frontiers | Research Topics

Strategies in the drug discovery and development for leishmaniasis: Immunomodulators, natural products, synthetic compounds, and drug repositioning

Edited by
Taís Fontoura de Almeida, Edezio Ferreira Cunha-Junior, Juliana Da Silva Pacheco, Gabriela Santos-Gomes, Joao Luiz Mendes Wanderley, Suzana Passos Chaves, Valter Viana Andrade-Neto and Lucia Helena Pinto da Silva

Published in
Frontiers in Cellular and Infection Microbiology
Frontiers in Medicine

ISSN 1664-8714
ISBN 978-2-8325-4899-8
DOI 10.3389/978-2-8325-4899-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Strategies in the drug discovery and development for leishmaniasis: Immunomodulators, natural products, synthetic compounds, and drug repositioning

Topic editors

Taís Fontoura de Almeida — Federal University of Rio de Janeiro, Brazil
Edezio Ferreira Cunha-Junior — Federal University of Rio de Janeiro, Brazil
Juliana Da Silva Pacheco — University of Dundee, United Kingdom
Gabriela Santos-Gomes — New University of Lisbon, Portugal
Joao Luiz Mendes Wanderley — Federal University of Rio de Janeiro, Brazil
Suzana Passos Chaves — Federal University of Rio de Janeiro, Brazil
Valter Viana Andrade-Neto — St Michael's Hospital, Canada
Lucia Helena Pinto da Silva — Federal Rural University of Rio de Janeiro, Brazil

Citation

de Almeida, T. F., Cunha-Junior, E. F., Pacheco, J. D. S., Santos-Gomes, G., Wanderley, J. L. M., Chaves, S. P., Andrade-Neto, V. V., Pinto da Silva, L. H., eds. (2024). *Strategies in the drug discovery and development for leishmaniasis: Immunomodulators, natural products, synthetic compounds, and drug repositioning*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4899-8

Table of contents

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 13 March 2024
DOI 10.3389/fcimb.2024.1384244

OPEN ACCESS

EDITED AND REVIEWED BY
Tania F. De Koning-Ward,
Deakin University, Australia

*CORRESPONDENCE
Taís Fontoura de Almeida
taisfalmeida@macae.ufrj.br;
taisfa@hotmail.com

RECEIVED 08 February 2024
ACCEPTED 20 February 2024
PUBLISHED 13 March 2024

CITATION
de Almeida TF, Cunha-Junior EF, Wanderley JLM, Pacheco JDS, Pinto-da-Silva LH, Andrade-Neto VV and Chaves SP (2024) Editorial: Strategies in the drug discovery and development for leishmaniasis: immunomodulators, natural products, synthetic compounds, and drug repositioning.
Front. Cell. Infect. Microbiol. 14:1384244.
doi: 10.3389/fcimb.2024.1384244

Editorial: Strategies in the drug discovery and development for leishmaniasis: immunomodulators, natural products, synthetic compounds, and drug repositioning

Taís Fontoura de Almeida[1]*, Edézio Ferreira Cunha-Junior[2], João Luiz Mendes Wanderley[3], Juliana da Silva Pacheco[4], Lucia Helena Pinto-da-Silva[5], Valter Viana Andrade-Neto[6] and Suzana Passos Chaves[3]

[1]Laboratory of Physiopathology, Biosciences Applied to Health Department, Institute of Medical Sciences, Multidisciplinary Center of Federal University of Rio de Janeiro, Macaé, Brazil, [2]Laboratory of Immunoparasitology, Institute of Pharmaceutical Sciences, Multidisciplinary Center of Federal University of Rio de Janeiro, Macaé, Brazil, [3]Laboratory of Immunoparasitology, Biosciences Applied to Health Department, Institute of Medical Sciences, Multidisciplinary Center of Federal University of Rio de Janeiro, Macaé, Brazil, [4]Drug Discovery Unit, Department of Biological Chemistry and Drug Discovery, School of Life Sciences, University of Dundee, Dundee, United Kingdom, [5]Institute of Veterinary, Department of Veterinary Microbiology and Immunology, Seropédica, RJ, Brazil, [6]St Michael Hospital, Li ka Shing Knowledge Institute, Toronto, ON, Canada

KEYWORDS

treatment, chemotherapy, immunomodulation, nanoparticles, repositioning drugs, flavonoid, protozoa, *Leishmania*

Editorial on the Research Topic

Strategies in the drug discovery and development for leishmaniasis: immunomodulators, natural products, synthetic compounds, and drug repositioning

Leishmaniasis is a group of tropical diseases that affect millions of people worldwide. These diseases are transmitted by vectors and caused by protozoa of the genus *Leishmania*. The WHO reported that in 2020, Leishmaniasis was present in 98 countries or territories, and it posed a severe public health issue in four eco-epidemiological regions: Americas, East Africa, North Africa, West and Southeast Asia. That year, there were reports of 208,357 cases of Cutaneous Leishmaniasis (CL) and 12,838 cases of Visceral Leishmaniasis (VL) (Ruiz-Postigo et al., 2021). For the last 70 years, the available chemotherapy has been constituted by first-line (pentavalent antimonials) and second-line drugs (amphotericin B, pentamidine, paramomycin, and miltefosine). Existing drugs generally have disadvantages related to therapeutic approaches, such as route of administration, the need for trained professionals for administration, toxicity, high cost. Considering the number of pathogenic species concerning therapeutic options, this arsenal still needs to be improved (Burza et al., 2018). The lack of a vaccine or effective chemotherapy has stimulated many studies

involving the research of new molecules or drug repositioning and new strategies and technologies in treating Leishmaniasis with potential microbicidal and immunomodulators.

The approach to developing chemotherapeutic strategies for Leishmaniasis is a comprehensive understanding of the disease's immunology aspects, aiming to identify new drug targets and target the immune response against the protozoa. Magalhães et al. conducted a clinical trial treating patients with meglumine antimoniate plus N-acetylcysteine (SbV+NAC) and with meglumine antimoniate (SbV). The SbV+NAC group exhibited elevated soluble CD40-ligand levels, negatively correlated with interleukin-10 (IL-10). NAC reduced IL-10 production in monocytes and altered T-cell responses in stimulated peripheral blood mononuclear cells. Although the TNF-α/IL-10 ratio increased, suggesting an immunomodulatory role for NAC, it did not directly affect parasite load in infected macrophages. Despite immunological alterations, the pilot trial did not reveal significant clinical outcome differences between SbV and SbV+NAC groups. The study supports NAC's safety and immunomodulatory potential in VL but recommends further research on its clinical efficacy, optimal dosages, and mechanisms in *Leishmania* infection therapy. Conde et al.,discussed diverse aspects of the immune response in Leishmaniasis, emphasizing factors like antigenicity, host immunity, sandfly saliva, and parasite load. The work highlights the role of B cells and humoral immunity, suggesting that the balance between immunoglobulin classes indicates different disease stages. It underscores the complex role of B cell subpopulations and antibody subtypes since different B cell subpopulations may have detrimental or beneficial roles for disease progression and immune response evasion. This review presents challenges for vaccine development due to the inaccurate information regarding the dynamics of B cells during the infection, emphasizing the need for future research on both cellular and humoral adaptive responses. Noronha et al. investigated the role of leukotrienes, particularly cysteinyl leukotrienes LTC4 and LTD4, in resisting *L. amazonensis* infection. Previous data indicated LTB4 production's role in infection control. However, this study revealed that treatment with cysteinyl leukotrienes resulted in fewer amastigotes in macrophages and reduced cutaneous lesion progression in mice. The paper explores the link between ATP-induced cysteinyl leukotrienes (Cys-LTs) production triggered by the $P2X_7$ receptor, showing that *L. amazonensis* infection downregulates Cys-LTs production and $P2X_7$-deficient macrophages exhibit reduced Cys-LTs production. These findings highlight the importance of Cys-LTs as mediators for infection control and potential targets for CL treatment, suggesting the need for further mechanistic studies to understand the $P2X_7$-LT axis's leishmanicidal effects and its association with macrophage activation, cytokine production, and immune response.

It is also necessary to search for new alternatives for actual treatment and several natural products have been demonstrated to have antileishmanial activities. Quercetin is a polyphenolic flavonoid well known for its antioxidant activity in radical scavenging and other biological effects (Gervazoni et al., 2020). Dos Santos et al., demonstrated the therapeutic potential of oral quercetin in hamsters infected with *L. braziliensis*. Quercetin has both direct antileishmanial activity and the potential to modulate macrophages' activity since there was a reduction in the parasite load both in the lesion and the draining lymph node. In contrast, treatment with oral administration of apigenin controls infection but does not compromise the overall health of the infected mice. So, apigenin, another flavonoid, satisfies all eligibility's criteria of a new drug to treat VL and supports further studies to determine the ideal therapeutic and optimal drug dose regimen (Emiliano et al.). Rebouças-Silva et al. highlight the effects of lignans isomers, yangambin and epi-yangambin, as leishmanicidal and immunomodulatory molecules for *in vitro* infection with *L. amazonensis* and *L. braziliensis*. Either lignan attenuated the inflammatory profile of infected cells, but epi-yangambin was more potent in reducing the intracellular viability of *L. amazonensis* than yangambin. These leishmanicidal effects are related to a direct impact on intracellular parasites and macrophage activation. Pinheiro et al. showed that Farnesol, derived from farnesyl pyrophosphate in the sterols biosynthetic pathway, interferes with the proliferation of *L. amazonensis* promastigotes, inhibiting the cell cycle without causing DNA fragmentation or loss of mitochondrial functionality.

Another important approach is drug repurposing, which involves identifying new therapeutic uses for existing drugs developed for a different medical condition. Several drugs for treating Leishmaniasis have been repositioned (Andrade-Neto et al., 2018). Barroso et al. evaluated the effectiveness of meglumine antimoniate and liposomal amphotericin B in Brazil's *L. braziliensis* endemic area. The results demonstrated that meglumine antimoniate displayed a higher cure rate but presented a greater rate of adverse events than liposomal amphotericin B. van Henten et al. explored the treatment of CL using cryotherapy and miltefosine in Ochollo (Ethiopia). One hundred forty-seven patients were treated with these strategies, revealing that better clinical outcomes and lesion remission were shown for miltefosine. However, there was poor adherence to miltefosine treatment, which explained the lower cure rates observed in this study. These findings reinforce the importance of decentralizing therapy and the need for extensive efforts to sensitize and instruct rural communities on treatment adherence. In addition to drug repositioning, a combination of drugs can be used in leishmaniasis treatment. Borges et al. investigated the anti-*leishmania* activity of triclabendazole, used to treat fascioliasis, combined with amphotericin B against *L. amazonensis*. The treatment with triclabendazole demonstrated morphological alteration and synergic effect with amphotericin B against intracellular amastigotes. Meira et al. showed the activity of the synthetic lapachol derivative 3-phenyl-lawsone (3-PL) *in vitro* and its therapeutic potential in experimentally infected hamsters through subcutaneous and tattooing route compared with meglumine antimoniate. 3-PL exerts a dose-dependent effect on *L. braziliensis* and in previous studies against *L. amazonensis*. The use of tattooing for drug delivery proved efficacious in reducing parasite load for 3-PL and Glucantime, delivered by tattooing for the first time. Jara et al. studied a panel of *L. braziliensis* strains highly susceptible to potassium antimonyl tartrate (PAT). Exposed promastigotes to lethal PAT pressure and compared several cellular

and molecular parameters distinguishing temporary quiescence or survival drug tolerance from resistance against anti-leishmanial drugs. Resistance against anti-*Leishmania* drugs has been studied for years, giving meaningful insights into the long-term adaptations of these parasites to drugs through genetic modifications. However, microorganisms can also survive lethal drug exposure by entering temporary quiescence, a phenomenon called drug tolerance, which is rather unexplored in *Leishmania*.

These promising studies related to the discovery of new molecules or drug repositioning, new strategies, and alternative protocols in the treatment of CL and VL were welcome and a step forward to other efforts to treat this complex disease.

Author contributions

TdA: Writing – review & editing. EC-J: Writing – review & editing. JW: Writing – original draft. JP: Writing – original draft. LP: Writing – original draft. VA-N: Writing – original draft. SC: Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The authors are supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Fundação de Apoio à Pesquisa do Estado do Rio de Janeiro Carlos Chagas Filho (FAPERJ).

Acknowledgments

Thanks to all researchers who participated in this Research Topic. And especially to the editor Dr Gabriela Santos-Gomes.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Andrade-Neto, V. V., Cunha-Junior, E. F., Dos Santos Faioes, V., Pereira, T. M., Silva, R. L., Leon, L. L., et al. (2018). Leishmaniasis treatment: update of possibilities for drug repurposing. *Front. Biosci. (Landmark Ed).* 23 (5), 967–996. doi: 10.2741/4629

Burza, S., Croft, S. L., and Boelaert, M. (2018). Leishmaniasis. *Lancet* 392, 951–970. doi: 10.1016/S0140-6736(18)31204-2

Ruiz-Postigo, J. A., Jain, S., Maia-Elkhoury, A. M. A. N., Valadas, S., Warusavithana, S., Osman, M., et al. (2021). Global leishmaniasis surveillance: 2019-2020, a baseline for the 2030 roadmap/Surveillance mondiale de la leishmaniose: 2019-2020, une periode de reference pour la feuille de route a l'horizon 2030. *Weekly Epidemiological Record.* 96, 401–420.

Gervazoni, L. F. O., Barcellos, G. B., Ferreira-Paes, T., et al. (2020). Use of natural products in Leishmaniasis 128 chemotherapy: an overview. *Front. Chem.* 8. doi: 10.3389/fchem.2020.579891 (Accessed 8 February 2024).

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 23 September 2022
DOI 10.3389/fcimb.2022.993338

OPEN ACCESS

EDITED BY
Rubem Figueiredo Sadok Menna-Barreto,
Oswaldo Cruz Foundation (Fiocruz), Brazil

REVIEWED BY
Iraj Sharifi,
Kerman University of Medical Sciences, Iran
Kwame Kumi Asare,
University of Cape Coast, Ghana

*CORRESPONDENCE
Daniel Holanda Barroso
danielhbarroso@unb.br

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 13 July 2022
ACCEPTED 02 September 2022
PUBLISHED 23 September 2022

CITATION
Barroso DH, Gonçalves RT, Barbosa JS, Motta JOC, Freire GSM, Gomes CM and Sampaio RNR (2022) Meglumine antimoniate was associated with a higher cure rate than liposomal amphotericin B in the treatment of American tegumentary leishmaniasis: A retrospective cohort study from a *Leishmania braziliensis*-endemic area.
Front. Cell. Infect. Microbiol. 12:993338.
doi: 10.3389/fcimb.2022.993338

Meglumine antimoniate was associated with a higher cure rate than liposomal amphotericin B in the treatment of American tegumentary leishmaniasis: A retrospective cohort study from a *Leishmania braziliensis*-endemic area

Daniel Holanda Barroso[1,2]*, Renata Trindade Gonçalves[1], Joadyson Silva Barbosa[1], Jorgeth de Oliveira Carneiro da Motta[1], Gustavo Subtil Magalhães Freire[1], Ciro Martins Gomes[1,2,3] and Raimunda Nonata Ribeiro Sampaio[1,2,3,4]

[1]Hospital Universitário de Brasília, Universidade de Brasília, Brasília, Brazil, [2]Laboratório de Dermatomicologia da Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil, [3]Programa de Pós-Graduação em Ciências Médicas, Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil, [4]Pós-Graduação de Ciências da Saúde da Faculdade de Ciências Saúde, Universidade de Brasília, Brasília, Brazil

Background: Pentavalent antimonials (PAs) are the primary therapeutic option for American tegumentary leishmaniasis (ATL). However, the use of these drugs is complicated by adverse events (AEs), resistance and contraindications. Alternative therapies relative effectiveness is not well established.

Objective: This study compared the effectiveness of liposomal amphotericin B (LAB) with intravenous meglumine antimoniate (NMG) in the treatment of ATL. We also analysed and compared associated AEs and treatment interruption rates.

Methods: This was a retrospective cohort study from Brazil. The potential risk factors for the primary outcome were age, sex, total cutaneous lesion area, presence of mucosal lesions, AEs and treatment interruption. The primary outcome was lesion healing within 6 months of treatment. AEs and treatment interruption were also analysed. Multiple analytic strategies were employed to evaluate the reliability of the results.

Results: Before propensity score (PS) matching, patients in the LAB group were older and had a higher frequency of mucosal lesions. The NMG group had a higher cure rate than the LAB group (cure rate 88% versus 55% respectively) in the adjusted analysis (relative risk (RR)=1.55 95% CI: 1.19 - 2.02) and after PS matching (RR=1.63 95% CI: 1.20 - 2.21). NMG group had a higher AE rate (event rate 52% versus 44%) in the adjusted analysis (RR= 1.61, 95% CI: 1.06 - 2.43, p=0.02), but this result was not observed after PS matching (RR= 0.87, 95% CI: 0.49 -1.52, p= 0.61).

Conclusions: We observed that the NMG group had a higher cure rate than the LAB group, with an equivocally higher EV rate in the adjusted analysis.

KEYWORDS

therapy, liposomal amphotericin B (LAB), N-methyl glucamine antimoniate, adverse effect, American cutaneous leishmaniasis (ACL), mucosal leishmaniasis

Introduction

Leishmaniasis is a vector-borne disease caused by a protozoan in the *Leishmania* genus (Burza et al., 2018); it is known to cause a wide variety of clinical syndromes, with an estimated world incidence of 700,000 to 1 million cases each year (World Health organization, 2021). The disease burden is estimated to be higher than those of leprosy, dengue fever and Chagas disease (Hotez et al., 2004). American tegumentary leishmaniasis (ATL) is likely to have a greater impact on a patient`s quality of life due to the possible development of deforming mucosal lesions (Motta et al., 2007; Luz et al., 2014).

Although the therapeutic landscape is slowly changing, pentavalent antimonials (PAs) (including N-methyl glucamine, NMG) are currently the first-line treatment for ATL (González et al., 2009; Pinart et al., 2020). The use of these drugs is problematic because they can induce severe and potentially fatal adverse events (AEs), such as arrhythmias, renal toxicity, hepatitis and pancreatitis (Kopke et al., 1993; Oliveira et al., 2011; Lyra et al., 2016). Alternatives to PAs include amphotericin B formulations, pentamidine, miltefosine, fluconazole, and ketoconazole (Aronson et al., 2017). According to a recent systematic review, however, for ATL, none of these drugs can be considered equivalent to PAs with a high or moderate level of evidence (Pinart et al., 2020). This may reflect poor designs of and reporting in most studies (Pinart et al., 2020).

Amphotericin B is an antifungal agent that has been used to treat leishmaniasis since 1960 (SAMPAIO et al., 1960); it is generally considered a second-line treatment in cases of therapeutic failure, contraindications or intolerance to PAs (Berman, 1988; Lima et al., 2007). Despite its recommendation in therapeutic guidelines (Aronson et al., 2017; Transmissíveis, 2017), published studies have shown ambiguous results regarding its efficacy (González et al., 2009; Pinart et al., 2020). Additionally, amphotericin B use has been classically associated with moderate to severe AEs, but high-quality studies evaluating this topic are scarce (Oliveira et al., 2011). Furthermore, lipid formulations of this drug, with better safety and efficacy profiles, have been studied (Walsh et al., 1999; Kleinberg, 2006; Grazziotin et al., 2018), making it challenging to derive definitive conclusions about the optimal drug for treatment. Liposomal amphotericin B (LAB) is a currently available systemic antileishmanial agent that has been successfully used in case series to treat old world cutaneous leishmaniasis due to *Leishmania major* (Wortmann et al., 2010), *Leishmania tropica* (Solomon et al., 2011) and *Leishmania aethiopica* (Zanger et al., 2011). In ATL, this drug was initially proposed in Brazil by our group with World Health Organization (WHO) sponsorship (Sampaio and Marsden, 1997), being reportedly useful to treat the main new world species: *Leishmania braziliensis*, *Leishmania guyanensis* (Senchyna et al., 2020), *L. amazonensis*(Soares et al., 2020) and Leishmania panamensis(Cannella et al., 2011). The liposomal formulation of amphotericin has high potential for clinical benefit due to its well-known effective management of other infections (Guery et al., 2017) and better safety profile than its conventional form with less nephrotoxicity, infusion reactions and hypomagnesemia (Wade et al., 2013). However, due to its high cost, few data from clinical studies on the clinical benefit of this formulation in the treatment of ATL are available (Guery et al., 2017). Additional challenges in the completion of clinical trials comparing amphotericin B and PAs are the high dropout and interruption rates, which are possibly related to AEs and rigorous therapeutic schedules (Neves et al., 2011; Solomon et al., 2013).

