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Susceptibility to paromomycin in clinical isolates and reference strains of *Leishmania* species responsible for tegumentary leishmaniasis in Brazil

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ABSTRACT

Treatment of tegumentary leishmaniasis in Brazil is limited to pentavalent antimonial, amphotericin B and pentamidine. These drugs, administered parenterally, cause several side effects and have a varied clinical response, depending on the species of *Leishmania*. Urgent expansion of the therapeutic arsenal against the disease is therefore necessary. Paromomycin is an aminoglycoside antibiotic that has already been approved for the treatment of visceral leishmaniasis in Southeast Asia. Here, we provide an *in vitro* evaluation of the activity of paromomycin in fifteen clinical isolates from patients with tegumentary leishmaniasis at a reference center for the treatment of the disease. Furthermore, the *in vitro* susceptibility to this drug in reference strains of *Leishmania* species that are endemic in Brazil has also been evaluated. Among the clinical isolates, nine were typed as *Leishmania (Viannia) braziliensis*, five as *L. (Leishmania) amazonensis* and one as *L. (V.) guyanensis*. Although never exposed to paromomycin, we found variable susceptibility among these isolates and reference strains in promastigotes and intracellular amastigotes, with the drug being more active in the amastigote form of the parasite. This study provides a preclinical dataset that is useful for the evaluation of paromomycin in the treatment of tegumentary leishmaniasis caused by species that are endemic in Brazil.

1. Introduction

Leishmaniasis is a vector borne disease caused by parasites protozoan of the genus *Leishmania.* These parasites alternate between a female sandfly vector as flagellated promastigotes and the mammalian host as intracellular amastigotes. More than 20 species of parasite are responsible for two forms of disease in humans, visceral and tegumentary leishmaniasis (Burza et al., 2018). Tegumentary leishmaniasis may be classified in different clinical forms of disease. The most prevalent form is cutaneous leishmaniasis (CL) that may progress in more severe manifestations in up to 10% of cases (Burza et al., 2018). The other forms are mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and disseminated cutaneous leishmaniasis (Burza et al., 2018; Reithinger et al., 2007). In Brazil, CL is caused mainly by *Leishmania (Viannia) braziliensis*, the most prevalent species, followed by *L. (Leishmania) amazonensis* and *L. (V.) guyanensis* (Alvar et al., 2012). Other species responsible for CL are: *L. (V.) naiffi, L. (V.) lainsoni, L. (V.) shawi* and *L. (V.) lindenbergi*. These species have recently been linked to an increase in the number of human cases, mainly in the Amazon region (Cantanhede et al., 2019; Fagundes-Silva et al., 2015). Disfiguring and destructive lesions of the oronasopharyngeal mucosa, due to a strong immunopathological response, are a characteristic of MCL, a form of disease caused mainly by *L. (V.) brazilensis,* but also by *L. (V.) guyanesis* (Burza et al., 2018; Silveira, 2019). Finally, DCL is caused mainly by *L. (L.) amazonensis* in Brazil. There is no effective treatment for this clinical form, mainly due to an impairment in the cellular immune

Abbreviations: BMDM, bone marrow-derived macrophages; CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; ITS, internal transcribed ribosomal; MCL, mucocutaneous leishmaniasis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PM, paromomycin.

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response (Burza et al., 2018; Silveira, 2019).

Pentavalent antimonials, pentamidine and amphotericin B (available in deoxycholate and liposomal formulations) are the only drugs available for chemotherapy of leishmaniasis in Brazil (Uliana et al., 2018). These drugs have limitations related to toxicity, are parenterally administrated and, in the case of pentavalent antimonials, the efficacy in patients is not higher than 50% (Chrusciak-Talhari et al., 2011; Machado et al., 2018). An investigation of alternative drugs is both urgent and necessary, particularly against species that are endemic in Brazil. Although other drugs have already been approved for treatment of visceral leishmaniasis, such as, for example, miltefosine and paromomycin (PM) (Jamil et al., 2015; Sundar et al., 2002; Sundar et al., 2007; Sundar et al., 2012), studies are still limited with the regard to the potential of these drugs against species responsible for tegumentary disease.

