

In vitro activity of Surfactant Protein A against *Leishmania amazonensis*

Yuliannis Lugones¹, Odalys Blanco¹, Roberto Faure¹, ✉ Lianet Monzote²

¹Grupo de Química-Farmacología-Toxicología, Centro Nacional de Sanidad Agropecuaria, CENSA Mayabeque, Cuba

²Departamento de Parasitología, Instituto de Medicina Tropical Pedro Kourí, IPK La Habana, Cuba

E-mail: monzote@ipk.sld.cu.

ABSTRACT

Surfactant protein A (SP-A) is a hydrophilic pulmonary protein belonging to the collectin family, involved in lung homeostasis, the regulation of host defenses and inflammation. The present study addresses the activity of SP-A against promastigotes and amastigotes of *Leishmania amazonensis* and its cytotoxicity for host cells. SP-A exhibited similar activity against both parasite forms, with an IC₅₀ of 34.0 ± 3.1 and 33.6 ± 1.1 $\mu\text{g/mL}$ against promastigotes and amastigotes, respectively. It was moderately cytotoxic to peritoneal macrophages from BALB/c mice, exhibiting an IC₅₀ value of 172.0 ± 6.1 $\mu\text{g/mL}$. This is the first report on the anti-leishmanial activity of SP-A.

Keywords: *Leishmania amazonensis*, Pulmonary surfactant; Antimicrobial, SP-A

Biotechnología Aplicada 2012;29:35-37

RESUMEN

Actividad in vitro de la proteína A del surfactante pulmonar frente a *Leishmania amazonensis*. La proteína surfactante A (SP-A), es una proteína pulmonar hidrofílica, de la familia de las colectinas, que contribuye al mantenimiento de la homeostasis en el pulmón y la regulación de los mecanismos de defensa e inflamación. En este estudio se reporta la actividad de la SP-A frente a *Leishmania amazonensis* en su forma promastigote y amastigote. La SP-A muestra actividad similar contra ambas formas del parásito, con un valor de CI₅₀ de 34.0 ± 3.1 y 33.6 ± 1.1 $\mu\text{g/mL}$ frente a promastigotes y amastigotes, respectivamente. La proteína causó una citotoxicidad moderada en macrófagos peritoneales procedentes de ratones BALB/c, con un valor de CI₅₀ de 172.0 ± 6.1 $\mu\text{g/mL}$. Este es el primer reporte de la actividad anti-leishmanial de la SP-A.

Palabras clave: *Leishmania amazonensis*, surfactante pulmonar, antimicrobiano, SP-A

Introduction

Pulmonary surfactant is a mixture of phospholipids and proteins that reduces surface tension at the air-liquid interface of the alveolus, preventing alveolar collapse and reducing breathing work [1]. Four surfactant protein (SP) have been described four classes: SP-A, SP-B, SP-C and SP-D [2-3]. SP-B and SP-C are extremely hydrophobic proteins largely responsible, together with phospholipids, for the biophysical properties of pulmonary surfactant. Phospholipids and hydrophobic proteins are constituents of all available exogenous surfactant preparations, including Surfacten®, a formulation developed in Cuba to treat the Newborn Respiratory Distress Syndrome [4]. On the other hand, SP-A and SP-D are hydrophilic, oligomeric glycoproteins belonging to the collectin family that play a role in maintaining lung homeostasis and regulating host defenses and inflammation. None of the existing exogenous surfactant preparations developed for clinical use have SP-A or SP-D among its constituents [5].

Our group has been conducting research on the antioxidant properties of porcine SP-A [6-7]. This molecule is able to specifically bind many pathogens, aggregating and opsonizing a variety of microorganisms (bacteria, fungi and virus). Moreover, it is involved in the regulation of host defenses by modulating the production, in macrophages, of cytokines and oxidant compounds, which enhance the intracellular killing of mycobacteria and other microbes [5, 8, 9]. The aim of

the present study, therefore, was to assess the potential anti-protozoal activity of porcine SP-A, using the extracellular and intracellular forms of *Leishmania* as model targets and evaluating, in addition, its cytotoxicity towards host macrophages.

Materials and methods

Strain, protein and parasites

Strain MHOM/77BR/LTB0016 of *Leishmania amazonensis* was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation, Brazil. The parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26 °C in Schneider's medium (Sigma Chem Co, St. Louis, Mo, US), containing 10% heat inactivated fetal bovine serum (Sigma), penicillin 200 U/mL and streptomycin 200 g/mL (Sigma). Parasites were not used after the fifth passage.

SP-A was isolated as reported by Blanco *et al.* [10], resuspending the lyophilized protein at 20 mg/mL in distilled water. Meglumine antimoniate (Sb^{III}) (Glucantime®) obtained from Rhône-Poulenc Rorer, Mexico, was used as reference drug.