The main objective of the present study was to evaluate the ATL cure rate in patients receiving LAB and to compare the cure rate with that in those receiving intravenous meglumine antimoniate (IV-NMG) in a tertiary Brazilian leishmaniasis reference centre. We also aimed to compare the incidence rates of AEs and the rates of treatment interruption between the two treatment groups.

Materials and methods

Population and case definition

This retrospective cohort study included ATL patients treated with NMG or LAB at the University Hospital of Brasília, Brazil, from 1992 to 2017. Inclusion criteria was the presence of a clinical lesion compatible with ATL associated with a positive parasitological test (direct examination, culture, polymerase chain reaction or the presence of amastigotes in the histopathological exam) or at least two non-parasitological exams(serology, leishmanin skin test or compatible histopathological exam) (Gomes et al., 2014). We excluded patients who received treatment 6 months prior to the main evaluation, those with a follow-up period of less than six months. We also excluded patients in use of immunosuppressive drugs or with immunosuppressive diseases including HIV/AIDS, solid organ transplant, chronic kidney disease and cancer diagnosis. In the primary analysis one hundred and ten patients were included (63 in the NMG group and 47 in the LAB group).

Ethics

This study was approved by the research ethics committee of the faculty of medicine of the University of Brasília, with the following CAAE 62110616.8.0000.5558. The referred committee waived the requirement to obtain informed consent since the present real-world data involves no more than minimal risk to subjects.

Sampling

Sample size calculation was performed using Stata 17 software (College Station, TX: Stata Press. StataCorp, 2021) considering the response rates of 81% in the LAB group and 99.9% in the NMG group obtained in a previous pilot study (Motta and Sampaio, 2012). Based on these rates, a sample of 37 patients in each group would result in 80% power to identify significant differences between the groups, with a significance level of 5%. Additional evaluations including the analysis of other outcomes and the analysis of simultaneous predictors were accessed by a *post hoc* strategy.

Intervention

We compared the NMG and LAB interventions. NMG was used in accordance with the recommendations of Brazil`s Ministry of Health (10 to 20 mg SbV/kg/day for 20 days for the cutaneous form and for 30 days for the mucosal form). LAB was administered at a dosage of 1-3 mg/kg/day in at least 5 days.

Outcomes

The main outcome was cure, defined as complete healing (reepithelization without infiltrations or erythema) of the lesion by the 180th day after the first medication dose. Interruption of treatment for more than 7 days and AEs of any grade were secondary outcomes. According to the institutional protocol, patients were monitored at least weekly during treatment and at 2, 3 and 6 months after treatment. Laboratorial alterations in electrocardiogram results, liver enzyme levels or kidney function indicators were monitored at each visit to monitor for AEs.

Statistical analysis

The cure rate, occurrence of AEs and treatment interruption rate were individually considered dependent variables, and the treatment group (NMG or LAB) was considered an independent variable. Initially, we performed univariate analyses to identify associations between the independent and dependent variables. Sensitivity analysis was performed to evaluate whether methodological shortcomings could be responsible for the identified associations. To evaluate whether patient characteristics associated with the intervention allocation or with the outcome could be responsible for the results, Poisson regression with robust variance was performed to obtain adjusted relative risks (RRs) based on sex (male; female), age (years), presence of mucosal lesions and total area of lesions, including no cutaneous lesions. To evaluate the outcome of cure, we added treatment interruption and AE rates into the model. To evaluate the outcome of treatment interruption, the presence of AEs was also added. To further evaluate cure, patients were matched in a 1:1 ratio based on propensity scores (PS) using the "greedy" strategy considering a calibration of 0.2 standard deviations (SDs) using the same variables analysed in the multivariate analysis. Univariate analysis of the associations of predictors variables using Poisson regression with robust variance were also done in the whole population. To evaluate whether LAB dosage variation could explain the cure rates observed in this group, a univariate Poisson regression with robust variance model was constructed considering cure as a dependent variable and LAB dosage as the independent variable. Statistical analysis was performed in SAS 9.4 (SAS Institute,

Cary, NC) and Stata 17 (StataCorp, College Station, TX). The results were considered statistically significant if p<0.05.

Results

The NMG group received the standard dosage recommended by the Brazilian Ministry of Health (15 mg SbV/kg/day for 20 days if there was no mucosal disease or for 30 days if there was mucosal disease). The total LAB dosage administered was 21.61 mg/kg ± 17.37 (SD). Patients who received LAB were older, had a higher frequency of mucosal lesions and had a lower cure rate than patients who received NMG (Tables 1, 2).

We were able to match 33 patients in each treatment arm based on their PSs, obtaining well-balanced groups (Figure 1). The NMG group had a higher cure rate than the LAB group (cure rate 88% versus 55% respectively) in the adjusted analysis (RR=1.55 95% CI: 1.19 - 2.02) and after PS matching (RR=1.63 95% CI: 1.20 - 2.21). NMG group had a higher AEs rate (event rate 52% versus 44%) in the adjusted analysis (RR= 1.61, 95% CI: 1.06 - 2.43, p=0.02), but this result was not observed after PS matching (RR= 0.87, 95% CI: 0.49 -1.52, p= 0.61) (Table 3).

The LAB dosage was not associated with cure in the dosage range applied in this study. In the whole population, we found significant association between age 60 or greater and the outcome interruption of treatment (RR= 3.68, 95% CI: 1.75-7.73, p<0.01) and adverse events (RR= 1.75, 95% CI: 1.23- 2.48, p<0.01). Other relevant influences on the tested outcomes were not detected.

Discussion

High-quality clinical trials comparing the use of PAs with amphotericin B in ATL patients are lacking, and the recommendations of health agencies are based on case series and retrospective studies (Aronson et al., 2016; Pinart et al., 2020). The reported efficacy of amphotericin B in the literature is greater than 90% (Sampaio et al., 1971; Wortmann et al., 2010; Amato et al., 2011; Rocio et al., 2014). In a study from Bolivia, LAB had a superior cure rate when compared with sodium stibogluconate (SSG) (84% versus 70%), but the results were nonsignificant (Solomon et al., 2013). We expected that increasing the sample size would lead to significant differences between the groups. The better effectiveness of NMG observed in our study is not surprising since this is the standard drug for ATL treatment and the treatment with which other treatments are compared (Pinart et al., 2020). A study from French Guyana also showed a lower cure rate in patients who received LAB than in those who received NMG (Senchyna et al., 2020), although the difference did not reach statistical significance. The overall cure rate in LAB patients in this study (55.32%) was similar to that in the study by Guery et al. (44%), which also included patients with Old-World leishmaniasis (Guery et al., 2017). In another case series that included only mucosal leishmaniasis patients from Brazil, the cure rate was 93.1% (Cunha et al., 2015). This difference may be explained by the fact that in their study, therapeutic failure was defined as the absence of clinical response after two successive therapeutic cycles (Cunha et al., 2015), whereas retreatment with the same therapeutic scheme has been reported to promote clinical cure in some patients (Nogueira and Sampaio, 2001).

We also investigated the associations between treatment interruption and AEs. A previous study showed a higher rate of treatment interruption in patients who received PAs (SSG) than in those who received LAB (Solomon et al., 2013). In the study by Senchyna et al., NMG was associated with a higher rate of moderate AEs, defined as those with clinical symptoms but that did not lead to treatment interruption (Senchyna et al., 2020). Despite the possible development of formulation-specific adverse reactions (Szebeni et al., 2000; Roden et al., 2003), LAB is

TABLE 1 Sample characteristics stratified by drug before and after PS matching.

	Before PS matching			After PS matching		
Characteristic *	**NMG (n = 63)**	**LAB (n = 47)**	**p value**	**NMG (n = 33)**	**LAB (n = 33)**	**p value**
Age	37.19 ± 18.68	51.70 ± 22.18	<0.0001#	41.82 ± 20.81	46.03 ± 24.14	0.4506#
Total area of cutaneous lesions	11.24 ± 17.51	9.37 ± 13.52	0.3130†	13.60 ± 21.46	8.57 ± 10.20	0.4649†
Sex						0.2284ϙ
Female	17 (26.98)	12 (25.53)	0.8642 ϙ	5 (15.15)	9 (27.27)	
Male	46 (73.02)	35 (74.47)		28 (84.85)	24 (72.73)	
Mucosal lesions						0.2840ϙ
No	45 (71.43)	25 (53.19)	0.0492 ϙ	25 (75.76)	21 (63.64)	
Yes	18 (28.57)	22 (46.81)		8 (24.24)	12 (36.36)	

* values expressed as the mean ± standard deviation or frequency (%).
Univariate analysis using #Student's T test, (†)Mann–Whitney or ϙ chi-square test.
NMG, meglumine antimoniate; LAB, liposomal amphotericin B; PS, propensity score; n, number of patients.

TABLE 2 Outcomes before and after PS matching.

	Before PS matching			After PS Matching		
Outcome*	**NMG (n = 63)**	**LAB (n = 47)**	**p value#**	**NMG (n = 33)**	**LAB (n = 33)**	**p value#**
Interruption						0.7412
No	54 (85.71)	34 (72.34)	0.0828	27 (81.82)	28 (84.85)	
Yes	9 (14.29)	13 (27.66)		6 (18.18)	5 (15.15)	
Adverse events						0.6184
No	30 (47.62)	26 (55.32)	0.4242	20 (60.61)	18 (54.55)	
Yes	33 (52.38)	21 (44.68)		13 (39.39)	15 (45.45)	
Cure						0.0006
No	7 (11.11)	21 (44.68)	<0.0001	2 (6.06)	14 (42.42)	
Yes	56 (88.89)	26 (55.32)		31 (93.94)	19 (57.58)	

* values expressed as the mean ± standard deviation or frequency (%).
P value calculated using chi-square test.
NMG, meglumine antimoniate; LAB, liposomal amphotericin; B, PS, propensity score; n, number of patients.

known to have a better safety profile than the other formulations (Wasan et al., 1994; Walsh et al., 1999; Wade et al., 2013). Accordingly, in this study, the adjusted RR for adverse events was higher in patients who received NMG than patients who received LAB. Although this result reached statistical significance, it was not reproduced after matching. Thus, the higher AE rate in the NMG group should be interpreted with caution and deserves further evaluation in larger studies specifically powered to evaluate comparative AEs between medication groups.

The age of patients and proportion of mucosal lesions in the current study are likely to be different from those in the overall population of ATL patients since older people and those with mucosal lesions are more likely to be referred to a tertiary care centre. In an epidemiological study performed in a primary care setting in Bahia, Brazil, only 4.3% of patients had mucosal lesions, and the average age of cutaneous leishmaniasis patients was 21 years (Jirmanus et al., 2012), which was younger than 37 years (NMG group) and 51 years (LAB group), as reported in our study. Thus, the convenience sample used in this study may limit the generalizability of our results. Additionally, in the primary analysis, patients who received LAB were significantly older and had a significantly higher frequency of mucosal lesions. Again, this may be explained by the increased risk of mucosal lesions with age (Machado-Coelho et al., 2005) and by the recommendation that people aged 50 years or older should be treated with amphotericin B according to the national guidelines(Transmissíveis, 2017). The lower cure rate observed in patients who received LAB, however,

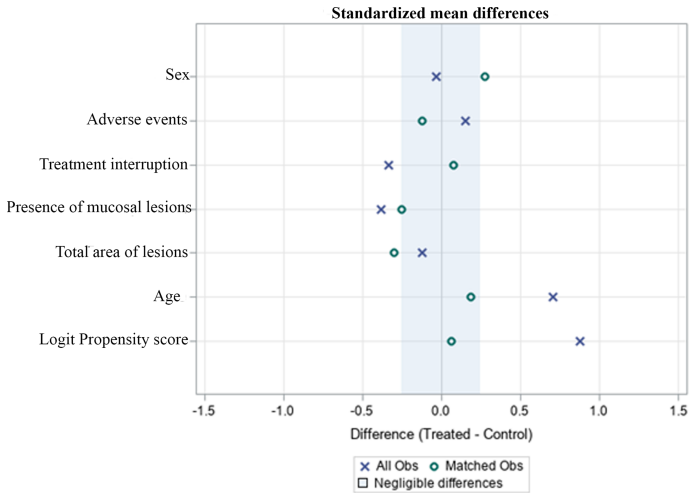


FIGURE 1
Standardized differences before and after PS matching comparing variables for patients treated with NMG and LAB drugs.

TABLE 3 Unadjusted and adjusted relative risks (RRs) and 95% confidence intervals (CIs) for cure, interruption and AEs before and after propensity score matching.

			Before PS matching			After PS matching	
Outcome	**Drug**	**RR (95% CI)**	**p value**	**Adjusted RR* (95% CI)**	**p value**	**RR (95% CI)**	**p value**
Cure	**NMG**	1.61 (1.22; 2.11)	0.0006	1.55 (1.19; 2.02)	0.0013	1.63 (1.20; 2.21)	0.0017
Interruption	**NMG**	0.52 (0.24; 1.11)	0.0890	0.60 (0.30; 1.19)	0.1401	1.20 (0.41; 3.55)	0.7417
Adverse events	**NMG**	1.17 (0.79; 1.74)	0.4310	1.61 (1.06; 2.43)	0.0252	0.87 (0.49; 1.52)	0.6194

* For all the outcomes, the relative risks was adjusted by the variables in Table 1 using Poisson regression analysis. For the outcome cure, interruption and AEs were added to the model, and for the outcome interruption, AEs were added to the model.
RR, Relative risk; 95% CI, 95% confidence interval; PS, Propensity score; NMG, meglumine antimoniate.

is unlikely explained by their basic characteristics since these results were consistent across multiple analytic strategies that included controlling for confounders.

One of the limitations of this study is its observational design. Although randomized clinical trials (RCTs) are the gold standard for analysing the intended effects of therapies, observational studies are as valid as RCTs to investigate AEs associated with medications (Vandenbroucke, 2008).Additionally, Interruption and dropout rates can be a treat to internal validity of clinical trials (Ravani et al., 2007) and have been important in studies of LAB for the treatment of leishmaniasis (Neves et al., 2011; Solomon et al., 2013). LAB studies have been limited by the cost of the medication, especially considering that ATL is highly prevalent in low-income countries (Guery et al., 2017). Thus, the relatively low cost, wide range of patients and rapidly obtained conclusions make observational studies an interesting approach to investigate the effects of LAB in ATL patients (Benson and Hartz, 2000). The main limitation of observational studies is related to treatment allocation, but the strategy used by our team, propensity analysis, is known to offset this issue (Feneck, 2007). Provided that important confounders are controlled for (Vandenbroucke, 2008), it has been shown that observational studies can produce results similar to those of RCTs (Benson and Hartz, 2000).

We therefore attempted to overcome the limitations of previous studies using an observational design coupled with an adequate analytical strategy. To do this we performed *post hoc* adjusted and PS matched analysis including clinical and individual characteristics to try to explain the associations found in the unadjusted analysis. As shown by other studies, age is associated with increased adverse events rate (Araujo-Melo et al., 2010; Diniz et al., 2012; do Lago et al., 2018) and immunological responses (Carvalho et al., 2015). As expected, we have found that elderly patients had a higher rate of adverse events and interruption of treatment but, as previously suggested, we were not able to find an association between age and treatment response (do Lago et al., 2018). Although sex (de Araújo Albuquerque et al., 2021), total area of lesions (Valencia et al., 2012) and the presence of mucosal lesions (García-Bustos et al., 2021) were all previously related with treatment failure, we were not able to find significant relationship with the outcome in our data.

In this cohort study from a *L. braziliensis*-endemic area (Gomes, 2014), NMG was associated with a higher cure rate than LAB, although it also had an equivocally higher AE rate. The consistency of the primary results across multiple analysis and their applicability in the real world setting of a Brazilian reference centre are the main strengths of this study. Is important to state, however, that their validity in the overall population is limited. Possible known confounders were controlled for in the analysis, but the presence of unknown covariates is a limitation in any observational study. As randomization is the only way to balance these covariates, our results should be confirmed in a large RCT.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de Brasília, CAAE 62110616.8.0000.5558. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because: The referred committee waived the requirement to obtain informed consent since the present real-world data involves no more than minimal risk to subjects.

Author contributions

DB – conception, design, data acquisition, analysis, interpretation of data, drafting; RG - data acquisition, analysis, interpretation of data; JB - data acquisition, analysis, interpretation of data; JM - data acquisition, analysis; GM- data acquisition, analysis and interpretation; CG - conception, design, data acquisition, analysis, interpretation of data, drafting; RS -

conception, design, data acquisition, analysis, interpretation of data, drafting, supervision. All: Final approval.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Amato, V. S., Tuon, F. F., Camargo, R. A., Souza, R. M., Santos, C. R., and Nicodemo, A. C. (2011). Can we use a lower dose of liposomal amphotericin b for the treatment of mucosal American leishmaniasis? *Am. J. Trop. Med. Hygiene* 85 (5), 818. doi: 10.4269/ajtmh.2011.11-0287

Araujo-Melo, M., Meneses, A., Schubach, A., Moreira, J., Conceição-Silva, F., Salgueiro, M., et al. (2010). Risk factors associated with dizziness during treatment of mucosal leishmaniasis with meglumine antimoniate: 16-year retrospective study of cases from Rio de Janeiro, Brazil. *J. Laryngology Otology* 124 (10), 1056–1060. doi: 10.1017/S0022215110001325

Aronson, N., Herwaldt, B. L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., et al. (2016). Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the infectious diseases society of America (IDSA) and the American society of tropical medicine and hygiene (ASTMH). *Clin. Infect. Dis.* 63 (12), e202–e264. doi: 10.1093/cid/ciw670

Aronson, N., Herwaldt, B. L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., et al. (2017). Diagnosis and treatment of leishmaniasis: Clinical practice guidelines by the infectious diseases society of America (IDSA) and the American society of tropical medicine and hygiene (ASTMH). *Am. J. Trop. Med. Hygiene* 96 (1), 24–45. doi: 10.4269/ajtmh.16-84256

Benson, K., and Hartz, A. J. (2000). A comparison of observational studies and randomized, controlled trials. *New Engl. J. Med.* 342 (25), 1878–1886. doi: 10.1056/NEJM200006223422506

Berman, J. D. (1988). Chemotherapy for leishmaniasis: biochemical mechanisms, clinical efficacy, and future strategies. *Rev. Infect. Dis.* 10 (3), 560–586. doi: 10.1093/clinids/10.3.560

Burza, S., Croft, S. L., and Boelaert, M. (2018). Leishmaniasis. *Lancet* 392 (10151), 951–970. doi: 10.1016/S0140-6736(18)31204-2

Cannella, A. P., Nguyen, B. M., Piggott, C. D., Lee, R. A., Vinetz, J. M., and Mehta, S. R. (2011). A cluster of cutaneous leishmaniasis associated with human smuggling. *Am. J. Trop. Med. Hygiene* 84 (6), 847. doi: 10.4269/ajtmh.2011.10-0693

Carvalho, A. M., Amorim, C. F., Barbosa, J. L., Lago, A. S., and Carvalho, E. M. (2015). Age modifies the immunologic response and clinical presentation of American tegumentary leishmaniasis. *Am. J. Trop. Med. Hyg* 92 (6), 1173–1177. doi: 10.4269/ajtmh.14-0631

Cunha, M. A., Leão, A. C., de Cassia Soler, R., and Lindoso, J. A. (2015). Efficacy and safety of liposomal amphotericin b for the treatment of mucosal leishmaniasis from the new world: A retrospective study. *Am. J. Trop. Med. Hyg* 93 (6), 1214–1218. doi: 10.4269/ajtmh.15-0033

de Araújo Albuquerque, L. P., da Silva, A. M., de Araújo Batista, F. M., de Souza Sene, I., Costa, D. L., and Costa, C. H. N. (2021). Influence of sex hormones on the immune response to leishmaniasis. *Parasite Immunol.* 43 (10-11), e12874. doi: 10.1111/pim.12874

Diniz, D. S., Costa, A. S. V., and Escalda, P. M. F. (2012). The effect of age on the frequency of adverse reactions caused by antimony in the treatment of American tegumentary leishmaniasis in governador valadares, state of minas gerais, Brazil. *Rev. da Sociedade Bras. Medicina Trop.* 45, 597–600. doi: 10.1590/S0037-86822012000500011

do Lago, A. S., Nascimento, M., Carvalho, A. M., Lago, N., Silva, J., Queiroz, J. R., et al. (2018). The elderly respond to antimony therapy for cutaneous leishmaniasis similarly to young patients but have severe adverse reactions. *Am Trop Med Hygiene* 98 (5), 1317–1324. doi: 10.4269/ajtmh.17-0736

Feneck, R. (2007). Clinical research in anaesthesia; randomized controlled trials or observational studies? *Eur. J. Anaesthesiology* 24 (1), 1–5. doi: 10.1017/S0265021506001967

García-Bustos, M. F., González-Prieto, G., Paniz-Mondolfi, A. E., Parodi, C., Beckar, J., Monroig, S., et al. (2021). Risk factors for antimony treatment failure in American cutaneous leishmaniasis in northwestern-Argentina. *PLoS Negl. Trop. Dis.* 15 (1), e0009003. doi: 10.1371/journal.pntd.0009003

Gomes, C. M. (2014). *Acurácia da reação em cadeia da polimerase em amostras de saliva, swab nasal e papel filtro oral no diagnóstico da leishmaniose tegumentar americana: estudo clínico, revisão sistemática da literatura e meta-análise*. (Brasília, Brazil: Universidade de Brasília).

