Amphotericin B in poly(lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) nanoparticles against paracoccidioidomycosis

Andre´ C. Amaral1,2, Anamélia L. Bocca1, Alice M. Ribeiro1, Janayna Nunes1, Danielle L. G. Peixoto1, Andrea R. Simioni3, Fernando L. Primo3, Zulmira G. M. Lacava1, Ricardo Bentes1, Ricardo Titze-de-Almeida4, Antonio C. Tedesco3, Paulo C. Morais5 and Maria Sueli S. Felipe1*

1Biological Sciences Institute, Universidade de Brasília, Brasília DF 70910-900, Brazil; 2Genomic Science and Biotechnology, Universidade Católica de Brasília, Brasília DF 70790-160, Brazil; 3Chemistry Institute, Universidade de São Paulo, Ribeirão Preto SP 14040-901, Brazil; 4Faculty of Agronomy and Veterinary Medicine, Universidade de Brasília, Brasília DF 70910-900, Brazil; 5Physics Institute, Universidade de Brasília, Brasília DF 70910-900, Brazil

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Objectives: The present study reports on the preparation and testing of a desoxycholate amphotericin B (D-AMB) sustained delivery system based on poly(lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) polymeric blends (Nano-D-AMB) aimed at reducing the number of AMB administrations required to treat mycosis.

Methods: BALB/c mice were infected with the yeast Paracoccidioides brasiliensis intravenously to mimic the chronic form of paracoccidioidomycosis. At 30 days post-infection, the animals were treated with Nano-D-AMB [6 mg/kg of encapsulated D-AMB, intraperitoneally (ip), interval of 72 h] or D-AMB (2 mg/kg, ip, interval of 24 h). Drug efficacy was investigated by the fungal burden recovery from tissues. Toxicity was assessed by renal and hepatic biochemical parameters, physical appearance of the animals and haematological investigation. The control groups used were non-infected and the infected mice mock treated with PBS.

Results: Nano-D-AMB presented results comparable to free D-AMB, with a marked antifungal efficacy. The Nano-D-AMB-treated group presented lower loss of body weight and absence of stress sign (piloerection and hypotrichosis) observed after D-AMB treatment. No renal [blood urea nitrogen (BUN), creatinine] or hepatic (pyruvic and oxalacetic glutamic transaminases) biochemical abnormalities were found. The micronucleus assay showed no significant differences in both the micronucleus frequency and percentage of polychromatic erythrocytes for Nano-D-AMB, indicating the absence of genotoxicity and cytotoxic effects.

Conclusions: The D-AMB-coated PLGA–DMSA nanoparticle showed antifungal efficacy, fewer undesirable effects and a favourable extended dosing interval. Nano-D-AMB comprises an AMB formulation able to lessen the number of drug administrations. Further studies would elucidate whether Nano-D-AMB would be useful to treat systemic fungal infections such as paracoccidioidomycosis, candidiasis, aspergillosis and cryptococcosis.

Keywords: nanobiotechnology, antifungal, polymers, drug delivery
AMF-PLGA–DMSA nanoparticles for mycosis

Introduction

Amphotericin B (AMB) is a polyene antifungal agent used to treat systemic mycoses.1,2 Despite the therapeutic efficacy provided by AMB, its potentially toxic effects have limited its clinical use.3,4 Lipid-based preparations have been developed recently and include AMB lipid complex (ABLC; Abelcet®), AMB colloidal dispersion (ABCD; Amphotec®) and liposomal AMB (L-AMB; Ambisome®), which have enabled some of the toxic effects of AMB to be ameliorated.3

Recent findings in the field of nanotechnology have dramatically increased the research on drug delivery systems for the pharmaceutical industry. Among them, polymeric nanoparticles composed of poly(D, L-lactide-co-glycolide) (PLGA) have shown several technological advantages such as biocompatibility, biodegradability, sustained release and safety.6 The antibiotics ciprofloxacin and rifampicin and the antifungals fluconazole and voriconazole are examples of drugs incorporated in PLGA preparations recently.7–10

