

Pharmacokinetic and parasitological evaluation of the bone marrow of dogs with visceral leishmaniasis submitted to multiple dose treatment with liposome-encapsulated meglumine antimoniate

D.A. Schettini¹,
A.P. Costa Val³,
L.F. Souza², C. Demicheli⁴,
O.G.F. Rocha⁵, M.N. Melo²,
M.S.M. Michalick²
and F. Frézard¹

¹Departamento de Fisiologia e Biofísica, ²Departamento de Parasitologia, Instituto de Ciências Biológicas, ³Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, ⁴Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
⁵Laboratório de Análise de Traços Metálicos, CETEC, Belo Horizonte, MG, Brasil

Abstract

The aim of the present study was to evaluate the impact of a multiple dose regimen of a liposomal formulation of meglumine antimoniate (LMA) on the pharmacokinetics of antimony in the bone marrow of dogs with visceral leishmaniasis and on the ability of LMA to eliminate parasites from this tissue. Dogs naturally infected with *Leishmania chagasi* received 4 intravenous doses of either LMA (6.5 mg antimony/kg body weight, N = 9), or empty liposomes (at the same lipid dose as LMA, N = 9) at 4-day intervals. A third group of animals was untreated (N = 8). Before each administration and at different times after treatment, bone marrow was obtained and analyzed for antimony level (LMA group) by electrothermal atomic absorption spectrometry, and for the presence of *Leishmania* parasites (all groups). There was a significant increase of antimony concentration from 0.76 µg/kg wet organ (4 days after the first dose) to 2.07 µg/kg (4 days after the fourth dose) and a half-life of 4 days for antimony elimination from the bone marrow. Treatment with LMA significantly reduced the number of dogs positive for parasites (with at least one amastigote per 1000 host cells) compared to controls (positive dogs 30 days after treatment: 0 of 9 in the LMA group, 3 of 9 in the group treated with empty liposomes and 3 of 8 in the untreated group). However, complete elimination of parasites was not achieved. In conclusion, the present study showed that multiple dose treatment with LMA was effective in improving antimony levels in the bone marrow of dogs with visceral leishmaniasis and in reducing the number of positive animals, even though it was not sufficient to achieve complete elimination of parasites.

Key words

- Liposome
- Meglumine antimoniate
- Pharmacokinetics
- Leishmaniasis
- Dogs
- Bone marrow

Correspondence

F. Frézard
Departamento de Fisiologia e
Biofísica, ICB, UFMG
Av. Antônio Carlos, 6627
Caixa Postal 486
30123-970 Belo Horizonte, MG
Brasil
Fax: +55-31-3499-2924
E-mail: frezard@mono.icb.ufmg.br

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The leishmaniasis are a group of diseases produced by invasion of the reticulo-endothelial system of a mammalian host by a parasite of the genus *Leishmania*. This parasite is found as a motile promastigote in the sandfly and transforms into an amastigote when engulfed by host macrophages (1). Visceral leishmaniasis (VL) is the most severe form of the disease, causing death of humans if not treated. The pentavalent organoantimonial complexes, meglumine antimoniate and sodium stibogluconate, are the first line drugs for the treatment of all forms of leishmaniasis (2). The achievement of a complete cure in dogs with VL is currently a great challenge, since dogs are the main reservoir for the transmission of VL to humans and respond poorly to conventional treatment with pentavalent antimonials (3). In order to improve the efficacy of treatment, we recently developed a novel liposomal formulation for meglumine antimoniate (LMA), prepared by rehydration of freeze-dried empty liposomes with an aqueous solution of the antimonial (4). Furthermore, we have reported the distribution of antimony (Sb) in healthy dogs, following a single intravenous (*iv*) bolus injection of LMA. The latter study suggested that the critical organ for the treatment with LMA could be the bone marrow, since it showed very low antimony levels (5), and that a multiple dose regimen would be required to achieve therapeutic levels of antimony in this tissue. On the other hand, the impact of infection on the capture of liposomes by this tissue has not yet been documented.

