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Combined topical paromomycin and oral miltefosine treatment of mice experimentally infected with *Leishmania (Leishmania) major* leads to reduction in both lesion size and systemic parasite burdens

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Objectives: This study aimed to investigate the activity of the combination of topical paromomycin gel and oral miltefosine for the treatment of experimental cutaneous leishmaniasis caused by *Leishmania (Leishmania) major*.

Methods: The efficacy of the combination, evaluated by measuring lesion size and parasite burden in the skin and spleen, was assessed in BALB/c mice infected by *L. (L.) major*. Miltefosine was administered orally at 25 mg/kg/day for 10 days, while 10% paromomycin gel was applied topically twice a day for 10 days.

Results: Treatment of the experimentally infected animals with topical paromomycin + oral miltefosine combination induced a statistically significant reduction in lesion size and parasite burden in the skin, with complete healing of ulcers, as compared with those treated with oral miltefosine or placebo. Furthermore, topical paromomycin + oral miltefosine combination was as effective as topical paromomycin alone to reduce the lesion size and parasite load in lesions. However, the efficacy of the combination was significantly higher than that observed for the other treatments, including topical paromomycin alone, in reducing the parasite burden in spleen.

Conclusions: The combination of topical paromomycin gel and oral miltefosine provides an enhanced efficacy in the treatment of *L. (L.) major*-infected mice, thus presenting a significantly higher activity than that observed for the monotherapeutic regimens.

Keywords: cutaneous leishmaniasis, therapy, efficacy evaluation, animal model

Introduction

Leishmaniasis is a group of disease syndromes caused by different species of flagellate protozoa belonging to the *Leishmania* genus.[1,2] There are two main clinical manifestations of leishmaniasis: cutaneous and visceral. Cutaneous leishmaniasis (CL) most commonly appears first as a localized papule, which then evolves into an ulcer upon the loss of the epidermis, resulting in a great impairment of the skin barrier. Parenteral administration of pentavalent antimony organic compounds remains as the first choice therapy for all leishmaniasis syndromes. However, resistance and the high frequency of side effects (anorexia, myalgias, arthralgias, chemical pancreatitis, leucopenia,

cardiotoxicity, etc.) are still relevant problems associated with this treatment.[3,4]

Over the past few decades, major emphasis has been given to the development of alternative therapies, including the identification of formulations for both oral and topical treatment of CL.[4,5] Topical treatment represents an interesting approach, offering several advantages in comparison with parenteral route: easy administration; lower adverse reaction incidence; and an attractive cost–benefit ratio.[6]

Paromomycin, an aminoglycoside antibiotic, is the most commonly studied drug for the topical treatment of CL. Petrolatum ointments containing paromomycin, associated with methylbenzethonium chloride, have been evaluated with favourable results.[7]

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However, the local side effects of the ointment containing paromomycin and methylbenzethonium chloride[8,9] have given rise to the search for alternative topical formulations. Other formulations have been developed, in which the methylbenzethonium chloride was replaced by urea[10] or gentamicin,[11] but no significant difference in cure rates has been reported in the clinical trials of these formulations when compared with the placebo.[12,13] Recent studies have shown that a new paromomycin hydrophilic formulation was highly effective in *Leishmania (Leishmania) major*, *Leishmania (Leishmania) amazonensis*-infected mice or *Leishmania (Viannia) braziliensis*-infected hamsters.[14,15] This formulation was recently tested in patients in whom CL was caused by *L. (V.) braziliensis* and who could not be submitted primarily to meglumine antimoniate therapy.[16]

A new agent—miltefosine—has been successfully implemented in the oral treatment of New World CL.[17] Early studies on the treatment of CL included pilot dose-ranging studies, which proved that miltefosine, at a dose of 2.5 mg/kg/day over a 28 day period, was as effective as a standard therapy using antimony. However, lower doses of this substance were associated with a lower rate of cure.[18] Subsequently, in controlled clinical trials aimed at testing miltefosine at 2.5 mg/kg/day in the treatment of CL caused by *Leishmania (Viannia) panamensis* in Colombia and *L. (V.) braziliensis* in Bolivia, a high rate of cure was observed, which was comparable to the cure rates observed in patients treated with antimony.[19] In the Old World, miltefosine was successfully used in an HIV-infected patient who had contracted *L. (L.) major* diffuse CL.[20] Therapies based on the application of miltefosine, however, are lengthy, lasting in general 28 days, and generate concerns regarding the existence of toxicity, resistance and teratogenicity.

