



US 20150306035A1

(19) **United States**

(12) **Patent Application Publication**

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(10) **Pub. No.: US 2015/0306035 A1**

(43) **Pub. Date: Oct. 29, 2015**

(54) **PHARMACEUTICAL COMPOSITION  
CONTAINING CONVENTIONAL LIPOSOMES  
AND PROLONGED-CIRCULATION  
LIPOSOMES FOR THE TREATMENT OF  
VISCERAL LEISHMANIASIS**

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(21) Appl. No.: **14/384,155**

(22) PCT Filed: **Mar. 11, 2013**

(86) PCT No.: **PCT/BR2013/000079**

§ 371 (c)(1),  
(2) Date: **Dec. 24, 2014**

(30) **Foreign Application Priority Data**

Mar. 9, 2012 (BR) ..... BR1020120052652

Mar. 8, 2013 (BR) ..... BR1020130056014

**Publication Classification**

(51) **Int. Cl.**

*A61K 9/127* (2006.01)

*A61K 31/29* (2006.01)

(52) **U.S. Cl.**

CPC ..... *A61K 9/1271* (2013.01); *A61K 31/29*  
(2013.01)

(57) **ABSTRACT**

The present invention relates to a pharmaceutical composition for treating visceral leishmaniasis, characterized in that it comprises the association of conventional liposomes and prolonged-circulation liposomes with a leishmanicidal-drug delivery system. Said composition may be used in the preparation of a medicinal product for treating leishmaniasis and may be administered intramuscularly, subcutaneously, intra-peritoneally or intravenously. The use of this system enables the drug efficiently to reach all sites infected by the parasite, the pegylated liposomes promoting more effective targeting of the leishmanicidal drugs on the bone marrow and spleen, whilst the conventional liposomes contribute to the targeting of the drug on the liver.

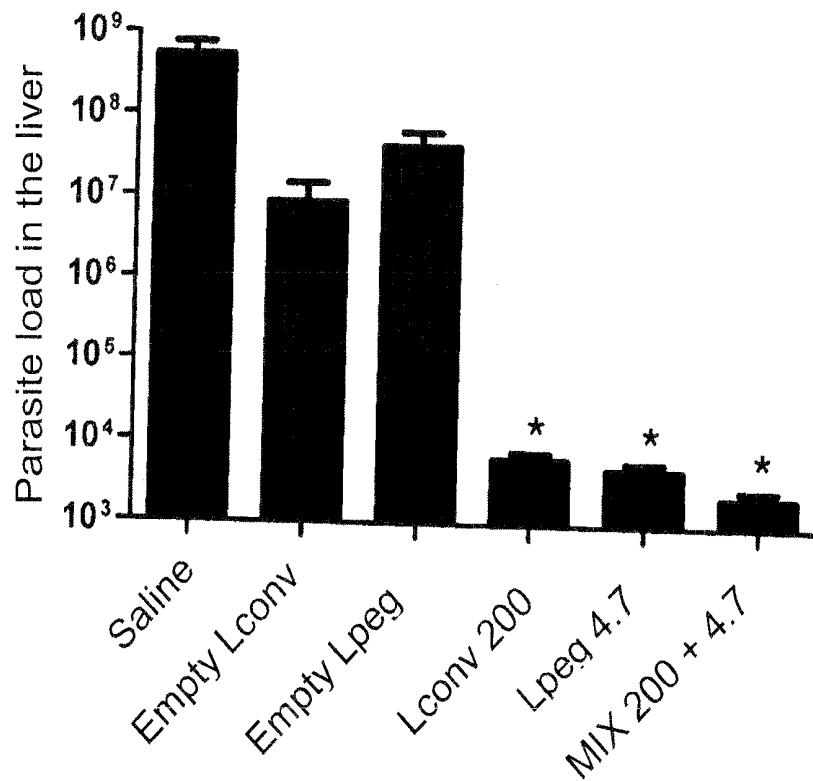


Figure 1

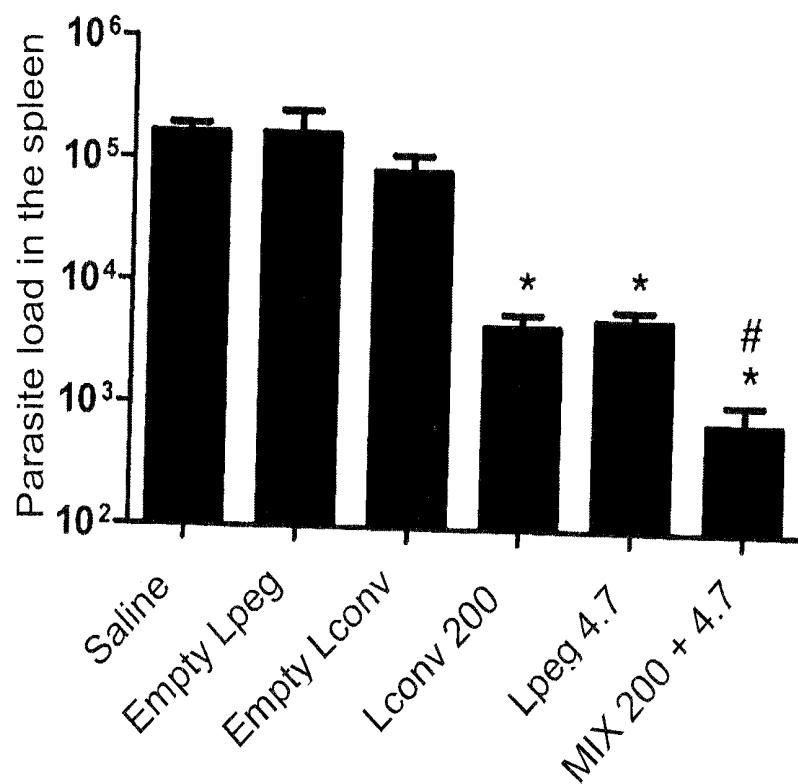


Figure 2

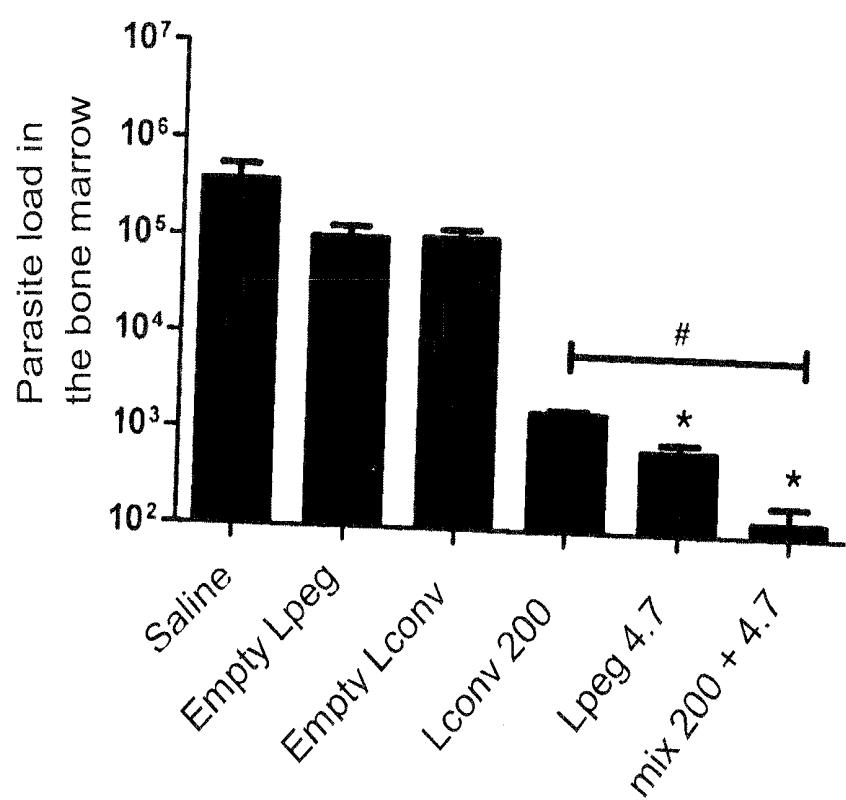


Figure 3

**PHARMACEUTICAL COMPOSITION  
CONTAINING CONVENTIONAL LIPOSOMES  
AND PROLONGED-CIRCULATION  
LIPOSOMES FOR THE TREATMENT OF  
VISCERAL LEISHMANIASIS**

[0001] The present invention relates to a pharmaceutical composition for the treatment of visceral leishmaniasis, characterized by comprising the association of conventional liposomes and prolonged-circulation liposomes as a leishmanicidal-drug delivery system. This composition can be used in preparing a medicament for the treatment of leishmaniasis, and may be administered by intramuscular, subcutaneous, intraperitoneal or intravenous route. The use of this system enables the drug efficiently to reach all the sites infected by the parasite. The pegylated liposomes promote more effective targeting of leishmanicidal drugs to the bone marrow and the spleen, whereas conventional liposomes act on directing the drug to the liver.

[0002] Leishmaniasis are parasite diseases that affect about 12 million people all over the world, being caused by flagellate protozoans belonging to the order Kinetoplastida, family Tripanosomatidae and genus *Leishmania* (UNDP/World Bank/WHO Special Programme for Research & Training in Tropical Diseases (TDR). In Tropical Disease Research Programme Report, 13; progress 1995-96; World Health Organization: Geneva, 1997, chap. 8]. In Brazil, all recent data report the occurrence of about 30,000 new annual cases of the disease.

[0003] Leishmaniasis are transmitted to vertebrate hosts by the sting of an insect that regurgitates the parasite in the promastigote form. These parasites are phagocytized by macrophages, where they change into amastigotes. Amastigotes multiply freely in the acidic compartment of phagolosomes and escape from the defense systems of the host. *Leishmania* corresponds to a complex of various different species that cause various types of clinical manifestations, which include cutaneous, mucocutaneous and visceral forms. In Brazil, the species that causes visceral leishmaniasis is *Leishmania chagasi*. Dogs appear as the vertebrate host of the cutaneous and visceral forms and, particularly in the case of visceral leishmaniasis, they play an important role as reservoir and source of infection of the disease in an endemic area. Visceral leishmaniasis exhibits lethality rate of 100% in cases that are not treated clinically.

[0004] Starting in the 1940's, pentavalent antimony complexes (SB(V) began to be used in the therapy of Leishmaniasis [Marsden, P. D.; Rev. Soc. Bras. Med. Trop. 1985, 18,187; Berman, J. D.; Clin. Infect. Dis. 1997, 24, 684; Rath, S.; Trivelin, L. A.; Imbrunito, T. R.; Tomazela, D. M.; de Jesus, M. N.; Marzal, P. C.; Junior H. F. A.; Tempone, A. G.; Quim. Nova 2003, 26, 550]. The main antimonials in use at presente are Sb(V) complexes with N-methyl-glucamine (meglumine antimoniate) and with sodium gluconate (sodium stibogluconate). It was suggested that Sb(V) was a pro-drug, being reduced in the host organism to Sb(III), which was said to be the active and toxic form. Although pentavalent antimonials continue to be the first-choice medicaments in the treatment of all the forms of leishmaniasis, the clinical use thereof has various limitations. These compounds should be administered by parenteral route (intravenous or intramuscular injection), daily, in a period of 20-40 days. In this context, the side effects are frequent. The appearance of cases of resistance to the treatment also represents a serious problem in the therapy of leishmaniases. Another difficulty encoun-

tered in controlling visceral Leishmaniasis is that so far there is no effective treatment for naturally infected dogs.

[0005] Among the new medicaments that might replace pentavalent antimonials are: (1) AmBisome®, a formulation of amphotericin B based on liposomes that was recently approved by the US Food and Drug Administration (FDA) for the treatment of visceral leishmaniasis [Meyerhoff A. 1999 U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin) for treatment of visceral leishmaniasis. Clin Infect Dis 28, 42-48]; (2) L'Impavido®, which is the hexadecylphosphocholin or mitephosine, initially developed under the name of Miltex® for the treatment of cancer and which exhibited high efficacy by oral route in the treatment of visceral leishmaniasis in clinical tests in India [Sundar S, Jha T K, Thakur C P, Engel J, Sendermann H, Fischer C, Junke K, Bryceson A, Berman J. 2002 Oral miltefosine for Indian Visceral leishmaniasis. N Engl J Med 347, 1739-1746]; and (3) a topical formulation of paromomycin (or aminosidin), which proved to be effective in the experimental treatment of cutaneous leishmaniasis [Gamier T, Croft SL. 2002 Topical treatment for cutaneous leishmaniasis. Curr Opin Investig Drugs 3, 538-544]. However, the prospect of use of these medicaments is remote, due to the high cost of the AmBisome®, the side effects of Miltefosina and the low efficacy of the paromomycin formulation.

[0006] In view of these limitations, the World Health Organization recommends, also with incentives to other related entities like TDR (Special Programme for Research and Training in Tropical Diseases), the research on new medicaments [Remme, J. H. F.; Blas, E.; Chitsulo, L.; Desjeux, P. M. P.; Engers, H. D.; Kanyok, T. R.; Kayondo, J. F. K.; Kiyo, D. W.; Kumaraswami, V.; Lasdins, J. K.; Nunn, P. P.; Oduola, A.; Ridley, R. G.; Toure, Y. T.; Zicker, F.; Morel, C. M.; Trends Parasitol. 2002, /8, 421; Ridley, R. G. In: Drug against Parasitic Diseases; Failamb, A. H.; Ridley, R. G.; Vial, H. J., eds.; UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR): Geneva, 2003, p. 13].

[0007] Essentially, three different strategies are being studied at present for improving the therapy of visceral leishmaniasis. One of them consists in combining different leishmanicidal agents with synergistic action. For instance, the association of gentamicin to paromomycin has increased the efficacy of paromomycin in topical application (WO9406439-A1). The combination of aminosidin with sodium stibogluconate, in turn, proved to be an effective measure in the treatment of the visceral form which does not respond to the conventional treatment. Similarly, the association of this antimonial with another drug in clinical evaluation, namely allopurinol, proved to be effective even in cases of resistance to antimonials [Martinez, S.; Gonzalez, M.; Vernaza, M. E.; C/in Infect Dis 1997, 24, 165]. Immunotherapy, which associates immunomodulating substances to pentavalent antimonials, proved to be a way to reduce the applied dose of antimonial, keeping the efficacy of the treatment [Murray, H. W.; Berman, J. D.; Wright, S. D.; J Infect Dis 1988, 157, 973; Machado-Pinto, J.; Pinto, J.; da Costa, C. A.; Genaro, O.; Marques, M. J.; Modabber, F.; Mayrink, W.; Int. J. Dermatol. 2002, 41, 73].

[0008] A second strategy involves the planning/synthesis of new active substances or of known drugs, with chemical modifications. However, the long time and high cost of the development of a new drug limit considerably the success of this strategy.

[0009] The third strategy involves the reversible strategy of drugs already in use to a carrying system, with a view to lead the drug to a target cell in a better manner and prevent the undesirable sites where the drug exerts toxicity. This strategy, known also as "rejuvenation of drugs", provides a gain in time in the phase of developing the product, since it uses a drug that is already characterized from the pharmacological point of view. Among the medicament carrying systems available at present, liposomes occupy an outstanding position for the therapy of leishmaniasis forms.

[0010] The use of liposomes as drug carriers has been a tendency in the pharmaceutical industry and has been opening prospects for the development of new leishmanicidal medicaments. These spherical vesicles, constituted by one or more concentric bi-layers of lipids, can store, in their internal aqueous compartment, water-soluble active principles, or have lipophilic or amphiphilic active principles incorporated in their membranes. Thus, the medicament is released slowly, which prevents the rapid elimination thereof by the organism. The result is an increase of the bioavailability of the medicament, with potentiation of the action and reduction of toxicity.

[0011] Conventional liposomes were widely studied for carrying leishmanicidal drugs, aiming at the treatment of visceral leishmaniasis [Frézard, F., Demicheli, F. 2010. New delivery strategies for the old pentavalent antimonial drugs. Expert Opin. Drug Deliv. 7(12), 1343-58]. Conventional liposomes are typically formed from a phospholipide such as phosphatidylcolin, or from a non-ionic surfactant. They may also include, in its composition, cholesterol, a phospholipide with a negative charge, as for example, phosphatidylglycerol, phosphatidic acid, dicetylphosphate and/or a phospholipide with a positive charge, such as sterylamine. Since they are rapidly cleared from the blood circulation by the macrophages of the mononuclear phagocytic system, mainly the liver, the spleen and the bone marrow, the conventional liposomes carry the drug to the sites of infection of visceral leishmaniasis, which makes available a larger amount of the drug to interact with the parasite.

[0012] In this context, an amphotericin B formulation was developed in conventional liposomes (AmBisome®) [WO9640060-a1], which was used successfully in the treatment of patients who did not respond to antimonial drugs, and the same thing happened in the treatment of patients with the PKDL form, without report of side effects. The efficacy of 100% on immunocompetent patients earned it the approval by the US Food and Drug Administration (FDA) with the first presentation based on liposomes to be recognized for the treatment of Calazar (visceral leishmaniasis) [Meyerhoff A. 1999 U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin) for treatment of visceral leishmaniasis. Clin Infect Dis 28, 42-48].

[0013] In the case of the antimonial compounds, encapsulated forms in conventional liposomes were also developed [U.S. Pat. No. 4,186,183A; EP72234A; W09604890-A1; U.S. Pat. No. 4,594,241]. In an experimental model of visceral leishmaniasis, these preparations proved to be at least 200 times as effective as the non-encapsulated antimonial in eliminating the parasite in the liver [New, R. R.; Chance, M. L.; Thomas, S. C.; Peters, W.; Nature 1978, 25 272(5648), 55; Alving, C. R.; Steck, E. A.; Chapman, W. L.; Waits, V. B.; Hendricks, L. D.; Swartz, G. M.; Hanson W. L.; Proc. Natl. Acad. Sci. U.S.A. 1978, 75, 2959; Black, C. D. V.; Watson, G. J.; Ward, R. J.; Trans. Roy. Soc. Trop. Med. Hyg. 1977, 71, 550]. Studies on biodistribution/pharmacokinetics of anti-

mmony on mice and dogs showed that the conventional liposomal form promotes much higher and prolonged levels of antimony and in the liver and in the spleen of the animals, as compared with the free form [Collins, M., Carter, K. C., Baillie, A. J., O'Grady, J.; J. Drug Targeting 1993, 1, 133; Frezard e Demicheli. 2010 New delivery strategies for the old pentavalent antimonial drugs. Expert Opin Drug Deliv 7, 1343-58]. However, the low stability of these first formulations explains, at least in part, that none of them came to be marketed.

[0014] More recently, efforts were made with a view to obtain novel formulations of the meglumine antimoniate in conventional liposomes with more favorable technological characteristics. Thus, a novel preparation process was developed which consist in re-hydrogenating lyophilized empty liposomes formed by phosphatidylcoline, cholesterol and dicetylphosphate with an antimonial solution [Frézard F, Michalick M S M, Soares C F, Dermicheli C. 2000. Novel methods for the encapsulation of meglumine antimoniate in liposomes. Braz J. Med Biol Res 33, 841-6]. A significant technological advantage of the method of preparing this formulation, as compared to convention methods, is that the resulting formulation may be stored in the form of lyophilized empty liposomes, and the re-hydration is carried out shortly before administration, which facilitates the preservation and transport thereof.

[0015] A single endovenous injection of the liposomal formulation into healthful dogs resulted in high levels of antimony in the liver and in the spleen, corresponding to about 40% of the injected dose, for a period of 4 days [Schettini D A, Costa Val A P, Souza L F, Demicheli C, Rocha O G F, Melo M N, Michalick M S M, Frezard F. 2003. Distribution of liposome-encapsulated antimony in dogs. Braz J Med Biol Res 36, 269-72]. On the other hand, the levels of antimony in the bone marrow were much lower, about 10 times as low. Besides, after treatment of dogs naturally infected with multiple doses of this formulation, a significant reduction in the number of parasites in the bone marrow was observed, but there was no complete elimination of the parasites in that tissue [Schettini D A, Costa Val A P, Souza L F, Demicheli C, Rocha O G F, Melo M N, Michalick M S M, Frezard F. 2005. Pharmacokinetic and parasitological evaluation of the bone marrow of dogs with visceral 30 leishmaniasis submitted to multiple dose treatment with liposome-encapsulated meglumine antimoniate. Braz J Med Biol Res 38, 1879-83]. These data suggested that the increase in the concentration of antimony in the bone marrow is probably critical to obtain the cure with this formulation. On the basis of the prior art [Carter, K. C.; Dolan, T. F.; Alexander, J.; Baillie, A. J.; McColgan, C.; J. Pharm. Pharmacol. 1989, 41, 87], one raised the hypothesis that the large size of these conventional liposomes (average hydrodynamic diameter larger than 1000 nm) might be responsible for the low routing of the vesicles to the bone marrow.

[0016] Thus, in a subsequent study, the process of preparing the above-mentioned liposomes was modified by introducing a cryoprotector sugar, so as to obtain liposomes of reduced size [Frezard F, 10 Demicheli C, Schettini D A, Ribeiro R R, Melo M N, Michalick M S M. A process for the preparation of pharmaceutical formulations from meglumine antimoniate in liposomes and use of the pharmaceutical formulations on animals attacked by visceral leishmaniasis. Patent application filing at the INPI PI0405489-0, Nov. 11, 2004]. The use of sucrose in the sugar/lipid ratio 3:1 (p/p),

enabled the average diameter of the vesicles to be reduced from 1200 nm to 400 nm. The two formulations of different sizes were administered to naturally infected dogs in the same doses of lipid and of antimony, and were compared by capability of routing the antimony to the bone marrow. The "medium" liposomes (average diameter of 400 nm) promoted, in this tissue, level of antimony 3 to 4 times as high as those achieved with the "big" liposomes (average diameter of 1200 nm) [Schettini D A, Ribeiro R R, Demicheli C, Rocha O G F, Melo M N, Michalick M S M, Frezard F. 2006. Improved targeting of antimony to the bone marrow of dogs using liposomes of reduced size. Int J Pharm 315, 140-7]. This result is of great interest, since it suggests higher efficacy of the reduced-size liposome formulation for eliminating the parasite in the bone marrow of the animals.

[0017] In more recent studies [Ribeiro R R, Moura P, Pimentel V M, Sampalo W M, Silva S M, Schettini D A, Alves C F, Melo F A, Demicheli C, Tafuri W L, Melo 30 M N, Frezard F, Michalick M S M. 2008 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 52, 2564-72], one evaluated the therapeutic efficacy and the toxicity of formulation of the meglumine antimoniate in liposomes of medium size on dogs naturally infected by *Leishmania chagasi*. Groups of 12 animals received, by endovenous route, 4 doses (with intervals of 4 days), either of the meglumine antimoniate encapsulated in liposomes (GI, 6.5 mg Sb/kg/dose), of empty liposomes (GII) or of saline solution (GIII). Parameters marking the renal, hepatic and hematopoietic functions, when evaluated before and right after the treatment, did not exhibit significant alterations, indicating absence of toxicity of the formulation. The parasite load in the bone marrow of the GI showed a significant reduction 4 days after the treatment, as compared to that of the control groups. Immunocytochemical evaluations of the parasite load in the skin, in the lymphnodes, in the liver, in the spleen and in the bone marrow, 5 months after treatment, showed a significant reduction of the lymphnodes, in the liver and in the spleen of the animals of the GI, as compared with the animals of the GII and GIII. When flebotomides *Lutzomyia* (*Lutzomyia*) *longipalpis* were fed to the animals in the different groups 5 months after treatment, a significantly lower infection rate was found in GI, as compared with groups GII and GIII. It should be pointed out that this high therapeutic efficacy was achieved by using a cumulated dose of antimony 25 times as low as that used in the conventional treatment.

[0018] However when cultures of bone marrow of the animals of GI were carried out 5 months after treatment, the presence of the parasite was evidenced in all the animals.

[0019] It should be pointed out that the treatment with AmBisome was not capable of curing dogs naturally infected with *Leishmania infantum* either [Oliva G, Gradoni L, Ciaramella P, et al. Activity of liposomal amphotericin B (AmBisome) in dogs naturally infected with *Leishmania infantum*. J Antimicrob Chemother 1995; 36:1013-19].

[0020] Therefore, the prior art shows that the technologies based on conventional liposomes, available at present for providing the routing of the drug to the infection sites of affected animals, mainly dogs with visceral leishmaniasis, do not enable one to achieve sufficient levels of the drug to eliminate the parasite at determined infection sites. More specifically, the bone marrow seems to be a critical tissue, in which it is necessary to increase the concentration of the drug.

[0021] In the Nineties, different researchers simultaneously reported that prolonged-circulation liposomes can be obtained by incorporating into the membrane of the vesicles a lipid with polar head constituted of ethylene glycol polymer (PEG). In this case, this new class of liposomes was called pegylated, furtive or sterically-stabilized liposomes [Klibanov e Col. 1990. FEBS Letter 268:235-237; Allen e Col. 1991. Biochim Biophys Acta 1066, 29-36; Woodle M C, Lasic D D. 1992 Sterically stabilized liposomes. Biochim Biophys Acta 1113, 171-99]. A non-limiting example of these lipids is di-stearoylphosphatidylethanolamine-PEG (2000) (DSPE-PEG200), incorporated typically in a proportion of 5 to 10 mole % of the total lipids.

[0022] This chemical modification of the vesicle surface has the following impacts on the pharmacokinetics thereof [Frezard F, Schettini D A, Rocha O G F, Demicheli C. 2005 Liposomes: physicochemical and pharmacological properties, applications in antimony-based chemotherapy. Quim Nova 28, 511-20 518]: reduction of the level of opsonization of the liposomes; reduction of the velocity of capturing by the macrophages of the organs of the phagocytic mononuclear system (liver, spleen, bone marrow); prolonged circulation in the blood plasma; preferred accumulation at inflammation/infection sites due to the fact that these sites exhibit increased vascular permeability. This property is being exploited in the development of radiomarked liposomes for diagnose of inflammation/infection sites [Boerman e Col. 1997. Optimization of technetium-99m-labeled PEG liposomes to image focal infection: effects of particle size and circulation time. J Nucl Med 38, 489-493].

[0023] Studies in this area have established that the plasmatic half-life of liposomes containing PEG lipid depends on various factors. The ideal characteristics of the pegylated liposomes are: i) an average diameter of the vesicles ranging from 150 to 200 nm; ii) the use of a PEG having molecular weight of about 2000 Da; iii) incorporation of the PEG-lipid in the relation of 5 mole % with respect to the other lipids [Woodle M C, Lasic D D. 1992 Sterically stabilized liposomes. Biochim Biophys Acta 1113, 171-99].

[0024] Other chemical entities that may replace PEG and impart prolonged-circulation properties to the liposomes are polyvinylpyrrolidine, polyethyloxazolin, polyethyl-oxazolin, polyhydroxypropyl-metacrylamide, polylactic acid, polyglycolic acid and derivatized celluloses, such as hydroxymethylcellulose or hydroxyethylcellulose [Patente U.S. Pat. No. 7,666,674]. The use of lipids having partly fluorinated or perfluoroalkylated hydrophobic chains is another strategy that enables one to prolong the plasmatic half-life of the liposomes [Santaella C, Frezard F, Vierling P, Riess J G. 1993 Extended in vivo blood circulation time of fluorinated liposomes. FEBS Lett. 336:481-4].

[0025] Considering that the liver, the spleen and the bone marrow are the main infection sites in visceral leishmaniasis and that these tissues play a fundamental role in removing the liposomes from the blood circulation, the plasmatic half-life and the distribution of the pegylated liposomes after administration to affected animals cannot be predicted a priori, since the pathologic state may affect the liposome opsonization process and the recognition thereof by the tissue macrophages. In this regard, the efficacy of the camptotecin encapsulated in pegylated liposomes, as compared to its free form, exhibited a significant improvement with regard to the parasite load in the liver, in murine model of visceral leishmaniasis [Proulx, M.-E., Desormeaux, A., Marquis, J.-F.,

Olivier, M., Bergeron M. G. 2001 Treatment of Visceral Leishmaniasis with Sterically Stabilized Liposomes 25 Containing Camptothecin. *Antimicrob Agents Chemother* 45, 2623-7], suggesting that the pegylated liposomes do not accumulate in the liver and are little promising alone, for treating visceral leishmaniasis.

[0026] The present invention relates to a pharmaceutical composition for the treatment of visceral leishmaniasis, characterized by comprising the association of conventional liposomes and prolonged-circulation liposomes as a leishmanicidal-drug delivery system. This composition can be used in preparing a medicament for the treatment of the forms of leishmaniasis, and may be administered by intramuscular, subcutaneous, intraperitoneal or intravenous route. The use of this system enables the pegylated liposomes to promote the more effective routing of leishmanicidal drugs to the bone marrow and the spleen, whereas the conventional liposomes act in routing the drug to the liver.

[0027] According to the present invention, after administration to dogs naturally affected by visceral leishmaniasis, pegylated liposomes containing encapsulated meglumine antimoniate promote a longer half-life ion the blood than the medium-size (medium size of 410 nm) and reduced-size (medium size of 175 nm) (Table 2) conventional liposomes, which establishes the property of prolonged circulation of pegylated liposomes in infected dogs and the potential thereof to distribute the drug to tissues other than the liver. After evaluation of the distribution of antimony in the liver, in the spleen and in the bone marrow 24 hours after endovenous administration of each formulation, the pegylated liposomes promoted a more effective routing to the bone marrow, whereas conventional liposomes of medium size (medium size of 410 nm) resulted in higher levels of antimony in the liver of the animals (Table 3).

[0028] The set of this results validated in naturally infected dogs the model in which the pegylated liposomes enable one to achieve a higher concentration of antimony at infection sites other than the liver and that the conventional liposomes of medium size enable one to achieve a higher concentration of antimony in the liver.

[0029] According to the present invention, the composition characterized by the mixture of the two types of liposomes, that is, conventional liposomes and pegylated liposomes encapsulating meglumine antimoniate was more effective in eliminating parasites in the spleen of mice experimentally infected with *Leishmania infantum chagasi*, as compared with the compositions of each type of liposomes administered separately (Table 4 and FIG. 2). On the other hand, all the compositions exhibited the same efficacy in reducing the parasite load in the liver. Therefore, these results prove, for the first time, the superiority of the mixture of the two types of liposomes as compared with each type of liposomes, in reducing the parasite load in an infection site other than the liver.

[0030] Besides, the present study reports, for the first time, the efficacy of pegylated liposomes and of the mixture thereof with conventional liposomes in reducing the parasite load in the bone marrow of mice (FIG. 3). These results differ from those achieved in the literature with liposomes and niosomes containing antimonial, which did not prove to be capable of reducing the parasite load in this tissue (Carter K C, Baillie A J, Alexander J, Dolan T F. 1988 The therapeutic effect of sodium stibogluconate in BALB/c mice infected with *Leishmania donovani* is organ-dependent. *J Pharm Pharmacol* 40, 370373).

[0031] The proposed innovation differs from the existing technologies, which consist of conventional liposomes [U.S. Pat. No. 4,186,183A; EP72234A; W09604890-A1; U.S. Pat. No. 4,594,241 BR0405489] or of prolonged circulation liposomes [Proulx e Col. 2001 *Antimicrob. Agents Chemother* 45, 2623-7]. The new technology is more effective than those based on conventional liposomes, since it increases the distribution of the drug to other infection sites besides the liver. It is also more effective than those based in prolonged circulation liposomes, since it enables one to achieve a high concentration and action of the drug in the liver.

[0032] The proposed technology may be applied in the treatment of canine and human visceral leishmaniasis, resulting in a more effective and safe treatment. A greater adhesion of the patients to this treatment is also expected as a function of the lower incidence of side effects (because of the smaller total dose of drug administered), of the smaller number of doses and of the longer interval between the doses. It also opens new prospects for the therapeutics of visceral leishmaniasis, with the possibility of achieving parasitologic healing on naturally infected dogs.

#### BRIEF DESCRIPTION OF THE FIGURES

[0033] FIG. 1: Parasite load in the liver of BALB/c mice infected with the *Leishmania (Leishmania) infantum* 043 strain, determined by the limiting dilution technique 8 weeks after infection of the animals and 2 weeks after treatment with the different formulations in single dose by endovenous route (n=8). \*p<0.05 (Kruskal-Wallis test) for comparison between the groups that received liposomes containing encapsulated meglumine antimoniate (MA/AM) and the control group (saline and empty liposomes) (n=8).

[0034] FIG. 2: Parasite load in the spleen of BALB/c mice infected with the *Leishmania (Leishmania) infantum* 043 strain, determined by the limiting dilution technique 8 weeks after infection of the animals and 3 weeks after treatment with the different formulations in single dose by endovenous route (n=8). \* p<0.05 (Kruskal-Wallis test) for comparison between the groups that received encapsulated AM/MA the control group (saline). # p<0.05 (Kruskal-Wallis test) for comparison with the group MIX 200+4.7 with groups Lconv 200 and Lpeg 4.7 ( )n=8).

[0035] FIG. 3: Parasite load in the bone marrow of BALB/c mice infected with the *Leishmania (leishmania) infantum* 043 strain, determined by the limiting dilution technique 8 weeks after infection of the animals and 2 weeks after treatment with the different formulations in single dose by endovenous route (n=8). \* p<0.05 (Kruskal-Wallis test) for comparison with groups Lpeg 4.7 and MIX 200+4.7 and the control groups (saline and empty liposomes). # p<0.05 (Kruskal-Wallis test) for comparison with group MIX 200+4.7 WITH GROUP Lconv 200 (n=8).

#### DETAILED DESCRIPTION OF THE TECHNOLOGY

[0036] The present invention relates to a drug composition for the treatment of visceral leishmaniasis, characterized by comprising the association of conventional liposomes and prolonged-circulation liposomes with a leishmanicidal-drug delivery system. This composition can be used in preparing a medicament for the treatment of the leishmaniasis forms, and may be administered by intramuscular, subcutaneous, intraperitoneal or intravenous route. In this composition, the con-

ventional liposomes may comprise natural or synthetic phospholipids and/or surfactants and/or cholesterol and have an average hydrodynamic diameter ranging from 20 to 1000 nm. [0037] In this composition, the prolonged-circulation liposomes can be obtained by including in the lipid composition liposomes of one lipid or surfactant that results in the increase of the time of circulation of the liposomes in the blood stream. A non-limiting example is given by lipids or surfactants with polar head constituted by ethyleneglycol polymer (PEG), preferably di-stearoylphosphatidylethanolamine-PEG (5000).

[0038] Leishmanicidal drug(s) may be selected from the group consisting of drugs in clinical use at present, including antimony complexes, amphotericin B, pentamidine, miltefosine, allopurinol and paramomycin, but also from the group of pharmaceuticals with leishmanicidal activity, established experimentally, including sitamaquine, iminoquimod, fluconazole, ketoconazol, itraconazol, posaconazol, ticaresol, azitromicin, buparvaquona, tamoxifen, terbinafin, furazolidone, fluoroquinolone, domperidone, interleukin 12, interleukin 10, lipid A, derivatives of alkyl-lisophospholipid and of alkyl-phospholipide, azasterols, lichocalcone A, maesabadi III, trichothecenes, n-acetyl-cistein, 3-substituted quinolin [Monzote L. 2009 The Open Antimicrobial Agents Journal 1, 9-19].

[0039] The invention described herein can be better understood by means of the following non-limiting examples.

#### Example 1

##### Preparation and Characterization of Suspensions of Conventional Liposomes and Pergylated Liposomes Containing the Encapsulated Meglumine Antimoniate

[0040] A lipid film was formed from the di-stearoylphosphatidylcholine (DSPC), cholesterol (COL) and dicetylphosphate (DCP) at the mole ratio 5:4:1 (conventional liposomes) or from DSPC/COL/DCP/di-stearoylphosphatidylethanolamine-PEG(2000) at the mole ratio 4.53:1:4:0.47 (pergylated liposomes). The film was hydrated with distilled water at a temperature of 60° C. and subjected to mechanical stirring, which led to the obtainment of multilamellar-type liposomes.

[0041] Then, the resulting multilamellar-liposome suspension was subjected to ultrasonication (Probe-type Ultrasonic Processor, 500 Watts) at a temperature of 60° C., so as to obtain small uni- and oligo-lamellar liposomes. The resulting suspension was then filtered by using sterile filters having pores of 0.2 µm in diameter. To this suspension an aqueous solution of cryoprotective sugar was added at sucrose/lipid ratio of 1:1 (m/m) (pergylated liposomes and calibrated conventional liposomes) or 3:1 (m/m) (non-calibrated conventional liposomes). Right afterward, this mixture was frozen by immersion into liquid nitrogen and subjected to lyophilization (freeze-dryer, Labconco). At this stage, the lyophilized formulation may be stored at -20° C. for at least 6 months without impairing the final characteristics of size and encapsulation rate of the formulation.

[0042] One day prior to the administration, the lyophilizate was then hydrated with an aqueous solution of the meglumine antimoniate at the concentration of antimony of 80 g/L and using a mass ratio Sb/lipid of about 0.6. In this phase of hydration, the suspension was incubated for 30 min at 60° and subjected to mechanical stirring (use of a vortex) in the time 0 and every 10 min.

[0043] In the case of pegylated liposomes and of calibrated conventional liposomes, the liposome suspension was subjected to the extrusion process by repeated filtrations at 65° C. through polycarbonate membranes with pores having a diameter of 200 nm (Extruder, Lipex biomembrane, Canada). After this step, the liposome suspension was diluted with the sterile saline solution (NaCl 0.9% (m/v)) and subjected to centrifugation at 20,000xg for 40 min at 4° C. The supernatant of the centrifugation was eliminated and the liposome precipitate was washed twice in a sterile saline solution (re-suspension in a sterile saline solution and centrifugation at 20,000xg for 40 min at 4° C.). The liposome precipitate was finally re-suspended in the desired volume of sterile saline solution, typically at the concentration of 10 g/L antimony.

[0044] For determination of the encapsulated antimony rate, the sample was subjected to digestion with nitric acid and the antimony was dosed by graphite furnace atomic absorption spectrometry (ETASS).

[0045] The liposome size was determined by photon correlation spectrometry by using a particle size analyzer (Malvern Instrument, UK).

[0046] The results achieved, with antimony encapsulation rates and the average diameters of liposomes, are shown in Table 1 below.

TABLE 1

Characteristics of conventional liposomes and pergylated liposomes with respect to the meglumine antimoniate encapsulation and to the vesicle size			
Liposome	Encapsulation rate (%)	Average diameter of the vesicles (nm)	Polydispersion rate
Conventional liposomes (non-calibrated)	40	410	0.3
Conventional liposomes (calibrated)	34	175	0.07
Pergylated liposomes	16	154	0.09

[0047] Therefore, the present example shows simple processes for obtaining suspensions of conventional liposomes and of pegylated liposomes containing encapsulated meglumine antimoniate.

#### Example 2

##### Plasmatic Half-Life and Concentrations of Antimony in the Liver, in the Spleen and in the Bone Marrow of Dogs with Visceral Leishmaniasis after Endovenous Administration of the Meglumine-Antimoniate Formulations in Conventional and Pergylated Liposoms

[0048] With a view to determine and compare the plasmatic half-life of conventional liposomes and of prolonged-circulation liposomes, as well as their capability of routing an encapsulated drug to the spleen, liver and bone marrow of animals with visceral leishmaniasis, the suspensions of liposomes containing encapsulated meglumine antimoniate were prepared as described in Example 1 and administered in single dose by endovenous route to dogs naturally infected with *L. chagasi*.

[0049] Three groups of dogs of indefinite race, weight 5 to 15 kg, with proven infection by *L. chagasi*, were used in this experiment. The first group (3 animals) received by endovenous route the formulation of non-calibrated conventional liposomes at the dose of 4.2 mg of Sb/kg of body weight. The second group (5 animals) received by endovenous the formulation of calibrated conventional liposomes at the dose of 6.5 of Sb per kg of body weight. The third group (4 animals) received by endovenous route the formulation of pegylated liposomes at the dose of 3.7 mg of Sb per kg of body weight.

[0050] In the period of 24 hours after administration, blood samples were taken at different intervals of time for antimony dosage by graphite furnace atomic absorption spectrometry (GFAAS). In the period of 24 hours the dogs were sacrificed and samples of the liver, spleen and bone marrow were removed. The liver and spleen were weighed and homogenized. The tissue samples were then subjected to digestion with nitric acid and taken for later determination of antimony by atomic absorption spectrometry in a graphite furnace. The results of half-life of antimony in the blood are shown in Table 2. The results of percentages of antimony dose recovered in the organs are shown in Table 3.

TABLE 2

Half-life of antimony in the blood of dogs affected by visceral leishmaniasis, after endovenous administration of the formulations of conventional and pegylated liposomes		
Half-life of antimony in the blood of dogs with LV		
	After administration of conventional liposomes	After administration of pegylated liposomes
Non-calibrated (410 nm)	Calibrated (175 nm)	224 min
27 min	128 min	

TABLE 3

Percentage of the administered dose of antimony found in the liver, in the spleen and in the bone marrow of dogs affected by visceral leishmaniasis, 24 hours after endovenous administration of the formulations of conventional and pegylated liposomes (data shown as average $\pm$ DP).			
Tissue	Percentage of antimony (%)		
	Conventional liposomes	Pegylated	
Liver	50.0 $\pm$ 8.7	7.7 $\pm$ 2.9	9.2 $\pm$ 1.2
Spleen	6.1 $\pm$ 0.8	3.3 $\pm$ 1.7	6.7 $\pm$ 6.0
Bone marrow	1.4 $\pm$ 0.8	0.6 $\pm$ 0.4	2.0 $\pm$ 0.4

[0051] These data show that conventional liposomes exhibit a short half-life, as compared with pegylated liposomes (Table 2). Therefore, the results achieved confirm that properties of prolonged circulation of pegylated liposomes in naturally infected dogs, as compared with conventional liposomes. Besides, conventional liposomes of medium size promote a greater routing of the antimony to the liver of the animals, whereas pegylated liposomes exhibit a greater routing to the bone marrow while keeping a high routing to the spleen (Table 3).

[0052] The set of these results validates, in naturally infected dogs, the model that pegylated liposomes enable one to achieve a higher concentration of antimony ion other infection sites than the liver, due to their prolonged-circulation characteristic, and that conventional liposomes of medium size enable one to achieve a much higher concentration of antimony in the liver.

[0053] The data obtained suggest that the mixture of conventional liposomes with pegylated liposomes would enable one to enhance the routing of antimony to the bone marrow and to guarantee the routing of the antimony to the liver and spleen of animals affected by visceral leishmaniasis. Therefore, by using this combination, one expects a more effective combat of visceral leishmaniasis.

[0054] It should be further pointed out that the endovenous injection of these pharmaceutical formulations, even with the high dose of antimony administered, was well tolerated in the animals.

## Example 3

Antileishmania Activity of Formulations of  
Conventional liposomes and Pegylated Liposomes  
Containing Meglumine-Antimoniate and of the  
Association of the Two Types of Liposomes in a  
Murine Model of Visceral Leishmaniasis

[0055] BALB/c female mice were infected with *L. infantum chagasi* (M2682—MHM/BR/74/PP75 strain). 14 days after inoculation of the parasites through the caudal vein, the animals received different compositions of conventional liposomes and pegylated liposomes and mixtures thereof in single dose of 10 mg Sb/kg by endovenous route. The mixture consisted of conventional liposomes at the dose of 5 mg Sb/kg and pegylated liposomes at the dose of 5 mg Sb/kg. 14 days after treatment, the animals were killed and the liver and the spleen were taken for evaluation of parasite load by the limiting dilution technique.

[0056] Table 4 shows the proportions of negativated animals (without parasite detected) in the liver and in the spleen.

