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Liposomes containing an ASP49-phospholipase A_2 from *Bothrops jararacussu* snake venom as experimental therapy against cutaneous leishmaniasis

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ABSTRACT

The aim of this study was to evaluate *in vitro* anti-*Leishmania amazonensis* activity of a Phospholipase A_2 (Asp49-PLA_2) isolated from the venom of *Bothrops jararacussu* and its encapsulated form. Asp49-PLA_2 (2 mg/mL) was added to a lipid mixture, solubilized in chloroform and dried under nitrogen flow. The lipid vesicles were formed homogeneously using the extrusion method, physicochemically characterized by their diameters, zeta potentials, encapsulation rate and also submitted to a molecular docking *in silico* analysis. The activity of Asp49-liposomes was evaluated *in vitro* against promastigote forms of *L. amazonensis* and J774 macrophages. Parasite and macrophage viabilities using MTT method were assessed after 48 h incubation. *L. amazonensis*-infected macrophages were also incubated with encapsulated Asp49 and in solution form. The amastigote forms were counted inside the infected macrophages and the culture supernatants were collected for nitrites and TNF-α quantifications. Asp49-PLA_2 in solution form displayed an *anti-Leishmania* concentration-dependent effect. Asp49-liposomes were able to reduce 78% of promastigote forms and preserved 82% of J774 macrophages' viability. After 48 h of incubation with nanoencapsulated Asp49-PLA_2 there was a significant reduction in the number of amastigotes (55%; $p < 0.05$) compared to the control group. When the macrophages were infected and incubated with Asp49-liposomes a significant increase in the production of nitrites and TNF-α was observed when compared to infected cells alone. The results indicated that the liposomal system achieved in this study is a promising tool to enhance microbicidal activity of the infected macrophages, conferring a biotechnological therapeutic approach against experimental leishmaniasis.

1. Introduction

Leishmaniases are diseases caused by protozoa of the genus *Leishmania* and clinically appear as visceral, cutaneous, mucocutaneous and diffuse cutaneous forms [1]. *Leishmania amazonensis* is a major etiologic agent of a wide spectrum of clinical forms of leishmaniasis with a wide distribution in the Americas, coinciding with areas of transmission of other species of *Leishmania* [2]. Cutaneous leishmaniasis, the most common form is characterized by limited ulcerative or non-ulcerative skin lesions.

Even with the high incidence of leishmaniasis in Brazil and worldwide, there are few alternatives for treating it, apart from the shortcomings of currently available therapeutic agents, demonstrating the urgent need for new drug candidates for the treatment of this disease [3,4]. Despite global evolution, the first drug of choice for the treatment of leishmaniasis still remains pentavalent antimony, showing severe side effects such as cardiac arrhythmias, nephrotoxicity and hepatotoxicity. In addition, it needs to be administered parenterally, intramuscularly or intravenously, daily, over a period of 20 to 40 days [5,6]. Responses to treatment with pentavalent antimony are variable and may result in a clinical cure, treatment failure or recurrence of the disease after an initial favorable response [7,8].

In order to develop new drug candidates, various proteins isolated from snake venoms, such as phospholipases (svPLAs), matrix metalloproteinases, acidic serineproteases, L-amino oxidases, lectins and peptides have been investigated. These toxins are pure chemical substances that are present in venoms presenting specific action in biological systems [9]. Several studies demonstrate the antimicrobial potential of venoms of neotropical snakes, especially those from the genus *Bothrops* [10]. *Bothrops* toxin-II (BthTX-II) used in this study was isolated from *Bothrops jararacussu* venom, which also displays myotoxic action, whose amino acid sequence was further characterized and it was identified as a basic Asp49-PLA_2 [11]. Despite their toxicity, these toxins are used as efficient drugs in many diseases. Some studies have shown that $svPLA_2$s have biological activity against several species of protozoa

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(*Leishmania* spp., *Trypanosoma* ssp., *Plasmodium* ssp.), bacteria and tumor cells [10,12]. In addition to their primary catalytic function, svPLA$_2$s still have additional pharmacological activities such as anti-inflammatory, anticoagulant, myotoxic, hemolytic, neurotoxic (pre- and post-synaptic), cardiotoxic, bactericidal, anti-HIV, anti-tumor, anti-malarial and anti-parasitic effects [10]. *B. jararacussu* snake venom is able to induce a local inflammatory response [13] but no study was reported for the proinflammatory activity of the Asp49-PLA$_2$.

Regarding the nanobiotechnological approach for chemotherapy, liposomes have been investigated from various types of colloidal carriers, ranging from 50 nm to 500 nm. These nanostructures are able to encapsulate both hydrophilic and hydrophobic drugs [14]. These vesicular systems represent a more efficient therapy for certain diseases, such as for the treatment of leishmaniasis employing liposomal amphotericin B. In this context, liposomes control the release of the drug by increasing its pharmacokinetics to the tissue, reducing drug toxicity [15].

In this study, the developed liposomes containing an Asp49-PLA$_2$ from the snake *B. jararacussu* displayed relevant anti-*L. amazonensis* activity *in vitro*. The results achieved may contribute to the advancement in the search for new prototypes of drugs for the therapy of leishmaniasis, which still represents a major public health problem, especially in developing countries.

