

In Vitro Sensitivity of Cutaneous Leishmania Promastigote Isolates Circulating in French Guiana to a Set of Drugs.

Marine Ginouvès, Stéphane Simon, Mathieu Nacher, Magalie Pierre Demar, Bernard Carme, Pierre Couppié, Ghislaine Prévot

▶ To cite this version:

Marine Ginouvès, Stéphane Simon, Mathieu Nacher, Magalie Pierre Demar, Bernard Carme, et al.. In Vitro Sensitivity of Cutaneous Leishmania Promastigote Isolates Circulating in French Guiana to a Set of Drugs.. American Journal of Tropical Medicine and Hygiene, 2017, [Epub ahead of print]. 10.4269/ajtmh.16-0373. inserm-01481512

HAL Id: inserm-01481512 https://inserm.hal.science/inserm-01481512

Submitted on 2 Mar 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés. In order to provide our readers with timely access to new content, papers accepted by the American Journal of Tropical Medicine and Hygiene are posted online ahead of print publication. Papers that have been accepted for publication are peer-reviewed and copy edited but do not incorporate all corrections or constitute the final versions that will appear in the Journal. Final, corrected papers will be published online concurrent with the release of the print issue.

GINOUVÈS AND OTHERS

LEISHMANIA SENSITIVITY IN FRENCH GUIANA

In Vitro Sensitivity of Cutaneous *Leishmania* Promastigote Isolates Circulating in French Guiana to a Set of Drugs

Marine Ginouvès,^{1,2}* Stéphane Simon,^{1,2} Mathieu Nacher,^{1,3} Magalie Demar,^{1,2,4} Bernard Carme,¹ Pierre Couppié,^{1,5} and Ghislaine Prévot¹

¹Laboratoire des Ecosystèmes Amazoniens et Pathologie Tropicale (EPaT), Université de Guyane, Labex CEBA, DFR de médecine, Cayenne, French Guiana; ²Laboratoire Associé, Centre National de Référence Leishmania, Laboratoire Hospitalo-Universitaire de Parasitologie et Mycologie, General Hospital, Cayenne, French Guiana; ³Centre d'Investigation Clinique Epidémiologie Clinique Antilles Guyane CIC EC 1424, General Hospital, Cayenne, French Guiana; ⁴Laboratoire Hospitalo-Universitaire de Parasitologie et Mycologie, General Hospital, Cayenne, French Guiana; ⁵Service de Dermatologie, Institut Guyanais de Dermatologie Tropicale, General Hospital, Cayenne, French Guiana

* Address correspondence to Marine Ginouvès, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, EA 3593, Labex CEBA, University of French Guiana, Cayenne, French Guiana. E-mail: marine.ginouves@univ-guyane.fr

Abstract.

Anti-leishmaniasis drug resistance is a common problem worldwide. The aim of this study was to inventory the general in vitro level of sensitivity of *Leishmania* isolates circulating in French Guiana and to highlight potential in vitro pentamidine-resistant isolates. This sensitivity study was conducted on 36 patient-promastigote isolates for seven drugs (amphotericin B, azithromycin, fluconazole, meglumine antimoniate, miltefosine, paromomycin, and pentamidine) using the Cell Counting Kit-8 viability test. The IC₅₀ values obtained were heterogeneous. One isolate exhibited high IC₅₀ values for almost all drugs tested. Pentamidine, which is the first-line treatment in French Guiana, showed efficacy at very low doses (mean of 0.0038 μ g/mL). The concordance of the in vitro pentamidine results with the patients' clinical outcomes was 94% (*K* = 0.82).

INTRODUCTION

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis worldwide with 0.7–1.3 million new cases each year.¹ In the New World, it is mainly caused by *Leishmania (Viannia) braziliensis, Leishmania (Leishmania) amazonensis*, and *Leishmania (Viannia) guyanensis*. CL and disseminated cutaneous leishmaniasis (DCL) potentially cause disfiguring scars, and muco-cutaneous leishmaniasis (MCL) can potentially cause obstruction or destruction of the nose, pharynx, and larynx. Treatments are available to cure this infection, but therapeutic options are threatened by the emergence of resistant strains.

Many drugs used as first- or second-line treatment are approved by the World Health Organization (WHO) and are used in different world regions, depending on the species and the clinical presentation. The conventional treatments based on meglumine antimoniate, sodium stibogluconate, and pentamidine cause severe side effects and require parenteral administration. Visceral leishmaniasis (VL) has become increasingly drug resistant with a non-response rate to antimonials of over 60% in Bihar, India.² In CL, the efficacy of antimonials varies: 94.2% in Bolivia,³ 84% in Brazil,⁴ 75.6–78% in Peru,^{5,6} and 61–67% in Colombia.⁷ The efficacy of pentamidine is 35% in Peru.⁶ Other treatments have been used, such as amphotericin B as a first- or second-line treatment to cure VL, MCL, and CL. Trials have shown the effectiveness of a less toxic form, liposomal amphotericin B (Ambisome[®]), in the treatment of CL.^{8–11}

The efficacy of azithromycin against CL ranges from an 85% cure rate in *L. braziliensis* infections in Araçuaí and Varzelândia, Brazil¹² to 45.5% for the same species in Salta, Argentina.¹³ Fluconazole has good efficacy, which increases with the given dose, for the treatment of *L. major* infections.^{14,15} High doses of fluconazole are required for *L. braziliensis* infections.¹⁶ The miltefosine cure rate varies from 82% in Guatemala to 33% in Colombia.¹⁷ The cure rate is dose dependent and can reach 94%¹⁸ for New World strains. The use of injectable paromomycin is not effective against CL.^{19,20} However, its

topical use as an ointment cured CL caused by species of the New and Old World, especially when supplemented with gentamicin.^{21–25} However, the efficacy may vary.²⁶

Until 1980, meglumine antimoniate (Glucantime[®]) was the first intention treatment of leishmaniasis in French Guiana. It was replaced in 1980 by pentamidine (Lomidine[®]) and since 1992, by pentamidine isethionate (Pentacarinat[®]), for *L. guyanensis* infections. Recurrences were reported in French Guiana in two studies. One reported a relapse rate of 6.8% for *L. guyanensis* in 219 patients followed from 1981 to 1987.²⁷ The other reported a 33% recurrence rate in 21 military patients monitored between 2004 and 2005.²⁸ The mechanism of late recurring leishmaniasis is poorly understood. Several mechanisms may be involved, such as late onset reactivation of persistent living parasites or the presence of *Leishmania* clones with lower drug sensitivity within isolates.

