Nanoformulations of pentavalent antimony entrapped in phosphatidylserine-liposomes demonstrate highest efficacy against Experimental Visceral Leishmaniasis

Nanoformulações de antimônio pentavalente encapsuladas em lipossomos contendo fosfatidilserina demonstram maior eficácia contra Leishmaniose Visceral experimental

RIALA6/1165

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Recebido: 07/07/2008 – Aceito para publicação: 15/08/2008

ABSTRACT

Leishmaniasis is an endemic and tropical disease that afflicts mainly the developing countries. The limited and highly toxic therapeutic arsenal for leishmaniasis treatment still remains on antimony salts. The second line drugs as amphotericin B and pentamidine are also toxic**,** and no novel drug is available against *Leishmania* spp. Liposomes are effective drug delivery systems which can deliver high amounts of entrapped drugs to target cells. In this study, a strategic liposome formulation was developed in order to deliver the pentavalent antimony to *Leishmania*-infected macrophages through the *in vivo* interaction with scavenger receptors. Antimony-entrapped liposomes demonstrated a high efficacy *in vivo* reducing 133-fold the total antimony dose, with a 100% decrease in the liver parasite burden at 0.75 mg/kg dose. By transmission electron microscopy a stable formulation composed by oligolamellar vesicles with 0.2 μm was demonstrated. Zeta potential studies showed a negative charge attached to the external membrane of liposomes due to phosphatidylserine addition. This novel approach contributes to the study on novel liposomal formulations for reducing the toxic effects of drugs in Leishmaniasis therapy.

Key words. leishmania, therapy, liposomes, phosphatidylserine, antimony, nanoformulations.

RESUMO

A Leishmaniose é uma doença tropical endêmica que afeta principalmente países em desenvolvimento. O arsenal terapêutico da Leishmaniose é muito restrito e altamente tóxico, tendo como base o uso dos sais de antimônio. Os fármacos de segunda escolha como a anfotericina B e a pentamidina também apresentam elevada toxicidade e**,** assim, nenhuma terapia recente é efetiva contra *Leishmania* spp. Lipossomos são sistemas carreadores de fármacos, que podem direcionar altas doses a células alvo. No presente trabalho foi desenvolvida uma nova formulação lipossomal com o objetivo de direcionar o antimônio pentavalente aos macrófagos infectados com *Leishmania* (L.) *chagasi*, por meio da interação com receptores *scavengers in vivo.* A formulação de antimônio lipossomal demonstrou elevada eficácia *in vivo*, reduzindo 133 vezes a dose total de antimônio administrada, com diminuição de 100% da carga parasitária do fígado na dose de 0,75 mg/kg. Estudos em microscopia eletrônica de transmissão revelaram uma formulação estável e de aspecto oligolamelar. Estudos do potencial zeta demonstraram carga negativa acoplada à superfície dos lipossomos, derivada da adição de fosfatidilserina. Esta nova abordagem vem contribuir no estudo de novas formulações lipossomais para redução da toxicidade de fármacos no tratamento da Leishmaniose.

Palavras-chaves. Leishmania, terapia, lipossomos, fosfatidilserina, antimônio, nanoformulações.

INTRODUCTION

Neglected diseases, as Leishmaniasis, affect the poorest population in developing countries. The visceral form of Leishmaniasis (VL) is a progressive and fatal hepatosplenomegaly with 500 million cases/year. No effective drug exists if one considers the high toxicity of the current treatment[1]. Antimonial salts still remain as first-line drugs since 1912, when the Brazilian researcher, Gaspar O. Vianna, reported the efficacy of the trivalent salt of antimony[2] against Cutaneous Leishmaniasis. Pentamidine and amphotericin B have been used as second line drugs, but the toxicity has also been a limiting feature for many patients[3]. Miltefosine, an oral anticancer drug, has been reached clinical phase IV in India against *Leishmania donovani*[4], but in South-America, this drug failed to treat other *Leishmania* species[5]. New drugs and novel therapeutical approaches for Leishmaniasis are urgent.

The use of drug delivery systems for selective targeting has been the main goal of therapy of highly toxic drugs. Liposomes are vesicles composed of lipid layers that enclose an aqueous and lipid compartments that entrap drugs of different lipophilicities. They offer a substantial improvement in the therapeutic indices of entrapped drugs[6]. The first commercial liposomal forms in the market were AmBisome™ (amphotericin B), DaunoXome (daunorubicin citrate), and Doxil (doxorubicin)[7]. Ambisome have been used for Leismaniasis therapy with an excellent efficacy against many parasite strains, but the high costs for a single treatment limits the use in developing countries. Despite the inconvenient, the costs of hospitalization and laboratorial surveillance during treatment, may overcome the high costs of liposomal ampotericin B[1].