Paromomycin is an aminoglycoside antibiotic, isolated from filtrates of *Streptomyces krestomuceticus,* with activity against several infectious agents, from bacteria to intestinal protozoa (Davidson et al., 2009). Clinical trials in patients with visceral leishmaniasis in Southeast Asia showed that injectable PM has cure rates higher than 90% (Jamil et al., 2015; Sundar et al., 2007) and is more effective when compared to pentavalent antimonial treatment (Jha et al., 1998). The drug has a short half-life (around 2-3 hours) and has been proposed in combination therapies against visceral leishmaniasis (Davidson et al., 2009; van Griensven et al., 2010). In association with miltefosine or amphotericin B, combinations were effective and safe, with shorter duration of the treatment and lower doses administered (Rahman et al., 2017; Sundar et al., 2011).

Systemic or topical paromomycin, alone or in combination with other drugs has shown clinical efficacy of 80% to 85% for treatment of CL caused by different species of *Leishmania* (Arana et al., 2001; Ben Salah et al., 2013; Kim et al., 2009; Sosa et al., 2019). However, little is known about the action of PM in the different species that cause tegumentary leishmaniasis in Brazil. Studies on the activity of PM against species endemic in Brazil are scarce and this evaluation is important for a consideration of its potential use in the chemotherapy of disease. The activity has already been evaluated in the main species responsible for CL in Brazil (de Morais-Teixeira et al., 2014). In this study, we determine the PM *in vitro* susceptibility of species responsible for CL in Brazil and of a panel of fifteen isolates from patients of a reference center for the treatment of leishmaniasis in the city of São Paulo, Brazil.

2. Materials and Methods

2.1. Ethics statement

Procedures involving patients were approved by the Human Research Ethics Committee of the Instituto de Infectologia Emílio Ribas and were registered on the Plataforma Brasil (http://plataformabrasil.saude.gov.br) under CAAE number: 07801112.1.0000.0061. An informed consent was obtained from all patients before the procedures. Animal experiments were approved by the Ethics Committee for Animal Experimentation of the UNICAMP [Protocols: 4797-1/2018 and 4797-1 (A)/2019].

2.2. Parasite cultivation and animals

Promastigotes of reference strains of *L. (L.) amazonensis* (MHOM/BR/1973/M2269) and (IFLA/BR/1967/PH8), *L. (V.) braziliensis* (MHOM/BR/1994/H3227), *L. (V.) guyanensis* (MHOM/BR/1975/M4147), *L. (V.) shawi* (MCEB/BR/1984/M8408), *L. (V.) naiffi* (MDAS/BR/1979/M5533), *L. (V.) lindenbergi* (MHOM/BR/1996/M15732) and *L. (V.) lainsoni* (MHOM/BR/1981/M6426) and clinical isolates were grown at 25°C in M199 medium (Sigma-Aldrich) supplemented with HEPES 40 mM (pH 7.4), adenin 0.1 mM, hemin 0.25%, 10% heat-inactivated fetal bovine serum (Thermo Scientific), 50 U/mL penicillin and 50 μg/mL streptomycin (Kapler et al., 1990). For promastigotes of the subgenus *Viannia,* parasites were grown in the M199 medium supplemented with 2% sterile male human urine (Howard et al., 1991). Fifteen clinical isolates were obtained from patients with tegumentary leishmaniasis at the Instituto de Infectologia Emílio Ribas (Table 1). The probable site of infection of these patients were the following Brazilian States: Amazonas, Bahia, Mato Grosso, Minas Gerais, Pará and São Paulo (Table 1). Parasites of each isolate were obtained from an aspiration of skin lesions with the patient's consent. Initially, skin biopsies of the lesion were subjected to initial cultivation in the diphasic agar medium NNN (Novy, Mac Neal, Nicolle), followed by cultivation in the M199 medium at 25°C. For each isolate, the species of the parasite was typed through PCR amplification followed by sequencing of the internal transcribed ribosomal (ITS) DNA (Cupolillo et al., 1995) as previously described (Gosch et al., 2018). The GenBank accession number of the ITS sequence of each isolate is indicated in Table 1.

Bone marrow-derived macrophages (BMDM) were obtained from BALB/c mice (aged 4–6 weeks) as described by (Zamboni and Rabinovitch, 2003). All animals were acquired from Centro Multidisciplinar para Investigação Biológica of UNICAMP and were kept in mini-isolators, and received food and water *ad libitum*.

