Investigation of Calcium Channel Blockers as Antiprotozoal Agents and Their Interference in the Metabolism of Leishmania (L.) infantum

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Received 28 October 2015; Accepted 24 December 2015

Academic Editor: Juntra Karbwang

Leishmaniasis and Chagas disease are neglected parasitic diseases endemic in developing countries; efforts to find new therapies remain a priority. Calcium channel blockers (CCBs) are drugs in clinical use for hypertension and other heart pathologies. Based on previous reports about the antileishmanial activity of dihydropyridine-CCBs, this work aimed to investigate whether the in vitro anti-Leishmania infantum and anti-Trypanosoma cruzi activities of this therapeutic class would be shared by other non-dihydropyridine-CCBs. Except for amrinone, our results demonstrated antiprotozoal activity for fendiline, mibefradil, and lidoflazine, with IC50 values in a range between 2 and 16 μM and Selectivity Index between 4 and 10. Fendiline demonstrated depolarization of mitochondrial membrane potential, with increased reactive oxygen species production in amlopidine and fendiline treated Leishmania, but without plasma membrane disruption. Finally, in vitro combinations of amphotericin B, miltefosine, and pentamidine against L. infantum showed in isobolograms an additive interaction when these drugs were combined with fendiline, resulting in overall mean sum of fractional inhibitory concentrations between 0.99 and 1.10. These data demonstrated that non-dihydropyridine-CCBs present antiprotozoal activity and could be useful candidates for future in vivo efficacy studies against Leishmaniasis and Chagas’ disease.

1. Introduction

Leishmaniasis is a neglected infectious disease caused by several different species of protozoan parasites of the genus Leishmania. It affects 12 million people in 98 countries and territories and is mainly associated with poverty in developing nations. Current strategies to control this disease are mainly based on chemotherapy. Despite being available for the last 100 years, the chemotherapy is based on the use of few drugs, including the antimonial derivatives. High costs of treatment, elevated toxicity, parenteral administration, and the emergence of resistance are the main drawbacks [1]. Considering the few therapeutic options and lack of interest from private sector, the need for novel drugs is evident [2].

Calcium channel blockers (CCBs) are a class of drugs that act by selective inhibition of calcium influx through cellular membranes. They are among the most widely used drugs in cardiovascular medicine with roles not only in hypertension but also in angina and (for some CCBs) tachyarrhythmias [3]. Although often considered as a single class, CCBs can be subdivided into the following groups depending on chemical structure: dihydropyridines (e.g., nifedipine, nimodipine, and amlopidine), the benzothiazepines (e.g., diltiazem), and phenylalkylamines (e.g., verapamil) [4].
Dihydropyridines have been considered promising antiparasitic hits, especially against protozoan parasites. The in vivo oral efficacy of amlodipine and lacidipine has been shown in the treatment of Leishmania (L.) donovani infected mice [5]. Additionally, the in vitro antiparasitic activity of eight clinically used dihydropyridines (azelnidipine, amlodipine, cilnidipine, lercanidipine, nicardipine, nifedipine, nimodipine, and nitrrendipine) was demonstrated against a panel of Leishmania species and Trypanosoma cruzi [6, 7].

Based on previous reports about the antileishmanial activity of dihydropyridines, this work investigated the antiparasitic potential of other non-dihydropyridine-CCBs. For this, the in vitro activity of four non-dihydropyridine agents (amrinone, fendiline, mibebradil, and lidoflazine) was tested against different Leishmania species and their cytotoxicity to mammalian cells was evaluated. We also investigated the mechanism of action (MoA) involved with the antileishmanial activity of fendiline and the in vitro effect of its combination with antileishmanial standard drugs.

2. Material and Methods

2.1. Drugs and Chemicals. Dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, thiazol blue), sodium dodecyl sulfate (SDS), RPMI-1640 medium, and M199 medium were purchased from Sigma (St. Louis, MO, USA). Amrinone, fendiline, lidoflazine, and mibebradil were purchased from Sigma (St. Louis, MO, USA). Pentamidine was from Sideron. Pentavalent antimony (Glucantime®) and amrinone were kindly donated by Bayer (Brazil). Other analytical reagents were purchased from Sigma unless otherwise stated.

