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Amiodarone and Miltefosine Act Synergistically against *Leishmania mexicana* and Can Induce Parasitological Cure in a Murine Model of Cutaneous Leishmaniasis▽

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Leishmaniasis is parasitic disease that is an important problem of public health worldwide. Intramuscularly administered glucantime and pentostam are the most common drugs used for treatment of this disease, but they have significant limitations due to toxicity and increasing resistance. A recent breakthrough has been the introduction of orally administered miltefosine for the treatment of visceral, cutaneous, and mucocutaneous leishmaniasis, but the relative high cost and concerns about teratogenicity have limited the use of this drug. Searching for alternative drugs, we previously demonstrated that the antiarrhythmic drug amiodarone is active against *Leishmania mexicana* promastigotes and intracellular amastigotes, acting via disruption of intracellular Ca^{2+} homeostasis (specifically at the mitochondrion and the acidocalcisomes of these parasites) and through inhibition of the parasite's de novo sterol biosynthesis (X. Serrano-Martín, Y. García-Marchan, A. Fernandez, N. Rodriguez, H. Rojas, G. Visbal, and G. Benaim, Antimicrob. Agents Chemother. 53:1403–1410, 2009). In the present work, we found that miltefosine also disrupts the parasite's intracellular Ca^{2+} homeostasis, in this case by inducing a large increase in intracellular Ca^{2+} levels, probably through the activation of a plasma membrane Ca^{2+} channel. We also investigated the in vitro and in vivo activities of amiodarone and miltefosine, used alone or in combination, on *L. mexicana*. It was found that the drug combination had synergistic effects on the proliferation of intracellular amastigotes growing inside macrophages and led 90% of parasitological cures in a murine model of leishmaniasis, as revealed by a PCR assay using a novel DNA sequence specific for *L. mexicana*.

Leishmaniasis is a parasitic disease present in tropical and subtropical areas that generates high mortality and morbidity (15). The first line of treatment, based on pentavalent antimonials, is unsatisfactory due to high toxicity and the requirement of long periods of parenteral treatment. On the other hand, miltefosine, an orally active alkyl-lysophospholipid with potent anti-*Leishmania* activity, represents a major advance in the treatment of leishmaniasis (4). Nevertheless, miltefosine presents a series of adverse effects such as teratogenicity and potential development of resistance (4).

It has been suggested that miltefosine acts in *Leishmania mexicana* by inhibiting the alkyl-specific acyl coenzyme A-acyltransferase involved in the synthesis de novo of phosphatidylcholine (9), whereas in *Trypanosoma cruzi* and *Leishmania donovani*, miltefosine acts by inhibiting the Bremer-Greenberg pathway, specifically at level of phosphatidylethanolamine *N*-methyl-transferase (8, 17). In contrast, this drug inhibits phosphatidylcholine biosynthesis in humans by blocking phosphocholine citidyltransferase (24).

In a search for new alternative therapies, we previously demonstrated that amiodarone, a commonly used antiarrhythmic drug, affects the viability of *T. cruzi* epimastigotes and amastigotes (2), as well as *L. mexicana* promastigotes and amastigotes (20). In both cases, amiodarone induces an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by disrupting the Ca^{2+} regulation in the mitochondrion and the acidocalcisomes and also by blocking the ergosterol biosynthesis of these parasites (2, 20).

It has also been shown that miltefosine is able to affect intracellular Ca^{2+} homeostasis in human epithelial KB cells (7) and human myeloid leukemia cell lines (23). In this work, we sought to investigate the effects of miltefosine on the $[Ca^{2+}]_i$ in *L. mexicana* and its potential synergistic effects with amiodarone, using in in vitro and in vivo assays.

MATERIALS AND METHODS

Drugs. Amiodarone {(2-butyl-3-benzofuranyl)-[4-[2-(diethylamino)ethoxi]-3,5-diiodophenyl]methanone hydrochloride} and miltefosine (hexadecylphosphocholine) were obtained from Sigma Aldrich (St. Louis, MO). All other reagents were of the highest purity available.

Determination of in vitro antiproliferative activity. *L. mexicana* promastigotes were cultured in RPMI 1640 medium (Gibco) supplemented with 10% inactivated fetal bovine serum (FBS) in constant agitation at 29°C as described previously (20). *L. mexicana* susceptibility to amiodarone and miltefosine was evaluated by following up the proliferation of parasites in the absence or presence of these drugs, counting living parasites every day in a Neubauer chamber in triplicate cultures. Drugs were added to the growth medium 24 h after starting the cultures with 10^6 parasites/ml. At least three independent experiments were performed for each drug or drug combination.