The design of nanosized drug delivery systems to target tissues has gained increasing attention in the field of pharmacology. These novel preparations would reduce both the dose required to achieve a therapeutic level and the related toxicity.3,11 Besides the commonly used antibodies, new molecules have been found to target nanoparticles to particular tissues. The incorporation of dimercaptoesuccinic acid (DMSA) into delivery systems drives the nanoparticles preferentially to the lungs, which highlights its use in drug delivery strategies to treat pulmonary mycoses and other diseases that affect this organ. The mechanism supporting this tropism is still not described but previous studies in vivo showed clearly that nanoparticles associated with DMSA were extensively carried to the lungs.12,13

Paracoccidioidomycosis (PCM) is a health problem in Latin America where ~10 million individuals may be infected by its aetiological agent, the dimorphic human pathogenic fungus Paracoccidioides brasiliensis; 2% of them could develop fatal acute or chronic PCM.14 Chronic PCM is characterized by involvement of the lungs, presenting a granulomatous inflammatory response, which is an effective defence against the fungus.14–16 Therapy for PCM is based on polyenes, azaoles and sulfamethoxazole. AMB (2 mg/kg/day) is indicated for disseminated and severe cases and must be followed by a prolonged treatment response, which is an effective defence against the fungus.14–16

The present study reports on the preparation and testing of a deoxycholate AMB (D-AMB)-sustained delivery system based on nanoparticulate PLGA–DMSA polymeric blends, which was developed with the aim of reducing the number of AMB administrations required during treatment. The therapeutic efficacies and toxicities of this nanoparticulate formulation (Nano-D-AMB) were compared with those of the conventional D-AMB (Fungizone®) in a murine model of systemic PCM.

Materials and methods

Drugs and chemicals

D-AMB (Fungizone®) was purchased from Bristol-Myers Squibb (Princeton, NJ, USA). Aliquots containing D-AMB 40 µg in 100 µL PBS pH 7.4 (8 g of NaCl, 0.2 g of KCl, 0.122 g of KH2PO4, 1.716 g of Na2HPO4.7H2O, 1 L distilled water) were prepared daily from a 5 mg/mL stock solution. The polylactic acid (PLA), polyglycolic acid (PGA), DMSA and the D-AMB used to prepare Nano-D-AMB were purchased from Sigma (St Louis, MO, USA).

Preparation of PLGA–DMSA nanoparticles loaded with D-AMB

Since our main objective was to reduce the number of AMB injections, we prepared PLGA–DMSA polymeric nanoparticles loaded with D-AMB based on the drug dose per kg per day, with 3 days dosing interval (6 mg/kg every 3 days). This was the maximum AMB amount technically possible to encapsulate together with DMSA within nano- and microparticulate PLGA preparations. All the details presented here are under patent request in Brazil and must be protected according to the Brazilian agency regulation. The composite percentages were adjusted to provide a 72 h sustained release of AMB. The polymers were first dissolved in dichloromethane with 50 mg of PLA and 50 mg of PGA. We added an aqueous solution containing 1% polyvinyl alcohol to this PLA/PGA organic solution to obtain the initial water-in-oil emulsification. This solution received 120 mg of D-AMB with or without the additive DMSA, and the mixture was submitted to vigorous agitation in a blender (10000 rpm). The water-in-oil emulsification was also obtained by vigorous agitation in an ULTRA-TURRAX stirrer. The organic solvent was removed from the solution by stirring at room temperature and evaporation under reduced pressure. The nanoparticles were centrifuged (25°C, 5000 rpm) at intervals of 10 min. The preparation was washed three times in distilled water, suspended in 1.0 mL physiological PBS solution and stored at 4°C. All procedures were performed in a sterile room with all the manipulation in a sterile hood. The stability of the suspension was analysed over time and stability was maintained for 3 weeks. The process was protected by deposited patent in the INPI (Instituto Nacional de Propriedade Intelectual, Brazil) under PI # 0700446-0.

Mice

Female BALB/c mice weighing 20–22 g purchased from the University of São Paulo, Ribeirão Preto, Brazil, were used in this study. Mice were housed in polypropylene cages under controlled conditions of humidity and were provided with food and water ad libitum. All animal procedures performed in this study were approved by the Animal Care and Use Committee of the University of Brasília (UnB), Brasília DF, Brazil.

