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ORIGINAL PAPER

Antileishmanial activity and ultrastructural alterations of *Leishmania* (L.) *chagasi* treated with the calcium channel blocker nimodipine

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Abstract In a search for novel antileishmanial drugs, we investigated the activity of the calcium channel blocker nimodipine against *Leishmania* spp. and explored the ultrastructural damages of parasites induced by nimodipine after a short period of incubation. Nimodipine was highly effective against promastigotes and intracellular amastigotes of *Leishmania* (L.) *chagasi*, with 50% inhibitory concentration values of 81.2 and 21.5 μM, respectively. Nimodipine was about fourfold more effective than the standard pentavalent antimony against amastigotes and showed a Selectivity Index of 4.4 considering its mammalian cells toxicity. *Leishmania* (L.) *amazonensis* and *Leishmania* (L.) *major* promastigotes were also susceptible to nimodipine in a range concentration between 31 and 128 μM. Ultrastructural studies of *L.* (L.) *chagasi* revealed intense mitochondria damage and plasma membrane blebbing, resulting in a leishmanicidal effect as demonstrated by the lack of mitochondrial oxidative metabolism. The amastigote-killing effect suggests other mechanism than macrophage activation, as no upregulation of nitric oxide was seen. This calcium channel blocker is an effective in vitro antileishmanial compound and if adequately studied could be used as a novel drug candidate or as a novel drug lead compound for drug design studies against leishmaniasis.

Introduction

Neglected parasitic diseases affect millions of people in developing countries and protozoan parasites are among the most important pathogens, causing a high morbidity and mortality. Leishmaniasis affects 12 million people worldwide and was responsible for almost 59,000 deaths in 2003 (Davies et al. 2003) The visceral form, with more than 500,000 cases, is a fatal disease and is caused by *Leishmania* (L.) *chagasi* in Latin America and by *Leishmania donovani* in India. The therapy of leishmaniasis is particularly difficult, with a restricted therapeutic arsenal, very toxic drugs, and resistance cases especially in India (Santos et al. 2008; Croft et al. 2006). Despite the introduction of the oral anticancer drug Miltefosine in the clinical therapy in India, pentavalent antimonials remain the first line drugs for the New World's disease. Miltefosine is still under investigation, but it fails to treat the American cutaneous species (Soto and Berman 2006). Chemotherapeutic switching or "piggy-back therapy" has been one of the most important methods for the study and clinical introduction of novel drugs for leishmaniasis. This approach can deliver new drugs more quickly and at lower cost as much of the development work has already been done (Croft 2005). Some examples include the use of Miltefosine, amphotericin B, antifungal azoles for leishmaniasis.

Nimodipine (Fig. 1) is a 1,4-dihydropyridine calcium channel blocker that acts by relaxing the arterial smooth muscle (Blardi et al. 2002). Able to cross the blood-brain barrier, nimodipine can dilate the cerebral arterioles (Haws et al. 1983); thus, it is currently used to prevent and treat the ischemic damage caused by cerebral arterial spasm in subarachnoid hemorrhage (Allen et al. 1983). Nimodipine has also been used in other cerebrovascular disorders, such as ischemic stroke (Langley and Sorkin 1989) and multi-infarct dementia (Pantoni et al. 2000). Dihydropyridines have been presenting promising antimicrobial and antiparasitic activities. Dasgupta and coworkers (Dasgupta et al. 2007) demonstrated that lacidipine, a dihydropyridine, inhibited Gram-positive and negative bacteria in a range

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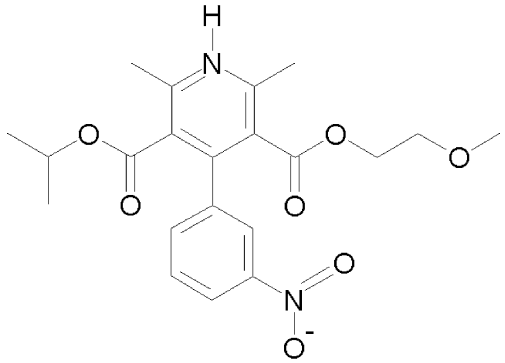