Determination of FIC index and isobologram construction. For the fractional inhibitory concentration (FIC) and isobologram experiments, we used promas-

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tigotes and amastigote-infected macrophages, as previously reported (20). Briefly, *L. mexicana* promastigotes were maintained at 29°C in RPMI 1640 (Gibco) medium supplemented with 10% FBS, and the J774G8 mouse macrophages were maintained at 37°C and 5% CO_2 in RPMI 1640 (Gibco) medium supplemented with 10% FBS. For parasite infection, a proportion of 10 promastigotes to 1 macrophage was employed and the infected cells were incubated for 24 h at 37°C and 5% CO_2. To determine the effects of amiodarone and miltefosine on intracellular amastigotes, the percentage of infection was determined at 72 h posttreatment, under a light microscope, using Giemsa stain. At least three independent experiments were performed for each condition. Isobologram construction and calculation of the FIC were carried out as described by Hallender et al. (6). Briefly, FIC was determined as the combined concentration divided by the single concentration. The interaction index was calculated as $\sum$FIC = (MIC combination A/MIC alone A) + (MIC combination B/MIC alone B), where MIC is the MIC that produces total death of parasite population in the culture, with A representing the amiodarone concentration and B the miltefosine concentration.

$[Ca^{2+}]_i$ determinations. For the $[Ca^{2+}]_i$ measurements, the fluorescent ratiometric Ca^{2+} indicator Fura 2 was used, because its excitation spectrum depends on the concentration of the cation, while its emission peak remains invariable. Parasites were loaded with Fura 2 as previously described (20). Briefly, parasites (4×10^6 promastigotes/1.5 ml of culture medium) were centrifuged at $600 \times g$ for 2.5 min and then washed twice with the following loading buffer: 137 mM NaCl, 4 mM KCl, 1.5 mM KH_2PO_4, 8.5 mM Na_2HPO_4, 11 mM glucose, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 20 mM HEPES-NaOH, pH 7.4. The parasite pellet was resuspended in the same buffer, and a mixture of 6 μM Fura 2-AM and 2.4 mM probenecid was added. Parasites were then incubated at 29°C for 45 min under continuous agitation. Following two washes with the loading buffer without Fura 2-AM, fluorescence measurements were performed in a Perkin-Elmer LS-55 spectrofluorimeter provided with an acquisition system for excitation ratio measurements at 29°C with continuous agitation in a stirred cuvette. $[Ca^{2+}]_i$s were evaluated by applying the equation $[Ca^{2+}]_i = K_d \times (R - R_{min}/R_{max} - R) \times F_{min}(380)/F_{max}(380)$, where K_d is the dissociation constant of Fura 2 (244 nM) as reported by Grynkiewicz et al. (5); R is the fluorescent emission ratio obtained after excitation at 340 nm/380 nm; R_{max} and F_{max} are the ratio of excitation fluorescence at 340 nm/380 nm and the fluorescence of Fura 2 at 380 nm, respectively, under saturated Ca^{2+} concentrations; and R_{min} and F_{min} are the ratio of excitation fluorescence at 340 nm/380 nm and the fluorescence of Fura 2 at 380 nm, respectively, in the absence of Ca^{2+}. Maximum and minimum values were obtained after the addition of 30 μM digitonin, which allows the flow of Ca^{2+} to the interior of the cell. Then, 8 mM EGTA was added to chelate all remaining Ca^{2+} outside the cells (5).