Combined therapy, as compared with monotherapeutic regimens, also represents an exciting alternative in the treatment of CL, joining new therapeutic modalities that offer several advantages, such as preventing the emergence of resistance, increasing efficacy or shortening the course of treatment.[21] Therefore, this study aimed to investigate the efficacy of the combination of topical paromomycin with oral miltefosine in the treatment of experimental CL. The experimental model selected for this study was mice infected by *L. (L.) major*.

Materials and methods

Materials

Paromomycin as sulfate (757 µg/mg; Antibióticos, Milan, Italy), hydroxyethylcellulose (HEC; Natrosol 250 HR, Aqualon), methylparaben (MP) and propylene glycol (PG; BASF, Ludwigshafen, Germany) were used to prepare the hydrophilic gel. Miltefosine was donated by Zentaris GmbH (Frankfurt, Germany).

Preparation of formulations

The paromomycin hydrophilic gel was prepared by heating 1.5% HEC, 10% PG and 0.2% MP in water to 60–70°C, under constant agitation, until a homogeneous and transparent gel had been obtained. After cooling, paromomycin, previously dissolved in water, was incorporated into the gel at a 10% concentration. Subsequently, the mixture was agitated until a homogeneous preparation had been attained. For oral treatment, miltefosine was dissolved in distilled water.

Parasites and infection of animals

L. (L.) major (MHOM/IL/80/Friendlin) promastigotes were maintained at 23°C in Schineider's medium (Merck, Germany), supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, St Louis, MO, USA).

BALB/c mice (females, 6–7 weeks old) were inoculated with 1×10^7 stationary growth phase promastigotes of *L. (L.) major* through subcutaneous injections at the base of the tail, after trichotomy. This study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFMG: 36/2008).

Treatment of infected animals

Two separate experiments were performed using *L. (L.) major*-infected BALB/c mice. First, a dose–effect study of oral miltefosine was carried out. After the development of ulcerated lesions (average diameter 7–9 mm), BALB/c mice were divided into four groups ($n = 4$) according to lesion size, to assure similar average lesion size among treated groups. The miltefosine was administered by oral gavage at 10, 25 or 50 mg/kg/day for 5 days a week, over a 2 week period. The control group received distilled water. The animals were maintained in abstinence from food 3 h pre-treatment and 1 h post-treatment. The number of deaths and body weight (before and after treatment) were recorded as indicators of systemic toxicity. For this first study, the treatment efficacy was evaluated considering the parasite quantification at the site of infection (see below).

The second study evaluated the efficacy of the combination of topical paromomycin (gel containing 10% drug) and oral miltefosine. After the development of ulcerated lesions, BALB/c mice were divided into four groups. For the paromomycin group, lesions were covered with 50 µL of 10% paromomycin gel twice a day for 5 days a week, over a 2 week period. The gel was applied using an Eppendorf® pipette. For the miltefosine group, miltefosine was administered orally at 25 mg/kg/day for 5 days a week, over a 2 week period. For the paromomycin + miltefosine group, the lesions received a topical treatment (10% paromomycin gel) in the same manner as did the paromomycin group; in addition, the animals were also treated with miltefosine administered orally, in the same manner as the miltefosine group. For the control group, animals were treated with a gel that did not contain paromomycin (placebo). Treatment efficacy was evaluated by measuring the size of the lesions as well as by determining parasite quantification of the skin and spleen after the interruption of treatment.

Parasite quantification

Three days after the interruption of treatment, the number of viable parasites at the site of infection was determined by a limiting dilution assay.[22] Skin fragments, consisting of ulcerated lesions, were weighed and homogenized with an Ultra-Turrax (Ika, Germany) in Schineider's medium supplemented with 10% bovine fetal serum and 1 mL of a 100 U/mL penicillin and 100 µg/mL streptomycin solution. The homogenate was submitted to serial dilutions in duplicate in sterile 96-well culture plates and incubated at 23°C. Each well was examined for the presence of parasites and the number of parasites was determined by the highest dilution at which parasites could grow over a 7 day period. The lowest dilution level at which parasites could be detected was 10^{-1}, which was considered the limit of quantification. The number of viable parasites was also determined in spleens as described above.