TABLE 4

Formulation medium diameter	Proportion of negativated animals	
	Liver	Spleen
Non-calibrated conventional liposomes (410 nm)	87.5%*	0%
Calibrated conventional liposomes (175 nm)	100*	12.5%
Pegylated liposomes (154 nm)	87.5%*	0*
Mixture of calibrated conventional liposomes (5 mg Sb/kg) + pegylated liposomes (5 mg Sb/kg)		
Empty non-calibrated conventional liposomes	0%	0%
Empty calibrated conventional liposomes	0%	0%
Saline	0%	0%

TABLE 4-continued

Antileishmania activity of conventional liposomes and of pegylated liposomes containing meglumine antimoniate in BALB/c mice infected with <i>Leishmania infantum chagasi</i> . The data shown are the proportions of negativated animals (without parasite detected) in the liver and in the spleen after treatment by endovenous route with single dose of 10 mg Sb/kg (n = 8 per group).		
	Proportion of negativated animals	
Formulation medium diameter	Liver	Spleen

\*Significant difference with respect to the groups treated with formulations without antimony;  
\*\* - Significant difference with respect to the other groups (P < 005, exact Fisher test).

[0057] The results of Table 4 show that all the formulations exhibited equivalent efficacy in reducing the parasite load in the liver of the mice with respect to the control groups (treated with empty or saline liposomes). However, in the spleen only the formulation associating conventional liposomes and pegylated liposomes proved to be capable of reducing the parasite load significantly.

[0058] This data demonstrates clearly and in a surprising manner the synergism of the two types of liposomes (conventional and pegylated) for reducing the parasite load in the spleen of animals.

[0059] Therefore, these results prove, for the first, time, the superiority of the mixture of the two types of liposomes as compared with each type of liposome in isolation, in reducing the parasite load in an infection site other than the liver.

#### Example 4

##### Efficacy of the Formulations of PEGylated Liposomes and Conventional Liposomes and of the Mixture Thereof in Reducing the Parasite Load in the Liver, in the Spleen and in the Bone Marrow, in a Murine Model of Visceral Leishmaniasis

[0060] The *Leishmania (leishmania) infantum* C43 strain, obtained from an isolate of symptomatic dog and characterized by RFLP, was used for its high capability of infecting the bone marrow of mice.

[0061] BALB/C mice (8 animals per group) were inoculated into the caudal vein with  $1 \times 10^7$  promastigote forms of *Leishmania infantum chagasi*. After six months, the animals received one of the following formulations in single dose by endovenous route;

[0062] 1) Formulation of meglumine antimoniate (MA) in pegylated liposomes of 200 nm (Lpeg 4.7, at the dose of 10 mg Sb/kg);

[0063] 2) Formulation of MA in conventional liposomes of 200 nm (Lconv 200, at the dose of 10 mg Sb/kg);

[0064] 3) Formulation of MA constituted by the mixture of Lpeg 4.7 (5 mg Sb/kg) with Lconv 200 (5 mg Sb/kg) (MIX 200+4.7);

[0065] 4) Formulation of empty conventional liposomes Lconv 200;

[0066] 5) Formulation of empty pegylated liposomes Lpeg 4.7;

[0067] 256) Phosphate buffer 10 mM containing NaCl 0.15 pH 7.4 (PBS).

The MA formulations in liposomes were prepared and characterized as described in Example 1. The Sb and lipid concentrations were adjusted to 3.77 g/L and 49.8 g/L, respec-

tively, with addition of PBS or of a suspension of empty liposomes of the same lipid composition and identical size. Table 5 shows the characteristics of size and rate of encapsulation of the formulations administered, as well as the adjustments made.

[0068] For the preparation of the liposome mixture, equal volumes of the formulations Lconv 200 and Lpeg 4.7 were mixed at the moment immediately preceding the administration of the formulation to the animals.

TABLE 5

Rate of antimony encapsulation and volume adjustments made for the MA/AM formulations in liposomes for efficacy experiment with the C43 strain						
Formulation	Encapsulation rate (%)	Concentration of Sb in the lipos. (mg/mL)	Volume With Sb Lipos. (μL)	Vol. empty lipos. (μL)	Vol. of PBS (μL)	Total volume (μL)
Lpeg 4.7	11	4.40	60.0	—	10	70
Lconv 200	29	11.72	22.5	37.5	10	70

[0069] Two weeks after treatment (8 weeks after inoculation of the parasites), the mice were killed by cervical dislocation, and the liver, spleen and bone marrow were collected for determination of the parasite load through the limiting dilution technique.

[0070] Table 6 shows the characteristics of liposome formulations administered in single dose to BALB/c mice infected with the *L. infantum chagasi* 043 strain. All the formulations exhibited populations with monodispersed vesicles (polydispersion rate <0.3) with an average hydrodynamic diameter close to 200 nm.

TABLE 6

Characteristics of size and doses of Sb and of lipid administered of different formulations of meglumine antimoniate in liposomes.				
Formulation	Diameter (nm)	Polydispersion rate	Dose of Sb administered (mg Sb/Kg)	Dose of lipid administered (mg lipid/Kg)
Lconv 200	193	0.066	10	132
Lpeg 4.7	208	0.084	10	132
MIX 200 + 4.7	205	0.090	10	132
Empty	205	0.035		132
Lconv				
Empty Lpeg	180	0.177		132

[0071] The determination of the parasite load in the liver, spleen and bone marrow of the mice was made 8 weeks after inoculation of the parasites and 2 week after administration of the treatment and, in order to coincide with the peak of the parasite load in the bone marrow.

[0072] FIGS. 1, 2, and 3 show the parasite loads ion the liver, in the spleen and in the bone marrow, respectively.

[0073] The results show that the MA formulation in pegylated liposomes and in the mixture of pegylated and conventional (MIX 200+4.7) promoted significant reductions of the parasite load in all the organs evaluated, as compared with the control treatment (saline or empty liposomes) (FIGS. 1, 2 and 3). On the other hand, the MA formulation ion conventional liposomes promoted a significant reduction of the parasite

load in the liver (FIG. 1) and in the spleen (FIG. 2) of the animals, but not in the bone marrow (FIG. 3).

[0074] When the formulations were compared with respect to the capability of reducing parasite load, the MA formulation in mixture of pegylated liposomes and conventional liposomes proved to be significantly more effective in the spleen, with respect to the formulations of pegylated or conventional liposomes (FIG. 2), and in the bone marrow with respect to the formulation of conventional liposomes (FIG. 3). On the other hand, here was no difference in efficacy between the MA/AM formulations in reducing the parasite load in the liver (FIG. 1).

[0075] This study establishes the superiority of the mixture of pegylated liposomes and conventional liposomes with respect to the individual formulations, in reducing the parasite load in the spleen and in the bone marrow in a model of visceral leishmaniasis.

**1-7. (canceled)**

8. A pharmaceutical composition for treatment of visceral leishmaniasis, characterized by comprising association of conventional liposomes and prolonged-circulation liposomes, incorporating one or more leishmanicidal drugs.

9. The pharmaceutical composition of claim 8, characterized in that the conventional liposomes comprise natural and/or synthetic phospholipid(s) and/or surfactant(s) and/or cholesterol and have an average diameter ranging from 50 nm to 1000 nm.

10. The pharmaceutical composition of claim 8, characterized in that the prolonged-circulation liposomes include a lipid or surfactant that results in increased time of circulation of the liposomes in the blood stream.

11. The pharmaceutical composition of claim 10, characterized in that the lipid or surfactant has its polar head formed of ethyleneglycol polymer.

12. The pharmaceutical composition of claim 8, characterized in that the one or more leishmanicidal drugs are selected from the group consisting of antiomonal compounds, amphotericin B, pentamidin, miltefosine, allopurinol, paromomycin, sitamaquin, sitamaquine, iminoquimod, fluconazole, ketoconazol, itraconazol, posaconazol, ticaresol, azitromicin, buparvaquone, tamoxifen, terbinafin, furazolidone, fluoroquinolone, domperidone, interleukin 12, interleukin 10, lipid A, derivatives of alkyl-lisophospholipid and of alkyl-phospholipide, azasterols, lichocalcone A, maesabadi III, trichothecenes, N-acetylcysteine, 3-substituted quinolin, and/or other drugs usually employed for treatment of leishmaniasis.

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13. The pharmaceutical composition of claim 8, which is formulated for administration by intramuscular, subcutaneous, intraperitoneal, and/or intravenous route.

14. A method for treatment of a form of leishmaniasis in a mammal, the method comprising administering a pharmaceutical composition, which is characterized by association of conventional liposomes and prolonged-circulation liposomes that incorporate one or more leishmanicidal drugs.

15. The method according to claim 14, wherein said conventional liposomes comprise natural and/or synthetic phospholipid(s) and/or surfactant(s) and/or cholesterol and have an average diameter ranging from 50 nm to 1000 nm.

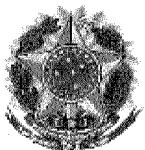
16. The method according to claim 14, wherein said prolonged-circulation liposomes include a lipid or surfactant that results in increased time of circulation of the liposomes in the blood stream.

17. The method according to claim 16, wherein said lipid or surfactant has its polar head formed of ethyleneglycol polymer.

18. The method according to claim 14, wherein said one or more leishmanicidal drugs are selected from the group consisting of antiomonal compounds, amphotericin B, pentamidin, miltefosine, allopurinol, paromomycin, sitamaquin, sitamaquine, iminoquimod, fluconazole, ketoconazol, itraconazol, posaconazol, ticaresol, azitromicin, buparvaquone, tamoxifen, terbinafin, furazolidone, fluoroquinolone, domperidone, interleukin 12, interleukin 10, lipid A, derivatives of alkyl-lisophospholipid and of alkyl-phospholipide, azasterols, lichocalcone A, maesabadi III, trichothecenes, N-acetylcysteine, 3-substituted quinolin, and/or other drugs usually employed for treatment of leishmaniasis.

19. The method according to claim 14, wherein said pharmaceutical composition is administered by intramuscular, subcutaneous, intraperitoneal, and/or intravenous route.

\* \* \* \* \*



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Ministério do Desenvolvimento, Indústria  
e do Comércio Exterior  
Instituto Nacional da Propriedade Industrial

(11) (21) PI 0405489-0 A



(22) Data de Depósito: 09/11/2004

(43) Data de Publicação: 13/06/2006

(RPI 1849)

(51) Int. Cl<sup>7</sup> :

A61K 9/127

A61K 33/24

A61P 33/02

(54) Título: PROCESSO PARA A PREPARAÇÃO DE FORMULAÇÕES FARMACÊUTICAS DO ANTIMONIATO DE MEGLUBINA EM LIPOSSOMAS E USO DAS FORMULAÇÕES FARMACÊUTICAS EM ANIMAIS ACOMETIDOS COM LEISHMANIOSE VISCERAL

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(57) Resumo: "PROCESSO PARA A PREPARAÇÃO DE FORMULAÇÕES FARMACÊUTICAS DO ANTIMONIATO DE MEGLUMINA EM LIPOSSOMAS E USO DAS FORMULAÇÕES FARMACÊUTICAS EM ANIMAIS ACOMETIDOS COM LEISHMANIOSE VISCERAL". A presente invenção se refere a novas formulações farmacêuticas do entimoniato de meglumina em lipossomas de tamanho reduzido, que apresentam um direcionamento aumentado para a medula óssea em cães acometidos com leishmaniose visceral. Refere-se ainda a um processo de preparação dessas formulações, que consiste na desidratação de uma mistura de lipossomas preparados em água com um açúcar crioprotetor, seguida pela reidratação do produto desidratado com uma solução aquosa do antimoniato de meglumina. A presente invenção é de uso eficaz em animais acometidos com leishmaniose visceral.

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As leishmanioses são doenças parasitárias causadas por protozoários  
15 flagelados pertencentes a Ordem *Kinetoplastida*, Família *Trypanosomatidae* e ao Gênero *Leishmania*. Estas atingem cerca de 12 milhões de pessoas no mundo [UNDP/World Bank/WHO Special Programme For Research & Training In Tropical Diseases (TDR). Em *Tropical Disease Research Programme Report, 13; progress 1995-96*; World Health Organization: Geneva, 1997, cap.  
20 8]. No Brasil, dados recentes relatam a ocorrência de cerca de 30.000 novos casos anuais da doença. As leishmanioses são transmitidas aos hospedeiros vertebrados pela picada de um inseto que regurgita o parasita na forma promastigota. Esses parasitas são fagocitados por macrófagos, no interior dos quais estes se transformam em amastigotas. Os amastigotas se multiplicam

livremente no compartimento ácido dos fagolisossomos e escapam dos sistemas de defesa do hospedeiro. A *Leishmania* corresponde a um complexo de várias espécies diferentes que causam vários tipos de manifestações clínicas que incluem formas cutânea, mucocutânea e visceral. No Brasil, a 5 espécie causadora da leishmaniose visceral é a *Leishmania chagasi*. O cão aparece como hospedeiro vertebrado nas formas cutânea e visceral e, particularmente no caso da leishmaniose visceral, tem papel importante como reservatório e fonte de infecção da doença em área endêmica. A leishmaniose 10 visceral apresenta índice de letalidade de 100% nos casos não tratados clinicamente.

A partir da década de 1940, complexos de antimônio pentavalente (Sb(V)) começaram a ser utilizados na terapêutica das leishmanioses [Marsden, P. D.; *Rev. Soc. Bras. Med. Trop.* 1985, 18, 187; Berman, J. D.; *Clin. Infect. Dis.* 1997, 24, 684; Rath, S.; Trivelin, L. A.; Imbrunito, T. R.; Tomazela, 15 D. M.; de Jesus, M. N.; Marzal, P. C.; Junior H. F. A.; Tempone, A. G.; *Quim. Nova* 2003, 26, 550]. Os principais antimoniais atualmente em uso são complexos de Sb(V) com o N-metil-glucamina (antimoniato de meglumina) e com o gluconato de sódio (estibogluconato de sódio). Foi sugerido que o Sb(V) seria uma pró-droga, sendo reduzido no organismo hospedeiro a Sb(III) que 20 seria a forma ativa e tóxica. Embora os antimoniais pentavalentes continuam sendo os medicamentos de primeira escolha no tratamento de todas as formas de leishmanioses, o seu uso clínico apresenta várias limitações. Esses compostos devem ser administrados por via parenteral (injeção intravenosa ou intramuscular), diariamente, num período de 20-40 dias. Nesse contexto,

efeitos colaterais são freqüentes. O aparecimento de casos de resistência ao tratamento representa também um problema sério na terapêutica das leishmanioses. Uma outra dificuldade encontrada no controle da leishmaniose visceral é que não existe até hoje tratamento eficaz para os cães naturalmente

5 infectados.

Em face dessas limitações, a Organização Mundial da Saúde recomenda, com incentivo inclusive a outras entidades afins como a TDR ("Special Programme for Research and Training in Tropical Diseases"), a pesquisa de novos medicamentos [Remme, J. H. F.; Blas, E.; Chitsulo, L.;  
10 Desjeux, P. M. P.; Engers, H. D.; Kanyok, T. R.; Kayondo, J. F. K.; Kiroy, D. W.; Kumaraswami, V.; Lasdins, J. K.; Nunn, P. P.; Oduola, A.; Ridley, R. G.; Toure, Y. T.; Zicker, F.; Morel, C. M.; *Trends Parasitol.* 2002, 18, 421; Ridley, R. G. Em *Drug against Parasitic Diseases*; Fallamb, A. H. ; Ridley, R. G. ; Vial, H. J., eds.;  
15 UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR); Geneva, 2003, p. 13].

Essencialmente três estratégias diferentes estão atualmente disponíveis para o desenvolvimento de novos medicamentos.

Uma primeira estratégia consiste na combinação de diferentes agentes leishmanicidas com ação sinérgica. Por exemplo, a associação da gentamicina  
20 à paromomicina aumentou a eficácia da paromomicina em aplicação tópica [WO9406439-A1]. A combinação de aminosidina com estibogluconato de sódio, por sua vez, mostrou ser uma medida efetiva no tratamento da forma visceral não responsiva ao tratamento convencional. De forma semelhante, a associação desse antimonal com outra droga em avaliação clínica, o

alopurinol, se mostrou eficaz até em casos de resistência à antimoniais [Martinez, S.; Gonzalez, M.; Vemaza, M. E.; *Clin Infect Dis* 1997, 24, 165]. A imuno-quimioterapia, que associa substâncias imunomoduladores aos antimoniais pentavalentes, se revelou ser uma forma de reduzir a dose 5 aplicada de antimonal, mantendo eficácia do tratamento [Murray, H. W.; Berman, J. D.; Wright, S. D.; *J Infect Dis* 1988, 157, 973; Machado-Pinto, J.; Pinto, J.; da Costa, C. A.; Genaro, O.; Marques, M. J.; Modabber, F.; Mayrink, W.; *Int. J. Dermatol.* 2002, 41, 73].

Uma segunda estratégia envolve o planejamento/síntese de novas 10 substâncias ativas ou de fármacos já conhecidos com modificações químicas. Entretanto, o tempo longo e o custo elevado do desenvolvimento de um novo fármaco representam importantes limitações dessa estratégia.

A terceira estratégia envolve a associação reversível de fármacos já em uso a um sistema transportador, visando direcionar melhor o fármaco para a 15 célula alvo e evitar os locais indesejáveis onde o fármaco exerce toxicidade. Esta estratégia, além de prolongar a validade de proteção por patente no uso do fármaco, oferece um ganho de tempo na fase de desenvolvimento do produto porque usa um fármaco já caracterizado do ponto de vista farmacológico. Entre os sistemas transportadores de medicamentos atualmente 20 disponíveis, os lipossomas ocupam uma posição de destaque, tanto para terapia das leishmanioses quanto da esquistossomose.

Os lipossomas são vesículas esféricas, constituídas de uma ou várias bicamadas concêntricas de lipídeos, que isolam um ou vários compartimentos aquosos internos do meio externo [Frézard, F.; *Braz. J. Med. Biol. Res.* 1999,

32, 181]. Uma grande vantagem dos lipossomas, com relação a outros sistemas transportadores de medicamento, é sua elevada biocompatibilidade, especialmente quando estes são formados de lipídeos pertencentes às famílias de lipídeos naturais. Além disso, são sistemas altamente versáteis, cujas 5 características, como tamanho, lamelaridade, superfície, composição lipídica, volume e composição do meio aquoso interno podem ser manipuladas em função dos requisitos farmacêuticos e farmacológicos. A taxa de encapsulação de uma substância em lipossomas e a relação substância encapsulada/lipídeo são dois parâmetros importantes que devem ser considerados na escolha do 10 processo de preparação/encapsulação, sobretudo quando se procura desenvolver uma composição farmacêutica. Esses parâmetros podem ser otimizados através da escolha do método de encapsulação e da manipulação da composição lipídica da membrana. A taxa de encapsulação deverá ser maximizada, pois é inversamente relacionada à quantidade de substância não 15 encapsulada que é perdida na maioria das vezes. A relação fármaco/lipídeo deverá também ser maximizada, visto que determina a quantidade de lipídeo a ser administrada ao paciente. Assim, quanto menor for a quantidade de lipídeo veiculada, menor serão os riscos de efeitos colaterais associados aos mesmos.

Em 1977-78, grupos de pesquisa inglês e americano propuseram o uso 20 de lipossomas convencionais contendo drogas leishmanicidas como uma nova abordagem para o tratamento da leishmaniose visceral [US4186183; New, R. R.; Chance, M. L.; Thomas, S. C.; Peters, W.; *Nature* 1978, 272(5648), 55; Alving, C. R.; Steck, E. A.; Chapman, W. L.; Waits, V. B.; Hendricks, L. D.; Swartz, G. M.; Hanson W. L.; *Proc. Natl. Acad. Sci. U.S.A.* 1978, 75, 2959;

Black, C. D. V.; Watson, G. J.; Ward, R. J.; *Trans. Roy. Soc. Trop. Med. Hyg.* 1977, 71, 550]. Esta proposta foi baseada na observação de que antimoniais pentavalentes encapsulados em lipossomas foram 200-700 vezes mais eficazes do que na forma livre, em modelo experimental de leishmaniose visceral (hamsters ou camundongos infectados por *Leishmania donovani*). O aumento espectacular de eficácia dessas drogas foi atribuído ao destino natural dos lipossomas quando administrados por via endovenosa: o fato de eles serem captados pelos mesmos órgãos (o fígado, o baço e a medula óssea) e pelas mesmas células (os macrófagos teciduais) nas quais se localiza o parasita. Assim, os lipossomas convencionais apresentam-se como sistema ideal para direcionar drogas leishmanicidas para o local de infecção. Estudos de biodistribuição/farmacocinética do antimônio em camundongos e cães mostraram que a forma lipossomal promove níveis de antimônio muito mais elevados e prolongados no fígado e no baço dos animais quando comparado à forma livre [Collins, M., Carter, K. C., Baillie, A. J., O'Grady, J.; *J. Drug Targeting* 1993, 1, 133; Schettini, D. A.; Costa Val, A. P.; Souza, L. F.; Demicheli, C.; Rocha, O. G. F.; Melo, M. N.; Michalick, M. S. M.; Frézard, F.; *Braz. J. Med. Biol. Res.* 2003, 36, 269].

Apesar da necessidade de aprimorar a quimioterapia antimonial atual e dos resultados extremamente promissores obtidos com os lipossomas em modelos experimentais de leishmaniose visceral, até hoje, nenhuma composição farmacêutica associando lipossomas e antimoniais chegou a ser comercializada. Este fato pode ser atribuído, pelo menos em parte, às dificuldades tecnológicas inerentes a obtenção de formulação estável de

compostos hidrossolúveis encapsulado em lipossomas.

O primeiro processo proposto para a encapsulação dos antimoniais pentavalentes em lipossomas foi o método de "hidratação do filme". Este consiste na hidratação de um filme lipídico com uma solução aquosa do antimonal, levando à formação de lipossomas multilamelares chamados de "MLVs" ("multilamellar vesicles"). O estado da técnica relata a preparação de lipossomas MLVs contendo antimoniais, seja a partir de fosfolipídeos convencionais [US4186183] ou de surfactantes não iônicos [WO9604890]. As maiores taxas de encapsulação e de retenção foram obtidas com lipossomas constituídos de lipídeo de alta temperatura de transição de fase, de colesterol e de lipídeo carregado negativamente.

Um outro processo proposto para a encapsulação do estibogluconato de sódio em lipossomas usa o método de "evaporação em fase reversa" [US4594241; US4235871] que leva à formação de lipossomas oligolamelares de tipo "REVs" ("reverse phase evaporation vesicles"). Esse último método permite alcançar maiores taxas de encapsulação que o método de hidratação do filme. Entretanto, lipossomas de tipo REV se mostraram menos estáveis que aqueles do tipo MLVs, sendo que 25-50% de antimônio foi liberado da preparação após uma semana de incubação em 25 °C. Por outro lado, o uso de solvente orgânico na preparação desses lipossomas representa um inconveniente, sendo que sempre permanecem resíduos potencialmente tóxicos na formulação final.

Um outro inconveniente dos dois métodos apresentados anteriormente é que as preparações resultantes só podem ser armazenadas na forma de

suspensão aquosa, o que limita consideravelmente o seu potencial como produto farmacêutico.

Recentemente, foi relatado no estado da técnica o emprego do método de “desidratação-reidratação”, desenvolvido por Kirby e Gregoriadis [Kirby, C.; Gregoriadis, G.; *Biotechnology* 1984, 2, 979], para a encapsulação do antimoniato de meglumina em lipossomas formados de diestearoilfosfatidilcolina, colesterol e dicetilfosfato [Frézard, F.; Michalick, M. S. M.; Soares, C. F.; Demicheli, C.; *Braz. J. Med. Biol. Res.*, 2000, 33, 841]. Este método consiste em misturar uma suspensão de lipossomas pequenos vazios (preparados em água) com uma solução aquosa de antimoniato de meglumina e em desidratar a mistura resultante. A reidratação dessa preparação em condições específicas de temperatura e de concentração lipídica leva à obtenção de lipossomas com elevada taxa de encapsulação (> 30%), chamados de “DRVs” (“dehydration rehydration vesicles”).

Uma alternativa a esse processo, encontrada no estado da técnica, consiste em liofilizar os lipossomas pequenos vazios (preparados em água) sem o antimoniato e em reidratar o liofilizado, chamado de “FDELs” (“freeze-dried empty liposomes”), com a solução de antimoniato [Frézard, F.; Michalick, M. S. M.; Soares, C. F.; Demicheli, C.; *Braz. J. Med. Biol. Res.*, 2000, 33, 841]. Esse método apresenta a vantagem de não submeter o antimoniato à liofilização, além de permitir uma elevada taxa de encapsulação (> 30%). Uma patente foi depositada para esse processo [US5376380], entretanto, refere-se especificamente à encapsulação de substâncias que apresentam carga elétrica efetiva oposta àquela dos lipídeos formando os lipossomas.

Uma grande vantagem desses dois últimos processos com relação aos primeiros é que a formulação pode ser armazenada por tempo prolongado na forma liofilizada intermediária e reidratada um pouco antes do uso.

Vale ressaltar que os diversos tipos de lipossomas obtidos por esses

5 diferentes processos apresentam diâmetro médio elevado (600 a 5000 nm) e distribuição de tamanho heterogênea. Essas características representam um problema potencial, pois a administração endovenosa de lipossomas de tamanho grande (>600 nm) pode levar a obstrução dos capilares pulmonares.

Além disso, o tamanho dos lipossomas é um parâmetro importante, pois tem  
10 influência tanto na taxa de eliminação do parasita [Carter, K. C.; Dolan, T. F.; Alexander, J.; Baillie, A. J.; McColgan, C.; *J. Pharm. Pharmacol.* 1989, 41, 87], quanto na concentração de antimônio ao nível da medula óssea [Schettini, D. A.; Costa Val, A. P.; Souza, L. F.; Demicheli, C.; Rocha, O. G. F.; Melo, M. N.; Michalick, M. S. M.; Frézard, F.; *Braz. J. Med. Biol. Res.* 2003, 36, 269].

15 Portanto, lipossomas pequenos (diâmetro < 600 nm) parecem ter acesso facilitado à medula óssea, um dos principais locais de infecção no caso da leishmaniose visceral, o que é de interesse para o controle da leishmaniose visceral, pois devem facilitar a eliminação dos parasitas que se encontram nesse tecido.

20 O estado da técnica relata processos para a obtenção de suspensão de lipossomas de tamanho reduzido e mais homogêneo, utilizando o método de desidratação-reidratação, baseados no uso de açúcares crioprotetores [Zadi, B.; Gregoriadis, G.; *J. Lipos. Res.* 2000, 10, 73-80; WO0156548; WO9965465; US5922350; US5837279; US5817334; US5578320; WO8601103; US4880635].

Entretanto, não se encontra descrito, no estado da técnica, um processo que associa o método de redução do tamanho dos lipossomas (diâmetro médio < 600 nm) pelo uso de açúcar crioprotetor com o método de reidratação de lipossomas vazios desidratados com uma a solução aquosa do antimoniato de meglumina.

Nesse contexto, a presente invenção compreende um novo processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas, que consiste na desidratação de uma mistura de lipossomas preparados em água com um açúcar crioprotetor, seguida pela reidratação do produto desidratado com uma solução aquosa do antimoniato de meglumina. O referido processo compreende as seguintes etapas: 1- preparação de uma suspensão de lipossomas uni- ou oligo-lamelares em água; 2- adição de açúcar crioprotetor à suspensão de lipossomas vazios; 3- desidratação da suspensão de lipossomas resultante; 4- reidratação da suspensão de lipossomas desidratada com uma solução aquosa do antimoniato de meglumina.

O processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas pode também incluir etapas adicionais, tais como a de reidratação com solução salina, a de calibração do tamanho das vesículas por filtração através de membrana porosa e a de purificação dos lipossomas formados por centrifugação, seguida pela resuspensão dos lipossomas em solução salina aceitável do ponto de vista fisiológico.

O processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas é caracterizado pelo uso da

liofilização, da evaporação sob vácuo ou da secagem por pulverização, como processo de desidratação.

De acordo com a presente invenção, o processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas 5 caracteriza-se também pelo uso dos seguintes lipídeos: fosfatidilcolina, tal como o dipalmitoilfosfatidilcolina ou diestearoilfosfatidilcolina, ou surfactante não iônico; colesterol; lipídeo apresentando uma carga elétrica negativa, tal como dicetilfosfato, dimiristoilfosfatidilglicerol, dipalmitoilfosfatidilglicerol ou diestearoilfosfatidilglicerol; com cada componente lipídico presente em relação 10 molar de 40-70%, 10-40%, e 10-50%, respectivamente.

O processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas é caracterizado ainda pelo uso de pelo menos um açúcar crioprotetor do grupo, trealose, maltose, sacarose, glicose, lactose, dextran e ciclodextrina, utilizado na relação açúcar/lipídeo 1:1 15 a 5:1 (m/m) e em concentração inicial de 10 a 500 mmol/L na solução submetida a desidratação.

Vale ressaltar que o processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas, objeto da presente invenção, ao contrário dos processos de preparação encontrados no estado da 20 técnica, combina as seguintes vantagens: elevada taxa de encapsulação do antimoniato de meglumina em lipossomas (tipicamente superior a 35%); elevada relação final Sb/lipídeo (tipicamente superior a 0,2:1 (m/m)); tamanho reduzido dos lipossomas (tipicamente, diâmetro médio < 600 nm); não-exposição do antimonial à desidratação e a possibilidade de armazenar a

preparação no estado desidratado intermediário e de reidratá-la pouco antes do uso.

Foi demonstrado que as formulações farmacêuticas do antimoniato de meglumina em lipossomas, obtidas segundo o processo descrito na presente invenção, quando administradas por via endovenosa em cães acometidos com leishmaniose visceral, produzem uma concentração de antimônio maior na medula óssea, com relação àquela produzida por formulações do antimoniato de meglumina em lipossomas preparadas segundo o mesmo processo mas sem açúcar crioprotetor. Como a medula óssea é um dos principais sitio de infecção na leishmaniose visceral, as formulações farmacêuticas da presente invenção oferecem um controle mais eficaz da leishmaniose visceral, especialmente em cães que não respondem à quimioterapia antimonial convencional.

As formulações farmacêuticas da presente invenção, ao contrário das várias outras relatadas no estado da técnica para o controle da leishmaniose visceral, dizem respeito ao rejuvenescimento de um princípio ativo (o antimonial) já em uso clínico há várias décadas, mas não ao desenvolvimento de um novo fármaco, o que representa uma vantagem por economizar tempo e dinheiro nas diversas fases de desenvolvimento de um produto. Outras vantagens dessas formulações, com relação às outras soluções relatadas no estado da técnica, são a reconhecida e elevada eficácia dos antimoniais no tratamento das leishmanioses e os benefícios sócio-econômicos, tais como a redução dos gastos públicos com o controle da leishmaniose visceral, a redução do número de casos de desistência do tratamento e a redução do risco

de aumento do número de casos de resistência ao tratamento.

A presente invenção está também relacionada ao uso das formulações farmacêuticas, obtidas segundo processo descrito na presente invenção, em animais acometidos com leishmaniose visceral, na ausência ou presença de 5 outros compostos farmacologicamente ativos, tais como substâncias apresentando atividade imunomoduladora.

A invenção, aqui descrita, pode ser melhor entendida pelos seguintes exemplos que não são limitantes.

Exemplo 1 – Processo de preparação de formulações farmacêuticas do 10 antimoniato de meglumina em lipossomas de tamanho reduzido.

Um filme lipídico foi formado a partir dos lipídeos diestearoilfosfatidilcolina, colesterol e dicetilfosfato, na relação molar 5:4:1. O filme foi hidratado com água destilada em temperatura de 50 a 65°C e submetido à agitação mecânica, o que levou a obtenção de lipossomas de tipo 15 multilamelar.

Em seguida, a suspensão de lipossomas multilamelares resultante foi submetida à ultrassonicação (Ultrasonic Processor, 500 Watts de tipo sonda) em temperatura de 50 a 65°C, de forma a obter lipossomas pequenos uni- e oligo-lamelares. A suspensão resultante foi então filtrada, usando filtros estéris 20 de poros de diâmetro de 0,2 µm.

À suspensão de lipossomas pequenos assim obtida, foi acrescenta uma solução aquosa de açúcar crioprotetor na relação açúcar/lipídeo 0:1 (m/m), 1:1 (m/m) ou 3:1 (m/m). Logo em seguida, essa mistura foi congelada por imersão em nitrogênio líquido e submetida à lyofilização (freeze-dryer, Labconco).

O liofilizado foi então hidratado com uma solução aquosa do antimoniato de meglumina na concentração de antimônio de 70 a 80 g/L e usando uma relação mássica Sb/lipídeo de aproximadamente 0,6. Nessa fase de hidratação, a suspensão foi incubada por 20 min a 50 a 65°C e submetida a agitação

5 mecânica (uso de um vortex) no tempo 0 e a cada 10 min.

Foi acrescentada ainda uma solução salina estéril em volume semelhante àquele da solução de antimonial acrescentada previamente ao liofilizado e a suspensão foi novamente incubada por 20 min em temperatura de 50 a 60°C e submetida à agitação mecânica.

10 Depois dessa etapa, a suspensão de lipossomas foi diluída com uma solução salina estéril (NaCl 0,9% (m/v)) e submetida a centrifugação em 10.000 x g por 40 min. O sobrenadante da centrifugação foi eliminado e o pelete de lipossomas foi lavado duas vezes em solução salina estéril (resuspensão em solução salina estéril e centrifugação em 10.000 x g por 40 min).

15 O pelete de lipossomas foi finalmente resuspendedido no volume desejado de solução salina estéril.

Para a determinação da taxa de antimônio encapsulada, a amostra foi submetida à digestão com ácido nítrico e o antimônio foi dosado por espectrometria de emissão de plasma (ICP-OES).

20 O tamanho dos lipossomas foi determinado por espectrometria de correlação de fótons, usando um analisador de tamanho de partícula (Malvern Instrument). Os resultados obtidos, com as taxas de encapsulação de antimônio e os diâmetros médios de lipossomas, são mostrados na Tabela 1 abaixo.

Tabela 1. Influência de açúcar crioprotetor nas características dos lipossomas

contendo o antimoniato de meglumina encapsulado

Tipo de lipossoma	Relação açúcar/lipídeo (m/m)	Diâmetro	Taxa encapsulação
		médio (nm) ±DP <sup>a</sup>	(% Sb) ±DP <sup>a</sup>
Lipossomas	0	1205±451	39±3
Lipossomas + sacarose <sup>b</sup>	1:1	514±100	43±4
Lipossomas + trealose <sup>b</sup>	3:1	312±30	40±4
Lipossomas + hidroxipropil-beta-ciclodextrina <sup>c</sup>	3:1	463±182	53±9
Lipossomas + hidroxipropil-beta-ciclodextrina <sup>c</sup>	3:1	197	7,5

<sup>a</sup>DP: desvio padrão ( $n = 2-3$ ); <sup>b</sup>Relação inicial Sb/lipídeo = 0,6:1 (m/m);

<sup>c</sup>Relação inicial Sb/lipídeo = 0,25:1 (m/m)

Vale ressaltar ainda que taxas de encapsulação de antimônio baixas, inferiores

5 a 15%, foram obtidas, quando não foi usado lipídeo carregado negativamente na composição dos lipossomas das formulações farmacêuticas da presente invenção.

Esses resultados mostram que a introdução de açúcar crioprotetor no processo de “desidratação de lipossomas vazios/reidratação com solução de antimoniato

10 de meglumina” resulta na obtenção de lipossomas de tamanho reduzido, de diâmetro médio tipicamente inferior a 600 nm. Além disso, a inclusão dos açúcares crioprotetores, sacarose e trealose, não alterou de forma significativa a taxa de encapsulação de antimônio, que permaneceu elevada (superior a 35%), nem a relação final Sb/lipídeo (superior a 0,2:1 (m/m)).

No caso das formulações farmacêuticas preparadas na presença de sacarose, observa-se que a relação açúcar/lipídeo 3:1 foi mais eficiente que a relação 1:1, na redução do tamanho dos lipossomas.

De acordo com esses resultados, fica comprovado que o processo, que  
5 consiste da desidratação de uma mistura de lipossomas preparados em água com um açúcar crioprotetor, seguida pela reidratação do produto desidratado com uma solução aquosa do antimoniato de meglumina, permite a obtenção de uma suspensão de lipossomas de tamanho reduzido (diâmetro médio < 600 nm) apresentando elevada taxa de encapsulação de antimônio e relação final  
10 antimônio/lipídeo.

Portanto, o presente exemplo mostra um processo simples, que permite a obtenção de formulações farmacêuticas, cujo tamanho de lipossomas é compatível com a administração endovenosa em animais e cujas características de encapsulação do antimoniato de meglumina são compatíveis  
15 com o desenvolvimento de um produto farmacêutico. O fato de poder armazenar essas formulações farmacêuticas no estado desidratado intermediário e reidratá-las pouco antes do uso e o fato de não submeter o antimonal à liofilização garantem a estabilidade da preparação e a integridade do antimonal.

20 Exemplo 2 – Nível de antimônio na medula óssea de cães acometidos com leishmaniose visceral, após administração endovenosa de formulação farmacêutica do antimoniato de meglumina em lipossomas da presente invenção.

Visando avaliar a capacidade das formulações farmacêuticas do antimoniato de

meglumina em lipossomas, preparadas como descrito no Exemplo 1, de direcionar o antimônio para a medula óssea de cães acometidos com leishmaniose visceral, foi realizado um estudo em cães naturalmente infectados com *L. chagasi*, comparando o nível de antimônio alcançado na medula óssea

- 5      após administração da formulação de lipossomas preparada na presença de sacarose (lipossomas de tamanho reduzido) com aquele após administração da formulação de lipossomas preparada na ausência de açúcar (lipossomas de tamanho grande).

Foram utilizados lipossomas preparados a partir de diestearoilfosfatidilcolina,

- 10     colesterol e dicetilfosfato, na relação molar 5:4:1, na ausência ou presença de sacarose (relação açúcar/lipídeo = 3:1 (m/m)), como descrito no Exemplo 1.

Dois grupos de cães sem raça definida, pesando de 5 a 15 kg, com infecção comprovada com *L. chagasi*, foram utilizados nesse experimento. O primeiro

- 15     grupo (10 animais) recebeu por via endovenosa a formulação de lipossomas preparada na ausência de açúcar (diâmetro médio = 1205 nm) na dose de 6,8 mg de Sb por kg de peso corporal. O segundo grupo (5 animais) recebeu por via endovenosa a formulação de lipossomas preparada na presença de sacarose (diâmetro médio = 312 nm) na dose de 6,8 mg de Sb por kg de peso corporal.

- 20     Noventa e seis horas após a administração, uma amostra foi retirada da medula óssea de cada cão. As amostras biológicas foram digeridas com ácido nítrico e levadas para dosagem de antimônio por espectrometria de absorção atômica em forno de grafite. Os resultados obtidos são mostrados abaixo na Tabela 2.

Tabela 2. Níveis de antimônio na medula óssea de cães acometidos com leishmaniose visceral, 96 horas após administração endovenosa da formulação de lipossomas de tamanho reduzido (preparada na presença de açúcar crioprotetor) e da formulação de lipossomas de tamanho elevado (preparada na ausência de açúcar).

Nível de antimônio na medula óssea de cães infectados ( $\mu\text{g de Sb/g de tecido}$ )	
Após lipossomas grandes	Após lipossomas pequenos
$0,76 \pm 0,76$	$2,19 \pm 0,61$

Esses dados demonstram claramente que as formulações farmacêuticas do antimoniato de meglumina em lipossomas, obtidas de acordo com a presente invenção, em consequência do uso de açúcar crioprotetor e do tamanho reduzido dos lipossomas, apresentam um direcionamento significativamente aumentado para a medula óssea em cães acometidos com leishmaniose visceral. Como a medula óssea é um dos principais sítios de infecção na leishmaniose visceral, as formulações descritas na presente invenção resultam num combate mais eficaz da leishmaniose visceral canina.

Vale ressaltar ainda que a injeção endovenosa dessas formulações farmacêuticas, mesmo com a elevada dose de antimônio administrada, foi bem tolerada nos animais, pois não provocou morte dos mesmos.

## REIVINDICAÇÕES

1. Processo para a preparação de formulações farmacêuticas do antimoniato de meglumina em lipossomas, caracterizado pelas seguintes etapas sucessivas: 1- preparação de uma suspensão de lipossomas uni- ou oligolamelares em água; 2- adição de açúcar crioprotetor à suspensão de lipossomas vazios; 3- desidratação da suspensão de lipossomas resultante; 5 4- reidratação da suspensão de lipossomas desidratada com uma solução aquosa do antimoniato de meglumina.
2. Processo de acordo com a reivindicação 1, caracterizado pelo uso de pelo 10 menos um açúcar crioprotetor do grupo, trealose, maltose, sacarose, glicose, lactose, dextran e ciclodextrina.
3. Processo de acordo com a reivindicação 2, caracterizado pelo uso do açúcar crioprotetor na relação açúcar/lipídeo 1:1 a 5:1 (m/m) e em concentração inicial de 10 a 500 mmol/L na solução submetida a 15 desidratação.
4. Processo de acordo com as reivindicações 1 a 3, caracterizado pelo uso de lipossomas constituídos dos seguintes lipídeos: fosfatidilcolina, tal como dipalmitoilfosfatidilcolina ou diestearoilfosfatidilcolina, ou surfactante não iônico; colesterol; um lipídeo apresentando uma carga elétrica negativa, tal como dicetilfosfato, dimiristoilfosfatidilglicerol, dipalmitoilfosfatidilglicerol ou diestearoilfosfatidilglicerol; com cada componente lipídico presente em relação molar de 40-70%, 10-40%, e 10-50%, respectivamente. 20
5. Processo de acordo com a reivindicação 4, caracterizado pelo uso dos componentes lipídicos: fosfatidilcolina, colesterol e dicetilfosfato na

proporção relativa de aproximadamente 5:4:1.

6. Processo de acordo com as reivindicações 1 a 5, caracterizado pela reidratação do liofilizado com uma solução de antimoniato de meglumina na concentração de antimônio de 40-80 g/L.

5 7. Processo de acordo com as reivindicações 1 a 6, caracterizado pelo uso da liofilização, da evaporação sob vácuo ou da secagem por pulverização, como processo de desidratação.

8. Processo de acordo com as reivindicações 1 a 7, caracterizado pela inclusão de mais uma etapa de reidratação da suspensão de lipossomas, 10 usando uma solução salina aceitável do ponto de vista fisiológico.

9. Processo de acordo com as reivindicações 1 a 8, caracterizado pela inclusão de uma etapa final de purificação dos lipossomas, usando centrifugação, e de resuspensão dos lipossomas em solução salina aceitável do ponto de vista fisiológico.

15 10. Processo de acordo com as reivindicações 1 a 9, caracterizado pela obtenção de vesículas de diâmetro médio na faixa de 200 nm a 600 nm.

11. Processo de acordo com as reivindicações 1 a 10, caracterizado pela obtenção de lipossomas contendo antimoniato de meglumina encapsulado com taxa superior a 35% e relação final Sb/lipídeo superior a 0,2:1 (m/m).

20 12. Processo de acordo com as reivindicações 1 a 9, caracterizado pela inclusão de uma etapa adicional que consiste na calibração do tamanho das vesículas por filtração através de membrana porosa.

13. Processo de acordo com as reivindicações 1 a 12, caracterizado por produzir formulações farmacêuticas do antimoniato de meglumina em

lipossomas com direcionamento aumentado para a medula óssea de cães acometidos com leishmaniose visceral, quando comparadas aquelas preparadas sem a presença de açúcar crioprotetor.