The aim of the present study was to evaluate the impact of a multiple dose regimen with LMA on the pharmacokinetics of antimony in the bone marrow of dogs with VL and on the ability of LMA to eliminate parasites from this tissue.

Meglumine antimoniate was synthesized as previously described (6) from equimolar amounts of N-methyl-D-glucamine and oxyhydrated pentavalent antimony. The re-

sulting product contained approximately 30% antimony by weight, as determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer (Boston, MA, USA) Optima 3000 plasma emission spectrometer.

Meglumine antimoniate was encapsulated in freeze-dried liposomes as previously described (4,5). Briefly, small unilamellar vesicles were prepared in water from L- α -distearoylphosphatidylcholine, cholesterol and dicetylphosphate (molar ratio of 5:4:1) at a final lipid concentration of 55 g/L. This suspension was frozen and subsequently freeze-dried overnight. The dried powder was rehydrated with an aqueous solution of meglumine antimoniate (antimony concentration of 80 g/L) and phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM phosphate, pH 7.2) as follows: 40% of the original small unilamellar vesicle volume of the meglumine antimoniate solution was added to the lyophilized powder and the mixture was incubated for 30 min at 55°C; the same volume of PBS was then added and the mixture was incubated for 30 min at 55°C. Drug-containing liposomes were separated from the non-encapsulated drug by centrifugation at 10,000 g for 30 min. The liposome pellet was then washed twice and finally diluted in PBS. The amount of antimony was determined in the resulting liposome suspension by ICP-OES. The encapsulation of meglumine antimoniate was achieved with a trapping efficiency of 42% and an Sb/lipid ratio of 0.25 (w/w). The vesicular suspension was sized by photon correlation spectroscopy at a 90° scattering angle using a channel correlator (Malvern Instruments type 4700, Worcestershire, UK) in combination with an He/Ne laser (wavelength, 633 nm; nominal power output, 32 mW). The mean hydrodynamic diameter of the vesicles was found to be equal to 1.2 μ m. Empty liposomes were also prepared using the same process, but replacing meglumine antimoniate solution with PBS.

To evaluate the pharmacokinetics of antimony in dogs with VL, male mongrel dogs (weighing 5-12 kg) were obtained from the Centro de Zoonoses, Prefeitura Municipal de Belo Horizonte (MG, Brazil), and tested for positivity to *L. chagasi* according to the following tests: indirect immunofluorescence, complement fixation test and enzyme-linked immunosorbent assay, demonstration of *Leishmania* amastigotes in Giemsa-stained bone marrow aspirates, and polymerase chain reaction using *L. donovani* complex-specific primers (7). Animals received *iv* 4 doses of either LMA (each corresponding to 6.5 mg Sb/kg body weight, 9 animals) or empty liposomes (at the same lipid dose as LMA; 9 animals) at 4-day intervals. A third group of animals was untreated (N = 8). Before each administration and at different times after treatment, bone marrow aspirates were recovered for the determination of antimony (LMA group), as previously described (5). Bone marrow samples were submitted to digestion with nitric acid in a microwave oven (CEM, MDS 200, Matthews, NC, USA). Antimony was then measured by electrothermal atomic absorption spectrometry using a Perkin-Elmer Z 5100 graphite furnace atomic absorption spectrometer. All analyses were performed using continuous background correction. All measurements reported in this study were found to be superior to the method detection limits. Bone marrow was also evaluated for *Leishmania* amastigotes in Giemsa-stained bone marrow aspirates 30 days after treatment. Animals were considered to be positive when bone marrow aspirates showed at least 1 amastigote per 1000 host cell nuclei.