Fig. 1 Chemical structure of nimodipine

concentration of 50–200 μg/mL. Antibacterial activity was also demonstrated for amlodipine (Kumar et al. 2003). Protozoan parasites as *Trypanosoma cruzi* were also susceptible to calcium channel blockers, as demonstrated by a significant activity of isradipine and lacidipine (Núñez-Vergara et al. 1998). Despite no antileishmanial activity, Misra and coworkers (Misra et al. 1991) demonstrated the inhibition effect of nifedipine and verapamil on *Leishmania*–macrophage attachment, suggesting a role of the Ca^{2+} ion on the invasion process. Recently, amlodipine and lacidipine were demonstrated to be in vitro and in vivo effective against *L. donovani*, the Indian agent of visceral leishmaniasis (VL). In this work, we report for the first time the antileishmanial activity of nimodipine, a 1,4-dihydropyridine calcium channel blocker, against cutaneous and visceral species of *Leishmania*. We have also investigated the ultrastructural damages caused by nimodipine in *Leishmania* parasites and the possible macrophage activation by nimodipine.

Materials and methods

Materials Lipopolysaccharide (LPS), nimodipine (chemical name *O*5-(2-methoxyethyl) *O*3-propan-2-yl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate), sodium dodecyl sulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazol blue), M-199, and RPMI-PR^{-} 1640 medium (without phenol red) were purchased from Sigma (St. Louis, MO, USA). Pentavalent antimony (Glucantime, Aventis-Pharma, Brazil) and pentamidine (Sideron, Brazil) were used as standard drugs. Other analytical reagents were purchased from Sigma (St. Louis, MO, USA) unless where stated otherwise.

Animals BALB/c mice and golden hamsters (*Mesocricetus auratus*) were supplied by the animal breeding facility at the Adolfo Lutz Institute of São Paulo and maintained in sterilized cages under a controlled environment, receiving water and food ad libitum. Animal procedures were performed with the approval of the Research Ethics Commission (project CCD-BM 16/2004), in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences (http://www.nas.edu).

Parasite maintenance L. (L.) *chagasi* (MHOM/BR/1972/LD) was maintained in golden hamsters, up to approximately 60 to 70 days postinfection. Promastigotes were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24°C (Tempone et al. 2004). Isolated promastigotes of *Leishmania* (L.) *amazonensis* (WHO/BR/00/LT0016) and *Leishmania* (L.) *major* (MHOM/1L/80/Fredlin) were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24°C.

Peritoneal macrophage collection Peritoneal macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI-1640 without phenol red, supplemented with 10% calf serum. Cells were dispensed in 96- or 24-well microplate and maintained for 1 h in the same medium at 37°C in a 5% CO_2 humidified incubator for attachment. Nonadherent cells were removed by two step washings with medium.

Determination of the 50% inhibitory concentration Promastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells/well in 96-well microplates using pentamidine as standard.[14] Nimodipine was dissolved in methanol, diluted in M-199 medium, and incubated with parasites in different concentrations (based on dry weight) for 24 h at 24°C. Parasite viability was determined using the MTT assay at 550 nm (Tada et al. 1986). Each assay was performed in triplicate. The activity against *L. chagasi* intracellular amastigotes was determined with infected macrophages, using pentavalent antimony as standard. Briefly, peritoneal macrophages were seeded at 4×10^5 cells/well in 13 mm glass cover slips in 24-well microplates for 24 h at 37°C in a 5% CO_2 humidified incubator. Amastigotes obtained from hamster spleen by differential centrifugation (Stauber et al. 1958) were added to macrophages at 10:1 ratio (amastigotes/macrophage) and incubated for 24 h. Noninternalized parasites were removed by washing and test compounds were then incubated for 120 h at the same conditions. Finally, glass cover slips were fixed with methanol, stained with Giemsa, and observed in a light microscope. The parasite burden was defined as the mean number infected macrophages out of 600 cells (Tempone et al. 2004).

