International Journal of Antimicrobial Agents 38 (2011) 341–347

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Uptake and antileishmanial activity of meglumine antimoniate-containing liposomes in *Leishmania* (*Leishmania*) *major*-infected macrophages

Samanta Etel Treiger Borborema[a,*], Reto Albert Schwendener[b], João Alberto Osso Junior[c], Heitor Franco de Andrade Junior[d], Nanci do Nascimento[a]

[a] *Instituto de Pesquisas Energéticas e Nucleares–CNEN/SP, Centro de Biotecnologia, Av. Lineu Prestes 2242, CEP: 05508-000, São Paulo, SP, Brazil*
[b] *Institute of Molecular Cancer Research, Laboratory of Liposome Research, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*
[c] *Instituto de Pesquisas Energéticas e Nucleares–CNEN/SP, Centro de Radiofarmácia, Av. Lineu Prestes 2242, CEP: 05508-000, São Paulo, SP, Brazil*
[d] *Instituto de Medicina Tropical, Laboratório de Protozoologia, Universidade de São Paulo, Av. Dr Enéas de Carvalho Aguiar 470, CEP: 05603-000 São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 10 February 2011
Accepted 11 May 2011

Keywords:
Leishmaniasis
Glucantime
Liposome
Antimony
Macrophage

ABSTRACT

Leishmaniasis is a parasitic disease caused by the intramacrophage protozoa *Leishmania* spp. and may be fatal if left untreated. Although pentavalent antimonials are toxic and their mechanism of action is unclear, they remain the first-line drugs for treatment of leishmaniasis. An effective therapy could be achieved by delivering antileishmanial drugs to the site of infection. Compared with free drugs, antileishmanial agent-containing liposomes are more effective, less toxic and have fewer adverse side effects. The aim of this study was to develop novel meglumine antimoniate (MA)-containing liposome formulations and to analyse their antileishmanial activity and uptake by macrophages. Determination of the 50% inhibitory concentration (IC_{50}) values showed that MA-containing liposomes were ≥10-fold more effective than the free drug, with a 5-fold increase in selectivity index, higher activity and reduced macrophage toxicity. The concentration required to kill 100% of intracellular amastigotes was ≥40-fold lower when MA was encapsulated in liposomes containing phosphatidylserine compared with the free drug. Fluorescence microscopy analysis revealed increased uptake of fluorescent liposomes in infected macrophages after short incubation times compared with non-infected macrophages. In conclusion, these data suggest that MA encapsulated in liposome formulations is more effective against *Leishmania*-infected macrophages than the non-liposomal drug. Development of liposome formulations is a valuable approach to the treatment of infectious diseases involving the mononuclear phagocyte system.

1. Introduction

The leishmaniases are a complex of diseases caused by at least 17 species of the protozoan parasite *Leishmania*. Human leishmaniasis occurs worldwide but is mainly localised to the tropics and subtropics, with a prevalence of 12 million cases and an incidence of 0.5 million cases of visceral leishmaniasis (VL) and 1.5 million cases of cutaneous leishmaniasis (CL). Globally there are an estimated 70 000 deaths each year and 350 million people are at risk of infection [1]. Generally, VL is fatal if untreated, whereas CL frequently self-cures, leaving disfiguring scars. Despite its increasing worldwide incidence, leishmaniasis has become one of the so-called neglected diseases, generating little interest from financial donors, public health authorities, pharmaceutical companies and professionals that is required for research and the prevention and control of a disease [2].

The mainstays of leishmaniasis treatment are pentavalent antimonials such as meglumine antimoniate (MA) (Glucantime®). These antimonials were first introduced in 1945 and remain effective treatments for some forms of leishmaniasis. However, long periods of parenteral administration, variable efficacy against VL and CL, and the emergence of significant resistance are all factors that limit the usefulness of these drugs [3]. Moreover, they can have severe side effects and their mode of action is still unclear. Furthermore, effective leishmaniasis vaccines currently do not exist [4]. The high prevalence of leishmaniasis and the emergence of resistance to conventional drugs demonstrate the need to develop and discover new, less toxic and more efficient treatments [5]. Development of formulations of antileishmanial agents using nanocarriers such as liposomes is an attractive alternative [6].

Liposomes are well established to be non-toxic, non-immunogenic and biocompatible. They have been shown to improve the efficacy and to reduce the systemic toxicity of drugs [7], specifically as carriers for antifungals, antibiotics [8]

* Corresponding author. Present address: Núcleo de Doenças Respiratórias–Centro de Virologia, Instituto Adolfo Lutz, Av. Dr Arnaldo 355, Cerqueira César, CEP: 01246-902, São Paulo, SP, Brazil. Tel.: +55 11 3068 2913.
E-mail address: samanta@usp.br (S.E. Treiger Borborema).

0924-8579/$ – see front matter © 2011 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.
doi:10.1016/j.ijantimicag.2011.05.012

and anticancer drugs [9]. Liposomes are preferentially taken up through the mononuclear phagocyte system, predominantly by the macrophages of the liver and spleen, which are the main reservoirs of parasites in VL. Therefore, use of liposomes represents a rational strategy to target these tissues with antileishmanial agents to treat this parasitic disease more efficiently. Several studies have demonstrated that liposome-encapsulated MA is significantly more effective in vitro [10] and in vivo than the free drug [11,12]. Conventional processes for the preparation of liposomal MA showed significant technological disadvantages, such as short-term stability and low encapsulation of the drug [13]. We tried to overcome these difficulties by incorporation of negatively charged phospholipids into liposomes of reduced size with increased macrophage uptake and drug encapsulation efficiency, and also to prepare liposome formulations that can be stored frozen with longer storage stability. Thus, the aims of this study were to develop MA-containing liposome formulations and to analyse both their antileishmanial activity and their uptake by macrophages in vitro, which have been the subject of only few studies by other research groups.

