In-vitro and in-vivo antileishmanial activity of inexpensive Amphotericin B formulations: Heated Amphotericin B and Amphotericin B-loaded microemulsion

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Running title: Antileishmanial activity of Amphotericin B formulations

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Abstract:

Amphotericin B (AmB) is effective against visceral leishmaniasis (VL), but the renal toxicity of the conventional form, mixed micelles with deoxycholate (M-AmB), is often dose-limiting, while the less toxic lipid-based formulations such as AmBisome® are very expensive. Two different strategies to improve the therapeutic index of AmB with inexpensive ingredients were evaluated on this work: (i) the heat treatment of the commercial formulation (H-AmB) and (ii) the preparation of an AmB-loaded microemulsion (ME-AmB). M-AmB was heated to 70°C for 20 min. The resulting product was characterized by UV spectrophotometry and circular dichroism, showing super-aggregates formation. ME-AmB was prepared from phosphate buffer pH 7.4, Tween 80®, Lipoid S100® and Mygliol 812® with AmB at 5 mg/mL. The droplet size, measured by dynamic light scattering, was about 40 nm and transmission electron microscopy confirmed a spherical shape. Rheological analysis showed low viscosity and Newtonian behavior. All the formulations were active *in vitro* and *in vivo* against *Leishmania donovani* (LV9). A selectivity index (CC_{50} on RAW/IC_{50} on LV9) higher than 10 was observed for ME-AmB, H-AmB and AmBisome®. Furthermore, no important *in vivo* toxicity was observed for all the samples. The *in-vivo* efficacy of the formulations after IV administration was evaluated in Balb/C mice infected with LV9 (three doses of 1 mg/kg AmB) and no significant difference was observed between H-AmB, M-AmB, ME-AmB and AmBisome®. In conclusion, these two inexpensive alternative formulations for AmB showing good efficacy and selectivity for *Leishmania donovani* merit further investigation.

INTRODUCTION

Leishmaniasis is a parasitic infectious disease caused by obligate intra-macrophage protozoa of the *Leishmania* species, transmitted to humans via the bite of female sand flies of the genera *Phlebotomus* and *Lutzomyia*. This disease is widespread in tropical and subtropical areas as three different clinical manifestations: mucocutaneous, cutaneous, and visceral leishmaniasis (VL), also known as kala-azar, which is fatal if untreated (1). Pentavalent antimonials are the most commonly used drugs to treat VL; however, they are no longer recommended due to their toxicity and the emergence of drug resistance (2). Amphotericin B (AmB) and miltefosine are the two alternative drugs able to replace antimonials, although neither drug is completely safe (3).This work is focused on the exploitation of AmB as low–cost and low-toxic formulations.

AmB has been used as a second-line drug for leishmaniasis treatment (4). Despite its high efficacy, toxic effects, such as cardiotoxicity and nephrotoxicity, limit its successful therapeutic use. The AmB selectivity and toxicity level depend on its aggregation state, which can exist as monomeric, aggregated and super-aggregated states. In order to reduce the side effects credited to AmB, several lipid formulations have been developed. These systems prevent the AmB self-aggregation and slowly release AmB monomers to the surrounding medium (5). An example of success on the lipidic approach is the liposomal AmB formulation (AmBisome®), which is less toxic than Fungizone®, the commercial available micellar AmB (M-AmB). Nevertheless, AmBisome® is not widely available due to its cost.

To summarize, the current visceral leishmaniasis treatment remains limited by the issues of drug resistance, toxicity and high cost (6). The therapeutic properties and

pharmacological profile of AmB can be optimized by modifying the aggregation state of the drug (7) and by developing new drug delivery systems.

M-AmB has good availability and relatively low cost; hence it is the most widely used AmB-based product. However, the presence of AmB in the aggregated state allows the formation of ion channels in membranes containing cholesterol, which causes toxicity (8). Studies have shown that the heating of M-AmB is capable of inducing a new type of aggregate, called super-aggregates, which is less selective for the cholesterol in the mammalian cell membranes. Therefore, the toxicity can be significantly reduced without loss of activity (5, 7, 9, 10). The molecular rearrangement that yields super-aggregate structures can be achieved by appropriate heating of M-AmB (5, 11).

An alternative formulation without the drawbacks of cost and toxicity could be a microemulsion (ME). Microemulsions are anisotropic, clear, small droplet sized and thermodynamically stable drug delivery systems, comprised of an oil and aqueous phase, stabilized by surfactants (12). The ME would be able to carry AmB (ME-AmB) in its dispersed phase, increasing its solubilization and bioavailability, and decreasing its toxicity (13, 14).

The aim of this work was to investigate Heated M-AmB (H-AmB) and ME-AmB in comparison with M-AmB and AmBisome® with regard to their *in-vitro* and *in-vivo* safety and antileishmanial activity against *Leishmania donovani*, in order to improve the therapeutic index of AmB while using inexpensive ingredients.