2.2. Experimental Animals. Golden hamsters and BALB/c mice were supplied by the animal breeding facility at the Adolfo Lutz Institute of São Paulo. They were maintained in sterilized cages under a controlled environment and received water and food ad libitum. Golden hamsters were infected each month with amastigotes from the spleen to maintain the strain of L. (L.) infantum. BALB/c mice were used for obtaining peritoneal macrophages. Animal procedures were performed with the approval of the Research Ethics Commission, in agreement with the Guidelines for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.3. Parasites and Macrophages. Promastigotes of L. (L.) amazonensis (WHO/BR/00/LT0016), L. (V.) braziliensis (MHO/BR/75/M2903), and L. (L.) infantum (MHOM/BR/1972/LD) [synonymous with L. (L.) chagasi] were maintained in M199 medium supplemented with 10% calf serum and 0.25% hemin at 24°C. L. (L.) infantum amastigotes were obtained from the spleen of infected hamster by differential centrifugation at the 60–70th days after infection. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing with RPMI-1640 medium supplemented with 10% fetal calf serum and were maintained at 37°C in a 5% CO₂ humidified incubator. Trypanosoma cruzi trypomastigotes (Y strain) were maintained in LLC-MK2 (ATCC CCL 7) cells using RPMI-1640 medium supplemented with 2% calf serum at 37°C in a 5% CO₂ humidified incubator.

2.4. Determination of the In Vitro Antileishmanial Activity. To determine the 50% inhibitory concentration (IC50) against Leishmania promastigotes, the drugs were dissolved in DMSO and diluted with M199 medium in 96-well microplates, with 100 μM as the highest concentration. Each drug was tested twice at eight concentrations prepared in twofold dilutions. Promastigotes were counted in a Neubauer hemocytometer and seeded at 1 × 10⁶ parasites/well with a final volume of 150 μL. Controls with DMSO and without drugs were performed. Pentamidine was used as a standard drug. The plate was incubated for 24 hours at 24°C and the viability of promastigotes was verified by the MTT assay. Briefly, MTT (5 mg/mL) was dissolved in PBS, sterilized through 0.22 μm membrane and 20 μL/well was added, for 4 hours at 24°C. Promastigotes were incubated without compounds and used as a viability control. Formazan extraction was performed using 10% SDS for 18 hours (80°C) at 24°C, and the optical density was determined in a Multiskan MS (UNISCIENCE) plate reader at 550 nm. The 100% viability was expressed based on the optical density of control promastigotes, after normalization. To determine the IC50 value against L. (L.) infantum intracellular amastigotes, peritoneal macrophages were collected from the peritoneal cavity of BALB/c as described above, and added to 16-well chamber slides (Lab-Tek, NUNC®) at 5 × 10⁴ cells/well. Plates were incubated at 37°C in a 5% CO₂ humidified incubator for 24 hours. L. (L.) infantum amastigotes extracted from spleens and separated by differential centrifugation were added to macrophages at a ratio of 10:1 (amastigotes : macrophage). After 24 hours, extracellular parasites were removed by washing, fresh medium containing the drugs and controls was added, and the cells were incubated at 37°C for a period of 120 hours. Further medium changes with fresh drugs were carried out after 72 hours. At the end of the assay, the slides were stained with Giemsa and observed using light microscopy. Glucantime was used as a standard drug. The IC50 was determined by the number of infected macrophages in 400 cells.

2.5. Determination of the Antitrypanosomal Activity. To determine the IC50 against T. cruzi trypomastigotes, drugs were dissolved in DMSO and diluted with RPMI-1406 medium in 96-well microplates, with the highest concentration at 100 μM. Trypanomastigotes obtained from LLC-MK2 cultures were counted in a Neubauer hemocytometer and seeded at 1 × 10⁶ parasites/well in 96-well microplates. Test drugs were incubated for 24 hours at 37°C in a 5% CO₂ humidified incubator and the viability of trypanomastigotes was verified by the MTT assay as described above. Benznidazole was used as a standard drug.

2.6. Cytotoxicity in Mammalian Cells. LLC-MK2 were seeded at 5 × 10⁴ cells/well in 96-well microplates and incubated with
2.7. Investigation of Mitochondrial Membrane Potential. L. (L.) infantum promastigotes were washed with PBS and deposited on a 96-well microplate (2 × 10^6 cells/well) and incubated with amiodopine or fendiline (20 μM) for 60 minutes at 24°C. Then MitoTracker® Red CM-H₂XROS (500 nM) was added and the incubation was continued for 40 minutes in the dark. Cells were washed twice with HBSS (Hanks Balanced Salt Solution) and the fluorescence was measured using a fluorimetric microplate reader (FilterMax F5 Multi-Mode Microplate Reader-Molecular Devices) with excitation and emission wavelengths of 540 and 595 nm, respectively [8]. Nitazoxanide (60 μg/mL) was used as a positive control [9] and untreated promastigotes were used as negative control.