2. Material and methods

2.1. Chemicals

Opti-MEM® I reduced serum medium, Schneider's *Drosophila* medium with L-glutamine, RPMI 1640 with L-glutamine and foetal bovine serum (FBS) were from Gibco (Basel, Switzerland). Cholesterol was from Fluka (Buchs, Switzerland) and resazurin (7-hydroxy-3h-phenoxazin-3-one 10-oxide sodium salt) and phosphatidylserine (3-sn-phosphatildyl-L-serine) were from Sigma (St Louis, MO).

Soy phosphatidylcholine (PC) was obtained from L. Meyer (Hamburg, Germany). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was from Roche Diagnostics GmbH (Mannheim, Germany) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes (Eugene, OR). D,L-α-Tocopherol was obtained from Merck (Darmstadt, Germany) and MA (Glucantime®, 300 mg/mL) was obtained from Sanofi-Aventis (São Paulo, Brazil).

2.2. Animals and cells

C57BL/6 mice were supplied by the animal breeding facility at the University of Zurich (Zurich, Switzerland) and were maintained in sterilised cages in a controlled environment. Mice received water and food ad libitum. All of the animal procedures were performed with the approval of the Veterinary Department of the Kanton Zürich (permit 215/2008). RAW 264.7 murine macrophage cells (TIB-71) (American Type Culture Collection, Manassas, VA) were cultivated in RPMI 1640 supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 as described by Tempone et al. [14]. Isolated *Leishmania* (*Leishmania*) *major* amastigotes (MHOM/IL/81/FEBNI) were a kind gift from Prof. M. Kopf (Institute of Integrative Biology, Molecular Biomedicine, Swiss Federal Institute of Technology, Zurich, Switzerland). Parasites were cultivated in Schneider's *Drosophila* medium supplemented with 1% antibiotics and 20% FBS. Cells were maintained at 27 °C as described by Iezzi et al. [15].

2.3. Preparation and characterisation of meglumine antimoniate-containing liposomes

Liposomes were prepared as described previously [16]. Briefly, lipid mixtures of either PC (200 mg/mL), cholesterol (40 mg/mL) and D,L-α-tocopherol (1.1 mg/mL; molar ratio 10:4:0.1) (L) or of PC, phosphatidylserine (PS) (20 mg/mL), cholesterol and D,L-α-tocopherol (molar ratio 10:1:4:0.1) (PS-L) were prepared. For the fluorescent liposomes, 0.02 μmol/mL of DiI was added to the lipid mixtures (DiI-L and DiI-PS-L, respectively). Dry lipid films were then dispersed in a physiological phosphate–mannitol buffer (PB-man) (20 mM, 230 mM mannitol, pH 7.4) and supplemented with either the corresponding amount of MA (10.4 mg/mL) or with PB-man for the empty control liposomes. The resulting multilamellar vesicles were freeze–thawed in three cycles of liquid nitrogen and 40 °C water and then subjected to repetitive extrusion through filter membranes (Nucleopore™; Sterico, Dietikon, Switzerland) with 400 nm (5×), followed by 100 nm (8×) pore sizes, using a LIPEX™ extruder (Northern Lipids Inc., Burnaby, BC, Canada) to obtain small unilamellar vesicles.

Non-encapsulated MA was removed by dialysis (Spectra/Por tube, 12–14 KDa molecular weight cut-off; Spectrum Laboratories Inc., Rancho Dominguez, CA) at 4 °C in PB-man buffer (1:100, v/v). Liposomal preparations were filter-sterilised using 0.45 μm filters and the aliquots were then frozen and stored at −80 °C. Liposome size and homogeneity were measured with a Nicomp 370 laser light-scattering particle sizer (Nicomp Inc., Santa Barbara, CA). MA-containing liposomes were classified as Gluc-L or Gluc-PS-L after complete processing.

2.4. Antimony (Sb) determination by instrumental neutron activation analysis (INAA)

Aliquots of either MA liposomes or Sb solutions were irradiated at the IEA-R1 nuclear reactor facility of IPEN-CNEN/SP (Sao Paulo, Brazil) at a thermal neutron flux of $0.8–1.0 \times 10^{12}$ n/cm^2/s for 15 min as described by Osso et al. [17]. Concentrations of Sb in the irradiated samples were determined after 2 days of cooling by measuring ^{122}Sb (564 keV). As a reference, Sb_2O_3 (Merck) concentrations were quantified to determine the accuracy of the INAA. Each sample was counted for 300–1800 s live time on an HPGe detector coupled to a Genie-PC software program (Canberra Industries Inc., Meriden, CT). Concentrations of Sb were determined by comparison with the standards that contained comparable amounts of the elements and were irradiated under the same conditions as the samples. Precision of the measurements was calculated as the statistical counting error of the net peak area.

Encapsulation efficiency was determined by measuring Sb concentrations in the liposomal dispersions before and after separation of the non-encapsulated drug. The values were calculated as the percentage of the drug that was incorporated into the liposomes.

2.5. Macrophage cytotoxicity assay

RAW 264.7 macrophages were seeded in 96-well microplates (Gibco) at a density of 1×10^4 per well and were grown under normal culture conditions. After 24 h, the medium was replaced with either the drug or liposomes at various concentrations. Cells were incubated for 48 h at 37 °C in 5% CO_2. The cytotoxic effects of the test compounds were determined using the resazurin assay according to the manufacturer's recommendations. Briefly, resazurin-containing medium (1:20, v/v) was added to each well. After 3 h incubation at 37 °C in 5% CO_2, fluorescence was measured at a 590 nm emission wavelength and a 560 nm excitation wavelength in a SpectraMax® M5 Microplate Reader (Molecular Devices, Sunnyvale, CA). Data analysis was performed using GraphPad Prism 5.03 software (GraphPad Software Inc., La Jolla, CA) using the mean of three experiments in duplicate.

Table 1
Liposome characterisation: initial weight ratio of lipid to encapsulated meglumine antimoniate (MA) ($Ratio_i$), mean size and encapsulation efficiency (*EE*).

Liposomes	$Ratio_i$ (w/w)	Mean diameter (nm)	E_e (%)
Gluc-L	0.04	142.3 ± 61.4	25
Gluc-PS-L	0.04	141.0 ± 96.2	38

Gluc-L, MA-containing liposomes composed of phosphatidylcholine/cholesterol; Gluc-PS-L, MA-containing liposomes composed of phosphatidylcholine/phosphatidylserine/cholesterol.

