Pharmacokinetic of meglumine antimoniate encapsulated in phosphatidylserine-liposomes in mice model: A candidate formulation for visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a fatal parasitic disease caused by the protozoan *Leishmania* spp. Meglumine antimoniate (MA) is the main treatment and has demonstrated a promising efficacy in a VL-model when encapsulated into negatively charged liposomes. Considering the current concept for the evaluation of pharmacokinetic parameters at early phases of drug discovery, we developed a formulation of MA-encapsulated into phosphatidylserine liposomes (MA-LP) and analyzed the in vitro antileishmanial activity, physicochemical properties, and pharmacokinetic profile in a mice model. The liposomal formulation had an internal mean diameter of 114 nm and a high stability in plasma. MA-LP was 23-fold more in vitro effective against *Leishmania infantum*-infected macrophages than the free drug, with a selectivity index higher than 220. The pharmacokinetic studies demonstrated that the liposomes increased the uptake of the drug by the liver and spleen and promoted sustained levels. MA-LP was first eliminated through renal excretion, followed by biliary excretion. In the blood, MA-LP followed a biexponential open model. This work emphasizes the importance of liposomes as potential drug delivery systems for visceral leishmaniasis.

1. Introduction

Leishmaniases are mosquito-borne infectious diseases caused by protozoan parasites of the genus *Leishmania*. Once inside the mammalian body, the *Leishmania* multiplies within the macrophage and infects cells of different tissues. The spectrum of disease range from self-healing cutaneous leishmaniasis to severe visceral leishmaniasis, fatal when left untreated [1]. Leishmaniases represent a major global health problem across 98 countries, many in tropical and subtropical areas and in some temperate settings. They are recognized by World Health Organization as one of the world's most neglected tropical diseases affecting 1.6 million of people each year [2].

Pentavalent antimonial agents, such as sodium stibogluconate or meglumine antimoniate (MA), have remained the mainstay of treatment for all forms of leishmaniasis for more than 70 years. However,

their pharmacokinetic profile and mechanism of action are not well understood. There is no available vaccine against leishmaniases, and chemotherapy remains as the major medical approach for managing the disease. Due to potential toxicities of drugs, doses are often limited, treatment failures are common and lower drug doses might be expected to promote resistant organisms. Then, there is a continued need for new therapies that are safe, effective in inducing long-term cure and easy to administer [3].

An alternative is to carry antileishmanial agents using drug delivery systems, such as liposomes. Liposomes have been reported to target the drug to *Leishmania*-infected tissue and reduce the parasite load [4,5]. Antimonial drug-containing liposomes were hundreds of times more effective than unencapsulated ones for the treatment of experimental visceral leishmaniasis [6], promoting the parasite suppression in the liver and/or spleen [7,8]. There are many properties that make

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liposomes the most appropriate carrier system for antimonial drugs as their ability to encapsulate and retain large amounts of water-soluble compounds; natural tendency to be taken up through the mononuclear phagocyte system, predominantly by the macrophages which are the same cells that harbor *Leishmania* parasites; relative safety and also high versatility with respect to lipid composition, volume, vesicle size and number of lamellae [9]. However, charged liposomes are known to be phagocytosed at higher rates owing to specific or electrostatic interactions between cells and vesicles [10]. Targeting *Leishmania*-infected macrophages via phosphatidylserine (PS) nanoliposomes has been a promising approach to deliver meglumine antimoniate (MA) and other drugs [11,12]. Our initial studies demonstrated that MA-entrapped into PS-liposomes were 133-fold more effective than free MA in a VL-mice model [13]. Although no efforts have been made for the evaluation of the in vivo pharmacokinetic (PK) parameters of this formulation, its efficacy suggested a satisfactory PK profile for experimental VL. A recent work from Novartis suggests pharmacokinetic and pharmacodynamic studies be implemented at early phases of drug discovery, to identify pharmacokinetic properties for further improvement and optimal compound and formulation design [14].

In the development of drug-containing liposomes, pharmacokinetic and biodistribution of such systems are major issues to be known. To investigate the fate of liposomes in vivo, a large variety of markers are available. Radiolabels provide a sensitive and powerful tool to determine liposome biodistribution [15]. Studying the biodistribution of MA is easier when the drug is radioactive, but there are no antimony radioisotopes commercially available. As we previously described, irradiating the MA was the best choice by which to support this goal *in vivo* studies [16].

In continuation of the investigation of MA-liposome properties [17,18], this study aimed to develop a meglumine antimoniate encapsulated in phosphatidylserine-liposome and analyze its physicochemical characteristics, antileishmanial activity and pharmacokinetic profile in a mice model.

2. Materials and methods

2.1. Chemicals

Hydrogenated phosphatidylcholine (PC) and phosphatidylserine (PS) were kindly given by Lipoid GmbH (Ludwigshafen, Germany). Glycerol, cholesterol, MTT, RPMI-1640 medium, sucrose, and phosphotungstic acid were purchased from Sigma-Aldrich Co (Sao Paulo, Brazil). Fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific, Inc (Sao Paulo, Brazil). Polycarbonate membranes were purchased from Avanti Lipids, Inc (Alabama, USA). Meglumine antimoniate was obtained from Sanofi-Aventis (Sao Paulo, Brazil).

2.2. Animals and cells

Female BALB/c mice (*Mus musculus*) and male golden hamsters (*Mesocricetus auratus*) were supplied by the animal breeding facility at the Faculty of Medicine of the University of São Paulo and maintained in sterilized cages in a controlled environment with free access to water and food. All of the animal procedures were performed with the approval of the Animal Care and Use Committee of the Institute of Tropical Medicine of the University of São Paulo (São Paulo, Brazil) [CEP-IMTSP 012/29/042008].

Leishmania (*Leishmania*) *infantum* (MHOM/BR/1972/LD) was maintained in golden hamsters for up to approximately 60–70 days post-infection. Amastigotes were obtained from spleens of previously infected hamsters by differential centrifugation. Peritoneal macrophages were collected from the peritoneal cavity of BALB/c mice by washing with 10% FBS-supplemented RPMI 1640.