Cytotoxicity against mammalian cells Kidney Rhesus monkey cells (LLC-MK2) were cultured using RPMI-1640 medium supplemented with 10% calf serum at 37°C in a 5% CO_2 humidified incubator. Cells were removed by

scrapping, seeded at 4×10^4 cells/well in 96-well microplates, and further incubated with drugs for 48 h at 37°C, using pentamidine and Glucantime® as control. The viability of the cells was determined using MTT assay at 550 nm (Tada et al. 1986).

Hemolytic activity The capacity of nimodipine to induce hemolysis at concentrations close to the 50% inhibitory concentration (IC_{50}) against *Leishmania* was verified using a 3% suspension of BALB/c mice erythrocytes. The drug was incubated for 2 h with cells at different concentrations in 96-well *U*-shape microplates at 25°C. The supernatant was removed and analyzed at 550 nm (Moreira et al. 2007) in a multiwell scanning spectrophotometer (Labsystems; Multiskan EX).

Macrophage nitric oxide production In order to detect the macrophage activation induced by nimodipine, the production of nitric oxide (NO) was evaluated using the Griess reaction (Panaro et al. 1999). Briefly, macrophages were dispensed in a 24-well microplate and incubated for 24 h with the drug at a nontoxic concentration ($<IC_{50}$) at 37°C. LPS (50 μg/mL) was used to induce NO upregulation. The absorbance was determined at 550 nm using a multiwell scanning spectrophotometer (Labsystems; Multiskan EX).

Transmission electron microscopy analysis The ultrastructural changes of *L.* (L.) *chagasi* promastigotes induced by nimodipine (21 μM) were evaluated in different periods (2, 4, 6 h). Subsequently, promastigotes were processed and observed in a JEOL transmission electron microscope (Duarte et al. 1992).

Inhibition of amastigotes invasion in macrophages The inhibitory effect of nimodipine on amastigote invasion in macrophages was evaluated using different drug concentrations and periods. Peritoneal macrophages (24-well microplates) were pretreated with nimodipine at 48, 24, and 2.4 μM for 1 to 3 h. Before infection, cells were washed twice with RPMI-1640 medium and further incubated with previously isolated amastigotes at 10:1 (amastigotes/macrophage) ratio for 1 h. Cells were washed to remove noninternalized parasites.

Statistical analysis The data obtained represent the mean and standard deviation of triplicate samples from two independent assays. The IC_{50} values were calculated using sigmoid dose–response curves performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA.

Results

In vitro antileishmanial activity of nimodipine Leishmania parasites were incubated for 24 h with nimodipine using the MTT assay for viability. The drug was effective against both *L.* (L.) *chagasi* forms, the axenic promastigotes and intracellular amastigotes. The evaluation of the IC_{50} demonstrated that promastigotes were less susceptible than amastigotes, with an EC_{50} value of 82.68 and 21.62 μM, respectively (Table 1). *L.* (L.) *major*, an etiological agent of the Old World Cutaneous Leishmaniasis, was the most susceptible to nimodipine with an IC_{50} value of 31 μM. Nimodipine was also effective against an etiological agent of Brazilian cutaneous leishmaniasis, *L.* (L.) *amazonensis*, showing an IC_{50} value of 128.16 μM. Based in the mitochondrial oxidation of MTT and by light microscopy, nimodipine presented a leishmanicidal activity as observed by the sigmoidal dose–response curves, with 100% of *L.* (L.) *chagasi* death at the highest concentration of 191 μM, at 37 μM for *L. major*, and at 358 μM for *L. amazonensis* (data not shown). Pentamidine was used as internal control against promastigotes and resulted in an IC_{50} range between 0.059 and 0.303 μM. Pentavalent antimony (Glucantime®) was used as control to the intracellular amastigote assay and resulted in an IC_{50} of 242 μM against *L.* (L.) *chagasi*.

