Rev. Inst. Med. Trop. Sao Paulo
53(4):235-238, July-August, 2011
doi: 10.1590/S0036-46652011000400012

TECHNICAL REPORT

In vitro ACTIVITY OF THE CLINICAL PULMONARY SURFACTANT SURFACEN® AGAINST *Leishmania amazonensis*

Odalys BLANCO(1), Yuliannis LUGONES(1), Elaine DÍAZ(1) & Lianet MONZOTE(2)

SUMMARY

Surfacen® is an exogenous natural lung surfactant, composed by phospholipids and hydrophobic proteins, which is applied successfully in Newborn Respiratory Distress Syndrome. In this paper, *in vitro* activity of Surfacen® against *Leishmania amazonensis* is described. The product showed activity against the amastigote form found in peritoneal macrophages from BALB/c mice, with an IC_{50} value of 17.9 ± 3.0 µg/mL; while no toxic effect on host cell was observed up to 200 µg/mL. This is the first report about the antileishmanial activity of Surfacen®.

KEYWORDS: *Leishmania amazonensis;* Pulmonary surfactant; Antibiotic activity; Antimicrobial, SP-B.

Leishmaniasis refers to a spectrum of diseases ranging from self-healing cutaneous lesion to debilitating mucocutaneous and lethal visceral infections. It is estimated that more than 12 million people are affected by leishmaniasis, and 350 million people are at risk, making *Leishmania* one of the most important parasitic diseases. Since there is no antileishmanial vaccine in clinical use, control of leishmaniasis relies almost exclusively on chemotherapy. Treatment of leishmaniasis is still complicated, due to the occurrence of different causative species and various clinical manifestations. For almost seven decades, pentavalent antimonials constituted the standard antileishmanial treatment worldwide. However, in the last 15 years, their clinical value has been jeopardized due to the widespread emergence of resistance[10].

In recent years, there has been a growing interest in alternative therapies such as the use of natural products[6]. Surfacen® is an exogenous natural lung surfactant, which has been applied successfully in Newborn Respiratory Distress Syndrome since 1990. The action mechanism of the exogenous surfactants is by replacing the missing endogenous surfactants. They accomplish this by reducing alveolar surface tension, increasing lung compliance, and stabilizing the alveoli to prevent atelectasis at end expiration[8]. Besides its property, the lung surfactant also plays a major role in the pulmonary defense, preventing the access of pathogens through the large alveolar surface exposed to the environment[16].

Recently, the antimicrobial effect of Surfacen® has been demonstrated against infectious agents; particularly as an antibacterial agent[3]. The aim of the present study was to assess the potential antileishmanial activity of Surfacen®. In addition, cytotoxicity in macrophage was tested.

The strain of *L. amazonensis* (MHOM/77BR/LTB0016) 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 ºC in Schneider's medium (Sigma Chem Co, St. Louis, Mo, USA), containing 10% heat inactivated fetal bovine serum (Sigma), 200 U penicillin/mL and 200 g streptomycin/mL (Sigma). The parasites were not used after the fifth passage.

Surfacen® was supplied by CENSA (Havana, Cuba). The preparation was suspended at 20 mg/mL of phospholipids in distilled water. Meglumine antimoniate (Sb^V) (Glucantime®), obtained from the Rhône-Poulenc Rorer, Mexico, was used as a reference drug.

Antipromastigote activity: Eleven concentrations of the products were assayed in quadruplicate. Exponentially growing cells (10^5 promastigotes/mL, 199 µL) were distributed in 96-well plates. One microliter of each concentration of the product was added between 0.2 and 200 µg/mL, to a final volume of 200 µL and then incubated at 26 ºC for 72 h. A distilled water control was included in each experiment. After three days of exposure, the parasite was incubated with p-nitrophenyl phosphate (20 mg/mL) dissolved in buffer of sodium acetate 1M (BDH, Poole, England), pH 5.5, with 1% Triton X-100 (BDH, Poole, England) at 37 ºC for 3h. The absorbance was determined in an EMS Reader MF Version 2.4-0, at a wavelength of 405 nm[4]. The endpoint was considered as the absorbance below of 3.0.