Fungal inoculum

Paracoccidioides brasiliensis, isolate Pb18, was used to challenge the animals. The isolate was sub-cultured in liquid YPD medium (10 g of yeast extract, 20 g of peptone, 20 g of dextrose, 1 L of distilled water) at 36°C in a rotary shaker (220 rpm). After 5 days of culture, the yeast cells were collected by centrifugation, the supernatant was discarded and the cells were washed twice in sterile PBS. We determined the cell counts with a haemocytometer and adjusted the inoculum suspension to 3 × 10⁷ viable fungi/mL. The cellular viability was determined by vital staining with 0.05% green-Janus. The animals were anaesthetized and challenged with 3 × 10⁶ viable fungi intravenously.

Intravenous infection

To evaluate the antifungal effects and toxicity of Nano-D-AMB, BALB/c mice were inoculated with P. brasiliensis virulent strain.
Pb18 by the intravenous route to mimic the chronic form of PCM, which represents more than 90% of the clinical cases for this mycosis. The disease involves mainly the lungs with granulomatous lesions, leukocyte aggregation and fibrous tissue.\textsuperscript{14,16} The dose of free D-AMB was chosen based on recommendations for the clinical treatment of human PCM and converted to the body weight of mice, which allows 2 mg/kg/day. Also, the dose of Nano-D-AMB at 6 mg/kg each 3 days is equivalent to the dose used for free D-AMB. The animals were randomly divided into four groups of 10 animals as follows: group I (non-infected control group and treated with PBS); group II (infected control group and treated with PBS); group III (infected group and D-AMB-treated, 2 mg/kg, intraperitoneally (ip), interval of 24 h, which is equivalent to 0.9 mg/kg of pure AMB); group IV (infected group and Nano-D-AMB-treated, 6 mg/kg ip, interval of 72 h, which is equivalent to 2.7 mg/kg of pure AMB). Treatment of the groups began 30 days post-infection and continued through the following 30 days, according to clinical recommendations to treat PCM. The treatment was continued for the next 30 days to evaluate toxicological parameters and the fungal burden results.

**Lung and liver fungal burden assay**

The Nano-D-AMB antifungal effects were assessed by the liver and pulmonary fungal burden. The animals from all experimental groups were euthanized by cervical rupture on days 30 and 60 from the beginning of treatment regimens, and their liver and lungs were aseptically removed. The organs were rinsed and homogenized in sterile PBS, pH 7.4, with a tissue grinder. The homogenates were cultured on BHI agar (Acumedia\textsuperscript{\textregistered}, Baltimore, MD) supplemented with 4% horse serum, 5% P. brasiliensis 192 culture filtrate and 40 mg/L gentamicin (gentamicin sulphate, Schering-Plough) in duplicate. The Pb192 culture filtrate was prepared according to a previous report and added to the medium to promote the colony growth for the cfu fungus assays.\textsuperscript{18} The plates were incubated at 36°C and cfu were counted at day 21 post-plating to determine the cfu/g of tissue.

**Histopathology**

The lungs were aseptically removed, fixed in 10% paraformaldehyde solution and prepared by routine techniques for paraffin embedding. Histological 5 \mu m sections were stained with haematoxylin/eosin (H&E) and the slides were evaluated by light microscopy. The lung injury was evaluated by the presence of both fungi cells and inflammatory cells within tissue granulomatous lesions.

**Biochemical and clinical parameter analyses**

Toxic side effects and physical appearance of Nano-D-AMB-treated animals were analysed.

**Biochemical parameters.** BUN and creatinine, pyruvic and oxaloacetic glutamic transaminases (GPT; GOT) levels were determined by colorimetric and Jaffé modified assays from blood samples collected under anaesthesia.

**Clinical parameters.** Mice were monitored daily for changes in their physical appearance; they were weighed and photographed before starting the antifungal therapy and also at treatment days 30 and 60.