Table 1 Effect of nimodipine on *Leishmania* parasites and mammalian cytotoxicity

Drug	IC_{50} (μM; 95%CI)					HA	SI
	L. (L.) *chagasi* promastigotes	*L.* (L.) *amazonensis* promastigotes	*L.* (L.) *major* promastigotes	*L.* (L.) *chagasi* amastigotes	LLC-MK2		
Nimodipine	82.68 (83.33 to 197.11)	128.16 (83.33 to 197.11)	31.04 (24.68 to 39.05)	21.62 (15.05 to 30.99)	96.19 (95.87 to 96.50)	0	4.4
Pentamidine	0.16 (0.099 to 0.264)	0.050 (0.033 to 0.050)	0.269 (0.236 to 0.303)	nd	14.72 (12.21 to 17.75)	nd	nd
Glucantime	>8,213	>8,213	>8,213	80.74 (76.75 to 84.95)	>2,732,38	nd	>33

IC_{50} 50% inhibitory concentration, *95% CI* 95% confidence interval, *HA* hemolytic activity (%) at 179.23 μM, *SI* Selectivity Index, *nd* not determined

Nimodipine cytotoxicity The mammalian cytotoxicity of nimodipine was evaluated using Kidney Rhesus monkey cells in a close concentration to the antileishmanial IC_{50} value. The in vitro assay was performed for 48 h and resulted in an IC_{50} of 96.19 μM. The hemolytic activity was also determined as a cytotoxic parameter, but no damages to mice erythrocytes could be observed after nimodipine incubation to the highest concentration of 179 μM (Table 1).

Inhibition of macrophage infection assay The probable blockage of the amastigote penetration into the host cells was evaluated using nimodipine-treated macrophages. Peritoneal macrophages were pretreated with the drug for 1 to 3 h at three concentrations, but nimodipine could not inhibit the penetration of amastigotes even at the highest tested concentration. The number of parasites/macrophages was also unaltered in treated groups (data not shown).

Nitric oxide production As a consequence of a leishmanicidal effect of nimodipine in the intracellular amastigote assay, the possible activation of macrophages was investigated through the Griess reaction. The data showed no considerable upregulation of the nitric oxide production after 24 h incubation when compared to controls (Fig. 2). Bacterial LPS was used as internal control (100% NO production) and demonstrated a high upregulation when compared to nimodipine.

Transmission electron microscopy study The ultrastructural damages of *Leishmania* caused by nimodipine incubation were investigated using a transmission electron microscopy. Promastigotes were previously treated with the drug for different periods at its IC_{50} value. After 2 h incubation, the overall morphology of the cell was not altered, but an increase in intracytoplasmatic vacuoles was clearly observed (Fig. 3b). The parasite plasma membrane was also affected by the drug, as shown by the membrane blebbing (arrow). No significant alterations were observed in the nucleus, but enlarged mitochondria of the kinetoplast was observed. After 4 h incubation, the nuclear membrane was clearly affected, with detachment of bilayer (Fig. 3e), despite the overall morphology of the cell was well preserved. A large number of intracytoplasmatic vacuoles and enlarged mitochondria (Fig. 3c) and an intense membrane blebbing effect was observed (Fig. 3f, g, arrows). After 6 h, the fusion of intracytoplasmatic vacuoles might have occurred, as large vacuoles were found inside the cells with an intense loss of intracytoplasmatic organelles (Fig. 3d). A nontreated promastigote was used as control (Fig. 3a).