2.6. *Leishmania (L.) major* cytotoxicity assays

Promastigotes were seeded in 96-well microplates at a density of 1×10^6 cells/well. Test compound solutions were diluted with growth medium using two-fold serial dilutions and were incubated with the parasites for 24 h at 24 °C. Parasite viability was determined using the resazurin assay as described previously. Viability (100%) was expressed based on the fluorescence units of the untreated positive controls after normalisation.

Activity against *L.* (*L.*) *major* intracellular amastigotes was determined in infected macrophages. Macrophages were isolated from the peritoneal cavities of C57BL/6 mice (eight males) by lavage with phosphate-buffered saline (PBS). Cells were then seeded into 24-well plates containing glass cover slips at a density of 4×10^4 cells/well and were incubated for 24 h prior to infection. *Leishmania* (*L.*) *major* promastigotes were added at a ratio of 1:10 (macrophage/promastigote) and were incubated for 24 h. Non-internalised parasites were removed by washing once with medium followed by incubation with the test compounds for 5 days at 37 °C in 5% CO_2. Cells were fixed in methanol, stained with Giemsa stain and observed under a light microscope to determine the number of intracellular parasites. The number of amastigotes was determined in 400 macrophages from drug-treated and control cultures. The number of counted amastigotes in the untreated cultures was considered to be 100% for calculation of the percentage of suppression of parasites in the drug-treated cultures [10]. The 50% inhibitory concentration (IC_{50}) was calculated with a sigmoid dose–response model that was generated with GraphPad Prism 5.03 software using the mean of two experiments in duplicate.

2.7. Uptake of fluorescent liposomes by RAW 264.7 macrophages

RAW 264.7 macrophages were infected with *L.* (*L.*) *major* promastigotes as described previously. Opti-MEM I medium (2% FBS) containing fluorescent liposomes (DiI-L or DiI-PS-L, 10 μL or 25 μL liposomes in 500 μL of medium) was then added and the plates were incubated up to 24 h at 37 °C in 5% CO_2. Cells were then washed twice with PBS, fixed with 3% formaldehyde and stained with DAPI (1 μg/mL). Finally, cells were washed with PBS and liposome uptake was examined using an Olympus BioSystems fluorescence microscope (OBS IX81; Olympus, Tokyo, Japan). To quantify the uptake of fluorescent liposomes by macrophages, the number of fluorescent cells in a total of 200 counted macrophages was determined. The number of macrophages without fluorescence was considered 100% when calculating the percentages of uptake. The figures were prepared using Adobe Photoshop and data analysis was conducted with GraphPad Prism 5.03 software using the mean of two experiments in duplicate.

3. Results

3.1. Liposome characterisation

The physicochemical parameters of the liposomes were evaluated following drug entrapment. Table 1 summarises the results of the analysis of the mean diameters of the liposome preparations following removal of non-encapsulated drug. All of the liposomes had mean diameters of <150 nm. Size reduction and population homogeneity were achieved by sequential repeated extrusion through polycarbonate membranes. The encapsulation efficiency was higher when the vesicles carried a negatively charged phospholipid such as PS. Whilst the Gluc-L liposomes had an encapsulation efficiency of ca. 25%, the efficiency increased to 38% with Gluc-PS-L. Preparation of the liposomes in a mannitol-containing buffer allowed them to be stored frozen and lyophilised with longer storage stability, an important property for distribution in remote areas with human *Leishmania* infections [18,19].

Table 2
Antileishmanial activity against *Leishmania* (*Leishmania*) *major* and cytotoxicity in non-infected RAW 246.7 macrophages of meglumine antimoniate (MA)-containing liposomes (Gluc-L or Gluc-PS-L) and free drug (MA).

Drug	IC_{50} (μM) (95% CI)[a]			SI
	Amastigotes	Promastigotes	Macrophages	
Gluc-L	10.5 (5–21)	>9000	9240 (7860–10 850)	880
Gluc-PS-L	≪25	>12 000	5890 (4940–7030)	≫235
MA	93 (65–132)	>234 000	16 420 (15 060–17 910)	177

Gluc-L, MA-containing liposomes composed of phosphatidylcholine/cholesterol; Gluc-PS-L, MA-containing liposomes composed of phosphatidylcholine/phosphatidylserine/cholesterol; IC_{50}, 50% inhibitory concentration; CI, confidence interval; SI, selectivity index (IC_{50} RAW 246.7 cells/IC_{50} amastigotes).
[a] Values (mean and 95% CI) derived from three independent experiments.

3.2. Antileishmanial and cytotoxic activity of meglumine antimoniate-containing liposomes

The cytotoxic activity of the different drug preparations against *Leishmania* promastigotes was analysed. As summarised in Table 2, evaluation of IC_{50} demonstrated that the promastigotes were not susceptible to either free MA or the liposome-encapsulated drug. The exact IC_{50} concentrations could not be determined because the small internal volume of liposomes limits both the capacity of drug transportation and the concentration of the drug.

Instead, *L.* (*L.*) *major* promastigotes were more susceptible to Sb^{3+} (potassium antimony tartrate trihydrate) and Sb^{5+} [potassium hexahydroxoantimonate (V)] solutions, with IC_{50} values of 0.017 mM (0.013–0.022 mM) and 1.56 mM (1.26–1.93 mM), respectively.

Analysis of the cytotoxic effects on *Leishmania* amastigotes revealed that the tested compounds were active in the micromolar concentration range. Gluc-L treatment inhibited intracellular amastigotes with an IC_{50} value of 10.5 μM, whereas the IC_{50} value for free MA was 93 μM (Table 2; Fig. 1). Gluc-PS-L had a leishmanicidal activity of 100%, resulting in the death of all *L.* (*L.*) *major* amastigotes at the lowest applied concentration of 25 μM. A reduction in the drug concentrations used to achieve the IC_{50} is required to improve this assay. This drug concentration effectively eliminated the intracellular parasite and was significantly more active than the standard drug. The dose–response curves for the determination of the IC_{50} values of MA and MA-containing liposome formulations in mouse peritoneal macrophages infected with *L.* (*L.*) *major* amastigotes are shown in Fig. 1.