**Bone-marrow micronucleus slide preparation and scoring.** The genotoxicity and cytotoxicity effects of Nano-D-AMB were assessed by the micronucleus assay, as previously described.\textsuperscript{19} This haematological investigation is recommended by the regulatory agencies to test new pharmaceuticals and has been widely used to screen for drugs that cause genetic damage.\textsuperscript{20} In brief, after euthanasia, we dissected the femurs and cut their epiphyses to expose the marrow channel. The bone marrow material was flushed with 1 mL of fetal bovine serum and centrifuged at 1000 rpm for 5 min. Then we prepared the smears with one drop of the material, in duplicate for each animal. The slides were allowed to air-dry for 24 h to avoid the loss of cells during fixation with methanol (5 min) and stained with Giemsa and PBS 1:14 for 8 min. The slides were evaluated by light microscopy for each animal, with the observer blind to the treatment group. The positive control group (n=6) received 40 mg/kg of cyclophosphamide ip 24 h before euthanasia. We used the following formula to calculate the percentage of multiple erythrocytes (%PCE): \[ \%\text{PCE} = \text{PCE}/(\text{PCE} + \text{NCE}) \times 100, \] where PCE are polychromatic and NCE are normochromatic erythrocytes. For the genotoxicity test, we determined the frequency of micronucleus in PCE (MN-PCE).

**Statistical analysis**

Statistical package for social sciences (SPSS) version 15 was used to analyse our data. All results were expressed as means \pm standard error of the means. A one-way analysis of variance (ANOVA) with Tukey’s post-test was applied to test inter-group differences in fungal tissue burden recovery, biochemical data, micronucleus assay and body weight changes. Differences between paired groups were analysed by the Mann–Whitney test. P values of <0.05 were considered significant.

**Results**

**Assessment of pulmonary and liver fungal burden assay**

In order to compare antifungal efficacy between Nano-D-AMB and D-AMB, we carried out in vivo drug antifungal experiments. We investigated the fungal load in the lungs and liver of the animals. Thirty days after intravenous (iv) infection, no significant number of fungi were detected in the liver. This result was expected since the liver infection is detected only early on in this animal model of mycosis (15 days after iv fungal inoculation). In contrast, the main focus of the infection was the lungs. In this organ, a decrease in cfu was obtained for both Nano-D-AMB- and free D-AMB-treated groups compared with untreated animals (PBS). Both groups showed a marked decrease in the lung cfu (n=40 \pm 2) in comparison with the PBS group (n=280 \pm 2) (P<0.05, ANOVA with Tukey’s post-test) (Figure 1). Nano-D-AMB produced therapeutic outcomes comparable to those of free D-AMB, which showed its antifungal activity and the advantage of administration at intervals of 3 days. Since we compared Nano-D-AMB with free D-AMB at the same dose, the latter was administered daily. Even knowing that free D-AMB is clinically recommended for 30 days and then followed by another antifungal, such as itraconazole, we decided to continue treatment for an extra 30 days to investigate the long-term effect of the formulation. No differences in cfu reduction were noticed for either treatment groups (Nano-D-AMB and free D-AMB) (Figure 1).

**Histopathology**

The pulmonary examination of the infected and untreated animals (group II) showed fungal cells within granulomas, in
There were five animals evaluated at each point, and the experiment was repeated every 72 h), compared with the infected control group treated with PBS. There were five animals evaluated at each point, and the experiment was repeated twice. Asterisks indicate $P<0.05$ for D-AMB versus PBS, as determined by one-way ANOVA followed by Tukey's test. No significant differences were found between D-AMB- and Nano-D-AMB-treated groups.

Biochemical and clinical parameter analyses

Biochemical parameters. The Nano-D-AMB-treated group showed levels of BUN, creatinine, GPT and GOT similar to the control groups. Thus, mice treated with Nano-D-AMB have their renal and hepatic functions preserved, even though they are receiving higher doses of AMB (2.7 mg/kg) (Table 1).