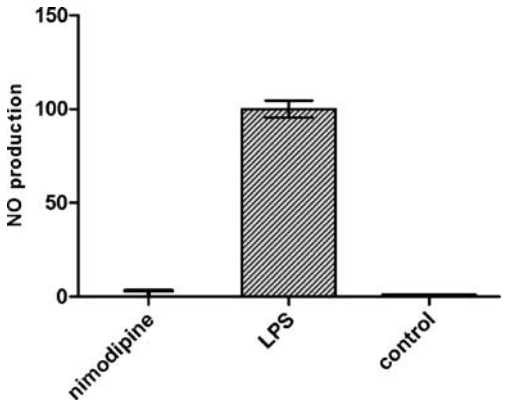

Fig. 2 Effect of nimodipine on macrophages nitric oxide (*NO*) production. The nitrite content was determined by the Griess reaction at 550 nm. LPS was used as control

Discussion

Previous work using dihydropyridines demonstrated potential antimicrobial (Kumar et al. 2003) and antiparasitic activities (Misra et al. 1991). Therapeutic switching or "piggy back chemotherapy" has been a promising alternative to provide novel antileishmanial drugs, reducing the time and costs of the research against neglected parasitic diseases. In a search for novel effective antileishmanial drugs, we demonstrated for the first time in the literature that nimodipine presents a specific activity against cutaneous and visceral species of *Leishmania*. The in vitro activity against promastigotes showed that *L. major* was the most susceptible to nimodipine, with an IC_{50} value 2.6-fold smaller than for *L.* (L.) *chagasi*. By using the most relevant clinical form, the amastigotes, nimodipine effectively eliminated the intracellular *L.* (L.) *chagasi* and demonstrated to be significantly ($p<0.001$) fourfold more active than the standard pentavalent antimony (Glucantime®). Other two dihydropyridines, amlodipine and lacidipine, have also been demonstrated antileishmanial activity. Both drugs effectively treated *L. donovani*-infected macrophages, with an in vitro IC_{50} value of 5.1 and 6.6 μM, respectively (Palit and Ali 2008). Other two calcium channel blockers, isradipine and lacidipine, have also been demonstrated antitrypanosomal activity, killing extracellular epimastigotes of *Trypanosoma cruzi* in a range concentration between 20 and 31 μM. Despite the promising activity, verapamil and diltiazem lacked antitrypanosomal effect (Núñez-Vergara et al. 1998), showing that protozoan parasites have different susceptibilities for calcium channel blockers. In our assays, the relationship between the toxicity toward the kidney Rhesus monkey cells (LLC-MK2) and the antileishmanial activity, given by the Selectivity Index (SI), demonstrated a considerable value of 4.4 for nimodipine. The capacity of nimodipine to induce hemolysis at concentrations close to the IC_{50} against *Leishmania* was verified using mice erythrocytes. Nimodipine showed no hemolytic activity to the highest concentration of 180 μM,