Table 1
Clinical form and typing of the isolates used in this study.

Clinical isolate	Clinical form [a]	Origin [b]	Typed species	GenBank accession number of ITS sequence
MHOM/BR/2008/ER054	CL	SP	*L. (L.) amazonensis*	MT940878
MHOM/BR/2009/ER117	MCL	BA	*L. (L.) amazonensis*	MT940881
MHOM/BR/2009/ER118	CL	MT	*L. (L.) amazonensis*	MT940882
MHOM/BR/2012/ER256	CL	Not specified	*L. (L.) amazonensis*	MT523027 [c]
MHOM/BR/2008/UB017	CL	SP	*L. (L.) amazonensis*	MT940889
MHOM/BR/2007/ER004	CL	PA	*L. (V.) braziliensis*	MT940876
MHOM/BR/2007/ER010	MCL	BA	*L. (V.) braziliensis*	MT940877
MHOM/BR/2009/ER098	CL	PA	*L. (V.) braziliensis*	MT940879
MHOM/BR/2009/ER114	CL	MG	*L. (V.) braziliensis*	MT940880
MHOM/BR/2010/ER135	CL	BA	*L. (V.) braziliensis*	MT940883
MHOM/BR/2011/ER215	CL	MG	*L. (V.) braziliensis*	MT940885
MHOM/BR/2013/ER267	CL	BA	*L. (V.) braziliensis*	MT940886
MHOM/BR/2013/ER269	CL	AM	*L. (V.) braziliensis*	MT940887
MHOM/BR/2013/ER271	CL	AM	*L. (V.) braziliensis*	MT940888
MHOM/BR/2013/ER192	MCL	PA	*L. (V.) guyanensis*	MT940884

[a] CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis.
[b] Brazilian States: AM, Amazonas; BA, Bahia; MG, Minas Gerais; MT, Mato Grosso; PA, Pará; SP, São Paulo.
[c] Nucleotide sequence of ITS previously determined in Coser *et al.*, 2020.

2.3. Paromomycin susceptibility at the promastigote and at intracellular amastigote stages

Stock solution of PM sulfate (100 mM) was prepared in Milli-Q ddH_2O, followed by filtering through a 0.2 μm syringe filter and then kept at -20°C until use. The susceptibility of clinical isolates and reference strains to PM against promastigotes was determined using the MTT colorimetric assay, after incubation of 2×10^6 parasites per well in a 96-well plate in the M199 medium for 24 h in the presence of increasing concentrations of PM as previously described (Espada et al., 2017). Counting of the promastigotes was performed in a Neubauer haemocytometer. Experiments were carried out in triplicate and the EC_{50} and EC_{90} values were determined from sigmoidal regression of the concentration-response curves using the GraphPad Prism 7.

The cytotoxicity of PM against BMDM after incubation of 72 h was described previously (CC_{50} = 536.6 ± 27.1 μM) (Coser et al., 2020). BMDM were plated at a density of 3×10^5 macrophages per well in a complete RPMI 1640 medium (Thermo Scientific) supplemented with 10% heat-inactivated fetal bovine serum on round glass coverslips 24-well culture dishes in a 5% CO_2 atmosphere for 24 h at 37°C. Promastigotes of reference strains and clinical isolates of *Leishmania* spp. at stationary phase growth (6^{th} day in culture) were used to infect macrophages at a ratio of 1:5 (macrophage/parasites) for *L. (L.) amazonensis*, while for strains and isolates of the subgenus *Viannia,* a ratio of 1:30 was employed. Four hours after infection at 34°C and 5% CO_2, non-internalized promastigotes were removed by washing with PBS. Infected macrophages were maintained in RPMI 1640 medium containing increasing PM concentrations for 72 h at 34°C and 5% CO_2. Afterwards, infected macrophages were fixed with metanol (Sigma-Aldrich) and stained with a panoptic haematological method (Laborclin, Brazil). The percentage of infection was determined by counting 100 macrophages in three independent experiments in duplicate and the EC_{50} and EC_{90} values were determined as described above. The activity index (AI) corresponds to the ratio of the EC_{50} of a clinical isolate to the EC_{50} of the reference strain (Yardley et al., 2006) and was determined for intracellular amastigotes of the clinical isolates, allowing comparison of the *in vitro* susceptibility of a particular isolate with the other isolates tested of the same species of parasite.