Clinical parameters

Body weight. As weight loss is a sensitive and objective sign of health problems, all treatment groups were monitored. The changes in median body weight of animals for each group are shown in Table 2. As expected, the median body weight increased in the non-infected control group (group I, 8.4%) and decreased in the infected and untreated animals (group II, 14.6%). The weight loss observed in group II and in the D-AMB-treated animals (group III, 12.4%), on day 30 post-treatment, was not observed in the Nano-D-AMB-treated group, which presented a slight variation in weight (group IV, $-2.5\%$, $P<0.05$). The median body weight of this group decreased only after 60 days of treatment (group IV, $-9.2\%$).

Physical appearance. The pelage of the non-infected control group displayed homogeneity, smooth coat, brightness and seamness. Infected and untreated (group II) and D-AMB-treated (group III) animals showed worst appearance, characterized by piloerection and hypotrichosis (lower hair density), which indicates signs of stress and prejudice of their health status.

In contrast, the Nano-D-AMB-treated animals showed appearance similar to the non-infected control group, which suggests its protective effect on animal health. Changes in animal behaviour were also noted. Daily, during the treatment administration, mice from all groups except the Nano-D-AMB, presented signs of distress, such as hiding and staying in groups, even among the non-infected group that received only PBS as treatment control, probably as a consequence of a daily PBS injection.

Micronucleus tests

The %PCE found for the D-AMB- and Nano-D-AMB-treated groups was similar to the non-infected control animals, which indicated the absence of cytoxic effects of AMB in both formulations (Table 3). Also, the frequency of micronucleus in NCE (MNNCE) and MNPC (MNPC) was explored (Table 3). For D-AMB-treated animals, we found a statistically significant increase in MNNCE when compared with the non-infected animals, suggesting damage to DNA ($P<0.05$, Mann–Whitney test). In contrast, for Nano-D-AMB-treated mice, no significant difference was observed, suggesting an absence of genotoxicity. These results were corroborated using cyclophosphamide as positive control for genotoxicity, which yielded the expected positive results.

Discussion

The present work examined the antifungal effects of D-AMB encapsulated into nanoparticulate PLGA–DMSA (Nano-D-AMB) with the expectation that the number of AMB injections for PCM treatment could be reduced by this formulation. The efficacy and toxicity of this formulation were examined in a murine model of chronic PCM. AMB is the drug of choice to treat severe cases of PCM and other fungal diseases. Its clinical effectiveness, broad range of antifungal activity and the lower rates of resistance keep AMB in the therapeutic arsenal against invasive mycosis. Nephrotoxicity is the most frequent dose-limiting adverse reaction, which affects at least 30% of treated individuals and results in higher mortality rates and extended lengths of stay in hospital.

In this work, nanoparticulate PLGA containing D-AMB was developed to promote a sustained and gradual release of the drug after the first injection. A decrease in the number of injections of Nano-D-AMB was possible by a sustained loading of D-AMB sufficient for three doses in a single injection. This is an important advantage that should be tested in clinical trials and could reduce discomfort and suffering for patients who also need to be under medical supervision during the administration of the D-AMB. PLGA is a co-polymer formed from PLA and PGA, which causes controlled drug release affected by the physicochemical properties of the polymer (molecular weight, hydrophilicity) and the concentration ratio of PLA:PGA. PLGA nanoparticles have presented satisfactory results for the controlled delivery of different classes of drugs, including antimicrobials, antifungals and hormone antagonists. As an example, ciprofloxacin-loaded PLGA nanoparticles released the antibiotic for 2 weeks, with a superior efficacy to inhibit Escherichia coli in comparison with free ciprofloxacin.

Nanoparticulate PLGA as a drug delivery system has been used for a long time because it offers many advantages, such as...
high stability, easy uptake into the cell by endocytosis and the ability to target specific organs, with many different compositions of PLA and PGA mixtures. The drug delivery system itself is non-toxic, and its use has long been approved by many regulatory agencies, including, for instance, the US FDA.\textsuperscript{26}–\textsuperscript{28} In vitro tests using different cell lines showed no toxicity for nanoparticulate PLGA.\textsuperscript{3,29} Considering all the reports available in the literature, an extra control group with unloaded nanoparticulate PLGA was not included in our experimental protocol. Moreover, the behaviour observed with this type of drug delivery system is similar to the studies performed by Mittal \textit{et al.},\textsuperscript{31} revealing a sustained release of the active component in an average time of 72 h. The results of preliminary studies using the PLGA composition associated with AMB were analysed by UV/visible spectroscopy and fluorimetric detection of AMB in PBS and plasma medium at 36 °C. The observed average time of release was 72 h, with lower burst release. Similar results were observed in studies using the same procedure to prepare the drug delivery system and the composition of the polymeric blend.\textsuperscript{4,29,30}–\textsuperscript{32}