2.3. Preparation and characterization of meglumine antimoniate-containing liposomes

Liposomes were prepared as previously described [19]. The PC, cholesterol, and PS (molar ratio of 5:4:1) were prepared at the final lipid concentration of 55 g/L. The dry lipid film was dispersed in an isotonic glycerol solution (IGS). After, the solution was sonicated and extruded through 0.2 μm pore size polycarbonate membrane. The liposome suspension was mixed with a sucrose solution at a mass ratio of 3:1 (sugar/lipid) and a final sugar concentration of 0.3 M. The mixture was frozen in liquid nitrogen and lyophilized. For rehydration, 40% of the original liposome volume of MA was added to the lyophilized powder and the mixture was vortexed and incubated for 30 min at 55 °C, the same procedure was repeated adding 40% volume of IGS, and 120% volume of IGS. Drug-containing liposomes were separated from the non-encapsulated drug by centrifugation (14,000g, 30 min). The liposome pellet was then washed and resuspended in IGS at a final antimony concentration of about 10–16 g/L.

Irradiated MA was previously produced [16] for radiolabeled liposome (MA-LP). The rehydration of the lyophilized powder was performed adding 20% of the volume of MA and 20% of irradiated MA following the same procedure. Non-encapsulated irradiated MA was removed by 24 h dialysis at 4 °C in IGS (1:1000, v/v).

The amount of antimony was determined in the resulting liposome suspension by instrumental neutron activation analysis (INAA) [17]. Encapsulation efficiency was determined by measuring antimony concentrations in the liposomal dispersions before and after separation of the non-encapsulated drug. The values were calculated as the percentage of the drug entrapped in the liposomes. The final phospholipids concentration was determined by the Stewart assay [20].

The morphological characterization was determined by negative-staining transmission electron microscopy using 1% phosphotungstic acid and imaged in a JEOL 1011 microscope (Peabody, Massachusetts, USA).

Antimony release was assessed in the storage condition, using an IGS (pH 7.4) at 4 °C and in physiological condition, using normal hamster pooled plasma at 37 °C. After 15, 60, and 120 min samples were removed and centrifuged (14,000g, 30 min, at 4 °C) to remove the leaked drugs. Drug concentrations in the pellet and supernatants were determined by INAA. Drug release was expressed as a percentage of the concentration of the encapsulated antimony [18].

2.4. Antileishmanial and cytotoxic activity

The 50% inhibitory concentration (IC_{50}) against *L. infantum* intracellular amastigotes was determined in infected macrophages. Macrophages were seeded for 24 h at 4×10^5 cells/well into 24-well plates. Amastigotes were isolated as described previously and added at a ratio of 10:1 (amastigotes: macrophages) for 24 h at 37 °C. Non-irradiated free MA and MA-LP were incubated for 120 h at 37 °C in 5% CO_2. Cells were fixed in methanol, stained with Giemsa and observed under a light microscope. The number of infected macrophage among 400 macrophages in the untreated cultures was considered to be 100% for calculating the percentage of infection in the drug-treated cultures [17].

Peritoneal macrophages were seeded at 4×10^5/well in 96-well microplates and incubated with the drugs for 48 h at 37 °C in 5% CO_2. The IC_{50} was determined using a colorimetric MTT assay [21]. The selectivity index (SI) was calculated using the following equation: SI = IC_{50} (Macrophages)/IC_{50} (*Leishmania* amastigotes).

2.5. Biodistribution of meglumine antimoniate-containing liposomes in BALB/c mice

Two experimental groups of mice were used: (i) MA injected mice (n = 40) and (ii) MA-LP injected mice (n = 40). In each group, eight subgroups of five animals were randomly distributed. All animals

received a single intraperitoneal injection of irradiated MA-LP containing 1.0 mg $Sb^{+5}/100\,\mu L$ with the activity of 1×10^3 Bq of ^{122}Sb and 250 Bq of ^{124}Sb or irradiated free MA containing 0.081 mg of $Sb^{+5}/100\,\mu L$ with the activity of 2.2×10^4 Bq of ^{122}Sb and 518 Bq of ^{124}Sb [16]. The animals were euthanized 0.08, 0.25, 0.5, 1, 2, 5, 24 and 48 h after administration by cervical dislocation, which was followed by blood sampling and their organs removed, washed, and weighed. The injected activity (IA) was measured in a NaI(Tl) scintillation counter (Cobra Auto-Gamma; Canberra Inc, Connecticut, USA). The data were expressed as the percentage of IA per gram of organ (IA/g) and of IA per milliliter of blood (IA/mL).

2.6. Pharmacokinetic analysis

A non-compartmental analysis of the blood concentration was performed using PK Solutions 2.0 software (Summit Research Services, CO, USA). Peak concentrations in the blood (Cmax) and the time at which these concentrations were observed (Tmax) were determined from the concentration-time data. The classical trapezoidal rule was used to compute the area under the drug concentration vs. time curve (AUC). The AUC was extrapolated to infinity by the addition of Clast/Kel, where Clast was the drug concentration in the last blood sample investigated and Kel was the terminal elimination rate constant. Kel was determined from the linear regression of the last three data points on each of the plots, and the blood half-lives (t1/2) were calculated as 0.693/Kel. The area under the first moment (AUMC) was determined using the same rules as for the AUC calculation. The mean residence time (MRT) was estimated as AUMC/AUC. The method of residuals (or curve-stripping) was applied to define the underlying exponential terms that best describe the current concentration-time data set. This procedure determines the PK parameters of half-life, rate and concentration intercept for each phase of the blood level curve.

2.7. Statistical analysis

The IC_{50} values were calculated using sigmoid dose-response curves generated by GraphPad Prism 5.03. Pharmacokinetic parameters were derived from the mean concentrations of 5 animals at each time point. For the biodistribution data, the mean $\pm$ SD of the measurements from 5 animals at each time point are shown. The unpaired two-tailed Student t-test was used to compare the two groups and defined as a $P < 0.05$.

3. Results

3.1. Liposome characterization

Liposomes were obtained through rehydration of freeze-dried empty liposomes with MA. The physicochemical parameters were evaluated following drug entrapment. The mean of phospholipid content was 10.26 ± 0.81 mg/mL. The encapsulation efficiency of MA and irradiated MA resulted in a mean of $14.70 \pm 1.95\%$ and $9.29 \pm 2.62\%$ ($p < 0.05$), respectively. The mean of antimony mass in liposomes was 9.41 ± 1.25 mg. Round-like vesicles were predominant, the liposomes were multilamellar (Fig. 1A) and had an internal mean diameter of 114 ± 43 nm (Fig. 1B). The drug release from liposome in isotonic glycerol solution and in plasma was similarly (Fig. 2); with a leakage rate of 6–8% of its content at 15 min, retaining approximately 90% of the entrapped drug at 2 h.