These results demonstrate that the in vitro activity against amastigotes of the liposome-encapsulated MA was ≥10-fold more effective than free MA. The cytotoxicity assay also revealed that MA-containing liposomes were two to three times more toxic towards macrophages than the free drug, resulting in IC_{50} values of 9.2 mM and 5.9 mM, respectively. Furthermore, the antimony salt solutions, Sb^{3+} with an IC_{50} of 40.7 μM (19.9–83.1 μM) and Sb^{5+} with an IC_{50} of 64.3 μM (33.5–123.4 μM), were more active in the amastigotes than MA, which had an IC_{50} of 93 μM.

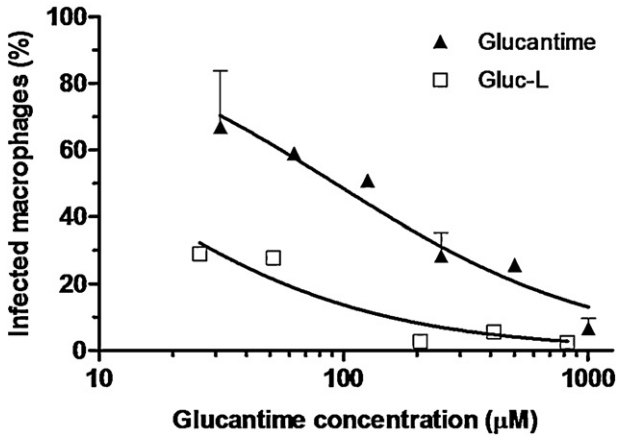

Fig. 1. Dose–response curve for determination of the 50% inhibitory concentration (IC_{50}) of meglumine antimoniate (MA) (Glucantime) or MA-containing liposome formulations (Gluc-L) in mouse peritoneal macrophages infected with *Leishmania* (*Leishmania*) *major* amastigotes. Cells were treated for a total of 5 days. Values (mean ± standard deviation) are derived from three independent experiments in duplicate.

Microscopic observation of the macrophages confirmed the leishmanicidal activity of the liposome-encapsulated MA on the *L.* (*L.*) *major* amastigotes, showing a complete absence of amastigotes in the macrophages after treatment with 13 μM of Gluc-L. Fig. 2A shows two Giemsa-stained control macrophages that were infected with *Leishmania* amastigotes and received no drug treatment, showing the characteristic localisation of the parasites around the nucleus. MA at a concentration of 31 μM (Fig. 2B) was not active enough to eliminate all of the parasites. Fig. 2C and D are representative examples showing the absence of amastigotes in the macrophages after treatment with 13 μM of Gluc-PS-L and Gluc-L, respectively. The absence of amastigotes or a significant reduction in number compared with the untreated control suggests leishmanicidal activity.

3.3. Uptake of fluorescent liposomes by Leishmania (L.) major-infected RAW 264.7 macrophages

Using fluorescence microscopy, high cell fluorescence of both types of DiI-labelled liposomes (DiI-L and DiI-PS-L) was observed at the onset of incubation, indicative of liposome uptake by *L.* (*L.*) *major*-infected RAW 264.7 macrophages. As shown in Fig. 3A and B, >80% of the infected macrophages had taken up fluorescent liposomes after 10 min of incubation. Saturation occurred after 1 h of incubation when almost 100% of the cells displayed fluorescence. In contrast, liposome uptake by non-infected macrophages was slower during the first 2–4 h but reached levels similar to those of the infected cells at later incubation times. Moreover, accumulation of fluorescent liposomes of all types remained high in the macrophages after 24 h of incubation (Fig. 3C). Significant differences in uptake between the empty PS liposomes (DiI-PS-L), empty liposomes (DiI-L) and the MA-containing liposomes (DiI-Gluc-L) were not observed. *Leishmania* infection appeared to change the liposome association and uptake profile, probably by activation of the phagocytic processes of the macrophages. These observations are in accordance with the results of a previous study of liposome uptake in infected macrophages by Tempone et al. [10].