Lesion size

During and after treatment, lesion size was followed up weekly using a caliper to measure its diameter (Mitutoyo, Brazil). The lesion size was determined by obtaining the average value between the longest line that could be traced from one border of the lesion to another and the line that bisected this distance at a 90° angle. Further evaluations, through careful observation of paws and tails, included the appearance of relapses, nodules and metastasis in other locations on the skin. Infected mice were observed for an additional 49 day period after the interruption of treatment. Animals were considered cured only if nodules and ulcers were completely absent after 49 days (end of experiment).

In vivo percutaneous absorption

In the other set of experiments, *in vivo* percutaneous absorption of paromomycin after application of the topical gel was investigated in plasma from BALB/c mice that presented ulcerated lesions. The 10% paromomycin gel was applied as previously described, and after the final application (10th day of treatment) the animals ($n=4$) were anaesthetized with a mixture of ketamine and xylazine (80 and 8 mg/kg, respectively) at intervals of 1, 3 and 6 h, intraperitoneally. Through an incision in the brachial artery, ∼1 mL of blood was collected and added to tubes containing EDTA (1.8 mg/mL). Subsequently, the blood was centrifuged at 1500 **g** for 5 min (Himac, Hitachi) and the plasma was separated.

The paromomycin concentration in the plasma was determined by a microbiological assay of agar diffusion inhibition of the growth of *Bacillus subtilis* (ATCC 6633), as previously described,[23] with some modifications. After 18 h of incubation of the plates at $36\pm0.5°C$, the zone of inhibition was measured for each sample. A straight-line relationship was obtained between the log drug concentration ($\mu g/mL$) and the zone of inhibition (mm). The concentration of paromomycin was determined from a standard curve ($y=3.34x+10.22$), which was obtained with paromomycin concentrations of 1.0, 2.0, 4.0, 8.0 and 16.0 $\mu g/mL$.

The assay procedure was validated to establish the accuracy, precision, quantification limit and selectivity. A reference standard (USP paromomycin sulfate: 730 $\mu g/mg$) was used for the validation. To investigate the accuracy, plasma samples ($n=3$) were spiked with known amounts of paromomycin (1.0, 4.0 and 16.0 $\mu g/mL$). The percentage of recovery was 106%$\pm$7.8%, 101%$\pm$2.4% and 96%$\pm$5.7%, respectively, showing that the plasma components did not interfere with the drug diffusion and that the assay is accurate. Next, selectivity experiments were conducted. Plasma from animals treated with placebo gel (without paromomycin) did not show a zone of inhibition. Therefore, the blank plasma was unable to inhibit the microorganism growth and, therefore, to interfere with the method of analysis. The intraday precisions were determined with the above solutions, and the data obtained were 1.1 ± 01, 4.0 ± 0.1 and 15.3 ± 0.9 $\mu g/mL$, respectively. The quantification limit of paromomycin in plasma was 1.0 $\mu g/mL$.

Statistical analysis

The Kruskal–Wallis non-parametric test was used to compare lesion size. The numbers of animals cured (complete healing of lesions and absence of nodules) in each group was compared by Fisher's exact test. The parasite quantification among groups and the weights of animals were evaluated using the one-way analysis of variance test followed by Tukey's test. The difference was considered significant when the P value was <0.05.

Results

### Dose–effect study of oral miltefosine

The development of lesions in control BALB/c mice infected with *L. (L.) major* followed the prognosis described in the literature. The ulcers progressed in size and shape, presenting a 7–9 mm average diameter 6 weeks after inoculation of the animals, when the treatment was started.