3.2. Antileishmanial and mammalian cytotoxicity

The activity of free MA and MA-LP was evaluated in intracellular *Leishmania* amastigotes. MA-LP inhibited parasite with an IC_{50} value of $2.65\,\mu g/mL$, whereas the IC_{50} value for MA was $60.28\,\mu g/mL$ (Table 1). MA-containing liposomes were more toxic towards peritoneal
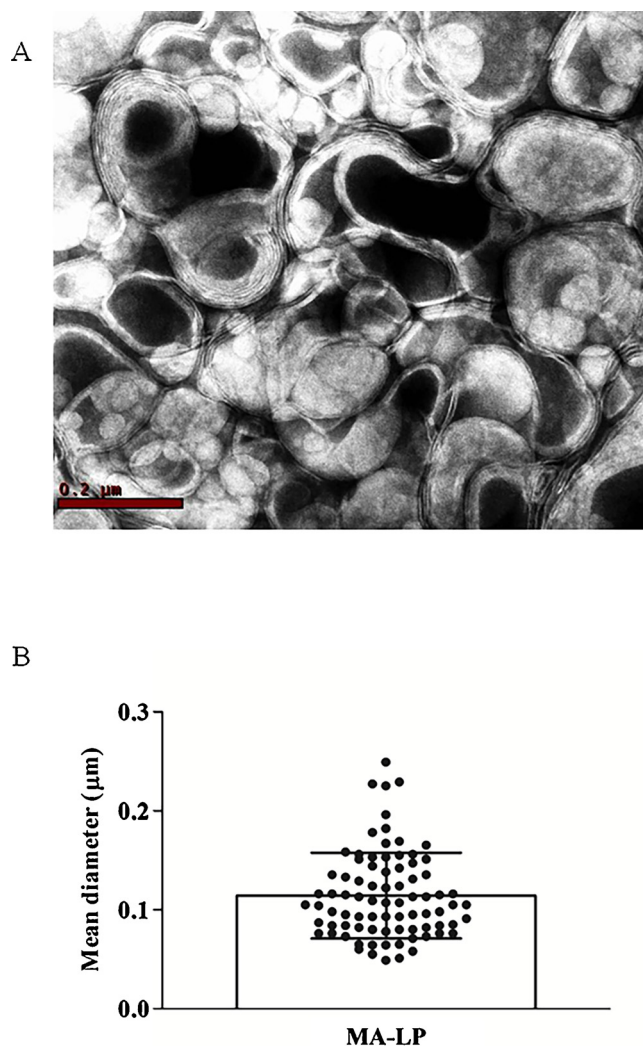

Fig. 1. Negative-staining transmission electron microscopy (TEM) analysis of meglumine antimoniate-containing liposomes (MA-LP). (A) Representative TEM image of liposomes. (B) Histogram showing the particle size distribution.
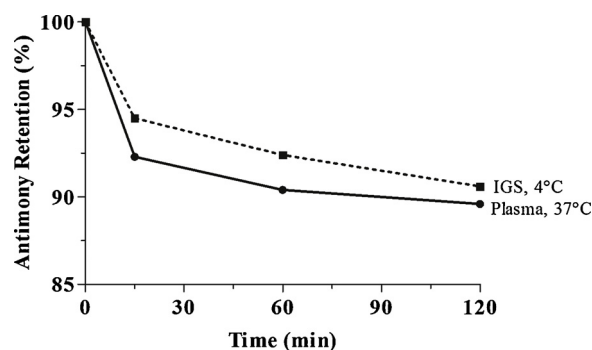

Fig. 2. In vitro release of meglumine antimoniate-containing liposomes (MA-LP). Liposomes were incubated in isotonic glycerol solution (IGS) at 4 °C (■) and plasma at 37 °C (●). At times indicated, samples were centrifuged and antimony concentration was determined by instrumental neutron activation analysis (INAA). The results are expressed as the mean of a single experiment, representative of two.

macrophages than the free drug, resulting in IC_{50} values of $593.6\,\mu g/mL$. Furthermore, the selectivity index was 224 and > 332, respectively. Empty liposomes (free of drug) showed neither cytotoxicity nor antileishmanial activity.

Table 1

In vitro antileishmanial and cytotoxic activity of non-irradiated free meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP).

Drug	IC50 (95% CI) (μg/mL)		
	L. (L.) infantum amastigotes	Peritoneal macrophages	SI
MA-LP	2.65 (2.17 − 3.23)	593.6 (481.9 − 731.2)	224
MA	60.28 (56.22 − 64.64)	> 1000	> 332
LP	NA	NT	–

IC50, 50% inhibitory concentration; CI, confidence interval; SI, selectivity index; LP, empty liposome; NA, not active; NT, non toxic.

3.3. Biodistribution of meglumine antimoniate-containing liposomes

The concentration of antimony in the brain, heart, lungs, and muscle are shown in Fig. 3. In the brain, a low uptake of MA-LP was observed with 0.11% IA/g at 0.08 h and 0.42% IA/g at 48 h, and 0.22% IA/g at 0.08 h and 0.06% IA/g at 48 h for MA (Fig. 3A). In the heart, the antimony activity of MA-LP was 2.66% IA/g at 0.08 h to 0.54% IA/g at 48 h and, MA decreased from 5.86% IA/g at 0.08 h to 0.27% IA/g at 48 h (Fig. 3B). In the lungs (Fig. 3C), the antimony activity of MA-LP and MA decreased from 3.48% IA/g at 0.08 h to 0.37% IA/g at 48 h, and 5.40% IA/g at 0.08 h to 0.21% IA/g at 48 h, respectively. In the skeletal muscle, free MA was significantly higher with 1.20% IA/g at 0.08 h and 0.15% IA/g at 48 h than the liposome, with 0.11% IA/g at 0.08 h and 0.16% IA/g at 48 h (Fig. 3D).

The concentration of antimony in the liver and spleen are shown in Fig. 4. The liver had rapidly taken up the antimony by 0.5 h from MA with a higher Cmax (60.96% IA/g) than the MA-LP by 5 h (34.44% IA/g) (Fig. 4A). However, MA-LP showed sustained level with longer MRT of 62 h compared to MA of 41 h. Also, MA-LP showed higher AUC

values than free MA (2059,21% IA.h/g vs 1462,7% IA.h/g). These are explained by the accumulation and retention of antimony in the liver combined with slow clearance rate, reaching levels of 16.4% IA/g form MA-LP and 10.1% for MA at 48 h.

The uptake of the antimony in the spleen was faster by 0.08 h with Cmax of 23.0% IA/g for MA compared to MA-LP by 5 h (54.14% IA/g), and its activity rapidly decreased reaching 0.74% IA/g at 48 h. The MA-LP was 60 times slower to reach the Cmax compared to MA but was 2.4-fold higher. In contrast, liposome promoted significantly higher and sustained antimony level, with 20.30% IA/g at 48 h (Fig. 4B). The AUC was 19-fold higher for MA-LP (2418,2% IA.h/g vs. 126,3% IA.h/g). The MRT of the antimony was prolonged from 44 h to 47 h after administration of MA and MA-LP, respectively.

