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La anfotericina B normalmente es subdosificada en el tratamiento de la leishmaniasis cutánea experimental

Amphotericin B is usually underdosed in the treatment of experimental cutaneous leishmaniasis

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Conflict of interest

Authors do not declare any conflict of interests.

Resumen

Introducción: La anfotericina B es un fármaco eficaz para el tratamiento de las distintas formas de leishmaniosis. Sin embargo, existen informes sobre su ineficacia en animales de laboratorio infectados experimentalmente con *Leishmania* spp. Es por ello que el objetivo del presente trabajo fue evaluar el balance de actividad-toxicidad a dosis de Anfotericina B superiores a 1 mg/kg, de modo que su uso como fármaco leishmanicida control positivo sea adecuado.

Método: Se infectaron experimentalmente ratones BALB/c con *L. amazonensis* y se trataron con anfotericina B por vía intraperitoneal a dosis desde 5 mg/kg hasta 12,5 mg/kg, comenzando 21 días después de la infección. Durante once semanas a partir del comienzo del tratamiento se evaluó el tamaño de las lesiones y el peso corporal de los ratones. Tres días después de concluido el tratamiento se determinó el número de parásitos en las lesiones.

Resultados: La anfotericina B a 5 mg/kg retrasó el crecimiento de las lesiones, pero no redujo su tamaño ni disminuyó significativamente el número de parásitos en la lesión. Dosis de 7,5 mg/kg a 10 mg/kg, cada 48 h durante 14 días (7 dosis) causaron una reducción significativa del tamaño de la lesión y de la carga parasitaria sin pérdida manifiesta de peso corporal y sin signos de toxicidad. La anfotericina B a 12,5 mg/kg fue más eficaz, pero produjo una toxicidad inaceptable.

Conclusiones: Los resultados avalan el uso de la anfotericina B como control positivo en ratones BALB/c infectados experimentalmente con *L. amazonensis* en dosis de 7,5 mg/kg a 10 mg/kg para lograr un efecto comparable al observado en la práctica clínica.

Palabras clave: *Leishmania amazonensis*; anfotericina B; BALB/c; dosis

Abstract

Introduction: Amphotericin B is an effective drug for the treatment of the different clinical forms of leishmaniasis. However, there are reports of its ineffectiveness in animals experimentally infected with *Leishmania* spp. That is why, the objective of the present work was to evaluate the balance of activity-toxicity at amphotericin B doses over 1 mg/kg, so that its use as a positive control antileishmanial drug were adequate.

Method: BALB/c mice were experimentally infected with *L. amazonensis* and treated with amphotericin B by intraperitoneal route at doses from 5 mg/kg to 12.5 mg/kg, beginning 21 days after infection. The size of the lesions and the body weight of the mice were measured for eleven weeks after the commencement of treatment. The number of parasites was also determined three days after the end of treatment.

Results: Amphotericin B at 5 mg/kg retarded lesions growth but neither reduced lesion size nor the parasite load at lesion site. Doses of 7.5 mg/kg to 10 mg/kg, every 48 h for 14 days (7 doses) caused a significant reduction of lesion size and parasite load without evident loss of body weight and without signs of toxicity. Amphotericin B at 12.5 mg/kg was more effective but produced unacceptable toxicity.

Conclusions: The results support the use of amphotericin B as a positive control drug in BALB/c mice experimentally infected with *L. amazonensis* at doses of 7.5 mg/kg to 10 mg/kg to achieve an effect comparable to that observed in clinical practice.

Keywords: *Leishmania amazonensis*; amphotericin B; BALB/c; dose

Highlights

Amphotericin B is used as positive control drug in experiments testing new compounds for leishmaniasis due to its high clinical efficacy. However, only partial efficacy, limited to retardation of lesion growth, reduction of the number of parasites in the infected tissue and eventual relapse is reported in many experimental cases.

The present study demonstrates that higher amphotericin B doses compared to those previously reported increased the treatment efficacy without evident signs of toxicity.

Doses of amphotericin B in the range of 7.5 mg/kg to 10 mg/kg are recommended to achieve the best experimental outcome.

Introduction

Amphotericin B is an antifungal compound produced by *Streptomyces nodosus*, which is characterized by a macrocyclic lactone ring. Its primary use was as an antifungal drug on the basis of its greater affinity for cell membranes containing ergosterol (fungal cells) with respect to cholesterol-containing membranes (mammalian cells)[1]. Cell membranes of trypanosomatids also contain ergosterol, hence the successful treatment of leishmaniasis with amphotericin B. It acts through binding to ergosterol, leading to changes in cell-membrane permeability, formation of pores, leakage of ions, induction of metabolic shock, and promoting cell death[2].