Anti-promastigote activity

Eleven different concentrations of SP-A and Glucantime® were assayed in quadruplicate. Exponentially growing cells (promastigotes 10⁵/mL,

1. Wright JR. Pulmonary surfactant: a front line of lung host defense. *J Clin Invest.* 2003;111(10):1453-5.

2. Creuwels LA, van Golde LM, Haagsman HP. The pulmonary surfactant system: biochemical and clinical aspects. *Lung.* 1997;175(1):1-39.

3. Possmayer F. A proposed nomenclature for pulmonary surfactant-associated proteins. *Am Rev Respir Dis.* 1988;138(4):990-8.

4. Manzanares DA, Díaz E, Alfonso WC, Escobar AC, Colomé H, Muñoz MC, *et al.*, inventors; Centro Nacional de Sanidad Agropecuaria, assignee. Surfactante Pulmonar Natural Porcino. Cuban Patent; CU 22479 A1. 1994 Dec 29.

5. Awasthi S. Surfactant protein (SP)-A and SP-D as antimicrobial and immunotherapeutic agents. *Recent Pat Antiinfect Drug Discov.* 2010;5(2):115-23.

6. Blanco O, Catala A. Surfactant protein A inhibits the non-enzymatic lipid peroxidation of porcine lung surfactant. Prostaglandins Leukot Essent Fatty Acids. 2001;65(4):185-90.

7. Sanchez J, Blanco O, Martínez G, De Armas E, Fernández O, Faure R. La proteína A del surfactante porcino (SP-A) exhibe propiedades antioxidantes en el sistema Fenton. *Rev Mex Cienc Farm.* 2003;34(3):19-23.

199 $\mu\text{L}/\text{well}$) were added to 96-well plates, then adding one microliter of each product concentration to each well, to final concentrations ranging 0.2–200 $\mu\text{g}/\text{mL}$ in a final volume of 200 μL . Plates were incubated at 26 °C during 72 h. A distilled water control was included in each experiment. After 3 days of exposure, the parasites were incubated for 3 h with p-nitrophenyl phosphate (20 mg/mL) dissolved in 1 M Sodium acetate buffer (BDH, Poole, England), pH 5.5, with 1% Triton X-100 (BDH, Poole, England) at 37 °C. Absorbance was determined in an EMS Reader MF Version 2.4-0, at a wavelength of 405 nm [11], taking as endpoint an absorbance below 3.0.

Anti-amastigote activity

Macrophages were harvested from the peritoneal cavities of normal BALB/c mice in RPMI medium (Sigma). A volume of 1 mL (10^6 cells) was plated and incubated at 37 °C under a 5% CO_2 atmosphere for 2 h. After removing non-adherent cells, stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio. The cultures were incubated for 4 h, washed to remove free parasites, and then 10 μL of different drug concentrations were added to obtain final concentrations from 6.25 to 50 $\mu\text{g}/\text{mL}$, in duplicate, for 48 h. Control cultures that were treated only with 10 μL of distilled water were also included. The cultures were fixed afterwards with absolute methanol, stained with Giemsa, and examined by light microscopy [12]. The number of intracellular amastigotes and the percentage of infected macrophages every 100 macrophages were determined, and infection rates by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophage. Results were expressed as percentage reductions of infection rate (% IR) compared to that of the controls, estimating IC_{50} by lineal regression [13].

Cytotoxicity assay

The cytotoxicity of the compound was determined on mouse peritoneal macrophages, as host cells for the amastigote form of the parasite. Peritoneal macrophage monolayers were treated with concentrations of 0.001 to 200 $\mu\text{g}/\text{mL}$ for 48 h, estimating their viability afterwards with a colorimetric assay based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA). MTT solutions were prepared at 5 mg/mL and added to each well, and after incubation for 3 h, the obtained formazan crystals were dissolved by adding 100 μL of DMSO. Viability was then estimated by measuring the resulting optical density with an EMS Reader MF Version 2.4-0, using test and reference wavelengths of 560 nm and 630 nm, respectively [14].

Results and discussion

Leishmaniasis is caused by parasites of the *Leishmania* genus, affecting more than 12 million of persons in 88 countries [16]. This disease remains a serious problem. As a zoonotic infection, transmission is difficult

to interrupt, although some attempts to reduce vector and mammalian reservoir populations have been successful. In addition, there are currently no vaccines against leishmaniasis. The available drugs are toxic, expensive and frequently ineffective [16]. These problems underscore the importance of developing new, effective drugs against this pathogen [17].

Previous experiments have uncovered no evidence of anti-protozoal activity for SP-A. However, the surfactant preparation Surfacten® has earlier been shown to have anti-leishmanial activity [18]; a result suggesting that surfactant proteins might have some potential against protozoal parasites, and particularly against *Leishmania*. The Tropical Diseases Program of the World Health Organization currently places a high priority on research into natural products for the treatment of *Leishmania*, as these offer an unlimited source of chemical diversity with potential biological activities [19].