14. Formulações farmacêuticas do antimoniato de meglumina em lipossomas,

5        caracterizadas por ser preparadas segundo processo de acordo com qualquer uma das reivindicações 1 a 13.

15. Formulações farmacêuticas de acordo com a reivindicação 14,

caracterizadas pelo fato de que os lipossomas são constituídos dos seguintes lipídeos: fosfatidilcolina, tais como dipalmitofosfatidilcolina ou

10      diestearoilfosfatidilcolina, ou surfactante não iônico; colesterol; um lipídeo apresentando uma carga elétrica negativa, tais como dicetilfosfato, dimiristoilfosfatidilglicerol, dipalmitoilfosfatidilglicerol ou diestearoilfosfatidilglicerol; com cada componente lipídico presente em relação molar de 40-70%, 10-40%, e 10-50%, respectivamente.

15      16. Formulações farmacêuticas de acordo com a reivindicação 14, caracterizadas por ser constituídas de vesículas de diâmetro médio na faixa de 100 nm a 600 nm.

17. Formulações farmacêuticas de acordo com as reivindicações 14 a 16,

caracterizadas pelo fato de aumentar a absorção de antimônio na medula óssea de cães acometidos com leishmaniose visceral, quando comparadas a formulações farmacêuticas preparadas com o mesmo processo mas sem a presença de açúcar crioprotetor.

18. Uso das formulações farmacêuticas, descritas nas reivindicações 14 a 17, em animais acometidos com leishmaniose visceral, caracterizado pela

administração endovenosa de uma quantidade terapêutica efetiva de lipossomas contendo antimoniato de meglumina obtidos segundo processo de acordo com qualquer uma das reivindicações 1 a 13.

19. Uso de acordo com a reivindicação 18, caracterizado pela administração

5 das formulações farmacêuticas descritas nas reivindicações 14 a 17, em  
associação com pelo menos um composto de atividade imunomoduladora  
ou um outro composto farmacologicamente ativo.

## RESUMO

"PROCESSO PARA A PREPARAÇÃO DE FORMULAÇÕES FARMACÊUTICAS DO ANTIMONIATO DE MEGLUMINA EM LIPOSSOMAS E USO DAS FORMULAÇÕES FARMACÊUTICAS EM ANIMAIS ACOMETIDOS

5 COM LEISHMANIOSE VISCERAL".

A presente invenção se refere a novas formulações farmacêuticas do antimoniato de meglumina em lipossomas de tamanho reduzido, que apresentam um direcionamento aumentado para a medula óssea em cães acometidos com leishmaniose visceral. Refere-se ainda a um processo de 10 preparação dessas formulações, que consiste na desidratação de uma mistura de lipossomas preparados em água com um açúcar crioprotetor, seguida pela reidratação do produto desidratado com uma solução aquosa do antimoniato de meglumina. A presente invenção é de uso eficaz em animais acometidos com leishmaniose visceral.

# Efficacy of Combined Therapy with Liposome-Encapsulated Meglumine Antimoniate and Allopurinol in Treatment of Canine Visceral Leishmaniasis

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An innovative liposomal formulation of meglumine antimoniate (LMA) was recently reported to promote both long-term parasite suppression and reduction of infectivity to sand flies in dogs with visceral leishmaniasis. However, 5 months after treatment, parasites were still found in the bone marrow of all treated dogs. In order to improve treatment with LMA, the present study aimed to evaluate its efficacy in combination with allopurinol. Mongrel dogs naturally infected with *Leishmania infantum* were treated with six doses of LMA (6.5 mg Sb/kg of body weight/dose) given at 4-day intervals, plus allopurinol (20 mg/kg/24 h *per os*) for 140 days. Comparison was made with groups treated with LMA, allopurinol, empty liposomes plus allopurinol, empty liposomes, and saline. Dogs remained without treatment from day 140 to 200 after the start of treatment. The drug combination promoted both clinical improvement of dogs and significant reduction in the parasitic load in bone marrow and spleen on days 140 and 200 compared to these parameters in the pretreatment period. This is in contrast with the other protocols, which did not result in significant reduction of the bone marrow parasite load on day 200. Strikingly, the combined treatment, in contrast to the other regimens, induced negative quantitative PCR (qPCR) results in the liver of 100% of the dogs. Both xenodiagnosis and skin parasite determination by qPCR indicated that the drug combination was effective in blocking the transmission of skin parasites to sand flies. Based on all of the parasitological tests performed on day 200, 50% of the animals that received the combined treatment were considered cured.

Visceral leishmaniasis (VL) is a systemic parasitic disease which leads to high rates of morbidity and mortality in humans worldwide. Even with scientific advances related to diagnosis, treatment, and prevention over the past 10 years, VL still is a neglected disease leading to ~60,000 human deaths/year. The clinical manifestations of VL are attributed to obligatory intracellular protozoa of the *Leishmania donovani* complex and, depending on the etiological agent, the disease presents two distinct forms: anthroponotic VL that is endemic in India and Central Africa, caused by *L. donovani*, and zoonotic VL that occurs in countries of the Mediterranean basin, Central Asia, and Americas, caused by *Leishmania infantum*. Domestic dogs are the most important urban reservoirs of *L. infantum*, which is transmitted to humans and dogs through bites of infected female sand flies of the genera *Lutzomyia* and *Phlebotomus* (Diptera: Psychodidae; Phlebotominae) in the New World and Old World, respectively (7, 33). The disease in dogs is characterized by a marked pleomorphism, and the clinical signs vary according to the immune response of the animals toward the infection. In general, the main clinical signs of canine visceral leishmaniasis (CVL) are various degrees of dermopathy, lymphadenopathy, onychogryposis, weight loss, abnormalities of the musculoskeletal system, eye lesions, hematopoietic disorders, renal disease, and lesions originating from immune complex deposition in tissues (e.g., vasculitis and arthritis) (30).

The treatment of dogs affected with VL has been practiced in Europe since the middle of the 20th century (1). Since then, the pentavalent antimonials, including meglumine antimoniate (MA) and sodium stibogluconate, have been the main class of drugs used to treat VL in both humans and dogs (1, 17). However,

treatment with antimonial drugs does not promote parasitological cure in infected dogs, leading to frequent relapses (23) and necessitating continuous administration of the drugs, which are both poorly tolerated and expensive.

The purine analog allopurinol is an alternative orally active drug (6) which presents low toxicity, is effective in reverting the clinical signs of CVL, and prevents recurrence of the disease (1, 8). However, allopurinol also is unable to promote parasitological cure in dogs with VL (23).

Currently, the combination of MA and allopurinol constitutes the first-line pharmaceutical protocol for CVL. The basis for the use of this combination is the synergism between these drugs against *Leishmania* parasites, as previously demonstrated *in vitro* (22) and *in vivo* (1, 15, 24, 27). However, although most dogs recover clinically after therapy, complete elimination of the parasite is usually not achieved, and infected dogs may eventually relapse. Because of its limited parasitcidal efficacy and the potential risk of long-term treatment for selection of *Leishmania* strains

Received 26 January 2012 Returned for modification 21 February 2012

Accepted 1 March 2012

Published ahead of print 12 March 2012

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doi:10.1128/AAC.00208-12

resistant to antimonial drug, WHO strongly supports the search for more effective therapeutic protocols (21, 23, 33).

In the 1970s, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than the free drugs against experimental VL based on parasite suppression in the liver (4). Since then, much effort has been devoted to the search for efficacious liposomal formulations in CVL (16). In this context, an innovative liposomal formulation of MA (LMA) was recently developed that has significant pharmaceutical and pharmacological advantages over conventional formulations (18, 29). Treatment of dogs naturally infected by *L. infantum* with four intravenous (i.v.) doses of LMA at 6.5 mg Sb/kg of body weight at 4-day intervals (28) promoted both long-term parasite suppression and reduction of infectivity to sand flies. However, parasites were still found in the bone marrow of all treated dogs.

With the aim of further improving the treatment efficacy of LMA in dogs, a new protocol was designed which combines LMA with allopurinol and takes advantage of the synergistic antileishmanial actions of allopurinol and antimonial drugs. In the present work, the efficacy of the combination of LMA with allopurinol as a mean of achieving parasitological cure in dogs naturally infected with *L. infantum* has been established for the first time, through clinical and parasitological parameters as well as assessment of the infectivity of dogs to sand flies.

## MATERIALS AND METHODS

**Materials and drugs.** Cholesterol and dicetylphosphate were purchased from Sigma Co. (St. Louis, MO in the supplemental material). Distearoyl-phosphatidylcholine was obtained from Lipoid (Ludwigshafen, Germany). N-Methyl-D-glucamine and antimony pentachloride ( $SbCl_5$ , 99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Allopurinol was purchased from a commercial laboratory for pharmaceutical products (Vetfarma, Brazil) as a formulation of oral capsules at individual dosages of 20 mg/kg of body weight. Meglumine antimoniate (MA) was synthesized, as previously described (14), from equimolar amounts of N-methyl-D-glucamine and pentavalent antimony oxyhydrate freshly prepared from the hydrolysis of antimony pentachloride in water. The resulting product contained 28% Sb by weight. As previously established (18), synthetic MA may be replaced by commercial MA (Glucantime; Sanofi-Aventis Farmacéutica Ltda., São Paulo, Brazil) in the preparation of the liposomal formulation.

**Animals.** The present study was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal de Minas Gerais (protocol number 211/2007), and all procedures were carried out according to the international guidelines (*Principles of Laboratory Animal Care* [26]).

Fifty-two mongrel dogs (32 males and 20 females) weighing  $12.3 \pm 5.2$  kg (mean  $\pm$  standard deviation), of unknown age, and naturally infected with *Leishmania infantum* were obtained by donation from the Center for Zoonosis Control of Ribeirão das Neves City (Minas Gerais State, Brazil), an area where canine visceral leishmaniasis is endemic. These dogs were previously identified by serological tests and captured as part of the activities of the municipality's Visceral Leishmaniasis Control Program.

The serological diagnosis was confirmed at the Serology Laboratory of the Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG) using indirect immunofluorescence assay (IFAT) and enzyme-linked immunosorbent assay (ELISA) (5). All animals were found to be positive by IFAT ( $\geq 1:40$  dilution) and ELISA (optical density,  $\geq 0.100$ ; 1:400 dilution). In addition, specific PCR of bone marrow aspirate was used to confirm the infection of the animals by *L. infantum* (13). The animals were kept in the experimental kennel of the ICB-UFMG with drinking water and balanced commercial food (Nero Refeição; Total Ali-

mentos, Brazil) *ad libitum* during the entire experimental period. Prior to treatment, dogs were treated against intestinal helminths (Helfine cães; Agener União, Brazil) and ectoparasite infestations (Frontline Top Spot; Merial, Brazil) and immunized against rabies (Defensor; Pfizer Saúde Animal, Brazil) and other infectious diseases (Vanguard HTLP 5/CV-L; Pfizer Saúde Animal, Brazil).

**Preparation and characterization of liposomes.** Liposome-encapsulated meglumine antimoniate (LMA) was prepared as previously described (29). Briefly, small unilamellar vesicles (SUVs) were obtained by mixing distearoylphosphatidylcholine, cholesterol, and dicetylphosphate (molar ratio of 5:4:1) following ultrasonication in deionized water at a final lipid concentration of 55 g/liter. Then, the SUV suspension was filtered with a sterile 0.22- $\mu$ m membrane and mixed with sucrose (sugar/lipid mass ratio of 3:1; final sugar concentration of 0.3 M), and the resulting mixture was immediately frozen in liquid nitrogen and subsequently dried (freeze dryer, 4.5 liters; Labconco, United Kingdom). At this point, the freeze-dried liposomal formulation may be stored at  $-20^{\circ}\text{C}$  for at least 6 months without any change of final vesicle size and drug encapsulation efficiency characteristics. The day before administration, the lyophilized powder was rehydrated with a solution of MA in water (antimony concentration of 0.65 M, corresponding to 40% of the original SUV volume) and the resulting suspension was vortexed and incubated for 30 min at  $55^{\circ}\text{C}$ . Then, the same volume of phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate, pH 7.2) was added to the mixture, followed by vortexing and incubation for 30 min at  $55^{\circ}\text{C}$ . The resulting liposome suspension was diluted in PBS and centrifuged ( $25,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ ) in order to separate LMA from nonencapsulated drug. After centrifugation, the liposome pellet was washed twice with isotonic saline and resuspended in sterile saline solution, and the final antimony concentration was adjusted to 10 g/liter. The liposome suspension was stored at  $4^{\circ}\text{C}$  until administration. The drug encapsulation efficiency was  $37\% \pm 5\%$ , as determined by atomic absorption spectroscopy (AA600; PerkinElmer, Inc., United States). The mean hydrodynamic diameter of the vesicles was  $350 \pm 58$  nm, with a polydispersity index of  $0.30 \pm 0.07$ , as determined by photon correlation spectroscopy (Malvern Zetasizer Nano ZS90; Malvern Instruments, United Kingdom). In parallel, lyophilized SUVs were rehydrated with a solution of N-methyl-D-glucamine (0.65 M, pH 7.2) instead of MA, using the same method as described above. The final suspension, called empty liposomes (LEMP), presented similar values of mean hydrodynamic vesicle diameter and polydispersity index.

**Treatment protocols.** Dogs were randomly distributed into six groups as follows. In the LMA + Allop group, eight animals were treated with six doses of LMA (6.5 mg Sb/kg of body weight/dose) given at 4-day intervals and allopurinol (20 mg/kg of body weight/24 h *per os*) for 140 days starting from the first dose of LMA. In the LMA group, eight animals were treated with six doses of LMA (6.5 mg Sb/kg of body weight/dose) given at 4-day intervals. In the Allop group, eight animals were treated with allopurinol (20 mg/kg of body weight/24 h *per os*) for 140 days and six doses of isotonic saline given at the same volume and time intervals as LMA in the LMA + Allop group. In the LEMP + Allop group, eight animals were treated with six doses of empty liposomes given at the same volume and time intervals as LMA in the LMA + Allop group and allopurinol (20 mg/kg of body weight/24 h *per os*) for 140 days. In the LEMP group, eight animals were treated with six doses of empty liposomes given at the same volume and time intervals as LMA in the LMA group. In the saline group, 12 animals received six doses of isotonic saline given at the same volume and time intervals as LMA in the LMA + Allop group.

Following day 140 after the beginning of treatment, all animals of the six experimental groups were kept in the kennel for 60 days without any intervention, representing a total experimental period of 200 days. Animals were clinically monitored during the experimental period and were submitted to clinical and parasitological evaluations just before and on days 140 and 200 after the beginning of treatment. At day 200, the animals were euthanized using an overdose of sodium thiopental (i.v.) under general anesthesia.

**TABLE 1** Staging system used for classification of dogs with visceral leishmaniasis

Clinical stage	IFAT titer	Clinical signs	Clinicopathological abnormalities	Score
Stage I: mild disease	Negative or $\leq 1:160$	Peripheral lymphadenopathy and/or one slight dermatological alteration, such as papular dermatitis, exfoliative dermatitis, seborrheic dermatitis, or onychogryposis	No clinicopathological abnormalities, creatinine $<1.4 \text{ mg/dl}$ , urea $<25 \text{ mg/dl}$ , and A/G ratio $\geq 0.6$	1
Stage II: moderate disease	$\geq 1:80$	Peripheral lymphadenopathy associated with two or more signs, as follows: cutaneous alterations (papular dermatitis, exfoliative dermatitis, seborrheic dermatitis, onychogryposis, or ulcerative dermatitis), anorexia, mucopurulent conjunctivitis, keratoconjunctivitis, fever, and epistaxis	Mild nonregenerative anemia and/or hypergammaglobulinemia, hypoalbuminemia, creatinine $<1.4 \text{ mg/dl}$ , urea $>25 \text{ mg/dl}$ , and A/G ratio $\leq 0.6$	2
Stage III: severe disease	$\geq 1:160$	Clinical signs of stage II and lesions associated with immune complex, such as vasculitis, arthritis, and uveitis	Mild nonregenerative anemia and hypergammaglobulinemia, hypoalbuminemia, creatinine $1.4\text{-}2 \text{ mg/dl}$ , urea $>25 \text{ mg/dl}$ , and A/G ratio $\leq 0.6$	3
Stage IV: very severe disease	$\geq 1:640$	Clinical signs of stage III and signs of chronic kidney disease, such as nephrotic syndrome and uremic crisis	Nonregenerative anemia and hypergammaglobulinemia, hypoalbuminemia, creatinine $>2 \text{ mg/dl}$ , urea $>25 \text{ mg/dl}$ , and A/G ratio $\leq 0.6$	4

During the experimental period, seven animals died: two dogs in the LMA + Allop group, two in the LMA group, and one in the LEMP group, due to wounds caused by fights, and two dogs (one in the saline group and one in the LEMP group), most probably due to the natural evolution of the disease.

**Clinical evaluation.** Just before treatment and on days 140 and 200 after the start of treatment, the dogs were inspected for the presence of clinical signs of CVL, and serum and plasma (using EDTA as an anticoagulant) were collected for quantitative evaluation of anti-*Leishmania* antibodies (IFAT), hemogram, and levels of serum urea, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total proteins, globulins, albumins, and albumin/globulin (A/G) ratio.

Based on the results of the physical examination, levels of anti-*Leishmania* antibodies determined by IFAT (5), and laboratory findings in hemogram and serum biochemistry, the animals were classified according to a previously proposed staging system (30), with a few modifications, as shown in Table 1. Each animal received a score ranging from 0 to 4, where score 0 corresponds to absence of clinical signs of CVL, negative serology (IFAT  $\leq 1:40$ ), and no abnormalities in hemogram and serum biochemistry, and score values from 1 to 4 correspond to the mild, moderate, severe, and extremely severe disease clinical stage, respectively.

**Parasitological evaluation.** Quantitative real-time PCR (qPCR) was used to determine the parasite loads in bone marrow and spleen just before treatment and on days 140 and 200 after the start of treatment. Dogs were submitted to general anesthesia using the combination of 2 mg/kg of body weight of xylazine chloride (Calmium; União Química Farmacêutica S/A, Brazil) and 11 mg/kg of body weight of ketamine chloride (Ketamina Agener; União Química Farmacêutica S/A, Brazil) by intramuscular injection. Then, 1.0 ml of sternal bone marrow aspirate was collected, followed by 1.0 ml of spleen aspirate after previous localization of the organ with portable ultrasound equipment (SonoSite SonoHeart Elite Superior 180 Plus; SonoSite Inc., United States). At euthanasia (day 200 after starting treatment), samples of liver and skin of the internal face of the right ear were collected to perform qPCR, in addition to the bone marrow and spleen aspirates. Skin samples were also obtained just before treatment to be used as controls. All samples were stored at  $-80^{\circ}\text{C}$  until required for further processing.

Total DNA extraction of the samples was carried out with the DNeasy

blood and tissue kit (Qiagen, Inc., United States), used according to the manufacturer's instructions. In order to quantify parasite burdens, primers (forward, 5' TGT CGC TTG CAG ACC AGA TG 3', and reverse, 5' GCA TCG CAG GTG TGA GCA C 3') that amplified a 90-bp fragment of a single-copy-number gene of DNA polymerase of *L. infantum* (GenBank accession number AF009147) were used (9). PCR was carried out with a final reaction mixture volume of 25  $\mu\text{l}$  containing 200 nM forward and reverse primers, 1× SYBR Green PCR master mix (Applied Biosystems, United States), and 5  $\mu\text{l}$  of template DNA. The PCR conditions were as follows: an initial denaturation step at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s and annealing/extension at  $60^{\circ}\text{C}$  for 1 min. Standard curves were prepared for each run using 10-fold serial dilutions in DNase-/RNase-free water ranging from 1 to  $10^6$  molecules/ $\mu\text{l}$  of pGEM-T Easy vector system plasmids (Promega, United States) containing the 90-bp fragment of the DNA polymerase gene of *L. infantum* (3, 9). The same procedure was carried out for the housekeeping  $\beta$ -actin gene using primers that amplified a 307-bp fragment (forward, 5' CTT CTA CAA CGA GCT GCG CG 3', and reverse, 5' TCA TGA GGT AGT CGG TCA GG 3') in order to verify the integrity of the samples and to normalize the initial concentrations of DNA. The number of copies of *L. infantum* in the samples was adjusted using the  $\beta$ -actin correction factor obtained for each sample. In all assays, the efficiency of the amplification was close to 100% and the standard curves presented correlation coefficients between plasmid concentrations and threshold cycle ( $C_T$ ) values ranging from 0.97 to 0.99. In our assays, standard curves allowed the detection of a limit of 0.8 parasites/ml of bone marrow and 0.25, 0.84, and 1.1 parasites/mg of liver, spleen, and skin, respectively. The reaction mixtures were processed and analyzed in the ABI Prism 7500 sequence detection system (Applied Biosystems, United States).

Bone marrow aspirates (1.0 ml) were collected on day 200 and immediately seeded into a biphasic culture medium, NNN (Novy, McNeal, and Nicole) medium, enriched with minimum essential medium (MEM  $\alpha$ ; Gibco, United States). Cultures were maintained at  $23 \pm 1^{\circ}\text{C}$  and examined each 10 days for 30 days in order to identify the presence of promastigote forms.

**Xenodiagnosis.** Xenodiagnosis was performed as previously described (12), with some modifications, in order to verify the ability of treated dogs to infect the sand flies. Briefly, just before treatment and on

days 140 and 200 after the start of treatment, animals were submitted to general anesthesia as described above and the internal surface of the right ear was shaved. Then, 40 to 50 4-day-old females of *Lutzomyia longipalpis* from the colony of the Laboratory of Physiology of Haematophagous Insects (Department of Parasitology, ICB/UFMG) were placed in a round plastic box called a FleboContainer (11), and the sand flies were allowed to feed directly on the right ear for 30 min in a dark room. After the blood meal, the sand flies were fed daily with a 50% fructose solution in distilled water and kept at 28°C in the insectary for 5 days. On the fifth day, the females of *L. longipalpis* were dissected in a drop of PBS solution and midguts were examined under an optical microscope at 400× magnification to verify the presence of promastigote forms and to determine the infection ratio. The estimated number of promastigotes in each midgut was determined as follows: −, absence of promastigotes; +, presence of 1 to 50 promastigotes; ++, 51 to 200 promastigotes; and +++, >201 promastigotes (32).

**Cure criteria.** The criteria established to consider an animal as cured of CVL on day 200 after the start of treatment, independent of the treatment protocol received, were (i) absence of parasites in bone marrow aspirate culture, (ii) negative results for parasitological evaluations by qPCR of bone marrow, spleen, liver, and ear skin, and (iii) negative results in xenodiagnosis.

**Statistical analysis.** Statistical analyses were performed with the aid of GraphPad Prism version 5.00 for Windows (GraphPad Software, United States). According to the Kolmogorov-Smirnov test, experimental data were not normally distributed. All data were not normally distributed. Then, the Kruskal-Wallis test followed by Dunn's multiple comparison test or the Mann-Whitney test was used to compare clinical stage and tissue parasitic loads. The Friedman test was used to compare the clinical stages and the tissue parasite loads in each group just before and on days 140 and 200 after the start of treatment. Comparison of qPCR results for skin samples before and after treatment was performed using Wilcoxon matched pairs. Fisher's exact test was used to compare the sand fly infection efficiencies and the proportions of cured dogs between different groups. A significance level of 95% was applied in all statistical tests.

## RESULTS

Treatment of mongrel dogs naturally infected with *Leishmania infantum* was performed with six doses of LMA (6.5 mg Sb/kg/dose) given at 4-day intervals plus allopurinol (20 mg/kg/24 h *per os*) (LMA+Allop) for 140 days starting from the first dose of LMA. The efficacy of the combined treatment was evaluated on the basis of clinical and parasitological parameters determined just before treatment and on days 140 and 200 after the start of treatment. Comparison was performed with experimental groups treated with LMA, allopurinol (Allop), or empty liposomes plus allopurinol (LEMP+Allop). Control groups received either saline or empty liposomes (LEMP).

**Clinical parameters.** Before treatment, all animals presented clinical signs of CVL, as established by the inclusion criteria. Dermatological alterations, such as exfoliative dermatitis, seborrheic dermatitis, localized alopecia, ulcerative dermatitis over joint prominences, and onychogryposis, were the main clinical signs observed (90.4% of the animals). The other most frequent clinical signs were lymphadenopathy and mucopurulent conjunctivitis/keratoconjunctivitis, observed in 75.0% and 32.7% of the animals, respectively. Normocytic normochromic anemia (31.0%), thrombocytopenia (23.0%), leukocytosis (24.5%), serum urea (84.6%), and globulins (32.6%) above and A/G ratio (69.2%) below the reference values were the main abnormalities found in hemograms and serum biochemistry of the animals. All animals were also positive according to IFAT, and 95% of these dogs presented high levels of anti-*Leishmania* antibody titers ( $\geq 1:640$ ).

Taking into account clinical, laboratory, and serological data and a recently proposed clinical classification of CVL (30), each animal received a specific score, the highest score corresponding to the most severe pattern of the disease. The distribution of animals was as follows: 7.7% of dogs were in stage I, 51.9% in stage II, and 40.4% in stage III.

Treatment of infected dogs with LMA in combination with allopurinol (LMA+Allop) promoted a marked reduction in anti-*Leishmania* antibody titers. Quantitative serology of this group, as determined by IFAT endpoint, showed a 20.3-fold reduction in antibody titer, ranging from 1:8,133 before treatment to 1:400 on day 200. For comparison, during the same period, the average titer reductions in the other groups were as follows: LMA, 1.9-fold (from 1:3,867 to 1:2,000); Allop, 1.3-fold (from 1:3,940 to 1:3,040); LEMP+Allop, 2.5-fold (from 1:2,800 to 1:1,140); LEMP, 1.4-fold (from 1:5,013 to 1:3,627); and saline, 2.5-fold (from 1:6,749 to 1:2,755). Interestingly, only in the LMA+Allop group was the median IFAT titer significantly lower on days 140 and 200 than prior to treatment ( $P < 0.05$ ; Friedman).

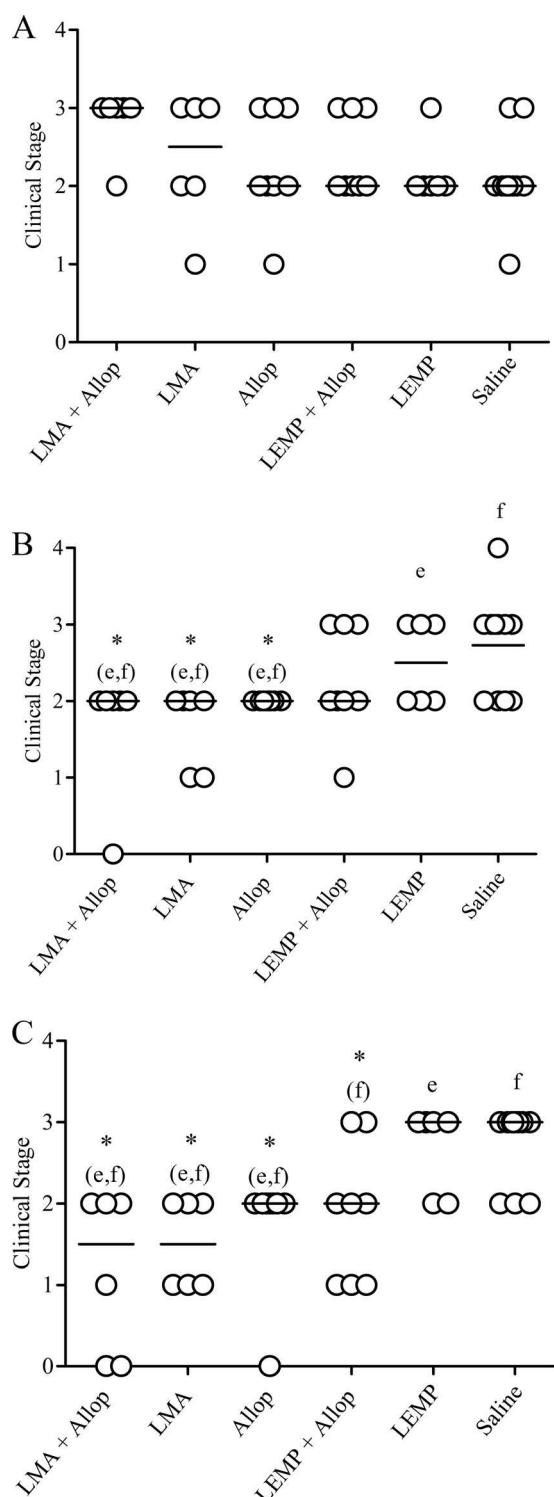
Figure 1 compares the more general and predictive clinical stage parameters between the different experimental groups, taking into account the results of quantitative serology, physical examination, and clinicopathological findings. The data indicate that the LMA+Allop, LMA, and Allop groups presented significantly lower clinical scores (severity of stages) on days 140 and 200 than control groups (LEMP and saline) ( $P < 0.05$ ; Kruskal-Wallis).

Among the different treatment protocols, the LMA-allopurinol combination was the one that promoted the most pronounced improvement of the clinical status of infected animals. The reduction in the median clinical score of the LMA+Allop group was significant on days 140 and 200 compared to the median score before treatment ( $P < 0.05$ ; Friedman). Importantly, all the animals of this group (100%) presented a lower clinical score at both times than prior to treatment. At day 200, two dogs were completely asymptomatic, with no alterations in physical examination, normal hemogram and serum biochemistry, and negative serology (score = 0). Another dog was classified as stage I, showing no clinicopathological alterations but medium levels of anti-*Leishmania* antibody titers (IFAT, 1:320). The other three animals of this group were classified in clinical stage II. They presented medium levels of anti-*Leishmania* antibody titers (IFAT, 1:320 to 1:640) and, associated with this alteration, one dog had a low platelet level, another showed seborrheic dermatitis and lymphadenopathy, and the third presented a low platelet level, hypoalbuminemia, and a low A/G ratio (data not shown).

The LMA group presented a significant reduction in the median clinical score on day 200 compared to the median score in the pretreatment period ( $P < 0.05$ ; Friedman). At day 200, 83.3% of these dogs presented a better clinical score than prior to treatment, and the animals were classified as stage I (50%) or stage II (50%).

The Allop and LEMP+Allop groups presented an improvement in general clinicopathological abnormalities, but no statistical difference was found when comparing the median clinical scores between the three time points.

In contrast, most dogs from the control groups presented with worse clinical stages on days 140 and 200 compared to the stage at the pretreatment period. In these animals, both a progressive increase of the number and severity of the lesions noted in the physical examination and worsening of hemogram and serum bio-



**FIG 1** Clinical staging of dogs naturally infected with *Leishmania (L.) infantum* before and after treatment with liposomal meglumine antimoniate (LMA), allopurinol (Allop), empty liposomes (LEMP), or the LMA+Allop or LEMP+Allop combination. (A) Staging prior to treatment. LMA (6.5 mg Sb/kg/dose), empty liposomes (same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals. Allopurinol was given at 20 mg/kg/24 h *per os* for 140 days. Clinical scores are defined in detail in Table 1. Score 0, absence of clinical signs and clinicopathological alterations suggestive of CVL and negative serology; score 1, mild clinical stage; score 2,

chemistry parameters were observed. Saline-treated animals presented a significantly greater median clinical score on day 200 than before treatment ( $P < 0.05$ ; Friedman).

**Parasitological evaluations in bone marrow and spleen.** Parasitological evaluation of dogs by qPCR indicated that the LMA-allopurinol combined treatment was the most effective protocol for reducing the parasite burden in bone marrow and spleen. As illustrated in Fig. 2, this combination promoted a significant reduction in bone marrow parasite loads on days 140 and 200 compared to the results of the LEMP+Allop, LEMP, and saline protocols ( $P < 0.05$ ; Kruskal-Wallis). Similarly, the LMA+Allop group presented with significantly lower parasite loads in the spleen on days 140 and 200 than the LEMP and saline groups ( $P < 0.05$ ; Kruskal-Wallis) (Fig. 2).

The effectiveness of the LMA-allopurinol combination for reducing the parasite burden in the bone marrow and spleen was also evident when the parasite loads were compared before and on days 140 and 200 after the start of treatment. The parasite burdens in both tissues were significantly reduced on days 140 and 200 compared to the burdens in the pretreatment period ( $P < 0.05$ ; Friedman). On day 140, five animals (83.3%) were negative by qPCR in both the bone marrow and spleen. At the end of the experimental period (day 200), three of these animals remained negative in both the spleen and bone marrow. Another dog was still negative only in the spleen. The median numbers of parasites in bone marrow and spleen aspirates, as determined by qPCR on day 140, were about 770 and 245 times lower, respectively, than before treatment. This substantial reduction in the parasite load was still observed at the end of the experimental period (parasite burdens were about 660 and 104 times lower, respectively).

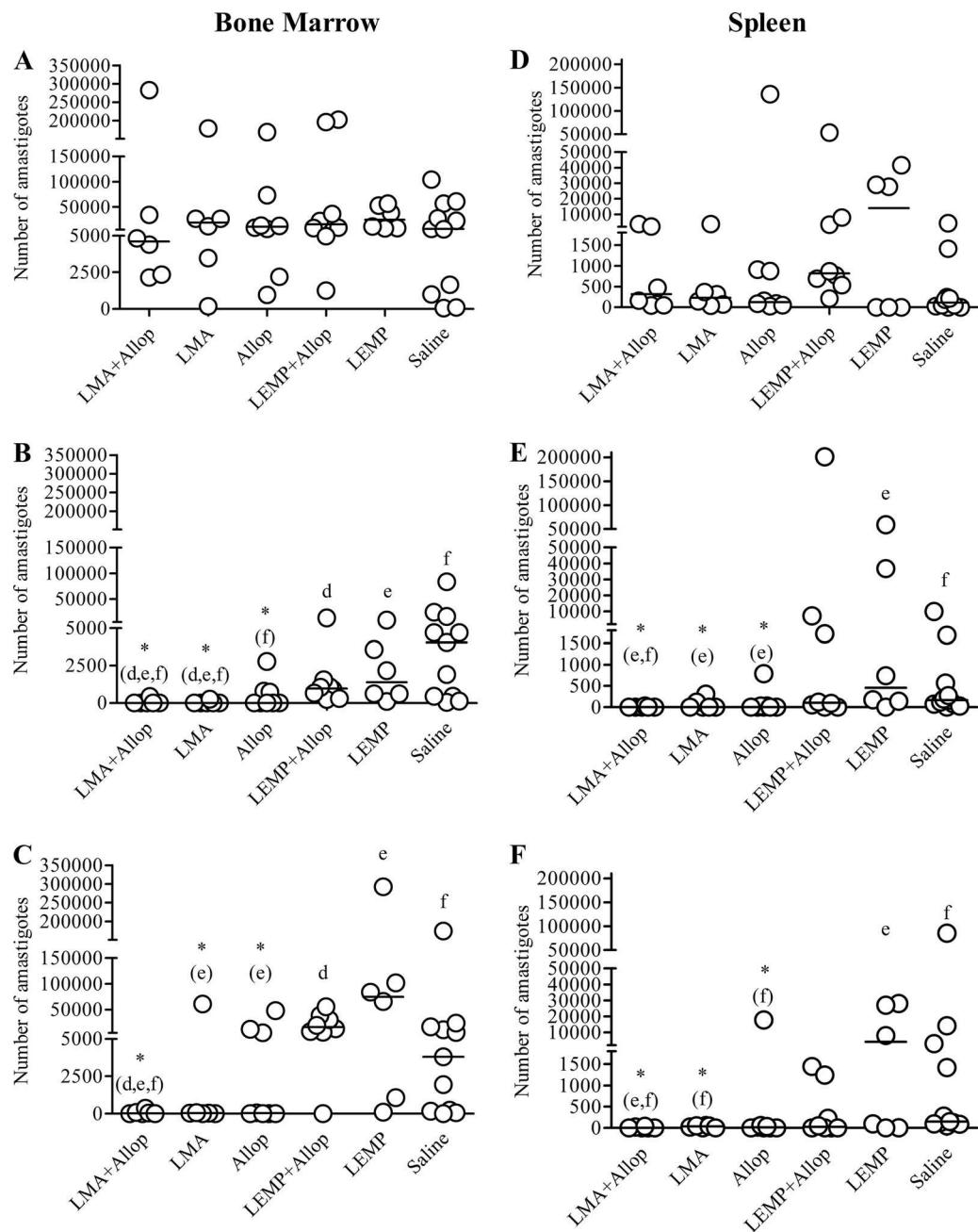
The LMA and Allop groups showed results similar to those for the LMA+Allop group with respect to the reduction of parasite loads in spleen and bone marrow on day 140 (Fig. 2). However, on day 200, treatment with LMA or allopurinol (with or without empty liposomes) did not show a significant reduction of the bone marrow parasite burden compared to that in the pretreatment period, confirming the inability of these drugs alone to maintain low parasite levels in this tissue after interruption of treatment.

**Parasitological evaluations in the liver.** The parasite loads in the liver of treated dogs on day 200 after the start of treatment were determined by qPCR. Significant parasite suppression in the liver of dogs in comparison to the parasite suppression in control groups was achieved in the groups that received LMA (with or without allopurinol) but not in those receiving allopurinol without LMA. All animals (100%) treated with the LMA-allopurinol combination presented negative qPCR results. As shown in Fig. 3, the parasite burdens in the livers of the LMA+Allop group were significantly lower than the parasite burdens in the LEMP and saline groups ( $P < 0.05$ ; Kruskal-Wallis). In the LMA group, five animals (87.3%) were found to be negative and the median number of parasites in this organ was significantly lower than that in the saline group ( $P < 0.05$ ; Kruskal-Wallis).

#### Parasitological evaluations in the skin.

Parasitological evalua-

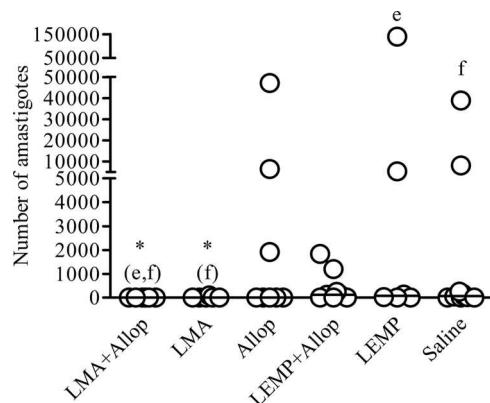
moderate stage; score 3, severe stage; score 4, extremely severe disease stage. Data are shown as dot plots, and lines correspond to the median of each group ( $n = 6$  to 11). \*(e,f) and \*(f),  $P < 0.05$  according to Kruskal-Wallis test followed by Dunn's multiple comparison test, for comparison with LEMP (e) and/or saline (f).



**FIG 2** Parasite burdens in the bone marrow (A, B, and C) and spleen (D, E, and F) of dogs naturally infected with *Leishmania* (*L.*) *infantum* before and after treatment with liposomal meglumine antimoniate (LMA), allopurinol (Allop), empty liposomes (LEMP), or the LMA+Allop or LEMP+Allop combination. Parasite burdens prior to treatment (A and D), at day 140 (B and E), and at day 200 (C and F) are shown. LMA (6.5 mg Sb/kg/dose), empty liposomes (same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals. Allopurinol was given at 20 mg/kg/24 h *per os* for 140 days, starting from the first dose of LMA. Parasite burdens were determined by qPCR as described in Materials and Methods. Data are shown as dot plots, and lines correspond to the median of each group ( $n = 6$  to 11). \*(d,e,f), \*(e,f), \*(e), and \*(f),  $P < 0.05$  according to Kruskal-Wallis test followed by Dunn's multiple comparison test, for comparison with LEMP+Allop (d), LEMP (e), and/or saline (f).

tion was carried out by qPCR in the ear skin of dogs before treatment and on day 200 after the start of treatment. No statistical difference was observed between the six groups in the median number of parasites in the ear skin on day 200 ( $P > 0.05$ ; Kruskal-Wallis). Nevertheless, as illustrated in Fig. 4, the LMA+Allop group was the only group to show a significantly lower parasite load than the saline group ( $P < 0.05$ ; Mann-Whitney). Furthermore, the parasite loads in the skin of

animals from the LMA+Allop, LMA, and LEMP+Allop groups were significantly lower on day 200 than before treatment ( $P < 0.05$ ; Wilcoxon matched pairs). Prior to treatment, one animal (16.7%) from the LMA+Allop group was negative according to skin qPCR. At day 200, the proportion of skin-negative dogs from this group was 83.3%, corresponding to the highest proportion of negative dogs among all experimental groups. In the dog whose qPCR remained positive, the



**FIG 3** Parasite burdens in the liver of dogs naturally infected with *Leishmania* (*L.*) *infantum* after treatment with liposomal meglumine antimoniate (LMA), allopurinol (Allop), empty liposomes (LEMP), or the LMA+Allop or LEMP+Allop combination. LMA (6.5 mg Sb/kg/dose), empty liposomes (same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals. Allopurinol was given at 20 mg/kg/24 h *per os* for 140 days, starting from the first dose of LMA. Parasite burdens were determined by qPCR as described in Materials and Methods. Data are shown as dot plots, and lines correspond to the median of each group ( $n = 6$  to 11). \*(e,f) and \*(f),  $P < 0.05$  according to Kruskal-Wallis test followed by Dunn's multiple comparison test, for comparison with LEMP (e) and/or saline (f).

median number of parasites decreased about 314-fold (from 69,776 amastigotes prior to treatment to 222 parasites on day 200).

**Xenodiagnosis.** The effect of the treatment of dogs on their infectivity to sand flies was investigated through xenodiagnosis in the different experimental groups just before and on days 140 and 200 after the start of treatment, using females of *Lutzomyia longipalpis*. As shown in Table 2, the LMA+Allop and Allop groups exhibited the best results in the xenodiagnosis evaluations.

In the LMA+Allop group, three animals (50%) infected sand flies just before treatment, with 38% infection efficiency (given by the ratio of infected to fed sand flies in positive dogs), whereas xenodiagnosis was negative in all animals (100%) on days 140 and 200. As shown in Table 2, significant reductions in the proportions of infected sand flies were observed on both day 140 and 200

compared to the proportions in the pretreatment period ( $P < 0.05$ ; Fisher's exact test).

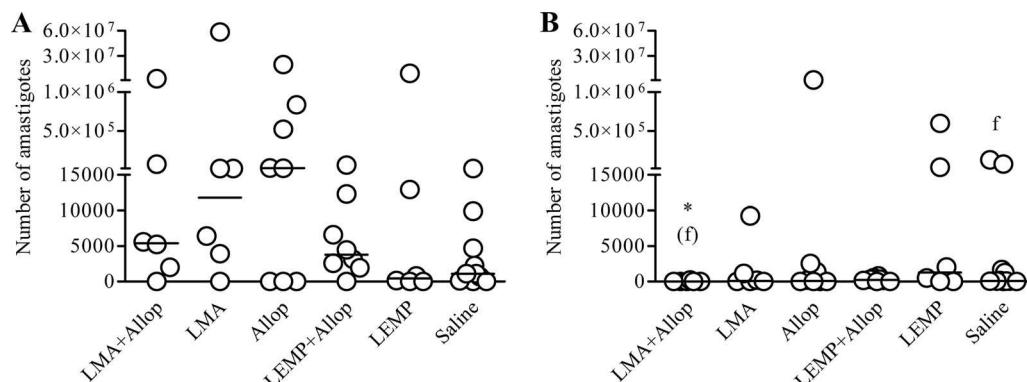
Among the other groups, the Allop but not the LMA and LEMP+Allop groups showed 100% noninfective dogs on day 200. In the LMA, Allop, and LEMP+Allop groups, significant reductions in the proportions of infected sand flies on days 140 and 200 were also observed compared to the proportions in the pretreatment period ( $P < 0.05$ ; Fisher's exact test) (Table 2). This is in contrast with the LEMP and saline groups, in which the proportions of infected sand flies were significantly increased on days 140 and 200 compared to the proportions in the pretreatment period.