Despite of its high clinical antileishmanial efficacy, amphotericin B has been used for years, but mostly as a second-line antileishmanial drug because of its side effects[3]. The main toxic effects of amphotericin B are nephrotoxicity and infusion-related toxicity. The latter is transient and can be treated with antihistamine drugs. In turn, renal function gradually returns to baseline upon discontinuation of therapy, although permanent damage remains in some patients, especially when the cumulative dose exceeds 5 g[4].

Various lipid formulations have been developed to reduce side effects while maintaining the effect. Among them, liposome amphotericin B (Ambisome®) has shown the best safety profile, probably because the large size of liposomes limits glomerulofiltration of the drug and exposure of renal distal tubules[5]. Consequently, higher doses of amphotericin B are tolerated, but unfortunately, higher doses are also required for adequate efficacy[2]. Even with liposomal amphotericin B, a relative high incidence of toxicity has been associated with its use[6].

Single dose liposomal amphotericin B has been suggested by the World Health Organization as the first line drug for the visceral leishmaniasis elimination program in the Indian subcontinent[7], but its high cost has limited its widespread use in other several highly endemic countries like Brazil[8]. Similar to the clinical situation, the routine use of liposomal amphotericin B has also been limited in rodent experiments due to cost.

Nevertheless, thanks to its high activity, amphotericin B is extensively used as reference drug (positive control) for both *in vitro* and *in vivo* studies of investigational anti-leishmanial compounds[9,10,11]. However, in many instances amphotericin B has only produced partial effect in animal models of cutaneous leishmaniasis: in most of the cases it causes a decrease in the rate of lesion growth and the parasite load, but rarely, a reduction of lesion size[11,12,13,14,15,16].

The studies using amphotericin B as reference drug are diverse regarding the moment of initiating drug administration, the doses and the period of administration, which are all potentially important issues for both the outcome of the experimental disease and the correct interpretation of the results[14,17]. In an attempt to improve the experimental antileishmanial efficacy of amphotericin B, we have previously tested the effect of the inoculum size and the time period elapsed between the infection and the commencement of treatment. We have found that the antileishmanial effect of amphotericin B increased with the size of the inoculum and that the earlier the onset of therapy, the better the results (unpublished results). But, still, with an optimum inoculum of 10^7 stationary-phase *L. amazonensis* promasti-

gotes and initiating treatment as early as one day post-infection, we could not achieve a reduction of cutaneous lesions with daily intraperitoneal administration of amphotericin B at 1 mg/kg.

Due to the suspicion that amphotericin B might be routinely underdosed in animal experiments, the objective of the present work was testing the efficacy and safety of larger doses of amphotericin B in BALB/c mice experimentally infected with *L. amazonensis*.

Methods

Parasites

The MHOM/77/LTB0016 reference strain of *Leishmania amazonensis* was kindly donated by the Department of Immunology of Fundação Oswaldo Cruz (Fiocruz), Brazil. The promastigotes were cultivated in Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% heat inactivated (56 ^{o}C, 30 min) fetal bovine serum (Gibco, USA) and incubated at 26 ^{o}C. Parasites were maintained in exponential multiplication by passage every 3-4 days. Cultures in stationary phase (5 days) were used for animal experiments.

Animals

Female, 16-18 g, 6-8 weeks old BALB/c mice were supplied by the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Mice were housed under controlled environmental conditions (room temperature 22-25 °C, relative humidity 60-65%, light cycle 10 h light-14 h dark) and handled by qualified personnel. At the end of the studies, they were sacrificed by an overdose of pentobarbital (100 mg/kg). The protocol of the study was approved (Code of approval: CEI-IPK 87-18) by the Ethics Committee for the Care and Use of Laboratory Animals of Instituto de Medicina Tropical "Pedro Kourí" (IPK), which functions in accordance with international standards on the topic[18]. Criteria for the selection of humane endpoints were established based on the Guidelines for Endpoints in Animal Study Proposal of the National Institute of Health Office of Animal Care and Use, USA[19].

Amphotericin B

Amphotericin B deoxycholate (Anfotericina B, La Habana, Cuba) was dissolved in double distilled sterile water and kept at 5-10 °C protected from light. Appropriate dilutions in water were made to adjust doses to the average body weights of the groups of mice. A dose adjustment was made after weighting mice at the end of the first week of treatment. Amphotericin B was administered by intraperitoneal route.