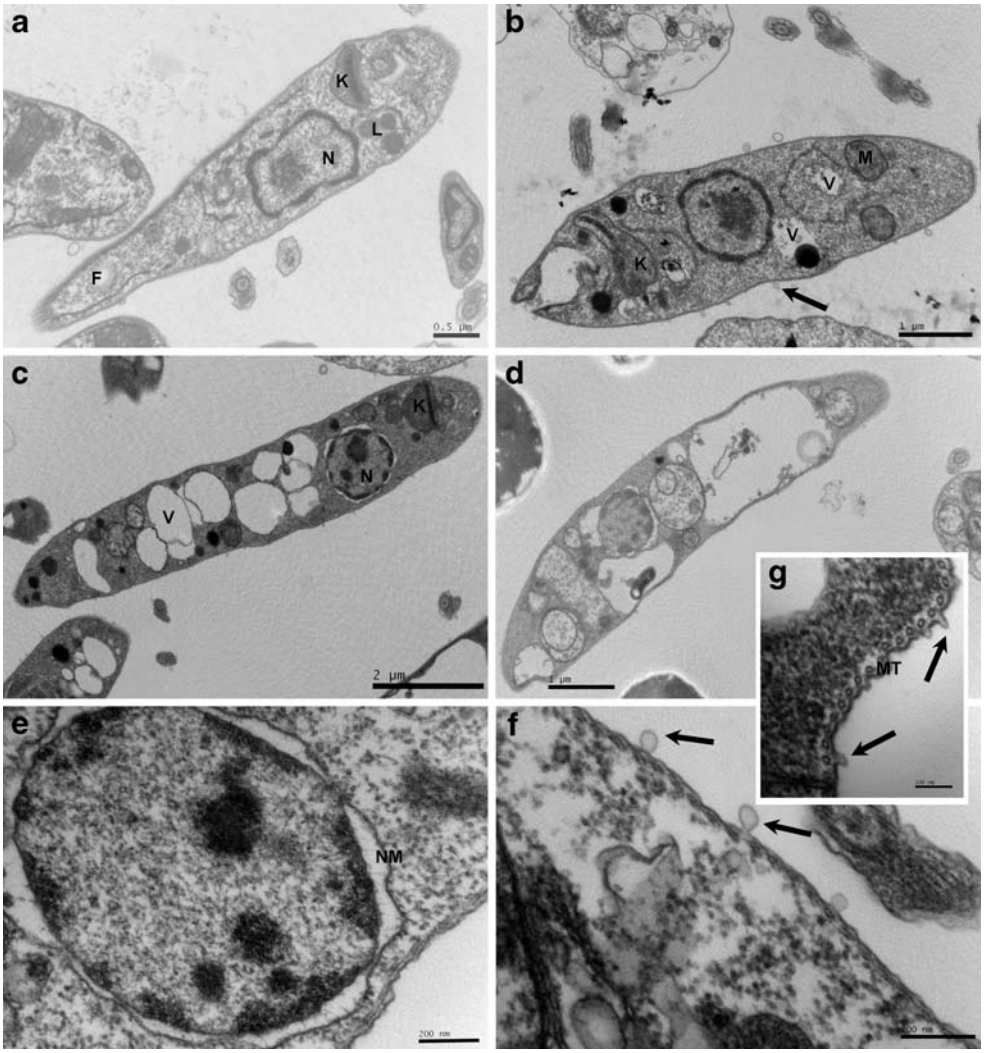

Fig. 3 Transmission electron microscopy of *L.* (L.) *chagasi* incubated with nimodipine. Promastigotes were incubated for different periods at 24 °C. **a** Control group. **b** 2 h incubation. **c** 4 h incubation. **d** 6 h incubation. **e** 4 h incubation. **f**, **g** 4 h incubation. *K* kinetoplast, *M* mitochondria, *N* nucleus, *F* flagellum, *L* lipid inclusion, *V* vacuoles, *MT* microtubules, *NM* nuclear membrane

being harmless to erythrocytes when one considers its in vitro IC_{50} value against intracellular amastigotes (21 μM). The use of drug delivery systems could be an important alternative to nimodipine, improving its SI through the delivery of high amounts of the drug to infected macrophages. Tempone et al. (2004) demonstrated that phosphatidylserine liposomes entrapping pentavalent antimony was in vitro 16-fold more effective against infected macrophages when compared to free drug. This formulation was also 133-fold more effective in experimental *L.* (L.) *chagasi* models (Tempone and Andrade 2008). In vivo assays should be carried out to investigate the nimodipine antileishmanial activity. Free and liposome-loaded nimodipine is under investigation in our laboratory for the treatment of hamster models of VL.

Macrophages are the host cells in leishmaniasis and play an important role in the immunological control of intracellular parasites through the production of cytokines and oxygen metabolites (Balaraman et al. 2004). Through the upregulation of the effective mediator nitric oxide inside the cell, macrophages trigger the amastigote killing mechanism (Mauel and Ransijn 1997). We have investigated the possible activation of macrophages induced by nimodipine using the Griess reaction (Panaro et al. 1999). Our results clearly demonstrated no upregulation of NO, suggesting that the antileishmanial effect of nimodipine might be other mechanism than NO activation by macrophages. In addition, the drug efficacy against the extracellular promastigotes supports the principle of a specific antiparasitic activity, without the need of macrophage intervention for the leishmanicidal effect. The dependency of macrophage activation for the pentavalent antimony leishmanicidal effect has been demonstrated, as an upregulation of NO and tumor necrosis factor-α contributes to the killing of intracellular amastigotes (Mookerjee Basu et al. 2006). Thus, an immunocompetent organism has been demonstrated to be important for the treatment of VL with antimonial drugs and coinfection with HIV clearly demonstrated the limitation of this therapy. Based in our in vitro data, nimodipine demonstrated a superior efficacy than pentavalent antimony against *L.* (L.)

chagasi, selectively eliminating the intracellular parasites without the requirement of macrophage activation.