In vivo studies. Drugs were tested in vivo using the murine model of leishmaniasis described previously (19) with minor modifications. Briefly, parasites were used and maintained by passage in hamsters every 8 weeks. Groups of 10 female BALB/c mice (6 weeks old, 25 g of weight) were inoculated (day 0) in the left hind footpad with 100 μl of phosphate-buffered saline (PBS) containing 1×10^6 amastigotes obtained from donor hamsters. One day after the inoculation, mice were randomly divided into 8 groups of 10 mice each and treatment was initiated 7 days after inoculation, when infection was well established and lesions were present. Each experimental group was treated daily with the drugs for 21 consecutive days, as follows: group 1 was treated with methylcellulose plus Tween 80 (0.2% [vol/vol]) used as vehicle, group 2 with glucantime at 100 mg/kg/day, group 3 with amiodarone at 25 mg/kg/day, group 4 with amiodarone at 50 mg/kg/day, group 5 with miltefosine at 10 mg/kg/day, group 6 with miltefosine at 20 mg/kg/day, group 7 with amiodarone at 25 mg/kg/day plus miltefosine at 10 mg/kg/day, and group 8 with amiodarone at 50 mg/kg/day plus miltefosine at 20 mg/kg/day. Glucantime was administered by the intraperitoneal route; all other drugs and the vehicle were administered orally through a stomach tube. Animals were observed 56 days after the end of treatment and then sacrificed to assess the parasite load in the infected footpad. Lesion growth was determined by measuring the diameter of both rear feet by direct reading with an electronic vernier scale. The size of the lesion in millimeters was calculated by subtracting the measurement of the uninfected foot from that of the infected foot. Measurements were made weekly and were continued for 84 days. For each experimental condition, the mean and standard error were calculated.

Parasite load determination. (i) Inoculation in cultures. Biopsy sections (of ~0.5 cm of diameter) from control and treated mice were placed in PBS, and after homogenization, 200 μl of the homogenate was added to 10 ml of culture medium supplemented with 10% FBS. Parasite growth was followed by microscopy for 3 weeks. Also, a smear impression from tissue sections was Giemsa stained and fixed with methanol to detect amastigotes or infected macrophages under light microscope.

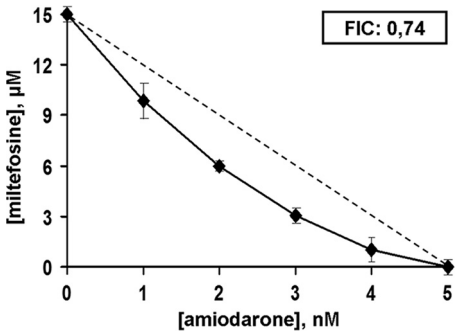

FIG. 1. Representative isobologram of in vitro interaction between amiodarone and miltefosine against *L. mexicana* promastigotes. The combined effect of amiodarone plus miltefosine upon *L. mexicana* promastigote viability is shown. FIC value was calculated as described in Materials and Methods. The dotted line corresponds to the predicted positions of the experimental points for a simple additive effect. This experiment was performed at least three times for each condition, and results are expressed as means ± standard errors.

(ii) PCR assay. DNA samples were prepared as previously described (16) and used in a novel *Leishmania* multiplex PCR assay based on a methodology previously described for these parasites (11). The assay combines the amplification of a 260-bp fragment from a DNA sequence specific for a *Leishmania* subgenus and a 900-bp fragment from the coding region of the β-tubulin gene. The PCR was conducted in a final volume of 50 μl and with the reaction mixture containing PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 80 ng DNA, 1.25 U Platinum *Taq* DNA, 2 mM $MgCl_2$, 2 mM each deoxynucleoside triphosphate (dNTP), and 50 pmol of each primer. The sequences of the *Leishmania* subgenus-specific oligonucleotide primers are as follows: F1, 5′-TGTGGGTGTGGGTGTGTGTGTATG-3′ (melting temperature [T_m],74°C); and B2, 5′-CGTGAAGAACATACAAAGCCTCCC-3′ (T_m, 72°C). The sequences of the β-tubulin primer set are as follows: Tub 1, 5′-ATGCGTGAGATCGTTTCC-3′ (T_m, 60°C); and Tub 2, 5′-GGCGGCCTGCATCAT-3′ (T_m, 62°C). The PCR was performed in an MJ Research PTC100 thermocycler, comprising 5 min of preincubation at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C, with a final extension for 5 to 10 min at 72°C. Each assay contained a positive control (0.10 μg parasite DNA) and a negative control (reaction mixture without DNA). PCR products were analyzed by electrophoresis on a 1% agarose gel with 1× TAE (4 mM Tris-acetate, 1 mM EDTA, pH 8).