Infection, treatment and evaluation

Seventy-five mice, allocated in five groups of 15 mice each, were infected by intradermal injection in the footpads with 10^7 stationary-phase *L. amazonensis* promastigotes as published elsewhere,[12] but with twice the infective dose based on previous unpublished works that showed higher amphotericin B activity with larger inoculum sizes. Three weeks later they were treated with 5; 7.5; 10 or 12.5 mg/kg amphotericin B every 48 h for 14 days. The fifth group (controls) of infected mice did not receive any treatment at all. Mice were weighed at the beginning of treatment and weekly then after for eleven weeks. The dorso-plantar thickness of the rear limbs was measured every week with a Vernier caliper and lesion sizes were calculated by subtracting the value of the non-infected limb (left) to that of the infected one (right). Five mice per group were sacrificed three days after the end of treatment and the number of parasites per gram of infected tissue was estimated by using the Limiting Dilution Assay (LDA) technique[20].

In order to record signs of toxicity associated to amphotericin B administration, mice were clinically observed 30 min and 4 h after each dosing, before the following administration, and daily for 14 days after the last dose. Attention was paid to changes in skin and fur, eyes and mucous membranes, the respiratory, circulatory, autonomic and central nervous systems, so as somatomotor activity and be-

havior pattern. Attention was specially directed to the occurrence of tremors, convulsions, salivation, diarrhea, lethargy, sleep, coma and death[21].

To assess the safety-activity profile of amphotericin B at the different dose levels, the relative percent body weight (RBW) and the activity were calculated at three weeks after the end of treatment by using the following formulas:

$$RBW\,(\%) = 100\left(1 - \frac{BW_{treated\ group}}{BW_{control\ group}}\right) \text{ and } Activity(\%) = 100\left(1 - \frac{LS_{treated\ group}}{LS_{control\ group}}\right);$$

where BW and LS stands for body weight and lesion size, respectively, for the treated and control groups.

Considering that experimental cutaneous leishmaniasis by *L. amazonensis* inoculation in BALB/c mice is progressive, leads to ulceration and, in most cases, causes eventual mutilation of the infected limb, the groups of mice with average lesion size over 4.0 mm were sacrificed before the end of the follow-up period to avoid unnecessary suffering. Moreover, groups of mice that showed evident clinical relapse after the fifth week of treatment onset were also sacrificed, since the primary objective of the follow up was to assess whether lesions relapsed or not with the highest doses of amphotericin B.

Statistics

Data were analyzed by using STATISTICA Software (Version 10.0, www.statsoft.com). Lesion sizes were compared by repeated measures analysis of variance and Fisher's least significant difference test. Parasite loads were compared by Kruskall-Wallis test and the distribution-free multiple comparison test. Values of p under 0.05 were considered significant.

Results

The doses of amphotericin B from 5 mg/kg to 12.5 mg/kg significantly reduced ($p<0.05$) the growth of the lesions compared to controls (Figure 1). At 5 mg/kg, although lesion growth was significantly reduced ($p <0.001$) compared to the control group, lesions did not show an absolute reduction of size. The doses of 7.5 mg/kg and 10 mg/kg caused a decrease in lesions that was proportional to the dose of amphotericin B. Such reduction in lesions lasted for up to three weeks after the end of treatment, showing a relapse then after. On the contrary, with the maximum dose tested (12.5 mg/kg), lesions diminished to be practically unnoticeable for up to 6-7 weeks after the end of treatment. From then on, lesion regrowth occurred in seven out of the nine surviving mice.

Mice treated with 5 mg/kg amphotericin B and control mice were sacrificed at the sixth week of follow-up to avoid unnecessary suffering. In the case of control mice, lesions had become too large and impaired ambulation. In relation to the mice treated with 5 mg/kg, it was evident that the lesions would not be reduced and that, on the contrary, they would grow rapidly, as had occurred until then. For the same reason, mice treated with 7.5 mg/kg and 10 mg/kg were sacrificed at week 10, since the purpose of the follow-up, which at that time for those groups was to assess relapse, was fulfilled.