**Parasitological cure.** Each animal was evaluated on day 200 using different parasitological tests, including culture from bone marrow aspirates, qPCR of bone marrow, spleen, liver, and skin samples, and xenodiagnosis. Animals that showed negative results in all the parasitological tests were considered cured of CVL. According to these cure criteria, the LMA-allopurinol combination promoted parasitological cure in 50% of treated animals. An additional immunohistochemistry test was performed, as described previously (28), in those tissues tested by qPCR. Strikingly, negative results were obtained in all samples collected from the six animals of this group (data not shown). Among the other five experimental groups, parasitological cure was achieved in only one animal that was treated with allopurinol.

As illustrated in Fig. 5, the proportion of cured dogs achieved after treatment with the LMA-allopurinol combination was significantly greater than that in the saline group ( $P < 0.05$ ; Fisher's exact test).

## DISCUSSION

With the aim of further improving the treatment efficacy of CVL with an innovative liposomal formulation of MA, a new therapeutic regimen was designed which combines this formulation with allopurinol, a well-tolerated and effective leishmanostatic drug capable of reaching all infected sites. In comparison to our previous protocol (28), LMA was given at the same dosage (6.5 mg Sb/kg/dose) and time intervals, but animals received six doses instead of four. Thus, a more effective protocol was also expected from the more prolonged treatment with LMA. The protocol used



**FIG 4** Parasite burdens in the ear skin of dogs naturally infected with *Leishmania* (*L.*) *infantum* before and after treatment with liposomal meglumine antimoniate (LMA), allopurinol (Allop), empty liposomes (LEMP), or the LMA+Allop or LEMP+Allop combination. Parasite burdens prior to treatment (A) and at day 200 (B) are shown. LMA (6.5 mg Sb/kg/dose), empty liposomes (same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals. Allopurinol was given at 20 mg/kg/24 h *per os* for 140 days, starting from the first dose of LMA. Data are shown as dot plots, and lines correspond to the median of each group ( $n = 6$  to 11). \*(f),  $P < 0.05$  for comparison with saline group according to the Mann-Whitney test, for comparison with saline (f).

**TABLE 2** Frequency of positive dogs in xenodiagnosis and proportion and intensity of infection of *Lutzomyia longipalpis* fed on dogs naturally infected with *Leishmania (L.) infantum* before and after treatment

Treatment group <sup>a</sup>	Frequency of positive dogs in xenodiagnosis (%) <sup>b</sup>			Proportion of infected sand flies (%) <sup>c</sup>			Intensity of infection (%) <sup>d</sup>								
	Prior to treatment		Day 140	Prior to		Prior to treatment	Prior to treatment			Day 140			Day 200		
	Prior to treatment	Day 140	Day 200	Prior to treatment	Day 140	Day 200	+	++	+++	+	++	+++	+	++	+++
LMA+Allop	50	0	0	19.1	0 <sup>e</sup>	0 <sup>e</sup>	42.5	40	17.5	0	0	0	0	0	0
LMA	50	16.7	33.3	11.9	1.4 <sup>e</sup>	1.9 <sup>e</sup>	28	12	60	33.3	66.7	0	75	25	0
Allop	50	0	0	35.0	0 <sup>e</sup>	0 <sup>e</sup>	11.2	11.2	77.6	0	0	0	0	0	0
LEMP+Allop	62.5	0	12.5	34.3	0 <sup>e</sup>	2.5 <sup>e</sup>	22.9	24	53.1	0	0	0	28.6	57.1	14.3
LEMP	33.3	33.3	50	16.7	26.2 <sup>f</sup>	30.0 <sup>f</sup>	28.6	22.9	48.5	23.7	14.5	61.8	22.2	20.6	57.2
Saline	36.4	45.5	63.6	3.9	11.4 <sup>f</sup>	16.4 <sup>f</sup>	46.7	33.3	20	45.8	25	29.2	41.6	27.7	30.7

<sup>a</sup> Liposomal meglumine antimoniate (LMA; 6.5 mg Sb/kg/dose), empty liposomes (LEMP; same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals; allopurinol (Allop) was given at 20 mg/kg/24 h per os for 140 days, starting from the first dose of LMA.

<sup>b</sup> Proportions of dogs in each group whose promastigotes were identified in the midgut of *Lutzomyia longipalpis* females 5 days after their blood meal on the internal surface of the right ear of these animals.

<sup>c</sup> Proportions of infected *Lutzomyia longipalpis* females in relation to total numbers of insects dissected 5 days after their blood meal on each experimental group of dogs.

<sup>d</sup> Distribution of midgut infections according to the estimated numbers of promastigotes, categorized as follows: +, 1 to 50 promastigotes; ++, 51 to 200 promastigotes; and +++, >201 promastigotes.

<sup>e</sup> P < 0.05 according to Fisher's exact test, showing a significantly lower proportion of infected sand flies than in the pretreatment period.

<sup>f</sup> P < 0.05 according to Fisher's exact test, showing a significantly greater proportion of infected sand flies than in the pretreatment period.

for the administration of allopurinol (20 mg/kg/24 h) differed slightly from the conventional one in that a frequency of dosing of 12 h was used (15, 20). Animals also remained without any therapeutic intervention from day 140 to 200, to uncover possible relapse of CVL.

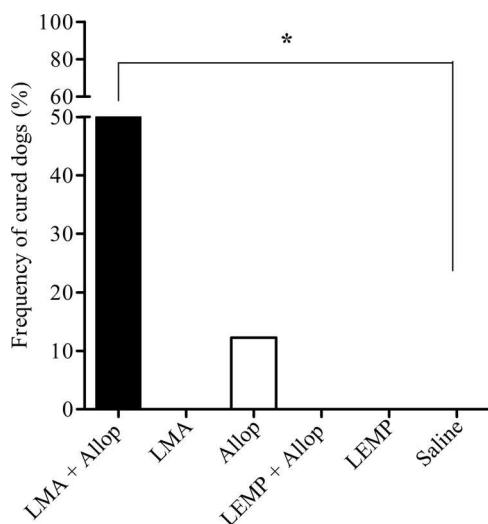
Analysis of the clinical, parasitological, and xenodiagnosis findings indicates at least an additive effect for LMA and allopurinol, since animals treated with this protocol presented much bet-

ter clinical and parasitological profiles than those treated with each drug alone.

Among the six experimental groups, the LMA+Allop group was the one that exhibited the most pronounced improvement of clinical signs and clinicopathological parameters. However, the improvements were gradual and improvements were also observed in the groups treated with LMA or allopurinol alone. During the experimental period, most of the animals (72% ± 19%) treated with these protocols showed weight gain, complete regression or marked reduction in the number and degree of skin lesions, absence of lymphadenopathy and eye lesions, and alterations in the color of the mucous membranes (data not shown). Improvement in the clinical condition was observed until day 140 and was also accompanied by the normalization of most hemogram and serum biochemistry parameters, such as protein electrophoresis, A/G ratio, number of erythrocytes, hemoglobin concentration, hematocrit, and platelets (data not shown).

From day 140 to the end of the experimental period, a tendency toward improvement was observed only in the LMA+Allop group, suggesting that, besides reversion of the physical and clinicopathological abnormalities, the drug combination promoted a long-term action resulting in the prevention of disease relapse. In contrast, the LMA, Allop, and LEMP+Allop groups presented a slight worsening of their clinical status from day 140 to 200. This fact can be attributed to the inability of allopurinol to prevent relapse after interruption of its use (10, 27) and to the transitory positive effect of LMA on the same parameters, as previously described by our group (28).

The clinical improvement observed in the LMA+Allop group was accompanied by a significant reduction in the parasitic load, as determined by qPCR. The choice of qPCR to assess the parasite burdens in the tissues of the dogs was based on the high sensitivity and specificity of the technique for the absolute quantification of parasites (25). The LMA-allopurinol combination had the greatest impact on parasite burden, reducing by hundreds of times the numbers of parasites in bone marrow, spleen, and skin following treatment.



**FIG 5** Proportions of dogs cured of *Leishmania (L.) infantum* infection after treatment with liposomal meglumine antimoniate (LMA), allopurinol (Allop), empty liposomes (LEMP), or the LMA+Allop or LEMP+Allop combination. LMA (6.5 mg Sb/kg/dose), empty liposomes (same dose of lipid), and saline (same volume) were given intravenously as six doses at 4-day intervals. Allopurinol was given at 20 mg/kg/24 h per os for 140 days, starting from the first dose of LMA. Dogs were considered cured when they showed negative results in all the parasitological tests performed on day 200, including culture from bone marrow aspirates, qPCR of bone marrow, spleen, liver, and skin, and xenodiagnosis. \*P < 0.05 according to Fisher's exact test.

Importantly, treatment with the drug combination resulted in significant decreases in the parasite loads in the bone marrow and spleen on days 140 and 200 compared to the parasite loads in the pretreatment period. This is in contrast with the other groups, which did not show significant reductions in the bone marrow parasite loads on day 200. Furthermore, the drug combination reduced the parasite loads in bone marrow to a significantly greater degree than the LEMP-allopurinol protocol. These results, taken together, indicate at least additive effects of LMA and allopurinol.

The importance of the use of splenic aspirates in monitoring treatment was previously demonstrated in dogs infected with *Ehrlichia canis* treated with doxycycline (19). To the best of our knowledge, the present study is the first to evaluate the efficacy of treatment of CVL according to the parasite burden in the spleen by using qPCR prior to and during treatment. The use of an ultrasound device to guide the aspiration of the spleen allowed the safe collection of samples from this organ without complications such as hemorrhages or ruptures. Given the importance of the spleen in the context of CVL (31), evaluation of the parasite burden in this organ during treatment could help in monitoring relapses and response to therapeutics.

A major benefit of LMA-allopurinol combination is its ability to produce negative qPCR results in the liver of all treated dogs. This remarkable effect is most probably due to the extremely high accumulation of antimony in the liver promoted by the liposome formulation (29). In accordance with this interpretation, the LMA group showed only one animal (18.7%) with positive qPCR of liver tissue, whereas the Allop and LEMP+Allop groups presented 37.5 and 62.5% positive animals.

Because of their hematophagous behavior, sand flies need direct contact with the skin of vertebrate hosts. The skin of dogs is the site where transmission of parasites occurs, both from infected dogs to noninfected sand flies and from infected sand flies to noninfected dogs, which spreads the disease to other dogs and humans (33).

Therefore, one of the most important objectives of the treatment of CVL is the blockade of transmission to sand flies by eliminating parasites in the skin or, at least, reducing the number of parasites to such a level that transmission does not occur (24). Thus, some authors (24) have proposed reduction in the infectivity of dogs to sand flies through treatment as a key measure in control programs designed to eradicate active foci of CVL.

In our study, the capacity of *L. longipalpis* to be infected with *L. infantum* after feeding in infected dogs was investigated through xenodiagnosis. Although it is the most accurate experimental tool for assessing the epidemiologic impact of treatment of CVL, xenodiagnosis is not a simple methodology and basically is restricted to research institutions (11, 24). Since our data show a positive correlation between the xenodiagnosis result and the number of parasites in the ear skin (Spearman's correlation, 0.6518;  $P < 0.0001$ ), in accordance with a recent study (5), skin qPCR may be used as an alternative protocol to assess the infectivity of dogs to the sand flies.

Both the xenodiagnosis and skin parasite load results showed that the LMA-allopurinol combination was the most effective protocol for inhibiting the transmission of skin parasites to *L. longipalpis*. Indeed, it promoted the blockade of transmission of parasites to sand flies on both days 140 and 200 and resulted in the highest percentage (83.3%) of dogs negative according to skin qPCR. This is in contrast with the results obtained with the LMA

and LEMP+Allop groups, which both presented infective animals on day 200 and lower percentages of negative dogs (50 and 25%, respectively).

An important question to be answered is whether LMA-allopurinol combined treatment is capable of promoting parasitological cure of infected dogs. This is a crucial question for the control of visceral leishmaniasis, since no fully effective treatment has been reported so far (2, 30) and an effective treatment would block the transmission of the parasite to sand flies and humans and reduce the risk of emergence of resistance to antimony. Based on the absence of parasites in bone marrow, spleen, liver, and ear skin and negative xenodiagnosis on day 200, the LMA-allopurinol combination was found to promote parasitological cure in 50% of the dogs treated. In the other groups, cured animals were found only in the Allop group, but at a much lower rate (8%). These findings confirm our previous report that LMA alone cannot promote parasitological cure (28).

The criteria used here for parasitological cure are based on the absence of parasites in critical sites of infection and on the blockade of parasite transmission to the sand flies at a specific time. Importantly, this evaluation was performed in treated dogs after a 60-day period without treatment, to allow the occurrence of possible relapses. Thus, in the LMA+Allop group, two of five animals initially negative in bone marrow and spleen (on day 140) became positive on day 200. In this context, our claim for achievement of parasitological cure should be taken cautiously, since not all tissues were evaluated and one cannot completely exclude the possibility of other relapses after a longer period of time without treatment.

As a major advance, the present study displays for the first time a new, highly effective therapeutic alternative for CVL, based on the combination of nanotechnology and conventional therapy, with the prospect of achieving parasitological cure. In future studies, new protocols based on this combination should be designed to confirm the achievement of cure in treated dogs and to further enhance the cure rate of CVL. A higher cure rate may be expected from an increase in the number of doses of LMA, from the use of allopurinol for a more prolonged period of time, and from the improvement of the liposomal formulation for more effectively reaching less accessible sites of infection.

## ACKNOWLEDGMENTS

We acknowledge the Brazilian agencies CNPq (grants 303046/2009-0, 473534/2010-0, and 473601/2009-5), FAPEMIG (grants REDE-221/08, REDE-40/11, APQ-01935-09, APQ-01355-09, PRONEX 2009, and PPM-00382-11), and CAPES for financial support. R.R.R. was the recipient of a postdoctoral fellowship from FAPEMIG.

We are also grateful to Oscar Bruna-Romero from ICB-UFMG for his helpful assistance with qPCR assays.

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RESEARCH

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# Long-term follow-up of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol

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## Abstract

**Background:** Visceral leishmaniosis is a potentially life-threatening illness caused by a protozoan parasite of the genus *Leishmania*. It is found mainly in areas where both the parasite and its vector are endemic and is one of the most challenging infectious diseases in the world to control. HIV infected patients are vulnerable to *Leishmania* infections, and the main reservoir hosts of *Leishmania infantum* parasites are domestic dogs. Here, we evaluated the long-term efficacy of treatment with meglumine antimoniate plus allopurinol (G1) compared to miltefosine plus allopurinol (G2) in dogs naturally infected *L. infantum*.

**Methods:** Eighteen dogs with leishmaniosis were divided into the following two groups: G1 ( $n = 9$ ) was treated subcutaneously with meglumine antimoniate (100 mg/kg/day/30 days) plus allopurinol (10 mg/kg/day/30 days), while G2 ( $n = 9$ ) was treated orally with miltefosine (2 mg/Kg/day/30 days) plus allopurinol (10 mg/kg/day/30 days). Thereafter, the same dose of allopurinol was administered to both groups for 6 years. *Leishmania* DNA in lymph node aspirates from the G1 and G2 dogs was quantified by real-time quantitative PCR at baseline and every 3 months for 24 months, and then at 28, 36, 48, 60 and 72 months. At each assessment, the dogs were examined for signs of disease, and their clinical scores were recorded.

**Results:** Both combination therapies produced significant clinical improvements in the dogs, with a significant reduction in the parasitic load in the lymph nodes of the dogs from both groups after 3 months of treatment. Clinical relapses were observed in four dogs from G2 (miltefosine/allopurinol), and just one dog from G1 (meglumine antimoniate/allopurinol). All dogs that relapsed had increased clinical scores, and increased anti-*Leishmania* antibody titers and parasitic loads in their lymph nodes.

**Conclusions:** Long-term, the clinical and laboratory findings of the G1 dogs were more stable than those of the G2 dogs, thus indicating that meglumine antimoniate had better clinical efficacy than miltefosine. The results suggest that treatment with allopurinol as a maintenance therapy is crucial for stabilizing the care of canine leishmaniosis.

**Keywords:** Dog, Leishmaniosis, Meglumine antimoniate, Miltefosine, RT-QPCR

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## Background

Leishmaniosis is a protozoan infection of dogs, and *Leishmania infantum* (syn. *L. chagasi*) is the most important etiological agent [1].

Several drugs used to treat the disease are able to temporarily improve the clinical signs or clinically cure dogs, but none of these treatments reliably eliminates the infection. The most commonly used treatments for CanL are a combination of meglumine antimoniate plus allopurinol, or miltefosine plus allopurinol. Some studies have shown that these drugs, alone or in combination, can clinically cure most dogs of the disease, but they do not lead to complete elimination of the parasite [2–4].

Miltefosine, a phospholipid (hexadecyl-phosphocholine) originally developed as an oral antineoplastic agent, has been registered in India for the treatment of human visceral leishmaniosis since March 2002 [5]. It is the first and still remains the only oral drug that can be used to treat leishmaniosis [6]. The drug was chosen for the elimination of leishmaniosis in India, Nepal and Bangladesh because of its ease of application in parasite control programs [7]. However, recent studies have shown that its efficacy appears to have declined because its relapse rate has doubled [8]. Additionally, our previous study showed that treatment with miltefosine alone reduced *Leishmania* replication but the parasite was not completely removed from the lymph nodes. For this reason, the action of miltefosine for the treatment of canine leishmaniosis appears to be ineffective [9].

In most parts of the world, the most widely used treatment for human and canine leishmaniosis is meglumine antimoniate [10]. Meglumine antimoniate, which has a parasitcidal activity and also potentiates the phagocytic capacity of macrophages [11], causes a marked decrease in the parasite load in dogs during the first four weeks of treatment [3–12]. In contrast, allopurinol has a parasitostatic activity and long-term administration keeps the parasite load low thereby preventing relapses [13, 14]. Previous studies have shown that in combination, miltefosine and allopurinol have similar efficacy as the “gold standard” treatment with meglumine antimoniate and allopurinol; however, the follow-up period for these studies was less than seven months [15].

In the present study, to evaluate the efficacy of the two treatments, meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol, we monitored 18 dogs with leishmaniosis for 6 years to determine if the disease could be eradicated in them.

## Methods

### Ethical statement

Dogs with leishmaniosis were treated and monitored according to the guidelines for the control of canine leishmaniosis issued by the Regione Campania, which provides

compulsory medical treatment for dogs and the periodic monitoring of dogs in this part of Southern Italy. All dogs in this study were infected with leishmaniosis and were, therefore, treated according to the regional legislation and the involvement of the Animal Welfare Committee. Written consent from all dog owners was obtained to allow us to perform clinical evaluations of the dogs, as well as collecting blood and lymph node aspirate samples at the time of diagnosis and during the post-therapy follow-up. All the procedures were performed in the presence of the dog owners and good veterinary practice was used to avoid suffering in the dogs. Also, in this retrospective study no dogs were sacrificed.

### Inclusion criteria

The trial was performed using 18 dogs with leishmaniosis hospitalized at the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II (Naples, Italy). All the dogs were enrolled between May 2001 and January 2013 and were followed-up for at least 6 years. The diagnosis of leishmaniosis in the dogs was based on the clinical manifestations, a positive immunofluorescence test (IFAT) the presence of anti-*Leishmania* serum antibodies, and a positive real-time quantitative PCR assay (RTQ-PCR) to determine the presence of the parasite and estimate its DNA load.

### Exclusion criteria

Dogs with renal failure or with a concomitant disease that might interfere with evaluation of treatment responses were excluded. Additionally, ehrlichiosis was ruled out by a specific *Ehrlichia canis* IFAT, and by clinical examination and hematological tests (thrombocytopenia and leukocytosis). Dogs previously treated with leishmanicidal or leishmanostatic drugs before inclusion were also excluded.

### Visit schedule and sample collection

Before therapy, all the dogs enrolled in the study showed clinical signs and clinic-pathological abnormalities characteristic of CanL at the time of diagnosis. Post-therapy assessments were performed on day 30 and thereafter at 3, 6, 9, 12, 15, 18, 21, 24, 28, 36, 48, 60, and 72 months.

Before therapy and during the post-therapy follow-up, and with the consent of the dog owners, blood and lymph node aspirate samples were collected from the dogs. Clinical evaluation of the dogs and their hematobiochemical profiles (including blood cell counts; aspartate transaminase, AST; alanine aminotransferase, ALT; total serum proteins, creatinine, urea, albumin/globulin ratio, urine analysis, and IFAT) were recorded. Dogs were scored based on the presence and severity of signs attributable to CanL (i.e., weight loss, dermatitis, skin ulcers, alopecia, ocular lesions, generalized lymphadenomegaly, splenomegaly, epistaxis, hemorrhagic diarrhea,

fever, apathy, anorexia, orchitis, lameness, hematuria, bone lesions, and liver involvement), the laboratory parameters, and the sum of the values were recorded to give a clinical score as described previously [9]. In addition, imaging techniques, such as radiography and/or echography, were appropriately performed in all cases in which other concomitant causes were suspected. The dogs were also classified following the clinical guide lines proposed by Solano Gallego *et al.* [16].

#### Treatment

With the agreement of their owners, the study dogs were allocated to two treatment groups. The orally administered drug treatment was preferable where there was a risk of a dog bite, while the parenteral drug treatment was preferred in dogs which were not controlled during the early stages of administration to avoid the possibility that nausea caused expulsion of the drug via regurgitation or vomiting.

The two treatment groups were as follows: Group 1 (G1) contained nine dogs with leishmaniosis treated with meglumine antimoniate (100 mg/kg/per day/30 days, subcutaneous) plus allopurinol (10 mg/kg/per day/sine die; per os, PO); Group 2 (G2) contained nine dogs with leishmaniosis treated with miltefosine (2 mg/Kg/per day/30 days, PO) plus allopurinol (10 mg/kg/per day/sine die, PO). After 30 days of combined therapy, allopurinol was continued at the same dose until the end of the study period (6 years). Thus, the dogs received allopurinol for the entire 72-month study period. G1 and G2 each contained one dog where itching was observed as a side-effect. In both cases we decided to discontinue the treatment for 1 month before recommencing allopurinol administration. Relapsing dogs were re-treated with meglumine antimoniate/miltefosine in combination with allopurinol for another 30 days.

#### Efficacy of treatment: clinical outcome and laboratory analyses

With the consent of the dog owners, the efficacy of each therapy was evaluated at each time-point based on the clinical and laboratory responses to treatment of each dog.

#### Efficacy of treatment: parasite burden

At each time point, the *Leishmania* DNA load in the lymph node aspirates was determined by RTQ-PCR analysis of the parasite DNA using a method described previously [3].

#### Statistical methods

All data are reported as the average, standard deviation (SD), and standard error (SE). Statistical analyses of the data were performed using a Student's *t*-test and an analysis of

variance (ANOVA); *p* values less than 0.05 were considered statistically significant.

#### Results

The clinical scores and serological data for the two populations in each treatment group were recorded over time. We also recorded changes in the parasitic load in the lymph nodes before and after therapy.

#### Clinical outcomes and laboratory analyses

Clinical examination of both study groups was carried out before therapy and at all times during the follow-up as indicated in the Material and Methods section. The clinical scores, IFAT results, and the *Leishmania* DNA load recorded in this study are shown in Tables 1, 2, and 3, respectively. The clinical score for each dog (obtained following the guide lines proposed by Solano Gallego [16] is shown in Additional file 1: Table S1, in which the basal state clinical evaluation was compared with that obtained by the Poot method [17], as was performed herein. The hemato-biochemical alterations observed most frequently included moderate anemia, thrombocytopenia, leukopenia, increased  $\beta$ - $\gamma$  globulins, and hepatic enzymes.

Table 1 shows the pre-therapy scores for the clinical basal state of both groups of dogs based on the presence and severity of the clinical signs and laboratory parameters. The scores for both groups were comparable and not statistically different (6.2 vs 6.0 as averages), while the inter-individual variability (standard deviation) of the groups was 1.6 for G1 and 1.7 for G2. The results of the ANOVA (with Bonferroni post-hoc test) among all scores showed that the basal state was significantly higher than all of the other time points (from 1 to 72 months) in both treatment groups. After thirty days of therapy with meglumine antimoniate or miltefosine, an improvement in the clinical condition of both groups was observed, even though the scores were significantly reduced in G1 dogs where the score decreased an average of 4.5 points (from 6.2 to 1.7) compared to the G2 dogs where the score reduced by 2.3 points (from 6.0 to 3.7). Conversely, the G2 clinical score by ANOVA one month after therapy was significantly higher than all the other time points (from 3 to 72 months). In this analysis, just one comparison was not statistically significant (1 month vs 6 months) and this was related to a relapse in two of the dogs. In G1 a dog relapsed after 12 months while the other subjects were asymptomatic. In contrast, there were no observable relapses in the G2 dogs after 12 months, and there was an improvement in their symptoms compared to the previous months; however, only six out of nine dogs were clinically cured. From month 15, all the G1 dogs were clinically cured. Contrastingly, two G2 dogs relapsed at 28 and 48 months. Hence, a second 30-day cycle of the drug,

**Table 1** Clinical score changes in G1 and G2 treated dogs during follow-up post-therapy. Data are reported as mean and (SD)

Time course months	G1 n = 9	G2 n = 9	p*
Basal	6.2 (1.6)	6.0 (1.7)	0.3894
1	1.7 (1.7)	3.7 (1.2)	0.0051
3	1.3 (1.0)	1.7 (0.5)	0.1922
6	0.6 (0.7)	2.3 (2.4)	0.0246
9	0.0 (0.0)	1.0 (1.6)	0.0380
12	0.9 (2.7)	0.4 (0.7)	0.3180
15	0.2 (0.7)	0.1 (0.3)	0.3304
18	0.1 (0.3)	0.3 (0.5)	0.1418
21	0.1 (0.3)	0.3 (0.5)	0.1418
24	0.2 (0.4)	0.3 (0.5)	0.3119
28	0.3 (0.7)	0.9 (0.9)	0.0862
36	0.0 (0.0)	0.4 (0.5)	0.0111
48	0.0 (0.0)	0.8 (1.1)	0.0243
60	0.0 (0.0)	0.4 (0.9)	0.0750
72	0.0 (0.0)	0.4 (0.7)	0.0426

\*Student's t-test probability significance between groups

**Table 2** IFAT score changes in G1 and G2 treated dogs during follow-up post-therapy. Data are reported as mean and (SD)

Time course months	G1 n = 9	G2 n = 9	p*
Basal	2.8 (1.1)	3.3 (0.7)	0.1093
1	1.3 (0.9)	3.3 (0.7)	0.0001
3	2.6 (0.5)	2.3 (1.0)	0.2818
6	1.6 (0.7)	3.2 (1.0)	0.0004
9	0.9 (0.3)	1.2 (0.8)	0.1408
12	1.1 (1.2)	1.0 (0.7)	0.4050
15	1.0 (0.5)	1.2 (0.7)	0.2177
18	1.0 (0.0)	0.9 (0.6)	0.2934
21	0.9 (0.6)	1.0 (0.5)	0.3377
24	0.9 (0.6)	1.3 (0.5)	0.0537
28	1.0 (0.7)	1.6 (1.4)	0.1550
36	1.0 (0.9)	1.4 (0.7)	0.1277
48	1.0 (0.5)	1.6 (1.1)	0.0982
60	1.0 (0.0)	1.2 (0.7)	0.1661
72	0.3 (0.5)	0.8 (0.7)	0.0646

\*Student's t-test probability significance between groups

antimoniate or miltefosine, was administered to the relapsed subjects in the G1 ( $n = 1$ ) and G2 ( $n = 4$ ) groups, respectively.

The IFAT scores in Table 2 show that in both groups the anti-*Leishmania* antibody titers in the blood decreased progressively from 2.8 (basal) to 0.3 (72 months) in G1 and from 3.3 to 0.8 in G2. However, the anti-*Leishmania* antibody levels in G1 were consistently lower than G2 as early as 6 months during the follow-up period. In G2, however, we observed a rise in the antibody titers in conjunction with the clinical relapses. A comparison of the clinical and IFAT scores is shown in

Fig. 1. The total score obtained from the sum of the clinical and IFAT values is also shown in Fig. 1, panel a.

The IFAT score for the basal state was significantly higher than all the other time points (from 1 to 72 months) in G1 and just one comparison was not significant (basal vs 3 months). The G2 IFAT score for the basal state was significantly higher than all the other time points (from 9 to 72 months); however, the basal score was not significantly different for the 1, 3 and 6 months post-therapy values (by ANOVA).

At 30 days post-therapy with meglumine antimoniate or miltefosine, the IFAT value decreased by 1.5 points

**Table 3** DNA load changes in G1 and G2 treated dogs during follow-up post-therapy. Data are reported as mean and (SD)

Time course months	G1 n = 9	G2 n = 9	p*
Basal	4952 (3341)	5222 (3935)	0.4385
1	386 (417)	1723 (2528)	0.0686
3	94 (84)	149 (120)	0.1356
6	91 (79)	944 (2148)	0.1255
9	76 (19)	184 (253)	0.1114
12	795 (2140)	88 (153)	0.1687
15	71 (17)	56 (63)	0.2475
18	43 (16)	32 (36)	0.2173
21	36 (19)	37 (36)	0.4520
24	33 (18)	778 (2112)	0.1529
28	16 (7)	1155 (3119)	0.1448
36	14 (5)	256 (667)	0.1464
48	4 (3)	756 (2220)	0.1622
60	7 (4)	114 (292)	0.1444
72	16 (27)	98 (243)	0.1616

\*Student's t-test probability significance between groups

(from 2.8 to 1.3) in G1, whereas the G2 score was unchanged (from 3.3 vs 3.3). The G2 IFAT value after 1 month of therapy was significantly higher (by ANOVA) than all the other time points (from 9 to 72 months), and just two comparisons were not significant (1 month vs 3 and 6 months).

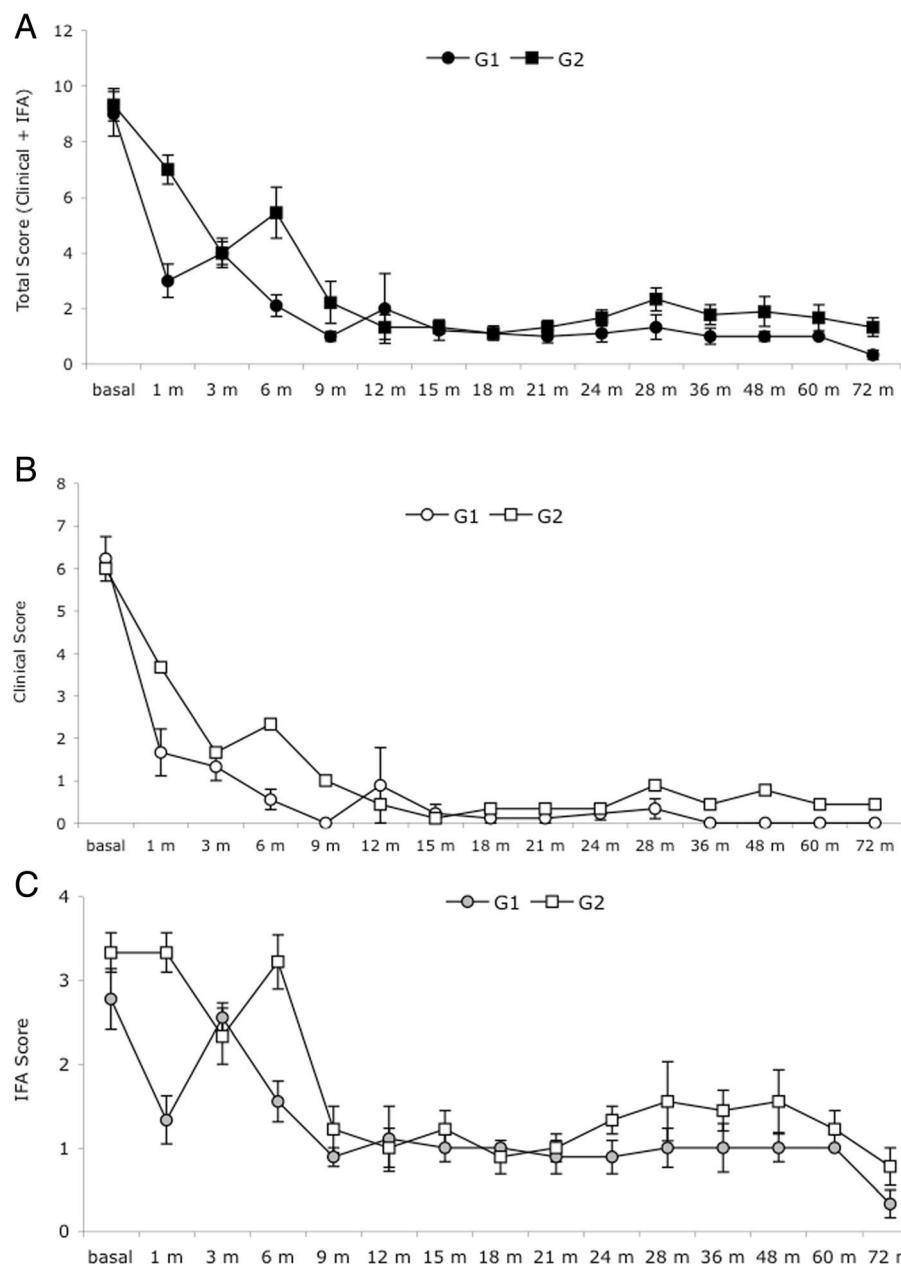
In G1, the IFAT after 3 months was significantly higher than all the other time points (from 9 to 72 months) and just one comparison was not significant (3 vs 6 months). The G2 IFAT after 3 months was not significantly higher than all the other time points (from 6 to 72 months) and just two comparisons were significant (3 vs 18 and

72 months). In G1, the IFAT score at 6 months compared to all the other scores showed just one value that was significantly higher (6 vs 72 months). In contrast, the IFAT scores of the G2 dogs at the 6-month evaluation were significantly higher than all the other time points (from 9 to 72 months).

The quantitative measurements of the DNA in the lymph nodes did not differ significantly in either group at baseline, and on average, the values of *Leishmania* DNA per ml of aspirate for the G1 and G2 dogs were 4952 (range 981–9100) and 5222 (range 159–10,000), respectively. Table 3 shows that the *Leishmania* load before therapy in both groups of dogs was not statistically different (4952 vs 5222, average values). Inter-individual variability between the groups (standard deviation) was 3341 in G1 and 3935 in G2 (Table 3). The score for the basal state compared to all the other time points (from 1 to 72 months) was significantly higher in both groups of dogs (by ANOVA). Figure 2 shows that in both groups the *Leishmania* load after therapy decreased strongly, particularly after 30 days of therapy. Additionally, the parasite load decreased on average by 4565 parasites (from 4952 to 386) in G1 compared to G2 where the load was reduced by 3500 parasites (from 5222 to 1723). The ANOVA also showed that the *Leishmania* load after 1 month of therapy in both groups of dogs did not differ significantly for all the time points (from 3 to 72 months). The decrease in parasite load for G1 was already statistically significant after just one month of treatment, with a linear decrease in the subsequent nine months reaching an average value of 76 *Leishmania* per ml of aspirate (range 42–98). In contrast, the G2 dogs showed higher variability within their group (compared with G1), and at 9 months had an average value of 184 *Leishmania* per ml of aspirate (range 35–698).

In both groups at 3 months after commencing treatment, the parasite load did not differ statistically from each other, and the decreased parasite load in G1 and G2 was about 50 and 35 times lower than the initial values, respectively. At 12 months an increase in the *Leishmania* DNA load was apparent in G1, the average value of which was higher than in G2. In particular, there was higher variability within G1; this was caused by a relapse in one of the dogs that had an increased DNA load (6501 L/ml of aspirate). From months 15 to 21, the *Leishmania* DNA load for both groups was stable and much lower than that of the basal state.

From month 24, the *Leishmania* DNA load in the G1 and G2 dogs differed from each other. In fact, the DNA load in G2 was consistently higher than that in G1, but not statistically different. Additionally, G2 showed more variability in the DNA load compared with G1. However, in all cases the DNA loads for G1 dogs from month 24 onwards were below 33 parasites per ml of aspirate. In



**Fig. 1** Clinical and laboratory scores for G1 and G2 dogs during the post-therapy follow-up period. The scores were recorded at baseline, then every three months for 24 months, and then at 28, 36, 48, 60 and 72 months. The data for (a) total score (clinical + IFAT score), (b) clinical score, and (c) IFAT score. Scores are reported as the average and standard deviation

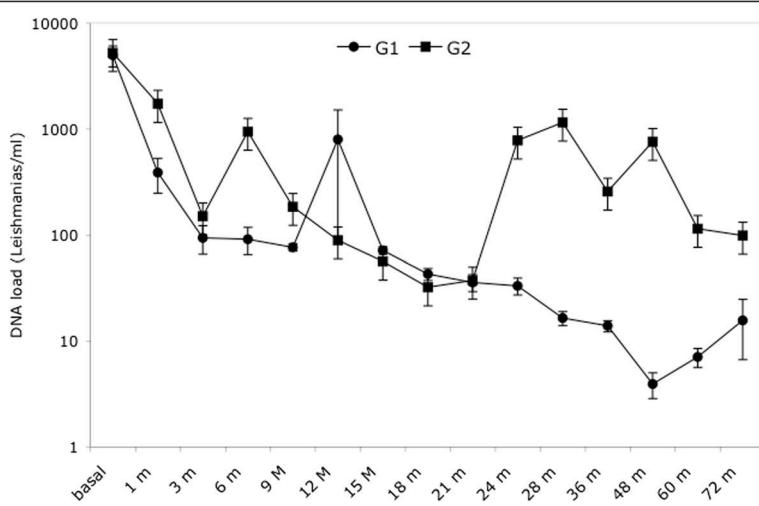
particular, for three of the G1 dogs during the 48 to 72 month period, the DNA load was below three parasites per ml of aspirate, and only one dog had an undetectable *Leishmania* load at 72 months post-treatment.

## Discussion

This longitudinal study involved constant and systematic monitoring of 18 dogs with leishmaniosis over 6 years in order to evaluate the efficacy of two different treatments,

meglumine antimoniate/allopurinol versus miltefosine/allopurinol. It is the first comparative study of two different drug combinations performed by evaluation of the clinical parameters, the hemato-biochemical profiles (urea, serum total protein, creatinine, globulins and albumin, AST, ALT), the serological (IFAT), and molecular (RTQ-PCR) data.

Clinical improvement occurred rapidly in the G1 dogs (30 days after meglumine antimoniate/allopurinol therapy) and less rapidly in the G2 dogs (3 months after miltefosine/



**Fig. 2** *Leishmania* DNA load in G1 and G2 dogs. The parasite load in the lymph node during the post-therapy follow-up was measured by RTQ-PCR. Data are reported as the logarithms of the average values and their standard deviations

allopurinol therapy). However, in agreement with previous reports [14, 18, 19], almost all the dogs in both groups were free of clinical signs at 90 days after drug treatment.

The incidence of clinical recurrence was higher in the G2 dogs than in the G1 dogs from 12 months after starting therapy. Indeed, in G1, eight of nine dogs were clinically cured, and by month 12 only one dog had relapsed. From month 15, all G1 dogs were clinically cured. However, in G2, two dogs relapsed at 6 months and two dogs relapsed at 28 and 48 months. RTQ-PCR analysis of parasite DNA in the lymph nodes of the dogs with leishmaniosis treated with a combination of meglumine antimoniate and allopurinol showed there was a significant decrease in the parasite load of the G1 dogs after only one month of therapy, with a linear decrease observed in the subsequent nine months. In particular, in three dogs from this group the DNA load in their lymph nodal aspirates was below 3 *Leishmania* parasites per ml of aspirate from 48 months to the end of the observation period (6 years). Furthermore, the DNA load of the parasite in one dog was undetectable at 72 months. These values are considered negligible when compared to the initial values, and are in agreement with those reported previously in infected dogs from areas where *Leishmania* is endemic; dogs from endemic regions react to the parasite by immune response activation, and thereby remain clinically healthy or asymptomatic for long periods [20]. In contrast, G2 dogs that received miltefosine plus allopurinol showed greater variability within the group, and although the parasite load in both groups was similar at 3 months, from month 24 the *Leishmania* DNA load showed a different trend between the two groups of dogs with the number of parasites in G2 being higher than that of G1 (not statistically significant).

In this study, we found a positive correlation between the clinical score and the antibody titer (IFAT score) in both groups of dogs (G1 and G2), with correlation factors (*r*) of 0.802 and 0.877, respectively. We also found a positive correlation between the total score (clinical score + IFAT score) and parasite load in both groups (G1 and G2) with correlation factors (*r*) of 0.917 and 0.861, respectively. Also, the IFAT score versus the parasite load showed a positive correlation for both G1 and G2 dogs, with correlation factors (*r*) of 0.679 and 0.705, respectively. Additionally, as has been suggested by Abrantes *et al.* (1991) [21], there was a positive correlation between the clinical score and parasite load in G1 and G2 dogs alike, with correlation factors (*r*) of 0.964 and 0.915, respectively. All the correlations were statistically significant ( $p < 0.05$ ). The supplementary data (Additional file 1: Figure S1 and S2) shows the correlations identified for the clinical and laboratory parameters that were investigated in the G1 and G2 dogs. These results are in agreement with some studies that have indicated the existence of a close correlation between the clinical response and IFAT score reduction after therapy [14, 22]. The results differ from other studies that have shown that IFAT titers can be high in clinically negative dogs [23]. Here, in both G1 and G2 groups, excluding the findings for G1 after 72 months, all the dogs had antibodies against *Leishmania* parasites, and the IFAT scores in both groups correlated positively with their clinical scores and parasite loads. Therefore, serology does not seem to be a reliable way to monitor treatment efficacy in the short-term [23]. There was no correlation between the serological titers and the severity of the clinical signs. It is concluded that the ELISA is a sensitive method for the diagnosis of canine leishmaniosis but is not satisfactory for monitoring the clinical

development of the disease [23]. High antibody levels are associated with high parasitism and disease [24]. However, the presence of low antibody levels is not necessarily indicative of the disease and further work-up is necessary to confirm or exclude clinical leishmaniosis by other diagnostic methods [4].

Notably, during the 72-month follow-up period, in all the relapsed dogs (1 dog in G1 and 3 dogs in G2) a rise in antibody titers was observed in conjunction with the clinical relapses. According to Reis *et al.* [24], our data confirm that IFAT is suitable for identifying *Leishmania*-infected dogs, irrespective of their clinical status. However, when the clinical signs are evident, the antibody levels increase significantly [21]. Based on these results, and on the direct correlation between parasite load and disease severity observed here, we suggest that the RTQ-PCR method is suitable for monitoring changes in the parasitic load during post-therapy follow-up, and may be an effective way to confirm accurately the presence of parasites in dog tissues. This study also begins to address the question of appropriate allopurinol dose during long-term follow-up.

Contrary to the study by Torres *et al.* [14], here, allopurinol was administered for the entire observation period at a dose of 10 mg/kg/day. No symptomatic dogs had urolithiasis, as determined by use of ecographic scanning of the uropoietic system and by urine analysis (for blood and microscopic sediment). Based on these findings, we suggest that a single daily administration of allopurinol at a dose of 10 mg/kg/day may promote greater tolerance of this drug. Furthermore, increasing the administration period by up to 6 years should not cause significant side effects, as was noted in the present study. In fact, only one dog in G1 and one in G2 had itching as a side effect due to a long allopurinol administration period, suggesting possible hypersensitivity of the two dogs as also observed in humans [25–27]. In both cases the treatment was discontinued for one month, after the allopurinol administration was started again.