MATERIALS AND METHODS

Chemicals

Micellar AmB (Anforicin B®) was a gift from Cristália (Itapira, Brazil). Liposomal AmB (AmBisome®) was purchased from Gilead (Foster City, USA). Miglyol® 812, used as the oil phase of the ME, was obtained from CONDEA Chemie GMBH (Hamburg, Germany). Lipoid® S100, used as surfactant, was purchased from LIPOID GMBH (Ludwigshafen, Germany). The AmB, used to load the ME, Tween® 80, used as surfactant, NaH_2PO_4 and Na_2HPO_4, used to prepare the phosphate buffer pH 7.4, were all acquired from Sigma Aldrich Inc (St. Louis, USA).

Sample preparation

AmBisome® was prepared according to the manufacturer's instructions. The commercial M-AmB was prepared by adding 10 mL of water for injection into the vial containing 50 mg of AmB (final concentration of $5x10^{-3}$ M), followed by vortex shaking until dissolution. In order to obtain the H-AmB, the M-AmB was heated at 70°C for 20 min.

In order to prepare the ME, 68 $\%_{(w/w)}$ of phosphate buffer pH 7.4, 14.7 $\%_{(w/w)}$ of Tween® 80, 6.3 $\%_{(w/w)}$ of Lipoid® S100 and 11 $\%_{(w/w)}$ of Miglyol® 812 were weighed. They were mixed under magnetic stirring followed by 3 cycles of probe sonication (80 watts power output) in a Branson Digital Sonifier S-250 (Branson Ultrasonic Corporation – Danbory, USA) for 1.5 min and followed by 3 min in a 1210E-MTH Bransonic Ultrasonic bath (Branson Ultrasonic Corporation – Danbory, USA). To incorporate AmB into the ME, solid AmB was added to a final concentration of $5x10^{-3}$ M. Then, the system was alkalinized with

NaOH 1M until complete AmB solubilization. After that the loaded ME was neutralized by addition of HCl 1M to a final pH of 7.4.

M-AmB and H-AmB characterization

AmB molecules exhibit absorbance bands correlated to their aggregation state in the UV/Vis range of 300 to 450 nm. A PerkinElmer Lambda 25 UV/VIS spectrometer (Waltham, MA, USA) was used to analyze AmB-containing samples in 1cm path length cuvettes. Samples were diluted in pure water and analyzed immediately after dilution. M-AmB and H-AmB were diluted to $5x10^{-6}$ M of AmB (5, 13).

Circular dichroism (CD) spectra were recorded with a Jasco J-180 dichrograph (Easton, MD, USA). Samples were diluted in water to 5.10^{-5} M AmB 24h before analysis. Measurements were conducted at room temperature using a 0.1 cm path length cuvette (15). Results are expressed as $\Delta\varepsilon$ (differential molar absorption dichroic coefficient).

Microemulsion characterization

Droplet size distribution analyses were carried out by Dynamic Light Scattering (DLS) using a Malvern-Zetasizer Nano ZS (Malvern, UK). Transmission electron microscopy (TEM) was used to investigate the ME morphology. The MEs was observed after staining with 2% phosphotungstic acid, using a JEOL 1400 apparatus (SamXPlus, France), operated at 80kV as the acceleration voltage, equipped with a high resolution CCD Gatan digital camera (SC1000 Orius, France). Before performing both types of analysis, the MEs were diluted 1:20 with water.

The rheological properties of the MEs were evaluated by a Haake Rheo Stress 600 rheometer equipped with 35mm cone-plate geometry (Thermo Scientific, USA). The flow

curve and the viscosity curve of the samples were performed by controlled shear rate rotation tests (from 0.1 s-1 to 1000 s-1).

The aggregation state of the AmB in the ME-AmB was also evaluated by spectrophotometry and CD as described above.

In vitro antileishmanial activity

The promastigote stages of *Leishmania donovani* (MHOM/IN/80/DD8) wild-type and of the AmB-resistant (AmB-R) strain obtained from the wild-type parasites by *in-vitro* drug pressure were grown in M-199 medium supplemented with 40 mM HEPES, 100 µM adenosine, 0.5 mg/L haemin, and 10% heat-inactivated fetal bovine serum (FBS) at 26 °C in a dark environment under an atmosphere of 5 % CO_2. Parasites in their logarithmic phase of growth were used for all experiments. Promastigotes were grown at 37 °C in 5% CO_2 atmosphere for 24 hours before treatment for differentiation. The axenic amastigotes were generated by differentiation of promastigotes. To achieve this, a 1×10^6 promastigote suspension was diluted in 5mL of axenic amastigote media (1 X M-199, 40 mM HEPES, 100 µM adenosine, 0.5 mg/L haemin, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 20% FBS) and the pH was adjusted to pH 6.