The quantification of parasites within lesions was used to select the oral dose of miltefosine. As shown in Figure 1, the number of parasites within the lesion decreased significantly when doses of oral miltefosine were increased. The number of parasites in the control group (1.4×10^6) was higher than that observed in the groups treated with miltefosine at 10 mg/kg/day (1.8×10^5), 25 mg/kg/day (2.6×10^4) or 50 mg/kg/day (5.5×10^3). Statistical analysis showed a significant reduction in parasite numbers in the animals treated with doses of 25 and 50 mg/kg/day when compared with the control group. Differences between these two doses were not significant ($P>0.05$). The lesion parasite load also diminished in the animals that received 10 mg/kg/day miltefosine when compared with the control group; however, this difference was not statistically significant ($P>0.05$). At the end of the treatment, as compared with at time zero, the animals that received oral miltefosine at a dose of 50 mg/kg/day presented a significant loss of body weight (25.0 ± 2.2 g reducing to 23.2 ± 2.5 g). However, no statistically significant differences were detected in body weight for the other groups (control, or 10 or 25 mg/kg/day miltefosine).

Therefore, 25 and 50 mg/kg/day oral miltefosine illustrated high activity, with a significant reduction in lesion parasite burden, in the treatment of *L. (L.) major*-infected mice. However, miltefosine administered orally at 50 mg/kg/day did, in fact, show evident signs of toxicity. Based on these results, the dose of 25 mg/kg/day was selected for further studies.

Efficacy of combination of topical paromomycin and oral miltefosine

The second study was carried out to evaluate the efficacy of the combination of oral miltefosine and topical paromomycin.
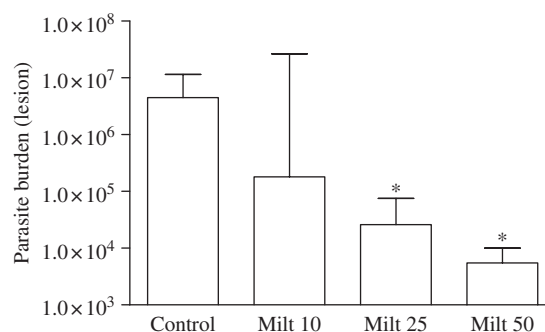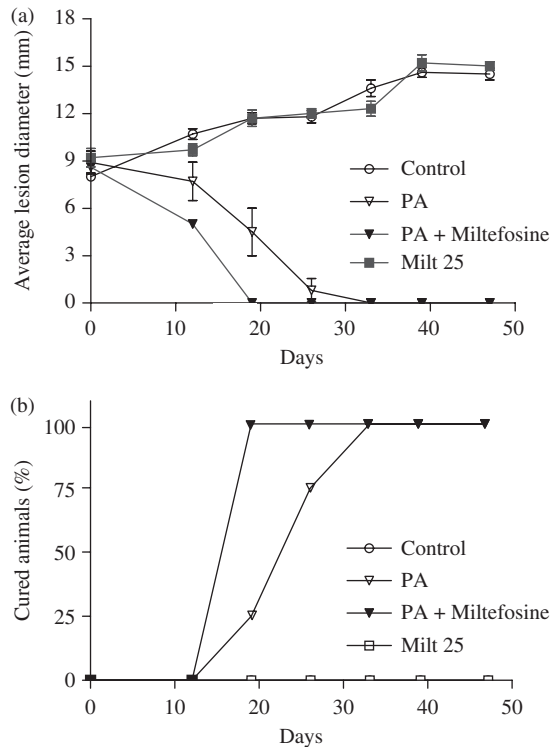

Figure 1. *In vivo* efficacy of oral miltefosine in *L. (L.) major*-infected mice. Female BALB/c mice were infected with *L. (L.) major* promastigotes in the base of the tail. Six weeks after inoculation, the animals were treated with distilled water (Control) and orally administered miltefosine at 10 mg/kg/day (Milt 10), 25 mg/kg/day (Milt 25) or 50 mg/kg/day (Milt 50) for 10 days. Three days after interruption of treatment, parasite numbers recovered from lesions were evaluated by limiting dilution assay. *$P<0.05$ when compared with the control group. $n=4$.