The ultrastructural damages of *L.* (L.) *chagasi* promastigotes caused by nimodipine revealed an intense swelling of mitochondrias, plasma membrane blebbing, and strong alterations in nuclear membrane. The overall mitochondrial damages were not accompanied by alterations of the kinetoplast, suggesting that this organelle might not be the preferential target of the drug. Despite the conserved subpellicular corset of microtubules, the parasite membrane was clearly affected, with a strong blebbing effect in a short period of incubation (2 h). Despite this marked alteration, no pore-forming activity in plasma membrane could be detected and in addition, no cell edema could be observed at the highest time of incubation, suggesting other mechanism than ions leakage during the cell death progression. Despite the prompt leishmanicidal effect, confirmed by the lack of mitochondrial oxidation of MTT, the overall shape and morphology of promastigotes were well preserved by the end of the assay.

In a previous report, the calcium channel blockers nifedipine and verapamil effectively inhibited the macrophage infection by *L.* (L.) *donovani*, but no antiparasitic effect was observed (Misra et al. 1991). The authors also suggested an important participation of calcium ions in the invasion process. In contrast, Ganguly et al. (1991) demonstrated an increase in the percentage of *L. donovani*-infected macrophages in the presence of nifedipine. The authors suggested that the availability of intracellular calcium is a factor in the defense mechanism of macrophages. In order to elucidate this controversial data using another calcium channel blocker (nimodipine), we carried out a similar assay with macrophages. Despite the related chemical structure between nifedipine and nimodipine, our data demonstrated no decrease in percentage of *L.* (L.) *chagasi*-infected macrophages compared to controls and also no enhance in the number of intracellular amastigotes per macrophage. Palit and Ali (2008) demonstrated that calcium channel blockers as lacipidine, amlodipine, verapamil, and diltiazem inhibited the Ca^{2+} uptake by *Leishmania*, but only the two dihydropyridines showed antiparasitic activity. This result contradicts the correlation between the leishmanicidal activity and the Ca^{2+} channel blocking action of these drugs, as verapamil and diltiazem are also effective calcium antagonists. Many antileishmanials that inhibit the respiratory chain complexes also induce apoptosis (Mehata and Shaha 2004). Our results demonstrated that nimodipine affects mitochondrial functions, as no oxidation of MTT by dehydrogenases could be detected. Inhibition of oxygen consumption causes an increase in the intracellular reactive oxygen species, leading to a loss of mitochondrial membrane potential (Palit and Ali 2008). This could have been a possible effect of nimodipine in *Leishmania*, resulting in enlarged mitochondria as observed in transmission electron microscopy. Further assays must be conducted in order to elucidate this mechanism and also to investigate the possibility of *Leishmania* apoptosis.

The 1,4-dihydropyridines have been considered promising antiparasitic drugs, mainly against protozoan parasites. Recently, it was demonstrated that verapamil could effectively reverses antimony resistance in *L.* (L.) *donovani* (Valiathan et al 2006). The 1,4-dihydropyridine nucleus belongs, in fact, to the class of "privileged structures", a term formally introduced by Evans et al (1988). It has served as a scaffold for second and third generation drugs of calcium channel blockers and also as a scaffold or "privileged" structure for molecules active at a diverse collection of ion channels and pharmacological receptors (Triggle et al. 1989; Triggle 2003a). Approximately, ten of these agents have been used in the clinical medicine for the treatment of a number of cardiovascular disorders (Triggle 2003b). Considering the high versatility of this pharmacophore, its further exploitation as a novel lead compound may yield selective and potent drug prototypes against neglected diseases as visceral leishmaniasis.

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