(1) Grupo de Química-Farmacología-Toxicología, Centro Nacional de Sanidad Agropecuaria (CENSA), Mayabeque, Cuba.
(2) Departamento de Parasitología, Instituto de Medicina Tropical "Pedro Kourí", Ciudad de la Habana, Cuba.
Correspondence to: Lianet Monzote. Departamento de Parasitología, Instituto de Medicina Tropical "Pedro Kourí", Apartado Postal 601, Marianao 13, Habana, Cuba. Tel.: 53.7.202 5061; fax: 53.7.204 6051. E-mail: monzote@ipk.sld.cu

Antiamastigote activity: Macrophages were harvested from peritoneal cavities of normal BALB/c mice in RPMI medium (Sigma). A volume of one mL of cells were plated at 10^6/mL and incubated at 37 ºC under an atmosphere of 5% CO_2 for two hours. Non-adherent cells were removed and stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio. The cultures were incubated for four hours, washed to remove free parasites and 10 µL of different drug concentrations were added between 6.25 and 50 µg/mL, in duplicate, for 48 h. Control cultures treated with 10 µL of distilled water were included. The cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy[15]. The number of intracellular amastigotes and the percentage of infected macrophage were determined in 100 macrophages. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages. The results were expressed as percent of reduction of the infection rate (%IR) in comparison with that of the controls and the IC_{50} was determined by lineal regression[7].

Cytotoxicity assay: The cytotoxicity of the compound was determined on mouse peritoneal macrophages, which are the host cells for the amastigote form of the parasite. The monocell layer of peritoneal macrophage was treated at concentrations between 0.2 and 200 µg/mL for 48 hours. The viability was determined using the colorimetric assay with 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. After an additional three hours incubation, the formazan crystals were dissolved by adding 100 µL DMSO. The optical density was determined using an EMS Reader MF, Version 2.4-0, at a test wavelength of 560 nm and a reference wavelength of 630 nm[14].

The results were compared by Mann-Whitney test using the Statistical for Windows Program (Release 4.5, StatSoft, Inc. 1993). Statistical differences were considered when $p < 0.05$.

Surfacen® showed activity against intracellular amastigotes forms, inhibiting the percent of infected macrophage and the average number of amastigotes per macrophage (Fig. 1A). The IC_{50} value against parasite was of 17.9 ± 3.0 µg/mL; while no toxic effect on mice macrophage