Combined topical paromomycin and oral miltefosine treatment

Figure 2(a) shows the evolution of the lesion size, after the beginning of the treatment, as a function of time. At the beginning of the treatment, the animals treated with topical paromomycin and a topical paromomycin+oral miltefosine combination presented lesions with an average diameter of 8.9 and 8.6 mm, respectively. The lesion size of these animals significantly diminished during the evaluation period until complete healing had been established, which could be observed 33 and 19 days after the onset of therapy for both the topical paromomycin and topical paromomycin+oral miltefosine groups, respectively. Animals treated with the combination presented faster healing rates than did those treated with topical paromomycin alone, but these differences were not statistically significant. All animal lesions remained cured throughout the entire observation period (49 days) and no relapse, characterized by the reappearance of ulcers, could be observed during this time interval. In the group treated with 25 mg/kg/day of miltefosine, none of the animals presented a reduction in lesion size. In addition, a gradual increase in the average lesion size could be observed, which was similar to that observed in the placebo group.
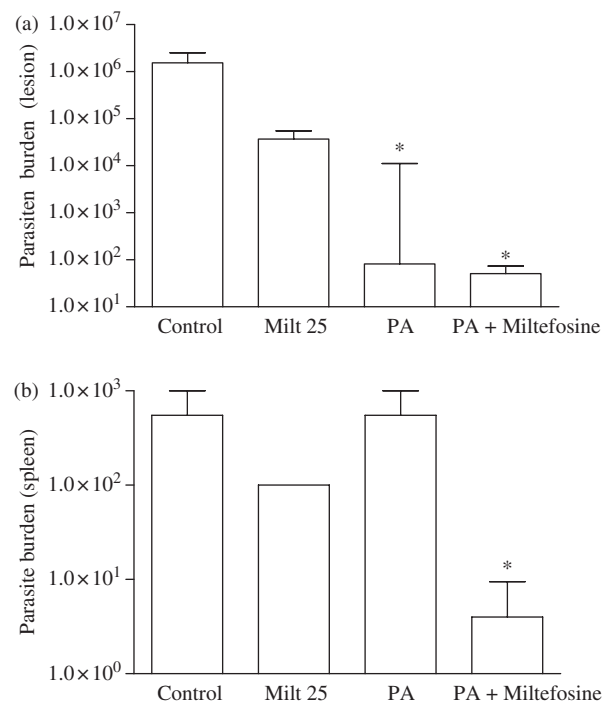
Data concerning the percentage of cured animals are shown in Figure 2(b). The cure criterion adopted was the complete healing of lesions and the absence of nodules. In the groups treated with topical paromomycin or a topical paromomycin+miltefosine combination, complete healing (100%) was observed in all animals 33 and 19 days after the beginning of the treatment, respectively. As shown above, animals treated with the combination presented faster healing rates than did those treated with topical paromomycin alone. No relapse, characterized by the reappearance of nodules or ulcers, could be observed for these two groups. Hair growth after the healing of lesions could be observed in all cured animals. In the group of animals treated with oral miltefosine alone, no cure was achieved for any animal (0% cure rate) throughout the evaluation period (49 days), which was similar to that observed in the placebo (control) group (Figure 2b). Therefore, under these conditions, the activity of topical paromomycin alone or the topical paromomycin+oral miltefosine combination was significantly higher than that observed for miltefosine alone.

The parasite burdens in the lesion and spleen were assessed 3 days after the end of the treatment. The data are presented in Figure 3. The parasite burden at the site of infection (lesion) was significantly reduced in animals treated with topical paromomycin (8.2×10^1) or the topical paromomycin+oral miltefosine combination (3.9×10^1) as compared with those that received either oral miltefosine (3.7×10^4) or the placebo gel (1.5×10^6). Topical paromomycin was as effective as the paromomycin+miltefosine combination, presenting a reduction of 99.99% in the lesion parasite load (Figure 3a). Significant

Figure 2. Evaluation of treatment efficacy in female BALB/c mice infected with *L. (L.) major*. Animals ($n=4$) were treated topically with 10% paromomycin gel (PA), 25 mg/kg/day oral miltefosine (Milt 25), a combination of topical paromomycin gel+25 mg/kg/day oral miltefosine (PA+Miltefosine) or placebo gel (Control) for 10 days. (a) Lesion diameters are shown as averages and standard errors. (b) Percentage of cured animals (complete healing of lesions and absence of nodules) evaluated for 49 days.