Evaluation of the in vitro antileishmanial activity on axenic amastigote stage

Axenic amastigotes were suspended at 5 x 10^6 cells/mL in a final volume of 200 µL. Serial dilutions from 100 to 0.39 µg AmB/mL was performed by distributing AmBisome®, M-Amb, H-AmB, ME-AmB, and ME in 96-well plates. Triplicates were used for each concentration. The viability of the axenic amastigote stage was assessed using the trypan blue exclusion method after a 3-day incubation period at 37 °C, in the dark, under a 5% CO_2

158 atmosphere. Parasite growth was determined by using SYBR Green I (Invitrogen, France), a
159 dye whose fluorescence is greatly enhanced upon contact with the parasite DNA. The plates
160 were frozen 3 times and the DNA was extracted from the plates according to the Direct PCR
161 (cell) protocol (Viagen Biotech Inc, Eurogentec, France). Into the PCR plate 10µL of Lysate
162 and 40 µL of direct PCR (cell) supplemented with SYBR green I (5 µL of SYBR Green I / 10
163 mL of lysis buffer) were added. Untreated parasites were used as control. The fluorescence
164 was directly evaluated on a Mastercycler® ep realplex real-time PCR system (Eppendorf,
165 France). The program was as follows: 90 °C, 1 min and ramp time of 5 min to obtain 10 °C,
166 during this period the fluorescence was obtained in continue and in a hold step at 10 °C. The
167 curves of fluorescence were analyzed and the fluorescence at 10°C was used to determine the
168 concentrations inhibiting parasite growth by 50 % (IC_{50}). Indeed, the IC_{50}, the concentration
169 inhibiting the parasite growth by 50%, was determined using the software in line at
170 http://www.antimalarial-icestimator.net/MethodIntro.htm.

171 *Evaluation of the in vitro antileishmanial activity on intramacrophagic amastigotes*

172 RAW 264.7 cells (mouse leukemic monocyte macrophage cells) cultured in DMEM
173 with L-glutamine (Life technologies) and 10% FBS (Life technologies) were suspended to
174 yield 1.5 x 10^5 cells/mL in 96-well plate, in a final volume of 100 µL. After a 24 h incubation
175 period, cells were infected by a suspension of 1 x 10^6 *Leishmania donovani* axenic
176 amastigotes/mL in DMEM, and incubated for 24 h at 37 °C in the dark and under a 5 % CO_2
177 atmosphere. The free parasites were, then, eliminated by washing and intramacrophagic
178 amastigotes were treated with the same AmB samples and concentrations as those in the assay
179 on axenic amastigotes. After 48 h of incubation under the same conditions, the experiment
180 was stopped and the plate was treated as for the axenic amastigote stage assay. Each
181 experiment was performed in triplicate (16). Fluorescence was compared to that obtained with

182 untreated infected and uninfected macrophages and untreated axenic parasites used as
183 controls.

184 ***In-vivo* evaluation**

185 All procedures involving animals were conducted in compliance with the standards for
186 animal experiments and were approved by the local committee for animal care (0858.01/2014,
187 Versailles, France).

188 ***In-vivo* antileishmanial activity**

189 A suspension of *Leishmania donovani* (LV9) amastigotes ($1x10^7$/100 µL M199
190 medium supplemented with 40 mM HEPES, 100 µM adenosine and 0.5 mg/L haemin, 10%
191 FBS), obtained from infected hamster spleen, was injected into female BALB/c mice
192 (weighing 20 g) by intravenous route. The mice were randomly sorted into six groups seven
193 days after the infection. Each group was treated with 100 µL of one formulation
194 (AmBisome®, M-AmB, H-AmB, ME-AmB and ME), administered by intravenous route for 3
195 alternate days (1mg/kg/day). Samples were diluted prior to use with 5% glucose to give a final
196 AmB concentration of $2x10^{-4}$ M. Animals were sacrificed three days after the end of
197 treatment. Livers and spleens were weighed and drug activity was estimated microscopically
198 by counting the number of amastigotes/500 liver cells in Giemsa stained impression smears to
199 calculate the *Leishmania donovani* units (LDU) for liver parasite burdens, using the Stauber's
200 formula (17). The mean number of parasites per gram of liver among treatment groups and
201 controls was compared. Three independent counting were performed and the results were
202 expressed as the mean values ± SD. The parasite burden of treatment groups and controls

were compared using the Kruskal–Wallis nonparametric analysis of variance test for comparing two groups. Significance was established for a P value < 0.05.

In vitro cytotoxicity assay

RAW 264.7 cells maintained in DMEM supplemented with 10% of heat-inactivated FBS (Life technologies) were seeded to early confluence in a 96-well plate at density of 7500 cell/well and incubated in 5 % CO_2 at 37 °C for 24 h in a final volume of 100 µL. Thereafter, M-AmB, H-AmB, ME-AmB, AmBisome® and ME were added to achieve final AmB concentrations from 0.1 to 108 µM and incubated for a further 24 h under the same conditions. In order to estimate background absorbance due to light scattering, wells without cells, but containing the same concentration of the samples, were analyzed. The cytotoxicity of the formulations was evaluated using the MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay, whereupon 20 µL of MTS solution were added into the wells and incubated for further 2 h. Finally, the absorbance was measured using a 492-nm high-pass filter in a Multiskan MS microwell plate reader (Labsystem, Ramat-Gan, Israel) and the IC_{50} for each formulation was calculated. For this assay 4 replicates were performed.

In vivo toxicity assay

Blood samples taken from the mice from the antileishmanial activity experiment were kept at room temperature for about 30 min and, then, centrifuged at 3,500 g for 10 min. In order to evaluate the renal and liver toxicity, serum was harvested from each blood sample and total serum cholesterol (CHO), alkaline phosphatase (ALP), aspartate aminotransferase

224 (AST), alanineaminotransferase (ALT), creatinine (CREA) and urea (URE) concentrations
225 were determined by commercially available kits according to the manufacturer's instruction.