2.8. Analysis of Reactive Oxygen Species (ROS). L. (L.) infantum promastigotes (2 × 10^6 cells/well) were washed in HBSS medium and incubated with amiodopine or fendiline (20 μM) for 60 minutes at 24°C. To these cells 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was added (5 μM) and incubation was prolonged for 15 minutes. Then the fluorescence was measured using a fluorimetric microplate reader (FilterMax F5 Multi-Mode Microplate Reader-Molecular Devices) with excitation and emission wavelengths of 485 and 520 nm, respectively. Nitazoxanide (60 μg/mL) was used as positive control [9] and untreated promastigotes were used as negative control.

2.9. Evaluation of the Permeability of the Cell Membrane. L. (L.) infantum promastigotes were washed with PBS, deposited on a 96-well microplate (2 × 10^6 parasites/well) and incubated with SYTOX® Green (1 μM) for 15 minutes at 24°C [10]. Amdlopine and fendiline were added at 20 μM and the fluorescence was measured up to 60 minutes. The fluorescence intensity was determined using a fluorimetric microplate reader (FilterMax F5 Multi-Mode Microplate Reader-Molecular Devices) with excitation and emission wavelengths of 485 and 520 nm, respectively. Triton X-100 (0.1%) was used as positive control and untreated promastigotes were used as negative control.

2.10. Determination of Drug Interactions. The interactions between drugs were in vitro evaluated by modified isobologram method [11, 12]. Fendiline was in vitro combined with amphotericin B, miltefosine, and pentamidine. IC₅₀ values of individual drugs were obtained against L. (L.) infantum promastigotes as described above. These values were used to determine the maximum concentrations of each drug in the combination assay, assuring the IC₅₀ in the fourth point of the serial dilution [11]. The highest concentrations of the solutions were prepared in proportions of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of fendiline and partner drug, respectively, which were serially diluted to the seventh well of the microplate in two intercalated serial dilutions (base 2).
| Drug      | L. (L.) infantum promastigotes | L. (L.) infantum amastigotes | L. (L.) amazonensis promastigotes | L. (V.) braziliensis promastigotes | T. cruzi trypomastigotes | LLC-MK2 cytotoxicity |
|-----------|-------------------------------|-------------------------------|----------------------------------|-----------------------------------|-------------------------|----------------------|
| Amrinone  | neb                          | neb                          | neb                              | neb                              | neb                     | >500                 |
| Fendiline | 16.15 ± 4.20                  | 12.20 ± 1.74                 | 8.66 ± 1.27                      | 9.15 ± 0.78                      | 12.13 ± 2.97            | 49.85 ± 8.16         |
| Lidoflazine| 17.67 ± 0.93                  | 16.29 ± 4.45                 | 11.54 ± 1.49                     | 14.48 ± 1.08                     | 10.39 ± 1.87            | 106.54 ± 57.99       |
| Mibebradil| 3.60 ± 0.11                   | neb                          | 2.23 ± 0.42                      | 2.75 ± 0.39                      | 2.99 ± 0.43             | 11.96 ± 1.03         |
| Pentamidine| 3.60 ± 0.12                   | neb                          | 1.14 ± 0.15                      | 0.69 ± 0.04                      | ndf                     | 23.48 ± 3.33         |
| Glucantime| ndf                          | 30.15 ± 1.18                 | ndf                              | ndf                              | ndf                     | >500                 |
| Benzimidazole| ndf                          | ndf                          | ndf                              | ndf                              | 440.18 ± 39.14         | >500                 |

aIC50: 50% inhibitory concentration ± standard deviation (SD).
bne: not effective.
cnd: not determined.
dConcentrations for Glucantime are expressed as µg/mL, as the molecular weight is unknown.

Table 2: Selectivity Index (SI) of CCBs, given by the ratio between the cytotoxicity to LLC-MK2 cells and the antiparasitic activity.

| Drug      | L. (L.) infantum amastigotes | T. cruzi trypomastigotes |
|-----------|-------------------------------|-------------------------|
| Amrinone  | nd                           | nd                      |
| Fendiline | 4.09                         | 4.11                    |
| Lidoflazine| 6.54                         | 10.25                   |
| Mibebradil| ndf                          | 4.01                    |

nd: not determined.