RESULTS

Susceptibility of *Leishmania* to amiodarone and miltefosine. In this work, we studied the effect of amiodarone on promastigotes alone and in combination with miltefosine. As found previously (20), amiodarone produced a marked reduction in the viability of *L. mexicana* promastigotes, with a 50% inhibitory concentration (IC_{50}) of 900 nM and a MIC of 5 μM. On the other hand miltefosine induced a dose-dependent effect on the parasites' proliferation, with an IC_{50} of 8 μM, and a MIC of 15 μM, both results being similar to that reported by Soto and Berman (21). In order to test whether the efficacy of the drugs was enhanced when used in combination, we constructed an isobologram from the MICs obtained for the drugs used alone or in combination: the results are shown in Fig. 1, where a weak synergism can be observed (FIC, 0.74).

We also studied the effects of both drugs on the clinically relevant form of the parasite, using amastigote-infected macrophages. We obtained an IC_{50} of 8 nM and a MIC of 20 nM for amiodarone and an IC_{50} of 1 μM and a MIC of 6 μM for miltefosine. When the drugs were used in combination, the

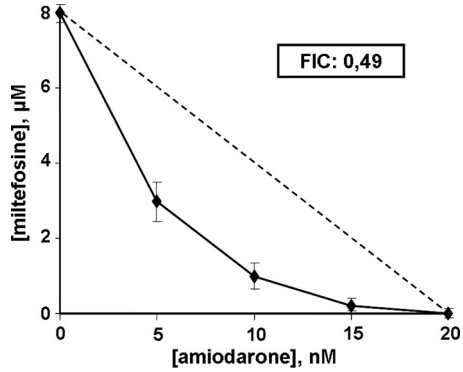

FIG. 2. Representative isobologram of in vitro interaction between amiodarone and miltefosine against intracellular *L. mexicana* amastigotes. The combined effect of amiodarone plus miltefosine upon *L. mexicana* amastigote viability is shown. The FIC value was calculated as described in Materials and Methods. The dotted line corresponds to the predicted positions of the experimental points for a simple additive effect. This experiment was performed at least three times for each condition, and results are expressed as means ± standard errors.

results indicated clear synergism, as shown by a concave isobologram and an FIC value of 0.49 (Fig. 2).

Effect of miltefosine on the $[Ca^{2+}]_i$ of *L. mexicana* promastigotes. *L. mexicana* promastigotes were initially loaded with the Ca^{2+} indicator Fura 2. Figure 3A shows that the addition of 2.5 μM (a sublethal concentration) miltefosine induced a marked increase in $[Ca^{2+}]_i$ in promastigotes, when the assay was performed in the presence of extracellular Ca^2. Under this condition, digitonin induced a further large increase in fluorescence (not shown), which is due to the permeabilization of the plasma membrane to Ca^{2+} and Fura 2. This result rules out the possibility that miltefosine induces an increase in Fura 2 permeability of the plasma membrane.

In order to study if the observed increase in the $[Ca^{2+}]_i$ was due to an influx of this cation from the extracellular milieu or if, instead, the cytoplasmic Ca^{2+} was elevated due to a release of the ion from intracellular compartments, we performed the same experiment, but in the absence of extracellular Ca^{2+} (in the presence of EGTA). Under this condition, miltefosine was unable to induce an increase in the $[Ca^{2+}]_i$ (Fig. 3B). This result demonstrated that the rise in the $[Ca^{2+}]_i$ level induced by miltefosine resulted from the entry of extracellular Ca^{2+}, probably through a Ca^{2+} channel at the plasma membrane of these parasites activated by the drug. Figure 4 shows a dose-response curve related to the effect of miltefosine on the $[Ca^{2+}]_i$. It can be seen that a plateau was reached with 2.5 μM of the drug. Concerning this effect of miltefosine on the $[Ca^{2+}]_i$, it has been demonstrated that, at least in one cancer cell line, namely, epidermoid carcinoma KB, miltefosine is also able to increase the $[Ca^{2+}]_i$ (7). Remarkably, the effect of miltefosine on the increase in the $[Ca^{2+}]_i$ observed in the present work for *L. mexicana* was at least 10 times more potent than in epidermoid carcinoma KB cells (25 μM). We have also tested the effect of miltefosine on other human cancer cell lines such as LoVo (colon cancer) and CarC (skin cancer), showing that miltefosine is also able to increase the $[Ca^{2+}]_i$ in these cells, but only using 50 μM of the drug, while 2.5 μM had no effects (data not shown).