226 **Statistical analysis**

227 Statistical analyses were performed by using analysis of variance or Kruskal-Wallis tests
228 (nonparametric), followed by the Tukey′s or the Dunn's multiple comparison test. GraphPad
229 Prism5 version (GraphPad Software Inc., La Jolla, CA) was used to perform the analysis.
230 Significance was established for a P value < 0.05.

231

RESULTS

M-AmB and H-AmB characterization

The UV-Vis spectrum for M-AmB (Figure 1a) showed four main peaks at 329, 367, 388 and 408 nm. The first peak is credited to the aggregated form of AmB whereas the one at 408 is ascribed to the monomeric form (15). After heat treatment it was observed that H-AmB (Figure 1a) presented similar peaks to M-AmB regarding the monomeric form, but the aggregate-related peak underwent a blue shift and became centered on 323 nm in this preparation. The UV-Vis results were in accordance with those observed by circular dichroism (Figure 1c), in which the doublet related to the AmB aggregation was also blue-shifted for H-AmB.

Microemulsion characterization

The produced ME was optically clear with a mean droplet size of 22 nm for the unloaded ME and 36 nm for the ME containing AmB, with polydispersity indexes of 0.170 and 0.594, respectively, indicating that the addition of AmB led to slightly larger particles. TEM revealed that the droplets were spherical in shape even after the addition of AmB (Figure 2). Furthermore, the average size was similar to the DLS results. The rheological analysis revealed that both the unloaded ME and the ME containing AmB (Figure 3) showed a linear relationship between shear stress and shear rate, which is characteristic of a Newtonian material. The apparent viscosity of the unloaded ME and ME containing AmB were 0.0623 Pas ± 0.0101 and 0.1141 Pas ± 0.0049, respectively, which is low because the ME was of the oil-in-water type. The low viscosity would ensure good syringeability as well as ease of mixing with intravenous fluids using minimum mechanical agitation. The AmB

molecule showed to be in both aggregated and monomeric forms (Figure 1b and 1d), since peaks at 323 nm (aggregated form), 367, 388 and 408 nm (monomeric form) were observed. All these results: the small spherical droplet size, the transparency and the low viscosity, allow these samples to be characterized as a true ME system. The incorporation of AmB did not affect these parameters.

Toxicity of the formulations

The CC_{50} was evaluated *in vitro* for each formulation (Table 1). M-AmB showed high toxicity towards RAW cells (CC_{50} 4.5 ± 0.4 μM). However, when this formulation was heated this value increased approximately 8-folds to 39.8± 3.2 μM, indicated a decrease in toxicity. In fact, in this study, the toxicity of H-AmB was similar to that of AmBisome®. On the other hand, although the incorporation of the AmB into the ME decreased the CC_{50} of the formulation compared to the unloaded ME, the cytotoxic effect of ME-AmB (CC_{50} 9.0 ± 0.3) was slightly lower than that of M-AmB.

The results of the evaluation of the toxicity *in vivo* are given in Table 2. There were no significant changes (p>0.05) among the samples for the two transaminase activities levels (AST and ALT) and the creatinine levels. However, all treatments caused a significant increase (P<0.05) in the urea level except for mice treated with unloaded ME. A significant decrease in this parameter was also observed in the mice treated with the ME and the ME-AmB compared to the AmBisome®. Furthermore, it is noteworthy that heating of M-AmB slightly decreased the blood urea levels. The ALP value increased significantly with all treatments compared to the untreated group. Nevertheless, the ALP level for the mice treated with the AmBisome® was significantly lower than that of the mice treated with the ME-AmB. The CHO results showed no difference between the ME, the AmBisome® and the untreated

277 group. However, M-AmB, H-AmB and ME-AmB caused a significant decrease in this
278 parameter, while the lowest values were observed in the mice treated with the ME-AmB.

279 **Antileishmanial activity**

280 The activity of the formulations against *Leishmania donovani* was evaluated *in vitro*
281 and *in vivo*. The *in-vitro* assays were performed on both Wild-type (WT) and AmB-R
282 intramacrophagic and axenic amastigotes (Table 1). All formulations, except ME (unloaded
283 microemulsion), showed high activity against WT parasites. ME-AmB and H-AmB yielded
284 lower IC_{50} values than the AmBisome® for both amastigote stages. As far as the activity on
285 axenic amastigotes was concerned, all the formulations exhibited lower IC_{50} compared to the
286 intramacrophage amastigote. However, the IC_{50} of the AmBisome® did not change
287 significantly (1.73 μM and 1.76 μM).

288 The H-AmB showed a higher IC_{50} than the M-AmB, when the *Leishmania* was within
289 macrophage, and similar antileishmanial activity (IC_{50} = 0.05 μM) against the axenic stage.
290 However, the selectivity index for the H-AmB was approximately 3-fold higher than that of
291 the M-AmB. Moreover, the ME-AmB showed a better selectivity index than the M-AmB,
292 although it remained lower than the AmBisome®.