2.4. Statistical analysis

Unpaired two-tailed Student's *t* test was used to compare the EC_{50} determined *in vitro* in promastigotes and intracellular amastigotes of reference strains and clinical isolates. Spearman's correlation coefficient was calculated to determine the correlation between the EC_{50} of promastigotes and intracellular amastigotes of the lines used in this study. All statistical analyses were performed in the GraphPad Prism 7. The value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Typing of the clinical isolates of Leishmania spp

For species identification of the clinical isolates, we typed isolates by sequencing of the ITS, as previously described (Gosch et al., 2018). The full nucleotide sequence of ITS of each one of the isolates indicated that nine isolates had more than 98% identity with ITS of the reference strain *L. (V.) braziliensis* (MHOM//BR/1975/M2903) (GenBank accession number AJ300483.1) and were typed as *L. (V.) braziliensis*. The respective sequences of five isolates have at least 99% identity with the reference strain *L. (L.) amazonensis* (MHOM/BR/73/M2269) (GenBank accession number AJ000316.1). Finally, one isolate presented more than 98% identity with the reference strain *L. (V.) guyanensis* (MHOM/BR/1975/M4147) (GenBank accession number AJ000299.1). The corresponding typed species, according to the nucleotide sequence of ITS for each clinical isolate, is indicated in Table 1. These isolates have a widespread origin, with six isolates from the Brazilian States of Amazonas, Pará and Mato Grosso in the Amazon region, whereas four isolates were from the Northeast region (Bahia) and the other four isolates were from the Southeast region (Minas Gerais and São Paulo) (Table 1).

3.2. In vitro susceptibility to PM in reference strains and clinical isolates of Leishmania spp

Twelve isolates from patients with localized CL were used in this study, while another three isolates were obtained from patients with MCL (Table 1). Isolation occurred before the treatment meaning there was no exposure to pentavalent antimony or to other drugs used in the chemotherapy of disease. First, we determined the rate of macrophage infection for the different clinical isolates and reference strains in the absence of PM. The rates of infection ranged between 44% and 83% for *L. (V.) braziliensis* isolates and the reference strain and between 9% and 66% for *L. (L.) amazonensis* isolates and the strains M2269 and PH8 (Table 2). The rate of infection of the clinical isolate *L. (V.) guyanensis* ER192 and the reference strain M4147 were 85% and 56% respectively (Table 2). Finally, the rate of macrophage infection of other reference strains of subgenus *Viannia* [*L. (V.) shawi; L. (V.) naiffi; L. (V.) lindenbergi* and *L. (V.) lansoni*] ranged from 31% to 72% (Table 2).

