, 25–34.
- Grevelink, S. A. and Lerner, E. A. (1996). Leishmaniasis. *Journal of the American Academy of Dermatology* **34**, 257–272.
- Havens, C. G., Bryant, N., Asher, L., Lamoreaux, L., Perfetto, S., Brendle, J. J. and Werbovetz, K. A. (2000). Cellular effects of leishmanial tubulin inhibitors on *Leishmania donovani*. *Molecular and Biochemical Parasitology* **110**, 223–236.
- Jiménez-Ruiz, A., Alzate, J. F., Macleod, E. T., Lüder, C. G. K., Fasel, N. and Hurd, H. (2010). Apoptotic markers in protozoan parasites. *Parasites and Vectors* **3**, 1–15.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Ledezma, E., Jorquera, A., Bendejú, H., Vivas, J. and Pérez, G. (2002). Antiproliferative and leishmanicidal effect of ajoene on various *Leishmania* species: ultrastructural study. *Parasitology Research* **88**, 748–753.
- Menna-Barreto, R. F. S., Salomão, K., Dantas, A. P., Santa-Rita, R. M., Soares, M. J., Barbosa, H. S. and de Castro, S. L. (2009). Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. *Micron* **40**, 157–168.
- Mitropoulos, P., Konidas, P. and Durkin-Konidas, M. (2010). New World cutaneous leishmaniasis: updated review of current and future diagnosis and treatment. *Journal of the American Academy of Dermatology* **63**, 309–322.
- Moreno, S. N. J. and Docampo, R. (2009). The role of acidocalcisomes in parasitic protists. *Journal of Eukaryotic Microbiology* **56**, 208–213.
- Nakamura, T. U., Mendonça-Filho, R. R., Morgado-Díaz, J. A., Maza, P. K., Dias Filho, B. P., Cortez, D. A. G., Alviano, D. S., Rosa, M. S. S., Lopes, A. H. C. S. and Nakamura, C. V. (2006). Antileishmanial activity of Eugenol rich oil from *Ocimum gratissimum*. *Parasitology International* **55**, 99–105.
- Nevalainen, T. J., Graham, G. G. and Scott, K. F. (2008). Antibacterial actions of secreted phospholipases A₂. Review. *Biochimica et Biophysica Acta* **1781**, 1–9.
- Neto, R. L. M., Sousa, L. M. A., Dias, C. S., Barbosa Filho, J. M., Oliveira, M. R. and Figueiredo, R. C. B. Q. (2011). Morphological and physiological changes in *Leishmania* promastigotes induced by yangambin, a lignan obtained from *Ocotea duckei*. *Experimental Parasitology* **127**, 215–221.
- Olivier, M., Atayde, V. D., Isnard, A., Hassani, K. and Shio, M. T. (2012). *Leishmania* virulence factors: focus on the metalloprotease GP63. *Microbes and Infection* **14**, 1377–1389.
- Paiva, R. M. A., Figueiredo, R. F., Antonucci, G. A., Paiva, H. H., Bianchi, M. L. P., Rodrigues, K. C., Lucarini, R., Caetano, R. C., Pietro, R. C. L. R., Martins, C. H. G., de Albuquerque, S. and Sampaio, S. V. (2011). Cell cycle arrest evidence, parasitocidal and bactericidal properties induced by L-amino acid oxidase from *Bothrops atrox* snake venom. *Biochimie* **93**, 941–947.
- Páramo, L., Lomonte, B., Pizarro-Cerda, J., Bengoechea, J. A., Gorvel, J. P. and Moreno, E. (1998). Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A₂ from *Bothrops asper* snake venom – synthetic Lys49 myotoxin II-(115–129)-peptide identifies its bactericidal region. *European Journal of Biochemistry* **253**, 452–461.
- Paris, C., Loiseau, P. M., Bories, C. and Bréard, J. (2004). Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* **48**, 852–859.
- Passero, L. F. D., Tomokane, T. Y., Corbett, C. E. P., Laurenti, M. D. and Toyama, M. H. (2007). Comparative studies of the anti-leishmanial activity of three *Crotalus durissus* ssp. venoms. *Parasitology Research* **101**, 1365–1371.