293 Some clinical isolates of *Leishmania donovani* have shown resistance against the AmB
294 (18). Therefore, the issue of AmB resistance should be considered early in the development of
295 any new AmB delivery system. With this in mind, the formulations were evaluated against
296 both AmB-resistant axenic and intramacrophagic amastigotes. The AmBisome® was active
297 against AmB-resistant intramacrophage amastigotes, but it did not show any activity on AmB-
298 resistant axenic amastigote. On the other hand, the other formulations were active in a range
299 from 0.8 to 4 μM on both axenic and intramacrophagic amastigotes. The drug pressure to get
300 the AmB-R line was previously carried out using M-AmB. Indeed, the IC_{50} value of M-AmB

on the AmB-R line was about 30 times higher than those of the WT, both on axenic and intramacrophage amastigotes. Interestingly, the IC_{50} values of H-AmB, AmBisome® and ME-AmB on the AmB-R intramacrophage amastigotes were only about twice as high as those on the WT. These results suggest that these formulations could be useful for the treatment of AmB-R leishmaniasis.

The *in-vivo* antileishmanial evaluation was performed on the *Leishmania donovani*/Balb/C mice model. The percentage of parasite burden reduction *in vivo* is shown in the Table 2. Whereas the unloaded ME alone did not show good activity *in vivo*, M-AmB, H-AmB, ME-AmB and AmBisome® all reduced the parasite burden greatly, with no significant difference between the formulations. No sign of acute toxicity was observed in the mice and the biological parameters did not show significant toxicity, as shown in Table 2.

DISCUSSION

The MEs prepared in this study showed a spherical shape and a small droplet size, which is in agreement with other studies that describe these systems as having diameters less than 150 nm (12, 14). Although the addition of AmB to the ME increased its droplet size, probably due to the large molecule size and its deposition on the ME interface, the ME-AmB droplet size remained within the acceptable range. Such small droplets on MEs produce only weak scattering of visible light, and as a result the system is transparent, as observed macroscopically in this work. Moreover, the probability of embolus formation after injection of MEs is insignificant due to their small droplet size (12).

Since the nanosized droplets do not interact with each other, the resulting MEs have low viscosity (19). The viscosity analysis is of utmost importance since the parenteral administration of very viscous samples could be painful for the patient. Furthermore, it is well known that the syringeability is affected by the viscosity of the parenteral formulations (20).

The ME and ME-AmB showed a constant low viscosity and proportionality between shear stress and shear rate, for which they were deemed to behave as Newtonian fluids. As a consequence, they can be considered suitable for the intravenous route. This low viscosity ensures ease of mixing with intravenous fluids with minimum mechanical agitation as well as good syringeability and, therefore, can ensure good patient compliance.

Spectrophotometric studies revealed that after the heating process, super-aggregates are formed from M-AmB, manifested as a blue-shift in the electronic spectra as well as a decrease on the dichroic doublet, which is also blue-shifted in the spectra of H-AmB (15). When AmB was loaded into the ME, a pattern similar to H-AmB was observed. Since the system was not heated in order to form AmB super-aggregates, the peak at 323 nm probably represents the association of the AmB with the ME-oil phase. Pham et al, (2014) (21) have shown blue-shifts in the AmB dichroic doublet when the molecule is loaded into liposomes. Our results are in agreement with Silva et al (2013) (22), who have developed ME systems to carry AmB and have demonstrated peaks of AmB aggregates and monomers at the same time. However, the aggregate peak is not the same as one observed in our work, supporting the theory of that such a peak is a result of a complex formed between the molecule and the system, which for instance depends on the used raw material, mainly the surfactants. Larabi et al (2004) (23) reported studies in which absorption spectrum and circular dichroism varied according to the proportion of lipid in the formulation. Knowing the aggregation state of the molecule inside the carrier is very important since it influences the drug efficacy and toxicity (5, 9, 13, 15, 24).

The treatment of visceral leishmaniasis was dominated by pentavalent antimonials for a long time. However due to their toxicity and adverse side effects, these drugs have progressively been replaced as the first-line treatment by the AmB, which is considered as the best existing drug against this disease. Among the different commercial formulations,

351 Fungizone® (a mixture of AmB with deoxycholate in a phosphate buffer) is the most
352 frequently used. However, the liposomal formulation is the most effective and safe, despite its
353 high cost (4).

354 In this work, we have proposed two new formulations of AmB which retain its activity
355 while reducing its toxicity, and are furthermore inexpensive, with costs accessible for patients
356 from the tropical endemic countries and easy to manufacture. Both H-AmB and ME-AmB
357 were thoroughly characterized and shown to be appropriate for parenteral use against visceral
358 leishmaniasis. In order to investigate the antileishmanial activity of these formulations, their
359 IC_{50} in axenic and intramacrophage amastigote were evaluated *in vitro*, as well as the
360 percentage of reduction of parasite burden *in vivo*. In addition, the cytotoxicity of the
361 formulations was examined *in vitro*, allowing calculation of the Selectivity Index, while the
362 toxic effects *in vivo* were analyzed by assessment of renal and liver function, because the most
363 serious side effect of AmB is nephrotoxicity (25).