Figure 1A shows the antimony levels determined in the bone marrow of dogs in the course of treatment with LMA, 4 days after each dose. There was a significant increase of antimony concentration from 0.76 $\mu\text{g/g}$ wet organ (4 days after the first dose) to 2.07 $\mu\text{g/g}$ (4 days after the fourth dose). These data show that a multiple dose treat-

ment with LMA at 4-day intervals is effective in improving antimony levels in the bone marrow of dogs with VL. Figure 1B shows the pharmacokinetics of antimony elimination from the bone marrow following treatment with LMA. A half-life of about 4 days for antimony elimination could be estimated. These data are consistent with the increase of antimony concentration in this tissue during treatment, when an interval of 4 days between doses was used.

Comparison of the level of infection in the bone marrow of LMA-treated dogs with that of untreated dogs and that of dogs treated with empty liposomes revealed significant differences 30 days after treatment. As shown in Figure 2, the number of positive dogs (with at least 1 amastigote per 1000 host cell nuclei) was 0 of 9 in the LMA group, 3 of 8 in the untreated group and 3 of 9 in the group treated with empty liposomes. This difference in the number of positive dogs was found to be significant by the Fisher exact test ($P < 0.01$). On the other hand, no significant difference between groups was observed before treatment (data not shown). Never-

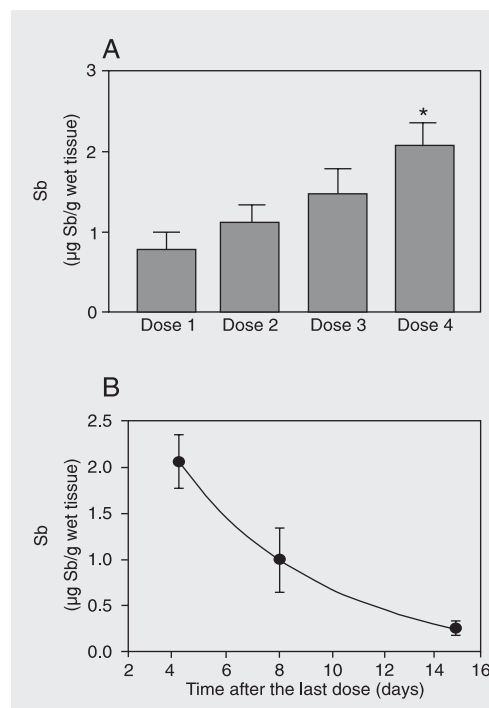


Figure 1. Bone marrow antimony levels determined in infected dogs, during (A) and after (B) treatment with liposome-encapsulated meglumine antimoniate. A, Antimony (Sb) was determined in bone marrow aspirates 4 days after each dose of 6.5 mg Sb/kg injected intravenously every 4 days. * $P < 0.05$ for comparison of antimony levels after the first dose and the fourth dose (one-way ANOVA with Tukey's post-test); all other comparisons were nonsignificant. B, Data were fitted to a monoexponential decay. Data are reported as means \pm SD (N = 4-9 animals).

theless, none of the treated animals showed complete elimination of *Leishmania* parasites, as evidenced by *in vitro* culture of bone marrow aspirates 120 days after treatment (data not shown).

The antimony levels in the bone marrow of dogs following treatment with free and liposome-encapsulated antimonials have been reported in two previous studies (5,8). However, these studies used healthy animals and did not evaluate the effect of multiple dose treatment. Therefore, the present study has the advantage of using conditions closer to those usually employed in therapeutics.

The levels of antimony achieved in the present study can be compared to those reported previously following administration of the same liposome preparation at a slightly lower dose (3.8 mg Sb/kg), but in healthy dogs instead of infected ones. An antimony concentration of 2.8 $\mu\text{g/g}$ was found 3 h after administration, decreasing to about 0.4 $\mu\text{g/g}$ after 96 h (5). We may infer that the levels of antimony achieved in the bone marrow of dogs following LMA are within the same range for healthy and infected animals, suggesting that liposome capture by this tissue is not significantly affected by *L. chagasi* infection.