## Conclusions

After the 6-year follow-up in each treatment group (G1 and G2), the average clinical scores and *Leishmania* loads were significantly lower than before starting therapy. The effects of meglumine antimoniate plus allopurinol seem better than miltefosine plus allopurinol for treating leishmaniosis in dogs, because during the follow-up we observed a decrease in the incidence of disease recurrence. Indeed, one of nine dogs had a recurrence in the meglumine antimoniate plus allopurinol group (G1), compared to four of nine dogs that received miltefosine plus allopurinol (G2). Because a relatively small number of dogs were used in this study, it would be worth repeating it with a larger number of dogs.

## Additional file

### Additional file 1: Additional data.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

LM designed and performed the study and wrote the manuscript; RC helped with molecular work and database searches; GG, AC, and PM participated in performing analyses; AEG provided active supervision and input in study design. All authors read and approved the final version of the manuscript.

#### Acknowledgements

We acknowledge Mr. Ciro Laperuta for his valuable nursing activity in this study.

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Received: 7 December 2014 Accepted: 12 May 2015

Published online: 28 May 2015

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REVIEW

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# LeishVet guidelines for the practical management of canine leishmaniosis

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## Abstract

The LeishVet group has formed recommendations designed primarily to help the veterinary clinician in the management of canine leishmaniosis. The complexity of this zoonotic infection and the wide range of its clinical manifestations, from inapparent infection to severe disease, make the management of canine leishmaniosis challenging. The recommendations were constructed by combining a comprehensive review of evidence-based studies, extensive clinical experience and critical consensus opinion discussions. The guidelines presented here in a short version with graphical topic displays suggest standardized and rational approaches to the diagnosis, treatment, follow-up, control and prevention of canine leishmaniosis. A staging system that divides the disease into four stages is aimed at assisting the clinician in determining the appropriate therapy, forecasting prognosis, and implementing follow-up steps required for the management of the leishmaniosis patient.

## Background

Canine leishmaniosis (CanL) due to *Leishmania infantum* is a major global zoonosis potentially fatal to humans and dogs, which comprise the main reservoir of infection to humans [1]. CanL is endemic in more than 70 countries in the world. It is present in regions of southern Europe, Africa, Asia, South and Central America [2] and has been reported also in the United States of America (USA) [3]. It is also an important concern in non-endemic countries where imported sick or infected dogs constitute a veterinary and public health problem [4].

CanL is manifested by a broad spectrum of clinical signs and degrees of severity, and there is insufficient scientific agreement on the management of this disease [2]. LeishVet is a group of veterinary scientists from academic institutes in Europe and the Mediterranean basin with a main clinical and scientific interest in CanL. The main goal of LeishVet is to develop consensus recommendations that would represent the most current understanding of *L. infantum* infection in dogs based on recent evidence-based literature and clinical experience [2]. The objective of these guidelines is to help

practitioners in the clinical management of CanL with emphasis on diagnosis, clinical staging, treatment, clinical monitoring, prognosis and prevention.

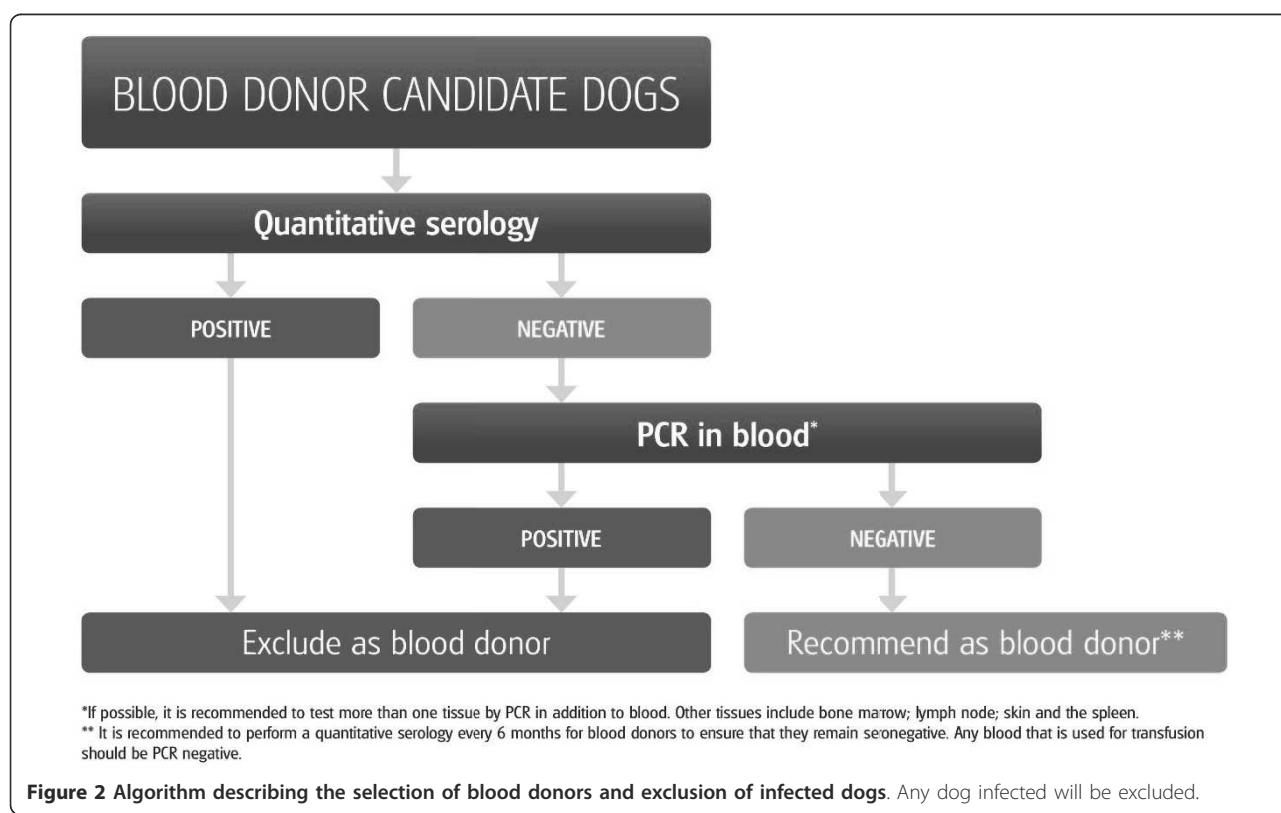
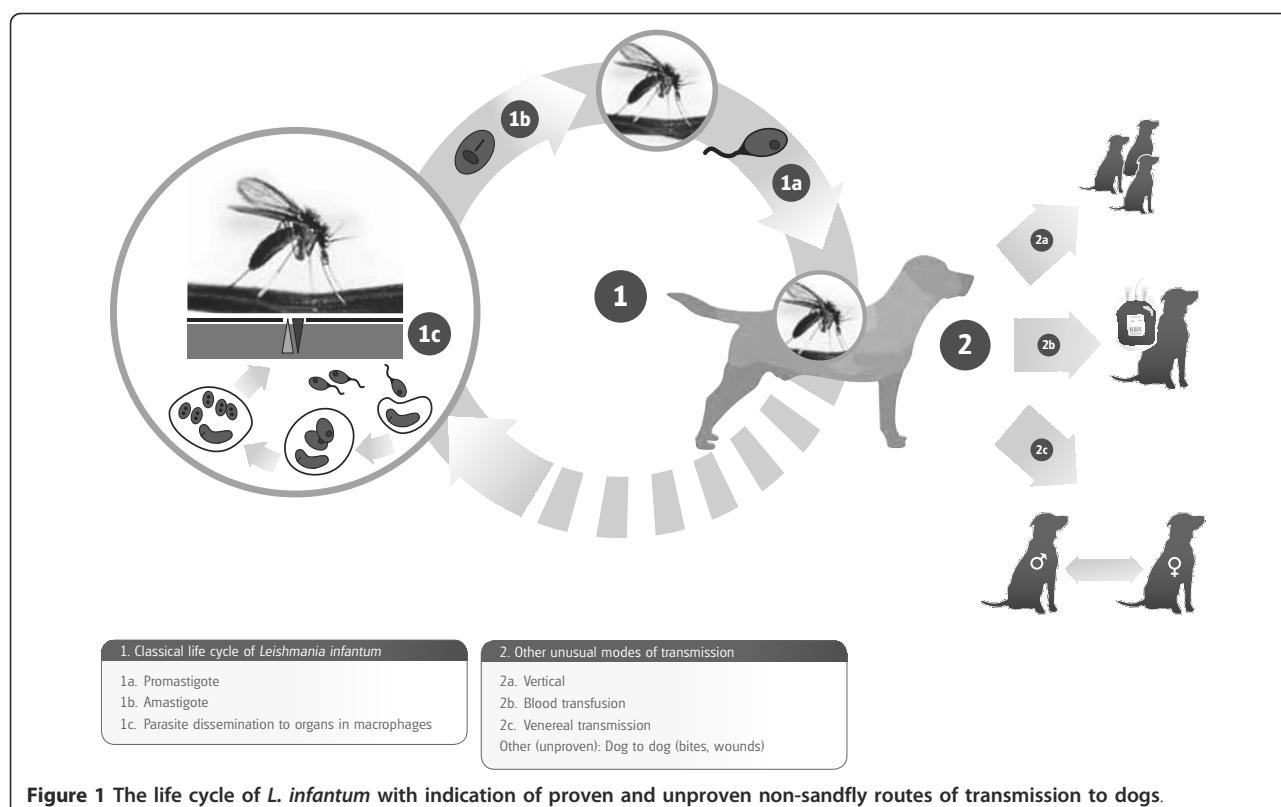
## Life cycle and transmission

*Leishmania* completes its life cycle in two hosts, a phlebotomine sand fly vector, which transmits the flagellated infective promastigote form, and a mammal, where the intracellular amastigote form develops and replicates (Figure 1). Sand flies are the only arthropods that are adapted for biological transmission of *Leishmania*. The relatively low proportion of sand flies harbouring *L. infantum* (0.5 - 3%) is sufficient for maintaining the infection in endemic areas. Non-sand fly modes of transmission have also been described but their role in the natural history and epidemiology of leishmaniosis remains unclear (Figure 1). Proven modes of non-sand fly transmission include infection through transfused blood products [5] from blood donors which are carriers of infection [6,7], vertical [8-10] and venereal transmission [11]. The adequate selection of canine blood donors is of great importance for the prevention of *L. infantum* infection and recommendations on donor selection are graphically summarized in Figure 2. Suspected yet unproven modes of transmission include: 1) direct dog-to-dog transmission through bites or wounds, which could explain the presence of autochthonous CanL

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clinical cases [12] in non-endemic areas in the absence of apparent vectors, as described in foxhounds in the USA [13] or in breeding kennels in Europe [14], and 2) transmission by other hematophagous arthropods such as ticks and fleas [15-21] (Figure 1).

## Distribution and epidemiology

Socioeconomic and possible climate factors have led to changes in the distribution of CanL in Europe (Figure 3).

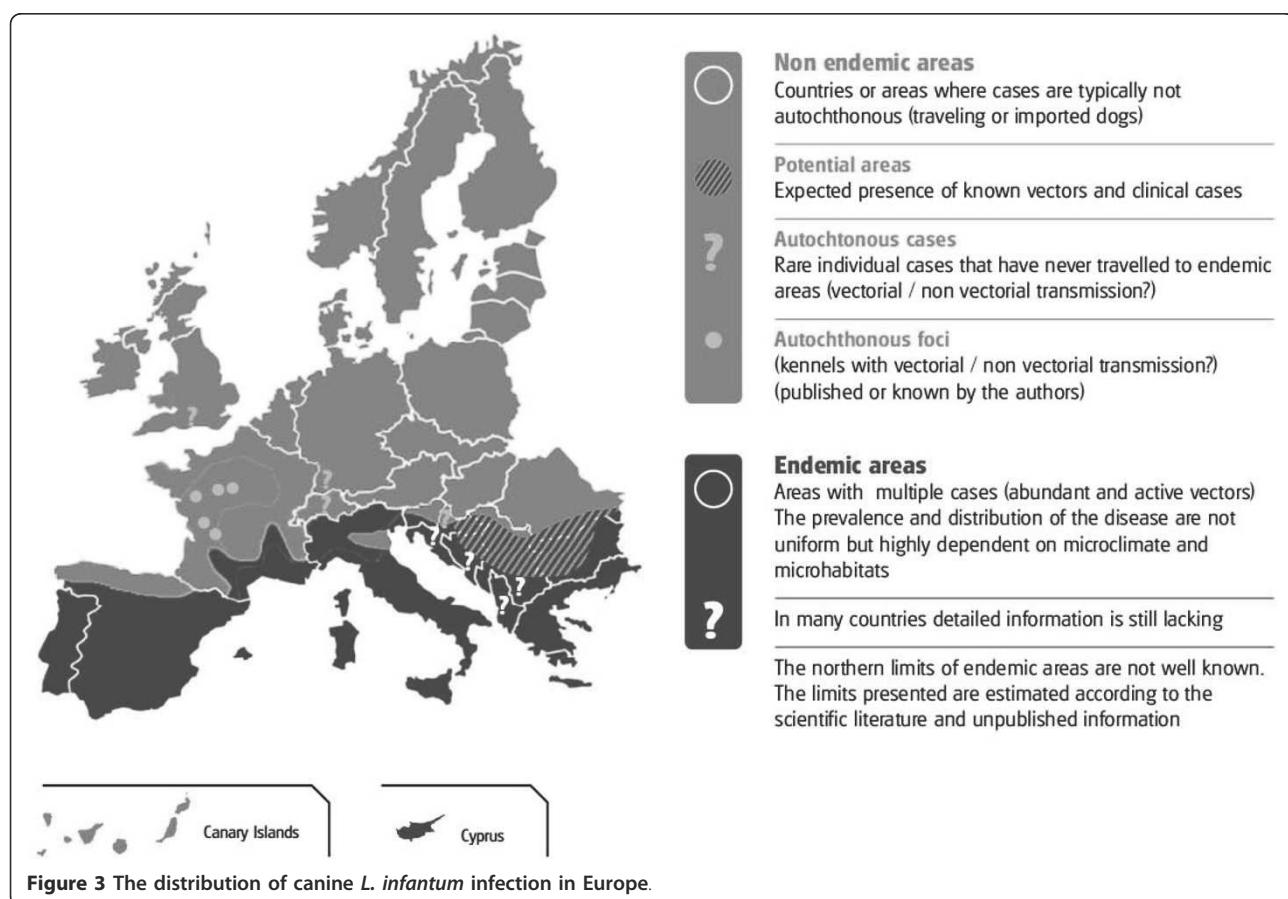
*Leishmania infantum* infection has spread northward reaching the foothills of the Alps in northern Italy [22] and of the Pyrenees in France [14] and northern Spain [23]. The large numbers of dogs travelling to southern Europe or imported as companion animals from areas where CanL is endemic have increased the number of clinical cases reported in non endemic countries such as the United Kingdom [12] and Germany [24].

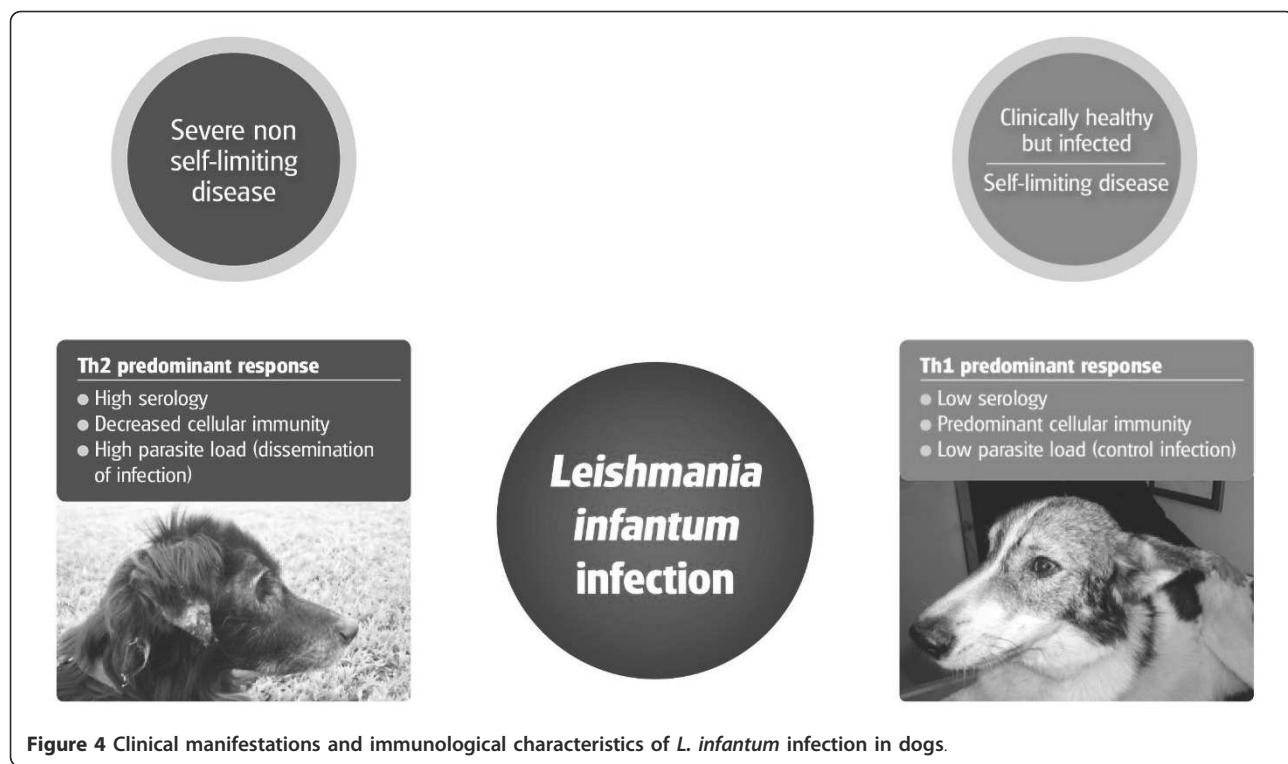
*Leishmania infantum* frequently follows an insidious and chronic pattern of infection [25]. Therefore, CanL is a disease in which infection does not equal clinical illness resulting in a high prevalence of subclinical infection [2,26].

A broad range of immune responses and clinical manifestations have been described in CanL (Figure 4).

Infection in dogs may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness [27]. Subclinical infection is not necessarily permanent and factors such as immunosuppression or concomitant diseases could break the equilibrium and lead to the progression of clinical disease in dogs [2,27] as observed in humans coinfected with human immunodeficiency virus and *Leishmania* [28].

Several predisposing factors for the development of disease have been described including breed, age and genetic background. Some dog breeds such as the Boxer, Cocker Spaniel, Rottweiler and German Shepherd seem to be more susceptible to the development of disease [29,30], while others such as the Ibizian Hound rarely develop clinical signs of CanL [31]. The Slc11c1 (Solute carrier family 11 member a1) gene, formerly named N-RAMP1, and certain alleles of the MHC II genes have been associated with susceptibility to CanL [32,33]. Age seems to be an important factor. The distribution of the disease is bimodal, with the highest prevalence reported in dogs younger than 3 years and older than 8 years [34,35].





### Clinical manifestations and laboratory abnormalities

CanL is a systemic disease that may potentially involve any organ, tissue or body fluid and is manifested by nonspecific clinical signs. The most common clinical manifestations and clinicopathological abnormalities found in CanL are listed in Table 1[2,36,37]. Skin lesions are the most frequent manifestation among them (Figure 5) and may be seen along with other clinical signs or clinicopathological abnormalities. However, dogs can be presented with other clinical signs unrelated to cutaneous lesions as the main presenting complaint [36,37] (Figure 6). Renal disease may be the sole clinical manifestation of CanL and it can progress from mild proteinuria to the nephrotic syndrome or to an end stage renal disease. Chronic renal failure is a severe result of disease progression and the main cause of mortality due to CanL. Despite the high prevalence of renal pathology in infected dogs [38,39], renal azotemia is relatively an uncommon laboratory finding. The common pathological findings detected by cytology (Figure 7) or histology in CanL [40-43] are listed in Table 2. However, the variable and nonspecific clinical signs make the list of differential diagnoses wide and extensive.

### Diagnosis

The purposes for which diagnosis of *L. infantum* infection is carried out are outlined in Figure 8. Due to these different diagnostic indications, it is important to

separate *Leishmania* infection from disease and to apply different diagnostic techniques for each state. The definitions of sick *versus* clinically healthy infected dogs are shown in Figure 9 [27].

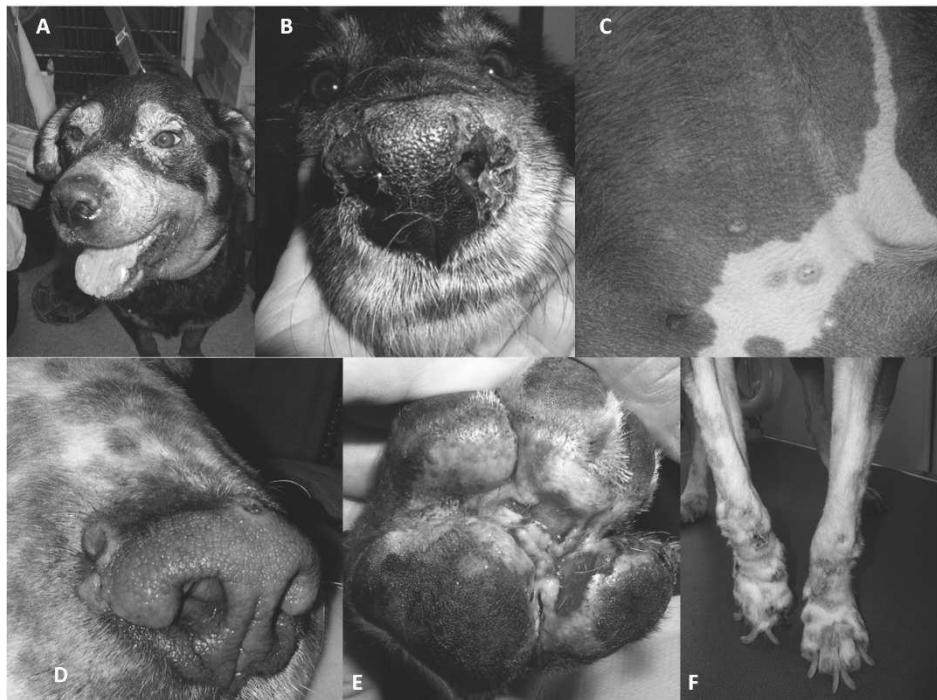
The diagnosis of CanL is complex as the clinical spectrum is broad and the range of clinicopathological abnormalities based on at least a complete blood count (CBC), biochemical profile and urinalysis can be both wide and non-specific. A thorough clinicopathological diagnostic approach needs to be adapted for each patient when assessing the suspicion of this disease. In addition, dogs with leishmaniosis might be co-infected with other vector borne diseases or suffering from other concomitant infectious or non-infectious diseases making the differential diagnoses more complicated and diverse. Therefore, based on the clinicopathological problem list, a differential diagnosis and specific diagnostic approach would be made for each patient.

Different specific diagnostic methods have been described for the detection of *L. infantum* infection in dogs and these are shown in Figure 10. Valid diagnostic tests are essential for the detection of *Leishmania* infection in sick dogs although they lack 100% sensitivity and specificity [27]. The advantages and disadvantages of the different diagnostic methods are summarized in Table 3.

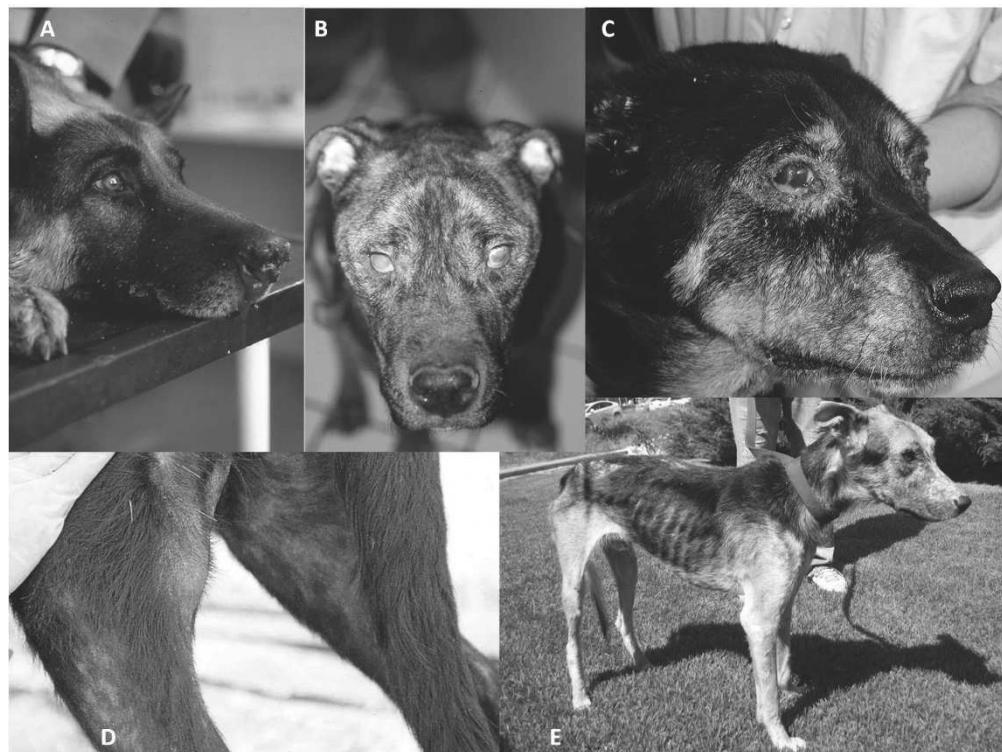
The diagnosis of CanL can be made by the detection of specific serum antibodies (IgG) using preferably quantitative serological techniques, such as the

**Table 1 Clinical manifestations and laboratory abnormalities found in canine leishmaniosis due to *L. infantum***

Clinical manifestations	Laboratory abnormalities
<b>General</b> <ul style="list-style-type: none"><li>○ Generalized lymphadenomegaly</li><li>○ Loss of body weight</li><li>○ Decreased or increased appetite</li><li>○ Lethargy</li><li>○ Mucous membranes pallor</li><li>○ Splenomegaly</li><li>○ Polyuria and polydypsia</li><li>○ Fever</li><li>○ Vomiting</li><li>○ Diarrhea (including chronic colitis)</li></ul>	<b>Serum proteins and electrophoretogram</b> <ul style="list-style-type: none"><li>○ Hyperglobulinemia</li><li>○ Polyclonal beta and/or gammaglobulinemia</li><li>○ Hypoalbuminemia</li><li>○ Decreased albumin/globulin ratio</li></ul>
<b>Cutaneous</b> <ul style="list-style-type: none"><li>○ Non-pruritic exfoliative dermatitis with or without alopecia</li><li>○ Erosive-ulcerative dermatitis</li><li>○ Nodular dermatitis</li><li>○ Papular dermatitis</li><li>○ Pustular dermatitis</li><li>○ Onychogryphosis</li></ul>	<b>CBC/Hemostasis</b> <ul style="list-style-type: none"><li>○ Mild to moderate non-regenerative anemia</li><li>○ Leukocytosis or leukopenia</li><li>○ Thrombocytopathy</li><li>○ Thrombocytopenia</li><li>○ Impaired secondary hemostasis and fibrinolysis</li></ul>
<b>Ocular</b> <ul style="list-style-type: none"><li>○ Blepharitis (exfoliative, ulcerative, or nodular) and conjunctivitis (nodular)</li><li>○ Keratoconjunctivitis, either common or sicca</li><li>○ Anterior uveitis/Endophthalmitis</li></ul>	<b>Biochemical profile/urinalysis</b> <ul style="list-style-type: none"><li>○ Mild to severe proteinuria</li><li>○ Renal azotemia</li><li>○ Elevated liver enzyme activities</li></ul>
<b>Other</b> <ul style="list-style-type: none"><li>○ Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital and nasal)</li><li>○ Epistaxis</li><li>○ Lameness (erosive or non-erosive polyarthritis, osteomyelitis, polymyositis)</li><li>○ Atrophic masticatory myositis</li><li>○ Vascular disorders (systemic vasculitis, arterial thromboembolism)</li><li>○ Neurological disorders</li></ul>	



**Figure 5 Different patterns of cutaneous lesions in CanL:** A) Exfoliative periocular alopecia and blepharitis; B) Ulcerative nasal mucocutaneous lesions; C) Papular dermatitis in the inguinal region; D) Nodular crateriform lesions bordering the muzzle; E) Ulcerative erythematous lesions on the plantar surface of the paw and between pads; F) Onychogryphosis.



**Figure 6** Some clinical signs found in CanL: A) Epistaxis; B) Bilateral uveitis and corneal opacity; C) Purulent conjunctivitis and blepharitis; D) Exfoliative alopecia in the rear leg and popliteal lymphadenomegaly; E) Marked cachexia and generalized exfoliative alopecia.

immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). Immunochromatography-based assays are easy to use and provide rapid qualitative results on the spot, but their performance is still not optimal [44–46]. The interpretation of serological qualitative rapid tests is described in Figure 11. It is important to submit samples to a laboratory that runs quantitative serological assays and can provide an endpoint titer (IFAT) or an optical density reading (ELISA) and a classification of the level of antibodies [27].

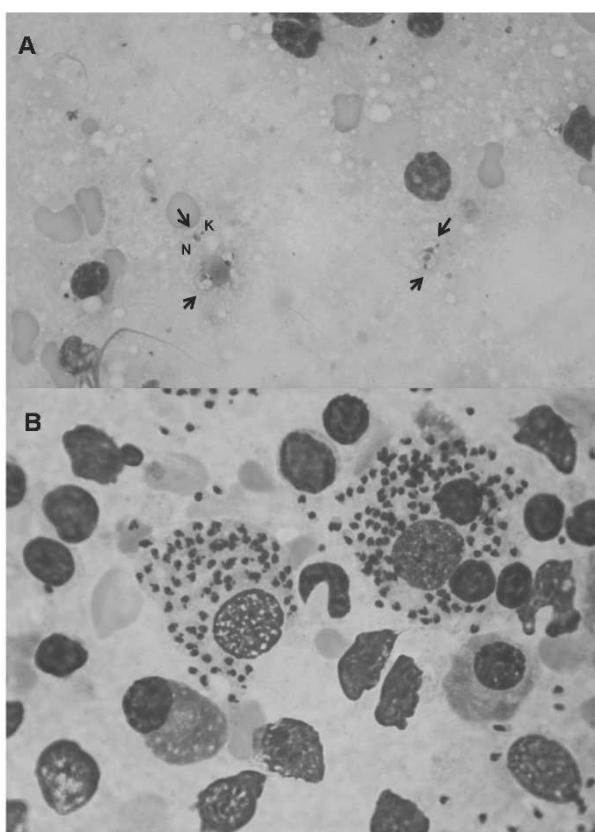
Detection of *Leishmania* DNA in tissues by PCR allows sensitive and specific diagnosis of infection. PCR can be performed on DNA extracted from tissues, blood, body fluids or even from histopathologic specimens. The different sensitivities of tissues for the detection of *L. infantum* by PCR [27,47,48] and variable sensitivities of PCR techniques are listed in Figure 12. Assays based on the detection of kinetoplast DNA (kDNA) appear to be the most sensitive for direct detection in infected tissues [49,50]. Real-time PCR allows quantification of the *Leishmania* parasite load in the tissues of infected dogs, which is useful for the diagnosis and the follow-up during treatment [51,52]. It is important to highlight that information provided by PCR should not be separated from the data obtained from clinicopathological and serological evaluations.

A high level of antibodies confirms the diagnosis of CanL in a dog with clinical signs and/or clinicopathological abnormalities compatible with leishmaniosis [53]. However, the presence of a low antibody level is not necessarily indicative of the disease and further work-up is necessary to confirm or exclude clinical leishmaniosis [27]. The diagnostic approach for sick dogs living in an endemic area is shown in Figure 13. The diagnostic approach for sick or healthy dogs living in a non-endemic area that have travelled to an endemic area, should include quantitative serology three months after the beginning of exposure in the endemic area.

#### Clinical staging, treatment and prognosis

A system of four clinical stages based on clinical signs, clinicopathological abnormalities and serological status was proposed by the LeishVet group in an effort to cover the wide spectrum of clinical manifestations and degrees of severity found in CanL [27]. Different treatment protocols and prognoses are suggested for each clinical stage as described in Table 4.

The most common drugs used for treatment of CanL, including dosage, combinations and side effects, are listed in Table 5. Several other candidate medications against CanL have been studied *in vitro* or in laboratory animals but rarely in controlled clinical trials and they



**Figure 7 Interpretation of cytology** A) Interpretation of cytology requires time and expertise for the detection of *Leishmania* amastigotes when parasites are in low numbers and freed from the cells. Note the nucleus (N) and the kinetoplast (K) of extracellular amastigotes (arrows) in a fine needle aspirate of a reactive lymph node from a dog with clinical leishmaniasis (x100, Diff-quick stain); B) High numbers of intracellular and extracellular *Leishmania* amastigotes in a fine needle aspirate of a reactive lymph node from a dog with clinical leishmaniasis (x100, modified Giemsa stain).

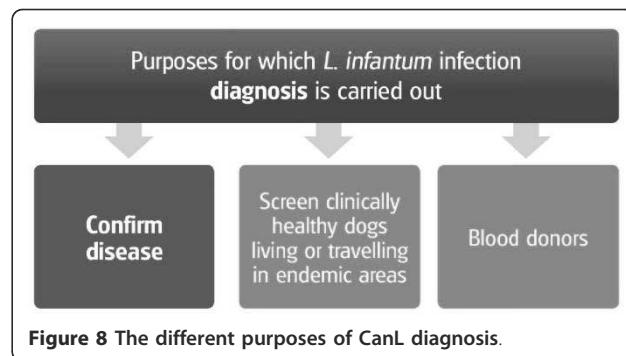
are, therefore, currently not recommended for the routine treatment of CanL [27].

The clinical response to treatment of sick dogs can vary from poor to good depending on their overall initial clinicopathological status and their specific response to therapy. Dogs with renal insufficiency are

**Table 2 Cytological and histopathological patterns suggestive of canine *L. infantum* infection found in organs or body fluids.**

**Pathological findings in organs or body fluids**

- ✓ Macrophagic inflammation (granulomatous)
- ✓ Neutrophilic-macrophagic inflammation (pyogranulomatous)
  - ✓ Lymphoplasmacytic inflammation
- ✓ Reactive hyperplasia in lymphoid organs
- ✓ No evidence or variable numbers of intracellular or extracellular *Leishmania* amastigotes



**Figure 8** The different purposes of CanL diagnosis.

expected to have a lower recovery rate in comparison to those without kidney compromise or only mild proteinuria. Therapy with antileishmanial drugs often leads to clinical cure [54] although treated dogs may continue to harbour the parasite and be infectious to sand flies, but to a lesser extent than pre-treatment [52,55-57].

The vast majority of dogs experience clinical improvement within the first month of therapy [51,52,58]; however, a longer period of therapy may be required for others before improvement is apparent. The frequencies of monitoring and clinicopathological parameters, including serology, to be followed up during treatment of CanL are summarized in Table 6.

The length of allopurinol treatment depends on the severity of the disease, the clinical and parasitological response to treatment and the individual tolerance to this drug. Some extremely susceptible dogs never reach a point that would allow the discontinuation of allopurinol, while others are capable of controlling infection without the need for extremely lengthy treatment [27]. Allopurinol can be discontinued when the combination of the following criteria is made:

- (1) The presence of complete physical and clinicopathological recovery evaluated by a thorough physical examination, CBC, full biochemistry panel and urinalysis.
- (2) A marked decrease of antibody levels (to negative or borderline by a quantitative serological assay).

In addition, allopurinol might be discontinued if it is not possible to control or decrease the xanthinuria with low purine diets or by reducing the drug's dosage, to avoid the risk of urolithiasis, if massive xanthine crystalluria is present [59].

#### **Management of clinically healthy infected dogs in endemic areas**

The management of clinically healthy infected dogs in areas where CanL is endemic is of great importance for practitioners.

## Clinical classification: physical exam and laboratory tests

### Clinically healthy infected

No clinical signs and  
clinicopath abnormalities  
Confirmed infection



### Sick

Clinical signs and/or  
clinicopath abnormalities  
Confirmed infection



**Figure 9 Definition of *L. infantum*-infected but healthy versus sick dogs.** Dogs with clinical leishmaniosis are defined as those presenting clinical signs and/or clinicopathological abnormalities and having a confirmed *L. infantum* infection. Dogs with subclinical infection, or clinically healthy but infected dogs, are defined as those that present neither clinical signs on physical examination nor clinicopathological abnormalities by routine laboratory tests (CBC, biochemical profile and urinalysis) but have a confirmed *L. infantum* infection.

The presence of *Leishmania* DNA in the blood or other tissues of clinically healthy dogs living in endemic areas indicates that these dogs harbour infection [26], but they may never develop clinical disease [60]. In contrast, a high positive antibody titer may indicate that an infected dog is heading towards the development of a widespread infection and future development of clinical disease [53]. Therefore, we recommend using serology alone or the combination of serology with PCR for screening healthy dogs. It is recommended to avoid screening clinically healthy dogs only by PCR.

Healthy dogs should be screened for *Leishmania* antibodies as an initial indication for the presence of infection if [27]:

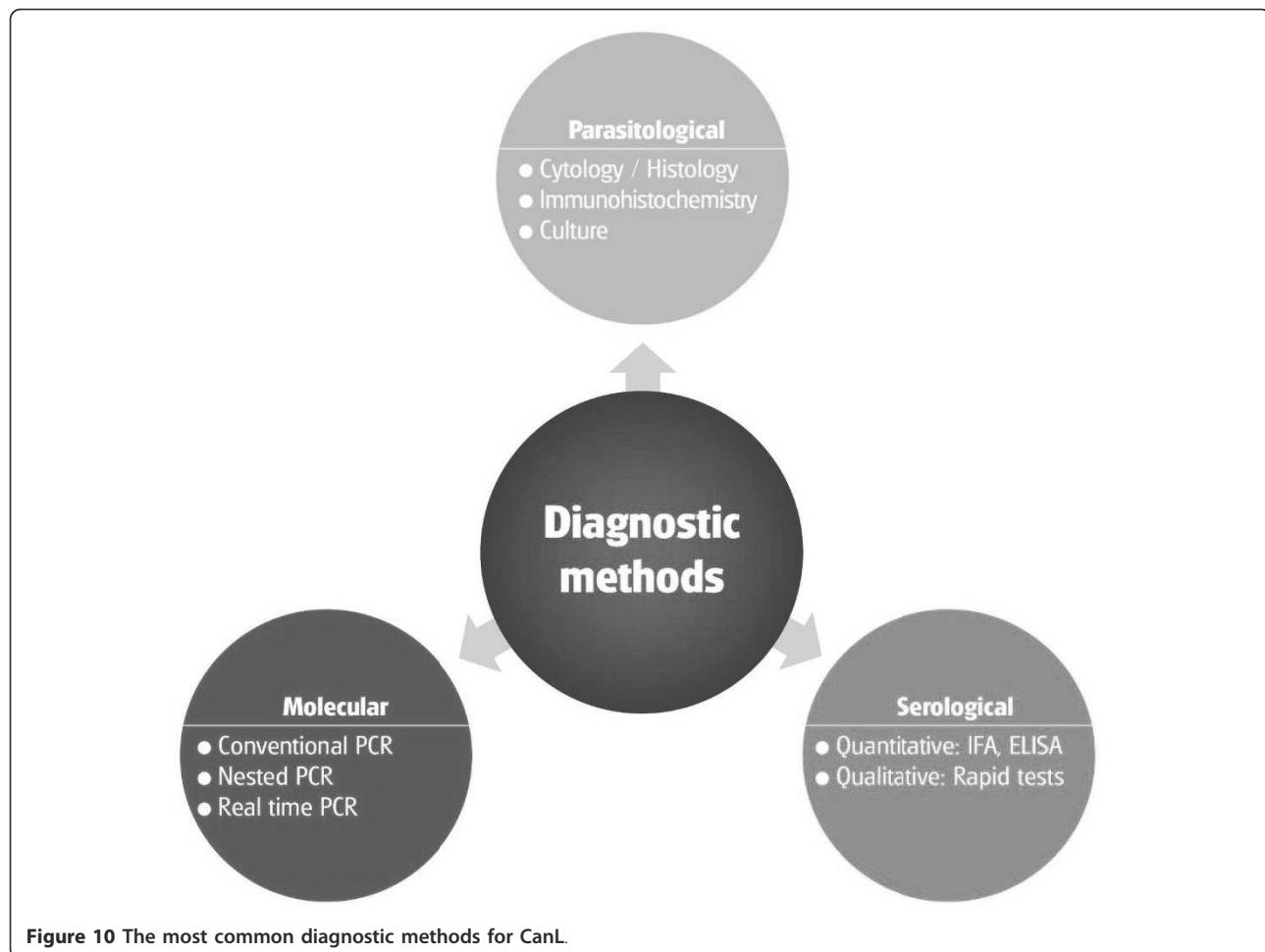
1. They are scheduled to travel or be exported to non-endemic areas (Figure 14)
2. They serve as blood donors (Figure 2)
3. Their owners wish to have them monitored at least every 12 months for early detection of infection and the potential to develop disease.

PCR should be used for the second above indication and as an ancillary test for the first and third above indications.

The management of a clinically healthy seropositive dog and a clinically healthy seronegative and PCR-positive dog is summarized in Figure 14.

### Prevention

Current preventative measures are mainly based on the use of veterinary registered products containing synthetic pyrethroids, permethrin, or deltamethrin with a repellent effect against sand flies whose efficacy has been demonstrated both experimentally [61-63] and in field studies [64-68]. These products are available in spot on formulation or in a collar form and they reduce the risk of new infections and the biting of sand flies on already infected dogs [64-68]. Other measures useful in the prevention of sand fly bites include [69,70]: 1) keeping the dog indoors during the sand fly season from dusk to dawn; 2) reducing microhabitats favourable to sand flies such as piles of wood and



**Figure 10** The most common diagnostic methods for CanL.

stones in the vicinity of the house and in other locations where dogs spend time; 3) usage of indoor insecticide treatment [27].

Long-acting topical insecticides evaluated in several published field studies [64-68] should be applied to dogs living or travelling to endemic areas as follows:

- Permethrin/Imidacloprid spot on formulation: Treatment provides repellent (anti feeding) activity against sand flies (*P. perniciosus*) for three weeks [61]. Repeat administration every 3 weeks. It should be applied at least 2 days before travelling
- Deltamethrin collars: Control of feeding by phlebotomine sand flies (*P. perniciosus*) for a period of 5-6 months [63]. Replace collar every 5-6 months. It should be applied at least 1-2 weeks before travelling.

Veterinarians and dog owners are advised to carefully check the product's label recommendations and follow the manufacturer's instructions for the correct application and frequency of reapplication. Client education on the maintenance of an appropriate insecticide [27]

throughout the period of sand fly activity in the Mediterranean basin (April-November) is also crucial for the protection of dogs [71].

Purified *Leishmania* fraction vaccines appear currently to be the most effective and promising vaccines for dogs. These include the "fucose mannose ligand" (FML)-based vaccine [72,73] and an excreted/secreted antigen purified from specific-medium culture supernatant of *L. infantum* based vaccine [74]. The FML-based vaccine is currently available commercially in Brazil. The same vaccine has also been proposed as a transmission-blocking vaccine [75]. Recently, another vaccine which contains the recombinant A2 protein and saponin as adjuvant has also been approved in Brazil [76]. In Europe, a different vaccine based on cultured *L. infantum* purified excreted/secreted antigens has been approved for vaccination of dogs [74].