3.2. Action of Amlodipine and Fendiline in the Mitochondrial Membrane Potential. The effect of amloidine and fendiline on the L. (L.) infantum mitochondrial membrane potential was evaluated in promastigotes using the fluorescent dye MitoTracker Red. Amlodipine and fendiline significantly (p < 0.05) affected the mitochondrial membrane potential of L. (L.) infantum, reducing the fluorescence levels by 7 and 18%, respectively, relative to untreated parasites. Nitazoxanide was used as positive control and resulted in a strong reduction of fluorescence intensity (Figure 1).

3.3. Generation of Reactive Oxygen Species (ROS) upon Treatment with Amlodipine or Fendiline. The regulation of ROS levels was examined using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) after incubation with the drugs. Amlodipine and fendiline promoted an intense and significant (p < 0.05) upregulation of ROS content when compared to untreated parasites (Figure 2). Amlodipine induced almost 3-fold higher ROS levels in L. (L.) infantum when compared to fendiline. Nitazoxanide was used as positive control and resulted in significant upregulation of ROS relative to untreated parasites, as previously reported [9]. Amlodipine presented 1.99-fold higher upregulation of ROS than nitazoxanide (Figure 2).

3.4. Effect of Amlodipine and Fendiline on Plasma Membrane Permeability of L. (L.) infantum. In order to evaluate if the mitochondrial dysfunction was a result of any alteration in
The interactions between fendiline and three antileishmanial standard drugs (amphotericin B, miltefosine, and pentamidine) were investigated in L. (L.) infantum promastigotes, demonstrating a wide range of promising biological activities, among them, in vitro experimental efficacy against Ebola virus [17], in vitro activity against filovirus [18], and also against protozoan parasites as Leishmania [12], T. cruzi [7, 19–21], and Plasmodium [22, 23].

In the present work, the in vitro activity of fendiline and lidoflazine against Leishmania spp. promastigotes, T. cruzi trypomastigotes, and L. (L.) infantum intracellular amastigotes was demonstrated. Mibebradil also presented activity against Leishmania spp. promastigotes and T. cruzi trypomastigotes but was ineffective against intracellular amastigotes, probably due to metabolic differences between the extracellular and intracellular parasites or even to poor penetration of the drug into the host cells. This is the first report of antiprotozoal activity of these compounds which are non-dihydropyridine-CCBs. In a previous work, the dihydropyridines isradipine and lacidipine demonstrated in vitro activity against T. cruzi epimastigotes, with IC$_{50}$ values of 20 and 33 µM, respectively [20] showing a similar activity to fendiline and lidoflazine against T. cruzi trypomastigotes in the present study.

Otherwise, amrinone, a positive inotropic cardiotonic with vasodilator properties, showed lack of activity against Leishmania spp. and T. cruzi. This result suggests that the antiparasitic activity of CCBs is rather ascribed to the chemical structure of individual compounds than to the CCB properties. Conversely, another closely related compound used as a potassium channel blocker, 4-aminopyridine, exhibited activity against L. (L.) amazonensis, with an IC$_{50}$ value of 46 µM, but also lacked activity against L. (L.) major (IC$_{50}$ > 400 µM) [24].

The mode of action of CCBs involves the blockage of calcium ions movement through calcium channels [3]. Calcium ions play an important role in regulation of many vital functions. By penetrating the cell, they activate bioenergetic processes, as the transformation of ATP into cyclic AMP and protein phosphorylation. In high concentrations, calcium ions cause different destructive changes [25].

### Table 3: Effect of combination of fendiline and antileishmanial standard drugs in L. (L.) infantum promastigotes.

| Combination                           | $x \sum \text{FIC}_{50}$ | $x \sum \text{FIC}_{90}$ |
|---------------------------------------|---------------------------|---------------------------|
| Fendiline + amphotericin B            | 1.18                      | 0.99                      |
| Fendiline + miltefosine               | 1.30                      | 1.02                      |
| Fendiline + pentamidine               | 1.22                      | 1.10                      |

*Overall mean sum.*
The presence of a voltage gated calcium channel sharing several characteristics with the human counterpart has been recently demonstrated in the plasma membrane of *Leishmania* [26]; then interference of CCBs in calcium channels of treated parasites cannot be ruled out.