Gomes, C. M., Paula, N. A., Morais, O. O., Soares, K. A., Roselino, A. M., and Sampaio, R. N. (2014). Complementary exams in the diagnosis of American tegumentary leishmaniasis. *Bras. Dermatol.* 89 (5), 701–709. doi: 10.1590/abd1806-4841.20142389

González, U., Pinart, M., Rengifo-Pardo, M., Macaya, A., Alvar, J., and Tweed, J. (2009). Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database Systematic Rev* (2), CD004834–CD004834. doi: 10.1002/14651858.CD004834.pub2

Gordón-Núñez, M. A., Ferreira, S. J., Andrade, A. L.D.L.D., Luz, K. G., Milan, E. P., and Galvão, H. C. (2014). New World Mucocutaneous Leishmaniasis with Oral Manifestations: Case Report and Damage Repair. *Am J Infectious Dis* 10 (4), 167–173. doi: 10.3844/ajidsp.2014.167.173

Grazziotin, L. R., Moreira, L. B., and Ferreira, M. A. P. (2018). Comparative effectiveness and safety between amphotericin b lipid-formulations: a systematic review. *Int. J. Technol. Assess. Health Care* 34 (3), 343–351. doi: 10.1017/S026646231800034X

Guery, R., Henry, B., Martin-Blondel, G., Rouzaud, C., Cordoliani, F., Harms, G., et al. (2017). Liposomal amphotericin b in travelers with cutaneous and muco-cutaneous leishmaniasis: Not a panacea. *PLoS Negl. Trop. Dis.* 11 (11), e0006094. doi: 10.1371/journal.pntd.0006094

Hotez, P. J., Remme, J. H., Buss, P., George, G., Morel, C., and Breman, J. G. (2004). Combating tropical infectious diseases: report of the disease control priorities in developing countries project. *Clin. Infect. Dis.* 38 (6), 871–878. doi: 10.1086/382077

Jirmanus, L., Glesby, M. J., Guimaraes, L. H., Lago, E., Rosa, M. E., Machado, P. R., et al. (2012). Epidemiological and clinical changes in American tegumentary leishmaniasis in an area of leishmania (Viannia) braziliensis transmission over a 20-year period. *Am. J. Trop. Med. Hygiene* 86 (3), 426–433. doi: 10.4269/ajtmh.2012.11-0378

Kleinberg, M. (2006). What is the current and future status of conventional amphotericin b? *Int. J. antimicrobial Agents* 27, 12–16. doi: 10.1016/j.ijantimicag.2006.03.013

Kopke, L. F. F., Café, M. E. M., Neves, L. B., Scherrer, M. A. R., Machado Pinto, J., Souza, M., et al. (1993). Morte após uso de antimonial pentavalente em leishmaniose tegumentar americana. *An. Bras. Dermatol.* 261, 259–260.

Lima, E., Porto, C., Motta, J., and Sampaio, R. N. R. (2007). Tratamento da leishmaniose tegumentar americana. *An. Bras. Dermatol.* 82 (2), 111–124. doi: 10.1590/S0365-05962007000200002

Lyra, M. R., Passos, S. R. L., Pimentel, M. I. F., Bedoya-Pacheco, S. J., Valete-Rosalino, C. M., Vasconcellos, E. C. F., et al. (2016). Pancreatic toxicity as an adverse effect induced by meglumine antimoniate therapy in a clinical trial for cutaneous leishmaniasis. *Rev. do Instituto Medicina Trop. São Paulo* 58 (1), 68–73. doi: 10.1590/S1678-9946201658068

Machado-Coelho, G. L., Caiaffa, W. T., Genaro, O., Magalhaes, P. A., and Mayrink, W. (2005). Risk factors for mucosal manifestation of American cutaneous leishmaniasis. *Trans. R Soc. Trop. Med. Hyg* 99 (1), 55–61. doi: 10.1016/j.trstmh.2003.08.001

Motta, A. C., Lopes, M. A., Ito, F. A., Carlos-Bregni, R., de Almeida, O. P., and Roselino, A. M. (2007). Oral leishmaniasis: a clinicopathological study of 11 cases. *Oral. Dis.* 13 (3), 335–340. doi: 10.1111/j.1601-0825.2006.01296.x

Motta, J., and Sampaio, R. (2012). A pilot study comparing low-dose liposomal amphotericin b with n-methyl glucamine for the treatment of American cutaneous leishmaniasis. *J. Eur. Acad. Dermatol. Venereology* 26 (3), 331–335. doi: 10.1111/j.1468-3083.2011.04070.x

Neves, L. O., Talhari, A. C., Gadelha, E. P., Silva Júnior, R. M., Guerra, J. A., Ferreira, L. C., et al. (2011). A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin b for the treatment of cutaneous leishmaniasis by leishmania guyanensis. *Bras. Dermatol.* 86 (6), 1092–1101. doi: 10.1590/s0365-05962011000600005

Nogueira, L. S. C., and Sampaio, R. N. R. (2001). Estudo hospitalar de leishmaniose tegumentar americana (LTA): epidemiologia e tratamento. *An. Bras. Dermatol.* 76 (1), 51–62.

Oliveira, L. F., Schubach, A. O., Martins, M. M., Passos, S. L., Oliveira, R. V., Marzochi, M. C., et al. (2011). Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the new world. *Acta Trop.* 118 (2), 87–96. doi: 10.1016/j.actatropica.2011.02.007

Pinart, M., Rueda, J.-R., Romero, G. A., Pinzón-Flórez, C. E., Osorio-Arango, K., Maia-Elkhoury, A. N. S., et al. (2020). Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database Systematic Rev.* 8, 1–337. doi: 10.1002/14651858.CD004834.pub3

Ravani, P., Parfrey, P. S., Dicks, E., and Barrett, B. J. (2007). Clinical research of kidney diseases II: problems of study design. *Nephrol. Dialysis Transplant.* 22 (10), 2785–2794. doi: 10.1093/ndt/gfm433

Rocio, C., Amato, V. S., Camargo, R. A., Tuon, F. F., and Nicodemo, A. C. (2014). Liposomal formulation of amphotericin b for the treatment of mucosal leishmaniasis in HIV-negative patients. *Trans. R. Soc. Trop. Med. Hygiene* 108 (3), 176–178. doi: 10.1093/trstmh/tru011

Roden, M. M., Nelson, L. D., Knudsen, T. A., Jarosinski, P. F., Starling, J. M., Shiflett, S. E., et al. (2003). Triad of acute infusion-related reactions associated with liposomal amphotericin b: analysis of clinical and epidemiological characteristics. *Clin. Infect. Dis.* 36 (10), 1213–1220. doi: 10.1086/374553

Sampaio, S. A. P., Castro, R. M., Dillon, N. L., and Costa Martins, J. E. (1971). Treatment of mucocutaneous (American) leishmaniasis with amphotericin b: report of 70 cases. *Int. J. Dermatol.* 10 (3), 179–181. doi: 10.1111/j.1365-4362.1971.tb01694.x

Sampaio, S. A., Godoy, J. T., Paiva, L., Dillon, N. L., and LACAZ, C. D. S. (1960). The treatment of American (mucocutaneous) leishmaniasis with amphotericin b. *Arch. Dermatol.* 82 (4), 627–635. doi: 10.1001/archderm.1960.01580040145026

Sampaio, R., and Marsden, P. (1997). Treatment of the mucosal form of leishmaniasis without response to glucantime, with liposomal amphotericin b. *Rev. da Sociedade Bras. Medicina Trop.* 30 (2), 125–128. doi: 10.1590/S0037-86821997000200007

Saúde, S.d.V.e. (2017). Manual for Surveillance of cutaneous leishmaniasis Biblioteca Virtual em Saúde do Ministério da Saúde: Editora MS.

Senchyna, A., Simon, S., Cissé, H., Ginouves, M., Prevot, G., Alcoba, G., et al. (2020). American Cutaneous leishmaniasis in French Guiana: a retrospective comparison between liposomal amphotericin b and meglumine antimoniate. *Br. J. Dermatol.* 183 (2), 389. doi: 10.1111/bjd.18964

Soares, G. H. C., da Silva, A. B. S., de Sousa Ferreira, L. S., Ithamar, J. S., de Alencar Medeiros, G., Pereira, S. R. F., et al. (2020). Case report: coinfection by leishmania amazonensis and HIV in a Brazilian diffuse cutaneous leishmaniasis patient. *Am. J. Trop. Med. Hygiene* 103 (3) 1076, 1076–1080. doi: 10.4269/ajtmh.20-0131.

Solomon, M., Pavlotsky, F., Leshem, E., Ephros, M., Trau, H., and Schwartz, E. (2011). Liposomal amphotericin b treatment of cutaneous leishmaniasis due to leishmania tropica. *J. Eur. Acad. Dermatol. Venereol* 25 (8), 973–977. doi: 10.1111/j.1468-3083.2010.03908.x

Solomon, M., Pavlotzky, F., Barzilai, A., and Schwartz, E. (2013). Liposomal amphotericin b in comparison to sodium stibogluconate for leishmania braziliensis cutaneous leishmaniasis in travelers. *J. Am. Acad. Dermatol.* 68 (2), 284–289. doi: 10.1016/j.jaad.2012.06.014

Szebeni, J., Baranyi, L., Savay, S., Bodo, M., Morse, D. S., Basta, M., et al. (2000). Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. *Am. J. Physiol. Heart Circ. Physiol.* 279 (3), H1319–H1328. doi: 10.1152/ajpheart.2000.279.3.H1319

Valencia, C., Arévalo, J., Dujardin, J. C., Llanos-Cuentas, A., Chappuis, F., and Zimic, M. (2012). Prediction score for antimony treatment failure in patients with ulcerative leishmaniasis lesions. *PLoS Negl. Trop. Dis.* 6 (6), e1656. doi: 10.1371/journal.pntd.0001656

Vandenbroucke, J. P. (2008). Observational research, randomised trials, and two views of medical science. *PLoS Med.* 5 (3), e67. doi: 10.1371/journal.pmed.0050067

Wade, R. L., Chaudhari, P., Natoli, J. L., Taylor, R. J., Nathanson, B. H., and Horn, D. L. (2013). Nephrotoxicity and other adverse events among inpatients receiving liposomal amphotericin b or amphotericin b lipid complex. *Diagn. Microbiol. Infect. Dis.* 76 (3), 361–367. doi: 10.1016/j.diagmicrobio.2013.04.001

Walsh, T. J., Finberg, R. W., Arndt, C., Hiemenz, J., Schwartz, C., Bodensteiner, D., et al. (1999). Liposomal amphotericin b for empirical therapy in patients with persistent fever and neutropenia. *New Engl. J. Med.* 340 (10), 764–771. doi: 10.1056/NEJM199903113401004

Wasan, K. M., Morton, R. E., Rosenblum, M. G., and Lopez-Berestein, G. (1994). Decreased toxicity of liposomal amphotericin b due to association of amphotericin b with high-density lipoproteins. *J. Pharm. Sci.* 83 (7), 1006–1010. doi: 10.1002/jps.2600830716

World Health organization (2021)Leishmaniasis. In: *World health organization webpage newsroom* (World Health organization). Available at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (Accessed 07/02/2021).

Wortmann, G., Zapor, M., Ressner, R., Fraser, S., Hartzell, J., Pierson, J., et al. (2010). Lipsosomal amphotericin b for treatment of cutaneous leishmaniasis. *Am. J. Trop. Med. Hygiene* 83 (5), 1028–1033. doi: 10.4269/ajtmh.2010.10-0171

Zanger, P., Kötter, I., Raible, A., Gelanew, T., Schönian, G., and Kremsner, P. G. (2011). Case report: Successful treatment of cutaneous leishmaniasis caused by leishmania aethiopica with liposomal amphothericin b in an immunocompromised traveler returning from Eritrea. *Am. J. Trop. Med. Hygiene* 84 (5), 692. doi: 10.4269/ajtmh.2011.10-0712

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 24 November 2022
DOI 10.3389/fcimb.2022.1045668

OPEN ACCESS

EDITED BY
Raimunda Nonata Ribeiro Sampaio,
University of Brasilia, Brazil

REVIEWED BY
Valdir Sabbaga Amato,
University of São Paulo, Brazil
Daniel Holanda Barroso,
University of Brasilia, Brazil

*CORRESPONDENCE
Roque Pacheco de Almeida
roquepachecodealmeida@gmail.com

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 15 September 2022
ACCEPTED 03 November 2022
PUBLISHED 24 November 2022

CITATION
Magalhães LS, Melo EV, Damascena NP, Albuquerque ACB, Santos CNO, Rebouças MC, Bezerra MdO, Louzada da Silva R, De Oliveira FA, Santos PL, da Silva JS, Lipscomb MW, da Silva ÂM, de Jesus AR and de Almeida RP (2022) Use of N-acetylcysteine as treatment adjuvant regulates immune response in visceral leishmaniasis: Pilot clinical trial and *in vitro* experiments. *Front. Cell. Infect. Microbiol.* 12:1045668. doi: 10.3389/fcimb.2022.1045668

Use of N-acetylcysteine as treatment adjuvant regulates immune response in visceral leishmaniasis: Pilot clinical trial and *in vitro* experiments

Lucas Sousa Magalhães[1,2,3], Enaldo Vieira Melo[4], Nayra Prata Damascena[4], Adriana Cardoso Batista Albuquerque[4], Camilla Natália Oliveira Santos[1,2], Mônica Cardozo Rebouças[1], Mariana de Oliveira Bezerra[1,4], Ricardo Louzada da Silva[1,5], Fabricia Alvisi de Oliveira[1], Priscila Lima Santos[1,2,5], João Santana da Silva[6], Michael Wheeler Lipscomb[7], Ângela Maria da Silva[1,4], Amélia Ribeiro de Jesus[1,2,4,8] and Roque Pacheco de Almeida[1,2,4,8]*

[1]Laboratory of Immunology and Molecular Biology, University Hospital, Federal University of Sergipe, Aracaju, Brazil, [2]Health Sciences Graduate Program, Federal University of Sergipe, Aracaju, Brazil, [3]Sector of Parasitology and Pathology, Biological and Health Sciences Institute, Federal University of Alagoas, Maceió, Brazil, [4]Department of Medicine, University Hospital-Empresa Brasileira de Serviços Hospitalares (EBSERH), Federal University of Sergipe, Aracaju, Brazil, [5]Department of Health Education, Federal University of Sergipe, Lagarto, Brazil, [6]Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [7]Department of Pharmacology, University of Minnesota, Minneapolis, MN, United States, [8]Immunology Institute of Investigation (iii), National Institute of Science and Technology (INCT), Brazilian Research and Technology Council (CNPq), São Paulo, Brazil

This investigation aimed to assess the effect of N-acetylcysteine (NAC) as an adjuvant treatment to alleviate visceral leishmaniasis (VL). The present work includes both blinded randomized clinical intervention and experimental *in vitro* studies. The clinical trial included 60 patients with VL randomly allocated into two groups: a test group (n = 30) treated with meglumine antimoniate plus NAC (SbV + NAC) and a control group (n = 30) treated with meglumine antimoniate only (SbV). The primary outcome was clinical cure (absence of fever, spleen and liver sizes reduction, and hematological improvement) in 180 days. The cure rate did not differ between the groups; both groups had similar results in all readout indices. The immunological parameters of the patients treated with SbV + NAC showed higher sCD40L in sera during treatment, and the levels of sCD40L were negatively correlated with Interleukin-10 (IL-10) serum levels. In addition, data estimation showed a negative correlation between the sCD40L levels and the spleen size in patients with VL. For the *in vitro* experiments, peripheral blood mononuclear cells (PBMCs) or PBMC-derived macrophages from healthy donors were exposed to soluble *Leishmania* antigen (SLA) or infected with stationary promastigotes of

Leishmania infantum in the presence or absence of NAC. Results revealed that NAC treatment of SLA-stimulated PBMCs reduces the frequency of monocytes producing IL-10 and lowers the frequency of CD4+ and CD8+ T cells expressing (pro-)inflammatory cytokines. Together, these results suggest that NAC treatment may modulate the immune response in patients with VL, thus warranting additional investigations to support its case use as an adjuvant to antimony therapy for VL.

KEYWORDS

visceral leishmaniasis, N-acetyl-l-cysteine, adjuvant chemotherapy, meglumine antimoniate, drug therapy

1 Introduction

Visceral leishmaniasis (VL) is an infectious disease caused by digenetic parasites of the species *Leishmania (Leishmania) infantum* or *L. donovani*. If left untreated, the disease can be fatal. Further compounding, the challenge is that the disease largely affects neglected populations—those without adequate healthcare and infrastructure support (Burza et al., 2018).

It is well established that VL is a disease caused by dysregulation of immune response. Several studies demonstrate that *Leishmania* parasites actively induce alterations in immune cell responses. This is most notable during parasite internalization by macrophages, which includes the inability to adequately clear the pathogen load (Liévin-Le Moal and Loiseau, 2016). Results demonstrate that patients with better disease progression have antigen-specific immune responses; this is chiefly based on an early action of IL-12 and Interferon-γ (IFN-γ) to promote immune effector responses (Dayakar et al., 2019). However, patients with VL with severe disease burden have exacerbated inflammatory response, a phenomenon known as cytokine storm, a mixture of inflammatory and anti-inflammatory cytokines from innate and adaptive immune responses (Bhor et al., 2021).

Pentavalent antimonial compounds are the major drugs used to treat VL. The drug is relatively low in cost to manufacture and can induce robust pro-inflammatory responses (Aruleba et al., 2020). However, treatment can have adverse side effects, which can include cardiotoxicity, hepatotoxicity, nephrotoxicity, and pancreatitis (Kumari et al., 2021). Moreover, there are alternative drugs, such as Amphotericin B, that are efficient in curing VL in administered patients. However, the immediate drawbacks in use are the high cost and the associated toxicity (Kumari et al., 2022). Similarly, other drugs available for treatment of VL are miltefosine and paromomycin that also have good cure rates in patients with VL. Unfortunately, accessibility is limited in afflicted countries, and there are associated reports of treatment failure (Chakravarty and Sundar, 2019; Musa et al., 2022).

N-acetylcysteine (NAC) is a metabolite of L-cysteine amino acid widely used as antioxidant treatment (Dodd et al., 2008). NAC administration results in (1) increased levels of glutathione, (2) scavenging of free radicals like hydrogen peroxide, and (3) reduction of disulfide compounds (Pedre et al., 2021). Furthermore, NAC has been shown to regulate immune responses especially by downregulating Nuclear factor-κB (NF-κB) activation in dendritic cells (Giordani et al., 2002) and diminishing B cell activation (Bonnaure and Néron, 2014). Numerous works have demonstrated the protective effects of NAC against infectious diseases (Amaral et al., 2016; Gao et al., 2017).

Therefore, knowing that VL disease is associated with a dysregulation in immunity and that NAC treatment directly regulates immune responses, these studies aimed to evaluate whether NAC treatment as an adjuvant to antimony treatment in VL could course correct VL-induced deficiencies in immunity to improve clinical outcomes. Our data demonstrate that NAC has immune modulatory effect in *in vitro* experiments involving either T cells or monocytes and alters production of immunological biomarker as adjuvant during treatment of patients with VL. These data give evidence that NAC could be further explored as adjuvant treatment to patients with VL.