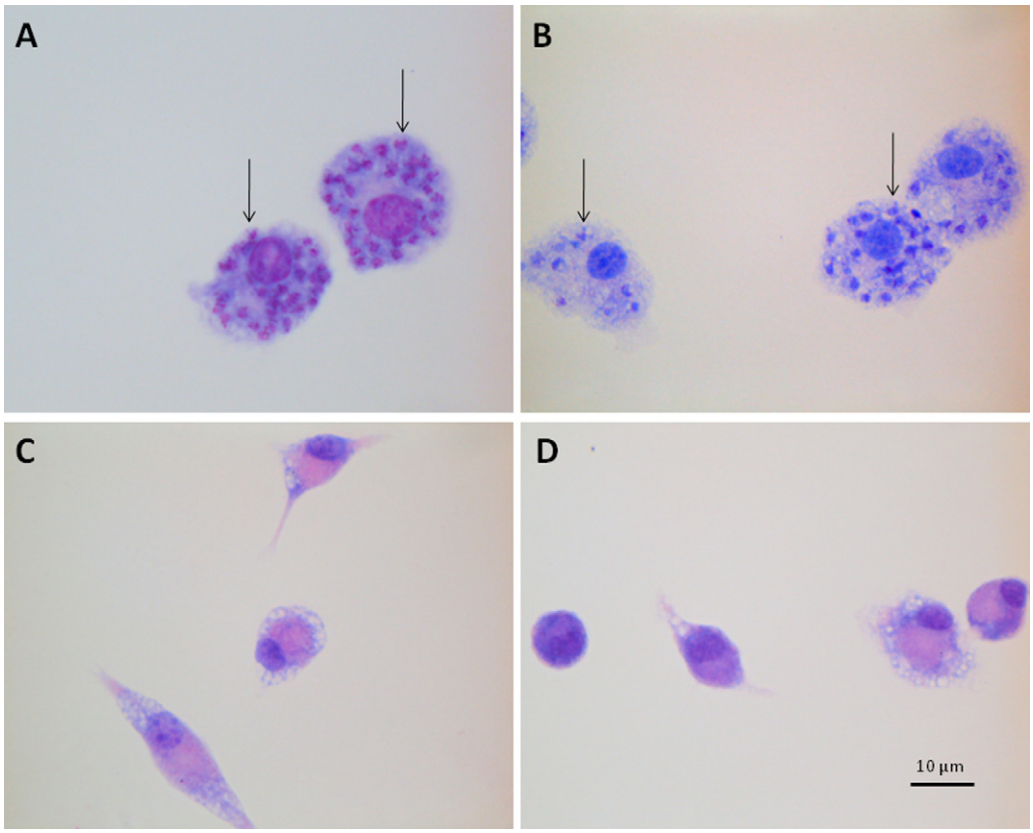

Fig. 2. Microscopic images of mouse peritoneal macrophages infected with *Leishmania* (*Leishmania*) *major* amastigotes in vitro and stained with Giemsa reagent, viewed under oil immersion (magnification ×1000): (A) control, untreated cells (macrophages infected with amastigotes, arrows); (B) macrophages following treatment with 31 μM of meglumine antimoniate (MA) (macrophages infected with amastigotes, arrows); (C) parasite-free macrophages after treatment with 13 μM of Gluc-PS-L; and (D) parasite-free macrophages after treatment with 13 μM of Gluc-L. Gluc-PS-L and Gluc-L indicate MA-containing liposomes composed of phosphatidylcholine/phosphatidylserine/cholesterol and phosphatidylcholine/cholesterol, respectively.

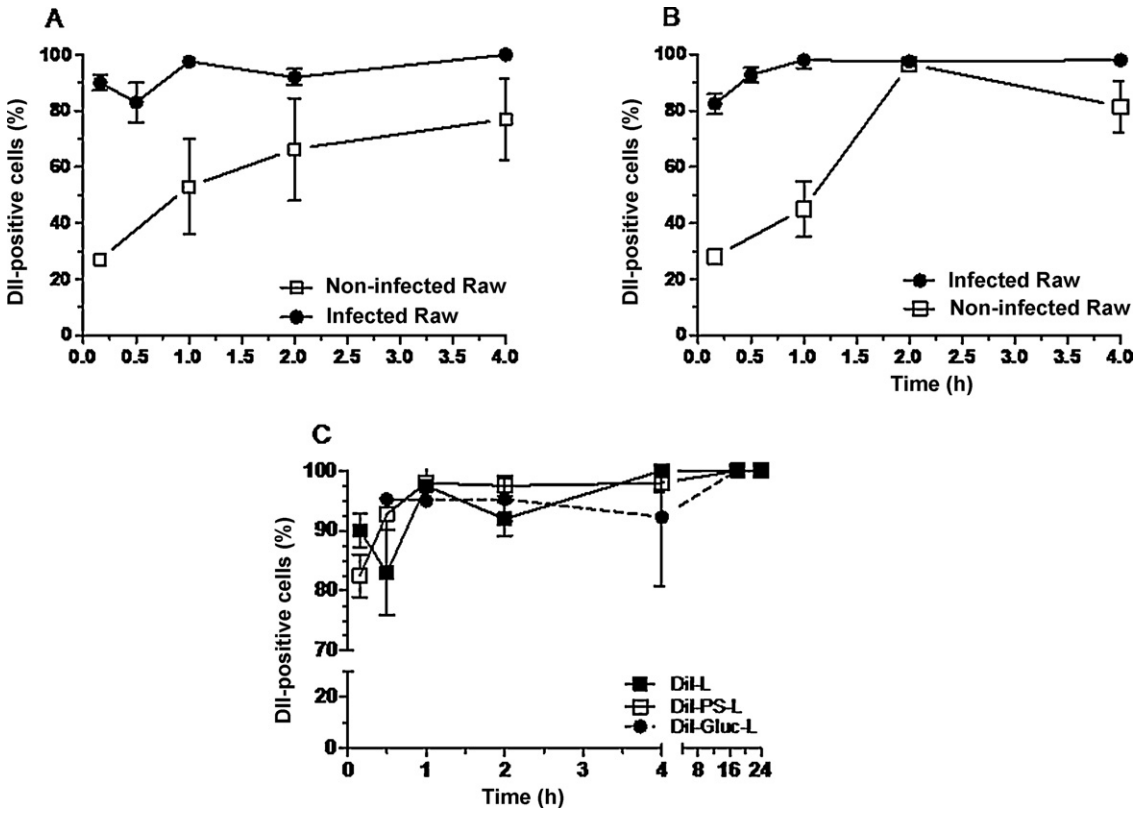

Fig. 3. Uptake of DiI-labelled liposomes by *Leishmania* (*Leishmania*) *major*-infected and non-infected RAW 264.7 macrophages incubated for various lengths of time and analysed by fluorescence microscopy. The percentage of fluorescence-positive cells was calculated as described in Section 2.7. (A and B) Comparison of the uptake of DiI-L (A) and DiI-PS-L (B) in infected versus non-infected macrophages; (C) uptake of DiI-liposomes (DiI-L, DiI-PS-L and DiI-Gluc-L) in infected macrophages. DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiI-L, empty DiI-labelled phosphatidylcholine/cholesterol liposomes; DiI-PS-L, empty DiI-labelled phosphatidylcholine/phosphatidylserine/cholesterol liposomes; DiI-Gluc-L, DiI-labelled meglumine antimoniate-containing liposomes.