In this report, SP-A showed activity against the promastigote and amastigote forms of *L. amazonensis* and moderate cytotoxicity against host cells (Table), requiring 5-fold higher concentrations in the latter case to reach the levels of cell death it exhibited against parasites. SP-A was less active than glucantime. It should be noted, however, that the anti-parasite toxicity of pentostam, another first-line option derivative from pentavalent antimonial, is similar to that of SP-A ($\text{IC}_{50} = 30 \mu\text{g}/\text{mL}$) [20].

We do not know a mechanism for SP-A-mediated death of *Leishmania* amastigotes and promastigotes, as described here. SP-A is a very versatile molecule, first shown to be an antimicrobial protein in 2003 in experiments where it directly inhibited the proliferation of Gram-negative bacteria in a macrophage- and aggregation-independent manner by increasing the permeability of the microbial cell membrane [21]. More recent reports have extended its anti-microbial range to *Mycoplasma pneumoniae* [22] and *Histoplasma capsulatum* [23]. Although nothing is known about its permeabilization mechanism(s), the crystal structure of SP-A reveals a hydrophobic cleft lined by charged residues which may play a role in membrane perturbation [9]. The anti-inflammatory and antimicrobial characteristics of SP-A [8, 21] turn this molecule into an attractive candidate for designing therapeutic agents.

In summary, this is the first report about the anti-leishmanial activity of SP-A. The results described here suggest that SP-A can be applied to design new formulations against the *Leishmania* parasite.

Table. Anti-leishmanial activity and cytotoxicity of the SP-A and glucantime

Tested Products	$\text{IC}_{50} \pm \text{SD} (\mu\text{g}/\text{mL})$		Peritoneal Macrophage
	Promastigote Parasite	Amastigote Parasite	
SP-A	34.0 ± 3.1	33.6 ± 1.1	172.0 ± 6.1
Glucantime	> 1500	11.0 ± 3.4	> 1500

IC_{50} : Concentration of drug causing 50% of mortality.
SD: Standard deviation.

8. Sano H, Kuroki Y. The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity. *Mol Immunol.* 2005;42(3):279-87.

9. McCormack FX. New concepts in collectin-mediated host defense at the air-liquid interface of the lung. *Respirology.* 2006;11 Suppl 1:S7-10.

10. Blanco O, Sanchez Y, Manzanares D, Fernández O. Isolation of surfactant protein A, SP-A, from bronchoalveolar porcine lavage. *Rev Salud Animal.* 2000;22(3):180-2.

11. Bodley AL, McGarry MW, Shapiro TA. Drug cytotoxicity assay for African trypanosomes and Leishmania species. *J Infect Dis.* 1995;172(4):1157-9.
12. Torres-Santos EC, Moreira DL, Kaplan MA, Meirelles MN, Rossi-Bergmann B. Selective effect of 2',6'-dihydroxy-4'-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. *Antimicrob Agents Chemother.* 1999;43(5):1234-41.
13. Delorenzi JC, Attias M, Gattass CR, Andrade M, Rezende C, da Cunha Pinto A, *et al.* Antileishmanial activity of an indole alkaloid from *Peschiera australis*. *Antimicrob Agents Chemother.* 2001;45(5):1349-54.
14. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. *J Immunol Methods.* 1993;157(1-2):203-7.
15. Desjeux P. The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg.* 2001;95(3):239-43.
16. Herwaldt BL. Leishmaniasis. *Lancet.* 1999;354(9185):1191-9.
17. Croft SL, Yardley V. Chemotherapy of leishmaniasis. *Curr Pharm Des.* 2002;8(4):319-42.
18. Blanco O, Lugones Y, Diaz E, Monzote L. *In vitro* activity of the clinical pulmonary surfactant Surfacten(R) against *Leishmania amazonensis*. *Rev Inst Med Trop Sao Paulo.* 2011;53(4):235-8.
19. Chan-Bacab MJ, Pena-Rodriguez LM. Plant natural products with leishmanicidal activity. *Nat Prod Rep.* 2001;18(6):674-88.
20. Callahan HL, Portal AC, Devereaux R, Grogg M. An axenic amastigote system for drug screening. *Antimicrob Agents Chemother.* 1997;41(4):818-22.
21. Wu H, Kuzmenko A, Wan S, Schaffer L, Weiss A, Fisher JH, *et al.* Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability. *J Clin Invest.* 2003;111(10):1589-602.
22. Ledford JG, Goto H, Potts EN, Degan S, Chu HW, Voelker DR, *et al.* SP-A preserves airway homeostasis during *Mycoplasma pneumoniae* infection in mice. *J Immunol.* 2009;182(12):7818-27.
23. McCormack FX, Gibbons R, Ward SR, Kuzmenko A, Wu H, Deepe GS, Jr. Macrophage-independent fungicidal action of the pulmonary collectins. *J Biol Chem.* 2003;278(38):36250-6.