In a previously published work, nimodipine, a dihydropyridine, has exhibited *in vitro* activity against *L. (L.) infantum*, causing extensive mitochondrial damage in treated parasites, as observed by transmission electron microscopy [6]. In our study, fendiline demonstrated the higher potency against *Leishmania* amastigotes and it was selected for the investigation of the mechanism of action and drug combination assays. For comparisons, amlodipine, a dihydropyridine-CCB, with previously reported activity against *Leishmania* parasites [7], was included in the assays. Amlodipine presented IC$_{50}$ values against *Leishmania* spp. close to mibefradil, but about 2- to 3-fold higher potency against *L. (L.) infantum* intracellular amastigotes than fendiline and lidoflazine.
Our data demonstrated that *Leishmania* promastigotes treated with amloidipine and fendiline exhibited reduced ability to concentrate the dye (MitoTracker Red), indicating a collapse of the mitochondrial membrane potential. This result is an indication that the energy-coupling system in the mitochondria is most likely inactivated, leading to parasite death. Another study demonstrated that *Leishmania* promastigotes treated with nimodipine also resulted in strong mitochondrial damage within 60 min incubation [7].

There is inherent relationship between ROS generation and respiratory chain in both mammals and *Leishmania*. The mitochondrial complex III was described as the main source of superoxide anion radicals [27]. Due to the observed effect of fendiline in the mitochondrial membrane potential, we also investigated the ROS levels of parasites treated with fendiline and amloidipine. We observed that depolarization in mitochondrial membrane potential was accompanied by an increase in ROS production when parasites were treated with both drugs. The single mitochondrion is one of the major sources of ROS in trypanosomatids, even under physiological conditions. These reactive species could play different roles in the parasites, involving signaling or cytotoxicity; to control the ROS levels trypanosomatids present mitochondrial antioxidant defenses [28]. The upregulation of ROS in *Leishmania* induced by fendiline and amloidipine might have contributed to a cellular toxicity, leading to an oxidative stress and parasite death.

In order to evaluate whether the fast and strong mitochondrial damage could be ascribed to the ability of amloidipine and fendiline to alter the plasma membrane of *Leishmania*, we investigated the permeability using the fluorescent probe SYTOX Green. Previous ultrastructural observation of nimodipine treated promastigotes revealed plasma membrane blebbing, although no pore forming activity could be observed [6]. In the present work, we observed that treatment with amloidipine and fendiline resulted in lack of significant changes in fluorescence intensity up to 60-minute incubation, suggesting no alteration in permeability levels. Parasites treated with Triton X-100 (positive control) showed early and increased penetration of the dye SYTOX Green and are indicative of membrane rupture.

The effects of several drugs that interfere directly with mitochondrial physiology in parasites such as *Leishmania* have been described. The unique mitochondrial features of *Leishmania* make this organelle an ideal drug target [29]. Taken together, our results demonstrate that amloidipine and fendiline exert their antileishmanial effect on *Leishmania* promastigotes due to the disruption in the mitochondrial function and to the generation of ROS.

Considering the need for new, potent, and safe treatments for Leishmaniasis, the use of monotherapy may not be the ideal future. Drug combinations are used to prevent resistance and increase safety of treatments. It has been widely studied for cancer [30], malaria, and also Leishmaniasis [31, 32]. Previous report demonstrated combinations of four dihydropyridine-CCBs (amlodipine, lercanidipine, nicardipine, and nimodipine) with antileishmanial drugs [12]. Here, we report the combination of a non-dihydropyridine drug, fendiline, with three antileishmanial clinically used drugs.

Drugs given in combination may produce effects that are similar to, higher or smaller than the effect predicted from their individual potencies [33]. Here, we observed that the effect of fendiline associated with amphotericin B, miltefosine, or pentamidine was equivalent, that is, equally effective when each drug was given separately, according to the $x \sum \text{FIC}$ and the isobologram graphic analysis. This behavior was similar to what was previously observed when using dihydropyridines and could be an indication that both dihydropyridine and non-dihydropyridine agents possess similar mode of action on *Leishmania*. However, additional studies are required to confirm this hypothesis.

5. Conclusions

The results of this work extend the investigation of CCBs as antiprotozoal agents and indicate that its leishmanicidal activity is related to mitochondria dysfunction and ROS generation. The combination of any of the drugs used did not show synergistic effects. On the contrary, all the isobolograms indicated indifferent/additive interaction. However, the drug combination assays indicated that the effect of fendiline plus amphotericin B, miltefosine, or pentamidine could be evaluated in future in animal models, since no in vitro antagonism was observed in any combination. Earlier, further assays must be conducted in order to verify the efficacy of fendiline in the treatment of *Leishmania* infected animals.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by São Paulo Research Foundation (FAPESP 2012/18756-1). The authors also acknowledge the CNPq scientific research award given to Andre Gustavo Temponi and the scholarships given to Juliana Tonini Mesquita (CNPq project number 471458/2012-0 and 132336/2011-1) and to Juliana Quero Reimão (FAPESP 08/11434-3 and 2011/21970-2).

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