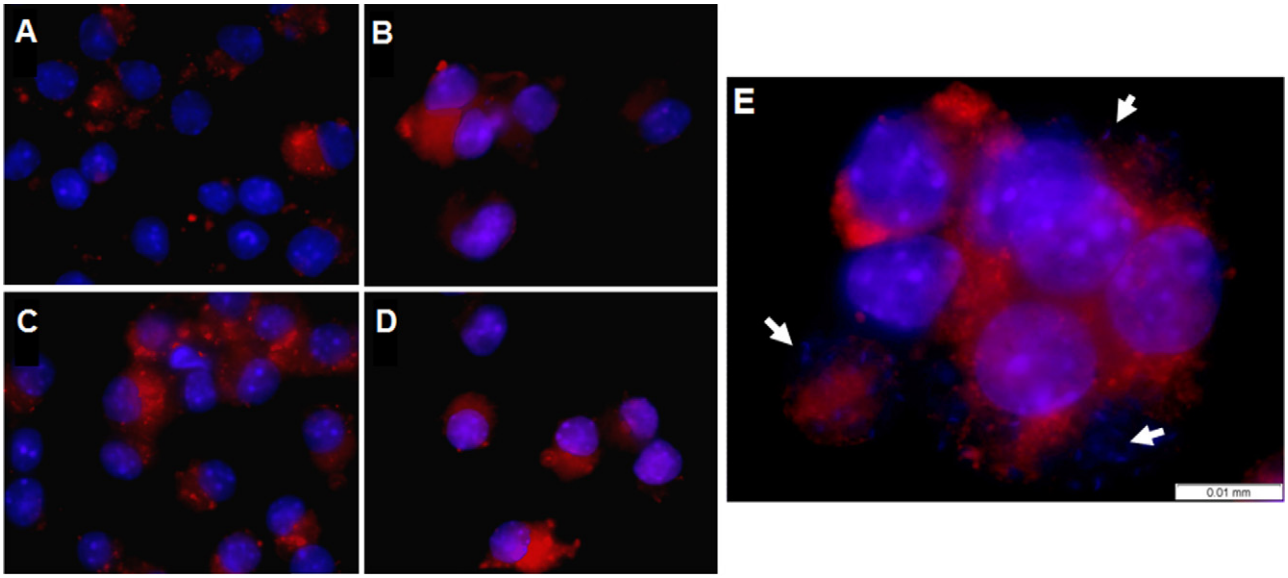

Fig. 4. Fluorescence microscopy images of infected and non-infected RAW 264.7 macrophages incubated with DiI-stained liposomes. Cells were incubated with DiI liposomes (red) for 1 h (1.83 μmol of lipid/well and labelled with 0.02 μmol of DiI) and were visualised with fluorescence microscopy (magnification ×600): (A) *Leishmania* (*Leishmania*) *major*-infected RAW 264.7 cells incubated with DiI-L; (B) non-infected RAW 264.7 cells incubated with DiI-L; (C) *L.* (*L.*) *major*-infected RAW 264.7 cells incubated with DiI-PS-L; (D) non-infected RAW 264.7 cells incubated with DiI-PS-L; and (E) *L.* (*L.*) *major*-infected RAW 264.7 cells incubated with DiI-Gluc-L (red) for 18 h (magnification ×1000). Images show cell nuclei staining using DAPI as a DNA marker (blue). The white arrowheads in (E) indicate areas with a high concentration of amastigotes. DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DiI-L, empty DiI-labelled phosphatidylcholine/cholesterol liposomes; DiI-PS-L, empty DiI-labelled phosphatidylcholine/phosphatidylserine/cholesterol liposomes; DiI-Gluc-L, DiI-labelled meglumine antimoniate-containing liposomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Comparison of liposome uptake by infected and non-infected macrophages is illustrated in Fig. 4. The concentration of fluorescent liposomes was higher in *L.* (*L.*) *major*-infected macrophages, and the fluorescence intensity in the cells was enhanced after a shorter incubation time. Such effects were markedly lower when non-infected macrophages were used as a reference. The fluorescence appeared as a heterogeneous population of red spots within the boundaries of the cell, presumably in the cytoplasm.

When liposome uptake was examined at a higher magnification, as shown in Fig. 4E, image analysis revealed that the fluorescent liposomes were predominantly stored inside the cells and distributed in a perinuclear fashion over the cytoplasm in an inhomogeneous pattern. We presume that they were localised to endosomes and lysosomes, confirming the intracellular distribution of the liposomes [20].

4. Discussion

In this study, it was verified that, when encapsulated in liposomes composed of either PC/cholesterol or PC/PS/cholesterol, the antileishmanial drug MA is able to target and eliminate intracellular amastigotes of *L.* (*L.*) *major* in infected macrophages. These data demonstrate that MA-containing liposomes have strong in vitro activity against intracellular amastigotes and are ≥10-fold more effective than the free drug. It was also observed that *L.* (*L.*) *major* promastigotes were not susceptible to the free drug and MA-containing liposomes. On the contrary, *L.* (*L.*) *major* amastigotes were ≥100 times more susceptible to the free and encapsulated drugs than the promastigote form (Table 2; Fig. 1). IC_{50} data of the free antimony solutions and MA are consistent with results described elsewhere [21,22]. These findings could be explained by the hypothesis that, to be active against *Leishmania*, the pentavalent antimony has to enter the host cell, cross the phagolysosomal membrane and act against the intracellular amastigotes. It is also highly likely that the Sb^{5+} has to be converted to the Sb^{3+} form to be active, acting as prodrugs [6,21]. The greater susceptibility of intracellular amastigotes to Sb^{5+} than promastigotes suggests that the reductive activation of the drug occurs within the intracellular amastigotes [23,24]. However, as parasites in both stages can take up Sb^{3+} and Sb^{5+}, the insensitivity of promastigotes to Sb^{5+} cannot be attributed to drug exclusion [25]. These results are not incompatible with these findings and it is conceivable that the reduction occurs in both the host and the parasite.