The future for CanL control should be an integrated approach to prevention including vaccination against *L. infantum* with an effective canine vaccine and the use of long-acting topical insecticide applications. A vaccine would prevent the establishment of infection introduced

**Table 3 Advantages and disadvantages of common diagnostic methods for the detection of *L. infantum* infection in dogs**

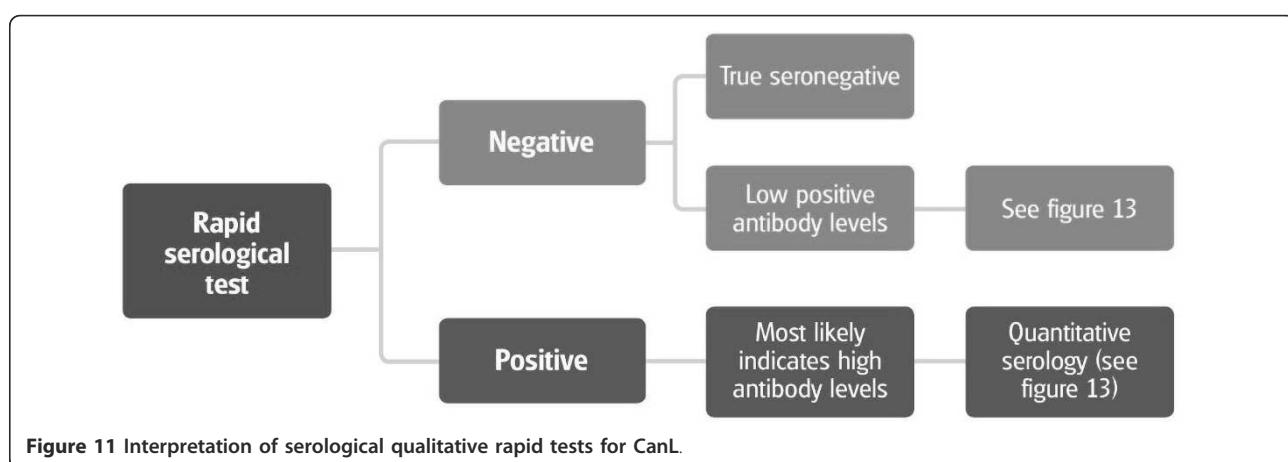
DIAGNOSTIC TECHNIQUES	ADVANTAGES	DISADVANTAGES
SEROLOGY	<ul style="list-style-type: none"> <li>Determination of antibody level which is essential for the diagnosis and establishing a prognosis</li> </ul>	<ul style="list-style-type: none"> <li>Does not detect the actual presence of the <i>Leishmania</i> parasite</li> <li>Serocrossreactions with trypanosomes</li> </ul>
QUALITATIVE	<ul style="list-style-type: none"> <li>Rapid in-clinic test</li> </ul>	<ul style="list-style-type: none"> <li>Provides only positive or negative result</li> <li>Variable sensitivities and performance with risk of false negatives</li> <li>A positive result needs to be further evaluated by a quantitative serology</li> </ul>
QUANTITATIVE (IFAT, ELISA)	<ul style="list-style-type: none"> <li>Determines the antibody level</li> <li>High antibodies levels in the presence of compatible clinical signs and/or clinicopathological abnormalities are conclusive of clinical leishmaniosis</li> </ul>	<ul style="list-style-type: none"> <li>Performance and accuracy of cut-off will depend on the laboratory</li> <li>Differences between laboratories and poor standardization of techniques</li> <li>Low antibody levels will require further work-up</li> </ul>
CYTOLGY/HISTOPATHOLOGY	<ul style="list-style-type: none"> <li>Permits direct detection of the parasite itself and the type of pathological findings:                             <ul style="list-style-type: none"> <li>- Pathological findings suspicious of infection</li> <li>- Allows exclusion of other differential diagnoses</li> <li>- Rapid and non invasive (cytology)</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>Low sensitivity for the detection of <i>Leishmania</i> amastigotes in tissues or body fluids</li> <li>Requires the performance of other diagnostic tests such as immunohistochemistry and/or PCR when parasites are not visualized</li> <li>Does not reveal the immunological status of the dog</li> <li>Needs expertise</li> </ul>
PCR	<ul style="list-style-type: none"> <li>Allows the detection of leishmanial DNA</li> <li>High sensitivity (kDNA) and specificity</li> <li>Parasitic load quantification (if Real time-PCR)</li> </ul>	<ul style="list-style-type: none"> <li>False positive results possible due to DNA contamination</li> <li>Different standardization and techniques used by different diagnostic laboratories</li> <li>Does not reveal immunological status</li> <li>It cannot be performed as the sole diagnostic technique for the confirmation of the disease because a positive result confirms <i>Leishmania</i> infection but not disease</li> </ul>
PARASITE CULTURE	<ul style="list-style-type: none"> <li>Permits the isolation of <i>Leishmania</i> parasites</li> <li>Facilitates the isoenzymatic identification of the parasite</li> </ul>	<ul style="list-style-type: none"> <li>Time-consuming and laborious diagnostic technique</li> <li>It can require one month to provide a result</li> <li>Performed only in research laboratories</li> </ul>

by the bites of those sand flies that escape the insecticide effect [50].

### Public health considerations

In Southern Europe, human visceral leishmaniosis caused by *L. infantum* is a zoonotic disease that affects

young children or adults suffering from the Acquired Immune Deficiency Syndrome (AIDS) or immunosuppressive conditions [77,78]. Dogs are considered the most important peridomestic reservoir of *L. infantum* infection for humans. However, the ownership of an infected dog does not appear to greatly increase the risk



**Figure 11 Interpretation of serological qualitative rapid tests for CanL.**

## What samples and techniques should be used for PCR?

### More sensitive

**Samples**

- Bone marrow
- Lymph node
- Spleen
- Skin
- Conjunctiva

**Techniques**

Real-time PCR

### Less sensitive

**Samples**

- Blood
- Buffy coat
- Urine

**Techniques**

Nested PCR; Conventional PCR

Figure 12 Selection of tissues to be used for PCR and types of PCR techniques when suspecting CanL.

## DIAGNOSTIC APPROACH

Dog with clinical signs and/or clinicopathological abnormalities compatible with CanL

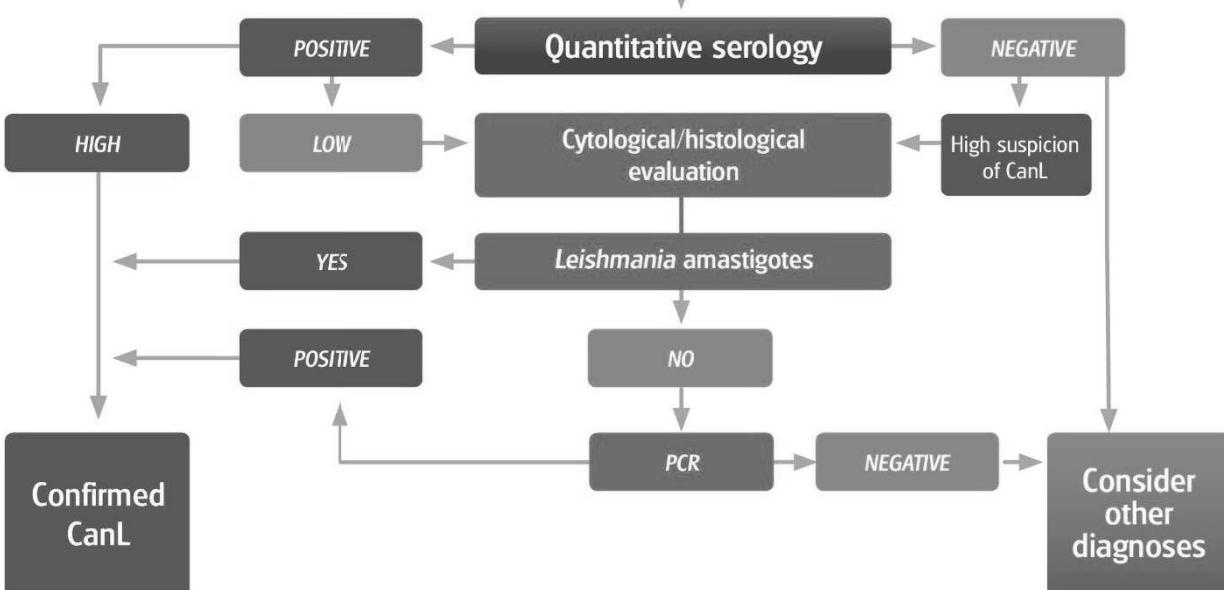


Figure 13 Flow chart for the diagnostic approach to dogs with suspected clinical signs and/or clinicopathological abnormalities consistent with CanL.

**Table 4 Clinical staging of canine leishmaniosis based on serological status, clinical signs, laboratory findings, and type of therapy and prognosis for each stage [27]**

Clinical stages	Serology *	Clinical signs	Laboratory findings	Therapy	Prognosis
<b>Stage I Mild disease</b>	Negative to low positive antibody levels	Dogs with mild clinical signs such as peripheral lymphadenomegaly, or papular dermatitis	Usually no clinicopathological abnormalities observed  Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5	Scientific neglect/allopurinol or meglumine antimoniate or miltefosine/allopurinol + meglumine antimoniate or allopurinol + miltefosine**	Good
<b>Stage II Moderate disease</b>	Low to high positive antibody levels	Dogs, which apart from the signs listed in stage I, may present: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), anorexia, weight loss, fever, and epistaxis	Clinicopathological abnormalities such as mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome  <b>Substages</b> a) Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 b) Creatinine < 1.4 mg/dl; UPC = 0.5-1	Allopurinol + meglumine antimoniate or allopurinol+ miltefosine	Good to guarded
<b>Stage III Severe disease</b>	Medium to high positive antibody levels	Dogs, which apart from the signs listed in stages I and II, may present signs originating from immune-complex lesions: vasculitis, arthritis, uveitis and glomerulonephritis.	Clinicopathological abnormalities listed in stage II  Chronic kidney disease (CKD) IRIS stage I with UPC > 1 or stage II (creatinine 1.4-2 mg/dl) [79]	Allopurinol + meglumine antimoniate or allopurinol + miltefosine  Follow IRIS guidelines for CKD [80]	Guarded to poor
<b>Stage IV Very severe disease</b>	Medium to high positive antibody levels	Dogs with clinical signs listed in stage III. Pulmonary thromboembolism, or nephrotic syndrome and end stage renal disease	Clinicopathological abnormalities listed in stage II  CKD IRIS stage III (creatinine 2-5 mg/dl) and stage IV (creatinine > 5 mg/dl) [79] Nephrotic syndrome: marked proteinuria UPC > 5	Allopurinol (alone)  Follow IRIS guidelines for CKD [80]	Poor

\*Dogs with negative to medium positive antibody levels should be confirmed as infected by other diagnostic techniques such as cytology, histology, immunohistochemistry or PCR. High levels of antibodies, defined as a 3-4 fold elevation above the cut off level of a well established reference laboratory, are conclusive of a diagnosis of CanL. \*\*Dogs in stage I (mild disease) are likely to require less prolonged treatment with one or two combined drugs or alternatively monitoring with no treatment. However, there is limited information on dogs in this stage and, therefore, treatment options remain to be defined.

of disease in the family when transmission is already present in the region [50].

## Conclusions

The complexity of CanL and the wide range of its clinical manifestations, from inapparent infection to severe disease, make the management of CanL challenging. Diagnosis is performed based on clinicopathological

manifestations and by confirmation of infection using mainly serological and molecular techniques. A staging system that divides the disease into four stages is aimed at assisting the clinician in determining the appropriate therapy, forecasting prognosis, and implementing follow-up steps required for the management of the leishmaniosis patient. Prevention should be an integrated approach including vaccination against *L. infantum* with

**Table 5 Current treatment protocols for canine leishmaniosis [27]**

Drugs	Dosages	Main side effects	References
Meglumine antimoniate*	75-100 mg/kg once a day or 40-75 mg/kg twice a day for 4 weeks, S.C.**	Potential nephrotoxicity Cutaneous abscesses/cellulitis	[52,55,57,81-83]
Miltefosine*	2 mg/kg/once a day for 28 days P.O.	Vomiting Diarrhea	[83-85]
Allopurinol	10 mg/kg twice a day for at least 6-12 months P.O.	Xanthine urolithiasis	[51,59,86-89]

\*Registered for veterinary use in most European countries; both drugs are commonly recommended in combination with allopurinol.

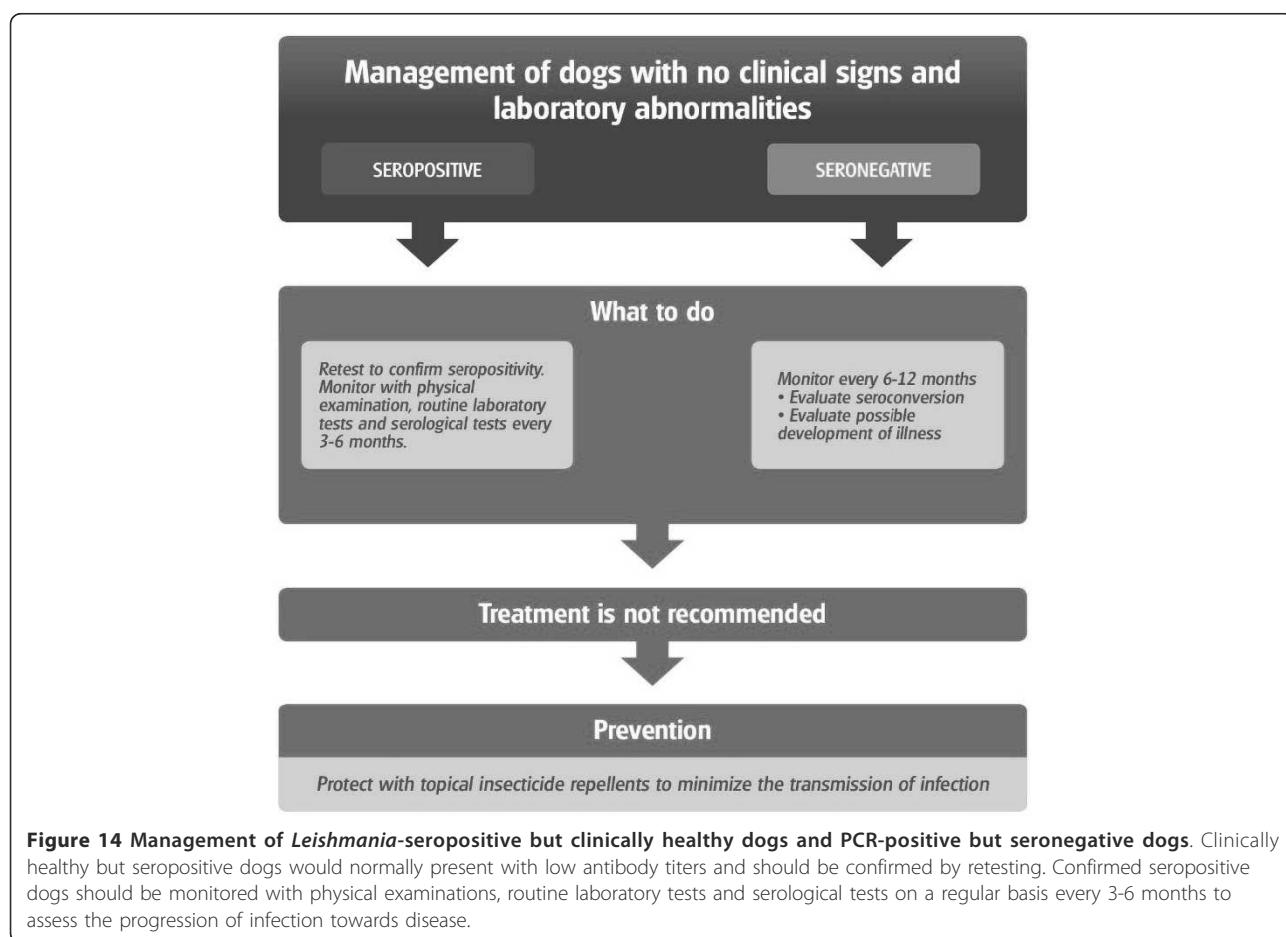
P.O.: per os; S.C.: subcutaneous

\*\*Treatment prolongation by 2-3 weeks may be considered if patient improvement is insufficient.

**Table 6 Treatment of canine leishmaniosis - recommended monitoring of clinicopathological parameters and serology including frequency of follow up [27]**

Parameters	Frequency
Clinical history and complete physical examination	After the first month of treatment and then every 3-4 months during the first year. Later on, if the dog is fully recovered clinically with treatment, a recheck would be recommended every 6 months or once a year.
Routine laboratory tests:	
Complete CBC, biochemical profile, serum electrophoresis (optional) and complete urinalysis including UPC in proteinuric dogs.	
Serology*	Not before 6 months after initial treatment and every 6 months or once a year thereafter.
Real time PCR	Can optionally be carried out at the same time as serology. The full usefulness of this assay for follow up during treatment is currently undetermined.

\*Some dogs present a significant decrease in antibody levels (more than a two-fold dilutions difference between the first and the following samples) associated with clinical improvement within 6 months to 1 year of treatment. Other dogs might not have a decrease in antibody levels despite clinical improvement. In contrast, a marked increase of antibody levels (more than two-fold elevation between monitoring samples) should be interpreted as a marker of relapse, especially in dogs following the discontinuation of treatment [27].



an effective canine vaccine and the application of a topical insecticide.

#### List of abbreviations

AIDS: Acquired Immune Deficiency Syndrome; CanL: Canine leishmaniosis; CBC: Complete blood count; CKD: Chronic kidney disease); DNA:

Deoxyribonucleic acid; ELISA: Enzyme-linked immunosorbent assay; FML: Fucose mannose ligand; IFAT: Immunofluorescence antibody test; IgG: Immunoglobulin G; IRIS: International Renal Interest Society; kDNA: Kinetoplast DNA; MHC II: Major histocompatibility complex type II; N-RAMP1: Natural Resistance-associated Macrophage Protein one; PCR: Polymerase chain reaction; P.O.: per os; S.C.: subcutaneous; Slc11a1: Solute carrier family 11 member a1; UPC: Urinary protein creatinine ratio; USA: United States of America

### Acknowledgements

The authors would like to acknowledge Dr. Norbert Mencke and the kind support of Bayer Animal Health GmbH. The authors would like to thank scientists, human physicians and veterinarians that have advanced the understanding of canine leishmaniosis. Publication of this manuscript has been sponsored by Bayer Animal Health GmbH. All authors are members of LeishVet. LeishVet address: Dpto. Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Spain e-mail: leishvet@vet.ucm.es

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### Authors' contributions

LSG, GM, AK, LC, MGP, LF, PB, GO and GB participated in the formation of the manuscript's content. LSG coordinated the preparation and writing of the manuscript. All authors contributed to helpful discussions, read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

Received: 25 April 2011 Accepted: 20 May 2011 Published: 20 May 2011

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doi:10.1186/1756-3305-4-86

Cite this article as: Solano-Gallego et al.: LeishVet guidelines for the practical management of canine leishmaniosis. *Parasites & Vectors* 2011 4:86.

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# In Vitro and In Vivo Interactions between Miltefosine and Other Antileishmanial Drugs

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Received 9 August 2005/Returned for modification 5 September 2005/Accepted 13 October 2005

The interaction of miltefosine with amphotericin B, sodium stibogluconate, paromomycin, and sitamaquine was assessed in vitro and additionally for the first three combinations in vivo. In vitro interactions were indifferent for miltefosine combined with amphotericin B (mean sums of fractional inhibitory concentrations [mean  $\Sigma$ FICs] ranging from 1.22 to 1.51 at the 50% effective concentration [ $EC_{50}$ ] level and 1.08 to 1.38 at the  $EC_{90}$  level), sitamaquine (mean  $\Sigma$ FICs from 1.33 to 1.38 and 1.0 to 1.02, respectively), and paromomycin (mean  $\Sigma$ FICs from 0.79 to 0.93 at the  $EC_{50}$  and 0.77 to 1.35 at the  $EC_{90}$  level). Some synergy was observed for miltefosine combined with sodium stibogluconate (mean  $\Sigma$ FICs from 0.61 to 0.75 at  $EC_{50}$  and 0.49 to 0.97 at  $EC_{90}$ ). Different interactions were found in vivo, where the highest potentiation of miltefosine activity was achieved with amphotericin B (activity enhancement index [AEI] of up to 11.3). No significant interaction was observed when miltefosine was combined with sodium stibogluconate (AEI of up to 2.38). The potentiation of miltefosine in vivo was also achieved with the combination of miltefosine and paromomycin (AEI of up to 7.22).

Visceral leishmaniasis (VL) is one of several manifestations of disease caused by protozoan parasites belonging to the genus *Leishmania*. The causative species of VL are *Leishmania donovani* on the Indian subcontinent and East Africa, *Leishmania infantum* in the Mediterranean region, and *Leishmania chagasi* in the New World, with the latter two species having a close genetic relationship (15). VL is endemic in 62 countries, with a total of 200 million people at risk (15), and coinfection with human immunodeficiency virus poses a major problem in chemotherapy (1). The yearly incidence is estimated at 0.5 million cases (11). Recommended first-line therapies include pentavalent antimonials ( $Sb^V$ ) (sodium stibogluconate [Pentostam] and meglumine antimoniate), the polyene antibiotic amphotericin B as well as its liposomal formulation, AmBisome, and the diamidine pentamidine. Drugs currently in clinical trials are the aminoglycoside paromomycin and the 8-aminoquinoline sitamaquine (10). All recommended treatments have limitations. Resistance to  $Sb^V$  has developed in some areas of endemicity, such as the high-prevalence epidemic region of the state of Bihar, where failure rates of up to 65% have been reported and where the use of antimony has been abandoned (45).

Miltefosine (Impavido), an alkylphosphocholine, is the most recent drug to come to the market for the treatment of VL. Cure rates of 95% and 94% were achieved after oral administration in phase II and phase III trials in Indian patients, leading to its registration in India in 2002 (17, 44). In a compassionate-use program involving 39 patients with human immunodeficiency virus-leishmaniasis coinfection, initial parasitological cure was observed in 41% of patients (41). Although miltefosine has the advantage of being an effective oral drug, its use in women of child-bearing age is restricted due to teratogenicity, which has been observed in one species (rat) with a no-effect dose level of 0.6 mg/kg. In addition, miltefosine

has a long half-life, which might encourage the emergence of resistance once its use becomes widespread (5). In laboratory studies, miltefosine-resistant clones of *L. donovani* were selected (40), and resistance was shown to be related to two mutations on an aminophospholipid translocase (32).

Drug combinations that aim to delay or prevent the emergence of resistance, increase efficacy, or shorten the course of treatment are the standard in the treatment of several viral, bacterial, and parasitic infections (31, 49). In VL, the combination of paromomycin plus sodium stibogluconate (8, 28, 39, 47) as well as sodium stibogluconate combined with allopurinol (7, 42) have been investigated experimentally and clinically. Combination therapy for leishmaniasis could have a role in delaying the development of resistance and shortening the duration of treatment (43). Here, we report on the in vitro and in vivo interactions between miltefosine and other antileishmanial drugs. The aim of this study was to acquire baseline data for a rational approach to identify miltefosine combinations for the therapy of VL.

## MATERIALS AND METHODS

**Parasites.** *Leishmania donovani* strain MHOM/ET/67/L82 was used throughout the study. The strain was maintained in Syrian hamsters (*Mesocricetus auratus*), and amastigotes were harvested from the spleen of an infected animal.

**Drugs.** Miltefosine was obtained from Zentaris GmbH (Frankfurt am Main, Germany) (formerly Asta Medica). Sodium stibogluconate was a gift from GlaxoSmithKline (Dartford, United Kingdom), and WR6026 (sitamaquine) was obtained from WRAIR (Washington, D.C.). Amphotericin B deoxycholate (Fungizone) was obtained from E.R. Squibb and Sons (Hounslow, United Kingdom), and paromomycin sulfate was purchased from Sigma (Poole, United Kingdom).

For in vitro assays, 10 mM stock solutions were prepared for miltefosine in deionized water and sitamaquine dihydrochloride in dimethyl sulfoxide (DMSO), and a 5.4 mM stock solution was prepared for amphotericin B deoxycholate in water. Sodium stibogluconate was suspended at 10 mg  $Sb^V/ml$  in DMSO, and paromomycin sulfate was suspended at 100 mM. All subsequent dilutions were prepared in the respective culture medium fresh on the day of the assay. Miltefosine and amphotericin B deoxycholate stock solutions were sterilized by passage through a filter (0.2- $\mu$ m membrane). Suspensions in DMSO were rotated on a Spiramax (Denley, Sussex, United Kingdom) apparatus with glass beads over-

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night prior to the assay to ensure solubility. The final DMSO concentration never exceeded 0.2% and had no deleterious effect on parasite growth.

For the *in vivo* part of the study, sodium stibogluconate was dissolved in phosphate-buffered saline, and miltefosine and paromomycin sulfate were dissolved in deionized water. Amphotericin B deoxycholate was reconstituted according to the manufacturer's directions by rapidly expressing 10 ml water into the lyophilized cake and shaking the vial until the colloidal suspension became clear. Further dilutions were prepared in 5% glucose.

**Determination of drug interactions against intracellular amastigotes in peritoneal macrophages *in vitro*.** *In vitro* drug interactions were assessed using a modified fixed-ratio isobogram method (14). Briefly, predetermined 50% effective concentration ( $EC_{50}$ ) values were used to decide the top concentrations of the individual drugs to ensure that the  $EC_{50}$  fell near the midpoint of a six-point twofold dilution series. Top concentrations used were 40  $\mu$ M for miltefosine and 0.2  $\mu$ M for amphotericin B deoxycholate in a 72-h assay, 20  $\mu$ M for miltefosine in a 120-h assay (interaction assays with miltefosine-sodium stibogluconate and miltefosine-sitamaquine dihydrochloride) and a 168-h assay (interaction assay with miltefosine-paromomycin sulfate), 20  $\mu$ M for sitamaquine (120-h assay), 200  $\mu$ M for paromomycin sulfate (168-h assay), and 20  $\mu$ g/ml Sb<sup>V</sup> for sodium stibogluconate (120-h assay). The top concentrations were used to prepare fixed-ratio solutions at ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of miltefosine and the partner drug, which were serially diluted six times in twofold dilutions.

Peritoneal macrophages from CD1 mice (Charles River Ltd., Margate, United Kingdom) were harvested by lavage with ice-cold RPMI 1640 medium (Sigma, Poole, United Kingdom) 24 h after induction with 2% soluble starch (Sigma, Poole, United Kingdom). Macrophages were diluted to  $5 \times 10^5$  cells/ml in RPMI 1640 medium plus 10% heat-inactivated fetal calf serum, plated in 16-well Lab-Tek tissue culture chamber slides (Scientific Laboratory Supplies, Wilford, United Kingdom) at a plating density of  $5 \times 10^4$  macrophages/well, and allowed to adhere overnight at 37°C in a 5% CO<sub>2</sub>-95% air mixture. Macrophages were infected with amastigotes at a macrophage-amastigote ratio of 1:5. Infected cultures were maintained at 37°C in a 5% CO<sub>2</sub>-95% air mixture. After 20 h, extracellular parasites were removed by washing, and fresh medium containing the different fixed-ratio solutions and their dilutions or no drug was added. Each point was tested in triplicate. Interaction assay mixtures were incubated at 37°C in a 5% CO<sub>2</sub>-95% air mixture for the times indicated above. Further medium changes with fresh drug were carried out after 72 h in the 120-h assays and after 72 h and 120 h in the 168-h assays. Drug activity was determined from the percentage of infected cells in drug-treated cultures in relation to nontreated cultures after methanol fixation and Giemsa staining. From the known concentrations of miltefosine and the partner drug in the fixed-ratio solutions,  $EC_{50}$  and  $EC_{90}$  values were calculated by sigmoidal analysis using MicroSoftx/fit (ID Business Solution, Guildford, United Kingdom). For each of the drugs, an  $EC_{50}$  and an  $EC_{90}$  on its own were obtained from the fixed-ratio solutions at ratios of 5:0 and 0:5. Solutions at ratios of 4:1, 3:2, 2:3, and 1:4 yielded the  $EC_{50}$  and  $EC_{90}$  of each of the drugs in combination (14).

Three separate experiments were performed *in vitro*.

**Determination of FIC index and isobogram construction.** Fractional inhibitory concentrations (FICs) and sum FICs ( $\Sigma$ FICs [FIC miltefosine + FIC partner drug]) were calculated as follows (2): FIC of miltefosine =  $EC_{50(90)}$  of miltefosine in combination/ $EC_{50(90)}$  of miltefosine alone. The same was applied to the partner drug. FICs and  $\Sigma$ FICs were calculated for all fixed-ratio solutions, and FICs were used to construct isobograms. Mean  $\Sigma$ FICs were used to classify the nature of the interaction.

**Assessment of drug interactions *in vivo* using a checkerboard design.** Female BALB/c mice (ordered at 20 g; Charles River Ltd., Margate, United Kingdom) were infected intravenously with  $2 \times 10^7$  *L. donovani* MHOM/ET/67/L82 amastigotes and randomly sorted into groups of five. Mice were dosed 7 days postinfection for five consecutive days and sacrificed 3 days after the completion of treatment (day 14 postinfection). Groups of mice were weighed before and after treatment, and the percent weight change was recorded. Impression smears were prepared from weighed livers, methanol fixed, and stained with 10% Giemsa stain in water. The number of amastigotes per 500 liver cell nuclei was determined and multiplied by the liver weight in milligrams to obtain Leishman-Donovan units (3). The percent inhibition was calculated for all drug-treated groups in relation to a nontreated group, and  $ED_{50}$ s were calculated by sigmoidal regression analysis using MicroSoftx/fit (ID Business Solution, Guildford, United Kingdom).

The dosing scheme followed a checkerboard design. Both miltefosine and the partner drugs were diluted threefold to obtain three different dose levels. All possible combinations of the doses used were tested with a group of five mice for every combined dose and control (16 groups of mice per combination in total).

Miltefosine was administered orally at 15, 5, and 1.6 mg per kg of body weight per dose in combination with sodium stibogluconate and at 9, 3, and 1 mg/kg/dose in subsequent combination experiments. Sodium stibogluconate was administered subcutaneously at 30, 10, and 3.3 mg of Sb<sup>V</sup>/kg/dose; amphotericin B deoxycholate was administered intravenously by slow infusion at 0.5, 0.16, and 0.05 mg/kg/dose; and paromomycin sulfate was administered subcutaneously at 63, 21, and 7 mg/kg/dose.

The miltefosine-sodium stibogluconate combination was tested twice. The top doses used in the first experiment were highly active (30 mg/kg for miltefosine and 45 mg/kg for Sb<sup>V</sup>), and doses were lowered in the subsequent experiment as described above. The combinations of miltefosine with amphotericin B and paromomycin sulfate were tested once.

**Analysis of drug combinations *in vivo*.** Where applicable, isobograms were constructed by plotting the  $ED_{50}$  of miltefosine against the respective dose of the partner drug and vice versa (33). Additionally, an activity enhancement index (AEI) was determined (34), which was calculated as follows:  $ED_{50}$  of miltefosine alone/ $ED_{50}$  of miltefosine in combination. The AEI was calculated for each dose level of the partner drug. An AEI of >1 indicates activity enhancement, and an AEI of <1 indicates activity reduction. An AEI of less than 2.0 was considered insignificant (34).

## RESULTS

**Sensitivities of intracellular amastigotes *in vitro*.** A summary of individual  $EC_{50}$  and  $EC_{90}$  values with the times of incubation required to give suitable dose response effects at both levels is given in Table 1.  $EC_{50}$ s of miltefosine ranged from 10.78  $\mu$ M at 3 days to 2.21  $\mu$ M and 2.68  $\mu$ M at 5 and 7 days, respectively, in repeated experiments. A representative value at 5 days is shown in Table 1. The infection level (percentage of infected macrophages) in untreated control cultures is indicated. Toxicity towards macrophages was observed with miltefosine above 40  $\mu$ M in a 3-day assay and 20  $\mu$ M in a 5-day assay and with sitamaquine above 20  $\mu$ M in a 5-day assay. No concentrations toxic to macrophages were used in this study.

**Drug interactions *in vitro* against intracellular amastigotes in peritoneal macrophages.** *In vitro* interactions were assessed using a modified fixed-ratio isobogram method, and data were analyzed at the  $EC_{50}$  and  $EC_{90}$  levels. Mean  $\Sigma$ FICs are presented in Table 2 for two independent experiments. Representative isobograms are shown in Fig. 1. Interactions were classified as synergistic with mean  $\Sigma$ FICs of  $\leq 0.5$ , as antagonistic with mean  $\Sigma$ FICs of >4, and as indifferent with mean  $\Sigma$ FICs between >0.5 and  $\leq 4$ . The interaction of miltefosine with amphotericin B deoxycholate and sitamaquine dihydrochloride was indifferent with mean  $\Sigma$ FICs of 1.22 to 1.51 and 1.33 to 1.38 at the  $EC_{50}$  level and 1.08 to 1.38 and 1.02 at the  $EC_{90}$  level, respectively. The interaction of miltefosine with sodium stibogluconate was classified as indifferent to synergistic with mean  $\Sigma$ FICs of 0.61 to 0.75 ( $EC_{50}$ ) and 0.49 to 0.97 ( $EC_{90}$ ). Miltefosine interaction with paromomycin sulfate displayed indifference (mean  $\Sigma$ FICs of 0.79 to 0.93 and 0.77 to 1.35, respectively, at both activity levels).

**Miltefosine in combination with selected partner drugs *in vivo*.** Interactions between miltefosine and sodium stibogluconate, amphotericin B deoxycholate, and paromomycin sulfate were determined *in vivo*. AEIs are summarized in Table 3. Miltefosine combined with the top dose of 0.5 mg/kg/dose amphotericin B deoxycholate gave the highest index of 11.31, followed by paromomycin sulfate at the top dose of 63 mg/kg/dose with an index of 7.22. The index for miltefosine combined with 30 mg Sb<sup>V</sup>/kg/dose was insignificant, with an index of 2.38. A graphical representation (isobogram) for the combinations

TABLE 1. Activity of individual drugs against intracellular amastigotes<sup>d</sup>

Drug	Infection level <sup>a</sup> (%)	Days <sup>b</sup>	EC <sub>50</sub> (μM)	EC <sub>90</sub> (μM)
Amphotericin B	84	3	0.033 (0.033–0.034)	0.080 (0.061–0.099)
Miltefosine	89	5	6.18 (6.01–6.36)	14.47 (11.74–17.2)
WR6026 (sitamaquine)	77	5	5.09 (4.78–5.41)	20.33 (18.48–22.18)
Sodium stibogluconate (Sb <sup>v</sup> )	89	5	2.39 <sup>c</sup> (2.06–2.72)	13.43 (10.47–16.39)
Paromomycin	71	7	40.60 (32.39–48.81)	136.8 (100.8–172.7)

<sup>a</sup> Infection level indicates the percentage of macrophages infected in untreated control cultures at the end point.

<sup>b</sup> Days indicate the number of days of incubation.

<sup>c</sup> Values for sodium stibogluconate are given in micrograms of Sb<sup>v</sup> per milliliter.

<sup>d</sup> All drugs were used in their salt form as stated in the text.

miltefosine-amphotericin B deoxycholate and miltefosine-paromomycin sulfate is given in Fig. 2. The interaction of miltefosine with amphotericin B deoxycholate displayed a concave curve consistently at all dose levels, demonstrating a gradual increase of miltefosine activity with escalating doses of amphotericin B. This is also reflected in the AEI (Table 3). The combination of miltefosine and paromomycin sulfate showed different interactions at different dose levels with a loss of activity when miltefosine was combined with 21 mg/kg of paromomycin, which is reflected in both the isobologram and AEI (Table 3). It has to be noted that the in vivo activity of paromomycin sulfate was low, with the ED<sub>50</sub> lying outside the dose range tested.

## DISCUSSION

This is the first systematic study of interactions between antileishmanial drugs. This study aimed to identify drug combinations that can potentiate the activity of miltefosine and described a matrix of patterns of interaction between antileishmanials. The nature of interaction was first determined in vitro, and selected combinations were further investigated in vivo. Analysis of drug interactions aimed to indicate whether the nature of the interaction could be categorized as synergistic, indifferent, or antagonistic. Definitions and discussions are available for in vitro studies for some microbes, but data obtained from animal models are less defined and more difficult to interpret (13, 18). In vitro data are based on an extended ratio and concentration range. In vivo, the number of manageable doses is limited. For detection of potentiation, choosing the right doses in relation to the level of analysis is crucial. Methods of analyzing the in vivo combinations were based on those used for antimalarial drug combinations (33, 34).

Different trends were demonstrated between in vitro and in vivo data. To classify the interactions, mean ΣFICs of 0.5 and 4 were used as cutoffs. These categories take experimental error ranges based on twofold dilutions into account, which raises questions about the biological relevance of values between 0.5 and 4 (18, 30). All in vitro interactions assessed displayed indifference. The same trend of in vitro interactions was observed in a study on promastigotes (unpublished data). The interaction of miltefosine and sodium stibogluconate was on the borderline of synergism in vitro but showed no poten-

TABLE 2. Mean ΣFICs of interactions between miltefosine and partner drugs towards intracellular amastigotes in vitro

Partner drug	Expt	Days <sup>a</sup>	Mean ΣFIC ± SD <sup>b</sup> at EC <sub>50</sub>	Mean ΣFIC ± SD <sup>b</sup> at EC <sub>90</sub>
Amphotericin B	1	3	1.22 ± 0.15	1.38 ± 0.13
	2	3	1.51 ± 0.44	1.08 ± 0.27
Sodium stibogluconate	1	5	0.61 ± 0.10	0.49 ± 0.09
	2	5	0.75 ± 0.05	0.97 ± 0.21
WR6026 (sitamaquine)	1	5	1.38 ± 0.29	1.02 ± 0.29
	2	5	1.33 ± 0.13	1.00 ± 0.25
Paromomycin	1	7	0.79 ± 0.13	0.77 ± 0.22
	2	7	0.93 ± 0.14	1.35 ± 0.31

<sup>a</sup> Days of incubation.

<sup>b</sup> SD, standard deviation.

tiation in vivo. Conversely, the interaction of miltefosine and amphotericin B was the most positive in vivo. Differences between in vitro and in vivo data can be expected, as in vitro systems measure direct antiparasitic activity, but in vivo parasites are exposed to variable levels of drug and metabolites in different tissues.

The activity of miltefosine was also enhanced when it was combined with the top dose of paromomycin in vivo. There was a slight decrease in activity over the mid-range of doses. Different interactions at different dose levels have been reported for antimalarial combinations (16, 48). In the case of drugs with pleiotropic modes of action, as is likely for miltefosine, the nature of the interaction could differ between doses. When the data obtained were ranked, combinations with the maximal tolerable drug exposure seem more relevant than combinations with suboptimal ones (18).

There are various accepted mechanisms underlying the different interactions (18).

However, the mechanisms of action of antileishmanial drugs are still poorly understood. Suggested targets of miltefosine in *Leishmania* include perturbation of ether-lipid metabolism, glycosylphosphatidylinositol anchor biosynthesis, and signal transduction (21) as well as inhibition of the glycosome-located alkyl-specific acyl coenzyme A acyltransferase, an enzyme involved in lipid remodeling (22). Recently, it has been demonstrated that miltefosine inserts into the membrane by miscibility and interacts with sterols (36). The toxicity of amphotericin B is through its binding to sterols in the cell membrane and formation of aqueous pores (4). A higher affinity for 24-substituted sterols, predominant in *Leishmania* and fungi, than for cholesterol, predominant in mammalian cell membranes, determines its selectivity, and C-24-alkylated sterols are absent from *L. donovani* promastigotes resistant to amphotericin B (35). However, a similar content of 24-alkylated sterols was found in wild-type and miltefosine-resistant promastigotes at the plasma membrane level (37). Still, the membrane could be the site of the interaction between miltefosine and amphotericin B.

Studies on the mode of action of paromomycin in *Leishmania* are sparse, with mitochondrial ribosomes (24), induction of respiratory dysfunction, and mitochondrial membrane depolarization implicated as suggested targets (23). Pentavalent antimonials are generally regarded as prodrugs requiring conversion to the trivalent form (Sb<sup>III</sup>), and suggested targets have

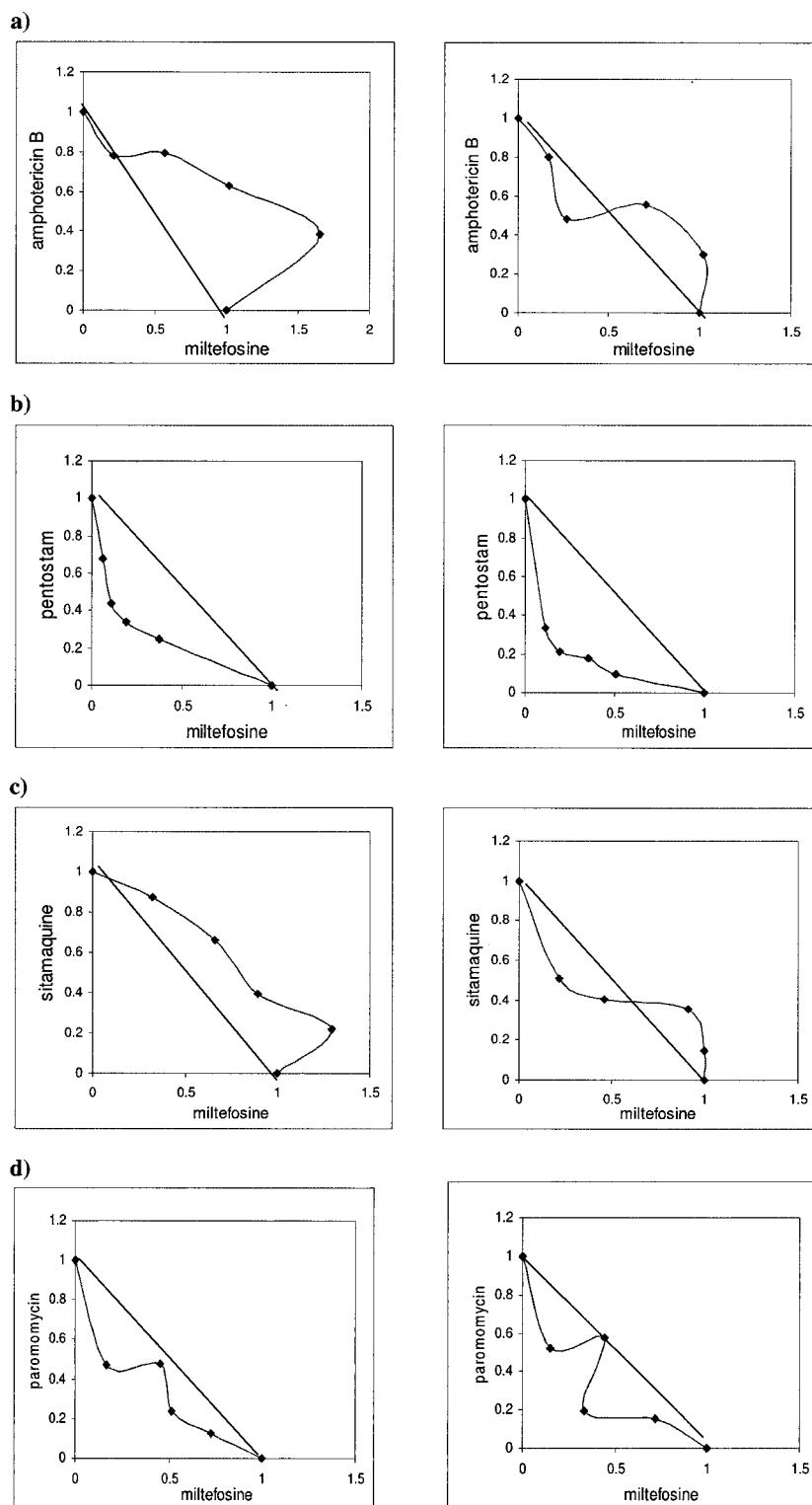


FIG. 1. Representative isobolograms of in vitro interactions against intracellular amastigotes. Interactions are given at the EC<sub>50</sub> (left) and EC<sub>90</sub> (right) levels. Numbers on the axes represent normalized FICs of miltefosine (x axis) and of the respective partner drugs (y axes). (a) Miltefosine-amphotericin B; (b) miltefosine-sodium stibogluconate; (c) miltefosine-sitamaquine; (d) miltefosine-paromomycin. Drugs were used in their salt forms as stated in the text.