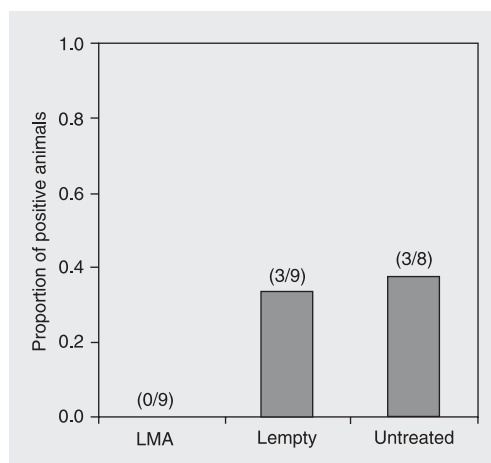
Our results can also be compared to those obtained following a single *iv* injection in healthy dogs of another vesicular system

given at 0.65 mg Sb/kg body weight (8). In the latter study, antimony levels of 5-30 μg Sb/g and 1-8 μg Sb/g were found in the bone marrow 3 and 48 h after administration, respectively. Although 10-fold higher and multiple doses were given in the present study, antimony levels in the bone marrow still remained relatively low. These low antimony levels may be attributed to the large size (1.2 μm) of the liposomes used in the present study when compared to that of the liposomes previously used (0.12 μm) (8), and to the fact that bone marrow is expected to be more accessible to smaller liposomes (9).

The extent of drug targeting achieved with the present liposome formulation can be further assessed by comparing our results to those obtained in a previous study following *iv* administration of free sodium stibogluconate at a therapeutic dose (45 mg Sb/kg) to healthy dogs. Even though sodium stibogluconate and meglumine antimoniate are different pentavalent antimony complexes, the fact that both drugs showed identical pharmacokinetics in humans (10) justifies the present comparison. In the bone marrow, antimony levels in the range of 2 $\mu\text{g/g}$ were found 3 and 48 h after administration (8). Strikingly, these levels are in the same range as those found in the present study. Considering that the dosage regimen recommended in dogs with the free antimonial drug consists of repeated injections twice daily for at least 10 days (11), the expected benefit of the present therapeutic protocol, when compared to conventional therapy, would be rather a reduced risk of toxicity, as a result of a lower antimony dose and a smaller number of doses, than an enhanced efficacy.

The present therapeutic protocol can be compared to that used by Valladares et al. (11). In the latter work, dogs with VL were treated daily for 10 days with a liposome formulation of meglumine antimoniate at 9.8 mg Sb $\text{kg}^{-1} \text{ day}^{-1}$ (the first 2 doses admin-

Figure 2. Proportion of dogs positive for *Leishmania* parasites 30 days after multiple dose treatment with liposome-encapsulated meglumine antimoniate (LMA group) or empty liposomes (Lempty group), and in the untreated group. Animals were considered to be positive when bone marrow aspirates showed at least one amastigote per 1000 host cell nuclei.



istered *iv* and the subsequent 8 doses administered subcutaneously). This treatment was repeated after a resting period of 10 days. Long-term efficacy for at least one year was claimed on the basis of gamma-globulin levels. Although the protocol used in the present study seems, at first sight, less effective than that used by these investigators, several aspects impair the comparison of efficacy between the two protocols. First, different models of VL were used. We used mongrel dogs naturally infected with *L. chagasi*, whereas the cited authors used beagle dogs experimentally infected with *L. infantum*. The two models may present different sensitivity to antimony treatment. Second, a parasitological criterion was used in the present study to evaluate treatment efficacy, which is more demanding than the criterion used by the other authors. Third, different lipo-

some formulations (lipid composition and vesicle size) and dosage regimen were used. With respect to the dosage regimen, the main differences were the number of doses (4 doses in the present study instead of 20 doses in the previous study) and the interval between doses (4 days instead of one day).

The present study documents the antimony levels in the bone marrow of infected dogs following multiple dose treatment with LMA. It indicates that this treatment was effective in improving antimony levels in this tissue and reducing the number of positive animals, even though it was not sufficient to achieve complete elimination of *Leishmania* parasites. Further improvement may be achieved through the use of more prolonged treatments and/or liposomes of reduced size.

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