The selectivity index also increased five-fold for MA-containing liposomes, showing higher antileishmanial activity with reduced macrophage toxicity. The concentration required to kill 100% of the intracellular amastigotes was ≥40-fold lower with MA encapsulated in PS-containing liposomes than with the free drug. Thus, these data suggest that liposomal MA is more effective than the non-liposomal drug against *Leishmania*-infected macrophages. Similar results were also found in that MA encapsulated in PS-containing liposomes was 16-fold more effective than the free drug against infected macrophages [10]. Jaafari et al. [26] evaluated liposomes containing paromomycin sulfate (PM) against *L. major* promastigotes and amastigotes and reported that they were three to four times more effective than PM in solution and infected mice were completely cured after topical treatment. Miltefosine, the first effective oral drug against VL, exhibited an IC_{50} at 7.8 μM against *Leishmania donovani* amastigotes, whereas entrapment in liposomes of PC-stearylamine (SA) was completely inactive [27].

The small size of the liposomes and the inclusion of negative charges may influence the targetability of the liposomes, possibly due to the ligand-binding properties to macrophage scavenger receptors (SRs). Tempone et al. [10] previously demonstrated that PS-based liposomes targeted intracellular *Leishmania* (*L.*) *chagasi* amastigotes via SRs in macrophages to deliver antimony. *Leishmania*-infected macrophages have been shown to upregulate the SRs CD36, SRB-1 and MARCO. Therefore, SRs may represent a promising target for PS liposomes [10,28].

These results further demonstrate that liposomes are able to target drugs to macrophages and deliver them to phagolysosomes. Negatively charged liposomes may target the parasitophorous vacuoles inside of macrophages by a mechanism related to annexins, the hydrophilic proteins that reversibly bind negatively charged phospholipids and act as bridging molecules in the fusion of vesicles [10]. Inside of this compartment, the liposomes are disrupted by an acid-phase phospholipase and undergo phagolysosomal degradation, ultimately releasing their contents into the cytoplasm [20,29].

Surprisingly, we did not observe significant differences between the uptake of negatively charged and uncharged liposomes. However, charged liposomes are known to be phagocytosed at higher rates owing to specific or electrostatic interactions between cells and vesicles [30]. Indeed, inclusion of PS can lead to the preferred recognition of liposomes by cells of the mononuclear phagocytic system [31,32].

In contrast, Dey et al. [33] reported that cationic liposomes with PC and SA had leishmanicidal activity both in vitro and in vivo. Entrapment of sodium stibogluconate (PC-SA-SSG) enhanced their potentiality against chronic VL in an in vivo murine model [34]. More recently, Roychoudhury et al. [35] discussed that PC-SA-SSG conferred cure against SSG-unresponsive *L. donovani* in mice by complete suppression of disease-promoting interleukin-10 and transforming growth factor-beta (TGFβ), upregulation of Th1 cytokines and expression of macrophage microbicidal nitric oxide.

Moreover, the physicochemical characteristics of liposomes can strongly influence their targetability. Dynamic laser light scattering analysis showed that all of the liposome formulations had a mean diameter size of <150 nm. Schettini et al. [11] evaluated the effects of treatment with a 400 nm liposomal formulation of MA in dogs with VL and observed three-fold higher antimony levels in the bone marrow of dogs when compared with a formulation with large-sized (1200 nm) liposomes. Ribeiro et al. [36] treated infected dogs with this smaller-sized liposomal MA and also observed a significant reduction in bone marrow parasite burden after 5 months of treatment. They also observed significantly lower parasite loads in the cervical lymph nodes, liver and spleen of the animals. In addition, liposomes containing antimonial drugs with mean diameters of <100 nm were more effective than the large-sized vesicles in reducing the number of *Leishmania* parasites in the bone marrow of mice [37].

The results of fluorescence microscopy studies showed an increased uptake of fluorescence-labelled liposomes by infected macrophages after short incubation times compared with non-infected macrophages. The parasitic infection may alter both the metabolic activity of the macrophages and their ability to ingest particulate material, thereby modifying the phagocytic activity and intracellular trafficking of liposomes. Liposomes were still observed in the cells >24 h after addition, demonstrating the persistent availability of the drug in the host cell, a factor that may be essential for antileishmanial activity.

In conclusion, these data demonstrate the increased effectiveness of liposome-encapsulated MA against the intracellular amastigotes of *L.* (*L.*) *major* parasites compared with the free standard therapeutic drug, resulting in an improvement of the selectivity index. Uptake of MA encapsulated in various types of liposomes was significantly increased in infected macrophages. Nevertheless, further in vivo studies are required to evaluate both the specific interactions of the liposomes with the parasites and host cells as well as the therapeutic activity of liposomal MA. In addition, this study emphasises the potential use of drug delivery

systems, such as the use of liposomes, to treat infectious diseases involving the mononuclear phagocytic system and, more specifically, to target antileishmanial agents to infected macrophages.

Funding: This work was supported by CNPq (Conselho Nacional de Pesquisa e Desenvolvimento) (142839/2005-1 and 201308/2008-8).

Competing interests: None declared.

Ethical approval: All of the animal procedures were performed with the approval of the Veterinary Department of the Kanton Zürich (permit 215/2008).