2 Material and methods

2.1 Study design and ethics statement

This work has two interconnected parts: (1) a pilot blinded and randomized clinical intervention study, with patients with VL treated with antimony plus NAC compared with patients with VL treated with antimony; and (2) an *in vitro* experimental study to evaluate the effect of antimony plus NAC in immunomodulation of monocytes, macrophages, and T-cell effector responses.

All patients and healthy donors included in the studies were recruited and signed informed consent for participations in the studies. The Research Ethical Committee from the Federal University of Sergipe approved this study (advice number 1.353.887). The clinical trial pilot was registered in clinicaltrials.gov database (NCT01138956).

2.2 Pilot blinded and randomized clinical trial study to evaluate the effect of N-acetylcysteine as adjuvant therapy to antimony for VL treatment

All patients included in this study were invited to participate after VL diagnostics. All patients or their legal guardians signed an informed consent. Patients were consecutively recruited between 2008 and 2010 at the University Hospital of the Federal University of Sergipe in northwestern Brazil, which is an endemic area of VL, where approximately 64 new cases of VL are reported per year (Campos et al., 2017). On the basis of the number of new patients with VL, the participation of 60 patients was estimated for this clinical trial study, adopting a significance level of 0.05 and a power of 0.8.

VL diagnosis was confirmed with positive serology to rK39 antigen and positive culture from bone marrow. The clinical criteria that are utilized included fever, liver and spleen (increased) sizes, diarrhea, epistaxis, jaundice, cough, and signs of anemia. Additional laboratory criteria included anemia, leukopenia, thrombocytopenia, hypoalbuminemia, and hyperglobulinemia. Patients with other chronic or acute diseases, who were pregnant, had severe neutropenia, or who used immunosuppressors were excluded from the study's participation pool. All patients were HIV-negative and showed no cardiac alterations in electrocardiogram. The protocol used in the study does not present risks to the patients.

Two groups were defined to the study, and all patients included were randomized using a computer randomization table: (1) test group (n = 30): received meglumine antimoniate or Glucantime® as standardized plus NAC, three doses of 600 mg/day (a dose every 8 h), oral route, for the same period (SbV + NAC); (2) control group (n = 30): received Glucantime® (SbV) as standardized by Brazilian Ministry of Health, 20 mg/kg/day for 20 days, intravenously.

The medical team assessing outcomes was blinded during patient's follow-up. Patients included in control group did not receive placebo treatment. A follow-up of clinical and laboratorial change over time was performed by the clinical staff for 180 days. The data included in this study were at the following time points: D0, before treatment; D10 and D20, during treatment; and D30, after treatment. Blood samples were obtained in these time points and used for the laboratorial tests: blood count, dosage of proteins, and alanine and aspartate aminotransferases; and for serum cytokines, measurement at D0, D15, and D30.

The primary outcome was defined as the cure of VL 180 days after treatment and based on the following key readout indices: reduction of liver and spleen sizes, absence of fever, weight gain, and improvement of leucocytes and erythrocytes numbers.

2.2.1 Parasite load

Parasite load was evaluated in blood sample of patients during treatment according to previous published works (Schulz et al., 2003; de Oliveira et al., 2013).

2.2.2 Cytokines quantification

Cytokines in sera were measured in three different time points: D0, D15, and D30. The serum levels of Tumor Necrosis Factor-α (TNF-α), IL-12p40, IL-10, and sCD40L were measured using Luminex multiplex assay, according to manufacturer's recommendations (MILLIPLEX MAP Human Magnetic Bead Panel) (Merck Millipore, USA), read in a Luminex 200, and analyzed by a Milliplex Analyst software.

2.3 *In vitro* study to evaluate the effect of NAC plus antimony in *L. infantum* infection

2.3.1 Obtention of PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized whole blood of the healthy donors following previously published approach(es) (Barrios et al., 2019). Briefly, PBMCs were isolated by density gradient using Histopaque® 1077 (Sigma-Aldrich, USA). Cells were then washed and resuspended with supplemented RPMI 1640 media (10% of inactivated fetal bovine serum plus 1% of penicillin/streptomycin) (Gibco, USA).

2.3.2 N-acetylcysteine concentration

To development of *in vitro* experiments using human cells, the concentration of NAC was determined in 10 mM on the basis of the previously published data in literature (Giodani et al., 2002; Cruz et al., 2008).

2.3.3 PBMC stimulation, treatment, and flow cytometry protocol

PBMC assays were based on the previously published methods (Santos et al., 2017). Briefly, PBMCs were seeded in 48-well plates at a density of 1×10^6/well. Cells were treated under different conditions: (a) unstimulated; (b) stimulated with Soluble *Leishmania* Antigen (SLA; 1 mg/ml); (c) stimulated with SLA (1 mg/ml) plus antimoniate meglumine (20 μg/ml); (d) stimulated with SLA (1 mg/ml) plus 10 mM of NAC (Sigma-Aldrich, USA) or stimulated with SLA (1 mg/ml) plus antimoniate meglumine (20 μg/ml) plus 10 mM of NAC. After 6 h of incubation, Brefeldin A (BD Biosciences, USA) was added,

and the cells were then seeded for an additional 12 h. The plates were washed with 1× PBS prior to blocking with 2% fetal bovine serum and 2% of fetal goat sera. After 30 min, plates were washed and incubated with the following fluorescent antibodies for cell surface markers: CD3 PE-Cy7 and CD4 V500, or CD14 PE-Cy5 and CD40L V500 (BD Pharmigen, USA). After 20 min, cells were washed, permeabilized, and incubated with the following fluorescent antibodies for cytokines for intracellular detection: IL-2 BV421, TNF-α Alexa Fluor 488, and IFN-γ APC or IL-10–APC and TNF-α PE (BD Pharmigen, USA). The cells were then washed and resuspended in 1× PBS. A BD FACSCanto II (BD Biosciences, USA) flow cytometry was used to acquire a minimum of 50,000 cells. Datasets were analyzed using FlowJo software (BD Biosciences, USA).

2.3.4 Transcription factor expression and qPCR

RNA was isolated from PBMCs using TRIzol (Thermo Fisher Scientific, USA) following the manufacturer's recommended protocol. Total RNA was used for qPCR assays using TaqMan probes (Thermo Fisher Scientific, USA) to TBX21, GATA3, and FOXP3 genes. Amplification was performed using a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, USA).

2.3.5 Parasite culture

One strain of *L. infantum* parasite was used in the macrophage infection assays (LVHSE17, isolated from patient not included in the studies). *L. infantum* strain was cultivated at 24°C in B.O.D. incubator in Schneider's Insect Medium (Gibco, USA) supplemented with 10% inactivated bovine fetal sera and 1% penicillin/streptomycin (Gibco, USA).

Macrophage infection and treatment with NAC plus antimony

Macrophage differentiation, infection, and treatment followed prior published works (Barrios et al., 2019). Briefly, healthy donors' PBMCs were seeded in Nunc LabTek™ chamber slides (Thermo Scientific, USA) at a density of 5×10^5 cell per well. After 2 h of incubation, non-adherent cells were removed by washing. Adherent monocytes were differentiated for 6 days in an incubator (at 5% CO_2 and 37°C). Next, macrophages (1×10^5 per well) were co-cultured with stationary promastigotes of *L. infantum* at a 10:1 ratio for 2 h. Next, plates were washed to remove the non-internalized parasites and were incubated for 2, 24, and 72 h under different conditions as afore-described. Supernatants were removed, and cells were fixed with absolute methanol prior to staining with rapid panoptic (LaborClin, Brazil). A number of amastigotes and infected macrophages were determined at different time points by microscopy, and the parasite index was calculated (i.e., number of infected macrophages × amastigotes/100 macrophages). All experiments were performed in triplicate.

2.3.6 Parasite viability analysis after NAC treatment

The ability of NAC in killing *L. infantum* parasites was tested using a dose–response curve, as previously published (Magalhães et al., 2018). Briefly, mid-log phase promastigotes of *L. infantum* isolate used in the experiments were washed (described above) and exposed to crescent concentrations of NAC (0.1 to 80 mM) in Schneider Insect's Medium for 48 h. Parasites' viability was determined by counting motile promastigotes in a Neubauer chamber.

2.4 Statistical analysis

D'Agostino-Pearson normality test was used for testing the Gaussian distribution. Differences among groups were considered statistically significant when $p < 0.05$. Friedman test followed by Dunn's test was used for multiple comparisons. Wilcoxon test was used for the simple paired comparison, and Mann–Whitney test was used for simple unpaired comparison. R software was used in the construction of heatmaps (Heatmaply and ComplexHeatmap packages). Graphs were made using GraphPad Prism. Correlations were tested using Pearson's r. Bootstrapping method was used to resample data and test correlation between cytokine levels and clinical parameters. Effect sizes of the differences between groups were calculated in the pilot clinical trial (Martins et al., 2021). Using IBM® SPSS Statistics, we calculated one-way multivariate analysis of variance (MANOVA) to compare age and group effects in the pilot clinical trial, and effect size r of Mann–Whitney was used to compare various quantitative variables at the end of the treatment.

3 Results

3.1 Treatment of patients with VL with antimony + NAC increased sCD40L and decreased IL-10 levels in sera

Sixty-eight patients were diagnosed with VL. Sixty patients were selected to participate of the clinical trial. Thirty patients were randomly allocated in the test group that administered meglumine antimoniate plus NAC treatment (SbV + NAC). For the control group, 30 (separate) patients received standard meglumine antimoniate treatment (SbV group), without the NAC treatment (Supplementary Figure 1). A summary of clinical and laboratory data of patients enrolled in the studies is presented in Supplementary Table 1. The clinical and laboratorial parameters were evaluated before treatment (D0, before treatment), during treatment (D10 and D20, days 10 of 20 after treatment initiation), and after completion of treatment regimen (D30, 28 days after treatment initiation). Figure 1 shows no differences between the SbV group and the SbV + NAC group in the clinical and laboratorial parameters: spleen and liver sizes, hepatic enzymes, hemoglobin levels, platelets, neutrophils, or eosinophil counting. Beyond that, data from 32 patients (16 from each group) were used to make a heatmap using the major

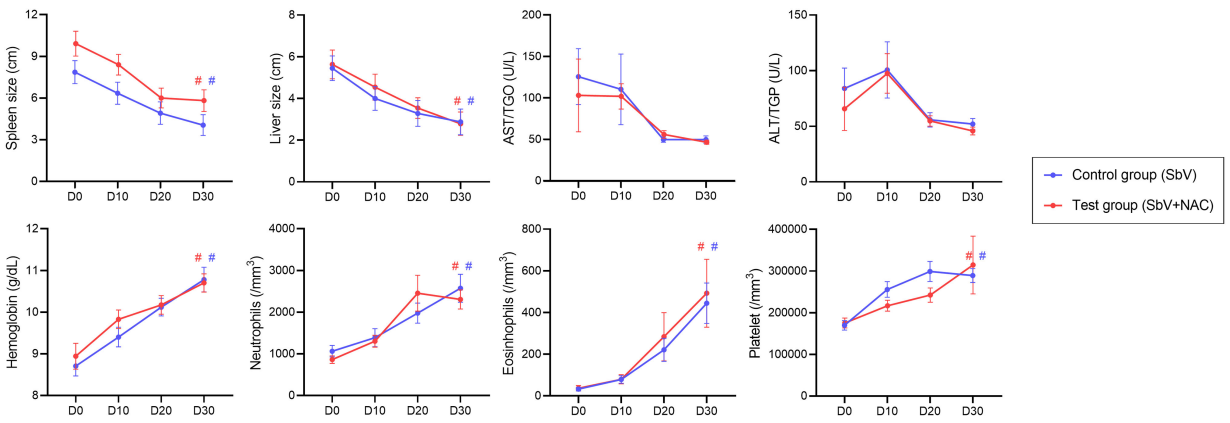


FIGURE 1
Clinical and laboratorial parameters measured from patients in the pilot clinical trial study. Data are obtained before (D0), during (D10 and D20), and after treatment (D30) of the control group (patients treated only with meglumine antimoniate for 28 days; SbV, blue) and the test group (patients treated with meglumine antimoniate plus NAC for 20 days; SbV + NAC, red). Dots and their lines represent mean ± SEM of patients in the group (n = 30). Kruskal–Wallis test followed by Dunn's test was used for comparisons between groups. #p < 0.001 (internal comparison between D30 and D0 for each group).

clinical and laboratorial parameters. Results show that there is no significant difference in patterns of responses between the groups independent of the treatment stage and parameter analyzed (Supplementary Figure 2).

To evaluate the immunological aspects from these patients, serum levels of IL-10, sCD40L, TNF-α, and IL-12p40 were measured at D0, D15, and D30. Observed results revealed that the test group, treated with SbV + NAC, had higher levels of sCD40L in all days evaluated when compared with the controls (Figure 2A). A significant difference between groups was observed when comparing the levels of sCD40L. Correlation analysis of sera during treatment shows that sCD40L of patients treated with SbV + NAC had a strong negative correlation with IL-10 levels (R = −0.809, p = 0.007) (Figure 2B). Moreover, IL-10 levels in sera during treatment of patients in the SbV + NAC group had a positive correlation with TNF-α (R = 0.851, p = 0.003). The sera from patients of the control group (SbV) show only a negative correlation between IL-12p40 and sCD40L (R = −0.700, p = 0.007). Supplementary Table 2 shows a comparison of clinical, laboratorial, and immunological parameters between the groups, as well as the effect sizes at D30. There is a significant difference between groups, and a strong effect size was observed when comparing levels of sCD40L. The cure rate is the same for both groups (100% of cure). There are no differences in clinical or laboratorial parameters between groups.

We also tested the association of sCD40L produced and released during treatment and the major clinical and laboratorial aspects evaluated in the study. Initial analysis shows the absence of significant correlation among sCD40L levels and the clinical and laboratorial aspects in SbV + NAC group or SbV group (Supplementary Figure 3). Using the bootstrapping procedure to resample data and to make correlations, a negative correlation between the spleen size and the levels of sCD40L in the SbV + NAC group (R = −0.351, p = 0.006), but not in the control SbV group, was determined (Figure 2C).

There was a negative correlation between sCD40L in sera and the parasite load in patients of the SbV + NAC group (R = −0.442, p = 0.007), but not observed in SbV group (R = −0.104, p = 0.6) (data not shown in figure). Beyond that, one-way MANOVA was conducted to determine a difference between SbV + NAC groups and controls of sCD40L on days 0, 15, and 30 (Supplementary Table 3). Notably, there was a significant difference in the rank of sCD40L in the group (F = 5.184, p = 0.008; Wilk's lambda = 0.425, partial eta squared = 0.425, observed power = 0.867). Furthermore, there was a significant effect on day 0 (F = 8.552, partial eta squared = 0.271, observed power = 0.800), on day 15 (F = 11,489, partial eta squared = 0.333, observed power = 0.901), and day 30 (F = 14,788, partial eta squared = 0.391, observed power =0.957). There was no significant difference in hemoglobin, amylase, platelets, and alanine aminotransferase (ALT/TGP) levels (data not shown).

3.2 *In vitro* experiments confirm that N-acetylcysteine reduces IL-10 production in monocytes and the activation of T CD4+ and CD8+ cells

The effect of NAC treatment in SLA-stimulated PBMCs was evaluated in monocyte cytokine production and T-cell functioning. The addition of NAC significantly reduced the

10.3389/fcimb.2022.1045668

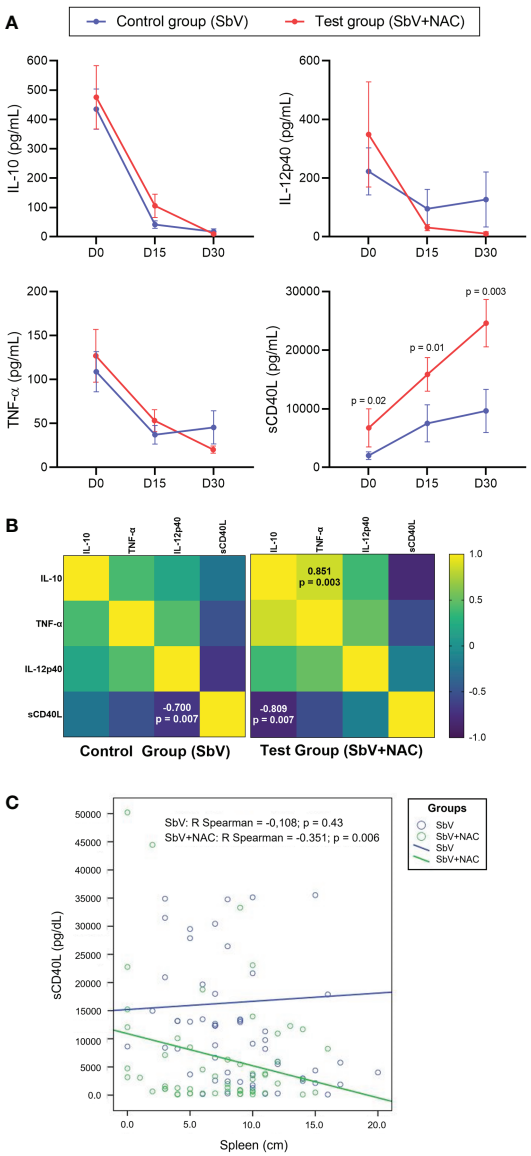


FIGURE 2
Immunological markers quantified in sera of patients included in pilot clinical trial study. Data are obtained before (D0), during (D15), and after treatment (D30) of the control group (patients treated only with meglumine antimoniate for 28 days; SbV, blue) and the test group (patients treated with meglumine antimoniate plus NAC for 20 days; SbV + NAC, red). **(A)** Dots and their lines represent mean ± SEM of patients in the group (n = 14 for SbV and n = 10 for NAC). Kruskal–Wallis test followed by Dunn's test was used for comparisons comparison between groups. **(B)** Correlation among levels of cytokines measured during treatment (D15) in patients of different groups was made using the Spearman correlation test. **(C)** For correlation between the sCD40L levels and the spleen size, data are resampled using bootstrapping. Dots represent resampled data, and lines represent correlation.

frequency of monocytes CD14+IL-10+ and not changed TNF-α+ cells frequency when compared to PBMCs treated only with meglumine antimoniate, leading to a higher TNF-α/IL-10 ratio in NAC-treated cells (Figure 3). In addition, NAC treatment of PBMC reduced the frequency of CD4+ T cells producing IL-2, TNF-α, and IFN-γ, either in the presence or the absence of meglumine antimoniate (Figure 4). Furthermore, results reveal a similar reduction of CD8+ T cells producing IL-2 or IFN-γ. Moreover, no effects were observed between the cells treated only with meglumine antimoniate, when compared to cells stimulated or not with SLA. The results of SLA-stimulated PBMCs treated with NAC reveal no significant reduction of GATA3 and FOXP3 or/and increased TBX21 transcripts (Supplementary Figure 4).

To evaluate the effect of NAC in the macrophage microbicidal activity, macrophages were infected with *L. infantum* and treated with NAC and/or SbV. Treatment of macrophages with NAC does not change the number of infected cells or parasite load in these cells. This is represented by unaltered parasite index at any time point (Figure 3). It was also tested whether NAC could reduce viability of *L. infantum* parasites. The data show that treatment of promastigotes with increasing concentrations of NAC for 48 h does not reduce parasites' viability (Supplementary Figure 5).

4 Discussion

The search of new drugs or alternative treatments for VL is an urgent goal for scientists and countries affected by this disease. In the present study, we aimed to evaluate the effect of NAC in *L. infantum* infection. A pilot clinical trial, using NAC as adjuvant to the standard VL treatment with meglumine antimoniate, shows that patients from the SbV + NAC group had higher sCD40L in sera during treatment, and these levels were negatively correlated with IL-10 levels. Data estimation show a negative correlation between the sCD40L levels and the spleen size and parasite load only in SbV + NAC group. However, the laboratorial and clinical parameters during disease evolution did not show any differences between the groups. *In vitro* experiments show that NAC treatment of stimulated PBMCs reduces the frequency of monocytes producing IL-10 and reduces the frequency of T CD4+ and CD8+ cells expressing inflammatory cytokines.