Antimony was rapidly eliminated by the kidneys (Fig. 5A), with Cmax of 10.68% IA/g at 0.08 h and 9.44% IA/g at 0.25 h for MA and MA-LP, respectively. A biphasic elimination profile was observed, corresponding to a fast elimination phase (E phase) for up 2 h postinjection, followed by a slow E phase that lasted at least until 48 h, with levels dropping to 0.46% IA/g and 0.79% IA/g for MA and MA-LP, respectively. A fraction of antimony was also absorbed and distributed by the gastrointestinal tract (Fig. 5B), with elimination occurring through hepatobiliary excretion after processing in the liver, eventually reaching the intestinal lumen. High levels of absorption occurred in the stomach by 0.08 h (8.02% IA/g for MA-LP and 13.34% IA/g for MA), then the level decreasing by 0.5 h and 2 h for MA and MA-LP, respectively. Next, increasing levels of antimony were observed within 1 h and 5 h, and the elimination process began. In the intestines, MA showed higher antimony elimination rates than the MA-LP with statistically significant differences. The peak of the fast E phase in the large intestine occurred at 5 h, with 14.4% IA/g and 4.93% IA/g for the MA and MA-LP, respectively. This was followed by a slow E phase until at least 48 h, with levels dropping to 0.91% IA/g for MA and 1.85% IA/g for MA-LP.
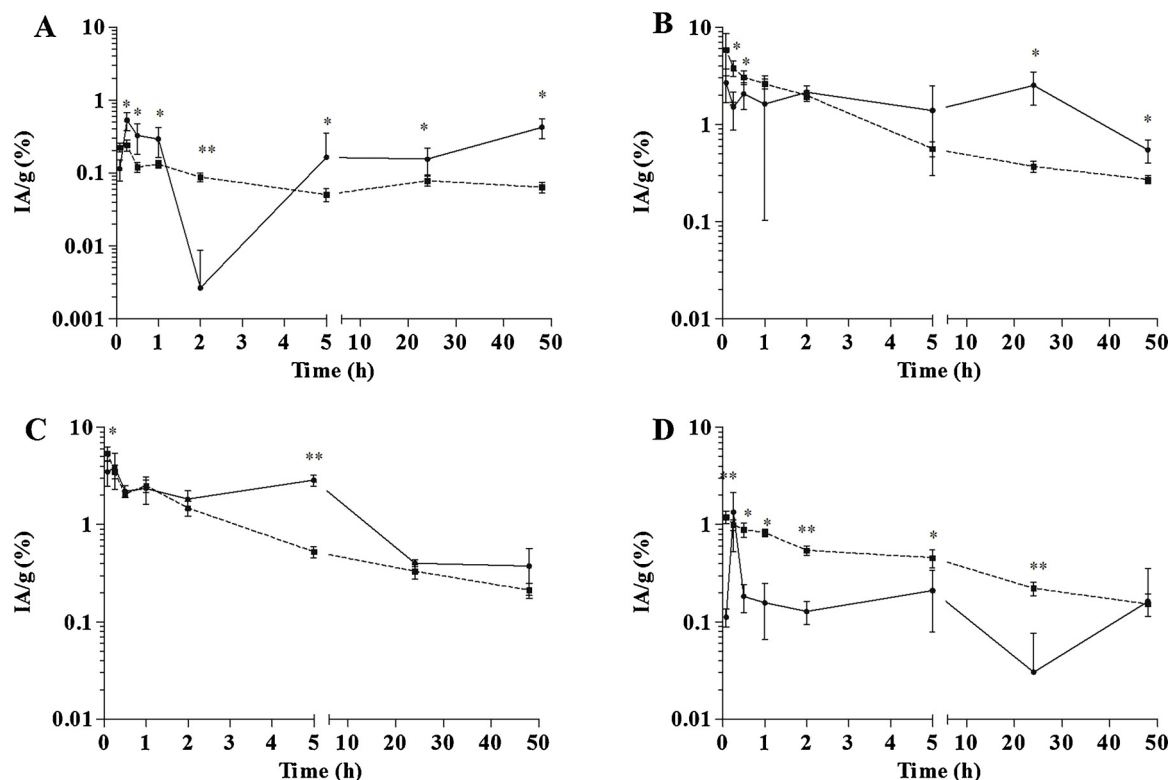

Fig. 3. Biodistribution of antimony in BALB/c mice after intraperitoneal administration of meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP). A: brain; B: heart; C: lung; D: muscle. Continuous line: MA-LP; dotted line: MA. Data are shown as the mean ± standard deviation (n = 5/time) of the percentage of injected activity (IA) per gram. Asterisk showed a significant difference: *p < 0.05.
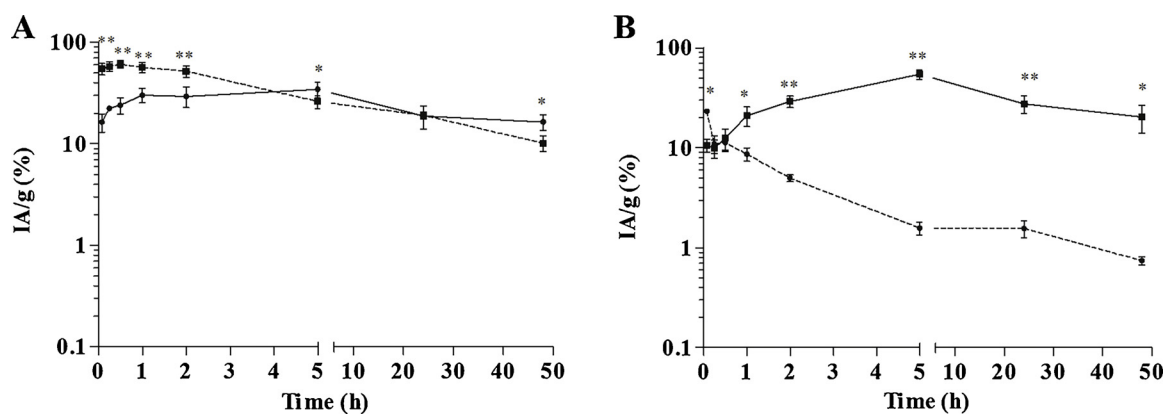
Fig. 4. Biodistribution of antimony in BALB/c mice after intraperitoneal administration of meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP). A: liver; B: spleen. Continuous line: MA-LP; dotted line: MA. Data are shown as the mean $\pm$ standard deviation (n = 5/time) of the percentage of injected activity (IA) per gram. Asterisk showed a significant difference: *p $<$ 0.05 and ** p $<$ 0.0001.
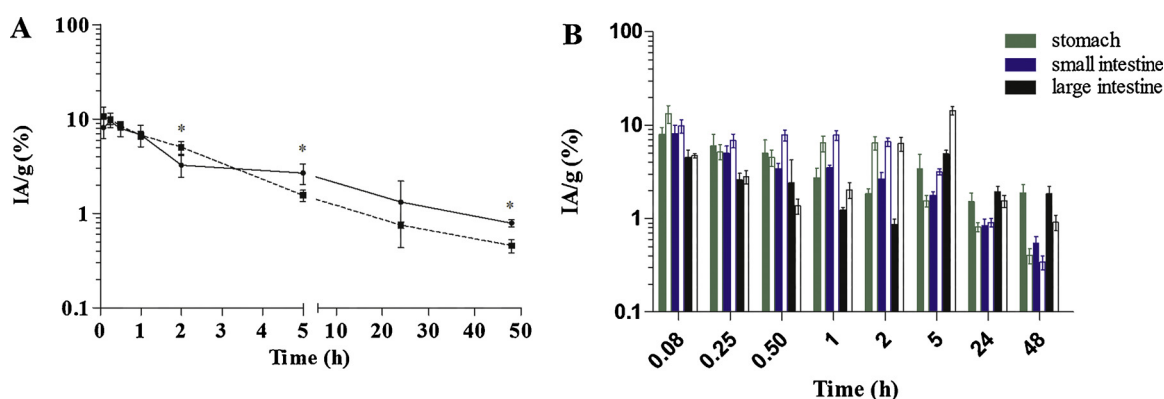