364 Both *in-vitro* and *in-vivo* experiments with the WT line demonstrated that, as expected,
365 all formulations containing AmB were effective against *Leishmania donovani*. On the other
366 hand, despite a moderate effect *in-vitro,* unloaded ME did not show significant *in-vivo*
367 antileishmanial activity, proving that it was the addition of AmB that produced this effect.

368 Some differences in the *in-vitro* effectiveness were observed when comparing the
369 axenic and intramacrophagic amastigotes. The axenic amastigote assay allowed us to evaluate
370 an intrinsic activity on parasites, whereas the intramacrophage amastigote assay demonstrates
371 the ability of the active drug to be delivered through the macrophage membrane, the
372 parasitophorous vacuole membrane and finally into the parasite (26, 27). It is noteworthy that
373 AmBisome® was not active against AmB-R axenic amastigotes while the other formulations
374 did have some activity. This result cannot be a result of the ME diameter being less than that
375 of AmBisome® (35 vs 80 nm, respectively) since H-AmB, AmBisome® and ME-AmB had

similar activities on the WT intramacrophage amastigotes. This observation could be related to the composition of the AmB-R membranes, poor in sterols, more fluid than the WT membranes and, therefore, more stable and less sensitive to a liposomal effect. With the intramacrophagic amastigotes, this difference was not observed since all the formulations after macrophage phagocytosis released AmB within the parasitiphorous vacuole, probably after fusing with lysosomes. Indeed, AmBisome® is a very stable formulation with cholesterol and a high-transition-temperature phosphatidylcholine making a very impermeable membrane and phosphatidylglycerol forming an electrostatic complex with AmB. It is possible that parasite-derived enzymes are necessary to release the AmB from the liposomes and that the AmB-R parasites have a different enzyme profile and are less efficient at this. On the other hand, AmB may be less strongly associated in M-AmB, H-AmB and ME-AmB. Further studies could be focused on the ME behaviour within the infected macrophages through confocal studies.

M-AmB showed the lowest selectivity index, while H-AmB presented the highest index. Therefore, the heating process was capable of considerably reducing the cytotoxicity of AmB i*n vitro* as demonstrated by the CC_{50} values. This result is supported by Gaboriau et al. (1997)(28) and Petit et al. (1998) (10) whose have found the mild heating as a simple way to decrease the toxicity of micellar AmB systems. In addition, the therapeutic window between the IC_{50} (1.53 µM) and CC_{50} (39.8 µM) of H-AmB is wider than that of M-AmB, thus improving its safety. It is interesting to mention that H-AmB have shown to favor drug uptake by macrophage-like cell line (9). The authors suggest that this fact could be interesting since the macrophage could act as a reservoir, releasing monomeric AmB on the infection site and, thus, improving antiparasitic effect (9). ME-AmB (selectivity index = 10.23) also showed a better selectivity compared to M-AmB. When the selectivity index is greater than 10, it is generally, considered that the pharmacological efficacy is not due to the *in-vitro* cytotoxicity

401 (29), therefore, the incorporation of AmB in ME systems was able to improve the
402 effectiveness of the drug.

403 The potential of the new formulations (H-AmB and ME-AmB) was confirmed by the
404 *in-vivo* studies, which showed no significant differences in their ability to reduce the parasite
405 burden compared to the AmBisome®. In addition, both formulations demonstrated minimal
406 cytotoxicity *in vivo*.

407 The biochemical evaluation of kidney and liver parameters showed that the untreated
408 infected mice had lower values than those with no parasite burden (reference values were
409 found in (30). Some studies have described a reduction of the serum cholesterol
410 concentrations as a function of the splenic parasite burden, since this organ is responsible for
411 cholesterol biosynthesis (31). An overview of all the biochemical results showed that despite
412 some values, which were statistically different, there was a minimal variation among the
413 groups, showing that none of the formulations induced toxicity at the doses used in this study.
414 Therefore, ME-AmB and H-AmB were considered safe regarding the limited damage to
415 macrophage and the *in vivo* evaluation.

416 Furthermore, ME-AmB, M-AmB and H-AmB showed efficiency against AmB-R
417 intramacrophagic amastigotes. AmB-unresponsive cases have been reported at the Rajendra
418 Memorial Research Institute of Medical Sciences (RMRIMS), Bihar, India. These drug-
419 resistant cases are to be expected due to the very high frequency of AmB use (18). Since
420 visceral leishmaniasis is fatal, the development of new systems, which do not demonstrate
421 drug resistance is very important to ensure that suitable treatment would be available.
422 Therefore, these two inexpensive alternative formulations for AmB showing good efficacy
423 and selectivity for *Leishmania donovani* merit further investigation. Unfortunately, it was not
424 possible to verify the *in-vivo* efficacy of the formulations on an AmB-R/ mice model since the
425 AmB-R parasites are poorly infectious for Balb/c mice (32).