Once the infectivity of the isolates and the reference strains had been determined, the susceptibility to PM was determined in *Leishmania* spp. the reference strains. A wide range of susceptibility was found at both stages of the parasite (Fig 1A-B and Table 2). The EC_{50} at the promastigote stage ranged from 28.58 ± 2.55 to 205 ± 7.04 μM, while in intracellular amastigotes of the reference strains, the EC_{50} values ranged from 0.62 ± 0.12 to 61 ± 9.48 μM (Fig. 1A-B and Table 2). Similarly, clinical isolates of *L. (L.) amazonensis, L. (V.) braziliensis* and *L. (V.) guyanensis* presented variation in susceptibility in both stages, in which the EC_{50} values were 4.95 ± 2.81 to 148.03 μM ± 18.9 and 0.2 ± 0.03 to 108.6 ± 5.43 μM for promastigotes and intracellular amastigotes respectively (Fig. 1C-D and Table 2). The reference strains and clinical isolates were significantly more susceptible to PM in the intracellular amastigote stage than in the promastigote stage ($p < 0.0001$ Student's *t* test), with the exception of the isolate ER192, in which the EC_{50} values were 45.73 ± 4.8 μM and 108.6 ± 5.43 μM for promastigotes and intracellular amastigotes respectively (Fig. 1C-D and Table 2). Isolates of *L. (L.) amazonensis* were highly susceptible to PM, when compared to the reference strains (M2269 and PH8) in both stages of the parasite (Fig. 1C-D and Table 2). In intracellular amastigotes of this species, the EC_{50} for the strain M2269 was 61 ± 9.48 μM, the median of EC_{50} of the isolates was 0.57 μM, and the AI of these isolates were, on average, 100-fold more susceptible than the reference strain M2269 (Table 2). Isolates and the reference strain of *L (V.) braziliensis* H3227 showed variation in the susceptibility to PM in promastigotes (between 4.95 ± 2.81 to 148.03 ± 18.9 μM) (Fig. 1C and Table 2); while in intracellular amastigotes a lower variation in susceptibility was found (between 0.67 ± 0.17 to 6.83 ± 0.8 μM) (Fig. 1D and Table 2). In intracellular amastigotes of *L. (V.) braziliensis*, the EC_{50} for the strain H3227 was 2.97 ± 0.74 μM and the median of EC_{50} of the isolates was 2.76 μM. The AI of the isolates of *L. (V.) braziliensis* ranged from 0.23 to 2.3 and the AI average of these isolates was 0.88 (Table 2). The isolate ER192 was more susceptible to PM than the reference strain M4147 in promastigotes, while at the intracellular amastigote stage, this isolate was less susceptible to PM than the M4147 strain (Fig. 1C-D and Table 2). The AI of the ER192 isolate was around 8-fold more resistant to the drug (Table 2).

Finally, a direct and slight but significant correlation between the susceptibility of the intracellular amastigote and promastigote stages of the isolates and reference strains was found (r = 0.418; p = 0.047 Spearman's correlation test).

Table 2
In vitro activity of PM against promastigotes and intracellular amastigotes of reference strains and clinical isolates of *Leishmania* spp.

Strain / clinical isolate	*Leishmania* species	Promastigotes			Intracellular amastigotes			AI [d]	Infection rate [e]
		EC_{50} [a]	EC_{90}	(n)[b]	EC_{50}	EC_{90}	(n)[c]		
M2269 [f]	*L. (L.) amazonensis*	145.23 ± 23.04	730.4	3	61 ± 9.48	111.9	3	-	66%
PH8	*L. (L.) amazonensis*	95.63 ± 15.45	556.7	3	42.93 ± 3.5	73.59	3	-	53%
ER054	*L. (L.) amazonensis*	37.2 ± 6.47	134	3	0.57 ± 0.08	9.23	3	0.009	9%
ER117	*L. (L.) amazonensis*	54.37 ± 7.34	219.9	3	0.73 ± 0.02	11.14	3	0.012	35%
ER118	*L. (L.) amazonensis*	29.2 ± 8.13	129.3	3	0.2 ± 0.03	4.40	3	0.003	45%
ER256 [f]	*L. (L.) amazonensis*	9.98 ± 2.97	101.8	5	0.54 ± 0.11	4.86	4	0.008	59%
UB017	*L. (L.) amazonensis*	17.46 ± 4.17	115.1	6	0.85 ± 0.13	7.74	3	0.014	45%
H3227	*L. (V.) braziliensis*	28.58 ± 2.55	101.6	3	2.97 ± 0.74	26.73	3	-	46%
ER004	*L. (V.) braziliensis*	40.1 ± 4.78	180.2	5	3.1 ± 0.53	42.33	3	1.04	67%
ER010	*L. (V.) braziliensis*	4.95 ± 2.81	27.2	3	0.81 ± 0.05	4.04	3	0.27	44%
ER098	*L. (V.) braziliensis*	30.12 ± 3.12	84.21	3	3.1 ± 0.4	31.46	3	1.04	72%
ER114	*L. (V.) braziliensis*	148.03 ± 18.9	373.9	3	6.83 ± 0.8	61.47	3	2.3	70%
ER135	*L. (V.) braziliensis*	96.76 ± 8.97	305.5	3	3.57 ± 0.42	32.13	3	1.2	82%
ER215	*L. (V.) braziliensis*	112.56 ± 6.04	332.7	3	2.76 ± 0.46	25.2	3	0.93	64%
ER267	*L. (V.) braziliensis*	53.05 ± 4.86	107.3	3	1.36 ± 0.3	12.33	3	0.46	78%
ER269	*L. (V.) braziliensis*	49.1 ± 2.6	95.33	3	0.67 ± 0.17	10.92	3	0.23	73%
ER271	*L. (V.) braziliensis*	33.31 ± 5.82	98.53	3	1.23 ± 0.26	6.43	3	0.41	83%
M4147	*L. (V.) guyanensis*	101.5 ± 6.68	280	3	13.32 ± 2.61	102.9	3	-	56%
ER192	*L. (V.) guyanensis*	45.73 ± 4.8	416.4	4	108.6 ± 5.43	337.8	3	8.15	85%
M8408	*L. (V.) shawi*	162.9 ± 14.3	386.7	3	5.8 ± 0.42	31.44	3	-	72%
M5533	*L. (V.) naiffi*	205 ± 7.04	634.9	3	0.62 ± 0.12	14.72	3	-	31%
M15732	*L. (V.) lindenbergi*	168.4 ± 19.5	476.3	3	2.65 ± 0.33	23.85	3	-	61%
M6426	*L. (V.) lainsoni*	41.93 ± 2.7	137.2	3	0.64 ± 0.13	16.03	3	-	37%