The annual incidence of CL in French Guiana is 0.5 ‰, with 86.2% of cases due to *L. guyanensis*, 9.7% due to *L. braziliensis*, 2.8% due to *L. amazonensis*, and 1.3% due to *L.(Viannia) lainsoni* (Simon and others, submitted). The first-line treatment against the predominant species is pentamidine²⁹ and the second-line treatment is meglumine antimoniate for *L. braziliensis* infections. Some cases of clinical resistance to these treatments have been reported in French Guiana.^{27,28}

This study aimed to determine the levels of in vitro sensitivity of *Leishmania* spp. isolates circulating in French Guiana to available treatments, and the pentamidine threshold resistance value. We performed in vitro *Leishmania* spp. sensitivity tests, using promastigote forms, for seven drugs: amphotericin B, azithromycin, fluconazole, meglumine antimoniate, miltefosine, paromomycin, and pentamidine.

MATERIALS AND METHODS

Parasites and cultures.

There were 221 patients consulting the dermatology department of Cayenne hospital or one of the health centers across French Guiana between April 2013 and May 2014, who were diagnosed as *Leishmania* positive using the polymerase chain reaction restriction fragment length polymorphism identification technique.³⁰ Biopsies collected from patients for diagnosis were cultured at 26°C in RPMI 1640 medium (Gibco[®]) containing L-glutamine, 20 mM HEPES, and phenol red, supplemented with 20% heat-inactivated fetal calf serum (Gibco), 50 IU/mL penicillin (Invitrogen[®]), 0.05 mg/mL streptomycin (Invitrogen), and nonessential amino acids (Gibco). Thirty-six culture isolates, representing approximately 28.7% of all annual cases, were suitable for drug-sensitivity tests.

The MHOM/GF/97/LBC6 reference L. guyanensis strain was originally from French Guiana.

Drugs.

The stock concentrations of drugs were 250 μ g/mL for amphotericin B (liquid solution, Sigma-Aldrich), 30 mg/mL for azithromycin (Sigma-Aldrich) diluted in ethanol, 100 mg/mL for fluconazole (Sigma-Aldrich) diluted in DMSO, 300 mg/mL for meglumine antimoniate (liquid solution supplied by the CHC, Glucantime, Aventis), 1.25 mg/mL for miltefosine (Sigma-Aldrich) diluted in ethanol, 50 mg/mL for paromomycin (Sigma-Aldrich) diluted in sterile water, and 100 mg/mL for pentamidine (Sigma-Aldrich) diluted in sterile water. Solutions were stored at -20° C.

The optimal concentration ranges were first determined for each drug. Based on these results, serial 2-fold dilutions were performed to obtain the final testing concentrations, which were $0.78-25 \ \mu g/mL$ for amphotericin B, $93.75-3,000 \ \mu g/mL$ for azithromycin, $312.5-10,000 \ \mu g/mL$ for fluconazole, $937.5-30,000 \ \mu g/mL$ for meglumine antimoniate, $3.9-125 \ \mu g/mL$ for miltefosine, $156.25-5,000 \ \mu g/mL$ for paromomycin, and $0.00039-0.0125 \ \mu g/mL$ for pentamidine.

In vitro promastigote sensitivity tests.

Leishmania promastigotes were cultured in different media: either in RPMI 1640 medium (Gibco) containing L-glutamine, 20 mM HEPES, without phenol red, supplemented with 10% heat-inactivated fetal calf serum (Gibco), 50 IU/mL penicillin (Invitrogen), 0.05 mg/mL streptomycin (Invitrogen), nonessential amino acids (Gibco), which was further supplemented with 0.6 mg/mL L-Biopterin (Santa Cruz Biotechnology[®]) and 5 mg/mL Hemin Chloride (Santa Cruz Biotechnology) (called R-BH medium) or not (called R medium); or in Schneider's drosophila medium (Sigma[®]) containing Lglutamine and supplemented with 10% heat-inactivated fetal calf serum (Gibco), 0.6 mg/mL L-Biopterin (Santa Cruz Biotechnology), and 5 mg/mL Hemin Chloride (Santa Cruz Biotechnology) (called S medium). Viability tests were performed in triplicate using the Cell Counting Kit-8 assay (Sigma), according to the procedure of Ginouves and others.³¹ Briefly, 10⁶ parasites/well, in the exponential growth phase, were placed in contact with different drug concentrations in a 96-well plate for 48 hours at 26°C. Then, 10% of WST-8 was added and the parasites were incubated for a further 24 hours at 26°C. Absorbance was measured at 450 nm using a Tristar LB941 spectrophotometer (Berthold Technologies[®]) or Multiskan (Thermo Scientific[®]). The percentage of inhibition was obtained as follows: % inhibition = $[(A_{\text{control}} - A_{\text{test}})/A_{\text{control}}] \times 100$. The 50% inhibitory concentration (IC₅₀) was calculated using GraphPad Prism6[®] software.

Ethical aspects.