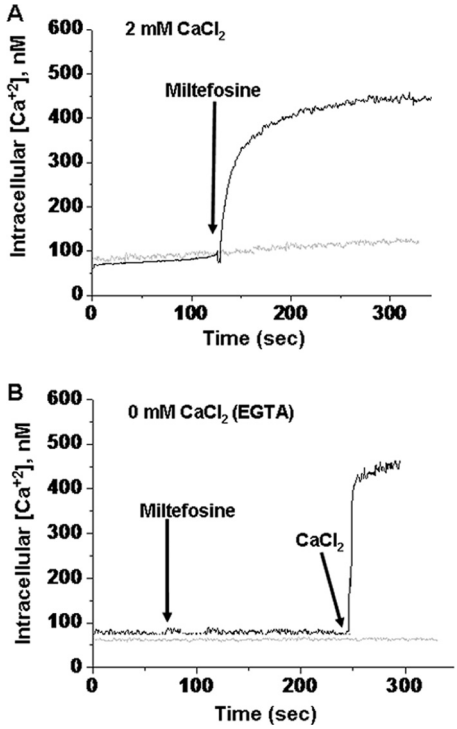

FIG. 3. Effect of miltefosine on the $[Ca^{2+}]_i$ of *L. mexicana* promastigotes. Populations of *L. mexicana* promastigotes (4×10^6 cells in 1.5 ml) were loaded with Fura 2 (using 6 μM Fura 2-AM) in a loading buffer, and $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. (A) Effect of 2.5 μM miltefosine on the parasite cytoplasmic Ca^{2+} concentration in the presence of 2 mM Ca^{2+}. The gray line represents the same experiment without miltefosine. (B) Effect of 2.5 μM miltefosine on promastigotes loaded with Fura 2 in the absence of external Ca^{2+} (1 mM EGTA). The gray line represents the same experiment without any addition.

Effect of amiodarone and miltefosine on the course of infection in BALB/c mice. We analyzed the anti-*Leishmania* efficacy of amiodarone and miltefosine, used alone or in combination, in a murine model of cutaneous leishmaniasis. BALB/c mice were infected with *L. mexicana* amastigotes on the footpad and

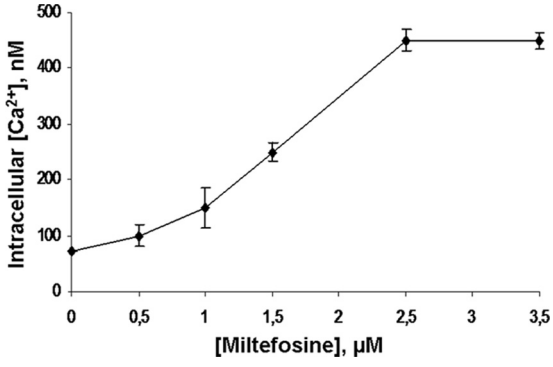

FIG. 4. Dose-response curve of the effect of miltefosine on the $[Ca^{2+}]_i$ of *L. mexicana* promastigotes. Populations of *L. mexicana* promastigotes (4×10^6 cells in 1.5 ml) were loaded with Fura 2 (using 6 μM Fura 2-AM) in a loading buffer, and $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. Each point represent the mean ± standard error of at least three independent experiments.

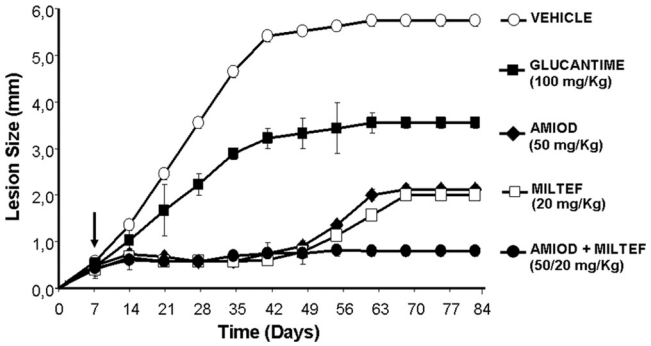

FIG. 5. Lesion evolution of mice infected with *L. mexicana* amastigotes, treated with amiodarone and miltefosine (alone or in combination). Shown are weekly measurements of lesion size (mm) versus time in days of mice infected with *L. mexicana* amastigotes and treated with amiodarone, miltefosine, glucantime, or different combinations of amiodarone plus miltefosine. Notice that the combination of amiodarone at 50 mg/kg/day plus 20 mg/kg/day miltefosine avoids the evolution of lesions. The arrow indicates the beginning of treatment.