Fig. 5. Elimination pathways of antimony in BALB/c mice after intraperitoneal administration of meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP). A: kidney; B: gastrointestinal tract. Continuous line: MA-LP; dotted line: MA. Data are shown as the mean $\pm$ standard deviation (n = 5/time) of the percentage of injected activity (IA) per gram. Asterisk showed a significant difference: * p $<$ 0.05.

Table 2

Mean pharmacokinetic parameters in the blood of BALB/c mice (n = 5/time) following intraperitoneal administration of meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP).

Parameter	MA-LP	MA
C_{max} (%IA/mL)	6.8*	12.7
T_{max} (h)	0.3	0.08
$t_{1/2}$ E phase (h)	50.58	48.91
$t_{1/2}$ D/A phase (h)	7.26	5.85
$AUC_{0-\infty}$ (%IA.h/mL)	74.7	62.8
AUMC (%IA.h^2/mL)	4770.5	3493.9
MRT (h)	63.9	55.7
CL (mL/h)	1.34	1.59

C_{max}, peak plasma concentration; T_{max}, time to C_{max}; $t_{1/2}$, plasma half-life; E phase, elimination phase; D/A phase, distribution or absorption phase; AUC, area under the concentration-time curve; AUMC, area under the first moment curve; MRT, mean residence time; CL, total clearance *P $<$ 0.05.

For the blood, the means of the pharmacokinetic parameters are summarized in Table 2. Antimony was rapidly absorbed, both for free and MA-containing liposome (Fig. 6). In the blood, MA showed a higher Cmax (12.7% IA/mL) than the MA-LP (6.8% IA/mL) (P $<$ 0.05). For MA the Tmax was achieved at 0.08 h, the first evaluation time. The determination of exponential terms corresponding to the absorption, distribution, and elimination phases occurring during the time course of the drug in the blood could characterize that absorption and distribution phase occurring at the same moment, with a predominance of distribution phase. The distribution phase was followed by a faster E
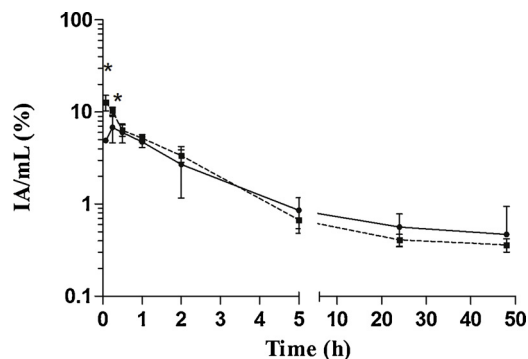

Fig. 6. Blood pharmacokinetics of antimony in BALB/c mice after intraperitoneal administration of meglumine antimoniate (MA) and meglumine antimoniate-containing liposomes (MA-LP). Continuous line: MA-LP; dotted line: MA. Data are shown as the mean $\pm$ standard deviation (n = 5/time) of the percentage of injected activity per milliliter of blood. Asterisk showed a significant difference: * p $<$ 0.05.

phase until 5 h. Thereafter, the slower E phase occurred at least 48 h, when it had reached 0.4% IA/mL. Despite the higher antimony levels for MA at the beginning of the study, no statistically significant differences were observed in other parameters.

4. Discussion

This study demonstrated that MA-containing liposome composed of

phosphatidylserine was able to eliminate intracellular amastigotes of *L. infantum* and was 23-fold more in vitro effective than the free drug. Furthermore, low in vitro cytotoxicity was detected, resulting in a selectivity index higher than 220. Similar results were previously observed using MA-liposomes, being ≥10-fold more effective against *L. major*-infected macrophages [17] and 63-fold more effective against *L. infantum*-infected macrophages than the free drug [18].

The clearance rate and biodistribution of liposomes are strongly influenced by their physicochemical characteristics. Liposomes had an internal mean diameter of 114 nm, indicating the presence of a homogeneous population. This small liposome size allows for a longer circulation time and substantial penetration into tissues such as the liver and spleen, sites where *Leishmania* resides. *L. infantum*-infected dogs treated with a MA-liposome of 400 nm targeted antimony to the bone marrow at a threefold-higher level than the liposome with 1200 nm, suggesting that it may more effectively clear parasites from this tissue [19]. Liposomes containing antimonial drugs with 100 nm were more effective than the large-sized in reducing the number of *Leishmania* parasites in the bone marrow of mice [7].