[a] EC_{50} ± standard deviation in μM.
[b] Number of experiments carried out in triplicate per strain or isolate.
[c] Number of experiments carried out in duplicate per strain or isolate.
[d] AI: Activity Index, which corresponds to the ratio between the EC_{50} of the clinical isolate and the EC_{50} of the corresponding reference strain species. Lines highlighted in gray correspond to reference strains and for determination of AI of the clinical isolates of *L. (L.) amazonensis*, the EC_{50} value of the reference strain M2269 was used.
[e] Percentage of infected macrophages.
[f] Data of susceptibility of these lines was previously described in Coser *et al.*, 2020.

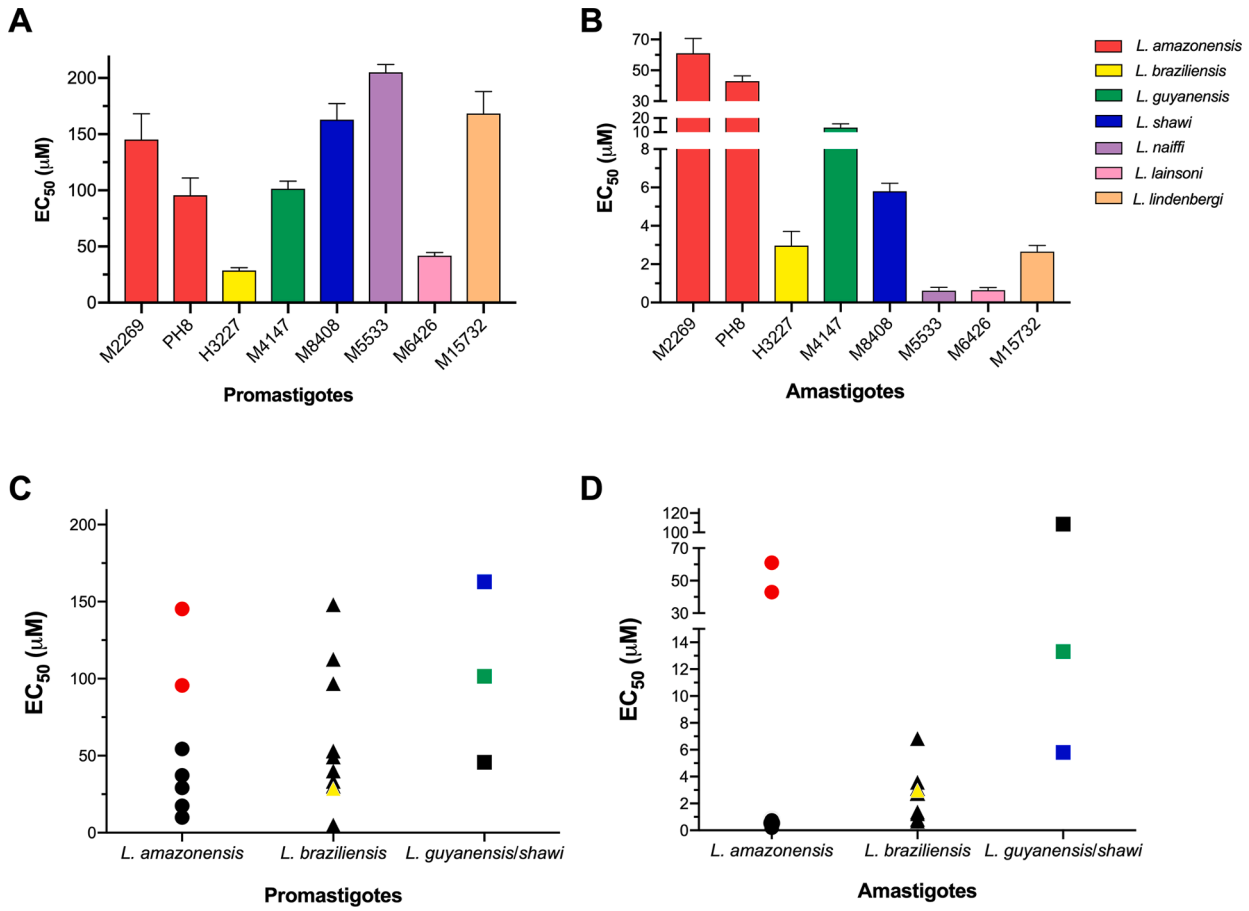

Fig. 1. Susceptibility of promastigotes (A and C) and intracellular amastigotes (B and D) of *Leishmania* spp. reference strains and clinical isolates to PM. EC_{50} values determined against (A) promastigotes and (B) intracellular amastigotes of eight reference strains that cause tegumentary leishmaniasis in Brazil. EC_{50} values determined against promastigotes (C) and intracellular amastigotes (D) of the clinical isolates and their corresponding reference strains: *L. (L.) amazonensis* M2269 and PH8 (red circles); *L. (V.) braziliensis* H3227 (yellow triangle); and *L. (V.) guyanensis* M4147 and *L. (V.) shawi* M8408 (green and blue squares respectively). Circles, triangles and the square in black correspond to clinical isolates of *L. (L.) amazonensis, L. (V.) braziliensis* and *L. (V.) guyanensis* respectively.

4. Discussion