The study of patient outcomes was retrospective and monocentric. All patients were informed using written documents and posters during consultation that case records and biological data might be further used in research and that they had the right to refuse. The monocentric audit of retrospective anonymized case record data is permitted by the CNIL (National Commission for Informatics and Liberties) (number 1805118v0), and the project did not raise any specific concerns by the Ethical Committee at Cayenne General Hospital.

Statistical analysis.

The 95% confidence interval (95% CI) was determined using GraphPad Prism6 software for each test. Kappa test values were determined using STATA[®], to assess the concordance between the in vitro phenotype "susceptible or resistant" to pentamidine and patient outcomes.

RESULTS

Leishmania sensitivity levels.

The in vitro sensitivity tests performed on the 36 promastigote isolates included 33 isolates of *L. guyanensis*, two of *L. braziliensis*, and one of *L. amazonensis* (Table 1). IC₅₀ values varied widely: they ranged from 1.03 (or even < 0.78) to 23.89 (> 25) μ g/mL for amphotericin B, 35.15 to 192 (or even > 3,000) μ g/mL for azithromycin, 830.7 to 4,638 (> 5,000) μ g/mL for fluconazole, 1,597 (< 937.5) to 18,699 (> 30,000) μ g/mL for meglumine antimoniate, 1.55 to 11.7 (> 125) μ g/mL for miltefosine, 48.12 to 4,461 (> 5000) μ g/mL for paromomycin, and 0.001 to 0.0094 (> 0.01) μ g/mL for pentamidine (Table 1).

The reference strain, a strain isolated in 1997 in French Guiana, allowed assessment of the evolution of the sensitivity of patient isolates. Moreover, this strain had the lowest IC_{50} and was, therefore, considered to be the sensitive reference strain for interpretation of the IC_{50} results.

 IC_{50} values for patient isolates were variable relative to the reference strain values, except for fluconazole, for which the values were close to those of the reference strain. One isolate, number 19, was of interest because it presented high IC_{50} values for four of the five drugs tested (amphotericin B, meglumine antimoniate, miltefosine, and pentamidine) and the corresponding patient was also very difficult to treat.

Various media were used in this study. RPMI medium was first used, because of its use in parasite cultures from diagnostic biopsies. The parasites are generally difficult to maintain in vitro. The culture medium was thus improved by adding essential factors, such as biopterin^{32,33} and hemin chloride,³⁴ to the RPMI medium and another *Leishmania* medium, Schneider's Drosophila medium. The addition of these essential factors improved parasite growth, but may have influenced the drug sensitivity of the isolates.³⁵ Indeed, we tested two patient isolates with the three different media and observed large differences depending on the drug tested for one of the two isolates, from 10 to more than 100 times (data not shown). These differences may result from the influence of the medium composition on drug activity.³⁵ However, the IC₅₀ values obtained in this study were globally in the same range, suggesting that the media were generally equivalent.

The lowest concentrations required for the in vitro tests were for pentamidine (average of 0.0038 μ g/mL), miltefosine, and amphotericin B (average of 3.00 and 5.81 μ g/mL, respectively).

Phenotypic variation and culture conditions.

We performed sensitivity tests at two random time intervals with four *L. guyanensis* isolates in R-BH medium (Table 2) to estimate the temporal phenotypic variability of *Leishmania* isolates. The random variability of the IC_{50s} did not appear to result from the time in culture (each with low passages of 1–4), but was associated with the drug tested. This variability was particularly pronounced for azithromycin (up to a 1.107-fold difference).

Comparison of in vitro sensitivity to pentamidine and clinical outcome.

The in vitro sensitivity of *Leishmania* to pentamidine was related to clinical features (Table 1). Isolates from patients cured after a single course of pentamidine were considered to be sensitive. Patients who received a single course of pentamidine isethionate and did not consult again were considered to be cured (in previous studies conducted at the reference center for leishmaniasis treatment in French Guiana, patients indicated that they were better and did not see the point in returning when asked why they failed to come to their control consultation³⁶) and the isolate to be sensitive. Isolates from patients who were cured after two courses of pentamidine were considered to be intermediate. Isolates from patients who were cured after three or more courses of pentamidine were considered to be resistant.

There was a strong correlation (r = 0.94 [17/18 without considering the intermediate status; K = 0.82]) between the in vitro results and patient outcomes.

DISCUSSION

This first study on the anti-leishmanial drug sensitivity of cutaneous *Leishmania* isolates from French Guiana showed great heterogeneity between isolates, and revealed one in vitro-resistant isolate to four of the five drugs tested.

We performed the tests using the promastigote form, because it was the easiest form to handle on a large scale and allowed us to make a first assessment of the drug sensitivity of the circulating isolates. The promastigote form model is not recommended in the literature for in vitro sensitivity tests because several parameters can influence the sensitivity results (such as cell density, growth rates, the drug tested, medium composition)³⁵; it is not the mammalian form, and is generally less sensitive to some drugs or plant compounds, unlike the intracellular amastigote or axenic amastigote forms. Indeed, it appears that intracellular amastigote forms better reflect the observed sensitivity in patients, especially to pentavalent antimonials,³⁷ which require conversion by the host cell to a trivalent form.³⁸ Moreover, promastigote sensitivity has been shown to be variable for the drugs tested, with low sensitivity to paromomycin and higher sensitivity to pentamidine than amastigote^{39,40} or axenic amastigote forms.⁴¹ However, the promastigote and amastigote forms display similar sensitivity to miltefosine and amphotericin.⁴² Though, there is no correlation between in vitro results using promastigotes and patient

clinical outcomes for visceral leishmaniasis antimonial assays, unlike for the amastigote form^{43,44} for which in vitro tests correlate well with clinical outcome. In contrast, Grogl and others showed an 86– 89% correlation coefficient for the patient response to sodium stibogluconate and meglumine antimoniate treatment and in vitro susceptibility of promastigotes from CL and MCL *Leishmania* isolates.⁴⁵ Here, we observed a 94% correlation between the in vitro pentamidine results and patient outcomes. Moreover, there is concordance between in vitro promastigotes and intracellular amastigotes⁴⁶ for antimonials, when they are in the identical environment.⁴⁷