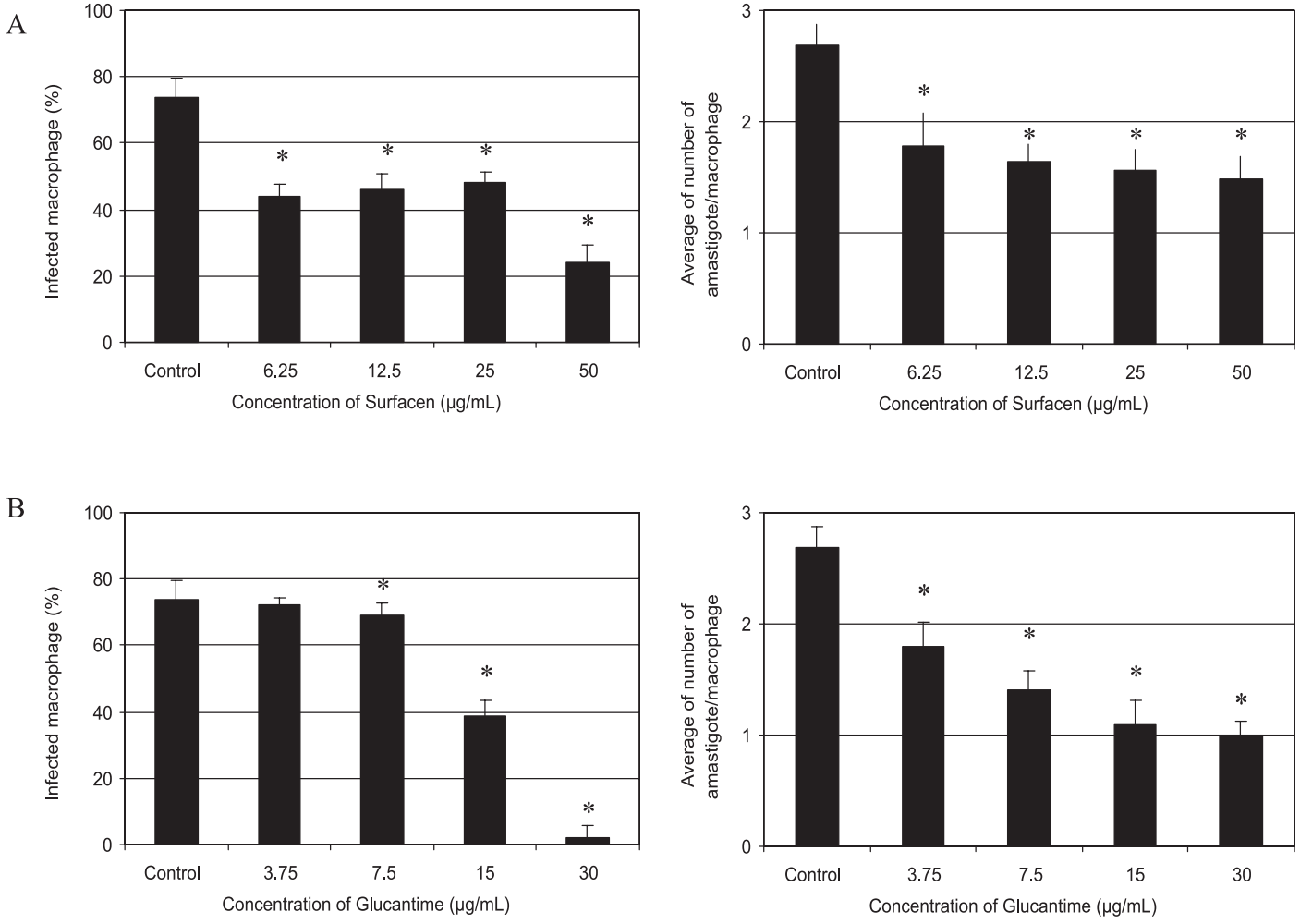

Fig. 1 - Percent of infected macrophage and the average number of amastigotes per macrophage in treated *L. amazonensis*-infected mouse peritoneal macrophage. Results are from three experiments in duplicate and are shown as percentages and standard deviations. **A:** treatment with Surfacen®; **B:** treatment with Glucantime; *: Statistical differences compared with untreated cells ($p < 0.05$).

was observed at the highest concentration evaluated (200 µg/mL). The product showed a similar activity ($p > 0.05$) to a drug used as first line, glucantime, which showed an IC_{50} value of 11.0 ± 3.4 µg/mL (Fig. 1B). Similar results are found with other drugs used in clinical studies such as posaconazole, which has decreased the survival of *L. amazonensis* amastigotes in 50 % at 16 µg/mL[1].

This surfactant did not cause inhibitory activity on promastigote forms of the parasite (results not shown). The specific activity observed on the intracellular form could be a consequence of the direct action of the compounds on probable specific targets on the amastigote, or an interference with defense mechanisms of the amastigote within the hostile environment of the macrophage. The pentavalent antimonials, drugs of the first line, do not show activity against the promastigotes, but they are active against the amastigote form, because the drug is transformed into the active compound, the trivalent antimonials, in the host cell[13].

The mechanism by which Surfacen® kills *Leishmania* is still unknown. Surfacen®, as another clinical lung surfactant, is a complex mixture composed by phospholipids and specific proteins, called surfactant proteins (SP) B and C. It has been suggested that SP-B has important and significant homologies with some eukaryotic antibiotic peptides and, in fact, it has been demonstrated that a synthetic SP-B-like peptide inhibits the growth of *Escherichia coli* cultures[9]. Recently, it has also been demonstrated that SP-B and SP-B-related peptides induce aggregation and death of bacteria in clinical isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Staphylococcus aureus* and group B *Streptococcus*. It was related that this protein promoted increase of the permeability of bacterial membranes[12]. These authors showed that antimicrobial activity of SP-B was inhibited by surfactant phospholipids; however, Curosurf, a clinical pulmonary surfactant with similar composition to Surfacen®, was bactericidal to group B streptococcal and *E. coli* strains[5]. Additionally, several virulence properties of GBS®-h/c can be blocked by dipalmitoyl phosphatidylcholine (DPPC), the major phospholipid component of the pulmonary surfactant[11]. SP-B is a member of the saposin-like proteins (SAPLIP), which include potent antimicrobial peptides such as Nk-lysin, a peptide with antimicrobial and cytolitic activities. All SAPLIPs interact with lipids and have membranolytic activity[2,12]. Recently, it was demonstrated the presence of a proSPB-derived saposin-like protein with antimicrobial activity at acidic pH in the lung surfactant. It was proposed to stimulate defense actions by macrophages[17]. All these findings could support the antipathogenic actions found in Surfacen®.