This study investigated the activity of PM in reference strains of *Leishmania* spp. that are responsible for tegumentary disease in Brazil and in a panel of fifteen clinical isolates obtained from patients at a reference center for the treatment of leishmaniasis in the city of São Paulo. The susceptibility to PM of these isolates was compared to the reference strains of the corresponding species of these isolates. These isolates were previously typed through the sequencing of ITS, a molecular target already used for typing *Leishmania* species (Gosch et al., 2018; Van der Auwera and Dujardin, 2015). Most of the isolates were typed as *L. (V.) braziliensis*, followed by *L. (L.) amazonensis* and *L. (V.) guyanensis*. Among these species, *L. (V.) braziliensis* is the most prevalent species responsible for tegumentary leishmaniasis in Brazil, followed by *L. (L.) amazonensis* and *L. (V.) guyanensis* (Alvar et al., 2012).

Paromomycin has been used in the treatment of VL caused by *L. (L.) donovani* in Southeast Asia, since 2006 (Sundar et al., 2007; Sundar and Rai, 2005). For CL due to *L. (L.) mexicana* or *L. (L.) major*, PM was already approved for use in combination with methylbenzethonium in a local treatment regimen (Burza et al., 2018). In combination with sodium stibogluconate, PM was evaluated parenterally in patients with DCL caused by *L. (L.) aethiopica*. In this study, results demonstrated complete healing of the lesions, with minimal side effects and no relapse was reported after the end of the treatment (Teklemariam et al., 1994). Effectiveness of PM was evaluated against *L. (V.) panamensis* using a topical formulation with gentamicin in skin lesions, with a cure rate of approximately 80% (Sosa et al., 2019). Although the drug has not yet been evaluated in patients infected with *L. (L.) amazonensis* or *L. (V.) braziliensis* in clinical studies, a topical formulation of PM was able to reduce the lesion size in animals experimentally infected with these two species (Goncalves et al., 2005).