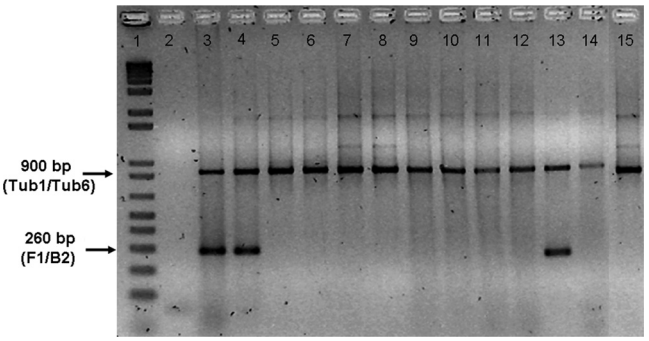

FIG. 6. Parasitological evaluation of infected BALB/c mice treated with amiodarone plus miltefosine by a *Leishmania* multiplex PCR assay. PCRs were performed as described in Materials and Methods. Lane 1, molecular weight marker (1 Kb Plus; Invitrogen); lane 2, PCR mixture without DNA (negative control); lane 3, *L. mexicana* genomic DNA (positive control); lane 4, untreated lesion DNA from infected mouse footpad; lanes 5 to 14, DNA from lesions of infected mouse footpads treated with 50 mg/kg/day amiodarone plus 20 mg/kg/day miltefosine for 49 days; lane 15, genomic DNA from a healthy footpad. Arrows indicate the β-tubulin amplification products (900 bp) and the specific fragment from *L. mexicana* amplified with the F1 and B2 primers (260 bp). The absence of a 260-bp band indicates the absence of *Leishmania* in the lesion.

then treated with amiodarone or miltefosine alone or in combination, according to the protocol described in Materials and Methods. Treatment evaluation showed that amiodarone at 50 mg/kg/day or miltefosine at 20 mg/kg/day given orally was more effective than glucantime (100 mg/kg/day intraperitoneal), fully preventing the development of lesions during the course of the treatment (Fig. 5). However, when the pressure of any of the drugs disappeared, a relapse of the infection was observed, as suggested by a sustained increase of the lesion size, indicating therapeutic failure (Fig. 5). In contrast, mice treated with the drug combination (amiodarone plus miltefosine at 50 and 20 mg/kg/day, respectively) showed a permanent control of the lesion size. The presence of parasites on infected mice after treatment was evaluated at day 84, using biopsy samples from the lesions and a novel multiplex PCR assay developed during this work (Table 1). All control (untreated) infected mice showed positive results by this method. Miltefosine was the most effective drug when given alone, with 50 to 60% of parasitological cures, depending on the drug concentration. Interestingly, the combination treatment at low doses of the individual drugs (amiodarone at 25 mg/kg/day plus miltefosine at 10 mg/kg/day) led to high (70%) values of apparent parasite elimination in comparison with the effects of the drugs given alone (Table 1), while the drug combination at higher concentrations (50 mg/kg/day of amiodarone plus 20 mg/kg/day of miltefosine) led to parasitological cure in 90% of the infected animals (Table 1 and Fig. 6).

TABLE 1. Conditions and healing tests applied to *L. mexicana*-infected mice treated with amiodarone and miltefosine[a]

Group (n = 10)	Treatment group	Dose of amiodarone or miltefosine (mg/kg body wt)	No. of mice negative/total by:		
			Culture	Giemsa staining	PCR
1	Drug vehicle	None	0/10	0/10	0/10
2	Glucantime	100	6/10	3/10	2/10
3	Amiodarone	25	2/10	0/10	0/10
4	Amiodarone	50	5/10	2/10	2/10
5	Miltefosine	10	8/10	7/10	5/10
6	Miltefosine	20	8/10	7/10	6/10
7	Amiodarone + miltefosine	25/10 (amiodarone/miltefosine)	10/10	10/10	7/10
8	Amiodarone + miltefosine	50/20 (amiodarone/miltefosine)	10/10	10/10	9/10

[a] Female BALB/c mice (body weight, 25 g) were infected with *L. mexicana* amastigotes (1×10^6). Drug treatment was started 7 days later, at the doses and frequencies indicated, followed by culture of lesion homogenates as described in Materials and Methods.