The axenic amastigote form has been suggested to be a possible alternative, because of its morphological and metabolic similarity to the intracellular macrophage amastigote form,⁴⁸ but it shares the same drawback with the promastigote form because of its inability to accumulate drugs as macrophages do.⁴¹ The mammalian intracellular amastigote model has been recommended as the gold standard. However, several factors may bias the response to drugs in this form also, including the type of macrophage used,⁴⁹ the variable macrophage infection rate,⁴⁹ macrophage infectivity depending on the *Leishmania* species,⁵⁰ incomplete intracellular transformation into the amastigote³⁷ and, as with axenic amastigote forms, the long process of adaptation to the environment and transformation, which leads to the selection of subpopulations.^{51,52} In vitro amastigote intracellular results also do not always correlated with the clinical outcome of the patients,⁵³ particularly due to host factors. Finally, this model is inappropriate for large-scale in vitro monitoring of drug efficacy. Overall, each model has its benefits and drawbacks.

As mentioned above, there are some potential limitations in this study. Comparison tests using the intracellular amastigote form may be informative. A larger number of isolates would refine and confirm the promastigote sensitivity threshold for pentamidine (determined to be $\geq 0.009 \ \mu g/mL$ in this study), as well as the in vitro and in vivo consistency. Another important limitation was the large variation in the results of the same isolate when tested in different media, depending on the drugs used, making it challenging to compare the results from one study to another.

Despite these limitations, this study may provide the first baseline to monitor the evolution of the drug sensitivity of *Leishmania* isolates in French Guiana.

Received May 10, 2016.

Accepted for publication November 19, 2016.

Financial support: This work was supported by the University of French Guiana and the Ministère Français de l'Enseignement Supérieur et de la Recherche Scientifique, the Conseil Régional de la Guyane and the European Union (FEDER-Presage N° 31454), and the "Investissement d'Avenir" grant managed by Agence Nationale de la Recherche (CEBA, ref. ANR-10-LABX-25-01).

Conflicts of interest: Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Authors' addresses: Marine Ginouvès and Stéphane Simon, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cavenne, French Guiana, and Laboratoire Associé, Centre National de Référence Leishmania, Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mails: marine.ginouves@univ-guyane.fr and stephane.simon@guyane.univ-ag.fr. Mathieu Nacher, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cayenne, French Guiana, and Centre d'Investigation Clinique Epidémiologie Clinique Antilles Guyane CIC EC 1424, Cayenne General Hospital, Cayenne, French Guiana, E-mail: mathieu.nacher@ch-cayenne.fr. Magalie Demar, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cayenne, French Guiana, Laboratoire Associé, Centre National de Référence Leishmania, Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cavenne, French Guiana, E-mail: magalie.demar@ch-cavenne.fr. Bernard Carme, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cayenne, French Guiana, and Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mail: carme.bernard@wanadoo.fr. Pierre Couppié, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cayenne, French Guiana, and Guianan Institute of Tropical Dermatology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mail: pierre.couppie@ch-cayenne.fr. Ghislaine Prévot, Medicine Department, Ecosystemes Amazoniens et Pathologie Tropicale, Labex CEBA, University of French Guiana, Cayenne, French Guiana, Email: fac.prevot@gmail.com.

REFERENCES

- <eref>1. WHO, 2015. Geneva, Switzerland: World Health Organization. Available at: http://www.who.int/mediacentre/factsheets/fs375/en/. </eref>
- <jrn>2. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, Kumar PCK, Murray HW, 2000. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis 31:* 1104–1107.
- <jrn>3. Bermudez H, Rojas E, Garcia L, Desjeux P, Dujardin JC, Boelaert M, Chappuis F, 2006. Generic sodium stibogluconate is as safe and effective as branded meglumine antimoniate, for the treatment of tegumentary leishmaniasis in Isiboro secure park, Bolivia. *Ann Trop Med Parasitol* 100: 591–600.
- <jrn>4. Oliveira-Neto MP, Schubach A, Mattos M, Goncalves-Costa SC, Pirmez C, 1997. A low-dose antimony treatment in 159 patients with American cutaneous leishmaniasis: extensive follow-up studies (up to 10 years). *Am J Trop Med Hyg* 57: 651–655.
- <jrn>5. Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, Miranda-Verastegui C, Santamaria-Castrellon G, Ramirez L, Lazo M, De Doncker S, Boelaert M, Robays J, Dujardin J, Arevalo J, Chappuis F, 2008. Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clin Infect Dis 46:* 223–231.
- <jrn>6. Andersen EM, Cruz-Saldarriaga M, Llanos-Cuentas A, Luz-Cjuno M, Echevarria J, Miranda-Verastegui C, Colina O, Berman JD, 2005. Comparison of meglumine antimoniate and pentamidine for Peruvian cutaneous leishmaniasis. *Am J Trop Med Hyg 72*: 133–137.
- <jrn>7. Palacios R, Osorio LE, Grajalew LF, Ochoa MT, 2001. Treatment failure in children in a randomized clinical trial with 10 and 20 days of meglumine antimonate for cutaneous leishmaniasis due to *Leishmania Viannia* species. *Am J Trop Med Hyg 64:* 187–193.
- <jrn>8. Brown M, Noursadeghi M, Boyle J, Davidson RN, 2005. Successful liposomal amphotericin B treatment of *Leishmania braziliensis* cutaneous leishmaniasis. *Br J Dermatol 153*: 203–205.
- <jrn>9. Yardley V, Croft SL, 1997. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. Antimicrob Agents Chemother 41: 752–756.
- <jrn>10. Wortmann G, Zapor M, Ressner R, Fraser S, Hartzell J, Pierson J, Weintrob A, Magill A, 2010. Lipsosomal amphotericin B for treatment of cutaneous leishmaniasis. *Am J Trop Med Hyg 83:* 1028–1033.
- <jrn>11. Neves LO, Talhari AC, Gadelha EP, Silva RM Junior, Guerra JA, Ferreira LC, Talhari S, 2011. A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by *Leishmania guyanensis*. *An Bras Dermatol* 86: 1092–1101.
- <jrn>12. Prata A, Silva-Vergara ML, Costa L, Rocha A, Krolewiecki A, Silva JC, de Paula EV, Pimenta FG Junior, Giraldo LE, 2003. Efficacy of azithromycin in the treatment of cutaneous leishmaniasis. *Rev Soc Bras Med Trop 36:* 65–69.
- <jrn>13. Krolewiecki AJ, Romero HD, Cajal SP, Abraham D, Mimori T, Matsumoto T, Juarez M, Taranto NJ, 2007. A randomized clinical trial comparing oral azithromycin and meglumine antimoniate for the treatment of American cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis. Am J Trop Med Hyg* 77: 640–646.
- <jrn>14. Alrajhi AA, Ibrahim EA, De Vol EB, Khairat M, Faris RM, Maguire JH, 2002. Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. N Engl J Med 346: 891– 895.