Macrophages, the host cell for *Leishmania* spp., are phagocytic cells which participate in the immune defense system through the recognition and elimination of pathogens and also self senescent cells. Macrophages clearly discriminate between apoptotic cells and viable cells, through the recognition of the different phospholipid asymmetry and exposure of phosphatidylserine in plasma membrane of senescent cells[8]. Based in this study, Tempone and co-workers[9], showed a high *in vitro* efficacy of antimony entrapped in phosphatidylserine-liposomes against *Leishmania*-infected macrophages and also demonstrated its targeting ability to macrophage scavenger receptors. In this work, we have studied the *in vivo* efficacy of this novel formulation of antimony-entrapped liposomes, using mice models against VL. We have also characterized the liposomal formulation through transmission electron microscopy and its membrane surface charge, by using ζ potential studies.

MATERIAL AND METHODS

Materials lipids

Hydrogenated phosphatidylcholine and phosphatidylserine were kindly donated by Lipoid GmbH, Ludwigshafen, Germany. Glycerol, dodecyl sodium sulphate, methanol and chloroform were purchased from Merck. RPMI-PR-1640 medium (without phenol red) were purchased from Sigma. Glucantime was obtained from Aventis.

Animals and parasites

L. (L.) *chagasi* (MHOM/BR/1972/LD) was maintained in Golden Hamsters. Amastigotes were obtained from the spleen by differential centrifugation and the parasite burden determined with the method of Stauber[10], at 60-70 th day post infection. Animals were supplied by the Animal Breeding Facility at the Faculty of Medicine of São Paulo and maintained in sterilized cages with absorbent environment, receiving water and diet *ad libitum*. Golden Hamsters (*Mesocricetus auratus*) were infected every two months by i.p. injection with *L.* (L.) *chagasi* in order to obtain amastigotes. Animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences (http://www.nap.edu).

Liposomes

Liposomes were prepared as described elsewhere[9]. Liposomes were composed of saturated egg phosphatidylcholine, phosphatidylserine and cholesterol (5:1:4 molar ratio). Briefly, the lipids were mixed in chloroform-methanol (1:1 v/v) solution, dried at 55°C by rotary evaporation. Pentavalent antimony was added at 55°C and mixed with lipids for 1h. After vesicle rehydration, the solution was sonicated for 5 min in a sonicating bath, under a stream of nitrogen at 55°C and extruded through 0.2μm pore size polycarbonate membranes using a mini-extruder device (Avanti Polar Lipids, Inc., Alabaster). The removal of non-encapsulated drug was carried out by 24h dialysis against an isotonic glycerol solution and the sterilization of the liposomal formulation was done with 0.22μm membranes prior to use. The final phospholipid concentration was determined by the Stewart Assay[11] and resulted in 69mg/mL of phospholipids. The concentration of encapsulated antimony was determined in an Atomic Absorption Spectrophotometer- Hydrate Generation (Intralab GeminiAA12/1475) at λ= 217.6nm, after liposomes lyses with 0.1 % SDS and resulted in 6.92 mg Sb^{v}/mL (final volume of 2mL).

Characterization of liposomes

Transmission Electron Microscopy- In order to determine the mean diameter of liposomes after antimony entrapment, an aliquot of the material was fixed in the microscopy grid, stained with 1% phosphotungstic acid for 2min and observed in a transmission electron microscopy – JEOL[12].

Determination of the membrane surface charge

The zeta potential (mV) of liposomes was determined using an aliquot of the material dissolved in 3M KCl. The equipment used for this assay was a Zeta Potential Analyzer – Zeta Plus (BrookHaven Instr. Corp.) - conductivity - 263 μS,

electric chain - 1,54mA[13]. Liposomes free of surface charge and antimony (phosphatidylcholine/cholesterol – 6:4 molar ratio) were used as internal controls.