Arginase pathway in macrophages not only permits parasites to diminish the production of free radicals but also enhances the capacity of parasites to grow by the production of polyamines (Muxel et al., 2018). Moreover, dysregulated production of cytokines by immune response leads to unspecific activation of inflammatory cells that cause several tissues damage. Among the cytokines of higher importance in the VL pathogenesis, the production of IL-10, an important down regulatory cytokine of immune response, followed by production of pro-inflammatory cytokines as IL-6 and TNF-α, is associated to VL severity (Costa et al., 2013; dos Santos et al., 2016). Whereas, well-balanced secretion of sCD40L, IFN-γ, and IL-12 is associated to better

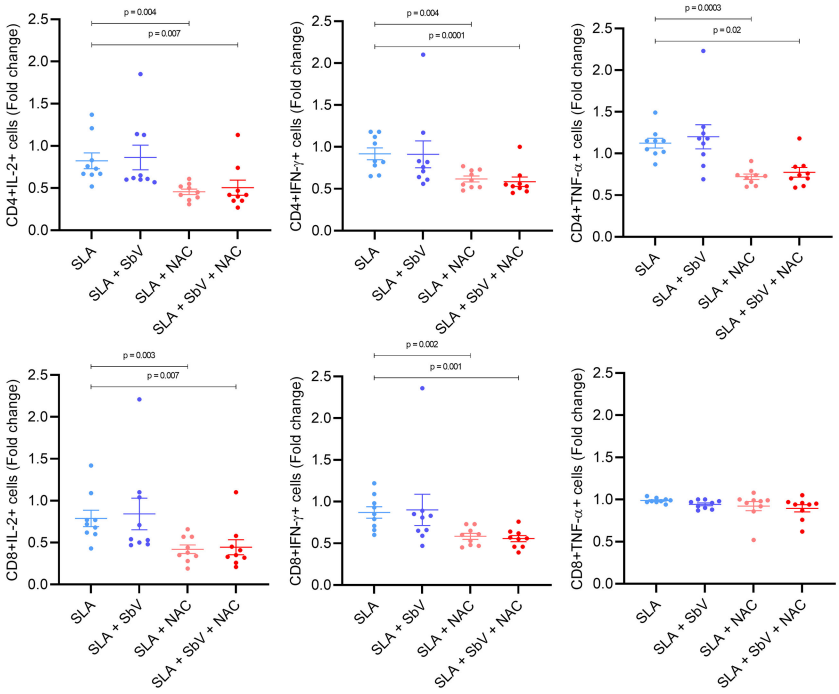


FIGURE 3
Frequency of CD4+ and CD8+ T cells producing IL-2, IFN-γ, or TNF-α in PBMCs stimulated with SLA and treated or not with SbV, NAC, and SbV + NAC. Graphs represent a fold change from unstimulated cells. Dots represent each healthy donor (n = 9), and lines represent mean ± SEM. Friedman test followed by Dunn's test was used for comparisons.

clinical evolution in patients with VL (Badaro et al., 1990; Bacellar et al., 2000; de Oliveira et al., 2013). Therefore, the effect of NAC reduction of IL-10 *in vitro*—together with the demonstration that, in the pilot clinical trial described here, the NAC treatment induces a higher production of sCD40L and that this production negatively correlates with IL-10 levels—suggests that the effect of NAC is associated with a better regulation of the innate and adaptive immune responses. Although IL-10 is important to downregulate an exacerbated immune response, the presence of IL-10 in concomitance with pro-inflammatory cytokines leads to a non-specific and non-resolutive immune response and has been associated to worse clinical evolution (Gautam et al., 2011; Singh et al., 2021).

Unfortunately, the use of NAC as treatment adjuvant does not change clinical evolution and laboratorial parameter when compared to standard protocol. In further analysis, the sCD40L levels also negatively correlated with the spleen size in the SbV + NAC group, but not in the control SbV group. sCD40L has been previously demonstrated as an important biomarker of VL disease, associated with better outcomes, stimulating resolutive immune response (Okwor and Uzonna, 2016; Adriaensen et al., 2018). Previous data of our group, using patients from the same local of this study, demonstrate that sCD40L is an important biomarker in VL, indicating a favorable evolution during and after treatment, negatively correlating with the spleen sizes and parasite load in patients with VL (de Oliveira et al., 2013).

Previous data in experimental animals also demonstrated that BALB/c mice infected with different *Leishmania* species and treated with NAC have lower parasitism in footpad lesion without affect lesion swelling (Cruz et al., 2008; Monteiro et al., 2008), reduced oxidative stress in liver without diminished production of inflammatory profile (Gasparotto et al., 2017), and reduced oxidative stress associated to lower levels of cytokines and pain in infected mice (Crupi et al., 2020). These data are in concordance with results demonstrated in the present work, showing a variation in *in vivo* disease evolution, mixing enhanced response to parasite infection, but not always associated to a better evaluation in markers of disease.

In accordance with our results of the pilot clinical trial, the *in vitro* study described here shows that the addition of NAC in the presence or absence of meglumine antimoniate reduces the frequency of monocytes producing IL-10 without a change in frequency of TNF-α+ cells. The higher ratio of TNF-α/IL-10 in NAC-treated monocytes than that in non-treated cells suggests that NAC has an important role as a modulator of immune response, leading to the activation of microbicidal pathway of TNF-α. In contrast to this, the treatment with NAC in

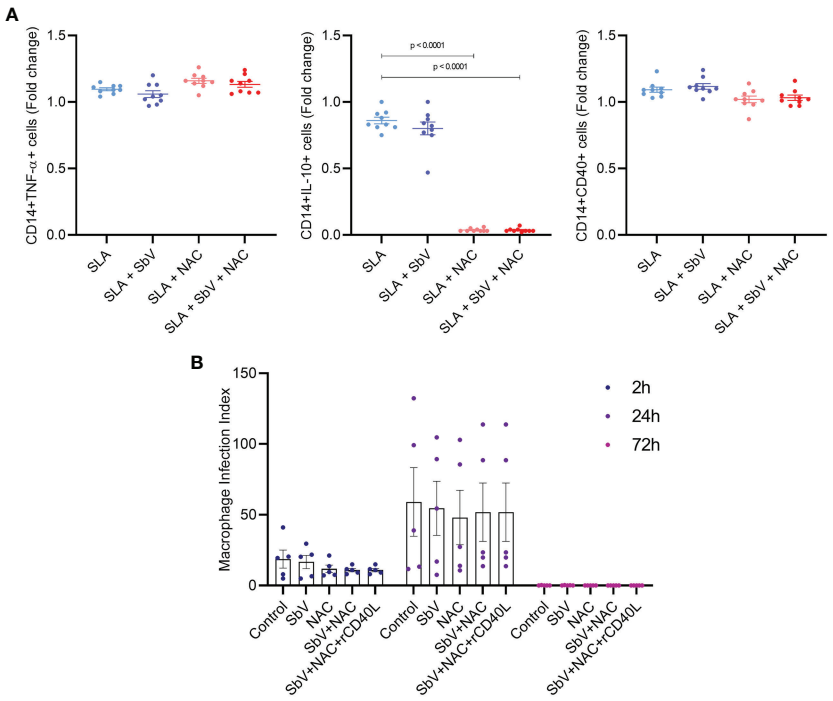


FIGURE 4
(A) Frequency of CD14+ monocytes producing TNF-α, IL-10+, CD40+ in PBMCs stimulated with SLA and treated or not with SbV, NAC, and SbV + NAC and obtained by flow cytometry. Graphs represent a fold change from unstimulated cells. Dots represent each healthy donor (n = 9), and lines represent mean ± SEM. **(B)** Macrophage infection index obtained from counting macrophages differentiated from PBMC and infected with *L. infantum* isolate (n = 5, in duplicate). Friedman test followed by Dunn's test was used for comparisons.

macrophage infected with *L. infantum* parasites does not reduce the parasite load. Our previous study has shown that sCD40L *in vitro* reduces the parasite numbers in macrophage cultures infected by *L. infantum* (de Oliveira et al., 2015). These data suggest that a combination of IL-10 downregulation and activation of CD40 pathway is needed. In addition to reducing the suppression of monocytes, NAC also reduces the presence of the effector T cells producing IL-2, TNF-α, and IFN-γ cytokines. These data suggest that NAC induces a resolutive immune response, especially when monocytes are in contact with lymphocytes, that could contribute to a better clinical evolution of patients with VL.

Although NAC could act as antioxidant donor to human cells and *Leishmania* parasites could be favored by antioxidant pathways in macrophage, the reduction of IL-10 might overcome the antioxidant effect. In addition, it is demonstrated that glutathione molecules, stimulated by NAC, could reduce activation of pro-inflammatory profile in human macrophages by inactivation of NF-κB transcription factor (Verhasselt et al., 1999; Fraternale et al., 2013). The use of glutathione donors decreases the production of TNF-α, IL-12, IL-6, IL-8, and IL-1β (Gosset et al., 1999; Haddad et al., 2001; Mazzeo et al., 2002), cytokines that are associated to the cytokine storm described in active VL, and some associated with worse prognosis, such as IL-6 and TNF-α. This mechanism of action together with our data is an evidence that NAC should be further tested as adjuvant in treatment of patients with VL, especially in the severe cases with cytokines storm. Therefore, on the basis of numerous studies in literature that demonstrate the importance of macrophage in the pathogenesis of VL disease and evolution (Liu and Uzonna, 2012), NAC as adjuvant therapy to antimony treatment might contribute to create a favorable immunological microenvironment in patients with VL. It is important to emphasize that the use of compound like NAC does not abrogate, but change the inflammatory response, making resolutive immune response and limiting the release of controversy cytokines, such as IL-10, and induces release of microbicidal agents as nitric oxide.

Although we have some positive results that demonstrate the effect of NAC in VL treatment, we could not demonstrate a clinical effect of this treatment, but there is no deleterious effect in any of the clinical parameters analyzed, demonstrating the safety of NAC even in association with antimony therapy. This study also did not observe the *in vitro* effect of NAC itself in the microbicidal activity of macrophages. Thus, more studies are needed, aiming to tests the use of NAC as adjuvant therapy in patients with VL, to compare its effect in different doses and in patients with

different disease severities, and to elucidate *in vitro* mechanisms of action of NAC in *Leishmania* infection.

Together, the results obtained here show that NAC as adjuvant therapy of patients with VL is safe, has altered some aspects of the immune response, such as higher levels of sCD40L, with a strong negative correlation with IL-10 levels, and confirmed *in vitro* the control of IL-10 release by monocytes. These data suggest that NAC treatment might be a candidate to be further studied as adjuvant therapy to antimony for patients with VL.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Universidade Federal de Sergipe. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Recruitment of patients and clinical evaluation: EM, ND, AA, AS, and MOB. Performing *in vitro* experiments: LM, CS, MR, RS, FO, and PS. Data analysis: EM, LM, AJ, and RA. Scientifical revision and analysis: JS and ML. Conceptualization and supervision of all research: AJ and RA. Writing of the manuscript: LM, CS, ML, AJ, and RA.

Funding

This work was supported by CHAMADA UNIVERSAL MCTI/CNPq n. 01/2016 (grant number: 429246/2016-1, RA) and by Universal, FAPITEC/SE, 2008. LM was sponsored by Postdoctoral Fellowship from INCT Imuno/CAPES. CS is sponsored by CAPES. JS, AJ, and RA are scientists supported by the Brazilian Research and Technology Council (CNPq). CS has Doctorate fellowship by CAPES.

Acknowledgments

All authors are grateful to the participating patients of this study. We also recognize the contributions of the medical team in administration and clinical monitoring at HU-EBSERH/UFS and the researchers and students of LIBM from UFS for support in the experimental studies.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer VA declared a shared affiliation with the author MR to the handling editor at the time of review.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1045668/full#supplementary-material

References

Adriaensen, W., Abdellati, S., van Henten, S., Gedamu, Y., Diro, E., Vogt, F., et al. (2018). Serum levels of soluble CD40 ligand and neopterin in HIV coinfected asymptomatic and symptomatic visceral leishmaniasis patients. *Front. Cell. Infect. Microbiol.* 8. doi: 10.3389/fcimb.2018.00428

Amaral, E. P., Conceição, E. L., Costa, D. L., Rocha, M. S., Marinho, J. M., Cordeiro-Santos, M., et al. (2016). N-acetyl-cysteine exhibits potent anti-mycobacterial activity in addition to its known anti-oxidative functions. *BMC Microbiol.* 16, 251. doi: 10.1186/s12866-016-0872-7

Aruleba, R. T., Carter, K. C., Brombacher, F., and Hurdayal, R. (2020). Can we harness immune responses to improve drug treatment in leishmaniasis? *Microorganisms* 8, 1–20. doi: 10.3390/microorganisms8071069

Bacellar, O., D'Oliveira, A., Jerônimo, S., and Carvalho, E. M. (2000). IL-10 and IL-12 are the main regulatory cytokines in visceral leishmaniasis. *Cytokine* 12, 1228–1231. doi: 10.1006/cyto.2000.0694

Badaro, R., Falcoff, E., Badaro, F. S., Carvalho, E. M., Pedral-Sampaio, D., Barral, A., et al. (1990). Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. *N. Engl. J. Med.* 322, 16–21. doi: 10.1056/NEJM199001043220104

Barrios, M. R., Campos, V. C., Peres, N. T. A., de Oliveira, L. L., Cazzaniga, R. A., Santos, M. B., et al. (2019). Macrophages from subjects with isolated GH/IGF-I deficiency due to a ghrh receptor gene mutation are less prone to infection by leishmania amazonensis. *Front. Cell. Infect. Microbiol.* 9. doi: 10.3389/fcimb.2019.00311

Bhor, R., Rafati, S., and Pai, K. (2021). Cytokine saga in visceral leishmaniasis. *Cytokine* 147, 155322. doi: 10.1016/j.cyto.2020.155322

Bonnaure, G., and Néron, S. (2014). N-acetyl cysteine regulates the phosphorylation of JAK proteins following CD40-activation of human memory b cells. *Mol. Immunol.* 62, 209–218. doi: 10.1016/j.molimm.2014.06.027

Brasil. Ministério da Saúde, Secretaria de Vigilância em Saúde (2022) *Leishmaniose visceral - casos confirmados notificados no sistema de informação de agravos de notificação – brasil.* Available at: http://tabnet.datasus.gov.br/cgi/tabcgi.exe?sinannet/cnv/leishvbr.def (Accessed 03/10/2022).

Burza, S., Croft, S. L., and Boelaert, M. (2018). Leishmaniasis. *Lancet* 392, 951–970. doi: 10.1016/S0140-6736(18)31204-2

Chakravarty, J., and Sundar, S. (2019). Current and emerging medications for the treatment of leishmaniasis. *Expert Opin. Pharmacother.* 20, 1251–1265. doi: 10.1080/14656566.2019.1609940

Costa, D. L., Rocha, R. L., Carvalho, R. M. A., Lima-Neto, A. S., Harhay, M. O., Costa, C. H. N., et al. (2013). Serum cytokines associated with severity and complications of kala-azar. *Pathog. Glob. Health* 107, 78–87. doi: 10.1179/2047773213Y.0000000078

Crupi, R., Gugliandolo, E., Siracusa, R., Impellizzeri, D., Cordaro, M., Di Paola, R., et al. (2020). N-acetyl-L-cysteine reduces leishmania amazonensis-induced inflammation in BALB/c mice. *BMC Vet. Res.* 16, 1–12. doi: 10.1186/s12917-020-2234-9

Cruz, K. K., Fonseca, S. G., Monteiro, M. C., Silva, O. S., Andrade, V. M., Cunha, F. Q., et al. (2008). The influence of glutathione modulators on the course of leishmania major infection in susceptible and resistant mice. *Parasite Immunol.* 30, 171–174. doi: 10.1111/j.1365-3024.2007.01014.x

Dayakar, A., Chandrasekaran, S., Kuchipudi, S. V., and Kalangi, S. K. (2019). Cytokines: Key determinants of resistance or disease progression in visceral leishmaniasis: Opportunities for novel diagnostics and immunotherapy. *Front. Immunol.* 10. doi: 10.3389/fimmu.2019.00670

de Oliveira, F. A., Vanessa Oliveira Silva, C., Damascena, N. P., Passos, R. O., Duthie, M. S., Guderian, J., et al. (2013). High levels of soluble CD40 ligand and matrix metalloproteinase-9 in serum are associated with favorable clinical evolution in human visceral leishmaniasis. *BMC Infect. Dis.* 13, 331. doi: 10.1186/1471-2334-13-331

de Oliveira, F. A., Barreto, A. S., Bomfim, L. G. S., Leite, T. R. S., dos Santos, P. L., de Almeida, R. P., et al. (2015). Soluble CD40 Ligand in Sera of Subjects Exposed to Leishmania infantum Infection Reduces the Parasite Load in Macrophages. *PLoS One* 10, e0141265. doi: 10.1371/journal.pone.0141265

Dodd, S., Dean, O., Copolov, D. L., Malhi, G. S., and Berk, M. (2008). Drug evaluation n -acetylcysteine for antioxidant therapy : pharmacology and. expert. *Opin.Biol.Ther* 8, 1955–1962. doi: 10.1517/14728220802517901

dos Santos, P. L., de Oliveira, F. A., Santos, M. L. B., Cunha, L. C. S., Lino, M. T. B., de Oliveira, M. F. S., et al. (2016). The severity of visceral leishmaniasis correlates with elevated levels of serum IL-6, IL-27 and sCD14. *PloS Negl. Trop. Dis.* 10, e0004375. doi: 10.1371/journal.pntd.0004375

Fraternale, A., Crinelli, R., Casabianca, A., Paoletti, M. F., Orlandi, C., Carloni, E., et al. (2013). Molecules altering the intracellular thiol content modulate NF-kB and STAT-1/IRF-1 signalling pathways and IL-12 p40 and IL-27 p28 production in murine macrophages. *PloS One* 8 (3), e57866. doi: 10.1371/journal.pone.0057866

Gao, X., Lampraki, E. M., Al-Khalidi, S., Qureshi, M. A., Desai, R., and Wilson, J. B. (2017). N-acetylcysteine (NAC) ameliorates Epstein-Barr virus latent membrane protein 1 induced chronic inflammation. *PloS One* 12, 1–20. doi: 10.1371/journal.pone.0189167

Gasparotto, J., Kunzler, A., Senger, M. R., de Souza, C., da, S. F., de Simone, S. G., et al. (2017). N-acetyl-cysteine inhibits liver oxidative stress markers in BALB/c mice infected with leishmania amazonensis. *Mem. Inst. Oswaldo Cruz* 112, 146–154. doi: 10.1590/0074-02760160403

Gautam, S., Kumar, R., Maurya, R., Nylén, S., Ansari, N., Rai, M., et al. (2011). IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. *J. Infect. Dis.* 204, 1134–1137. doi: 10.1093/infdis/jir461

Giordani, L., Quaranta, M. G., Malorni, W., Boccanera, M., Giacomini, E., and Viora, M. (2002). N-acetylcysteine inhibits the induction of an antigen-specific antibody response down-regulating CD40 and CD27 co-stimulatory molecules. *Clin. Exp. Immunol.* 129, 254–264. doi: 10.1046/j.1365-2249.2002.01897.x

Gosset, P., Wallaert, B., Tonnel, A. B., and Fourneau, C. (1999). Thiol regulation of the production of TNF-α, IL-6 and IL-8 by human alveolar macrophages. *Eur. Respir. J.* 14, 98–105. doi: 10.1034/j.1399-3003.1999.14a17.x

Haddad, J. J. E., Safieh-Garabedian, B., Saadé, N. E., and Land, S. C. (2001). Thiol regulation of pro-inflammatory cytokines reveals a novel immunopharmacological potential of glutathione in the alveolar epithelium. *J. Pharmacol. Exp. Ther.* 296, 996–1005.