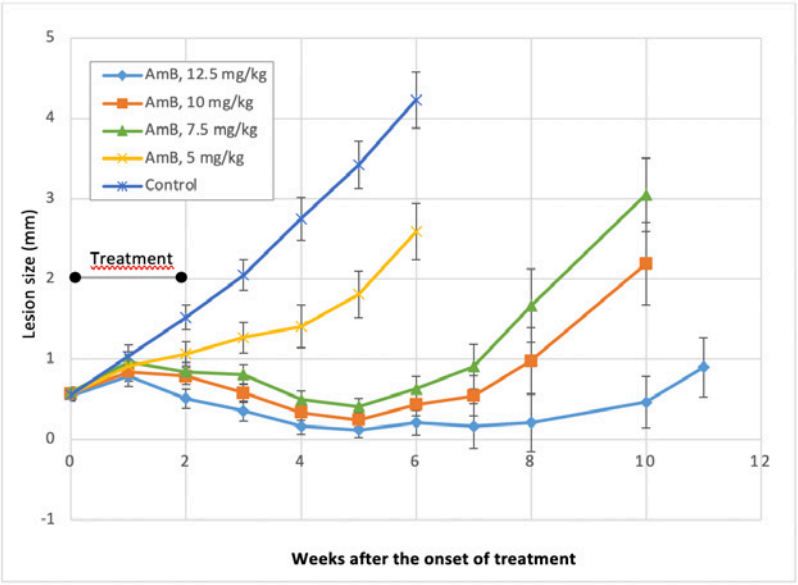

Figure 1: Dose-effect of amphotericin B in the model of cutaneous leishmaniasis caused by *L. amazonensis* in BALB/c mice. All treatments showed statistically significant differences with respect to the control group ($p < 0.01$) after the second week of starting therapy until the moment that control mice were sacrificed.

The number of parasites per gram of infected tissue three days after the end of treatment showed a progressive decrease as the dose of amphotericin B was increased. The doses from 7.5 mg/kg to 12.5 mg/kg resulted in statistically ($p<0.05$) lower parasite loads compared to that of the infected not treated control group, representing relative reductions of 90 % - 99 % compared to the control mice (Figure 2).

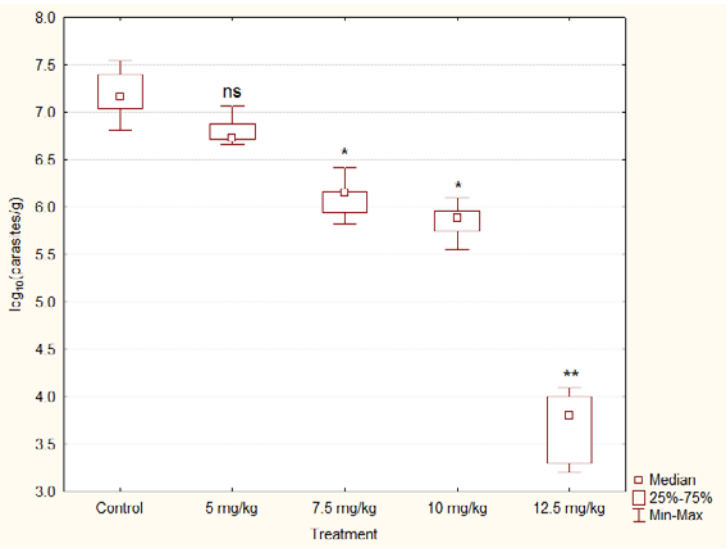

Figure 2: Number of parasites in the infected tissue.

The efficacy of amphotericin B (Figure 3) was calculated based on lesion sizes of treated mice compared to controls at three weeks after finishing therapy that was the moment of maximum observable effect. Doses from 7.5 mg/kg resulted in an efficacy close to 90 %. On the other hand, body weight loss was low and comparable for the dose range of 5 mg/kg to 10 mg/kg. A higher dose (12.5 mg/kg) significantly increased weight loss and caused 10 % mortality.

Body weight losses occurred once the administration of amphotericin B was started. Also, control mice, to which amphotericin B was not administered, showed a progressive body weight increase during the whole experiment (data not shown), which supports the assumption that weight losses were due to the administration of amphotericin B. A concurrent thorough toxicological assessment of amphotericin B in the dose range tested in the present study might have been desirable from the experimental design point of view but was not considered essential since the preclinical and clinical toxicology of amphotericin B is well-known.

Only mice treated with the maximum dose of amphotericin B showed unspecific signs of toxicity consisting of reduced muscle mass, rough hair coat and hunched posture. One mouse of this group died the week following the end of treatment. Clinical signs subsided one week after finishing amphotericin B administration, but mice did not totally recover their body condition and weight.