- <jrn>15. Emad M, Hayati F, Fallahzadeh MK, Namazi MR, 2011. Superior efficacy of oral fluconazole 400 mg daily versus oral fluconazole 200 mg daily in the treatment of cutaneous *Leishmania major* infection: a randomized clinical trial. *J Am Acad Dermatol 64:* 606–608.
- <jrn>16. Sousa AQ, Frutuoso MS, Moraes EA, Pearson RD, Pompeu MM, 2011. High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to *Leishmania (Vianna) braziliensis*. *Clin Infect Dis* 53: 693–695.
- <jrn>17. Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, Luz M, Gutierrez P, Arboleda M, Berman JD, Junge K, Engel J, Sindermann H, 2004. Miltefosine for new world cutaneous leishmaniasis. *Clin Infect Dis 38:* 1266–1272.
- <jrn>18. Soto J, Toledo J, Gutierrez P, Nicholls RS, Padilla J, Engel J, Fischer C, Voss A, Berman J, 2001. Treatment of american cutaneous leishmaniasis with miltefosine, an oral agent. *Clin Infect Dis* 33: E57–E61.</jrn>
- <jrn>19. Hepburn NC, Tidman MJ, Hunter JA, 1994. Aminosidine (paromomycin) versus sodium stibogluconate for the treatment of American cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg* 88: 700–703.
- <jrn>20. Soto J, Grogl M, Berman J, Olliaro P, 1994. Limited efficacy of injectable aminosidine as single-agent therapy for Colombian cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg 88:* 695– 698.
- <jrn>21. Ben Salah A, Ben Messaoud N, Guedri E, Zaatour A, Ben Alaya N, Bettaieb J, Gharbi A, Belhadj Hamida N, Boukthir A, Chlif S, Abdelhamid K, El Ahmadi Z, Louzir H, Mokni M, Morizot G, Buffet P, Smith PL, Kopydlowski KM, Kreishman-Deitrick M, Smith KS, Nielsen CJ, Ullman DR, Norwood JA, Thorne GD, McCarthy WF, Adams RC, Rice RM, Tang D, Berman J, Ransom J, Magill AJ, Grogl M, 2013. Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *N Engl J Med 368:* 524–532.
- <jrn>22. Ben Salah A, Buffet PA, Morizot G, Ben Massoud N, Zaatour A, Ben Alaya N, Haj Hamida NB, El Ahmadi Z, Downs MT, Smith PL, Dellagi K, Grogl M, 2009. WR279,396, a third generation aminoglycoside ointment for the treatment of *Leishmania major* cutaneous leishmaniasis: a phase 2, randomized, double blind, placebo controlled study. *PLoS Negl Trop Dis 3:* e432.
- <jrn>23. El-On J, Jacobs GP, Witztum E, Greenblatt CL, 1984. Development of topical treatment for cutaneous leishmaniasis caused by *Leishmania major* in experimental animals. *Antimicrob Agents Chemother 26:* 745–751.
- <jrn>24. Grogl M, Schuster BG, Ellis WY, Berman JD, 1999. Successful topical treatment of murine cutaneous leishmaniasis with a combination of paromomycin (aminosidine) and gentamicin. J Parasitol 85: 354–359.
- <jrn>25. Armijos RX, Weigel MM, Calvopina M, Mancheno M, Rodriguez R, 2004. Comparison of the effectiveness of two topical paromomycin treatments versus meglumine antimoniate for new world cutaneous leishmaniasis. *Acta Trop 91*: 153–160.
- <jrn>26. Soto J, Fuya P, Herrera R, Berman J, 1998. Topical paromomycin/methylbenzethonium chloride plus parenteral meglumine antimonate as treatment for American cutaneous leishmaniasis: controlled study. *Clin Infect Dis* 26: 56–58.
- <jrn>27. Dedet JP, Pradinaud R, Gay F, 1989. Epidemiological aspects of human cutaneous leishmaniasis in French Guiana. *Trans R Soc Trop Med Hyg 83:* 616–620.
- <jrn>28. Gangneux JP, Sauzet S, Donnard S, Meyer N, Cornillet A, Pratlong F, Guiguen C, 2007. Recurrent American cutaneous leishmaniasis. *Emerg Infect Dis 13:* 1436–1438.