CONCLUSION

These experiments show a novel approach to AmB therapy, addressing both the cost of formulations and the AmB-resistance. The formulations H-AmB and ME-AmB proved to be very successful in the treatment of a *Leishmania donovani* Balb/c mouse model. Both *in-vivo* and *in-vitro* evaluations showed good efficacy and low toxicity for these formulations. Since the method of preparing the H-AmB is just the heating of the reconstituted M-AmB for a few minutes, it appears to be a low cost alternative to the commercially available products. The cost of producing MEs is also lower than that of producing the liposomal AmB, since the components are commonly used pharmaceutical excipients rather than expensive phospholipids. Therefore, the formulation developed in this work is a promising way to reduce toxicity while maintaining the efficacy of AmB.

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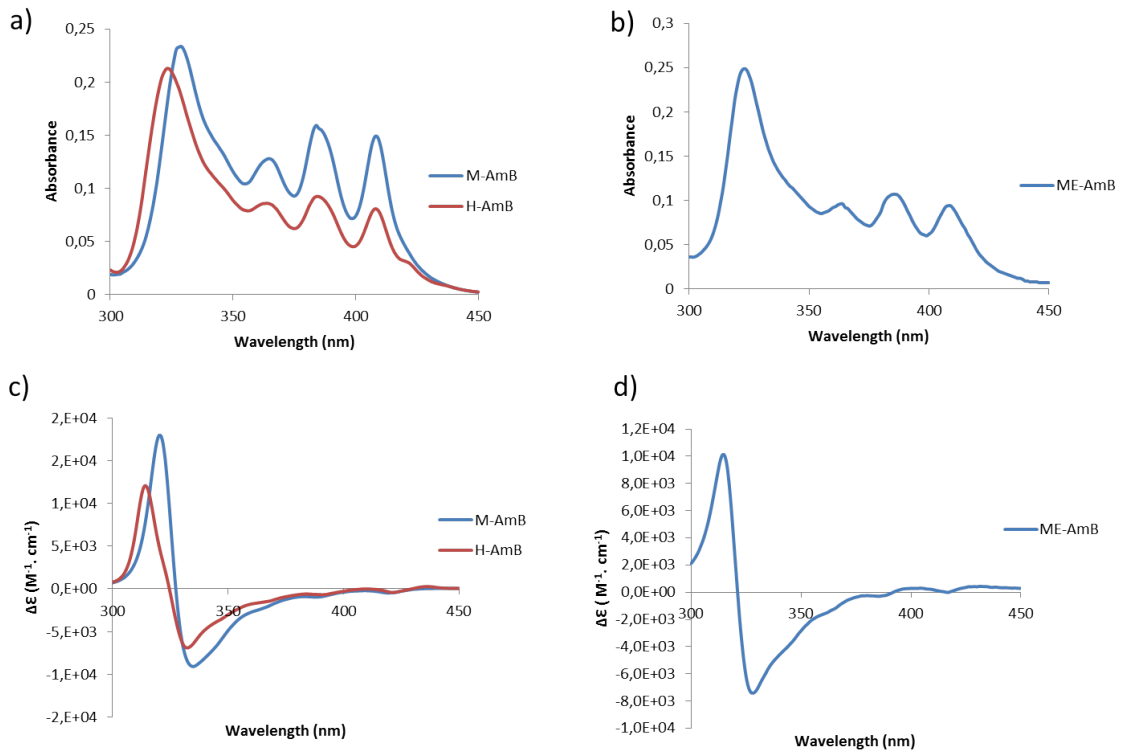

Figure 1: Spectroscopy of M-AmB and H-AmB (a) and of ME-AmB (b) and circular dichroism of M-AmB and H-AmB (c) and of ME-AmB (d).

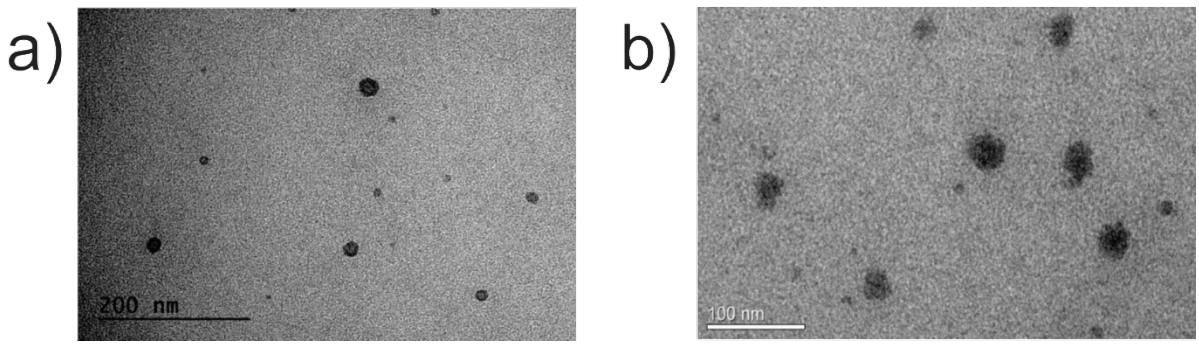

Figure 2: Transmission electronic microscopy of ME (a) and ME containing AmB (b).