***In Vivo* treatment with antimony-entrapped liposomes**

The *in vivo* efficacy of antimony-entrapped liposomes was determined using female BALB/c mice (18 - 22g), which was previously infected (tail vein) with *L*. (L.) *chagasi* amastigotes. Briefly, spleen isolated amastigotes were intravenously inoculated at 2 x 10^7 amastigotes/mouse, using groups of 5 animals[14]. Seven days after the infection, mice were intraperitoneally treated with antimony-entrapped liposomes in a range dose between 0.75 to 75mg/kg, and compared with free-antimony (non-liposomal formulation) in a range dose between 1 to 100mg/kg. The treatment was administered for 4 consecutive days and 14 days post infection, animals were sacrificed and spleen/liver infection analyzed using Giemsa stained imprints by light microscopy. Both organs were weighted and compared with untreated group (infected group). The number of amastigotes per 500 liver cell nuclei was determined and multiplied by the liver weight in mg to obtain Leishman Donovan Units (LDUs)[15]. Liposomes were developed prior to each inoculum and empty-liposomes (free of antimony) were developed as control.

Statistical analysis

Data represents the means (± S.D.) from duplicate independent assays. Data were obtained using Graph Pad Prism 3.0 software.

RESULTS AND DISCUSSION

The present data clearly demonstrated an improvement in the treatment of *L*. (L.) *chagasi*-infected mice by the pentavalent antimony entrapped in phosphatidylserine-liposomes. The formulation of liposomes entrapping the pentavalent antimony was evaluated under *in vivo* conditions, using BALB/c mice previously infected with *L*. (L.) *chagasi*. All treated groups showed 100% reduction in the number of liver amastigotes, even at doses of 0.75mg/kg (Figure 1). In contrast, free-pentavalent antimony showed only a 56 % reduction in parasite burden at 1mg/kg. Free-antimony presented a dose-dependent suppressive effect, with 100% reduction of liver parasite burden only at the highest tested doses (100mg/kg).

This liposomal formulation fulfilled most of the requirements for an efficient drug delivery system. Essentially, two primordial requisites should be considered: it should protect the drug from degradation in the blood environment and should