In conclusion, this is the first report about the antileishmanial activity of Surfacen®. Its effects and low toxicity suggest that this product could be explored in the design of new molecules against *Leishmania* parasite. Further experiments must be performed in animal models to corroborate the potential antileishmanial activity of Surfacen®.

RESUMEN

Actividad *in vitro* de surfactante pulmonar clínico Surfacen® frente a *Leishmania amazonensis*

Surfacen® es un surfactante natural exógeno extraído del pulmón, formado por fosfolípidos y proteínas hidrofóbicas, el cual es aplicado con éxito en el Síndrome de Distrés Respiratorio en Niños Recién Nacidos. En este trabajo, se describe la actividad *in vitro* del Surfacen® contra *Leishmania amazonensis*. El producto mostró actividad frente a amastigotes que se encuentran en macrófagos peritoneales de ratón BALB/c, con una CI_{50} de 17.9 ± 3.0 µg/mL, mientras no se observaron efectos tóxicos sobre la célula hospedera hasta 200 µg/mL. Este estudio constituye el primer reporte sobre la actividad antileishmania del Surfacen®.

ACKNOWLEDGEMENTS

We thank the assistance of Dr. Eduardo Sistachs in the revision of language.

REFERENCES

1. Al-Abdely HM, Graybill JR, Loebenberg D, Melby PC. Efficacy of the triazole SCH 56592 against *Leishmania amazonensis* and *Leishmania donovani* in experimental murine cutaneous and visceral leishmaniases. Antimicrob Agents Chemother. 1999;43:2910-4.

2. Andersson M, Curstedt T, Jornvall H, Johansson J. An amphipathic helical motif common to tumourolytic polypeptide NK-lysin and pulmonary surfactant polypeptide SP-B. FEBS Lett. 1995;362**:**328-32.

3. Blanco O, Riverón Y, de Armas E, Sánchez J, Faure R, Fernández O. SURFACEN® inhibe el crecimiento de bacterias causantes de infecciones respiratorias. Biotecnol Apl. 2005;22:279-81.

4. Bodley AL, McGarry MW, Shapiro TA. Drug cytotoxicity assay for African Trypanosomes and *Leishmania s*pecies. J Infect Dis. 1995;172:1157-9.

5. Bouhafs RK, Jarstrand C. Lipid peroxidation of lung surfactant by bacteria. Lung. 1999;177:101-10.

6. Brenzan MA, Nakamura CV, Prado Diaz Filho B, Ueda-Nakamura T, Young MC, Aparício Garcia Cortez D. Antileishmanial activity of crude extract and coumarin from *Calophyllum brasiliense* leaves against *Leishmania amazonensis*. Parasitol Res. 2007;101:715-22.

7. Delorenzi JC, Attias M, Gattass CR, Andrade M, Rezende C, Pinto AC, *et al.* Antileishmanial activity of an indole alkaloid from *Peschiera australis*. Antimicrob Agents Chemother. 2001;45:1349-54.

8. Goerke J. Pulmonary surfactant: functions and molecular composition. Biochim Biophys Acta. 1998;1408:79-89.

9. Kaser MR, Skouteris GG. Inhibition of bacterial growth by synthetic SP-B1-78 peptides. Peptides. 1997;18:1441-4.

10. Maltezou HC. Drug resistance in visceral leishmaniasis. J Biomed Biotech. 2010; 2010 (ID 617521). doi:10.1155/2010/617521.

11. Nizet V. Streptococcal B-hemolysins: genetics and role in disease pathogenesis. Trends Microbiol. 2002;10:575-80.

12. Ryan MA, Akinbi HT, Serrano AG, Perez-Gil J, Wu H, McCornack FX, *et al.* Antimicrobial activity of native and synthetic surfactant protein B peptides. J Immunol. 2006;176:416-25.

13. Singh N, Singh RT, Sundar S. Novel mechanism of drug resistance in kala azar field isolates. J Infect Dis. 2003;188:600-7.

14. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods. 1993;157:203-7.

15. 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;43:1234-41.

16. Wright JR. Pulmonary surfactant: a front line of lung host defense. J Clin Invest. 2003;111:1453-5.

17. Yang L, Johansson J, Ridsdale R, Willander H, Fitzen M, Akinbi HT, *et al*. Surfactant protein B propeptide contains a saposin-like protein domain with antimicrobial activity at low pH. J Immunol. 2010;184:975-83.

Received: 3 September 2010
Accepted: 1 June 2011