- Passero, L. F. D., Laurenti, M. D., Tomokane, T. Y., Corbett, C. E. P. and Toyama, M. H. (2008). The effect of phospholipase A₂ from *Crotalus durissus collilineatus* on *Leishmania (Leishmania) amazonensis* infection. *Parasitology Research* **102**, 1025–1033.
- Peichoto, M. E., Tavares, F. L., Dekrey, G. and Mackessy, S. P. (2011). Comparative study of the effects of venoms from five rear-fanged snake species on the growth of *Leishmania major*: identification of a protein with inhibitory activity against the parasite. *Toxicon* **58**, 28–34.
- Pereira, B. A. and Alves, C. R. (2008). Immunological characteristics of experimental murine infection with *Leishmania (Leishmania) amazonensis*. *Veterinary Parasitology* **58**, 239–255.

- Rodrigues, V. M., Soares, A. M., Mancin, A. C., Fontes, M. R. M., Homs-Brandeburgo, M. I. and Giglio, J. R. (1998). Geographic variations in the composition of myotoxins from *Bothrops neuwiedi* snake venoms: biochemical characterization and biological activity. *Comparative Biochemistry and Physiology Part A* **121**, 215–222.
- Samy, R. P., Gopalakrishnakone, P., Thwin, M. M., Chow, T. K. V., Bow, H., Yap, E. H. and Thong, T. W. J. (2007). Antibacterial activity of snake, scorpion and bee venoms: a comparison with purified venom phospholipase A₂ enzymes. *Journal of Applied Microbiology* **102**, 650–659.
- Santos, A. O., Ueda-Nakamura, T., Dias Filho, B. P., Veiga Junior, V. F., Pinto, A. C. and Nakamura, C. V. (2008). Effect of Brazilian copaiba oils on *Leishmania amazonensis*. *Journal of Ethnopharmacology* **120**, 204–208.
- Soares, A. M., Guerra-Sá, R., Borja-Oliveira, C. R., Rodrigues, V. M., Rodrigues-Simioni, L., Rodrigues, V., Fontes, M. R. M., Lomonte, B., Gutiérrez, J. M. and Giglio, J. R. (2000). Structural and functional characterization of BnSP-7 toxin, a Lys49 myotoxic phospholipase A₂ homologue from *Bothrops neuwiedi pauloensis* venom. *Archives of Biochemistry and Biophysics* **378**, 201–209.
- Stábeli, R. G., Amui, S. F., Sant'ana, C. D., Pires, M. G., Nomizo, A., Monteiro, M. C., Romão, P. R. T., Guerra-Sá, R., Vieira, C. A., Giglio, J. R., Fontes, M. R. M. and Soares, A. M. (2006). *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A₂ homologue: an example of function versatility of snake venom proteins. *Comparative Biochemistry and Physiology, Part C* **142**, 371–381.
- Tanaka, A. K., Valero, V. B., Takahashi, H. K. and Straus, A. H. (2007). Inhibition of *Leishmania (Leishmania) amazonensis* growth and infectivity by aureobasidin A. *Journal of Antimicrobial Chemotherapy* **59**, 487–492.
- Tempone, A. G., Andrade, H. F., Spencer, P. J., Lourenço, C. O., Rogero, J. R. and Nascimento, N. (2001). *Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase. *Biochemical and Biophysical Research Communications* **280**, 620–624.
- Torres, A. F. C., Dantas, R. T., Toyama, M. H., Filho, E. D., Zara, F. J., de Queiroz, M. G. R., Nogueira, N. A. P., de Oliveira, M. R., Toyama, D. O., Monteiro, H. S. A. and Martins, A. M. C. (2010). Antibacterial and antiparasitic effects of *Bothrops marajoensis* venom and its fractions: phospholipase A₂ and L-amino acid oxidase. *Toxicon* **55**, 795–804.
- van Deenen, L. L. M. and de Haas, G. H. (1963). The substrate specificity of phospholipase A₂. *Biochimica et Biophysica Acta* **70**, 538–553.