- <jrn>29. Nacher M, Carme B, Sainte Marie D, Couppie P, Clyti E, Guibert P, Pradinaud R, 2001. Influence of clinical presentation on the efficacy of a short course of pentamidine in the treatment of cutaneous leishmaniasis in French Guiana. *Ann Trop Med Parasitol 95:* 331–336.
- <jrn>30. Simon S, Veron V, Carme B, 2010. *Leishmania* spp. identification by polymerase chain reaction–restriction fragment length polymorphism analysis and its applications in French Guiana. *Diagn Microbiol Infect Dis* 66: 175–180.
- <jrn>31. Ginouves M, Carme B, Couppie P, Prevot G, 2014. Comparison of tetrazolium salt assays for evaluation of drug activity against *Leishmania* spp. *J Clin Microbiol* 52: 2131–2138.
- <jrn>32. Trager W, 1969. Pteridine requirement of the hemoflagellate *Leishmania tarentolae*. J Protozool 16: 372–375.
- <jrn>33. Jain M, Dole VS, Myler PJ, Stuart KD, Madhubala R, 2007. Role of biopterin transporter (BT1) gene on growth and infectivity of *Leishmania*. *AJBB 3:* 199–206.
- <jrn>34. Pal JK, Joshi-Purandare M, 2001. Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in *Leishmania donovani* promastigotes cultured in vitro. *J Biosci 26:* 225–231.
- <jrn>35. Moreira ES, Soares RM, Petrillo-Peixoto Mde L, 1995. Glucantime susceptibility of Leishmania promastigotes under variable growth conditions. Parasitol Res 81: 291–295.
- <jrn>36. Nacher M, Carme B, Sainte Marie D, Couppie P, Clyti E, Guibert P, Pradinaud R, 2001. Seasonal fluctuations of incubation, healing delays, and clinical presentation of cutaneous leishmaniasis in French Guiana. *J Parasitol 87:* 1495–1498.
- <jrn>37. Vermeersch M, da Luz RI, Tote K, Timmermans JP, Cos P, Maes L, 2009. In vitro susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother 53:* 3855–3859.
- <jrn>38. Croft SL, Sundar S, Fairlamb AH, 2006. Drug resistance in leishmaniasis. *Clin Microbiol Rev* 19: 111–126.
- <edb>39. Neal RA, 1989. Experimental chemotherapy. Killick-Kendrick R, Peters N, eds. *The Leishmaniases*. London, UK: Academic Press, 793–845.</edb>
- <jrn>40. Berman JD, Wyler DJ, 1980. An in vitro model for investigation of chemotherapeutic agents in leishmaniasis. J Infect Dis 142: 83–86.
- <jrn>41. Sereno D, Lemesre JL, 1997. Axenically cultured amastigote forms as an in vitro model for investigation of antileishmanial agents. *Antimicrob Agents Chemother 41:* 972–976.
- <jrn>42. Kumar D, Kulshrestha A, Singh R, Salotra P, 2009. In vitro susceptibility of field isolates of *Leishmania donovani* to miltefosine and amphotericin B: correlation with sodium antimony gluconate susceptibility and implications for treatment in areas of endemicity. *Antimicrob Agents Chemother 53*: 835–838.
- <jrn>43. Lira R, Sundar S, Makharia A, Kenney R, Gam A, Saraiva E, Sacks D, 1999. Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimonyresistant strains of *Leishmania donovani*. J Infect Dis 180: 564–567.
- <jrn>44. Ibrahim ME, Hag-Ali M, el-Hassan AM, Theander TG, Kharazmi A, 1994. *Leishmania* resistant to sodium stibogluconate: drug-associated macrophage-dependent killing. *Parasitol Res 80:* 569–574.

- <jrn>45. Grogl M, Thomason TN, Franke ED, 1992. Drug resistance in leishmaniasis: its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. *Am J Trop Med Hyg 47:* 117– 126.
- <jrn>46. Azeredo-Coutinho RB, Mendonca SC, Callahan H, Portal AC, Max G, 2007. Sensitivity of *Leishmania braziliensis* promastigotes to meglumine antimoniate (glucantime) is higher than that of other *Leishmania* species and correlates with response to therapy in American tegumentary leishmaniasis. *J Parasitol 93:* 688–693.
- <jrn>47. Berman JD, Edwards N, King M, Grogl M, 1989. Biochemistry of pentostam resistant Leishmania. Am J Trop Med Hyg 40: 159–164.
- <jrn>48. Gupta N, Goyal N, Rastogi AK, 2001. In vitro cultivation and characterization of axenic amastigotes of *Leishmania*. *Trends Parasitol 17*: 150–153.
- <jrn>49. Seifert K, Escobar P, Croft SL, 2010. In vitro activity of anti-leishmanial drugs against Leishmania donovani is host cell dependent. J Antimicrob Chemother 65: 508–511.
- <jrn>50. Zauli-Nascimento RC, Miguel DC, Yokoyama-Yasunaka JK, Pereira LI, Pelli de Oliveira MA, Ribeiro-Dias F, Dorta ML, Uliana SR, 2010. In vitro sensitivity of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* Brazilian isolates to meglumine antimoniate and amphotericin B. *Trop Med Int Health 15:* 68–76.
- <jrn>51. Grogl M, Oduola AM, Cordero LD, Kyle DE, 1989. *Leishmania* spp.: development of pentostam-resistant clones in vitro by discontinuous drug exposure. *Exp Parasitol 69:* 78–90.
- <jrn>52. Croft SL, 2001. Monitoring drug resistance in leishmaniasis. Trop Med Int Health 6: 899-905.</jrn>
- <jrn>53. Yardley V, Ortuno N, Llanos-Cuentas A, Chappuis F, Doncker SD, Ramirez L, Croft S, Arevalo J, Adaui V, Bermudez H, Decuypere S, Dujardin JC, 2006. American tegumentary leishmaniasis: is antimonial treatment outcome related to parasite drug susceptibility? *J Infect Dis 194:* 1168–1175.