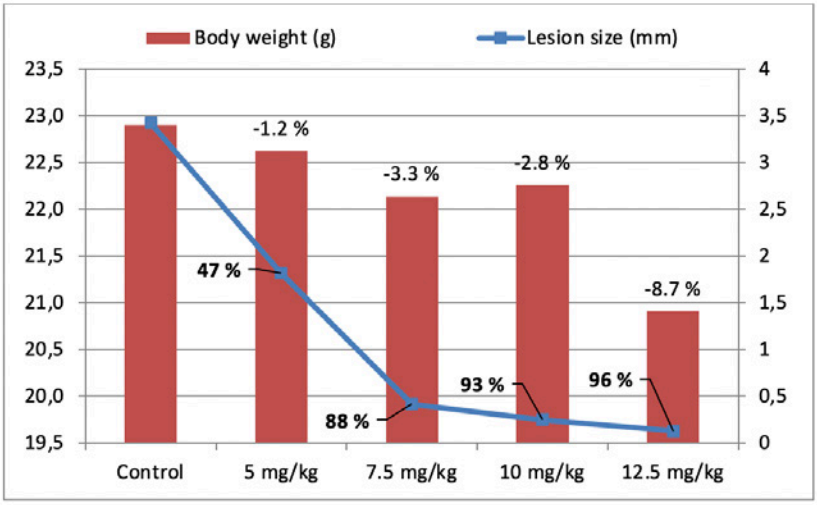

Figure 3: Combined analysis of dose-effect on the antileishmanial activity and toxicity. Primary axis, body weight; secondary axis, lesion size.

Discussion

Amphotericin B is the most clinically efficacious drug available for the treatment of any form of leishmaniasis, and it is even effective in cases refractory to other antileishmanial drugs[22,23]. Among registered antileishmanial drugs, pentavalent antimonial derivatives and amphotericin B are more frequently used as reference, positive control drugs in rodent experiments testing new compounds or treatments for leishmaniasis. However, due to the progressive development of antimonial drugs - resistant strains and the eventual need of testing not only reference strains but also recent clinical isolates when testing new drugs, the use of amphotericin B (for which drug resistance is rarely reported) is preferrable. Nevertheless, there are several reports on the inefficacy of amphotericin B in animals experimentally infected with a variety of *Leishmania* species[11-16,24] when it has been used at doses under

4 mg/kg; a fact that has been associated to the high multiplication rate of the parasites at the site of infection[15].

Although we have previously found that early treatment enhances the experimental antileishmanial efficacy of amphotericin B (unpublished results), in clinical practice, treatment usually starts several weeks after the infection and only after lesions are evident. Therefore, in the present study, we initiated treatment at 21 days post-infection, i.e., when lesions were evident in all mice.

Most authors have used amphotericin B as a positive control in doses of 1-5 mg/kg for 14-21 days. One milligram per kilogram coincides with the dose used in humans; therefore, the allometric relationship between both species is ignored in such cases. Based on body surface to body weight ratio, the human equivalent dose for amphotericin B in mice is 12.3 mg/kg[25], which is close to the highest dose tested in the present study and that also delayed disease relapse for longer. However, a combined analysis of the efficacy and toxicity of amphotericin B in the animal model used suggests that doses in the range of 7.5 mg/kg to 10 mg/kg (every 48 h) are the most appropriate for use as a positive control regimen.

Amphotericin B does not produce parasitological cure in patients with leishmaniasis, even though the clinical response in humans is evident. Coherently, the best expected scenario in BALB/c mice would be that observed with doses over 7.5 mg/kg; that is, a reduction of clinical signs (lesion size and parasite load) and relapse after a period of treatment withdrawal. Due to the inadequate immune response developed by BALB/c mice against *Leishmania* spp., the definitive clinical cure of BALB/c mice infected with *L. amazonensis* has rarely been reported as, generally, only a delay in the lesion growth rate or a transient reduction of the lesions has been demonstrated with most of the treatment tested[26,27,28,29].

An exception worth to mention was the combination of miltefosine and amiodarone, which produced long lasting recovery of the mice from *L. mexicana* infection with no clinical and parasitological evidence of relapse after 84 days of follow-up[26]. Interestingly, Glucantime® (a Sb^V derivative) at 100 mg/kg could not stop lesion growth at any time-point, it only caused a delay in its growth rate. In turn, amiodarone (50 mg/kg) and miltefosine (20 mg/kg), although they stopped lesion growth during treatment span and for two other weeks, lesion started to grow afterwards. That is consistent with what happened in this study with amphotericin B at 7.5 mg/kg and 10 mg/kg and supports the hypothesis that, even though relapse will eventually occur, evident, statistically significant lesion reduction is achievable shall proper doses of active compounds are administered.

In summary, results evidenced that doses of amphotericin B equal to or under 5 mg/kg are insufficient to reduce lesion size in BALB/c mice infected with *Leishmania amazonensis* (and probably with other *Leishmania* species as well). Consequently, doses in the range of 7.5 mg/kg to 10 mg/kg are recommended to achieve an effect comparable to that observed in clinical practice, since a proper balance of antileishmanial effect (reduction of lesion size and parasite load) and toxicity is achieved at such dose levels.

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