DISCUSSION

Despite its registration as an anti-*Leishmania* drug in several countries, the mechanism of action of miltefosine is not yet fully understood. Taking into account that this drug is able to induce an increase in the $[Ca^{2+}]_i$ in some cancer cell lines and considering the well-known relevance of calcium homeostasis in *Leishmania* spp. (1), in this work we investigated the possible effect of miltefosine on Ca^{2+} regulation in these parasites and found that the drug generates a large and rapid increase in $[Ca^{2+}]_i$ in *L. mexicana* promastigotes, a previously unreported finding that could explain the synergistic antiparasitic effects of the drug when used in combination with amiodarone. The specific increment in the $[Ca^{2+}]_i$ induced by miltefosine is probably due to the activation of a not-yet-characterized plasma membrane Ca^{2+} channel. Concerning this point, there are some reports related to the existence of Ca^{2+} channels in *Leishmania* spp. Thus, an increase in the cytosolic Ca^{2+} levels through the activation of nonselective cation channel induced by oxidative stress in *L. donovani* has been demonstrated (12), while Mehta and Shaha (10) demonstrated that some metalloids induced death in *L. donovani* promastigotes through activation of a T-type Ca^{2+} channel. Interestingly, Verma and Dey (22) concluded that miltefosine induces apoptosis-like death in *L. donovani*, a process that is associated with altered

Ca^{2+} homeostasis (3). Thus, it is conceivable that the disruption of Ca^{2+} homeostasis generated by miltefosine in *L. mexicana* could induce apoptotic-like phenomena seriously affecting the parasite's viability.

Based on these and previous findings (20), we evaluated the effect of the combination of miltefosine and amiodarone on the proliferation of *L. mexicana* in vitro and in vivo. Drug interactions were assessed through the construction of isobolograms for the in vitro effects on both promastigote and amastigote forms of the parasite. It was interesting to note that while the FIC value obtained for the promastigotes showed only a weak synergism, the FIC value of 0.49 obtained for intracellular amastigotes, the clinically relevant forms, indicated clear synergism. It has been previously demonstrated that miltefosine acts synergistically with other drugs used in combination on *Leishmania*, such as sodium stibogluconate (18), but the synergy observed with this combination in intracellular amastigotes was relatively low (FIC, 0.75). The studies on infected mice obtained in the present work showed a marked control of the lesion size during treatment with any of the drugs given alone (at 50 mg/kg/day/day for amiodarone or 20 mg/kg/day/day for miltefosine, given for 21 days), but the effects were only suppressive (leishmanistatic), as a clear relapse of the infections was observed when the drug pressure stopped. In contrast, the combined administration of the drug combination at the high dose levels led to curative (leishmanicidal) action, since no relapse of the lesion after drug withdrawal was observed and a high percentage of parasitological cures (90%) were verified by PCR. The level of cures obtained in this study with the combination of amiodarone plus miltefosine was higher than that (80%) reported for the treatment with amiodarone (50 mg/kg/day) plus posaconazole (20 mg/kg/day) in NMRI mice infected with *T. cruzi* (2).

In this work, we also developed a novel multiplex PCR assay using as targets the coding region of the β-tubulin gene, which is a highly conserved DNA sequence, and a novel *Leishmania* DNA sequence, L280, specific for *Leishmania* species, such as *L. mexicana*. The assay is straightforward and the sensitivity is, on average, 100 pg DNA, as determined by serial dilution experiments. The method proved to be very useful for tracking the cure of infected animals upon treatment with the different drugs used in this study and should be easily applicable in future investigations of experimental chemotherapy of leishmaniasis.

In closing, it is worthwhile to mention the results of a recent study on alternative therapies for human leishmaniasis treatment in which a patient with *L. mexicana* and *T. cruzi* mixed infection was treated with amiodarone (14). The amiodarone treatment (1,600 mg/day for the first 4 days and 800 mg/day for the next 21 days) led to the clinical cure of a borderline diffuse leishmaniasis in this patient (14). Based on the demonstration of the intrinsic activity of amiodarone against trypanosomatid parasites in experimental animals and human patients, it is possible that this drug could be used in combination with bonafide antiparasitic drugs to combat these infections at low doses, thus reducing its known adverse side effects such as cardiotoxicity, thyroid dysfunction, and pulmonary fibrosis (13). Accordingly, the present study represents the first evidence that the combination amiodarone plus miltefosine acts synergistically in controlling *L. mexicana* parasitic infections in vivo.

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