				0 5					Proma	stigote IC	50						
Species	Mediu m	Isolate numbe	Amphotericin B		Azithromycin		Fluconazole		Meglumine antimoniate		Miltefosine		Paromomycin		Pentamidine		Clinical
			μg/m L	95% CI	μg/m L	95% CI	μg/m L	95% CI	µg/mL	95% CI	μg/m L	95% CI	µg/mL	95% CI	µg/mL	95% CI	outcome§
		1	4.16	3.50– 4.94	> 3,000	_	3,759	3,252 - 4,345	9,510	8,768– 10,316	3.91	3.25– 4.34	< 156.25	_	0.0013	0.012– 0.014	Sensitive presumed
		2	11.51	11.17– 11.87	> 3,000	_	5,000 - 10,00 0	_	7,500– 15,000	_	3.9– 7.8	_	< 156.25	_	0.00078 -0.0015	_	Sensitive
		3	4.19	3.52– 4.98	192.4	175.9 _ 210.4	3,607	495.2 - 26,26 7	5,222	3,666– 7,438	< 3.9	_	< 156.25	_	0.0011	0.0009– 0.001	_
		4	1.87	1.63– 2.15	_	_	_	_	3,057	2,711– 3,447	< 3.9	_	1,211	982.7 - 1.492	_	_	Sensitive
L. guyanensis	R	5	1.56– 3.12	_	_	_	_	_	2,781	2,103– 3,676	< 3.9	_	> 5,000	_	_	_	Sensitive presumed
		6	1.58	1.43– 1.74	_	_	_	_	3,353	3,065– 3,669	< 3.9	_	701.8	542.7 - 907.4	_	-	_
		7	5.38	4.70– 6.16	_	_	_	_	3,034	2,860– 3,218	< 3.9	_	1,154	928.8 - 1,434	0.0065	_	Sensitive
		8	1.89	1.61– 2.21	_	_	_	_	2,562	1,009– 6,504	< 3.9	_	2,314	826.1 - 6,484	> 0.01	-	Resistant
		9	2.57	2.39– 2.77	_	_	_	_	2,802	2,458– 3,193	< 3.9	_	953.4	365.1 - 2,489	0.004	0.004– 0.005	Sensitive
		10	0.78– 1.56	-	-	_	-	-	< 937.5	_	5.27	4.69– 5.92	< 156.25	_	0.0025	0.002– 0.003	Sensitive
		11	9.81	8.86– 10.85	_	_	_	_	15,000 - 30,000	_	10.53	7.97– 13.90	96.27	53.84 	0.0056	0.005– 0.006	Sensitive presumed

		1 / 1 1	1 1.1 1 1
I cont Loishmania isolates and Loishmania	<i>nuvanensis</i> reference strain acco	ording to the medium use	d and the drug tested
1050 of Detstimatia Isolates and Detstimatia	<i>suyunensis</i> reference strain deeb	nume to the meanum use	a and the drug tested

TABLE 1

		12	6.25– 12.5	_	_	_	_	_	15,000 - 30,000	_	10.63	9.10– 12.42	359.7	338.5 - 382.2	0.006	0.003– 0.01	_
		13	1.56– 3.12	_	_	_	_	_	1,597	1,494– 1,708	_	_	269.7	237.1 _ 306.7	0.0094	0.003– 0.02	Resistant
		14	1.03	0.85– 1.23	_	_	_	_	< 937.5	_	< 3.9	_	56.47	39.93 _ 79.86	0.0059	0.005– 0.006	Sensitive
		15	3.76	3.12– 4.53	_	_	_	_	1,875– 3,750	_	6.64	5.33– 8.26	98.12	89.52 - 107.5	0.0057	0.005– 0.006	Sensitive
		Ref*	1.4	1.31– 1.492	< 93.75	_	3,117	3,016 - 3,222	10,705	9,830– 11,657	3.9– 7.8	-	110	75.5– 160.3	0.00211 9	0.0017- 0.0025	
Isolates interv medi	val value ium†	es in R	1.03– 12.5		192.4 -> 3,000		3,607 - 10,00 0		< 937.5– 30,000		< 3.9– 10.63		< 156.25 -> 5.000		0.001-> 0.01		
		16	6.25– 12.5	_	_	_	_	_	15,000 	_	> 125	_	48.12	14.82 - 156.3	0.0041	0.003– 0.004	_
		17	23.89	0.3079 -1,854	_	_	_	_	> 30,000	_	10.6	9.17– 12.26	< 156.25	_	0.00312 - 0.00625	-	Sensitive presumed
		18	6.25– 12.5	_	_	_	_	_	13339	10,841 - 16,412	7.6	6.89– 8.39	< 156.25	_	0.003	0.002– 0.003	Sensitive
L. guyanensis	S	19	> 25	_	_	_	_	_	> 30,000	_	> 125	_	267.3	241.9 295.2	> 0.01	_	Resistant
		20	_	_	_	_	_	_	_	_	< 3.9	_	< 156.25	_	0.00312 - 0.00625	-	Sensitive presumed
		21	3.12– 6.25	_	_	_	_	_	_	_	9.95	8.17– 12.13	< 156.25	_	0.0025	0.0022– 0.0027	Resistant
		22	3.1	_	_	_	_	_	< 937.5	_	6.66	5.92– 7.50	< 156.25	_	0.0033	0.002– 0.005	_
		23	3.12- 6.25		> 30,000	_	7.8– 15.6	_	< 156.25	_	0.0026	0.001– 0.004	Sensitive				
		24	3.12– 6.25	_	_	_	_	_	18,699	4,757– 73,501	11.7	10.81 - 12.66	< 156.25	_	0.0036	0.0033– 0.0039	Intermedia te