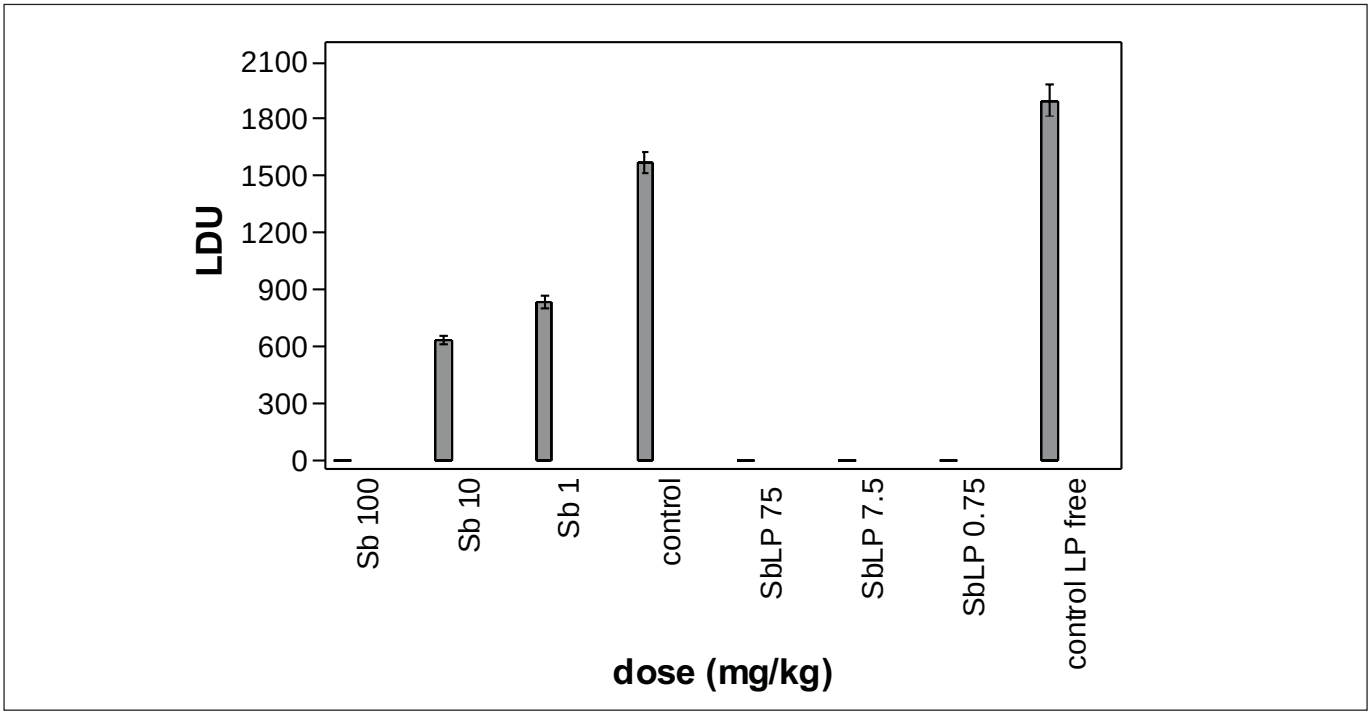

Figura 1. *In vivo* treatment of BALB/c mice infected with *L*. (L.) *chagasi* with free antimony (Sb) at 100, 10 and 1mg/kg, and antimony-entrapped liposomes (SbLP) at 75, 7.5 and 0.75mg/kg. Parasite burden was determined as Leishman Donovan Units (LDU). Groups without treatment (control) and treated with free-antimony liposomes (control LP) were used as controls.

enhance the drug uptake by the targeted cells, making the new formulation a cost-effective procedure[16]. In our previous data, a high *in vitro* efficacy (16-fold higher than free-antimony) of antimony-entrapped liposomes was demonstrated in *L.* (L.) *chagasi*-infected macrophages, with a particular interaction with macrophage scavenger receptors[9]. Our present experimental *in vivo* assay, confirmed the high efficacy of the phosphatidylserine liposomes, since at doses of 0.75mg Sb^5/kg, 100% reduction of liver parasite burden was observed. In contrast, free pentavalent antimony resulted in the same efficacy only at doses 133-fold higher than those used with antimony-entrapped liposomes. New and co-workers[16] showed a significant efficacy of antimony-entrapped in non-charged liposomes using mice models, with 61 % reduction of the parasite burden at 5 mg Sb^5/kg. The marked difference between phosphatidylserine liposomes (PS-liposomes) and other conventional formulations could be explained by the differencial pharmacokinetic of negatively-charged formulations[17]. Cationic liposomes have also been showed a superior efficacy in VL models when compared to free antimony. Pal and co-workers[18], have been demonstrated an efficient treatment of VL (*Leishmania donovani*) using single dose regimen with a non-extruded cationic formulation. Despite the considerable divergences in how surface charge of liposomes contribute to the *in vivo* interaction with macrophages, our previous work clearly demonstrated that the *in vitro* attachment of negatively-charged liposomes to macrophages is highly dependent on the interaction with scavenger receptors, resulting in a high *in vitro* and *in vivo* therapeutic improvement against *L.* (L.) *chagasi*[9]. Furthermore, a confocal microscopy study of fluorescent PS-liposomes suggested an intra-parasitophorous vacuole delivery, probably triggered by the interaction with annexins[9].

In VL therapy, the major difficulty is related to the considerable adverse effects caused by high doses of antimony[1]. The use of drugs entrapped in liposomes could significantly modify the drug pharmacokinetics, reducing the total amount of the administered drug. Additionally, it could enhance the Therapeutic Index of a drug by a reduced toxicity[19]. However, it is known that the chronic or acute phase of the disease could also contribute to a differential pharmacokinetics of a tested formulation[20]. Our data demonstrated a better efficacy of the liposomal formulation at the acute phase of VL. The study of chronic infections in experimental VL and the use of different treatment regimens could be an important tool for additional evaluation of this promising therapeutic approach.

In this work, it was evident that the treatment with high doses (75 mg/kg) of antimony-entrapped liposomes resulted in increased spleen and liver weights (data not shown), suggesting a possible toxicity. Despite this inconvenient, antimony-entrapped liposomes demonstrated a 100% reduction of parasite burden at 0.75 mg Sb^5/kg, with significant reduction of liver and spleen masses (compared to untreated group). These results demonstrate that small doses of the liposomal formulation could be adequate for the treatment, avoiding the usual high dose regimens. Hunter and co-workers[17] observed a suppressive effect on *Leishmania (L.) donovani*-infected mice using unloaded neutral liposomes (without drugs), suggesting an antiparasitic effect of the lipid formulation. Pal et al[18] have also demonstrated this same effect using unloaded cationic liposomes. Our results with unloaded liposomes (without antimony – used as internal control), demonstrated no interference in the parasite burden.