TABLE 3. AEIs for in vivo interactions

Partner drug	Dose (mg/kg)	% Inhibition <sup>a</sup> ± SEM	ED <sub>50</sub> of miltefosine (mg/kg)	AEI
Sodium stibogluconate	0		5.06	
	3.3	2.12 ± 6.13	4.41	1.15
	10	45.37 ± 5.94	4.68	1.08
	30	79.08 ± 5.33	2.13	2.38
Amphotericin B	0		11.08	
	0.05	20.58 ± 6.26	4.01	2.76
	0.16	24.89 ± 3.54	3.27	3.39
	0.5	50.22 ± 4.51	0.98	11.31
Paromomycin	0		7.36	
	7	17.07 ± 11.7	4.52	1.63
	21	36.23 ± 5.83	11.14	0.66
	63	41.88 ± 3.67	1.02	7.22

<sup>a</sup> % Inhibition, the percent inhibition of the partner drug alone at this dose level in the respective experiment.

been glycolysis, macromolecular biosynthesis, and trypanothione (50).

In addition to mechanistic interactions, pharmacokinetic interactions can occur where absorption, distribution, or elimination of a drug is altered, modifying the efficacy of component drugs (6). "Pharmacokinetic synergism" has been shown for rifampin and quinolones, where increased activity of the combination in vivo is due to improved tissue distribution rather than in vitro synergism (13). Matching pharmacokinetics are important in combination therapy that aims at preventing the emergence of drug resistance. Elimination half-life is one factor that is important for the mutual protection of combined drugs (20). In malaria combination therapy, the time to peak concentrations has been used to explain potentiation effects between atovaquone and proguanil (38). However, given the intracellular localization of the *Leishmania* parasite in organs of the reticuloendothelial system, factors like volume of distribution, tissue distribution, and uptake into macrophages are of equal importance. In this context, it is important to compare the pharmacokinetics of the administered drugs, as these fac-

tors contribute to the interactions in vivo. The plasma half-life of amphotericin B is 89 min, and the volume of distribution was 0.998 liters/kg in mice after intravenous administration (12). The plasma half-life of miltefosine was determined to be 96 h (25), and the volume of distribution was 0.7 liters/kg after oral administration in rats (19). Sodium stibogluconate had a longer half-life (1.46 h) and a smaller volume of distribution (0.25 liters/kg) when administered intravenously to dogs (29).

Combined treatments with various drugs showing some degree of efficacy against *Leishmania* have been reported previously (7, 27), but combination therapy is not standard in the treatment of leishmaniasis. This has been due to the limited availability of effective antileishmanial drugs. With miltefosine, one other effective and oral drug is added to the armory and might change this picture. Advantages of combination therapy have to be weighed against potential disadvantages, as combinations of drugs could increase the likelihood or severity of adverse effects. This is a special concern if overlapping toxicity between drugs exists in the first place. Advantages include the delay or prevention of the development of resistance (9) and shorter treatment regimens that could improve compliance and reduce cost. Leishmaniasis is a neglected disease, and there are few drugs in development; it is essential not to jeopardize the life span of new chemical entities. Potential problems arising from the unrestricted use of miltefosine for monotherapy in India have been pointed out recently (46).

In conclusion, (i) none of the drugs tested decreased miltefosine activity in vivo, (ii) no signs of toxicity were recorded for any of the combinations tested in vivo, and (iii) the in vivo data favor a combination of miltefosine and amphotericin B or paromomycin rather than miltefosine and sodium stibogluconate. This last point might be particularly relevant to treat antimony-resistant VL cases in India. Considering both toxicity and cost of the partner drug (26), paromomycin would appear to be a better option than amphotericin B.

However, further studies to extend the matrix of interactions of antileishmanials, including liposomal formulations of amphotericin B, are under way. Ultimately, any combination will

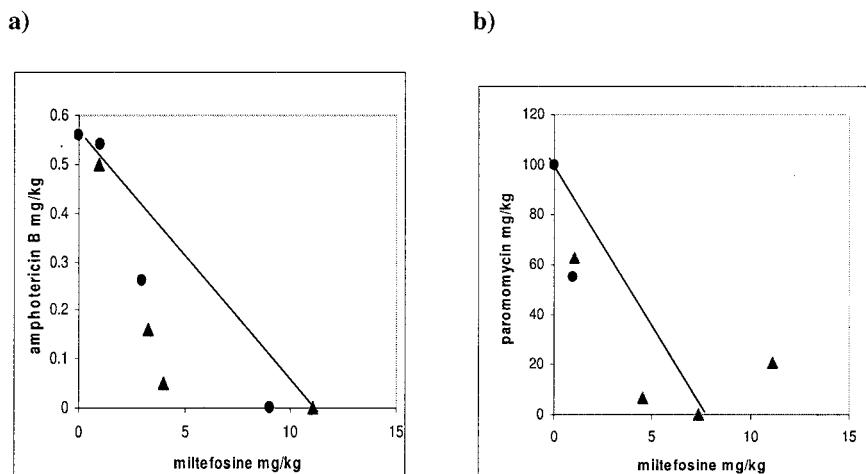


FIG. 2. Isobolograms of the in vivo interactions of (a) miltefosine-amphotericin B and (b) miltefosine-paromomycin. Triangles indicate ED<sub>50</sub>s of miltefosine on the x axis when given with the dose of amphotericin B or paromomycin indicated on the y axis. Conversely, closed circles indicate ED<sub>50</sub>s of amphotericin B or paromomycin on the y axis when given with the dose of miltefosine indicated on the x axis.

have to be tested in human patients and controlled clinical trials to prove useful for patients suffering from VL.

### ACKNOWLEDGMENTS

This work was supported by EC grant QLRT-2000-01404.

We are grateful to Zentaris for providing the miltefosine used in this study and to Quinton Fivelman for information on the fixed-ratio isobogram method in vitro.

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# EXPERT OPINION

1. Introduction
2. Materials and methods
3. Results
4. Discussion
5. Conclusion

## Mixed formulation of conventional and pegylated liposomes as a novel drug delivery strategy for improved treatment of visceral leishmaniasis

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**Objective:** Test the hypothesis that pegylated meglumine antimoniate-containing liposomes (LMA) and their mixture with non-pegylated (conventional) LMA may be more effective than conventional LMA against visceral leishmaniasis (VL), because of wider drug distribution among different mononuclear phagocyte system (MPS) tissues.

**Methods:** Sb was determined in the blood and MPS tissues after administration of pegylated or conventional LMA intravenously to mongrel dogs naturally infected with *Leishmania infantum* and Swiss mice. Pegylated and conventional LMA as well as their mixture were evaluated for their antileishmanial efficacy in BALB/c infected with *L. infantum* through determination of parasite load in liver, spleen and bone marrow.

**Results:** An improved targeting of Sb to the bone marrow of dogs was clearly evidenced, as an important impact of pegylation. In accordance with this data, pegylated LMA significantly reduced parasite load in bone marrow of infected mice, in contrast to conventional LMA. The mixed formulation of conventional and pegylated LMA promoted parasite suppression to a higher extent in both spleen and bone marrow, compared to pegylated or conventional LMA.

**Conclusions:** The present work establishes for the first time the potential of mixed formulations of conventional and pegylated liposomes as a drug delivery strategy for improved treatment of VL.

**Keywords:** antimony, dogs, leishmaniasis, liposomes, pharmacokinetics, visceral leishmaniasis

*Expert Opin. Drug Deliv. [Early Online]*

### 1. Introduction

The leishmaniases are a group of diseases produced by invasion of the mononuclear phagocyte system (MPS) 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 amastigote form when engulfed by host macrophages. Visceral leishmaniasis (VL) is the most severe form of the disease, causing death of humans if not treated [1].

Even though pentavalent antimonials, including meglumine antimoniate (MA), are still the first-line drugs in several developing countries for treatment of all forms of leishmaniasis, they have several limitations [2]. These drugs are administered parenterally, daily, for at least 3 weeks. Antimony therapy is often

accompanied by local pain during intramuscular injections and by systemic side effects, requiring very careful medical supervision [2].

The achievement of complete cure of dogs with VL, or at least the blockade of infectivity to the sandflies vector, is still a great challenge since dogs act as main reservoir for transmission of *Leishmania infantum* to humans and respond poorly to conventional drugs including pentavalent antimonials [3].

In the 1970s, a major advance occurred when it was found that antimonial drugs encapsulated in conventional liposomes were hundreds of times more effective than the free drugs against experimental VL, based on parasite suppression in the liver [4]. This effect of liposome encapsulation was attributed to the drug sustained-release property of liposomes and their natural tendency to be cleared from the circulation by the fixed macrophages of the liver and spleen, which are major sites of parasite infection [5]. In the 1990s, liposomal amphotericin B (AmBisome, Gilead Sciences, Inc., CA, USA) reached the market to treat human VL, showing reduced side effects, improved pharmacokinetic properties and higher cure rates, compared to conventional non-liposomal amphotericin B formulation [6]. However, treatment with AmBisome did not lead to parasitological cure in dogs with VL [7]. Since then, much effort has been devoted to the search for an effective liposomal drug in canine VL [8].

In this context, an innovative liposomal formulation of MA (LMA) has been developed with significant pharmaceutical and pharmacokinetic advantages over conventional formulations [9,10]. Treatment of dogs naturally infected by *L. infantum* with four or six intravenous (i.v.) doses of LMA at 6.5 mg Sb/kg of body weight at 4-day intervals promoted both long-term parasite suppression and reduction of infectivity to sandflies [11,12]. However, parasites were still found in the spleen, bone marrow and skin of treated dogs. These results suggest that improvement of the current liposome formulation and, more specifically, the prolongation of its blood circulation time and its retention time in the tissues are required to more effectively target the leishmanicidal drugs to the less accessible infected sites.

Our group recently reported that reduction of the vesicle diameter of LMA from 400 to 175 nm resulted in more sustained blood levels of Sb in dogs; this modification reduced the targeting of Sb to the liver, but did not improve significantly targeting to the spleen and bone marrow [13].

As an attempt to further prolong the blood circulation time of Sb, we have investigated here the impact of pegylation of LMA on the pharmacokinetics and distribution of Sb in mice and dogs. Pegylated LMA was further evaluated for its antileishmanial efficacy in a murine model of VL and compared to conventional LMA and a formulation consisting of a mixture of conventional and pegylated LMA.

Surprisingly, an improved efficacy for parasite suppression in the spleen and bone marrow was observed for the mixed formulation of conventional and pegylated liposomes.

## 2. Materials and methods

### 2.1 Materials

Cholesterol (CHOL, purity ≥ 99%), dicetylphosphate (DCP, purity ≥ 98%), N-methyl-D-glucamine (purity ≥ 99%) and antimony pentachloride ( $SbCl_5$ , purity ≥ 99%) were purchased from Sigma-Aldrich Co. Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG) were obtained from Lipoid (Ludwigshafen, Germany).

### 2.2 Synthesis of MA

MA was synthesized, as previously described [14], from equimolar amounts of N-methyl-D-glucamine and pentavalent antimony oxyhydrate. The resulting product contained 28% of Sb by weight.

### 2.3 Preparation and characterization of the liposome formulations of MA

Three different liposomal formulations of MA were prepared: conventional liposomes (Lconv) made from DSPC, CHOL and DCP at molar ratio of 5:4:1 and pegylated liposomes made from DSPC, CHOL, DCP and DSPE-PEG at molar ratio of 4.7:4:1:0.3 (Lpeg3) or DSPC, CHOL, DCP and DSPE-PEG at molar ratio of 4.53:4:1:0.47 (Lpeg4.7).

The encapsulation of MA in liposomes was performed as described previously [8], with the following modifications. Small unilamellar vesicles (SUV) were prepared by ultrasonication of a suspension of multilamellar vesicles in deionized water at the final lipid concentration of 55 g/l. After filtration through sterile 0.22 µm membrane, the SUV suspension was mixed with an aqueous sucrose solution at 1:1 sugar/lipid mass ratio. The resulting mixture was immediately frozen in liquid nitrogen and subsequently lyophilized (Freeze-Dryer 4.5 l, Labconco, UK). Rehydration of the dried powder was performed with an aqueous MA solution (Sb concentration of 80 g/l) as follows: 50% of the original SUV volume of MA solution was added to the lyophilized powder and the mixture was vortexed and incubated for 45 min at 60°C. Liposome suspensions were then extruded across two stacked 200-nm polycarbonate membranes at 65°C in order to reduce the mean vesicle diameter [15]. Fifty percent of the original SUV volume of phosphate buffer saline (PBS: 0.15 mol/l NaCl, 0.01 mol/l phosphate, pH 7.4) was then added and the mixture was vortexed and incubated for 15 min at 60°C. Liposome suspensions were diluted 1:10 with PBS and submitted to centrifugation (20,000 × g, 90 min, 4°C) in order to separate drug-containing liposomes from the non-encapsulated drug. The liposome pellet was then washed once (20,000 × g, 90 min, 4°C) and finally resuspended in PBS at a final Sb concentration of 10 g/l. The amount of Sb was determined in the resulting liposome suspension, after digestion of the sample with nitric acid, by electrothermal atomic absorption spectrometry (ETAAS) (Analyst AA600, Perkin Elmer, Inc., MA, USA).

Empty (drug-free) liposomes were also prepared using the same protocol, but replacing the MA solution by 0.15 mol/l NaCl.

The mean hydrodynamic diameter and polydispersity index of the vesicles in suspension were determined by photon correlation spectroscopy at 25°C using a particle size analyzer (Zetasizer S90, Malvern, UK).

The kinetic of release of encapsulated Sb from the different liposomal formulations was evaluated *in vitro*, as previously described [10] and the following modifications. The original formulations were diluted 1:10 in PBS and incubated at 37°C under constant stirring. After different times of incubation, an aliquot was centrifuged (20,000 × g, 90 min, 4°C), the pellet was recovered and Sb was determined as described above.

#### 2.4 Animals and ethics statement

Male mongrel dogs (weighing 5 – 10 kg), of unknown age, and naturally infected with *L. infantum* were obtained by donation from the Center for Zoonosis Control of Ribeirão das Neves City (Minas Gerais State, Brazil), an area where canine VL is endemic. These dogs were previously identified by serological tests and captured as part of the activities of the municipality's Visceral Leishmaniasis Control Program. Animals were found to be positive according to the following tests for *L. infantum* [12]: indirect immunofluorescence antibody test (IFAT), ELISA for antibodies to *Leishmania*, demonstration of leishmania amastigotes in Giemsa-stained bone marrow aspirates and polymerase chain reaction using specific oligonucleotide primers for the amplification of a repetitive DNA sequence of *L. infantum*. The animals were kept in the experimental kennel of the Institute of Biological Sciences at the Federal University of Minas Gerais (ICB/UFMG, Belo Horizonte, Brazil), with drinking water and balanced commercial food (Nero Refeição; Total Alimentos, Brazil) *ad libitum* during the entire experimental period.

Swiss mice (female, 4 – 6 weeks old) used in pharmacokinetic studies were obtained from the biotery of the ICB/UFMG (CEBIO). Free access to a standard diet was allowed, and water was supplied *ad libitum*.

Isogenic BALB/c mice (female, 4 – 6 weeks old), used as an experimental model of VL, were obtained and maintained at the Center for Animal Science of Federal University of Ouro Preto (CCA/UFOP). Animals received water and a commercial rodent diet (Nuvelab CR-II) *ad libitum*.

The present research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) and experimental protocols received approval from the Ethics Committees in Animal Experimentation of the UFMG and UFOP (protocol numbers: 211/2007, 115/2010, 2010/69 and 2012/03).

#### 2.5 Pharmacokinetic studies of Sb in dogs and mice

Infected dogs were divided into three groups, which received the following formulations of MA, as i.v. bolus injection: Lconv ( $n = 5$ ) at 6.5 mg Sb/kg of body weight, Lpeg3 ( $n = 5$ ) at 5.6 mg

Sb/kg and Lpeg4.7 ( $n = 4$ ) at 3.7 mg Sb/kg. The same lipid dose corresponding to 64 mg/kg of body weight was used in the three groups. Blood samples were collected with anticoagulant heparin from the jugular vein at the following time intervals: 5, 20, 60, 150 min, 6, 12 and 24 h. These samples were then frozen at -20°C. All animals were euthanized 24 h after the administration of the formulations. Liver and spleen were recovered, homogenized and frozen at -20°C. In the case of the bone marrow, samples (200 mg for each animal) were obtained by aspiration from both the sternal bone and iliac crest. Skin samples (70 mg for each animal) were also obtained from the ears' internal distal part.

Swiss mice were divided into three groups ( $n = 36/\text{group}$ ), which received 70 µl of the following formulations of MA, as i.v. bolus injection through the tail vein: Lconv; Lpeg4.7; and the mixture of Lconv and Lpeg4.7 at 1:1 lipid mass ratio. These formulations were given at the same dose of 7.5 mg of Sb/kg of body weight. To prevent the possible influence of the lipid dose on the pharmacokinetics, the lipid concentration was adjusted to the same value, through addition of PBS and/or of an empty liposome formulation with the same size and lipid composition, to give a lipid dose of 64 mg Sb/kg of body weight. Animals were then euthanized at the following time points (4 animals per time): 1 min, 5 min, 10 min, 30 min, 1 h, 2.5 h, 6 h, 15 h and 24 h. Blood samples were then collected from the brachial plexus using heparinized tip and frozen at -20°C.

For the determination of Sb in the tissues, the samples were subjected to digestion with nitric acid in a dry block (MA 4004; Marconi, São Paulo, Brazil), as described previously [10]. Antimony was determined in the diluted blood (1:40 in 0.2% nitric acid) and the digested tissue samples by ETAAS, as described previously [10,13]. The analytical methods for determination of Sb in the blood and organs were validated and showed suitable levels of precision (coefficient of variation < 5%), accuracy (80 – 120% analyte recovery) and linearity. The quantification limits of the analytical methods were 0.22 µg Sb/ml for the blood, 0.93 µg Sb/g for the liver and spleen, 2.0 µg Sb/g for the bone marrow and 1.6 µg Sb/g for the skin.

Experimental blood concentration-time data obtained in dogs were analyzed by iterative weighted nonlinear least-squares regression using the Rstrip 4.03 software. This data was best fitted to a monoexponential elimination model according to Akaike's information criterion [16]. Experimental data in mice were best fitted to a biexponential model and analyzed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Fitted parameters included maximum blood concentration of Sb ( $C_{\max}$ ), half-life of Sb in blood ( $t_{1/2}$ ) and AUC.

#### 2.6 Antileishmanial efficacy in a murine model of VL

##### 2.6.1 Infection

*Leishmania infantum* strain (MCAN/BR/2008/OPC43) used in this study, gently provided by Dr. Alexandre Barbosa Reis (Cipharma/UFOP), was originally isolated from a dog

with VL and subsequently typed by Random Amplification of Polymorphic DNA. Parasites were cultivated at  $25 \pm 1^\circ\text{C}$  in Grace's Insect Medium pH 6.5 supplemented with 10% heat-inactivated fetal bovine serum (SFB – LGC, Cotia, SP, Brazil), 2 mmol/l L-glutamine and 100 U/ml G penicillin. When the parasite culture reached the stationary phase of growth,  $1 \times 10^7$  promastigotes were obtained, washed and inoculated to each BALB/c mouse, by i.v. administration through the tail vein. Infected BALB/c mice were then maintained for 6 weeks prior to administration of the different treatment regimens.

### 2.6.2 Treatment regimens

Infected BALB/c mice were divided into six groups ( $n = 8$ ), which received the following formulations (70  $\mu\text{l}$ ), as a single i.v. bolus injection through the tail vein: 1– Lconv at 10 mg Sb/kg of body weight; 2 – Lpeg4.7 at 10 mg Sb/kg; 3 – the mixture of Lconv and Lpeg4.7 at 1:1 lipid mass ratio at 10 mg Sb/kg; 4 – empty Lconv; 5 – empty Lpeg4.7; and 6 – PBS. All mice received the same dose of lipid of 130 mg/kg of body weight. Mixing of Lconv and Lpeg4.7 to prepare the combined formulation was performed just before administration, to eliminate the possibility of transfer of the pegylated lipid from Lpeg4.7 to Lconv.

### 2.6.3 Parasite quantification

On the 14th day post-treatment, animals were euthanized and the parasite load associated to the liver, spleen and bone marrow was determined by the limiting dilution technique with minor modifications [17,18]. Briefly, each animal was submerged in 70% ethylic alcohol for 2 min, and under aseptic conditions, a liver fragment, the spleen and the left leg were collected. Both the remaining liver, its fragment and the spleen were weighed and further maintained in ice-cold DMEM medium pH 7.2 (Gibco<sup>®</sup>) supplemented with 10% SFB, 2 mmol/l L-glutamine and 100 U/ml G penicillin. The liver fragment and the spleen of each animal were individually macerated in DMEM, using a 2 ml potter device (Pyrex<sup>®</sup>, Corning, Inc. Life Sciences, Tewksbury, MA, USA). The whole supernatant obtained by maceration of the liver fragment and 1/5 of the volume obtained by maceration of the spleen were submitted to centrifugation at  $42 \times g$  for 1 min at  $4^\circ\text{C}$  for pelleting out any tissue debris. Parasite load in the bone marrow was evaluated by first dissecting the left leg for recovery of the femur and tibia and, after cutting out their epiphyses, approximately 5 ml of supplemented DMEM medium was injected until complete removal of the cellular content. All biological material obtained with the processing of liver, spleen and bone marrow were centrifuged at  $1540 \times g$  for 10 min at  $4^\circ\text{C}$  and the cell pellet suspended in 500  $\mu\text{l}$  of Grace's Insect Medium, pH 6.5, supplemented with 10% SFB. The obtained cell suspensions were distributed in duplicates using sterile 96 flat-well plate, followed by 1:5 serial dilutions until filling up the entire plate, using the same medium. Parasites were quantified after 14 days in culture at  $25^\circ\text{C}$ , through the first

observation of parasite growth in the most diluted well, considering in this case an estimate of three parasites.

### 2.7 Statistical analysis

Comparison of the results obtained between different experimental groups was performed using Kruskal-Wallis test (followed by Dunn's multiple comparison test) in the case of data with non-normal distribution. Comparison between normally distributed data was performed by one-way or two-way analysis of variance (ANOVA) (followed by Bonferroni post-test) or Student's *t* test. Differences with *p* value  $< 0.05$  were considered statistically significant.

## 3. Results

### 3.1 Preparation and characterization of conventional and pegylated liposomes

Liposome formulations of MA were obtained by reconstitution of empty liposomes initially freeze-dried in the presence of cryoprotective sugar with a concentrated solution of MA and then by extrusion through 200-nm-pore polycarbonate membrane [10]. The specific lipid composition (DSPC, CHOL and DCP at a molar ratio of 5:4:1) was selected on the basis of previous works, showing high MA encapsulation efficiency and stability, as well as good safety data in dogs after treatment with this liposome formulation [8,12,13]. Lconv, Lpeg3.0 and Lpeg4.7 formulations differed in the proportion of PEG-lipid, being, respectively, 0, 3.0 and 4.7% of total lipid. Table 1 shows the results of the characterization of vesicle size distribution and drug encapsulation efficiency. Pegylation of vesicles was found to decrease the drug encapsulation efficiency. Since the encapsulation of MA in DCP-containing liposomes seems to depend on electrostatic interactions between the cationic drug and the anionic membrane [8], one can propose that PEG moiety at the liposome surface may decrease the drug–membrane interaction. As expected from the use of extrusion process, the liposome suspensions showed similar mean diameters and were found to be monodisperse (polydispersity index  $< 0.2$ ).

Figure 1 shows the effect of pegylation on the kinetic of release of Sb from liposomes in isotonic saline for 48 h at  $37^\circ\text{C}$ . The kinetics of drug release were biphasic, with a first phase of fast drug release, followed by a second phase of sustained release. The extent and rate of drug release during the first phase were found to be significantly higher for the pegylated formulations. Thus, pegylation increased the initial rate of drug release from liposomes, suggesting a permeabilizing effect. After 48 h, the formulations showed comparable drug retention efficiencies, with values in the range of 45–58%.

### 3.2 Pharmacokinetics of Sb in infected dogs and in mice

The pharmacokinetics of Sb from conventional and pegylated liposomes was first evaluated in mongrel dogs naturally

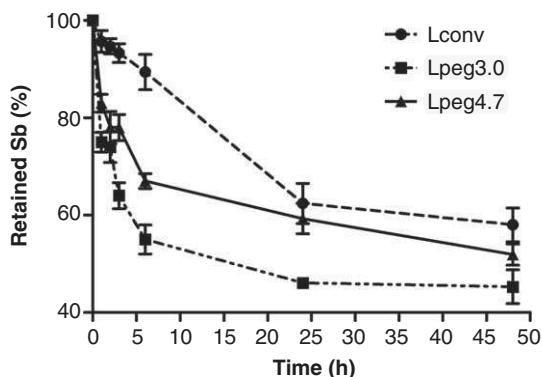
**Table 1.** Characteristics of liposome formulations\* in relation to drug encapsulation efficiency and vesicle size distribution (n = 6).

Formulation	Encapsulation		Vesicle size distribution (value ± SD)	
	%	Sb/lipid mass ratio	Mean diameter <sup>†</sup> (nm)	Polydispersity index
Lconv	34 ± 3	0.25	197 ± 40	0.067 ± 0.022
Lpeg3.0	20 ± 5	0.15	180 ± 33	0.083 ± 0.034
Lpeg4.7	18 ± 4	0.13	181 ± 13	0.086 ± 0.025

\*Liposomes were made from distearoylphosphatidylcholine, cholesterol and dicetylphosphate and included 0, 3.0 or 4.7% of PEG-lipid (Lconv, Lpeg3.0 and Lpeg4.7, respectively).

<sup>†</sup>Mean hydrodynamic diameter.

SD: Standard deviation.



**Figure 1.** *In vitro* stability of the different liposome formulations in isotonic saline at 37°C. Graph shows the time-course of change of percentage of encapsulated Sb, after 10-fold dilution of the formulations in PBS and incubation at 37°C under constant stirring for 48 h (n = 6). Liposomes were made from DSPC, CHOL and DCP and included 0, 3 or 4.7% of PEG-lipid: Lconv (circle), Lpeg3.0 (square) and Lpeg4.7 (triangle), respectively. CHOL: Cholesterol; DCP: Dicetylphosphate; DSPC: Distearoylphosphatidylcholine; PBS: Phosphate buffer saline.

infected with *L. infantum*. Infected dogs were chosen because of their epidemiological relevance and the fact that liposome accumulation in the MPS organs and the skin may be influenced by infection [19]. Indeed, the skin of infected dogs usually exhibits high parasite burden and represents the site where transmission of parasites occurs, both from infected dogs to non-infected sand flies and from infected sand flies to non-infected dogs, which spreads the disease to other dogs and humans [1].

To compare the different liposome formulations and evaluate the impact of pegylation on pharmacokinetics, we chose to administer the formulations at the same lipid dose and with comparable vesicle diameters. The reason for this choice is that lipid dose and liposome size are critical parameters that markedly influenced the pharmacokinetics of conventional liposomes [20]. Thus, by giving the liposome formulations at the same lipid dose and with similar mean vesicle diameters,

one can ensure that the differences observed are due exclusively to pegylation.

On the other hand, since the liposome formulations showed different drug encapsulation efficiencies, different doses of Sb were administered with the different formulations. In this case, antimony can be considered as a marker of liposomes in the pharmacokinetic study, where the half-life of Sb in the circulation (which is independent of the dose of Sb) reflects the half-life of liposomes.

The blood pharmacokinetics of Sb in dogs was best fitted with a monoexponential elimination model. Table 2 shows the pharmacokinetic parameters determined for Lconv, Lpeg3.0 and Lpeg4.7 formulations.

As expected, liposome pegylation was found to increase the half-life of Sb in the blood circulation. The half-life of Sb was significantly greater from conventional liposomes than the pegylated liposomes with highest content of PEG-lipid ( $p < 0.05$ , one-way ANOVA, followed by Bonferroni post-test).

Figure 2 shows the concentrations of Sb determined in the liver, spleen, bone marrow and skin of dogs 24 h after administration. The different liposome formulations promoted similar levels of Sb in the liver, spleen and skin. However, despite the lower dose of Sb given with Lpeg4.7, a twofold higher concentration of Sb was found in the bone marrow of dogs from this formulation, when compared to that of conventional liposomes.

The proportions of total dose of Sb recovered from the different organs were also calculated (Table 3) to compare more precisely the targeting properties of the different liposome formulations. From this data, the targeting effectiveness of Lpeg4.7 to the bone marrow was even more evident, with a 2.8-fold higher proportion of Sb encountered in the bone marrow, when compared to conventional formulation.

The pharmacokinetic properties of these liposome formulations were further evaluated in mice. This additional study was aimed to confirm the stealth characteristics of pegylated liposomes and to investigate for the first time the behavior of a mixture of conventional and pegylated liposomes. The precise knowledge of the pharmacokinetics of these formulations is also important to interpret the efficacy data obtained

**Table 2. Blood pharmacokinetic parameters\* in dogs with VL from conventional and pegylated liposome formulations<sup>†</sup> of MA.**

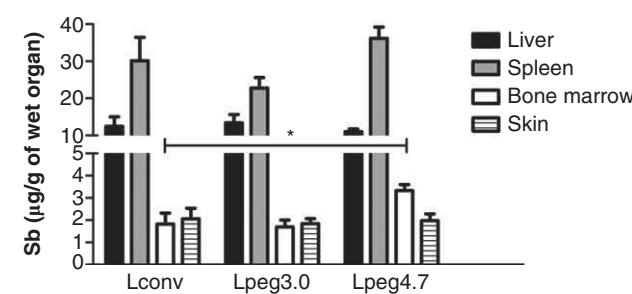
Formulation	Dose (mg Sb/kg)	$C_{max}$ (mg Sb/l)	$AUC_{0 - \infty}$ (g min/l)	$t_{1/2}$ (min)
Lconv	6.5	31.6 ± 6.3	5.7 ± 1.2	127.5 ± 24.1 <sup>§</sup>
Lpeg3.0	5.6	26.4 ± 6.2	6.2 ± 1.9	175.8 ± 54.8
Lpeg4.7	3.7	14.2 ± 1.6	4.8 ± 1.9	223.9 ± 74.1 <sup>§</sup>

\* $C_{max}$ , maximum blood concentration of Sb;  $AUC_{0 - \infty}$ , area under the curve projected to infinity;  $t_{1/2}$ , elimination half-life of Sb; data are shown as means ± SDs (n = 4 – 5).

<sup>†</sup>Liposomes were made from distearoylphosphatidylcholine, cholesterol and dicetylphosphate and included 0, 3.0 or 4.7% of PEG-lipid (Lconv, Lpeg3.0 and Lpeg4.7, respectively).

<sup>§</sup>p < 0.05 for comparison between Lconv and Lpeg4.7 by one-way ANOVA, followed by Bonferroni post-test.

MA: Meglumine antimoniate; VL: Visceral leishmaniasis.



**Figure 2. Concentrations of Sb determined in the liver, spleen, bone marrow and skin of dogs with VL, 24 h after intravenous bolus administration of conventional (Lconv) or pegylated MA-containing liposomes (Lpeg3.0 and Lpeg4.7).** Lconv was given at 6.5 mg Sb/kg of body weight. Lpeg3.0 and Lpeg4.7, containing 3.0 and 4.7% of PEG-lipid, were given at 5.6 and 3.7 mg Sb/kg, respectively. Antimony was determined by ETAAS after digestion of the tissue with nitric acid. Data are given as means ± SEMs (n = 4 – 5).

\*p < 0.05 for comparison of Sb levels in bone marrow between Lconv and Lpeg4.7 (Student's t-test).

ETAAS: Electrothermal atomic absorption spectrometry; MA: Meglumine antimoniate.

**Table 3. Proportions\* of the total dose of Sb recovered from liver, spleen and bone marrow of dogs with VL, 24 h after dosing with conventional and pegylated MA-containing liposomes<sup>†</sup>.**

Formulation	Liver (%)	Spleen (%)	Bone marrow (%)
Lconv	7.74 ± 2.90	3.28 ± 1.66	0.62 ± 0.38
Lpeg3.0	8.11 ± 0.44	5.12 ± 1.64	0.67 ± 0.27
Lpeg4.7	9.23 ± 1.11	6.67 ± 6.06	1.71 ± 0.76

\*%, means ± SDs (n = 4 – 5).

<sup>†</sup>Liposomes were made from distearoylphosphatidylcholine, cholesterol and dicetylphosphate and contained 0, 3.0 or 4.7% of PEG-lipid (Lconv, Lpeg3.0 and Lpeg4.7, respectively).

MA: Meglumine antimoniate.

in a murine model of VL. In this experiment, a strategy was adopted to allow administration of the same doses of Sb and lipid, with the different formulations. For this purpose, the concentrations of Sb and lipid in the formulations were adjusted to the same values, through addition of PBS and/or empty liposome formulation with the same vesicle size and lipid composition.

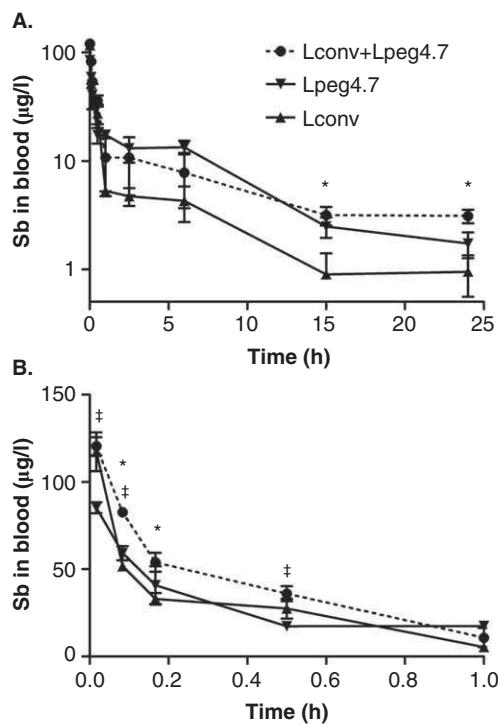
Figure 3 shows the pharmacokinetics of Sb in the blood of mice after i.v. administration of Lconv, Lpeg4.7 or their mixture at 1:1 lipid ratio at 7.5 mg Sb/kg. The blood pharmacokinetics of Sb in mice was best fitted with a biexponential elimination model. Table 4 displays the pharmacokinetic parameters determined for Lconv and Lpeg4.7 formulations and their mixture.

Lpeg4.7 showed greater values of elimination half-lives and of AUC, when compared to Lconv (Table 4), in accordance with the results obtained in dogs and the expected impact of pegylation. The Lconv + Lpeg4.7 mixture also exhibited greater AUC value, when compared to Lconv. In the case of the mixed formulation, the half-life of the first elimination phase was lower (Table 4) compared to that of Lconv, also evidenced by significantly higher concentrations of Sb during the 0 – 1-h period (Figure 3). Interestingly, Sb levels at 15 and 24 h were significantly higher after the mixed formulation than after Lconv. In addition, no significant variation of the Sb blood concentration was observed on the 1 – 24-h period, in contrast to Lpeg4.7 and Lconv that showed a significant decrease of Sb concentration on the same time period. This suggests that Lconv + Lpeg4.7 mixture promotes more sustained Sb levels in the blood, when compared to Lconv or Lpeg4.7.

### 3.3 Efficacy in a murine model of VL

The formulations of MA with conventional (Lconv) and pegylated (Lpeg4.7) liposomes and their mixture were compared for their efficacy in BALB/c mice infected with *L. infantum*. Animal groups treated with empty conventional, pegylated liposomes or saline were used as controls.

Figure 4 displays the parasite burdens determined in the liver, spleen and bone marrow, 2 weeks after a single i.v. dose of the liposome formulations.



**Figure 3. Pharmacokinetics of Sb in the blood of Swiss mice after bolus intravenous administration of different liposomal MA formulations: Lconv, conventional liposomes (upward triangles); Lpeg4.7, pegylated liposomes (downward triangles); Lconv + Lpeg4.7, mixture of conventional and pegylated liposomes (circles) at 1:1 lipid ratio. A. 24-h period. B. 1-h period.** The formulations were given by the tail vein at 7.5 mg Sb/kg and lipid dose of 64 mg/kg. Antimony was determined by ETAAS. Data are given as means  $\pm$  SEMs ( $n = 4 - 5$  per time point).

\* $p < 0.05$  for comparison between Lpeg4.7 + Lconv and Lconv, Student's t-test.

‡ $p < 0.05$  for comparison between Lpeg4.7 + Lconv and Lpeg4.7 and

\* $p < 0.05$  for comparison between Lpeg4.7 + Lconv and Lconv, Two-way ANOVA followed by Bonferroni post-test.

ETAAS: Electrothermal atomic absorption spectrometry; MA: Meglumine antimoniate.

**Table 4. Blood pharmacokinetic parameters\* in Swiss mice from MA-containing conventional or pegylated liposomes and their mixture.**

Formulations <sup>†</sup>	$C_{max}$ (mg Sb/l)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	$AUC_{0 - 24}$ (mg h/l)
Lconv	161.2	0.0254	0.442	82.4
Lpeg4.7	93.9	0.0966	12.16	186.9
Lconv + Lpeg4.7	137.9	0.0477	0.383	164.8

\* $C_{max}$ , maximum blood concentration of Sb;  $AUC_{0 - 24}$ , area under the curve from 0 to 24 h;  $t_{1/2\alpha}$ , disposition phase half-life;  $t_{1/2\beta}$ , terminal phase half-life.

<sup>†</sup>Lconv were made from distearoylphosphatidylcholine, cholesterol and diethylphosphate and Lpeg4.7 further included 4.7% of PEG-lipid; Lconv + Lpeg4.7 consisted of the mixture of Lconv and Lpeg4.7 at 1:1 lipid ratio.

MA: Meglumine antimoniate.

A significant reduction of the parasite burdens in the liver and spleen was observed after treatment with the three liposomal MA formulations, when compared to the control groups. In the bone marrow, only Lpeg4.7 and Lconv + Lpeg4.7 formulations promoted significant parasite suppression.

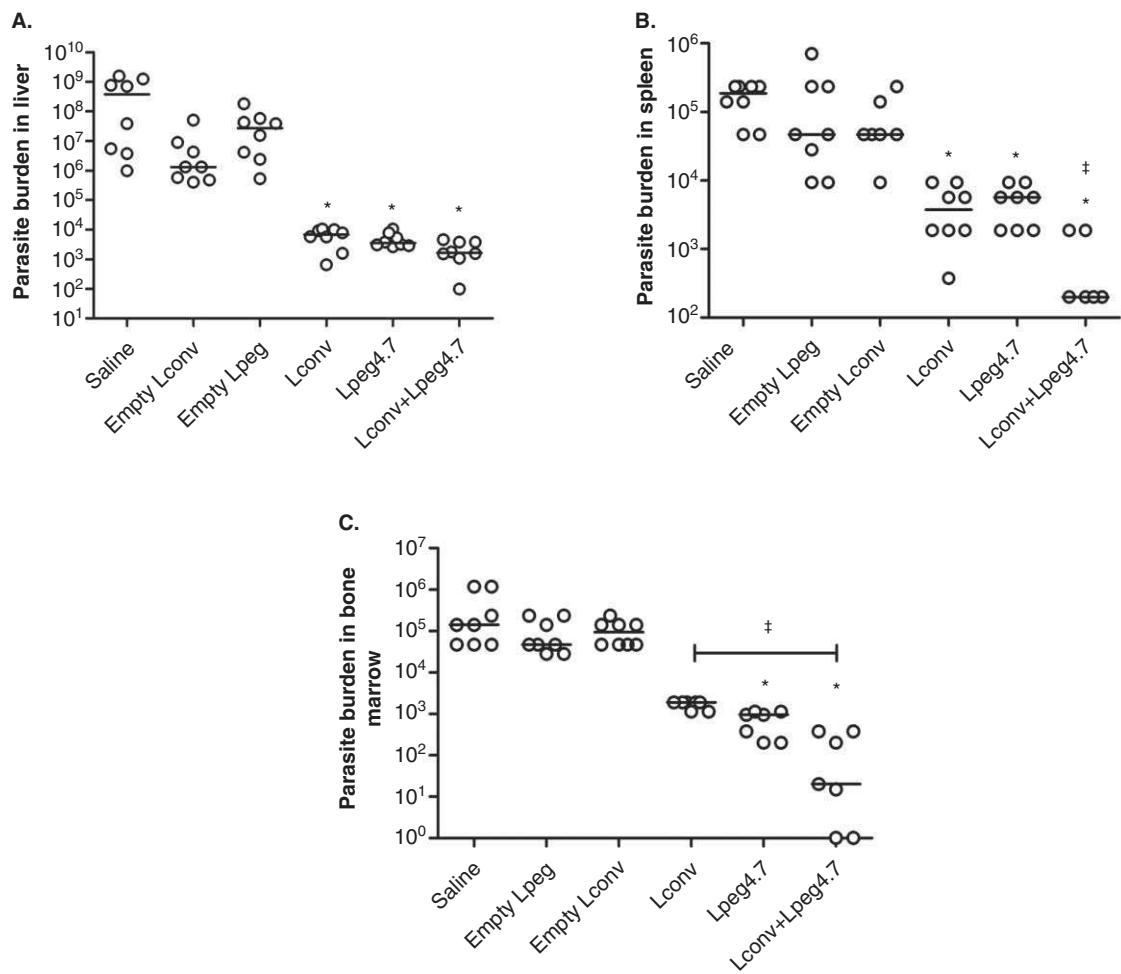
Comparison of the efficacy between the three liposomal MA formulations indicates a higher effectiveness of the mixed formulation for reduction of spleen parasite load, in relation to Lconv or Lpeg4.7, and of bone marrow parasite load, in relation to Lconv.

This data establishes the higher effectiveness of the mixed formulation, compared to conventional or pegylated formulations, for parasite elimination in the spleen and bone marrow. This improved efficacy is in good agreement with the more sustained level of Sb in the blood from the mixed formulation and the resulting higher targeting of bone marrow.

#### 4. Discussion

Previous attempts to achieve cure in dogs with VL through treatment with conventional LMA have failed [11,12]. Parasitological cure could only be reached when treatment with LMA was combined with the conventional drug allopurinol [12]. The latter work and a previous pharmacokinetic study have indicated that conventional LMA targets too specifically the liver and spleen [10]. It has been suggested that LMA may not deliver sufficient amount of Sb to less-accessible MPS tissues and skin, to allow for complete parasite elimination. This interpretation led us to investigate a means of reaching a more homogeneous distribution of Sb among different MPS tissues and delivering greater amount of Sb to the skin. This is why we have evaluated here the impact of pegylation of LMA on its pharmacokinetics in mice and dogs and on its efficacy in a murine model of VL. The mixed formulation of conventional and pegylated LMA was also investigated, with the idea that conventional liposomes may deliver Sb specifically to the liver and spleen and that pegylated liposomes may reach more effectively other MPS tissues due to lower rate of capture by Kupffer cells and more prolonged circulation time [20]. Furthermore, we hypothesized that pegylated liposomes may accumulate to a higher extent in the skin, because of possible enhanced vascular permeability in this infection site [19].