In our study, Nano-D-AMB at 6 mg/kg every 3 days controlled the clinical signs of PCM and pulmonary infection, as shown by the cfu decrease in lungs. The therapeutic effects were comparable to those obtained with D-AMB at 2 mg/kg daily.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Histopathological analysis of lungs from PBS-treated mice (a and b), D-AMB- (c and d) and Nano-D-AMB-treated (e and f) animals. Granulomas in D-AMB- and Nano-D-AMB-treated mice are more compact and organized than in untreated animals. Arrows indicate the fungal cells inside the granulomas. A colour version of this figure is available in the online version of this article (http://jac.oxfordjournals.org).}
\end{figure}
Nevertheless, a low number of fungal cells persisted in both Nano-D-AMB and free D-AMB-treated groups inside pulmonary granuloma structures, which could create a protective microenvironment for the pathogen against drug action. These structures are formed by fragmentation and reorganization of reticular fibres and are known to be an important mechanism to protect the organism against fungal dissemination.\textsuperscript{15,16} Although we believe that a control group receiving the same drug amount in the D-AMB form (6 mg/kg/day) every 3 days would have made our results stronger, it was not used because it would be extremely toxic to the animals (the recommended limit is up to 1 mg/kg/day of AMB). Another experiment from this laboratory (manuscript in preparation) showed that empty PLGA–DMSA nanoparticles do not treat paracoccidioidomycosis, indicating that this nanoparticle alone was not able to remove the fungus and control the disease.

Previous studies have described the effects of D-AMB on body weight and the respective improvements offered by the lipid-based formulations.\textsuperscript{23,33,34} In our study, Nano-D-AMB (2.7 mg/kg of AMB at intervals of 3 days) was able to prevent the loss of body weight observed in the free D-AMB-treated group during the first month. Following the treatment, the animals presented a slight decrease in body weight after 2 months, which suggests a delay in the onset of the adverse side effects as observed in other studies with lipid nanoparticles containing D-AMB (Abelcet\textsuperscript{®}).\textsuperscript{35} Also, Nano-D-AMB prevented the

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>BUN (mg/dL)</th>
<th>Cr (mg/dL)</th>
<th>GOT (IU/L)</th>
<th>GPT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) Non-infected</td>
<td>52 ± 6</td>
<td>0.10 ± 0.07</td>
<td>161 ± 30</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>(II) PBS</td>
<td>50 ± 12</td>
<td>0.14 ± 0.05</td>
<td>177 ± 40</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>(III) D-AMB</td>
<td>52 ± 7</td>
<td>0.14 ± 0.11</td>
<td>161 ± 46</td>
<td>22 ± 14</td>
</tr>
<tr>
<td>(IV) Nano-D-AMB</td>
<td>44 ± 3</td>
<td>0.08 ± 0.04</td>
<td>167 ± 72</td>
<td>18 ± 4</td>
</tr>
</tbody>
</table>

BUN, blood urea nitrogen; Cr, creatinine; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase. No significant differences were found for the renal and hepatic biochemical data of the animal groups.