The physical chemical characteristics of liposome formulations could strongly influence the targetability in living organism, and also determine the liposome efficacy. Surface charge, size and membrane fluidity are the major features[7]. The phase transition temperature (Tc) of lipids is an important aspect when one considers the cellular membrane interaction. At 37°C saturated lipids present a gel-state, conferring a superior cellular interaction with liposomes, when compared to other formulations using lipids of low phase-transition temperatures[21]. Thus, the strategic use of saturated lipids in our liposome formulation, besides promoting a protection against oxidative damages[22] and higher drug retention[23], might have also contributed to the observed efficacy.

The transmission electron microscopy of liposomes showed a medium diameter of 210 nm, with an oligolamellar aspect (Figure 2). Liposomes of different sizes have been

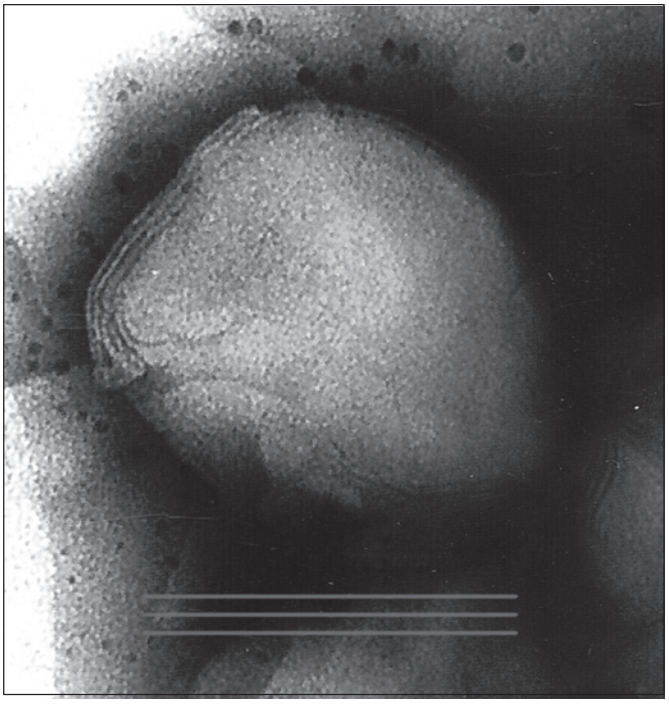

Figura 2. Electron transmission microsopy of pentavalent antimony entrapped into PS-liposomes. Negative staining using phosphotungstic acid. Bar= 200nm.

demonstrated diverse targetability to liver sites[24]. Carter and co-workers[25] have demonstrated a high efficacy of antimony-entrapped liposomes through size reduction from 850 nm to 116 nm, using neutral liposomes composed by L-α-phosphatidylcholine and cholesterol (7:3 molar ratio).

The surface charge of our PS-liposomes was determined by the ζ potential of the formulation and resulted in -88.0 mV (±20). Liposomes composed of neutral phospholipids were used as control and showed a ζ potential of + 1.25mV (±0,2). The membrane charge of liposomes is an essential characteristic for liposome-cell interaction. In addition, negatively-charged liposomes have been shown higher blood stability when compared to positively-charged formulations, as a consequence of a diminished adherence of serum proteins to liposome surfaces[26]. The determination of ζ potential of our PS-liposomes suggested a negative charge at the external liposome membrane, as a result of intentional addition of phosphatidylserine. Our previous *in vitro* data have clearly demonstrated that this feature contributed to a high interaction of liposomes with macrophage scavenger receptors[9]. According to Fadok and co-workers[27], ScavR are the major binding sites for PS-liposomes, as a consequence of the regular apoptotic clearance of senescent cells by macrophages. Besides the usual presence of ScavR in macrophage membranes, a high expression of CD36 and SR-BI has been demonstrated in *Leishmania*-infected macrophages[9]. This fact might also have contributed to the superior efficacy of our formulations.

Besides the elevated toxicity of antimonials, no novel drug fulfilled all requirements for the effective replacement of this old treatment, and consequently, this drug is still used as a first-choice therapy in the majority of endemic countries. The possible reduction of the high adverse effects of antimony therapy as a result of a diminished dose regimen and a consequent reduction of the treatment period may surpass the high costs of an antimony liposomal formulation against Visceral Leishmaniasis.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Suzane Rath (Unicamp) for pentavalent antimony quantification in liposomes. This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (99/08491-4) and LIM-49 FMUSP.

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