The surface charge of liposomes has an important role in the encapsulation efficiency of drugs and also in the interaction with the target cells. Higher encapsulation efficiency of MA was achieved when the vesicles carried a negatively charged phospholipid such as PS [17]. The inclusion of PS or phosphatidylglycerol in liposomes greatly enhances their binding to and phagocytosis by macrophages [22]. PS-based liposomes targeted intracellular *L. chagasi* amastigotes via scavenger receptors (SR) 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 [23]. PS MA-liposome was preferentially taken up by infected macrophages getting close to the amastigotes rather than by uninfected, probably due to changes in phagocytic behavior after infection [17].

The MA-containing lyophilized liposome suggests that this preparation is stable for storage at 4 °C for at least 2 h, the required time to rehydrate and administer the drug to the individual. Moreover, a significant technological advantage of this method over conventional ones is that liposomes may be stored as pre-formed freeze-dried empty vesicles and that rehydration may be performed just before use with longer storage stability [24], an important property for distribution in remote areas with *Leishmania* infections [25].

This work provides comprehensive pharmacokinetic data of MA-liposomes. Vital organs such as the heart, lungs, and brain showed no significant accumulation of antimony, suggesting no toxic properties, but additional studies are needed to confirm this hypothesis. The skeletal muscle showed higher levels of antimony from the MA than the MA-LP. It is possible that this tissue has no or low specific affinity for liposomes. A study of *L. braziliensis*-infected rhesus monkeys treated with MA found that these organs were among the tissues classified as having accumulated low to intermediate levels of antimony [26]. In our study, based on the antimony uptake from MA-LP, the levels in the organs can be classified as low (< 1%) in the brain, muscle; intermediate (> 1–10%) in the blood, heart, kidneys, large intestine, lungs, stomach, small intestine; and high (> 10%) in the liver, spleen.

The marked targeting and tissue persistence of antimony in the liver and spleen following administration of the liposome represent important findings of this study. The spleen showed the highest level of antimony at 5 h after administration of MA-LP, being 57-fold higher than free MA and, it is retained longer, a key factor for the antileishmanial activity. High levels of antimony in the spleen could be needed to achieve the elimination of the parasites [27] as low levels were associated with relapses and insufficiency for promoting the parasite suppression [28].

A higher level of antimony in the liver caused a reduction in parasite burden but was not observed in spleen and bone marrow, sites where *Leishmania* parasites were apparently less susceptible to antimony

therapy and should be the cause of treatment failure and relapse [29]. Hunter and coworkers [8] also observed a higher level of antimony and parasite suppression in the liver of mice treated with free, niosomal and liposomal antimony formulations.

However, MA-LP showed a lower value of Cmax than MA; a higher value of AUC and prolonged circulation time was observed. Our data suggest that part of antimony is retained in the liver into the portal system after administration before being released into the bloodstream. PS liposomes are efficiently eliminated from the blood by cells of the mononuclear phagocyte system, predominantly Kupffer cells in the liver [30]. Hepatocytes and liver endothelial cells have a significant contribution for the clearance of systemic administered PS-containing liposomes, presumably via a SR-mediated mechanism [31].

Liposome-encapsulated antimonial drugs were extremely effective in suppressing hepatic leishmaniasis. The dose required to obtain 50% suppression of hepatic parasites was approximately 350–900 times lower than the unencapsulated drug [4,32]. Similar results were also observed in mice [33,34] and in dogs [5]. Enhanced therapeutic effects were observed when liposomes were injected intracardially, intraperitoneally or intramuscularly and subcutaneously [11], but not orally [35].

The kidney and intestine uptake data support the hypothesis of two elimination pathways for antimony from both free and MA-liposome; initial elimination through renal excretion followed by biliary excretion. Moreover, the processes in both organs demonstrated a biphasic elimination profile classified as fast and slow. The fast renal clearance was followed by a concomitant fast enterohepatic clearance phase, and the slow elimination phase in the kidneys was followed by a slow biliary excretion phase. The slow terminal elimination phase by both routes may be related to the conversion of pentavalent antimony into trivalent antimony [36]. Similar findings were also reported in an experimental visceral leishmaniasis [16].

Few studies have reported on renal clearance being the predominant route of elimination of antimony [37,38]. Our data of rapid renal clearance during the first 2 h of administration is consistent with studies of Rees and coworkers [37]. Free MA was previously demonstrated to be absorbed by the gastrointestinal tract and entered the liver via the portal vein, where it was metabolized before reaching the rest of the body [16]. The biliary tree is known to be a major route of excretion for trivalent antimonial, but for the pentavalent drugs the kidney is the major route of excretion, and the biliary tree is only a minor route [39].

The clearance of the antimony from circulation also had a biphasic character. First, a rapid initial phase leading to 94% and 86% disappearance from the blood after 5 h was followed by slower elimination half-lives of approximately 50 h, for both free MA and MA-LP. The low antimony level after 5 h can be explained by both its rapid clearance by the liver and its rapid renal elimination. The small fraction of antimony that is more slowly eliminated may be related to its accumulation in the body during treatment. Similar profile for the MA-containing liposomes was observed in healthy dogs [40] and in experimental canine leishmaniasis [41].

The blood antimony concentration as a function of time is characterized by two phases until 48 h. The initial phase shows a rapid decrease of antimony concentration, corresponding to a distribution which is followed by a slower second phase describing drug elimination. A similar pharmacokinetic profile has been proposed for the unencapsulated drug in humans [38], as well as in healthy [40] and infected dogs [41] for the MA-containing liposome.

The large variability in patient response to the treatment of leishmaniasis with pentavalent antimonial drugs can be ascribed to various factors, including the ability of the antimony to reach the infected sites, which depends primarily on antimony's pharmacokinetic profile. Until now, there had been no consensus on whether a favorable response to treatment with pentavalent antimonial depends on achieving a high peak-drug concentration (smaller AUC) or on maintaining an inhibitory-drug concentration longer (larger AUC). A lower but more

sustained level of antimony would seem to be the more effective for treating leishmaniasis [38]. However, it appears that rather than the lower concentrations, which are readily sustained in most tissues, it is the peak tissue concentration of antimony achieved soon after dosing that is the most important factor in its antileishmanial activity [29,42].

Although animal models are useful to conduct preclinical evaluations of a drug and to study a disease, there are major structural and physiological differences with human beings that show a limitation of these models when testing drug to be used in human beings. Current animal models for the discovery of new potential antileishmanial drugs differ vastly in their immunological response to disease and clinical presentation of symptoms. However, animal models are used to ensure correlation of drug results in vivo before human clinical trials begin [43].

5. Conclusion

Meglumine antimoniate encapsulated in phosphatidylserine-containing liposomes enhances the uptake of the drug by liver and spleen, and promote sustained level for a longer period than free drug. This work emphasizes the importance of PS-liposomes as potential drug delivery systems for visceral leishmaniasis. Radiolabeled liposomes is a feasible approach to evaluate the biodistribution of drugs in animal models and these results could contribute to the development of new pharmacological studies using drug delivery systems for VL.