The *in vitro* susceptibility to PM was determined in both stages of parasite in species that are endemic and responsible for different clinical forms of tegumentary disease in Brazil. Strains and isolates were more susceptible to PM in intracellular amastigotes than promastigotes. Similar findings were described in other species of the parasite that also have higher susceptibility to PM in the form responsible for human disease (Kulshrestha et al., 2011; Rastrojo et al., 2018; Utaile et al., 2013). The only exception, described here, was the *L. (V.) guyanensis* ER192 isolate that was more susceptible in promastigotes than intracellular amastigotes. A direct correlation of *in vitro* susceptibility was found between the intracellular amastigote and promastigote stages of the isolates and reference strains, indicating that the promastigote form may eventually be used for determining the activity of PM against the parasite, although the intracellular amastigote stage must be prioritized.

Intraspecies variation in EC_{50} values was described in clinical isolates of *L. (L.) amazonensis* and *L. (V.) braziliensis*. These findings corroborate previous reports that describe variation in PM susceptibility in species and isolates of *Leishmania* (de Morais-Teixeira et al., 2014; Kulshrestha et al., 2011; Utaile et al., 2013). In a panel of isolates of *L. (L.) aethiopica*, a species responsible for different clinical forms of tegumentary leishmaniasis (van Henten et al., 2018), a significant

variation in susceptibility among the isolates was described (Utaile et al., 2013), as observed here in *L. (L.) amazonensis*. In *L. (L.) amazonensis* isolates, a significantly low AI was found, indicating that these isolates are highly susceptible to PM when compared to the reference strains (around 100-fold more susceptible). Isolates of *L. (V.) braziliensis* had an AI that ranged from 0.23 to 2.3 and a lower variation in susceptibility to PM was found in these isolates. In *L. (L.) donovani*, susceptibility to PM was similar among isolates from low and high resistance zones to antimony and from endemic and non-endemic regions of India (Kulshrestha et al., 2011; Prajapati et al., 2012). It is important to state that the susceptibility to drugs in intracellular amastigotes of *Leishmania* spp., including PM, is dependent on the host cell that affects the activity of the drug and also the rate of infection of the parasites (Maia et al., 2007; Seifert et al., 2010; Zauli-Nascimento et al., 2010). This fact can explain why in *L. (L.) donovani* for example, the susceptibility to PM using different host cells may range from 1.2 to $>$ 300 μM at the intracellular amastigote stage (Jhingran et al., 2009; Kulshrestha et al., 2011; Seifert et al., 2010). Here, BMDMs were used for *in vitro* studies and the rate of infection ranged from 9% to 85% of infection among isolates and reference strains.

It would also be interesting to evaluate whether there is a correlation between the low PM *in vitro* susceptibility and unresponsiveness to the *in vivo* treatment. Recently, we have described a direct correlation between the intrinsic *in vitro* susceptibility and the outcome of the treatment, using two lines of *L. (L.) amazonensis* used in this study, the M2269 reference strain and the isolate ER256 (Coser et al., 2020). These two lines have more than 100-fold difference between EC_{50} values in intracellular amastigotes (61 and 0.54 μM for the M2269 strain and the ER256 isolate respectively). Interestingly, in a strain of *L. (L.) mexicana* with low susceptibility to PM in intracellular amastigotes (EC_{50} $>$ 360 μM), no clinical or parasitological response was found (Wijnant et al., 2017). These findings indicate that the *in vitro* susceptibility in intracellular amastigotes may be an indicator of effectiveness of PM *in vivo*.

In conclusion, we have demonstrated the activity of PM against a panel of clinical isolates and reference strains of species responsible for tegumentary disease in Brazil. This drug is not approved for use in Brazil and this study may contribute to the evaluation of its potential use in the chemotherapy of the disease.

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CRediT authorship contribution statement

Elizabeth M. Coser: Conceptualization, Investigation, Methodology, Data curation, Writing - review & editing. **Bianca A. Ferreira:** Investigation, Data curation. **Edite H. Yamashiro-Kanashiro:** Investigation, Resources. **José Angelo L. Lindoso:** Investigation, Resources, Writing - review & editing. **Adriano C. Coelho:** Conceptualization, Methodology, Data curation, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors of this study declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105806.

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