References

[1] World Health Organization. Leishmaniasis. Geneva, Switzerland: WHO. http://apps.who.int/tdr/svc/diseases/leishmaniasis [accessed 22 December 2010].
[2] Reithinger R, Dujardin J, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis 2007;7:581–96.
[3] Roberts WL, McMurray WJ, Rainey PM. Characterization of the antimonial antileishmanial agent meglumine antimoniate (Glucantime). Antimicrob Agents Chemother 1998;42:1076–82.
[4] Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, Modabber F, et al. Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis: a meta-analysis. Vaccine 2009;27:4747–53.
[5] Santos DO, Coutinho CER, Madeira MF, Bottino CG, Vieira RT, Nascimento SB, et al. Leishmaniasis treatment—a challenge that remains: a review. Parasitol Res 2008;103:1–10.
[6] Frézard F, Demicheli C, Ribeiro RR. Pentavalent antimonials: new perspectives for old drugs. Molecules 2009;14:2317–36.
[7] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4:145–60.
[8] Owais M, Gupta CM. Targeted drug delivery to macrophages in parasitic infections. Curr Drug Deliv 2005;2:311–8.
[9] Schwendener RA. Liposomes in biology and medicine. Adv Exp Med Biol 2007;620:117–28.
[10] Tempone AG, Perez D, Rath S, Vilarinho AL, Mortara RA, de Andrade Jr HF. Targeting *Leishmania* (*L.*) *chagasi* amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine. J Antimicrob Chemother 2004;54:60–8.
[11] Schettini DA, Ribeiro RR, Demicheli C, Rocha OGF, Melo MN, Michalick MSM, et al. Improved targeting of antimony to the bone marrow of dogs using liposomes of reduced size. Int J Pharm 2006;315:140–7.
[12] Tempone AG, de Andrade Jr HF. Nanoformulations of pentavalent antimony entrapped in phosphatidylserine-liposomes demonstrate highest efficacy against experimental visceral leishmaniasis. Rev Inst Adolfo Lutz 2008;67:131–6.
[13] Frézard F, Demicheli C. New delivery strategies for the old pentavalent antimonial drugs. Expert Opin Drug Deliv 2010;7:1343–58.
[14] Tempone AG, da Silva ACMP, Brandt CA, Martinez FS, Borborema SET, da Silveira MAB, et al. Synthesis and antileishmanial activities of novel 3-substituted quinolines. Antimicrob Agents Chemother 2005;49:1076–80.
[15] Iezzi G, Fröhlich A, Ernst B, Ampenberger F, Saeland S, Glaichenhaus N, et al. Lymph node resident rather than skin-derived dendritic cells initiate specific T cell responses after *Leishmania major* infection. J Immunol 2006;177:1250–6.
[16] Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjällman AHM, Ballmer-Hofer K, Schwendener RA. Clodronate–liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer 2006;95:272–81.
[17] Osso Jr JA, Suzuki KN, Costa RF, Barrio G, Brambilla TP, Lopes PC, et al. Radioisotope production for nuclear medicine using the IEA-R1m reactor at IPEN-CNEN/SP-Brazil. In: Transactions of the Research Reactor Fuel Management (RRFM) Conference. 2009. p. 247–51.
[18] Chen C, Han D, Cai C, Tang X. An overview of liposome lyophilization and its future potential. J Control Release 2010;142:299–311.
[19] Stark B, Pabst G, Prassl R. Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: effects of cryoprotectants on structure. Eur J Pharm Sci 2010;41:546–55.
[20] Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci 2009;66:2873–96.
[21] Roberts WL, Berman JD, Rainey PM. In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother 1995;39:1234–9.
[22] Ephros M, Bitnun A, Shaked P, Waldman E, Zilberstein D. Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. Antimicrob Agents Chemother 1999;43:278–82.
[23] Yan S, Li F, Ding K, Sun H. Reduction of pentavalent antimony by trypanothione and formation of a binary and ternary complex of antimony(III) and trypanothione. J Biol Inorg Chem 2003;8:689–97.
[24] Frézard F, Demicheli C, Ferreira CS, Costa MAP. Glutathione-induced conversion of pentavalent antimony to trivalent antimony in meglumine antimoniate. Antimicrob Agents Chemother 2001;45:913–6.
[25] Brochu C, Wang J, Roy G, Messier N, Wang XY, Saravia NG, et al. Antimony uptake systems in the protozoan parasite *Leishmania* and accumulation differences in antimony-resistant parasites. Antimicrob Agents Chemother 2003;47:3073–9.
[26] Jaafari MR, Bavarsad N, Bazzaz BSF, Samiei A, Soroush D, Ghorbani S, et al. Effect of topical liposomes containing paromomycin sulfate in the course of *Leishmania major* infection in susceptible BALB/c mice. Antimicrob Agents Chemother 2009;53:2259–65.
[27] Papagiannaros A, Bories C, Demetzos C, Loiseau PM. Antileishmanial and trypanocidal activities of new miltefosine liposomal formulations. Biomed Pharmacother 2005;59:545–50.
[28] Gomes IN, Palma LC, Campos GO, Lima JGB, de Almeida TF, Menezes JPB, et al. The scavenger receptor MARCO is involved in *Leishmania major* infection by CBA/J macrophages. Parasite Immunol 2009;31:188–98.
[29] Ahsana F, Rivasb IP, Khana MA, Suarez AIT. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. J Control Release 2002;79:29–40.
[30] Schwendener RA, Lagocki PA, Rahman YE. The effects of charge and size on the interaction of unilamellar liposomes with macrophages. Biochim Biophys Acta 1984;772:93–101.
[31] Schroit AJ, Madsen J, Nayar R. Liposome–cell interactions: in vitro discrimination of uptake mechanism and in vivo targeting strategies to mononuclear phagocytes. Chem Phys Lipids 1986;40:373–93.
[32] Fidler IJ. Targeting of immunomodulators to mononuclear phagocytes for therapy of cancer. Adv Drug Deliv Rev 1988;2:69–106.
[33] Dey T, Anam K, Afrin F, Ali N. Antileishmanial activities of stearylamine-bearing liposomes. Antimicrob Agents Chemother 2000;44:1739–42.
[34] Pal S, Ravindran R, Ali N. Combination therapy using sodium antimony gluconate in stearylamine-bearing liposomes against established and chronic *Leishmania donovani* infection in BALB/c mice. Antimicrob Agents Chemother 2004;38:3591–3.
[35] Roychoudhury J, Sinha R, Ali N. Therapy with sodium stibogluconate in stearylamine-bearing liposomes confers cure against SSG-resistant *Leishmania donovani* in BALB/c mice. PLoS One 2011;6:e17376.
[36] Ribeiro RR, Moura EP, Pimentel VM, Sampaio WM, Silva SM, Schettini DA, et al. Reduced tissue parasitic load and infectivity to sand flies in dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi* following treatment with a liposome formulation of meglumine antimoniate. Antimicrob Agents Chemother 2008;52:2564–72.
[37] Carter KC, Dolan TF, Alexander J, Baillie AJ, McColgan C. Visceral leishmaniasis: drug carrier characteristics and the ability to clear parasites from the liver, spleen and bone marrow in *Leishmania donovani* infected BALB/c mice. J Pharm Pharmacol 1989;41:87–91.