Kumari, S., Kumar, V., Tiwari, R. K., Ravidas, V., Pandey, K., and Kumar, A. (2022). Amphotericin b: A drug of choice for visceral leishmaniasis. *Acta Trop.* 235, 106661. doi: 10.1016/j.actatropica.2022.106661

Kumari, D., Perveen, S., Sharma, R., and Singh, K. (2021). Advancement in leishmaniasis diagnosis and therapeutics: An update. *Eur. J. Pharmacol.* 910, 174436. doi: 10.1016/j.ejphar.2021.174436

Liévin-Le Moal, V., and Loiseau, P. M. (2016). Leishmania hijacking of the macrophage intracellular compartments. *FEBS J.* 283, 598–607. doi: 10.1111/febs.13601

Liu, D., and Uzonna, J. E. (2012). The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. *Front. Cell. Infect. Microbiol.* 2, 1–8. doi: 10.3389/fcimb.2012.00083

Magalhães, L. S., Bomfim, L. G., Mota, S. G., Cruz, G. S., Corrêa, C. B., Tanajura, D. M., et al. (2018). Increased thiol levels in antimony-resistant leishmania infantum isolated from treatment-refractory visceral leishmaniasis in Brazil. *Mem. Inst. Oswaldo Cruz* 113, 119–125. doi: 10.1590/0074-02760170289

Martins, S. S., Barroso, D. H., Rodrigues, B. C., da Motta, J., de, O. C., Freire, G. S. M., et al. (2021). A pilot randomized clinical trial: Oral miltefosine and pentavalent antimonials associated with pentoxifylline for the treatment of American tegumentary leishmaniasis. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.700323

Mazzeo, D., Sacco, S., Di Lucia, P., Penna, G., Adorini, L., Panina-Bordignon, P., et al. (2002). Thiol antioxidants inhibit the formation of the interleukin-12 heterodimer: A novel mechanism for the inhibition of IL-12 production. *Cytokine* 17, 285–293. doi: 10.1006/cyto.2002.1014

Monteiro, M. C., Marques, F. C. S., Blazius, R. D., Santos Da Silva, O., De Queiroz Cunha, F., Bento, D. B., et al. (2008). N-acetyl-L-cysteine reduces the parasitism of BALB/c mice infected with leishmania amazonensis. *Parasitol. Res.* 102, 801–803. doi: 10.1007/s00436-007-0827-x

Musa, A. M., Mbui, J.., Mohammed, R., Olobo, J., Ritmeijer, K., Alcoba, G, et al. (2022). Paromomycin and miltefosine combination as an alternative to treat patients with visceral leishmaniasis in eastern africa: A randomized, controlled, multicountry trial. *Clin. Infect. Dis.*. doi: 10.1093/cid/ciac643.

Muxel, S. M., Aoki, J. I., Fernandes, J. C. R., Laranjeira-Silva, M. F., Zampieri, R. A., Acuña, S. M., et al. (2018). Arginine and polyamines fate in leishmania infection. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.02682

Okwor, I., and Uzonna, J. E. (2016). Pathways leading to interleukin-12 production and protective immunity in cutaneous leishmaniasis. *Cell. Immunol.* 309, 32–36. doi: 10.1016/j.cellimm.2016.06.004

Pedre, B., Barayeu, U., Ezeriņa, D., and Dick, T. P. (2021). The mechanism of action of n-acetylcysteine (NAC): The emerging role of H2S and sulfane sulfur species. *Pharmacol. Ther.*, 228, 107916. doi: 10.1016/j.pharmthera.2021.107916

Santos, M. L. B., Nico, D., de Oliveira, F. A., Barreto, A. S., Palatnik-de-Sousa, I., Carrillo, E., et al. (2017). Leishmania donovani nucleoside hydrolase (NH36) domains induce T-cell cytokine responses in human visceral leishmaniasis. *Front. Immunol.* 8. doi: 10.3389/fimmu.2017.00227

Schulz, A., Mellenthin, K., Schönian, G., Fleischer, B., and Drosten, C. (2003). Detection, differentiation, and quantitation of pathogenic leishmania organisms by a fluorescence resonance energy transfer-based real-time PCR assay. *J. Clin. Microbiol.* 41, 1529–1535. doi: 10.1128/JCM.41.4.1529-1535.2003

Singh, O. P., Syn, G., Nylén, S., Engwerda, C., Sacks, D., Wilson, M. E., et al. (2021). Anti-interleukin-10 unleashes transcriptional response to leishmanial antigens in visceral leishmaniasis patients. *J. Infect. Dis.* 223, 517–521. doi: 10.1093/infdis/jiaa381

Verhasselt, V., Vanden Berghe, W., Vanderheyde, N., Willems, F., Haegeman, G., and Goldman, M. (1999). N-acetyl-L-cysteine inhibits primary human T cell responses at the dendritic cell level: association with NF-kappaB inhibition. *J. Immunol.* 162, 2569–2574.

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Mini Review
PUBLISHED 12 December 2022
DOI 10.3389/fcimb.2022.1063291

OPEN ACCESS

EDITED BY
Javier Moreno,
Carlos III Health Institute (ISCIII), Spain

REVIEWED BY
Ayan Kumar Ghosh,
Medical College of Wisconsin,
United States

*CORRESPONDENCE
Alexandre Morrot
alexandre.morrot@ioc.fiocruz.br

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 06 October 2022
ACCEPTED 14 November 2022
PUBLISHED 12 December 2022

CITATION
Conde L, Maciel G, de Assis GM, Freire-de-Lima L, Nico D, Vale A, Freire-de-Lima CG and Morrot A (2022) Humoral response in Leishmaniasis.
Front. Cell. Infect. Microbiol.
12:1063291.
doi: 10.3389/fcimb.2022.1063291

Humoral response in Leishmaniasis

Luciana Conde[1], Gabriela Maciel[1], Gustavo Meira de Assis[1], Leonardo Freire-de-Lima[1], Dirlei Nico[2], André Vale[1], Célio Geraldo Freire-de-Lima[1] and Alexandre Morrot[3,4]*

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [3]Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [4]Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil

Leishmaniasis presents different types of clinical manifestations that can be divided into cutaneous leishmaniasis and visceral leishmaniasis. The host's immune system, associated with genetic and nutritional factors, is strongly involved in the evolution of the disease or parasite escape. Humoral immunity is characterized by the production of antibodies capable of promoting neutralization, opsonization, and activation of the complement system. In this scenario, B lymphocytes produce antibodies that play an important role in Leishmania infection although neglected for a long time. Thus, relevant aspects in the establishment of Leishmania infection will be addressed, highlighting the importance of humoral immunity during the entire process of Leishmania infection.

KEYWORDS

***Leishmania*, humoral response, leishmaniasis, complement system, B lymphocytes**

Introduction

Leishmaniasis is a complex of diseases caused by flagellated protozoa of the genus *Leishmania* (order Kinetoplastida, family Trypanosomatidae) with different clinical manifestations (Kaufer et al., 2017). The protozoan is inoculated into the vertebrate host during the blood meal of infected female sandflies and can generate the disease symptomatically or asymptomatically (WHO, 2022). Leishmaniasis has two main forms with different clinical manifestations, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). The symptomatology is determined by a combination of factors, relating to the host, the parasite, and the vector, mainly the *Leishmania* species and the vertebrate host's immune response to infection (Kaufer et al., 2017).

CL is the most common form of leishmaniasis. It can occur in three different types: localized cutaneous leishmaniasis (LCL); diffuse cutaneous leishmaniasis (DCL); and mucocutaneous leishmaniasis (ML) (Reithinger et al., 2007). Collectively these three cutaneous forms can be called cutaneous leishmaniasis (CT). LCL is the mildest form of

leishmaniasis, its clinical manifestation is characterized by one or multiple lesions that can ulcerate in exposed parts of the body, usually rounded and with raised edges (Gontijo and de Carvalho, 2003). DCL is the least common form (Sampaio et al., 2021), characterized by the development of multiple nodules without ulceration that can affect the entire body. ML is a form restricted to Latin America, after skin lesions, the disease spreads to the mucous membrane of the nose, mouth, and throat, where subsequently there is the formation of ulcers in the mucosa that destroy the nasal septum, lips, and nose. palate, leading to deformations that disfigure the face of the infected individual (Steverding, 2017). The main species of *Leishmania* involved in the localized cutaneous manifestation are: *Leishmania braziliensis*, *Leishmania amazonensis*, *Leishmania panamensis*, *Leishmania lainsoni*, *Leishmania guyanensis*, *Leishmania tropica*, *Leishmania major*, *Leishmania mexicana*; in diffuse cutaneous manifestation: *Leishmania amazonensis*; in the mucocutaneous manifestation: *Leishmania braziliensis* (Desjeux, 2004; Reithinger et al., 2007).

Meanwhile, VL is the most severe form of the disease (Chapman et al., 2015). More than 90% of VL cases occur in the African Continent, in the Indian Subcontinent and in Latin America (Chappuis et al., 2007). Whereas in North Africa and in Latin America VL is commonly attributed to *Leishmania infantum*, cases in East Africa and in the Indian Subcontinent are usually linked to Lesihmania donovani (Lukes et al., 2007). VL is also known as Kala-azar, an Indian name for "black fever", due to the prolonged febrile manifestation and hyperpigmentation associated with the disease. It is characterized by the infection of phagocytes and of the reticuloendotelial system, leading to the infection of many anatomically associated sites, such as lymph nodes, spleen and liver (WHO, 2022).

Although most research groups focus on the study of cellular responses to Leishmaniasis for reasons that will be discussed subsequently, the challenges in control, treatment and vaccine formulation highlight the necessity of better understanding and discussing aspects of humoral immunity.

General aspects and immune system cells involved in the initiation of *Leishmania* infection

The immune response against the infection is dependent on several factors, such as its antigenicity, the host's immune system, and the parasite load (Santos-Gomes et al., 2002). After inoculation of the parasite by the vector into the vertebrate host, *Leishmania* benefits from a pro-inflammatory environment induced by the vector's saliva for its intracellular infection, which, through chemoattraction, attracts phagocytic cells to the site of infection (Chagas et al., 2014). The first cells to arrive at the site of infection and actively phagocytose the promastigote forms of *Leishmania* are neutrophils (Muller et al., 2001; Mollinedo et al., 2010). However, the most important cells for parasite replication and the establishment of infection are macrophages. Macrophages confine phagocytosed *Leishmania* in a phagolysosome, a low pH organelle filled with lytic enzymes (Podinovskaia and Descoteaux, 2015). The main leishmanicidal mechanisms of the macrophage are the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), these processes are extremely important for the elimination of the parasite without damage to the host cell (Iles and Forman, 2002; Fang, 2004). The inhibition of these mechanisms is the main evasion strategy of the parasite. The *Leishmania* metalloproteinase gp63 inhibits oxidative stress by interfering with the induced nitric oxide synthase (iNOS) and NADPH oxidase 2 (NOX2) signaling pathways of macrophages (Olivier et al., 2012).

Although innate immunity is associated with the elimination of the intracellular parasite, recently important aspects regarding humoral immunity have been raised during *Leishmania* infection.

Humoral response in leishmaniasis

Humoral immunity is mediated by antibodies secreted by B cells (Mauri and Bosma, 2012). During the immune response, antibodies are capable of neutralization, opsonization, and activation of the complement system (CS). In leishmaniasis, the importance of CS activation is commonly highlighted since it is the first barrier faced by *Leishmania* in the vertebrate host. The parasite can evade the CS, preventing its lysis through surface molecules such as LPG and gp63 (Gurung and Kanneganti, 2015).

For a long time, B lymphocytes were neglected in *Leishmania* infection, as these parasites are obligatorily intracellular (Bates and Rogers, 2004). However, studies have already demonstrated the exacerbation of the B lymphocyte's response to infection by some *Leishmania* species favoring the parasite (Firmino-Cruz et al., 2019). Studies with B-cell-deficient mice have shown that symptoms appear later and with less severe lesions than in control mice (Smelt et al., 2000; Wanasen et al., 2008).

Deak and colleagues demonstrated that polyclonal activation of B cells in the course of infection leads to disease exacerbation. Through the use of B-cell-deficient mice and adoptive transfer of specific or non-specific IgM and IgG, a return to the susceptibility phenotype was observed in JnD Balb/c resistant mice (Deak et al., 2010). The correlation between B cells and a poor prognosis in leishmaniasis was also evidenced by the work of Omachi et al., demonstrating that animals deficient in B cell activating factor (BAFF) can suppress the splenomegaly characteristic of the disease in the experimental model of VL with *L. donovani*, but not hepatomegaly (Omachi et al., 2017). In

a previous study, the same group demonstrated an increase in serum levels of BAFF in patients with visceral leishmaniasis, where the mean value of BAFF in Brazilian patients was 4.3 higher than the mean of controls (Goto et al., 2014). The magnitude of this increase in serum BAFF levels is equivalent to the increase demonstrated in patients with systemic sclerosis (Matsushita et al., 2006) and Sjorgen syndrome (Groom et al., 2002).

Prevalence of different immunoglobulin classes may point to different stages of the disease and different clinical outcomes

One of the most discussed immunological aspects of both cutaneous (Castellano et al., 2009) and visceral (Heinzel et al., 1989; Wang et al., 1994) leishmaniasis is the dynamics between Th1 and Th2 responses, in which a dominant Th2 response, stimulated by the preponderant presence of cytokines such as interleukin-10 (IL-10) and interleukin-4 (IL-4), would suppress the effector profile of a Th1 response and clamp down the classical activation of macrophages (M1). This would favor the parasite with the dominance of anti-inflammatory/pro-resolutive M2 macrophages, which not only block more aggressive responses that could help parasite clearance but are also susceptible cells in which the entry of *Leishmania* promastigotes is facilitated (Heinzel et al., 1993; Farrow et al., 2011; Lee et al., 2018).

In view of a such well established paradigm and the unfavorable effects of B cell responses to the host that will be discussed below, humoral responses in Leishmaniasis have not been a big focus of interest in this field of research. However, B cells can function not only as antibody-secreting cells, but they can also modulate the immune response through antibody-independent mechanisms, such as antigen presentation and secretion of cytokines and chemokines (Myers, 1991; Lund, 2008).

The presence of B cells and their polyclonal activation has been directly correlated with a poor prognosis of the disease, recent studies have directed efforts to demonstrate the role of regulatory B cells (Breg) in the course of the disease (Soares et al., 2017). Recently, it was demonstrated that the incubation of B cells with amastigote forms of *L. infantum* is capable of activating subpopulations of human B cells with an immunoregulatory phenotype that secretes IL-10 in a dose-dependent manner, inhibiting the activation and proliferation of CD4+ T cells (Andreani et al., 2015). Type 1 B cells (B-1) have also been implicated in susceptibility in experimental visceral leishmaniasis infection with increased IL-10 and it has been shown that Balb/XID mice (B-1 cell deficient) have lower serum IL-10 and less parasite load in the spleen compared to the control. The transfer of B-1 cells to IL-10 knockout animals led to increased susceptibility to *L. chagasi* infection (Gonzaga et al., 2015). In other infections with protozoa, such as *Trypanosoma cruzi*, studies have shown that antibodies are responsible for the survival of susceptible animals in the early stages of the disease and the maintenance of low levels of parasitemia in the chronic phase (Umekita et al., 1988; Bermejo et al., 2011). Thus, parasite-specific immune response is insufficient to eradicate the disease, allowing infection in the chronic phase.

The dominant cytokine profile also impacts antibody production both in quality and quantity, as it can direct B cells to engage in class switch recombination of the immunoglobulin gene (Snapper and Paul, 1987). Although high titers of antileishmanial antibodies are characteristically present in the visceral forms of the disease (Behforouz et al., 1976; Carvalho et al., 1985; Sacks and da Silva, 1987), a feature accompanied by diminished cellular response (Castes et al., 1983; Cillari et al., 1988), this antibody abundance is usually not capable of promoting protection of the host (Nylen and Gautam, 2010). In fact, B cell activity is often described as detrimental in the context of leishmaniasis: IgG-coated *L. major* amastigotes could be internalized more efficiently by murine macrophages, subsequently inducing IL-10 production (Kane and Mosser, 2001) which has been described as detrimental for, among other effects, supposedly aiding the shift from a predominantly Th1 profile to a predominantly Th2 (Ghalib et al., 1995; Revaz-Breton et al., 2010). Additionally, Fc deficient mice infected by *L. amazonensis* were observed to produce less IL-10 and to be less susceptible to infection (Buxbaum and Scott, 2005). High antibody titers have also been reported in association with disease severity in mice experimentally infected with *L. amazonensis* (Wanasen et al., 2008).

On the other hand, the adoptive transfer of IL-10-producing B-1 cells to infected mice did not impact disease outcomes (Firmino-Cruz et al., 2020). It has been shown that T cells themselves can be a source of IL-10 during visceral leishmaniasis in an antigen-dependent manner, determining infection aftermath in mice (Schwarz et al., 2013). This, of course, impacts vaccine development, as it would be necessary to induce a response that would exclude the activation of IL-10-producing T cells while still promoting the adaptive cellular response.

Still, more attention has been given to the potentially detrimental contribution of B cells in the context of leishmaniasis. While high anti-*Leishmania* IgG titers have been correlated to mucosal leishmaniasis severity (de Lima et al., 2021), a correlation between high levels of *Leishmania*-specific IgA and IgE seem to have contributed to more severe forms of American tegumentary leishmaniasis in the context of *L. panamensis* infection (O'Neil et al., 1993). Likewise, abundant IgG and IgM, forming immune complexes with complement factors of the classical and terminal pathways, have been

implicated in Leishmaniasis-Associated Membranoproliferative Glomerulonephritis (Sethi et al., 2016). Furthermore, a case of *L. infantum* reactivation with secondary IgA nephropathy has recently been described (Grewe et al., 2022).

Nevertheless, understanding the dynamics of antibody production in leishmaniasis may be an important prognostic tool. Steady levels of IgM, IgE, and IgG4 following drug therapy can be suggestive of disease persistence and potential clinical relapse (Anam et al., 1999). This is especially important considering the rise of strains resistant to pentavalent antimonials (Thakur et al., 1997; Rugani et al., 2019; Andrade et al., 2020).

Conversely, the importance of B cells and humoral response in protective responses to *Leishmania* should not be completely discarded. Studies characterizing B cell clones (through the sequencing of the rearranged, and potentially somatically hypermutated, immunoglobulin gene segments) and the subset to which these clones belong are still needed. For instance: while IgG1 against *L. infantum* was correlated to asymptomatic infection and IgG2 to disease manifestation by one group (Reis et al., 2006), another group observed that even though asymptomatic dogs infected with *L. infantum* had lower levels of anti-*Leishmania* IgG2, dogs protected against the disease through vaccination with Leishimmune® (Fort Dodge Animal Health) had high levels of anti-*Leishmania* IgG2 (Oliveira et al., 2009). Interestingly, although high titers of IgE have been implicated in disease activity in the context of VL, high titers of IgE in CL have been observed to be correlated with a diminished number of skin ulcers, although positively correlated with bigger Montenegro's reaction size (Atta et al., 2002). These data suggest that protective action of IgG2 and IgE is context dependent and that maybe the binding site characteristics of the antibodies is more decisive than immunoglobulin class.

Complement system

The complement system plays an important role in innate immune defense, consisting of about 35 proteins that may be present in the plasma or on the plasmatic membrane surface of some cell types (Trouw and Daha, 2011; Ambrosio et al., 2021). Previous studies discuss that several proteins that constitute the complement system are synthesized in the liver, about 7 proteins can be synthesized by human skin fibroblasts (Katz et al., 1989) and it is currently known that dendritic cells are capable of synthesizing C1q, C3, Factor I, Factor B and complement receptors 3 and 4 (Reis et al., 2007). Rcent studies shed light on the contribution of adipose tissue to the activation of the complement system through the production of complement factor D. Factor D is a serine protease that will play a fundamental role in generating the C3 convertase, after cleaving factor B, activating the alternative complement pathway (Sekine et al., 2022).

In addition to its role as an effector mechanism of the innate immune system, complement also plays an important role in the formation of the adaptive immune response. This occurs because these proteins can interact with each other, triggering a proteolytic cascade or with other molecules, such as antibodies. Activation of the complement system can occur through three distinct pathways: the classical pathway (Cooper, 1985), the lectin pathway (Sato et al., 1994), and the alternative pathway (Soothill and Harvey, 1977). All these pathways converge to a common point resulting in the activation of the C3 component and its deposition on the surface of a pathogen (Walport, 2001). Regardless of the pathway of activation of the complement system, all 3 lead to the formation of the C3-convertase complex that will then initiate the proteolytic cascade favoring the formation of the Membrane Attack Complex (MAC) that causes osmotic lysis of the pathogen (Trouw and Daha, 2011).