2. Materials and methods

2.1. Asp49-solid dilution and characterization of the liposomes

Asp49-solid (2 mg) was diluted in 1 mL PBS pH 7.4. This solution (2 mg/mL) was used to resuspend the lyophilized lipids for liposome preparation. Liposomes were prepared as described in the literature with modifications [16]. The lipids used were dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS) and cholesterol (all reagents from Sigma-Aldrich) at ratio 5:1:3 (w/w/w). They were dissolved in a glass tube with 2 mL chloroform/methanol (Dinamica, São Paulo, Brazil) (1:1, v/v). Then, the tube was vortexed and subjected to an ultrasonic bath repeatedly until the lipids dissolved completely. After the dissolution of lipids, a solution of chloroform/methanol was removed by evaporation, yielding a film of lipids on the inner wall of the tube. This film was vacuum dried in a desiccator for about 18 h and subsequently the lipids were resuspended in 2 mL of PBS 1×, pH 7.4. The solution was then incubated for 1 h at 57 °C with agitation every 10 min. The emulsion was subjected to an extruder (LiposoFastTM, Avestin, Ottawa, Canada) with a polycarbonate membrane with pores of 100–200 nm 20 times to produce a population of liposomes of uniform size. This unloaded liposome was called the liposome control. For the preparation of liposomes containing the PLA$_2$ (Asp49-liposomes), 1 mL (2 mg/mL) of the toxin in solution was added to the lipids' solution, at the beginning of the preparation.

The mean particle size and the zeta potential were determined by dynamic light scattering (using volume distribution) and laser-Doppler anemometry, respectively, in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), equipped with a 173° scattering angle.

The measurement of liposomes was carried out at 25 °C after appropriate dilution of the samples in water. The size distribution of the particles was represented by the polydispersity index (PI). For the measurement of the zeta potential, the liposome samples were placed in a specific cell, where a potential of ±150 mV was established. The values obtained were calculated from the mean electrophoretic mobility using Smoluchowski's equation.

2.2. Molecular docking

The molecular docking is a method, which provides for a preferred orientation to a second molecule when connected together to form a stable complex. The knowledge of preferred orientation can be used to predict the strength of association or binding affinity between molecules. These parameters become a useful tool to be employed for the *in silico* analysis between Asp49-PLA2 (BthTX-II) and the lipids used to prepare the liposomes. The Protein Data Bank (PDB) (http://www.pdb.org) was used to search and retrieve the structure of the protein. The selected target was the toxin's structure deposited in PDB (ID: 3JR8). To identify potential binding sites of compounds, we performed an automated molecular-docking procedure using the SwissDock program [17,18]. The docking was performed using the "Accurate" parameter, with no region of interest defined (blind docking). The structures of the compounds were extracted from the database ChEB (https://www.ebi.ac.uk/chebi/init.do) and Pubchem (https://pubchem.ncbi.nlm.nih.gov/). Interactive visualization, analysis and conversions of molecular structures were carried out in Swiss-PDB viewer [19] and UCSF Chimera [20] and structure images were created with Persistence of Vision Raytracer (POV-ray) 3.62 (http://www.povray.org).

2.3. Quantification of Asp49-PLA$_2$ from the liposomes

To determine the encapsulation rate of Asp49-PLA$_2$ in liposomes the method described was used [21]. The protein concentration in the samples was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). Asp49-solid (2 mg) was diluted in 1 mL PBS pH 7.4 and Asp49-solution (2 mg/mL) was used to be encapsulated (theoretical concentration). In order to quantify the concentration of encapsulated Asp49-PLA$_2$, Triton X-100® (Sigma-Aldrich, Chemical Co., St. Louis, MO, USA) was added to break the vesicles under centrifugation. A standard concentration curve was obtained with bovine serum albumin (BSA, Thermo Fisher Scientific, MA, USA). The absorbance values were performed using a spectrophotometer (Biotek®) at 595 nm.

2.4. Determination of the IC50 and selectivity index (SI)

The IC50 and SI values were obtained employing promastigotes of *L. amazonensis* (5×10^5/well) and J774A.1 macrophages (2.5×10^5/well) cell line (ATCC®TIB-67™) incubated with serial dilutions (ranging from 100 to 6.25 μg/mL) of PLA$_2$ (Asp49-solution), control liposomes and Asp49-liposomes. The MTT assay was used to measure cytotoxicity. The calculations were determined using the mean absorbance (OD) of the controls and the values expressed as a percentage according to the formula below:

$$\%inibition = 1 - \frac{D.O.sample}{D.O.control} \times 100$$

The SI values were obtained using the formula = LC50 (macrophages) ÷ IC50 (promastigotes) calculated using Probit analysis (MINITAB Release 14.1; Minitab Inc. 2003 Statistical Software).

2.5. Animals and parasites

BALB/c mice, male, 10–12 weeks old, weighing 20–30 g, were obtained from the Fundação Oswaldo Cruz – RO, Brazil facility. The animals were kept under standardized animal house conditions. The parasites, maintained in BALB/c mice, were isolated and expanded in RPMI-1640 medium (Sigma Chemical ™ Co., St. Louis, MO, USA) supplemented with Fetal Bovine Serum (Thermo Fisher Scientific, MA, USA). 10% (v/v) heat-inactivated calf serum, 0.25 mM HEPES, 5 mg/mL of gentamicin, and maintained at 24 °C. The experiments were performed in accordance with standards established by the Ethics Committee on Animal Use upon approval of the research project under no. 2013/1.

2.6. In vitro infection and treatments of peritoneal macrophages

The methodology employed to obtain mice peritoneal macrophages was described [16,22]. BALB/c mice were i.p. inoculated with 2 mL of 3% (w/v) thioglycolate (Sigma-Aldrich). After 5 days, the animals were