Figure 3. *In vivo* efficacy of the combination 10% paromomycin gel with 25 mg/kg/day oral miltefosine in *L. (L.) major*-infected mice. Female BALB/c mice ($n=5$) were infected with *L. (L.) major* promastigotes in the base of the tail. Six weeks after inoculation, the animals were treated with 25 mg/kg/day oral miltefosine (Milt 25), 10% paromomycin topical gel (PA), a combination of topical paromomycin gel+25 mg/kg/day oral miltefosine (PA+Miltefosine) or placebo gel (Control) for 10 days. Three days after interruption of treatment, parasite numbers recovered from lesions and spleen were evaluated by limiting dilution assay. (a) Parasite burden quantified in lesions. *$P<0.05$ when compared with control and 25 mg/kg/day oral miltefosine groups. (b) Parasite burden quantified in spleen. *$P<0.05$ when the topical paromomycin+oral miltefosine combination group was compared with the other groups.

decreases in the lesion parasite burden were also detected in mice treated with oral miltefosine alone as compared with the control group.

To investigate the systemic efficacy of the paromomycin + miltefosine combination, the spleen parasite burden was evaluated. The data are presented in Figure 3(b). The parasite burden in the spleen was significantly reduced in animals treated with the topical paromomycin + oral miltefosine combination as compared with the control group. Nevertheless, treatment with topical paromomycin alone did not change the parasite load in the spleen, while oral miltefosine, as compared with the control group, induced an insignificant reduction.

Evaluation of the paromomycin concentration in plasma

In the other set of experiments, the *in vivo* percutaneous absorption of paromomycin after the application of the topical gel was investigated in plasma from BALB/c mice that presented ulcerated lesions. The data were obtained after treating the animals for 10 days. The paromomycin concentration in the plasma after 1 h ranged from 2 to 22 µg/mL, while the values obtained after 3 and 6 h were negligible (below the detection limit). Therefore, paromomycin could be detected in the plasma of animals up to 1 h after the application of the topical gel, suggesting that topically applied paromomycin can be absorbed systemically. The fact that paromomycin could not be detected after 3 and 6 h may well be attributed to the elimination of the drug. The elimination half-life of paromomycin in plasma was found to be ~2 h.[24] These findings are consistent with previous studies, which demonstrated that *in vitro* skin permeation of paromomycin on stripped mice skin without stratum corneum (a damaged skin model) was high.[25]

Discussion

The development of alternative therapies, including the identification of formulations for both the oral and topical treatment of CL as well as drug combinations, is emerging.[3,5] Recently, studies have shown that a new paromomycin formulation can be highly effective against *L. (L.) major*, *L. (L.) amazonensis* and *L. (V.) braziliensis* in infected animals.[14,15] The efficacy of an orally applied antileishmanial drug (miltefosine) for the treatment of CL has also been reported.[19] Thus, this study aimed to investigate the efficacy of the topical paromomycin + oral miltefosine combination for the treatment of CL in experimentally infected mice.

Since there are no previous reports on the activity of oral miltefosine against the species causing CL in experimental models, the first step of this study was to evaluate its efficacy in mice that had been experimentally infected by *L. (L.) major*. Thus, we initially investigated a dose–effect curve of miltefosine. The miltefosine doses were chosen based on previous reports, which showed that effective dose 50% (ED_{50}) and 90% (ED_{90}) values for *Leishmania (Leishmania) donovani*-infected mice ranged from 14 to 27 mg/kg/day, respectively.[26] Data from the present study clearly showed that the administration of oral miltefosine at doses of 25 and 50 mg/kg/day led to a significant reduction in the lesion parasite burden. However, animals treated with miltefosine at a dose of 50 mg/kg/day showed signs of toxicity, with

a significant loss of body weight. Based on these results, the dose of 25 mg/kg/day was selected for further studies.