Protozoa of the genus *Leishmania* are obligate intracellular parasites that need to be phagocytosed and survive within phagocytic cells of mammals, such as neutrophils and macrophages (Podinovskaia and Descoteaux, 2015). To survive the hostile environments faced throughout its life cycle, *Leishmania spp* expresses unique molecules such as glycoinositolphospholipids (GIPLs), which are glycoconjugates known to be the main component of the parasite's surface, lipophosphoglycan (LPG) and metalloprotease GP63 (Davies et al., 1990). More recent studies with *L. infantum* investigated how the LPG molecule influences the initial establishment of infection during interaction with human neutrophils in an *in vitro* experimental environment. They observed that mutant parasites that did not express LPG had a reduced viability and that this was related to an increased lysosomal fusion in the neutrophils evaluated by confocal microscopy (Quintela-Carvalho et al., 2022). Other remarkable adaptive mechanisms include inhibition of phagosome-endosome fusion (Desjardins and Descoteaux, 1997), expression of hydrolytic enzymes, modulation of cell signaling pathways (Eilam et al., 1985), nitric oxide production (Wei et al., 1995), and cytokine induction (Barral-Netto et al., 1992).

While in the bloodstream, the escape of the complement system is an important step in the establishment of the infection, and a mechanism developed by this parasite, both in metacyclic promastigotes and in amastigotes, involves the inactivation of C3b, converting it to its inactive form iC3b by the action of the membrane protease GP63, which is the subject of clinical studies for a therapeutic approach (Brittingham and Mosser, 1996; Mosser and Brittingham, 1997). In addition, inactive C3b continues to play the role of opsonization and its deposit on the surface of the parasite increases the chance of phagocytosis, since macrophages and related cells have CR1, CR3, and CR4 receptors that recognize the intact C3b component but also its inactive form (Mosser and Edelson, 1987; Tausk and Gigli, 1990; Brittingham and Mosser, 1996; Mosser and Brittingham, 1997; Lukacsi et al., 2017).

Thus, recent studies have evaluated other mechanisms that the parasite could play to achieve immune escape and maintain opsonization through the inactivation of C3b in iC3b by a pathway other than GP63. It was seen that the parasite can recruit factor H (complement system regulatory protein), which in turn recruits factor I, which acts by cleaving the C3 deposited on the surface of the parasite, promoting its inactivation in iC3b without compromising opsonization and subsequent phagocytosis of the protozoan (Filho et al., 2021).

Another immune escape mechanism was demonstrated *via* the lectin pathway, where mannose-binding lectin (MBL), collectin-11 (CL-11), and ficolins-1 and -3 were shown to bind to the surface of *L. infantum* promastigotes (both LPG and GIPL) when exposed to 20% NHS (normal human serum). These molecules can recognize pathogen-associated molecule patterns (PAMPs) on the surface of *L. infantum* promastigotes triggering the activation of the lectin pathway, suggesting a role in promoting the host/parasite interaction, leading to important events such as phagocytosis and macrophage activation in the initial infection (Ambrosio et al., 2021). Furthermore, it has been shown that high levels of serum mannose-binding lectin (MBL) are associated with modulation in macrophage function, increasing the susceptibility to Leishmaniasis infection (Santos et al., 2001). It has been shown that *Leishmania donovani* is able to inhibit the lectin pathway through the activity of its Inhibitor of Serine Proteases 2 (LdISP2), preventing the formation of the MAC by reducing the formation of C3 and C5 convertases (Verma et al., 2018). Finally, studies have shown that genetic modifications in complement genes, such as single nucleotide polymorphisms (SNPs), can influence host susceptibility to these parasites (Tirado et al., 2021).

Conclusion

Divergent observations, such as that IgG2 can be protective or detrimental in the context of *L. infantum* infecting dogs, may point to the possibility that the complementarity-determining regions of the antibodies are more relevant than the immunoglobulin class itself in determining disease outcome. Although some of the studies show that mice lacking B cells are resistant to several forms of leishmaniasis, B cell depletion brings a huge variety of physiological imbalances, which would not be desirable in therapeutic applications to humans and animal companions. In the light of the fact that any vaccine that acts on T cells will affect B cell activity through cognate interactions, screening of the protective B cell repertoire and subpopulation distribution in the context of leishmaniasis is of the utmost importance.

The unique adaptive mechanisms developed by *Leishmania spp* to evade immune responses includes the ability to inhibit the complement system of mammalian hosts. It is not clear, however, if antibodies targeting surface molecules and enzymes involved in this process could impair such evasion. In light of the recent discovery that *Leishmania donovani*'s LdISP2 is able to inhibit C3 and C5 convertase formation, an interesting question would be if antibodies that bind to LdISP2 would be protective or attenuate the disease *in vivo*. In case such antibodies are protective, another interesting question would be if Immunoglobulin class is determinant in such protection. Understanding if specific B cell subpopulations are more implicated in the secretion of antibodies would also be compelling, as this may determine what would be the best adjuvants for an immunization protocol. These types of data may be useful in future vaccine designs that take not only cellular, but also humoral adaptative responses into consideration.

Author contributions

LC, GaM, GuM, LF-D-L, DN, AV, CF-D-L, AM wrote the manuscript equally. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by Faperj, CNPq and Capes.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ambrosio, A. R., Bavia, L., Hiraiwa, P. M., Tirado, T. C., Figueiredo, F. B., and de Messias-Reason, I. J. (2021). The lectin pathway of complement and the initial recognition of *leishmania infantum promastigotes*. *Life Sci.* 282, 119793. doi: 10.1016/j.lfs.2021.119793

Anam, K., Afrin, F., Banerjee, D., Pramanik, N., Guha, S. K., Goswami, R. P., et al. (1999). Differential decline in leishmania membrane antigen-specific immunoglobulin G (IgG), IgM, IgE, and IgG subclass antibodies in Indian kala-azar patients after chemotherapy. *Infect. Immun.* 67 (12), 6663–6669. doi: 10.1128/IAI.67.12.6663-6669.1999

Andrade, J. M., Gonçalves, L. O., Liarte, D. B., Lima, D. A., Guimarães, F. G., de Melo Resende, D., et al. (2020). Comparative transcriptomic analysis of antimony resistant and susceptible *leishmania infantum* lines. *Parasit. Vectors* 13 (1), 600. doi: 10.1186/s13071-020-04486-4

Andreani, G., Ouellet, M., Menasria, R., Gomez, A. M., Barat, C., and Tremblay, M. J. (2015). Leishmania infantum amastigotes trigger a subpopulation of human b cells with an immunoregulatory phenotype. *PloS Negl. Trop. Dis.* 9 (2), e0003543. doi: 10.1371/journal.pntd.0003543

Atta, A. M., Sousa-Atta, M. L., D'Oliveria, A., Almeida, R. P., Araújo, M. I., and Carvalho, E. M. (2002). IgG anti-IgE autoantibodies in visceral leishmaniasis. *Mem Inst Oswaldo Cruz.* 97(1):101–103. doi: 10.1590/s0074-02762002000100017

Barral-Netto, M., Barral, A., Brownell, C. E., Skeiky, Y. A., Ellingsworth, L. R., Twardzik, D. R., et al. (1992). Transforming growth factor-beta in *leishmanial infection*: A parasite escape mechanism. *Science* 257 (5069), 545–548. doi: 10.1126/science.1636092

Bates, P. A., and Rogers, M. E. (2004). New insights into the developmental biology and transmission mechanisms of leishmania. *Curr. Mol. Med.* 4 (6), 601–609. doi: 10.2174/1566524043360285

Behforouz, N., Rezai, H. R., and Gettner, S. (1976). Application of immunofluorescence to detection of antibody in *leishmania infections*. *Ann. Trop. Med. Parasitol.* 70 (3), 293–301. doi: 10.1080/00034983.1976.11687125

Bermejo, D. A., Amezcua Vesely, M. C., Khan, M., Acosta Rodríguez, E. V., Montes, C. L., Merino, M. C., et al. (2011). Trypanosoma cruzi infection induces a massive extrafollicular and follicular splenic b-cell response which is a high source of non-parasite-specific antibodies. *Immunology* 132 (1), 123–133. doi: 10.1111/j.1365-2567.2010.03347.x

Brittingham, A., and Mosser, D. M. (1996). Exploitation of the complement system by leishmania promastigotes. *Parasitol. Today* 12 (11), 444–447. doi: 10.1016/0169-4758(96)10067-3

Buxbaum, L. U., and Scott, P. (2005). Interleukin 10- and fcgamma receptor-deficient mice resolve *leishmania mexicana lesions*. *Infect. Immun.* 73 (4), 2101–2108. doi: 10.1128/IAI.73.4.2101-2108.2005

Carvalho, E. M., Johnson, W. D., Barreto, E., Marsden, P. D., Costa, J. L., Reed, S., et al. (1985). Cell mediated immunity in American cutaneous and mucosal leishmaniasis. *J. Immunol.* 135 (6), 4144–4148.

Castellano, L. R., Filho, D. C., Argiro, L., Dessein, H., Prata, A., Dessein, A., et al. (2009). Th1/Th2 immune responses are associated with active *cutaneous leishmaniasis* and clinical cure is associated with strong interferon-gamma production. *Hum. Immunol.* 70 (6), 383–390. doi: 10.1016/j.humimm.2009.01.007

Castes, M., Agnelli, A., Verde, O., and Rondón, A. J. (1983). Characterization of the cellular immune response in American cutaneous leishmaniasis. *Clin. Immunol. Immunopathol.* 27 (2), 176–186. doi: 10.1016/0090-1229(83)90068-5

Chagas, A. C., Oliveira, F., Debrabant, A., Valenzuela, J. G., Ribeiro, J. M., and Calvo, E. (2014). Lundep, a sand fly salivary endonuclease increases leishmania parasite survival in neutrophils and inhibits XIIa contact activation in human plasma. *PloS Pathog.* 10 (2), e1003923. doi: 10.1371/journal.ppat.1003923

Chapman, L. A., Dyson, L., Courtenay, O., Chowdhury, R., Bern, C., Medley, G. F., et al. (2015). Quantification of the natural history of visceral *leishmaniasis* and consequences for control. *Parasit. Vectors* 8, 521. doi: 10.1186/s13071-015-1136-3

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., et al. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat. Rev. Microbiol.* 5 (11), 873–882. doi: 10.1038/nrmicro1748

Cillari, E., Liew, F. Y., Lo Campo, P., Milano, S., Mansueto, S., and Salerno, A. (1988). Suppression of IL-2 production by cryopreserved peripheral blood mononuclear cells from patients with active visceral leishmaniasis in Sicily. *J. Immunol.* 140 (8), 2721–2726.

Cooper, N. R. (1985). The classical complement pathway: activation and regulation of the first complement component. *Adv. Immunol.* 37, 151–216. doi: 10.1016/S0065-2776(08)60340-5

Davies, C. R., Cooper, A. M., Peacock, C., Lane, R. P., and Blackwell, J. M. (1990). Expression of LPG and GP63 by different developmental stages of leishmania major in the sandfly phlebotomus papatasi. *Parasitology* 101 Pt 3, 337–343. doi: 10.1017/S0031182000060522

Deak, E., Jayakumar, A., Cho, K. W., Goldsmith-Pestana, K., Dondji, B., Lambris, J. D., et al. (2010). Murine visceral *leishmaniasis*: IgM and polyclonal b-cell activation lead to disease exacerbation. *Eur. J. Immunol.* 40 (5), 1355–1368. doi: 10.1002/eji.200939455

de Lima, C. M. F., Magalhães, A. S., Costa, R., Barreto, C. C., Machado, P. R.L., Carvalho, E. M., et al. (2021). High anti-leishmania IgG antibody levels are associated with severity of mucosal leishmaniasis. *Front. Cell Infect. Microbiol.* 11, 652956. doi: 10.3389/fcimb.2021.652956

Desjardins, M., and Descoteaux, A. (1997). Inhibition of phagolysosomal biogenesis by the *leishmania lipophosphoglycan*. *J. Exp. Med.* 185 (12), 2061–2068. doi: 10.1084/jem.185.12.2061

Desjeux, P. (2004). Leishmaniasis: Current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27 (5), 305–318. doi: 10.1016/j.cimid.2004.03.004

Eilam, Y., El-On, J., and Spira, D. T. (1985). Leishmania major: Excreted factor, calcium ions, and the survival of amastigotes. *Exp. Parasitol.* 59 (2), 161–168. doi: 10.1016/0014-4894(85)90068-2

Fang, F. C. (2004). Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nat. Rev. Microbiol.* 2 (10), 820–832. doi: 10.1038/nrmicro1004

Farrow, A. L., Rana, T., Mittal, M. K., Misra, S., and Chaudhuri, G. (2011). Leishmania-induced repression of selected non-coding RNA genes containing b-box element at their promoters in alternatively polarized M2 macrophages. *Mol. Cell Biochem.* 350 (1-2), 47–57. doi: 10.1007/s11010-010-0681-5

Filho, A. A. P., Nascimento, A. A.S., Saab, N. A.A., Fugiwara, R. T., D'Ávila Pessoa, G. C., Koerich, L. B., et al. (2021). Evasion of the complement system by leishmania through the uptake of factor h, a complement regulatory protein. *Acta Trop.* 224, 106152. doi: 10.1016/j.actatropica.2021.106152

Firmino-Cruz, L., Decote-Ricardo, D., Gomes, D. C.O., Morrot, A., Freire-de-Lima, C. G., and de Matos Guedes, H. L. (2019). How to b(e)-1 important cell during leishmania infection. *Front. Cell Infect. Microbiol.* 9, 424. doi: 10.3389/fcimb.2019.00424

Firmino-Cruz, L., Ramos, T. D., da Fonseca-Martins, A. M., Oliveira-Maciel, D., Oliveira-Silva, G., Dos Santos, J. S., et al. (2020). B-1 lymphocytes are able to produce IL-10, but is not pathogenic during *leishmania (Leishmania)* amazonensis infection. *Immunobiology* 225 (1), 151857. doi: 10.1016/j.imbio.2019.10.006

Ghalib, H. W., Whittle, J. A., Kubin, M., Hashim, F. A., el-Hassan, A. M., Grabstein, K. H., et al. (1995). IL-12 enhances Th1-type responses in human leishmania donovani infections. *J. Immunol.* 154 (9), 4623–4629.

Gontijo, B., and de Carvalho, L. (2003). American Cutaneous leishmaniasis. *Rev. Soc. Bras. Med. Trop.* 36 (1), 71–80. doi: 10.1590/S0037-86822003000100011

Gonzaga, W. F., Xavier, V., Vivanco, B. C., Lopes, J. D., and Xander, P. (2015). B-1 cells contribute to susceptibility in experimental infection with *leishmania (Leishmania)* chagasi. *Parasitology* 142 (12), 1506–1515. doi: 10.1017/S0031182015000943

Goto, Y., Omachi, S., Sanjoba, C., and Matsumoto, Y. (2014). Elevation of serum b-cell activating factor levels during visceral leishmaniasis. *Am. J. Trop. Med. Hyg.* 91 (5), 912–914. doi: 10.4269/ajtmh.14-0260

Grewe, I., Brehm, T. T., Kreuels, B., Steinmetz, O. M., Dumoulin, B., Asemissen, A. M., et al. (2022). *Leishmania infantum* reactivation with secondary IgA nephropathy. *J. Travel Med*, 29. doi: 10.1093/jtm/taac038

Groom, J., Kalled, S. L., Cutler, A. H., Olson, C., Woodcock, S. A., Schneider, P., et al. (2002). Association of BAFF/BLyS overexpression and altered b cell differentiation with sjogren's syndrome. *J. Clin. Invest.* 109 (1), 59–68. doi: 10.1172/JCI0214121

Gurung, P., and Kanneganti, T. D. (2015). Innate immunity against leishmania infections. *Cell Microbiol.* 17 (9), 1286–1294. doi: 10.1111/cmi.12484

Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L., and Locksley, R. M. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169 (1), 59–72. doi: 10.1084/jem.169.1.59

Heinzel, F. P., Schoenhaut, D. S., Rerko, R. M., Rosser, L. E., and Gately, M. K. (1993). Recombinant interleukin 12 cures mice infected with leishmania major. *J. Exp. Med.* 177 (5), 1505–1509. doi: 10.1084/jem.177.5.1505

Iles, K. E., and Forman, H. J. (2002). Macrophage signaling and respiratory burst. *Immunol. Res.* 26 (1-3), 95–105. doi: 10.1385/IR:26:1-3:095

Kane, M. M., and Mosser, D. M. (2001). The role of IL-10 in promoting disease progression in leishmaniasis. *J. Immunol.* 166 (2), 1141–1147. doi: 10.4049/jimmunol.166.2.1141

Katz, Y., Revel, M., and Strunk, R. C. (1989). Interleukin 6 stimulates synthesis of complement proteins factor b and C3 in human skin fibroblasts. *Eur. J. Immunol.* 19 (6), 983–988. doi: 10.1002/eji.1830190605

Kaufer, A., Ellis, J., Stark, D., and Barratt, J. (2017). The evolution of trypanosomatid taxonomy. *Parasit. Vectors* 10 (1), 287. doi: 10.1186/s13071-017-2204-7

Lee, S. H., Charmoy, M., Romano, A., Paun, A., Chaves, M. M., Cope, F. O., et al. (2018). Mannose receptor high, M2 dermal macrophages mediate nonhealing leishmania major infection in a Th1 immune environment. *J. Exp. Med.* 215 (1), 357–375. doi: 10.1084/jem.20171389

Lukacsi, S., Nagy-Baló, Z., Erdei, A., Sándor, N., and Bajtay, Z. (2017). The role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in complement-mediated phagocytosis and podosome formation by human phagocytes. *Immunol. Lett.* 189, 64–72. doi: 10.1016/j.imlet.2017.05.014

Lukes, J., Mauricio, I. L., Schönian, G., Dujardin, J. C., Soteriadou, K., Dedet, J. P., et al. (2007). Evolutionary and geographical history of the *leishmania donovani* complex with a revision of current taxonomy. *Proc. Natl. Acad. Sci. U.S.A.* 104 (22), 9375–9380. doi: 10.1073/pnas.0703678104

Lund, F. E. (2008). Cytokine-producing b lymphocytes-key regulators of immunity. *Curr. Opin. Immunol.* 20 (3), 332–338. doi: 10.1016/j.coi.2008.03.003

Matsushita, T., Hasegawa, M., Yanaba, K., Kodera, M., Takehara, K., and Sato, S. (2006). Elevated serum BAFF levels in patients with systemic sclerosis: Enhanced BAFF signaling in systemic sclerosis b lymphocytes. *Arthritis Rheum.* 54 (1), 192–201. doi: 10.1002/art.21526

Mauri, C., and Bosma, A. (2012). Immune regulatory function of b cells. *Annu. Rev. Immunol.* 30, 221–241. doi: 10.1146/annurev-immunol-020711-074934

Mollinedo, F., Janssen, H., de la Iglesia-Vicente, J., Villa-Pulgarin, J. A., and Calafat, J. (2010). Selective fusion of azurophilic granules with leishmania-containing phagosomes in human neutrophils. *J. Biol. Chem.* 285 (45), 34528–34536. doi: 10.1074/jbc.M110.125302

Mosser, D. M., and Brittingham, A. (1997). Leishmania, macrophages and complement: A tale of subversion and exploitation. *Parasitology* 115 Suppl, S9–23. doi: 10.1017/S0031182097001789

Mosser, D. M., and Edelson, P. J. (1987). The third component of complement (C3) is responsible for the intracellular survival of leishmania major. *Nature* 327 (6120), 329–331. doi: 10.1017/s0031182097001789

Muller, K., van Zandbergen, G., Hansen, B., Laufs, H., Jahnke, N., Solbach, W., et al. (2001). Chemokines, natural killer cells and granulocytes in the early course of leishmania major infection in mice. *Med. Microbiol. Immunol.* 190 (1-2), 73–76. doi: 10.1007/s004300100084

Myers, C. D. (1991). Role of b cell antigen processing and presentation in the humoral immune response. *FASEB J.* 5 (11), 2547–2553. doi: 10.1096/fasebj.5.11.1907935

Nylen, S., and Gautam, S. (2010). Immunological perspectives of *leishmaniasis*. *J. Glob. Infect. Dis.* 2 (2), 135–146. doi: 10.4103/0974-777X.62876

Oliveira, T. M., Mineo, T. W., Bason, M., Day, M. J., and Machado, R. Z. (2009). IgG subclass profile of serum antibodies to *leishmania chagasi* in naturally infected and vaccinated dogs. *Vet. Parasitol.* 162 (1-2), 16–22. doi: 10.1016/j.vetpar.2009.02.018

Olivier, M., Atayde, V. D., Isnard, A., Hassani, K., and Shio, M. T. (2012). Leishmania virulence factors: focus on the metalloprotease GP63. *Microbes Infect.* 14 (15), 1377–1389. doi: 10.1016/j.micinf.2012.05.014

Omachi, S., Fujii, W., Azuma, N., Morimoto, A., Sanjoba, C., Matsumoto, Y., et al. (2017). B-cell activating factor deficiency suppresses splenomegaly during leishmania donovani infection. *Biochem. Biophys. Res. Commun.* 489 (4), 528–533. doi: 10.1016/j.bbrc.2017.06.005

O'Neil, C. E., Labrada, M., and Saravia, N. G. (1993). *Leishmania (Viannia)* panamensis-specific IgE and IgA antibodies in relation to expression of human tegumentary leishmaniasis. *Am. J. Trop. Med. Hyg.* 49 (2), 181–188. doi: 10.4269/ajtmh.1993.49.181

Podinovskaia, M., and Descoteaux, A. (2015). Leishmania and the macrophage: a multifaceted interaction. *Future Microbiol.* 10 (1), 111–129. doi: 10.2217/fmb.14.103

Quintela-Carvalho, G., Goicochea, A. M.C., Mançur-Santos, V., Viana, S. M., Luz, Y. D.S., Dias, B. R.S., et al. (2022). *Leishmania infantum* defective in lipophosphoglycan biosynthesis interferes with activation of human neutrophils. *Front. Cell Infect. Microbiol.* 12, 788196. doi: 10.3389/fcimb.2022.788196

Reis, A. B., Teixeira-Carvalho, A., Vale, A. M., Marques, M. J., Giunchetti, R. C., Mayrink, W., et al. (2006). Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *leishmania (Leishmania)* chagasi. *Vet. Immunol. Immunopathol.* 112 (3-4), 102–116. doi: 10.1016/j.vetimm.2006.02.001

Reis, E. S., Barbuto, J. A., and Isaac, L. (2007). Complement components, regulators and receptors are produced by human monocyte-derived dendritic cells. *Immunobiology* 212 (3), 151–157. doi: 10.1016/j.imbio.2006.11.010

Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B., and Brooker, S. (2007). Cutaneous *leishmaniasis*. *Lancet Infect. Dis.* 7 (9), 581–596. doi: 10.1016/S1473-3099(07)70209-8

Revaz-Breton, M., Ronet, C., Ives, A., Torre, Y. H., Masina, S., Tacchini-Cottier, F., et al. (2010). The MyD88 protein 88 pathway is differently involved in immune responses induced by distinct substrains of leishmania major. *Eur. J. Immunol.* 40 (6), 1697–1707. doi: 10.1002/eji.200939821

Rugani, J. N., Gontijo, C. M.F., Frézard, F., Soares, R. P., and Monte-Neto, R. L.D. (2019). Antimony resistance in leishmania (Viannia) braziliensis clinical isolates from atypical lesions associates with increased ARM56/ARM58 transcripts and reduced drug uptake. *Mem. Inst. Oswaldo Cruz* 114, e190111. doi: 10.1590/0074-02760190111

Sacks, D. L., and da Silva, R. P. (1987). The generation of infective stage leishmania major promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *J. Immunol.* 139 (9), 3099–3106.