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References

[1] M. den Boer, D. Argaw, J. Jannin, J. Alvar, Leishmaniasis impact and treatment access, Clin. Microbiol. Infect. 17 (2011) 1471–1477, http://dx.doi.org/10.1111/j.1469-0691.2011.03635.x.

[2] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, Leishmaniasis worldwide and global estimates of its incidence, PLoS One 7 (2012) e35671, , http://dx.doi.org/10.1371/journal.pone.0035671.

[3] S.R.B. Uliana, C.T. Trinconi, A.C. Coelho, Chemotherapy of leishmaniasis: present challenges, Parasitology (2017) 1–17, http://dx.doi.org/10.1017/S0031182016002523.

[4] C.R. Alving, E.A. Steck, W.L. Hanson, S. Peter, W.L. Chapman Jr, V.B. Waits, Improved therapy of experimental leishmaniasis by use of a liposome-encapsulated antimonial drug, Life Sci. 22 (1978) 1021–1026.

[5] C.R. Alving, G.M. Swartz, L.D. Hendricks, W.L. Chapman, V.B. Waits, W.L. Hanson, Liposomes in leishmaniasis: effects of parasite virulence on treatment of experimental leishmaniasis in hamsters, Ann. Trop. Med. Parasitol. 78 (1984) 279–286, http://dx.doi.org/10.1080/00034983.1984.11811817.

[6] C.R. Alving, Liposomes as drug carriers in leishmaniasis and malaria, Parasitol. Today 2 (1986) 101–107, http://dx.doi.org/10.1016/0169-4758(86)90039-6.

[7] K.C. Carter, T.F. Dolan, J. Alexander, A.J. Baillie, C. Mccolgan, Visceral Leishmaniasis: drug carrier system characteristics and the ability to clear parasites from the liver, spleen and bone marrow in Leishmania donovani infected BALB/c mice, J. Pharm. Pharmacol. 41 (1989) 87–91, http://dx.doi.org/10.1111/j.2042-7158.1989.tb06399.x.

[8] C.A. Hunter, T.F. Dolan, G.H. Coombs, A.J. Baillie, Vesicular systems (niosomes and liposomes) for delivery of sodium stibogluconate in experimental murine visceral leishmaniasis, J. Pharm. Pharmacol. 40 (1988) 161–165, http://dx.doi.org/10.1111/j.2042-7158.1988.tb05210.x.

[9] F. Frézard, C. Demicheli, R.R. Ribeiro, Pentavalent antimonials: new perspectives for old drugs, Molecules 14 (2009) 2317–2336, http://dx.doi.org/10.3390/molecules14072317.

[10] R.A. Schwendener, P.A. Lagocki, Y.E. Rahman, The effects of charge and size on the interaction of unilamellar liposomes with macrophages, Biochim. Biophys. Acta 772 (1984) 93–101.

[11] T.A. da Costa-Silva, A.J. Galisteo, J.A.L. Lindoso, L.R.S. Barbosa, A.G. Tempone, Nanoliposomal buparvaquone immunomodulates Leishmania infantum-infected macrophages and is highly effective in a murine model, Antimicrob. Agents Chemother. 61 (2017) e02297–16, http://dx.doi.org/10.1128/AAC.02297-16.

[12] A.G. Tempone, R.A. Mortara, H.F. de Andrade, J.Q. Reimão, Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis, Int. J. Antimicrob. Agents 36 (2010) 159–163, http://dx.doi.org/10.1016/j.ijantimicag.2010.04.006.

[13] A.G. Tempone, H.F. Andrade Jr., Nanoformulations of pentavalent antimony entrapped in phosphatidylserine-liposomes demonstrate highest efficacy against experimental visceral leishmaniasis, Rev. Inst. Adolfo Lutz. 67 (2008) 131–136.

[14] T. Tuntland, B. Ethell, T. Kosaka, F. Blasco, R.X. Zang, M. Jain, T. Gould, K. Hoffmaster, Implementation of pharmacokinetic and pharmacodynamic strategies in early research phases of drug discovery and development at Novartis Institute of Biomedical Research, Front. Pharmacol. 5 (2014) 1–16, http://dx.doi.org/10.3389/fphar.2014.00174.

[15] J.A.A.M. Kamps, G.L. Scherphof, Biodistribution and uptake of liposomes in vivo, Methods Enzymol. (2004) 257–266, http://dx.doi.org/10.1016/S0076-6879(04)87016-2.

[16] S.E.T. Borborema, J.A. Osso Junior, H.F. de Andrade Junior, N. do Nascimento, Biodistribution of meglumine antimoniate in healthy and Leishmania (Leishmania) infantum chagasi-infected BALB/c mice, Mem. Inst. Oswaldo Cruz. 108 (2013) 623–630, http://dx.doi.org/10.1590/0074-0276108052013014.

[17] S.E. Treiger Borborema, R.A. Schwendener, J.A. Osso Junior, H.F. de Andrade Junior, N. do Nascimento, Uptake and antileishmanial activity of meglumine antimoniate-containing liposomes in Leishmania (Leishmania) major-infected macrophages, Int. J. Antimicrob. Agents. 38 (2011) 341–347, http://dx.doi.org/10.1016/j.ijantimicag.2011.05.012.

[18] S.E.T. Borborema, J.A. Osso Junior, H.F. de Andrade Junior, N. do Nascimento, Antimonial drugs entrapped into phosphatidylserine liposomes: physicochemical evaluation and antileishmanial activity, Rev. Soc. Bras. Med. Trop. 49 (2016) 196–203, http://dx.doi.org/10.1590/0037-8682-0041-2016.

[19] D.A. Schettini, R.R. Ribeiro, C. Demicheli, O.G.F. Rocha, M.N. Melo, M.S.M. Michalick, F. Frézard, Improved targeting of antimony to the bone marrow of dogs using liposomes of reduced size, Int. J. Pharm. 315 (2006) 140–147, http://dx.doi.org/10.1016/j.ijpharm.2006.01.048.

[20] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14, http://dx.doi.org/10.1016/0003-2697(80)90269-9.

[21] E.G. Pinto, I.O. Santos, T.J. Schmidt, S.E.T. Borborema, V.F. Ferreira, D.R. Rocha, A.G. Tempone, Potential of 2-hydroxy-3-phenylsulfanylmethyl-[1,4]-naphthoquinones against Leishmania (L.) infantum: biological activity and structure-activity relationships, PLoS One 9 (2014) e105127, http://dx.doi.org/10.1371/journal.pone.0105127.