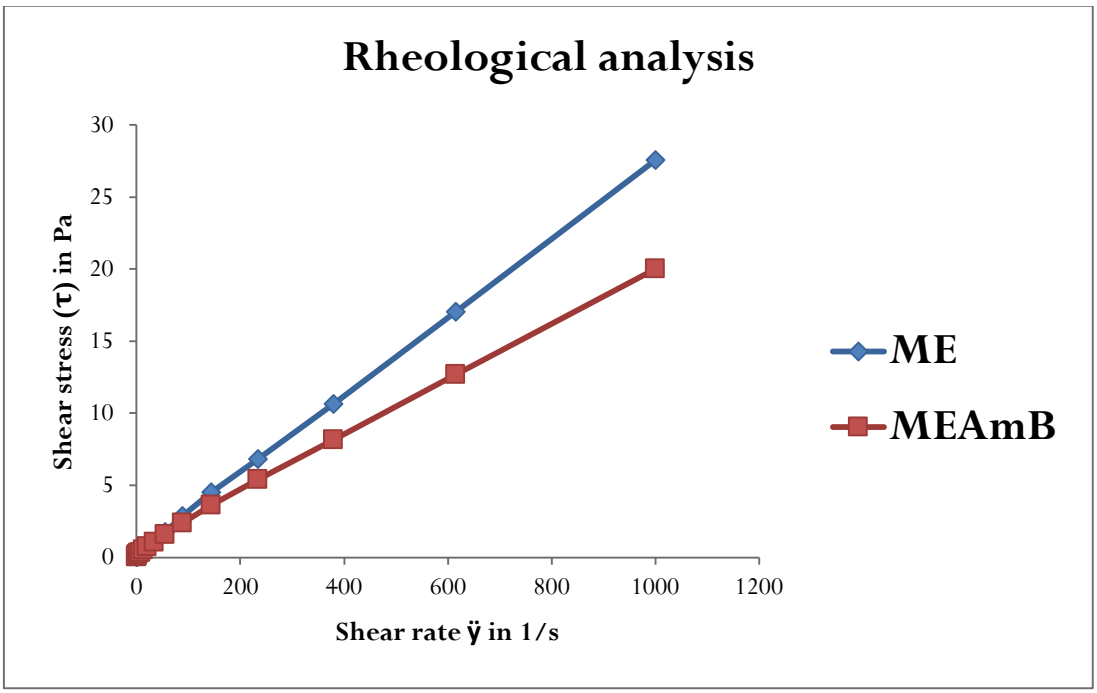

Figure 3: Rheological behavior of ME and ME containing AmB.

Table 1: *In vitro* evaluation of the cytoxicity and the anti-leishmanial activity on *L. donovani* WT and AmB-R.

Formulations	**Intramacrophage amastigotes** IC_{50} (µM) ± SD		**Axenic amastigotes** IC_{50} (µM) ± SD		**Cytotoxicity RAW 264.7 macrophages** CC_{50} (µM) ±SD	**Selectivity index** SI= CC_{50}/IC_{50}
	WT	AmB-R	WT	AmB-R		WT
M-AmB	0.06 ± 0.01	2.15 ± 0.18	0.05 ± 0.01	1.69 ± 0.04	4.5 ± 0.4	6.7
H-AmB	1.53 ± 0.33	2.31 ± 0.32	0.05 ± 0.01	0.82 ± 0.03	39.8 ± 3.2	26.0
AmBisome®	1.76 ± 0.69	3.54 ± 0.10	1.73 ± 0.12	>100	36.5 ± 2.3	20.7
ME-AmB	0.88 ± 0.26	1.56 ± 0.01	0.22 ± 0.03	3.38 ± 0.08	9.0 ± 0.3	10.2
ME	28.01 ± 2.70	27.34 ± 0.24	6.82 ± 0.72	27.06 ± 0.52	22.8 ± 2.9	0.8

Table 2: *In-vivo* antileishmanial activity and acute toxicity of H-AmB and ME-AmB compared to M-AmB and AmBisome®. Mice were treated intravenously at 1 mg/kg equivalent AmB at Day 8, Day 10 and Day 12 post-infection.

	***In vivo* antileishmanial activity**	***In vivo* toxicity**					
	% Reduction of parasite burden	[a]AST U/L	[a]ALT U/L	[b]CHO mM	[a]CREA µM	[b]ALP	[b]URE
M-AmB	72	233.0	134.0	2.515**	19.5	225.5	10.70
H-AmB	78	189.5	171.5	2.575**	18.0	241.0	9.65
AmBisome®	83	242.5	167.0	*2.755	20.0	229.0	10.15
ME-AmB	78	224.5	169.5	2.415**	18.5	246.0**	8.35**
ME	*33	293.0	181.0	*2.710	16.0	225.0	*7.60**
Untreated	-	313.0	239.0	2.810	18.0	199.0**	7.40**

AST: aspartate aminotransferase. Normal value: 456 (U/L) (30).
ALT: alanine aminotransferase. Normal value: 304 (U/L) (30).
CHO : Cholesterol. Normal value: 3.5 mM (30).
CREA : Creatinine. Normal value: <18 µM (30).
URE : Urea. Normal value 7.4 mM.

* There was no significant difference in comparison to untreated (P>0.05)
** There was significant difference in comparison to AmBisome® (P<0.05)
[a] P > 0.05
[b] P < 0.05