As expected, the blood levels of Sb were more prolonged after pegylated than conventional LMA in both the mice and dogs. In mice, a biexponential elimination was observed, the late phase being much slower for pegylated than conventional liposomes. In dogs, only a single elimination phase of Sb could be detected, the half-life of pegylated LMA being about twofold greater. On the other hand, the concentrations of Sb in the liver and spleen of dogs after 24 h of administration were very similar, when comparing pegylated and conventional LMA. In contrast to these findings, an improved targeting of Sb to the bone marrow was clearly evidenced, as an important impact of pegylation. Thus, despite the modest



**Figure 4. Antileishmanial efficacy of different liposome formulations of MA given as a single intravenous dose of 10 mg Sb/kg to BALB/c mice infected with *Leishmania infantum*.** Formulations were Lconv, conventional liposomes made from DSPC, CHOL and DCP; Lpeg4.7, pegylated liposomes further including 4.7% of PEG-lipid; Lconv + Lpeg4.7, mixture of conventional and pegylated liposomes at 1:1 lipid ratio. Control groups received saline, empty Lconv or Lpeg4.7. Parasite loads were determined by limiting dilution in the liver (A), spleen (B) and bone marrow (C). The parasite burden is expressed as the number of parasites per organ. Data are shown as dot plots and lines correspond to the medians of the groups ( $n = 6 - 8$ ).

\* $p < 0.05$  for comparison between the groups receiving liposomal MA and control groups.

† $p < 0.05$  for comparison between the group receiving the mixed formulation and the groups receiving Lconv or Lpeg4.7; Kruskal-Wallis followed by Dunn's multiple comparison post-test.

CHOL: Cholesterol; DCP: Dicetylphosphate; DSPC: Distearoylphosphatidylcholine; MA: Meglumine antimoniate.

influence of liposome pegylation on the blood pharmacokinetics and distribution of Sb to the liver and spleen, a significant improvement of its distribution to less-accessible MPS tissue was achieved. As a possible explanation for the surprisingly high capture of pegylated liposomes by the liver and spleen, the specific lipid composition used, that is, the high proportion of negatively charged lipid, may result in high extent of opsonization [21].

Even though pegylated and conventional liposomes did not promote significantly different levels of Sb in the skin, the 1.7-fold lower dose of Sb administered with pegylated liposomes suggests a higher targeting effectiveness of the pegylated carrier. Evidence for liposome targeting of Sb to the skin was

obtained from the observation that the level of Sb achieved in the skin from liposomal MA was equivalent to that achieved in the skin of dogs 24 h after i.v. bolus injection of free MA at 100 mg Sb/kg (data not shown).

From this biodistribution data, it is not surprising that conventional and pegylated LMA were equally effective in reducing parasite load in the liver and spleen of mice infected with *L. infantum*.

Importantly, the present study establishes for the first time the efficacy of pegylated LMA in reducing parasite load in the bone marrow. This is in contrast with conventional LMA that did not produce significant parasite suppression in this tissue, in accordance with previous studies [22,23].

Another remarkable result is the antileishmanial efficacy of the mixed formulation of conventional and pegylated LMA in the murine model of VL. Indeed, the mixed formulation promoted parasite suppression to a higher extent in both the spleen and bone marrow, when compared to pegylated or conventional LMA. The higher efficacy of the mixed formulation may be attributed to its ability to promote more sustained blood levels of Sb, as suggested by the higher blood concentration of Sb after 24 h (Figure 3). The fact that the mixed formulation was more effective than pegylated LMA also suggests that pegylated and conventional liposomes may not behave independently when administered as a mixture and that the distribution of one type of liposomes may be influenced by the presence of the other type. Therefore, it would be interesting to investigate in future studies the distribution of each type of liposomes when administered as a mixture.

## 5. Conclusion

In conclusion, the present work establishes for the first time that pegylated liposomes containing antimonial drug are more effective than conventional liposomes for treatment of experimental VL. It also introduces a novel and effective strategy that consists in the use of a mixed formulation of pegylated and conventional liposomes. This study opens new perspectives for an improved treatment of dogs with VL.

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## Acknowledgments

This work was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (303227/2013-3, 402634/2013-6, and studentship), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (REDE 40/11, APQ-01542-13) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (2447/2009 and studentship) for financial support. F.F. and C.D. are recipients of a research fellowship from CNPq.

## Declaration of interest

This work was funded by the Brazilian public agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo a Pesquisa do Estado de Minas Gerais and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. EG Azevedo and CS Ferreira received studentship from Conselho Nacional de Desenvolvimento Científico e Tecnológico. Frédéric Frézard is supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (303227/2013-3, 402634/2013-6, fellowship), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (REDE 40/11, APQ-01542-13) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (2447/2009 and studentship).

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## Research Article

## Open Access

# Prolonged Blood Circulation Time of Antimony in Dogs with Visceral Leishmaniasis from Liposomes with 175-nm Diameter

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## Abstract

The achievement of parasitological cure of dogs with visceral leishmaniasis (VL) remains a great challenge, since dogs act as main reservoir for transmission of *Leishmania infantum* to humans and respond poorly to conventional drugs including pentavalent antimonials. Liposome-encapsulated antimonials are hundreds of times more effective than the free drugs against VL based on parasite suppression in the liver. However, complete parasite elimination in dogs seems to depend on the ability of liposomes to reach less accessible infection sites such as the bone marrow and the skin. Recently, the reduction of liposome size from 1200- to 400-nm diameter was found to improve the targeting of Sb to the bone marrow of dogs with VL. In the present work, the influence of further reduction of vesicle diameter from 400- to 175-nm on the pharmacokinetics of Sb in dogs with VL and on the distribution of Sb in the liver, spleen and bone marrow were investigated. For this purpose, two liposome formulations of meglumine antimoniate with the same lipid composition but different mean hydrodynamic diameters were prepared. The formulations were given to mongrel dogs with VL as a single intravenous bolus injection and Sb concentrations were determined by graphite furnace atomic absorption spectroscopy. Surprisingly, much more prolonged blood levels of Sb were achieved from small size (175 nm) than medium size (400 nm) liposomes. Small size vesicles were also less effective than medium size ones in targeting Sb to the liver. On the other hand, similar Sb concentrations were achieved in both spleen and bone marrow. In conclusion, the prolonged blood circulation time of liposomes with 175-nm diameter makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

**Keywords:** Liposomes; Size; Pharmacokinetics; Dogs; Antimony; Visceral leishmaniasis

## Introduction

The leishmaniases are a group of diseases produced by invasion of the mononuclear phagocyte system (MPS) of a mammalian host by a parasite of the genus *Leishmania* (*L.*). This parasite is found as a motile promastigote in the sandfly and transforms into an amastigote when engulfed by host macrophages. Visceral leishmaniasis (VL) is the most severe form of the disease, causing death of humans if not treated [1].

The achievement of complete cure of dogs with VL, or at least the blockade of infectivity to the sandfly vector, is currently a great challenge since dogs act as main reservoir for transmission of *L. infantum* to humans and respond poorly to conventional drugs including pentavalent antimonials [2].

In the 1970s, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than the free drugs against experimental VL based on parasite suppression in the liver [3]. This effect of liposome encapsulation was attributed to the drug sustained release property of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the liver and spleen, which are major sites of parasite infection. In this context, much effort has been devoted to the search for effective liposomal formulations in dogs with VL [4]. However, complete parasite elimination in dogs seems to depend on the ability

of liposomes to reach less accessible infection sites, such as the bone marrow and the skin.

Recently, liposome size reduction from 1200- to 400-nm diameter was found to improve the targeting of Sb to the bone marrow of infected dogs [5]. High anti-leishmanial activity of medium size (400 nm) liposomes containing meglumine antimoniate drug was also reported after treatment of infected dogs with four doses of 6.5 mg of Sb/kg body weight [6]. Significant parasite suppression was found in the spleen and liver of dogs, however, blockade of infectivity of dogs to the sandfly was not achieved, indicating the presence of parasite in the skin of treated animals. These results suggest that improvement of the

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**Received** November 21, 2011; **Accepted** December 08, 2011; **Published** December 13, 2011

**Citation:** Azevedo EG, Ribeiro RR, Ferreira CS, da Silva SM, Schettini DA, et al. (2011) Prolonged Blood Circulation Time of Antimony in Dogs with Visceral Leishmaniasis from Liposomes with 175-nm Diameter. J Nanomedic Biotherapeu Discover 1:101. doi:10.4172/2155-983X.1000101

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actual liposome formulation, and more specifically the prolongation of its blood circulation time, is needed to more effectively target drugs to less accessible infected sites.

As an attempt to prolong the blood circulation time of our liposome formulation, vesicle diameter was reduced from 400- to 175-nm. The impact of vesicle size reduction on the blood pharmacokinetics of Sb in dogs with VL and on the metal distribution in the liver, spleen and bone marrow were investigated.

Even though the influence of the size of conventional liposomes on their *in vivo* fate is well established for comparison between very small liposomes (SUVs, with diameter < 100 nm) and larger ones (LUVs, with diameter > 100 nm) [7], the effect of size reduction from medium size liposomes (200 nm < diameter < 500 nm) to smaller LUVs (150 nm < diameter < 200 nm) has not yet been reported. Furthermore, most studies about the influence of vesicle size on liposome pharmacokinetics were carried out in the rat or mouse model, but those may not apply to dogs [8].

The present study shows that reduction of liposome diameter from 400- to 175-nm resulted in more prolonged blood Sb levels and a marked reduction of Sb concentration in the liver of dogs with VL. The prolonged blood circulation time of liposomes with 175-nm diameter makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

## Materials and Methods

### Materials

Cholesterol (CHOL) and dicetylphosphate (DCP) were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphatidylcholine (DSPC) was obtained from Lipoid (Ludwigshafen, Germany). N-methyl-D-glucamine and antimony pentachloride ( $SbCl_5$ , 99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

### Animals

Male mongrel dogs (weighing 5–15 kg), naturally infected with *Leishmania (L.) infantum chagasi* and destined to euthanasia, were obtained from the Centro de Zoonoses of the Prefeitura Municipal de Santa Luzia (MG, Brazil). Animals were found to be positive according to the following tests for *L. infantum*: indirect immunofluorescence (IFAT), complement fixation test (RFC) and enzyme-linked immunosorbent assay (ELISA), demonstration of *Leishmania* amastigotes in Giemsa-stained bone marrow aspirates and polymerase chain reaction (PCR) using specific oligonucleotide primers for the amplification of a repetitive DNA sequence of *L. infantum*.

### Synthesis of meglumine antimoniate

Meglumine antimoniate was synthesized, as previously described [10, 11], from equimolar amounts of N-methyl-D-glucamine and pentavalent antimony oxyhydrate. The resulting product contained 28% of Sb by weight.

### Preparation and characterization of meglumine antimoniate-containing liposomes

Small unilamellar vesicles (SUVs) were prepared by ultrasonication of a suspension of multilamellar vesicles made from DSPC, CHOL and DCP (molar ratio of 5:4:1) in deionized water, at the final lipid concentration of 55 g/L. After filtration through sterile 0.22  $\mu$ m membrane, the SUVs suspension was mixed with an aqueous sucrose solution at a sugar/lipid mass ratio 3:1 (Lip 400) or 1:1 (Lip 175).

The resulting mixture was immediately frozen in liquid nitrogen and subsequently lyophilized (freeze-dryer 4.5 L, Labconco, UK).

Rehydration of the dried powder was performed with an aqueous meglumine antimoniate solution (Sb concentration of 80 g/L) as follows: 50% of the original SUVs volume of meglumine antimoniate solution was added to the lyophilized powder and the mixture was vortexed and incubated for 45 min at 60°C. Only Lip 175 was further extruded across two stacked 200-nm polycarbonate membrane at 60°C in order to reduce the mean diameter of vesicles. In both formulations, the same volume of phosphate buffer saline (PBS: 0.15 mol/L NaCl, 0.01 mol/L phosphate, pH 7.4) was then added and the mixture was vortexed and incubated for 15 min at 60°C. Drug-containing liposomes were separated from the non- encapsulated drug by centrifugation (20,000×g, 45 min). The liposome pellet was then washed and finally resuspended in PBS at final Sb concentration of 10 g/L.

The amount of Sb was determined in the resulting liposome suspension by graphite furnace atomic absorption spectroscopy (Analyst AA600, Perkin Elmer Inc., MA, USA), after digestion of the sample with nitric acid [5].

The size of the vesicles in suspension was investigated by photon correlation spectroscopy at 25°C using particle size analyzer (Zetasizer S90, Malvern, UK). The mean hydrodynamic diameter and polydispersity index were determined.

### Kinetics of release of antimony from liposomes

Liposomal formulation was diluted 1:10 in PBS and incubated at 37°C under constant stirring. After different times of incubation, an aliquot was centrifuged (20,000×g, 45 min), the pellet was recovered and Sb was determined as described above.

### Pharmacokinetics and tissue distribution of antimony in dogs

Animals were divided into three groups which received different formulations of meglumine antimoniate, as intravenous bolus injection. The first group (5 dogs) received meglumine antimoniate-containing liposomes with 400-nm mean diameter (Lip 400) at 4.2 mg Sb/kg of body weight. The second group (4 dogs) received meglumine antimoniate- containing liposomes with 175-nm mean diameter (Lip 175) at 6.5 mg Sb/kg of body weight. A third group (5 dogs) received a meglumine antimoniate solution (Sb concentration of 0.66 mol/L) at 100 mg Sb/kg of body weight. Blood samples were collected from the jugular vein at the following time intervals: 5, 20, 60, 150 min, 6, 12 and 24 h. All animals were sacrificed 24 h after administration. Liver and spleen were recovered, homogenized and frozen at -20°C. In the case of the bone marrow, samples were obtained by aspiration from both the sternal bone and iliac crest.

A barbituric drug, sodium thiopental, was used to perform the humane euthanasia of the dogs, as described previously [12] and routinely used at the Veterinary Hospital of the Federal University of Minas Gerais. The present research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) and received approval from the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (123/05 protocol).

Antimony was determined in the tissues by graphite furnace atomic absorption spectroscopy after digestion of the tissues with nitric acid, as described previously [5]. The proportion of total Sb dose recovered from the liver and spleen were calculated using the actual weight of

the organs. In the case of bone marrow, tissue weight was estimated as 2.2% of the total body weight [8].

**Pharmacokinetic analysis:** Pharmacokinetic parameters were determined using compartmental analysis. Iterative weighted nonlinear least-squares regression with the Rstrip 4.03 computer program was used and model selection was guided by Akaike's information criterion [13]. Experimental blood concentration-time data were best fitted by a mono-compartment open model with i.v. bolus input. Fitted parameters included the blood half-life ( $t_{1/2}$ ), the volume of distribution at steady state ( $V_{ss}$ ), the total body clearance ( $CL$ ) and the mean residence time projected to infinity ( $MRT_{0-\infty}$ ).

**Statistical analysis:** Comparison of the levels of Sb between different tissues was performed using Kruskal-Wallis test (followed by Dunn's multiple comparison test) or by Mann-Whitney test. A two-tailed  $P$  value of  $<0.05$  was considered statistically significant.

## Results

### Characterization of the different liposome formulations of meglumine antimoniate

Two different liposome formulations of meglumine antimoniate were obtained: Lip 400 whose vesicle size was controlled by the use of cryoprotective sugar [5] and Lip 175 that was further extruded through 200-nm pore polycarbonate membrane to achieve vesicle size reduction.

Lip 400 and Lip 175 exhibited mean hydrodynamic diameters of  $410 \pm 75$  and  $175 \pm 25$  nm and drug entrapment efficiencies of  $40 \pm 4$  and  $34 \pm 3\%$ , respectively. The polydispersity index in the case of both formulations was lower than 0.3, indicating monodisperse vesicle dispersions.

Figure 1 shows the kinetic of release of Sb from Lip 175 in isotonic saline for 24 h at 37°C.

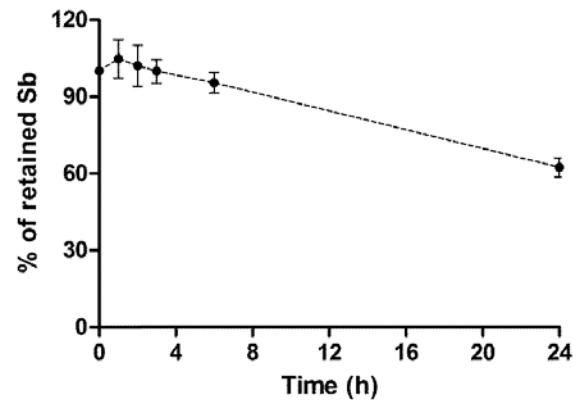
No significant release was detected during the first 3 h of incubation, however, release of Sb was observed from 6 to 24 h, reaching a value of about 40%. This percentage of drug release is greater than that reported previously for Lip 400 in the same conditions [5]. In the latter case, 12% of encapsulated drug was released after 24 h.

### Blood pharmacokinetics and tissue distribution of antimony in dogs

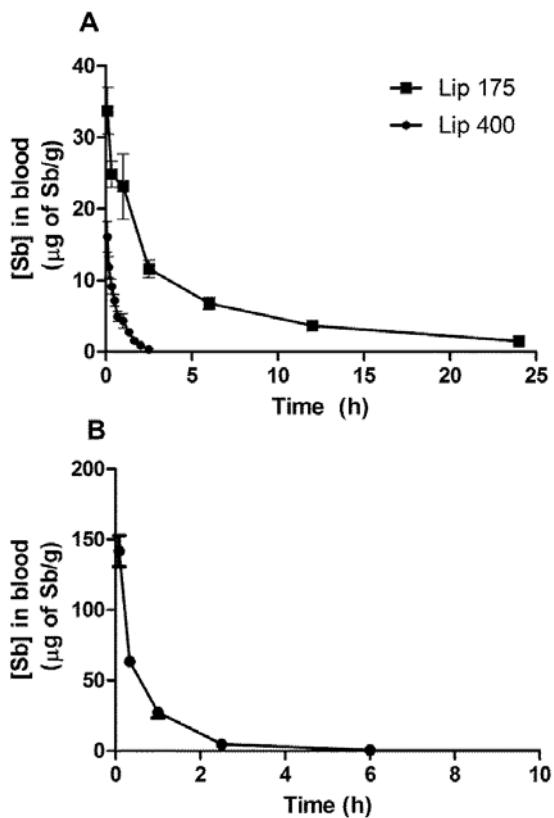
Lip 175 and Lip 400 were given to dogs with VL as intravenous bolus injections of 6.5 mg Sb/kg and 4.2 mg Sb/kg of body weight, respectively. The free drug was also given intravenously at a therapeutic dose of 100 mg Sb/kg for comparison purpose.

Figure 2 displays the pharmacokinetics of Sb in the blood of dogs from the free drug and their liposome formulations. The elimination of Sb from blood circulation occurred within 6 hours for the free drug and Lip 400, whereas significant levels of Sb were still detected

After 24h in the case of Lip 175. Data were consistent with monoexponential eliminations. Table 1 summarizes the main pharmacokinetic parameters determined for the three formulations. Interestingly, the values of elimination half-life and mean residence time obtained for small size liposomes (Lip 175) were at least 5-fold greater than those of medium size ones. On the other hand, Lip 400 and the free drug exhibited similar values of these parameters. The lower value of  $CL$  from Lip 175 is also consistent with the more prolonged blood levels of Sb from these liposomes.



**Figure 1:** Kinetic of release of Sb from small size liposomes (Lip 175) in isotonic buffer at 37°C. Liposome formulation was diluted at 1 g of Sb/L in PBS and incubated at 37°C under constant stirring. After different times of incubation, an aliquot was centrifuged, the pellet was digested with nitric acid and Sb was determined by graphite furnace atomic absorption spectroscopy. Data are given as mean  $\pm$  SD ( $n = 3$ ).

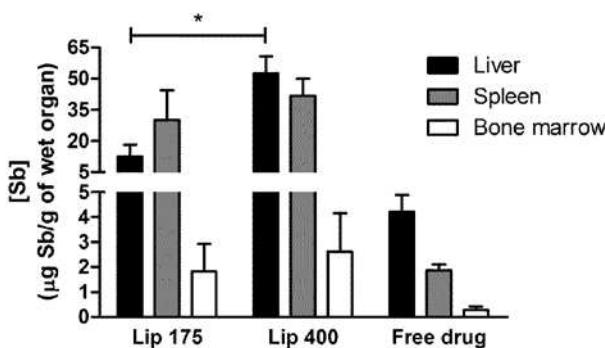


**Figure 2:** Pharmacokinetics of Sb in the blood of dogs with VL after intravenous bolus injection of liposomal (A) or free (B) meglumine antimoniate. Dogs received small size liposomes (Lip 175, 6.5 mg Sb/kg), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or the free drug (100 mg Sb/kg). After 24 h organs were collected, weighed, homogenized and submitted to digestion with nitric acid. Antimony was determined by graphite furnace atomic absorption spectroscopy. Data are given as mean  $\pm$  SD ( $n = 4-5$ ).

Drug/Parameters	C <sub>max</sub> (mg/L)	t <sub>1/2</sub> (min)	MRT <sub>0-∞</sub> (min)	V <sub>ss</sub> (L/kg)	AUC <sub>0-∞</sub> (g·min/L)	CL (L/h/kg)
Lip 175	31.6 ± 6.3	127 ± 25	184 ± 35	0.21 ± 0.04	5.7 ± 1.2	1.2 ± 0.3
Lip 400	17.3 ± 5.4	26.5 ± 7.9	38.3 ± 11.5	0.26 ± 0.07	0.63 ± 0.14	7.0 ± 1.6
Free Drug	172 ± 37	15.2 ± 9.8	25.5 ± 9.8	0.60 ± 0.16	4.1 ± 0.5	24.4 ± 2.5

<sup>a</sup>Dogs (n= 4-5) received small size liposomes (Lip 175, 6.5 mg Sb/kg body weight), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or free drug (100 mg Sb/kg). Blood samples were collected at different time intervals for determination of Sb by graphite furnace atomic absorption spectroscopy. Pharmacokinetic parameters were determined using Rstrip 4.03 computer program. C<sub>max</sub>, maximum blood concentration of Sb; t<sub>1/2</sub>, half-life of blood elimination of Sb; MRT<sub>0-∞</sub>, mean residence time of Sb projected to infinity; V<sub>ss</sub>, volume of distribution at steady state; AUC<sub>0-∞</sub>, area under the blood concentration-time curve projected to infinity; CL, total body clearance of Sb.

**Table 1:** Blood pharmacokinetics parameters<sup>a</sup> (mean ± SD) in dogs with VL after intravenous bolus injection of different formulations of meglumine antimoniate.



**Figure 3:** Concentrations of Sb determined in the liver, spleen and bone marrow of dogs with VL, 24 h after intravenous administration of liposomal or free meglumine antimoniate. Dogs received small size liposomes (Lip 175, 6.5 mg Sb/kg), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or the free drug (100 mg Sb/kg). Blood samples were collected at different time intervals for determination of Sb by graphite furnace atomic absorption spectroscopy. Data are given as mean ± SD (n = 3-5). \*P<0.05 for comparison of Sb levels in liver between Lipo 175 and Lipo 400 (Mann-Whitney test). In the spleen and bone marrow, no significant difference was found between both liposome formulations. The free drug showed significantly lower Sb levels in all organs when compared to Lipo 400.

Figure 3 shows the concentrations of Sb determined in the liver, spleen and bone marrow of dogs 24 h after administration. Despite the higher dose of Sb given with Lip 175 compared to Lip 400, a 4-fold lower Sb concentration was found in the liver. On the other hand, the concentrations of Sb in the spleen and bone marrow did not differ significantly between both liposome formulations. Interestingly, the concentration of Sb achieved in the liver after a therapeutic dose of the free drug was 3-fold and 12-fold lower than those achieved after Lip 175 and Lip 400, respectively, given at 15- and 20-fold lower doses of Sb.

The proportions of total dose of Sb recovered from the liver, spleen and bone marrow were also calculated (Table 2) to compare the targeting effectiveness of both liposome formulations. Strikingly, whereas 50% of the amount of administered Sb was found in the liver and spleen 24 h after Lip 400, only 10% was encountered in the same organs after Lip 175. Accordingly, medium size liposomes targeted more effectively the liver than small size liposomes. On the other hand, similar targeting effectiveness was observed towards the bone marrow and spleen.

## Discussion

In the present study, two liposome formulations of meglumine antimoniate with the same lipid composition, but differing in their mean vesicle diameter, were prepared and characterized in order to investigate the influence of vesicle size on the blood pharmacokinetics and tissue distribution of Sb in dogs with VL.

The choice of this experimental model is justified by its relevance in the field as the main reservoir of VL and by the fact that liposome pharmacokinetics is expected to depend on the animal species [8] and on the infected state of organs involved in liposome capture.

As main results of this work, reduction of liposome size from 400- to 175-nm resulted in more prolonged blood levels of Sb in infected dogs and a marked reduction of Sb concentration in the liver.

The marked increase of elimination half-life and reduction of hepatic uptake of Sb following liposome size reduction strongly suggests a less effective liposome capture by the Kupffer cells, the main cell involved in removal of vesicles from the circulation.

Both vesicle size and lipid dose are known critical parameters that strongly influence the rate of blood clearance of conventional liposomes [7, 14]. It has been proposed that liposomes of differing size and surface characteristics may attract different arrays of plasma proteins, called "opsonins", the content and conformation of which may account for the different pattern in the rate and site of vesicle clearance from the blood [15]. In this regard, it is clearly established that reduction of vesicle size from LUV (diameter > 100 nm) to SUV (diameter < 100 nm) results in more prolonged blood circulation time of the vesicles. However, the influence of curvature in terms of changes to the lipid packing may apply to radii of less than 150 nm, but differences are not expected above this size [14]. Thus, the increased blood residence time of Sb, as reported here, is probably not due to a direct size effect.

Even though Lip 175 was administered at about 2-fold greater lipid dose (60 mg lipid/kg body weight) when compared to Lip 400 and this dose may approach the saturation threshold of the liver, this factor cannot account solely for the marked pharmacokinetic changes observed. The increased total exposed surface area of Lip175 as a result of size reduction may be the main contributing factor. Accordingly, possible consequences of the increased surface area may be that the pattern of bound opsonins was altered reducing the rate of vesicle capture by Kupffer cells and/or the amount of exposed lipid reached the saturation threshold of the liver. Interestingly, this proposal is consistent with the fundamental nanotechnology concept that significantly modified properties of a system are observed, when the size of this system is reduced from to the nanometer range, as a result of increased surface area.

In conclusion, liposomes with reduced diameter (175-nm), in

	Spleen	Liver	Bone Marrow
Lip 175	3.3 ± 1.7	7.7 ± 2.9*	0.62 ± 0.38
Lip 400	6.1 ± 0.8	49.9 ± 8.7*	0.85 ± 0.50
Free drug	0.060 ± 0.017	0.51 ± 0.12	0.021 ± 0.013

Dogs (n= 3-5) received intravenous bolus injection of either small size liposomes (Lip 175, 6.5 mg Sb/kg body weight), medium size liposomes (Lip 400, 4.2 mg Sb/kg) or free drug (100 mg Sb/kg). After 24 h, organs were collected, weighed, homogenized and submitted to digestion with nitric acid. Antimony was determined by graphite furnace atomic absorption spectroscopy. \*P<0.05, for comparison between both liposome formulations (Mann-Whitney test).

**Table 2:** Proportions (%), mean ± SD) of the total dose of Sb recovered from liver, spleen and bone marrow of dogs with VL, 24 h after dosing with liposomal and free meglumine antimoniate.

addition to high drug encapsulation efficiency and retention, promote prolonged Sb levels in the blood of dogs with VL. This property makes this nanosystem suitable for passive drug targeting to the less accessible infection sites in dogs with VL.

### Acknowledgements

We acknowledge the Brazilian agencies CNPq, FAPEMIG and CAPES for financial support. R.R.R. and C.S.F. were recipients of postdoctoral fellowship from FAPEMIG and CNPq, respectively.

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# Novel methods for the encapsulation of meglumine antimoniate into liposomes

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## Abstract

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Research supported by CNPq  
(No. 521010/97), FAPEMIG  
(Nos. CEXI079/95, CBS1721/95,  
and CBS2418/96) and PRONEX  
(No. 3075).

Received November 25, 1999

Accepted April 10, 2000

The antimonial drug, meglumine antimoniate, was successfully encapsulated in dehydration-rehydration vesicles and in freeze-dried empty liposomes (FDELs). High encapsulation efficiencies (from 28 to 58%) and low weight ratios of lipids to encapsulated antimony (from 1:0.15 to 1:0.3) were achieved. These formulations, contrary to those obtained by conventional methods, can be stored as intermediate lyophilized forms and reconstituted just before use. The efficacy of FDEL-encapsulated meglumine antimoniate was evaluated in hamsters experimentally infected with *Leishmania chagasi*. A significant reduction of liver parasite burdens was observed in animals treated with this preparation, when compared to control animals treated with empty liposomes. In contrast, free meglumine antimoniate was found to be inefficient when administered at a comparable dose of antimony. This novel liposome-based meglumine antimoniate formulation appears to be promising as a pharmaceutical product for the treatment of visceral leishmaniasis.

### Key words

- Liposomes
- Glucantime
- Meglumine antimoniate
- Leishmaniasis
- Encapsulation

In the seventies, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than unencapsulated ones for the treatment of experimental visceral leishmaniasis in mice, hamsters and dogs (1,2). Similar results were obtained with other antileishmanial agents (3,4) and other vesicular systems made from nonionic surfactants, instead of phospholipids (5). This spectacular effect of liposome encapsulation was attributed to the sustained release properties of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the liver, spleen

and bone marrow, which are the major sites of parasite infection. It was therefore expected that liposome formulations would improve the use of antimoniales, enabling a reduction in drug dose and therapy duration. However, much effort still has to be devoted to turn the experimental liposome preparations into pharmaceutical products (6).

Two different methods have been proposed so far for the encapsulation of antimonial drugs in liposomes. One method consists of the hydration of a thin film of lipids with a solution of the drug (7). The other method, known as reverse-phase evaporation procedure, involves the formation of a

water-in-oil emulsion using the drug solution as aqueous phase followed by evaporation of the organic solvent, which results in a phase change and the formation of a vesicle suspension (8). The main advantage of the latter method, compared to the former, is that it yields higher efficiencies of drug encapsulation and higher ratios of encapsulated drug to lipid. These characteristics are important since the efficacy of liposome-encapsulated antimonials was previously shown to depend on the quantity of drug entrapped (9). These characteristics also mean that a lower quantity of lipid has to be injected in order to introduce the same quantity of antimonial, which makes the treatment safer and more economical. Nevertheless, liposomes prepared by the reverse-phase evaporation procedure may be toxic at high doses due to unavoidable residual traces of organic solvent in the final liposome formulation. Furthermore, the resulting liposome preparations can be stored only as aqueous suspensions. In this condition, however, a significant leakage of the drug occurred with time from the encapsulated aqueous phase into the continuous aqueous phase. For instance, a typical liposomal formulation prepared by the reverse-phase evaporation procedure released more than 26-48% of the originally encapsulated drug when stored for 7 weeks at 25°C (8). Such instability is actually unacceptable from a pharmaceutical point of view (6). Attention should also be paid to the chemical stability of the antimonial compound in such conditions. Indeed, in the specific case of meglumine antimoniate, a recent study (10) has suggested that this drug is a complex mixture of various antimony complexes, which change as a function of time and dilution.

In the present study, we have investigated some novel methods for the encapsulation of meglumine antimoniate into liposomes. In addition to the conventional thin film hydration method (7), two alternative methods were evaluated: i) a method that

produces dehydration-rehydration vesicles (DRV's) (11), which involves mixing the drug solution with a suspension of pre-formed liposomes in water, freeze-drying the resulting mixture and rehydrating it in a controlled manner, and ii) a method that produces freeze-dried empty liposomes (FDEL's) (12), which involves a controlled hydration, with the drug solution, of a lyophilized powder of empty liposomes. We report that these alternative methods display several advantages over conventional ones. They allow for the encapsulation of meglumine antimoniate with high encapsulation efficiency and they avoid stability problems during storage. The most promising formulation has been tested and found to be highly effective against experimental visceral leishmaniasis in hamsters.

Meglumine antimoniate (Glucantime) was obtained from Rhodia Brasil Ltda. (São Paulo, SP, Brasil), as an aqueous solution sealed in an ampoule. The antimony concentration was assessed at  $75 \pm 7$  g/l by atomic absorption spectroscopy using a Hitachi Z8200 spectrophotometer.

The encapsulation of meglumine antimoniate into multilamellar vesicles (MLVs) was performed according to the thin film hydration procedure, as previously described (7), with the following modifications. A chloroform solution of a mixture of 72 mg L- $\alpha$ -distearoylphosphatidylcholine (DSPC; Sigma Chemical Co., St. Louis, MO, USA), 28 mg cholesterol (CHOL, Sigma) and 10 mg dicetylphosphate (DCP, Sigma) was added to a round bottomed flask in the presence of 3-mm glass beads. A DSPC/CHOL/DCP molar ratio of 5:4:1 was therefore chosen. Using a rotary evaporator, the organic solvent was removed at 50°C for 1 h. MLVs were produced by hydration at 55°C of the thin lipid film with the solution of meglumine antimoniate (0.8 ml) and rotation of the flask for 15 min. The resulting suspension was diluted with 2.5 ml phosphate-buffered saline (PBS; 0.15 M NaCl and 10 mM phosphate, pH 7.2) and then centrifuged (10,000

g, 30 min, 4°C) to separate unencapsulated drug from liposomes. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

The encapsulation of meglumine antimoniate into DRVs was performed as previously reported (11). Briefly, MLVs were prepared from DSPC/CHOL/DCP (molar ratio of 5:4:1), as described above, using distilled water instead of meglumine antimoniate as the hydration solution, at a final lipid concentration of 55 g/l. The suspension of MLVs was subsequently submitted to ultrasound at 55°C using a 3-mm probe-sonicator (ultrasonic liquid processor, Misonix Inc., Farmingdale, NY, USA). Remaining MLVs and contaminating metal from the probe were eliminated by centrifugation at 10,000 g for 10 min. The resulting suspension of small unilamellar vesicles (SUVs) was then mixed with the solution of meglumine antimoniate at the final lipid to Sb weight ratio of 1:0.58. The mixture was immediately frozen and then dried overnight. Rehydration of the dried powder was performed at 55°C as follows: 20% of the original SUV volume of distilled water was added, and the mixture was vortexed and incubated for 30 min at 55°C; 20% of the volume of PBS was similarly added, and the mixture was vortexed prior to the addition of 160% of the original SUV volume of PBS and incubated for 30 min at 55°C. Drug-containing DRVs were separated from the free drug by centrifugation at 10,000 g for 30 min at 4°C. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

The encapsulation of meglumine antimoniate into FDELs was performed as previously described (12) with the following modifications. SUVs were prepared from DSPC/CHOL/DCP (molar ratio of 5:4:1) as described above at a final lipid concentration of 55 g/l. This suspension was frozen and subsequently dried overnight. Rehydration of the dried powder was performed with

meglumine antimoniate solution as follows: 40% of the original SUV volume of meglumine antimoniate solution was added (corresponding to a lipid/Sb weight ratio of 1:0.58), and the mixture was vortexed and incubated for 30 min at 55°C; 40% of the PBS volume was similarly added, and the mixture was vortexed prior to the addition of 120% of the original SUV volume of PBS and incubated for 30 min at 55°C. Drug-containing FDELs were separated from the free drug by centrifugation at 10,000 g for 30 min at 4°C. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

To determine the amount of antimonial drug encapsulated in liposomes, antimony was evaluated in the liposome pellets and in the supernatant of the first centrifugation. These samples were placed in a solution of nitric acid and heated to dryness. This step was then repeated until the complete digestion of organic material. The final dry product was re-dissolved in a 1:1 mixture of concentrated hydrogen chloride and water, and boiled for 1 h. Finally, antimony content was determined by atomic absorption spectroscopy. The encapsulation efficiency (%E) of meglumine antimoniate was calculated as follows: %E = 100 x quantity of encapsulated antimony/(quantity of encapsulated antimony + quantity of unencapsulated antimony).

Young 60-80 g Golden Syrian hamsters (*Mesocricetus auratus*) were used to maintain the parasite and were used throughout the experiments. The *Leishmania chagasi* strain MHU/BR/70/BH46 was used in this study. Parasites were obtained by homogenizing fragments of liver and spleen from a freshly killed hamster which had been infected for approximately 90 days.

In order to evaluate the antileishmanial activity of the liposomal preparation, 4 groups of 10 hamsters were infected intraperitoneally with 0.1 ml of the suspension of *L. chagasi* amastigotes (approximately 10<sup>8</sup> para-

sites). Seventeen days after infection, animals were treated intraperitoneally with i) meglumine antimoniate entrapped in DSPC/CHOL/DCP FDELs (Lglu) at 60 mg Sb/kg, ii) free meglumine antimoniate (Glu) at 100 mg Sb/kg, iii) empty liposomes (Lemp), and iv) PBS (untreated). After 55 days, animals were sacrificed, and impression smears of liver and spleen were taken. Smears were fixed with methanol, stained with 10% Giemsa (Gibco) and the number of amastigotes per 1000 host cell nuclei was counted. For each animal, about 500 host cell nuclei in the liver as well as in the spleen were evaluated for the presence of amastigotes.

Entrapment data (Table 1) indicated that the 'DRV method' is the most efficient method for the encapsulation of meglumine

antimoniate into liposomes. The 'FDEL method' gave a lower encapsulation efficiency than the DRV one, but a higher level (2.5-fold) than the conventional thin film hydration method. These results were obtained using a lipid quantity of 14 mg and an initial lipid to Sb weight ratio of 1:0.58. When the lipid quantity was increased in the FDEL preparation while maintaining constant the drug to lipid ratio, an increase in encapsulation efficiency was observed. For instance, when 140 mg of lipid was used, the encapsulation efficiency of meglumine antimoniate into FDELs reached  $50 \pm 10\%$ .

Table 2 shows that treatment of *Leishmania chagasi*-infected hamsters with Lglu reduced parasite burdens significantly in the liver, when compared to treatment with Lemp. On the other hand, no significant difference in parasite burden was observed between the groups treated with either empty liposomes or free meglumine antimoniate (100 mg Sb/kg) and the control group. More strikingly, all animals treated with Lglu showed less than 70 amastigotes per 1000 host cell nuclei either in the liver or in the spleen, and some animals (4 out of 9) seemed to be free of parasites (Table 2). This observation is in contrast with the results obtained with the other groups in which all animals showed parasites, and at a much higher level.

The aim of the present study was to look

Table 1 - Glucantime encapsulation into multilamellar vesicles (MLVs), dehydration-rehydration vesicles (DRVs) and freeze-dried empty liposomes (FDELs).

<sup>a</sup>N = 3. <sup>b</sup>14 mg of lipid used.

Liposome preparation	Percentage of entrapped Glucantime (mean $\pm$ SD) <sup>a</sup>	Weight ratio of lipid to encapsulated Sb
MLVs	12.5 $\pm$ 1	1:0.074
DRVs <sup>b</sup>	42 $\pm$ 2	1:0.25
FDELs <sup>b</sup>	31 $\pm$ 3	1:0.18

Table 2 - The effect of liposome-encapsulated meglumine antimoniate (Lglu, 60 mg Sb/kg), free meglumine antimoniate (Glu, 100 mg Sb/kg) and empty liposomes (Lemp) on *L. chagasi* parasite burdens of liver and spleen in hamsters.

\* P<0.05 compared to the Lemp group (one-way ANOVA and Tukey's test).

Treatment	Mean number of amastigotes/1000 host cell nuclei $\pm$ SD		Number of animals with amastigotes/1000 host cell nuclei <70 (total number)		Number of animals with no parasite detected (total number)
	Liver	Spleen	Liver	Spleen	
Lglu	9 $\pm$ 15*	13 $\pm$ 25	10 (10)	9 (9)	4 (9)
Glu	1800 $\pm$ 2000	3200 $\pm$ 4800	1 (11)	0 (10)	0 (11)
Lemp	2500 $\pm$ 2600	6700 $\pm$ 9700	0 (8)	0 (7)	0 (8)
None	1600 $\pm$ 1900	7600 $\pm$ 8100	1 (8)	0 (7)	0 (8)

for alternative methods for the encapsulation of meglumine antimoniate into liposomes, that do not suffer from the limitations of conventional ones. From the point of view of encapsulation efficiency, the methods that produce DRVs and FDELs appeared to be as efficient as the reverse-phase evaporation method that was claimed to produce encapsulation efficiency in the range of 38 to 57% (8). Moreover, comparable ratios of lipid to Sb were achieved.

In order to avoid stability problems during storage, such as those encountered with aqueous liposome suspensions, the DRV and FDEL preparations may be stored as intermediate lyophilized products: DRVs as a liposome-drug mixture and FDELs as pre-formed empty liposomes. The final rehydration step may be performed just before administration, using i) water and saline in the case of DRVs and ii) meglumine antimoniate solution in the case of FDELs. A significant advantage of the 'FDEL method' is that it does not expose the drug to lyophilization, thereby reducing the risk of chemical alteration of the drug. Moreover, the rehydration step was found to be much easier in the case of FDELs (data not shown). Finally, the FDEL preparation did show the expected high antileishmanial activity in hamsters infected with *Leishmania chagasi*.

Conventional therapy with meglumine antimoniate involves a multiple dosing regimen of a minimum of 28 daily intramuscular or intravenous injections of 20 mg Sb/kg (13). According to pre-clinical studies in experimental animals, liposome-encapsulated meglumine antimoniate is at least 200-fold as active as the free drug (1). These data suggest that a treatment consisting of four doses of 2 mg Sb kg<sup>-1</sup> week<sup>-1</sup> with meglumine antimoniate encapsulated into lipo-

somes might be satisfactory, since each dose is 70-fold lower than the cumulative dose of antimony administered in one week by conventional treatment. It is noteworthy that if the preparation is used without eliminating the 50% fraction of unencapsulated drug, then the quantity of unencapsulated drug administered would represent 2 mg Sb kg<sup>-1</sup> week<sup>-1</sup>, which is insignificant when compared to the dose administered in the conventional treatment. This observation means that the process of preparation of DRVs and FDELs might be simplified by the omission of the last centrifugation/separation step.

Another important point that needs to be addressed is the cost of treatment with the liposomal drug when compared to conventional therapy. In a treatment using the FDEL preparation, much lower doses of antimony would be used and the cost would be determined mainly by lipids. Assuming that the cost of lipid is US\$50 per g and that of meglumine antimoniate is US\$2 per 5 ml ampoule, a four-week treatment with 2 mg Sb kg<sup>-1</sup> week<sup>-1</sup> of liposomal meglumine antimoniate would cost US\$120, whereas conventional treatment (20 mg Sb kg<sup>-1</sup> day<sup>-1</sup>) costs about US\$200.

In conclusion, we observed that FDEL-entrapped meglumine antimoniate is a suitable formulation for the treatment of visceral leishmaniasis: the preparation exhibited high antileishmanial activity, did not suffer from stability problems and was cost-effective. It is expected that treatment with this preparation would allow for a lower number of injections and a lower dose of antimony when compared to conventional treatment. Therefore, the treatment, in addition to its higher efficiency, should be safer and more comfortable.

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