Subsequently, the treatment efficacy of the topical paromomycin + oral miltefosine combination, evaluated by measuring the lesion size and the parasite burden in the skin and spleen, was investigated. Treatment of the experimentally infected animals with the topical paromomycin + oral miltefosine combination, as compared with those treated with oral miltefosine or a placebo, led to a statistically significant reduction in the lesion size as well as in the lesion parasite burden, consequently presenting a complete healing of ulcers. In addition, the topical paromomycin + oral miltefosine combination proved to be as effective as topical paromomycin alone in reducing the lesion size and the lesion parasite load. However, the efficacy of the combination was significantly higher than that observed for the other treatments, including topical paromomycin alone, in reducing the parasite burden in the spleen. It is interesting to note that oral miltefosine, as compared with the placebo, significantly reduced the parasite loads in the skin, but the healing of lesions was not observed. This can be explained by the fact that the reduction in the lesion parasite burden was not enough to decrease the lesion size.

The improved systemic efficacy of the topical paromomycin + miltefosine combination may well be attributed to the effects of both drugs. A plausible explanation for this combined effect would be the percutaneous absorption of paromomycin after topical application. In this light, our studies showed that the paromomycin can be absorbed when administered topically and is above the concentration established as inhibitory concentration 50% (IC_{50}; 0.6 µg/mL) for *L. (L.) major*-infected macrophages.[27] These data are consistent with our previous observations, which showed that the *in vitro* permeation of paromomycin from hydrophilic gel on stripped mice skin (a damaged skin model) was high.[25] Thus, in the absence of an important barrier for diffusion, as observed in animals presenting ulcerated lesions, the percutaneous absorption of paromomycin was high.

Combined treatments with different drugs showing some degree of activity against *Leishmania* have been reported previously. The association of miltefosine (oral) with paromomycin (subcutaneous) was evaluated for *L. (L.) donovani* in BALB/c mice.[21] Although no synergistic effect has been demonstrated *in vitro*, an improved efficacy was observed *in vivo*. Our data showed that the combination of topical paromomycin and oral miltefosine was effective in the treatment of BALB/c mice infected by *L. (L.) major*, leading to a reduction in the parasite loads of both the skin and spleen, and healing of the lesions. The improved efficacy of the combination of topical paromomycin and oral miltefosine therefore could be attributed to the systemic and lesion effect of both paromomycin and miltefosine.

Besides the fact that paromomycin and miltefosine may have different mechanisms of action,[4] they present different elimination half-lives, which could be an interesting feature when combining drugs.[28] In agreement, paromomycin has an elimination half-life of 2 h, while miltefosine presents a longer half-life (96 h).[21,24] By combining these data it was possible to establish a treatment protocol with different intervals of administration. The activity of these drugs can reach a level significant enough to eliminate parasites that have disseminated to other organs. This combined effect may lead, for example, to the elimination of parasites in infected peripheral immature macrophages. These

cells may act as a significant reservoir of parasites, since *L. (L.) major* infection in BALB/c mice is associated with a significant increase in the number of precursor cells of macrophage–granulocyte lineage, which may provide an immature immune environment for the multiplication of parasites.[29]

Finally, the present study was performed using an animal model—*L. (L.) major*-infected BALB/c mice—that is commonly used to evaluate antileishmanial drugs or formulations.[10,15] Our data showed an improved efficacy of the combination of topical paromomycin and oral miltefosine in this model and suggest that this investigation should be extended to other *Leishmania* species that cause CL, such as *L. (L.) amazonensis* and *L. (V.) braziliensis*, since sensitivity to both paromomycin and miltefosine varies considerably among species.[27,30] In addition, each species is linked to a different clinical presentation and disease syndrome. *L. (V.) braziliensis* infection, for instance, is associated with the dissemination of parasites, a clinical situation that certainly requires a systemic effect of antileishmanial drugs.

Conclusions

In summary, our data show that the combination of topical paromomycin gel and oral miltefosine provides an enhanced efficacy in the treatment of *L. (L.) major*-infected mice, which is a widely used model for the evaluation of drugs used in the treatment of CL, thus presenting a significantly higher activity than that observed for the monotherapeutic regimens. These findings suggest that the topical paromomycin + oral miltefosine combination represents a promising alternative for the treatment of CL caused by *L. (L.) major*.

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Transparency declarations

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