Table 2. Body weight changes (mean ± SD) according to each experimental group at days 30 and 60 post-treatment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weight (g) (mean ± SD)</th>
<th>day 30</th>
<th>day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
<td>change (%)</td>
</tr>
<tr>
<td>(I) Non-infected</td>
<td>26 ± 0.6</td>
<td>29 ± 0.6</td>
<td>2.2 (8.4)\textsuperscript{a}</td>
</tr>
<tr>
<td>(II) PBS</td>
<td>25 ± 1.8</td>
<td>22 ± 1.0</td>
<td>−3.7 (−14.6)</td>
</tr>
<tr>
<td>(III) D-AMB</td>
<td>25 ± 1.0</td>
<td>22 ± 0.7</td>
<td>−3.2 (−12.4)</td>
</tr>
<tr>
<td>(IV) Nano-D-AMB</td>
<td>25 ± 1.3</td>
<td>24 ± 2.0</td>
<td>−0.6 (−2.5)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P<0.05 for group I versus groups II, III and IV (one-way ANOVA with Tukey’s post-test).
\textsuperscript{b}P<0.05 for group IV versus groups II and III (Mann–Whitney test).

Table 3. Frequencies of MNPCE, MNNCE and PCE in bone marrow of mice after the treatment regimens

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MNPCE frequency (mean ± SD)</th>
<th>MNNCE frequency (mean ± SD)</th>
<th>%PCE (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
<td>change (%)</td>
</tr>
<tr>
<td>(I) Non-infected</td>
<td>0.8 ± 0.8</td>
<td>0.2 ± 0.5</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>(II) PBS</td>
<td>0.5 ± 0.6</td>
<td>0.8 ± 0.5</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>(III) D-AMB</td>
<td>0.2 ± 0.5</td>
<td>1.2 ± 0.5\textsuperscript{a}</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>(IV) Nano-D-AMB</td>
<td>1.0 ± 1.0</td>
<td>0.8 ± 0.5</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>39.0 ± 7.0\textsuperscript{b}</td>
<td>1.8 ± 1.6</td>
<td>49 ± 5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P<0.05 for group III versus group I as determined by the Mann–Whitney test.
\textsuperscript{b}P<0.05 for group III versus group I as determined by the Mann–Whitney test.

Cyclophosphamide was used as a positive control for genotoxicity. MNPCE, micronucleus in normochromatic erythrocytes; MNNCE, micronucleus in polychromatic erythrocytes; %PCE, the percentage of multiple erythrocytes was calculated from the following formula %PCE = [PCE/(PCE + NCE)] × 100.
occurrence of piloerection and hypotrichosis caused by free D-AMB. In addition, the preparation reduced the behavioural stress and improved the general physical appearance of the animals.

Both D-AMB- and Nano-D-AMB-treated animals showed normal levels of the biochemical parameters to evaluate kidney (BUN, creatinine) and liver (GOT, GPT) toxicities. The dose for AMB did not exceed the limit of 1 mg/kg/day for the D-AMB-treated group, which could explain the absence of toxicity. In contrast, Nano-D-AMB, even loading an initial AMB amount exceeding the tolerable limit (2.7 mg/kg/day), did not cause renal and liver toxicities in the animals, probably because of the low effective blood circulation of free D-AMB gradually releasing from Nano-D-AMB and by site-targeting provided by DMSA in the formulation. The biochemical parameter for nephrotoxicity in humans is the increase in serum creatinine levels (>2.5 mg/dL), which can be avoided by limiting the AMB dose to ≤1.0 mg/kg/day or switching from D-AMB to one of the newly developed lipid-based preparations AmBisome®, Abelcet® or Amphocite®.

Cytotoxicity and genotoxicity were evaluated through the micronucleus assay as recommended by regulatory agencies to test pharmaceuticals prior to commercialization. The results showed that Nano-D-AMB presented an absence of genotoxicity when compared with D-AMB. High concentrations of AMB in blood are associated with genotoxicity in normochromic cells; the gradual release of AMB from Nano-D-AMB may be responsible for the reduction of genetic toxicity. Also, the complexation of AMB with the polymeric blend may cause a reduction in tissue accumulation of the drug and consequently lower toxicity. In conclusion, the newly developed D-AMB-PLGA–DMSA preparation represents an advantageous delivery system that is able to reduce the dosing frequency of D-AMB 3-fold. Since this new formulation described is a lipid-free structure, this would also avoid any increase in plasma lipid levels. Also, this dosage regimen of Nano-D-AMB presented antifungal efficacy in the murine model of PCM without causing renal or hepatic toxicity.

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Transparency declarations

None to declare.

References