		25	6.25– 12.5	_	_	_	_	_	-	_	3.09	2.57– 3.72	98.52	34.45 - 281.7	0.0015	0.0013– 0.0019	Intermedia te
		26	3.92	_	_	_	_	_	< 937.5	_	1.55	0.17– 13.96	< 156.25	_	0.0033	0.002– 0.004	_
		27	4.93	4.82– 5.04	102.3	88.52 - 118.2	3,145	2,995 - 3,303	18,642	8,707– 39,913	< 3.9	_	738.9	425.7 - 1,283	0.00312 - 0.00625	_	-
		Ref	2.08	0.002– 2,044	< 93.75	-	< 312.5	-	< 937.5	-	8.85	4.9– 15.9	186.8	134.6 _ 259.2	0.00232 9	0.00084 -0.0064	
Isolates into m	erval value edium†	es in S	3.1-> 25		102.3		3,145		< 937.5– > 30.000		< 3.9–> 125		48.12– 738.9		0.0015- > 0.01		
		28	6.25– 12.5	_	187.5 -375	_	2,500 - 5,000	_	13,130	12,902 13,362	3.52	3.24– 3.82	159.8	134.5 _ 189.9	0.00312 - 0.00625	_	Sensitive presumed
		29	4.41	4.11– 4.73	35.15	17.68 _ 69.89	2,993	2,951 	7,349	4,966– 10,875	< 3.9	_	358.1	275.5 _ 465.4	0.0015– 0.00312	_	_
		30	13.31	12.71– 13.94	83.72	74.91 _ 93.58	3,327	3,244 	_	_	1.61	0.52– 5.01	714.3	611.7 - 834.2	0.0035	0.003– 0.004	Sensitive presumed
L. guyanensis	R-BH	31	> 25	_	177.7	139.3 _ 226.5	4,638	3,505 - 6,138	> 30,000	_	< 3.9	_	4,461	303.6 - 65,55 1	0.0028	0.002– 0.003	_
		32	3.29	3.21– 3.37	< 93.75	_	2,011	1,893 	4,744	3,343– 6,730	< 3.9	_	_	_	0.0015– 0.00312	_	_
		33	7.83	7.38– 8.31	< 93.75	_	2,500 - 5,000	_	_	_	_	_	_	_	-	_	Intermedia te
		Ref	2.39	2.27– 2.54	< 93.75	-	2,992	2,773 - 3,228	10,635	9,424– 12,001	6.2	5.9– 6.52	77.89	46.15 - 131.5	0.00253 7	0.0023- 0.0027	
Isolates inte BH	rval values medium†	s in R–	3.29– > 25		< 93.75 –375		2,011 5,000		4,744– > 30,000		1.61– 3.52		159.8– 4,461		0.001– 0.006		
Leishmani a braziliensi	R–BH	34	1.27	1.08– 1.48	83.31	59.73 - 116.2	830.7	687.6 _ 1,004	_	_	3	1.31– 6.85	_	_	0.0016	0.001– 0.002	_

s L. braziliensi s	R	35	< 0.78	_	_	_	_	_	_	_	< 3.9	_	< 156.25	_	0.00625 -0.0125	_	_
Leishmani a amazonens is	R–BH	36	8.35	7.77– 8.98	< 93.75	-	3,105	2,983 	9,822	9,384– 10,282	1.95	0.45– 8.29	129.6	122.1 137.5	0.00312 0.00625	_	_
Total isolates interval values‡			< 0.78– > 25		< 93.75 -> 3,000		830.7 - 10,00 0		4,744– > 30,000		< 3.9–> 125		< 156.25 -> 5,000		0.001-> 0.01		

 $IC_{50} = 50\%$ inhibitory concentration; R = RPMI 1640 medium; R-BH = RPMI 1640 medium + L-Biopterin and Hemin Chloride; S = Schneider medium (+L-Biopterin and Hemin Chloride).

* Ref = Leishmania guyanensis reference strain.

[†] Isolates interval values for each medium according to the drug tested.

‡ Isolates interval values for all medium included, according to the drug tested.

§ Sensitive, cured after one course of pentacarinat; sensitive presumed, presumed cured after one course of pentacarinat (did not consult again); intermediate, cured after two courses of pentacarinat; resistant, cured after three or more courses of pentacarinat.

|--|

Temporal phenotypic variability of Leishmania isolates

			Meglumine													
			Amphotericin B Azithromyc		omycin	ycin Fluconazole		antimoniate		Miltefosine		Paromomycin		Pentamidine		
Isolate	Interval time between 2 tests	Test number	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)	CI50 (µg/mL)	Ratio (test2/ test1)
А	1 month	Test 1 Test 2	4.935 9.147	1.9	102.3 81.5	1.3	3,145 4,076	1.3	18,642 8,357	2.2	0.963 3.12	3.2	738.9 611.7	1.2	0.00319 0.001458	2.2
В	1 month	Test 1 Test 2	4.412 3.79	0.9	35.15 0.000019	0.18.10 ⁷	2,993 3,494	1.2	7,349 4,269	1.7	3.103 0.18	17	358.1 1,006	2.8	0.00278 0.001379	0.5
С	1 month	Test 1 Test 2	3.294 11.968	3.6	9.684 6.1E–07	1.6.10 ⁷	2,011 4,039	2.0	4,744 11,951	2.5	0.1324 0.16	1.2	- < 156.25	_	0.002657 0.00133	0.5
D	3 month	Test 1 Test 2	2.059	-	_ 217.6	-	_ 3,334	-	6,559 16,574	2.5	5.457 4.357	0.8	105.6 568	5.3	$0.00704 \\ 0.00504$	0.7

In bold: highest values among each pair of test (ratio > 1.5).