[22] I.J. Fidler, Targeting of immunomodulators to mononuclear phagocytes for therapy of cancer, Adv. Drug Deliv. Rev. 2 (1988) 69–106, http://dx.doi.org/10.1016/0169-409X(88)90006-3.

[23] A.G. Tempone, D. Perez, S. Rath, A.L. Vilarinho, R.A. Mortara, H.F. de Andrade Jr., Targeting Leishmania (L.) chagasi amastigotes through macrophage scavenger receptors: the use of drugs entrapped in liposomes containing phosphatidylserine, J. Antimicrob. Chemother. 54 (2004) 60–68, http://dx.doi.org/10.1093/jac/dkh281.

[24] F. Frézard, C. Demicheli, New delivery strategies for the old pentavalent antimonial drugs, Expert Opin. Drug Deliv. 7 (2010) 1343–1358, http://dx.doi.org/10.1517/17425247.2010.529897.

[25] C. Chen, D. Han, C. Cai, X. Tang, An overview of liposome lyophilization and its future potential, J. Control. Release. 142 (2010) 299–311, http://dx.doi.org/10.1016/j.jconrel.2009.10.024.

[26] K. Friedrich, F.A. Vieira, R. Porrozzi, R.S. Marchevsky, N. Miekeley, G. Grimaldi, F.J.R. Paumgartten, Disposition of antimony in rhesus monkeys infected with Leishmania braziliensis and treated with meglumine antimoniate, J. Toxicol. Environ. Heal. Part A. 75 (2012) 63–75, http://dx.doi.org/10.1080/15287394.2012.624826.

[27] A. Gellhorn, H. van Dyke, The correlation between distribution of antimony in tissues and chemotherapeutic effect in experimental leishmaniasis, J. Pharmacol. Exp. Ther. 88 (1946) 162–172.

[28] K.C. Carter, A.J. Baillie, J. Alexander, T.F. Dolan, The therapeutic effect of sodium stibogluconate in BALB/c mice infected with Leishmania donovani is organ-dependent, J. Pharm. Pharmacol. 40 (1988) 370–373, http://dx.doi.org/10.1111/j.2042-7158.1988.tb05271.x.

[29] M. Collins, K.C. Carter, A.J. Baillie, Visceral leishmaniasis in the BALB/c mouse: antimony tissue disposition and parasite suppression after the administration of free stibogluconate, Ann. Trop. Med. Parasitol. 86 (1992) 35–40, http://dx.doi.org/10.1080/00034983.1992.11812628.

[30] G.L. Scherphof, J.A.A. Kamps, The role of hepatocytes in the clearance of liposomes from the blood circulation, Prog. Lipid Res. 40 (2001) 149–166, http://dx.doi.org/10.1016/S0163-7827(00)00020-5.

[31] C. Rothkopf, A. Fahr, G. Fricker, G.L. Scherphof, J.A.A.M. Kamps, Uptake of phosphatidylserine-containing liposomes by liver sinusoidal endothelial cells in the serum-free perfused rat liver, Biochim. Biophys. Acta. 1668 (2005) 10–16, http://dx.doi.org/10.1016/j.bbamem.2004.10.013.

[32] C.R. Alving, E.A. Steck, W.L. Chapman, V.B. Waits, L.D. Hendricks, G.M. Swartz, W.L. Hanson, Therapy of leishmaniasis: superior efficacies of liposome-encapsulated drugs, Proc. Natl. Acad. Sci. 75 (1978) 2959–2963, http://dx.doi.org/10.1073/pnas.75.6.2959.

[33] C.D. Black, G.J. Watson, R.J. Ward, The use of Pentostam liposomes in the chemotherapy of experimental leishmaniasis, Trans. R. Soc. Trop. Med. Hyg. 71 (1977) 550–552.

[34] R.R.C. New, M.L. Chance, S.C. Thomas, W. Peters, Antileishmanial activity of antimonials entrapped in liposomes, Nature 272 (1978) 55–56.

[35] C.R. Alving, E.A. Steck, W.L. Jr Chapman, V.B. Waits, L.D. Hendricks, G.M. Jr

Swartz, W.L. Hanson, Liposomes in leishmaniasis: therapeutic effects of antimonial drugs, 8-aminoquinolines, and tetracycline, Life Sci. 26 (1980) 2231–2238.

[36] W.L. Roberts, J.D. Berman, P.M. Rainey, In vitro antileishmanial properties of tri- and pentavalent antimonial preparations, Antimicrob. Agents Chemother. 39 (1995) 1234–1239, http://dx.doi.org/10.1128/AAC.39.6.1234.

[37] P.H. Rees, M.I. Keating, P.A. Kager, W.T. Hockmeyer, Renal clearance of pentavalent antimony (sodium stibogluconate), Lancet (1980) 226–229.

[38] J.D. Chulay, L. Fleckenstein, D.H. Smith, Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate, Trans. R. Soc. Trop. Med. Hyg. 82 (1988) 69–72, http://dx.doi.org/10.1016/0035-9203(88)90267-2.

[39] A. Gellhorn, N. Tupikova, H. van Dyke, The tissue-distribution and excretion of four organic antimonials after single or repeated administration to normal hamster, J. Pharmacol. Exp. Ther. 87 (1946) 169–180.

[40] J. Valladares, J. Freixas, J. Alberola, C. Franquelo, C. Cristofol, M. Arboix,

Pharmacokinetics of liposome-encapsulated meglumine antimonate after intramuscular and subcutaneous administration in dogs, Am. J. Trop. Med. Hyg. 57 (1997) 40–406.

[41] J.E. Valladares, C. Riera, P. González-Ensenyat, A. Díez-Cascón, G. Ramos, L. Solano-Gallego, M. Gállego, M. Portús, M. Arboix, J. Alberola, Long term improvement in the treatment of canine leishmaniosis using an antimony liposomal formulation, Vet. Parasitol. 97 (2001) 15–21, http://dx.doi.org/10.1016/S0304-4017(01)00389-2.

[42] J.D. Berman, J.F. Gallallee, J.V. Gallalee, Pharmacokinetics of pentavalent antimony (Pentostam) in hamster, Am. J. Trop. Med. Hyg. 39 (1988) 41–45.

[43] E.R. Mears, F. Modabber, R. Don, G.E. Johnson, A. Review, The current in vivo models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous Leishmaniasis, PLoS Negl. Trop. Dis. 9 (2015) e0003889, http://dx.doi.org/10.1371/journal.pntd.0003889.