Sampaio, R. N. R., Ferreira, M. F., Martins, S. S., and Motta, J. O.C.D. (2021). Successful treatment of diffuse cutaneous leishmaniasis caused by *leishmania amazonensis*. *Bras. Dermatol.* 96 (5), 602–604. doi: 10.1016/j.abd.2021.03.003

Santos, I. K., Costa, C. H., Krieger, H., Feitosa, M. F., Zurakowski, D., Fardin, B., et al. (2001). Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. *Infect. Immun.* 69 (8), 5212–5215. doi: 10.1128/IAI.69.8.5212-5215.2001

Santos-Gomes, G. M., Rosa, R., Leandro, C., Cortes, S., Romão, P., and Silveira, H. (2002). Cytokine expression during the outcome of canine experimental infection by *leishmania infantum*. *Vet. Immunol. Immunopathol.* 88 (1-2), 21–30. doi: 10.1016/S0165-2427(02)00134-4

Sato, T., Endo, Y., Matsushita, M., and Fujita, T. (1994). Molecular characterization of a novel serine protease involved in activation of the complement system by mannose-binding protein. *Int. Immunol.* 6 (4), 665–669. doi: 10.1093/intimm/6.4.665

Schwarz, T., Remer, K. A., Nahrendorf, W., Masic, A., Siewe, L., Müller, W., et al. (2013). T Cell-derived IL-10 determines *leishmaniasis* disease outcome and is suppressed by a dendritic cell based vaccine. *PloS Pathog.* 9 (6), e1003476. doi: 10.1371/journal.ppat.1003476

Sekine, H., Machida, T., and Fujita, T. (2022). Factor d. *Immunol. Rev.* 1–10. doi: 10.1111/imr.13155

Sethi, S., Fervenza, F. C., Siddiqui, A., Quint, P. S., and Pritt, B. S. (2016). Leishmaniasis-associated membranoproliferative glomerulonephritis with massive complement deposition. *Kidney Int. Rep.* 1 (3), 125–130. doi: 10.1016/j.ekir.2016.06.003

Smelt, S. C., Cotterell, S. E., Engwerda, C. R., and Kaye, P. M. (2000). B cell-deficient mice are highly resistant to *leishmania donovani* infection, but develop neutrophil-mediated tissue pathology. *J. Immunol.* 164 (7), 3681–3688. doi: 10.4049/jimmunol.164.7.3681

Snapper, C. M., and Paul, W. E. (1987). Interferon-gamma and b cell stimulatory factor-1 reciprocally regulate ig isotype production. *Science* 236 (4804), 944–947. doi: 10.1126/science.3107127

Soares, R. R., Antinarelli, L. M.R., Abramo, C., Macedo, G. C., Coimbra, E. S., and Scopel, K. K.G. (2017). What do we know about the role of regulatory b cells (Breg) during the course of infection of two major parasitic diseases, malaria and leishmaniasis? *Pathog. Glob. Health* 111 (3), 107–115. doi: 10.1080/20477724.2017.1308902

Soothill, J. F., and Harvey, B. A. (1977). A defect of the alternative pathway of complement. *Clin. Exp. Immunol.* 27 (1), 30–33.

Steverding, D. (2017). The history of leishmaniasis. *Parasit. Vectors* 10 (1), 82. doi: 10.1186/s13071-017-2028-5

Tausk, F., and Gigli, I. (1990). The human C3b receptor: Function and role in human diseases. *J. Invest. Dermatol.* 94 (6 Suppl), 141S–145S. doi: 10.1111/1523-1747.ep12876125

Thakur, C. P., Narain, S., Kumar, N., Hassan, S. M., Jha, D. K., and Kumar, A. (1997). Amphotericin b is superior to sodium antimony gluconate in the treatment of Indian post-kala-azar dermal leishmaniasis. *Ann. Trop. Med. Parasitol.* 91 (6), 611–616. doi: 10.1080/00034983.1997.11813179

Tirado, T. C., Moura, L. L., Shigunov, P., and Figueiredo, F. B. (2021). Methodological appraisal of literature concerning the analysis of genetic variants or protein levels of complement components on susceptibility to infection by trypanosomatids: A systematic review. *Front. Immunol.* 12, 780810. doi: 10.3389/fimmu.2021.780810

Trouw, L. A., and Daha, M. R. (2011). Role of complement in innate immunity and host defense. *Immunol. Lett.* 138 (1), 35–37. doi: 10.1016/j.imlet.2011.02.014

Umekita, L. F., Takehara, H. A., and Mota, I. (1988). Role of the antibody fc in the immune clearance of trypanosoma cruzi. *Immunol. Lett.* 17 (1), 85–89. doi: 10.1016/0165-2478(88)90106-X

Verma, S., Mandal, A., Ansari, M. Y., Kumar, A., Abhishek, K., Ghosh, A. K., et al (2018). Leishmania donovani inhibitor of serine peptidases 2 mediated inhibition of lectin pathway and upregulation of C5aR signaling promote parasite survival inside host. *Front Immunol.* 9, 63. doi: 10.3389/fimmu.2018.00063

Walport, M. J. (2001). Complement. first of two parts. *N. Engl. J. Med.* 344 (14), 1058–1066. doi: 10.1056/NEJM200104053441406

Wanasen, N., Xin, L., and Soong, L. (2008). Pathogenic role of b cells and antibodies in murine leishmania amazonensis infection. *Int. J. Parasitol.* 38 (3-4), 417–429. doi: 10.1016/j.ijpara.2007.08.010

Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K., and Locksley, R. M. (1994). CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with leishmania major. *J. Exp. Med.* 179 (4), 1367–1371. doi: 10.1084/jem.179.4.1367

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., et al. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375 (6530), 408–411. doi: 10.1038/375408a0

WHO (2022) *Leishmaniasis - fact sheets* (World Health Organization). Available at: https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis (Accessed November, 2022).

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 09 January 2023
DOI 10.3389/fcimb.2022.1044665

OPEN ACCESS

EDITED BY
Juliana Da Silva Pacheco,
University of Dundee, United Kingdom

REVIEWED BY
Valter Viana Andrade-Neto,
Oswaldo Cruz Foundation (Fiocruz), Brazil
Douglas Escrivani De Oliveira,
University of Dundee, United Kingdom
Edezio Ferreira Cunha-Junior,
Federal University of Rio de Janeiro, Brazil

*CORRESPONDENCE
Lia Carolina Soares Medeiros
lia.medeiros@fiocruz.br

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 14 September 2022
ACCEPTED 16 December 2022
PUBLISHED 09 January 2023

CITATION
Borges BS, Bueno GdP, Tomiotto-Pellissier F, Figueiredo FB and Soares Medeiros LC (2023) *In vitro* anti-*Leishmania* activity of triclabendazole and its synergic effect with amphotericin B. *Front. Cell. Infect. Microbiol.* 12:1044665. doi: 10.3389/fcimb.2022.1044665

In vitro anti-*Leishmania* activity of triclabendazole and its synergic effect with amphotericin B

Beatriz Santana Borges[1], Gislayne de Paula Bueno[1], Fernanda Tomiotto-Pellissier[2,3], Fabiano Borges Figueiredo[1] and Lia Carolina Soares Medeiros[1*]

[1]Instituto Carlos Chagas, Fundação Oswaldo Cruz (Fiocruz), Curitiba, Paraná, Brazil, [2]Laboratory of Immunopathology of Neglected Diseases and Cancer (LIDNC), Department of Pathological Sciences, State University of Londrina, Londrina, Paraná, Brazil, [3]Department of Medical Pathology, Federal University of Paraná, Curitiba, Paraná, Brazil

Introduction: Leishmaniasis is a neglected tropical disease, with approximately 1 million new cases and 30,000 deaths reported every year worldwide. Given the lack of adequate medication for treating leishmaniasis, drug repositioning is essential to save time and money when searching for new therapeutic approaches. This is particularly important given leishmaniasis's status as a neglected disease. Available treatments are still far from being fully effective for treating the different clinical forms of the disease. They are also administered parenterally, making it challenging to ensure complete treatment, and they are extremely toxic, in some cases, causing death. Triclabendazole (TCBZ) is a benzimidazole used to treat fasciolosis in adults and children. It presents a lower toxicity profile than amphotericin B (AmpB) and is administered orally, making it an attractive candidate for treating other parasitoses. The mechanism of action for TCBZ is not yet well understood, although microtubules or polyamines could potentially act as a pharmacological target. TCBZ has already shown antiproliferative activity against *T. cruzi, T. brucei*, and *L. infantum*. However, further investigations are still necessary to elucidate the mechanisms of action of TCBZ.

Methods: Cytotoxicity assay was performed by MTT assay. Cell inhibition (CI) values were obtained according to the equation CI = (O.D treatment x 100/ O.D. negative control). For Infection evaluation, fixated cells were stained with Hoechst and read at Operetta High Content Imaging System (Perkin Elmer). For growth curves, cell culture absorbance was measured daily at 600 nm. For the synergism effect, Fractional Inhibitory Concentrations (FICs) were calculated for the IC50 of the drugs alone or combined. Mitochondrial membrane potential (DYm), cell cycle, and cell death analysis were evaluated by flow cytometry. Reactive oxygen species (ROS) and lipid quantification were also

determined by fluorimetry. Treated parasites morphology and ultrastructure were analyzed by electron microscopy.

Results: The selectivity index (SI = CC50/IC50) of TCBZ was comparable with AmpB in promastigotes and amastigotes of *Leishmania amazonensis*. Evaluation of the cell cycle showed an increase of up to 13% of cells concentrated in S and G2, and morphological analysis with scanning electron microscopy showed a high frequency of dividing cells. The ultrastructural analysis demonstrated large cytoplasmic lipid accumulation, which could suggest alterations in lipid metabolism. Combined administration of TCBZ and AmpB demonstrated a synergistic effect *in vitro* against intracellular amastigote forms with cSFICs of 0.25.

Conclusions: Considering that TCBZ has the advantage of being inexpensive and administrated orally, our results suggest that TCBZ, combined with AmpB, is a promising candidate for treating leishmaniasis with reduced toxicity.

KEYWORDS

triclabendazole, drug repurposing, Leishmaniasis, benzimidazoles, *Leishmania amazonensis*

1 Introduction

Leishmaniasis is a neglected disease caused by at least 18 protozoan species of the genus *Leishmania*, leading to up to 1 million cases per year worldwide (Burza et al., 2018). The disease presents three clinical forms, including cutaneous and mucocutaneous forms, which can cause permanent skin scarring, and the visceral form, which leads to death in 90% of the cases if not treated, being the parasitic disease with the second highest mortality index (Pace, 2014).

Despite being less severe than the visceral form, cutaneous leishmaniasis frequently leads to a large degree of psychological suffering, social stigma, and isolation due to the lesions, which, although usually self-healing, can be a source of secondary infections if left untreated (Pires et al., 2019). Approximately 300,000 cases per year of cutaneous and diffuse cutaneous clinical forms in South America are caused by *Leishmania amazonensis* (Burza et al., 2018).

Available treatments of all clinical forms are toxic, expensive, and/or difficult to administrate and cause severe side effects. Meglumine antimoniate and amphotericin B (AmpB) are the most common treatments (first- and second-line drugs, respectively), and both require prolonged intravenous administration, making the patient less likely to comply with the treatment fully. In its liposomal formulation, AmpB has been indicated for patients after the failure of the first-line treatment, for those with severe disease, those presenting comorbidities or immunodeficiencies, and for pregnant women (Croft and Olliaro, 2011). Pentamidines, paromomycin and miltefosine (first orally administered drug for leishmaniasis) can also be used in endemic areas, despite also having a high level of toxicity and therapeutic failure (Santi and Murta, 2022).

The development of new drugs is a long and expensive process. Successful treatments should have a specific pathogen target in order to eliminate the pathogen without harming the host (Ioset et al., 2009). Due to the high costs involved in treating parasitic diseases, they are often neglected by pharmaceutical companies.

Drug repositioning is a quicker way of providing new compounds for a wide range of diseases for which no adequate treatment currently exists. These drugs have already been approved by US Food and Administration (FDA), saving a great amount of time and money, and their safety profile is well-known (Nosengo, 2016). A variety of repositioning drugs have been frequently reported for treating conditions, including bipolar disorder, migraines, type 2 diabetes and pulmonary hypertension (Alberca et al., 2016). Also, tropical neglected diseases present a range of repurposed drugs either from other approved treatments or veterinary use such as eflornithine for Human African Trypanosomiasis (HAT) and albendazole for lymphatic filariasis (Klug et al., 2016). Indeed, some drugs currently used for leishmaniasis treatment are repurposed, such as amphotericin B, paromomycin and miltefosine, originally used as antifungal, antibiotic and antineoplastic, respectively (Field et al., 2017). In this context, it is important to highlight the benzimidazole family of drugs, which has not

only demonstrated a satisfactory anti-parasitic effect but has also been shown to have good patient tolerability in a variety of benzimidazole derivates (Bansal and Silakari, 2012).

One member of the benzimidazole family is triclabendazole (6-chloro-5-(2,3-dichloro phenoxy)-2-(methylthio)-1H-benzimidazole) (TCBZ), a drug that has been used to treat fascioliasis in animals since the early 1980s and which was approved for adult use in the early 1990s. It was also approved for infantile fascioliasis (over six years) in 2019 (Terashima et al., 2021). TCBZ has already demonstrated a bactericidal effect as a successful repurposed drug (Pi et al., 2021), and antiproliferative *in vitro* activity against *Trypanosoma cruzi* (Alberca et al., 2016). Further experiments have not yet been performed to investigate the drug's mechanism of action and its inhibitory effects on *Leishmania* spp.

TCBZ's mechanism of action is still not properly understood, and it may target microtubules and/or protein synthesis (Robinson et al., 2002). Still, it appears to be a very safe drug. TCBZ has shown a well-tolerability, with mild side effects such as biliary colic and abdominal pain, which is consistent with the expulsion of dead flukes parasites (*Fasciola hepatica*) (Gandhi et al., 2019). TCBZ also has the advantage of being administered orally in one, two, or three doses, facilitating the continuation and conclusion of the patient's treatment (Terashima et al., 2021). Studies have shown that TCBZ absorption after oral administration is fast, with t_{max} (time to reach the maximum serum concentration) of 2 to 3 hours (Gandhi et al., 2019). In addition, treatment with liposomal AmpB costs over 1,200 USD for an individual weighing 70 kg (Datta et al., 2020), while treatment with TCBZ costs 3 to 5 USD for the same individual.

Given the current availability of treatments for leishmaniasis and the importance of drug repositioning, especially for neglected tropical diseases, we tested the anti-*Leishmania* activity of TCBZ against the *L. amazonensis* strain alone and in combination with AmpB.

2 Material and methods

2.1 In silico study

The AmpB and TCZB structures were used to evaluate their theoretical physicochemical properties, whether PAINS are present, and the drug interaction prediction. The predictions were calculated using the swissADME web tool (http://www.swissadme.ch) considering Lipinski's rule of five (Ro5) (Lipinski, 2004) followed by the additional rule proposed by Veber (Veber et al., 2002) and PAINS presence (Baell and Holloway, 2010). Drug interaction prediction was performed using the Drug Interaction Checker of DrugBank (https://go.drugbank.com/drug-interaction-checker).

2.2 Parasites

Leishmania amazonensis (strain MHOM/BR/73/M2269) promastigotes were kept at 25°C in M199 medium added with 40 mM HEPES, 26 mM NaOH, 5 µg/mL of hemin supplemented with 10% of fetal bovine serum (FBS, Gibco®). The parasites were cultivated for three days for logarithm growth stage parasite assays and seven days for stationary growth stage parasites assays.

2.3 Cytotoxicity assay

Cytotoxicity assay was performed as previously described (Ceole et al., 2017). Cell viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Optical density (O.D.) was read in an ELISA plate reader (Biotek Model EL-800, VT, USA) at 570 nm. In order to calculate the 50% cytotoxic concentration index (CC_{50}), THP-1 (ATCC TIB-202) cells were seeded in 96-well plates at density of $25x10^4$ cells/mL in RPMI 1640 medium containing 50 ng/mL of PMA (Phorbol 12-myristate 13-acetate) for 48h as previously described (Alcântara et al., 2020). The plates were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2 to induce differentiation into adherent macrophages. After this, macrophages were incubated with increasing concentrations of TCBZ or AmpB for 48 or 72h in a CO_2 incubator. Concentrations used for AmpB ranged from 0 to 10 µM (0, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM), while concentrations used for TCBZ ranged from 0 to 100 µM (0, 0.097, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM). Then, 50 µL of MTT at 5 mg/mL was added per well, and the plates were incubated for 4h at 37°C. The plates had their medium removed, and 25 µL of 10% SDS solution in HCL was added for cell lysis. Subsequently, 50 µl of DMSO was added for the elution of formazan crystals. The colorimetric assay was read using optical density at 570 nm. Cell inhibition (CI) values were obtained from three experiments performed in triplicate according to the equation CI = (O.D treatment x 100/O.D. negative control) (Escobar et al., 2010; Ceole et al., 2017).

2.4 Infection evaluation

Infection assays were performed as previously described (Alcântara et al., 2020). Differentiated macrophages ($25x10^4$ cells/mL) were infected with metacyclic promastigotes (5 to 7-days-old metacyclic promastigote cells culture) at a ratio of 50:1 in RPMI media for 24h using 96 well-plates placed in a humidified incubator at 34°C with an atmosphere of 5% CO_2. Previously to cells treatment, infection tests were made in three different time-points (24, 48 and 72h) for determination of higher infection index.

For cells treatment, increasing concentrations of AmpB and TCBZ were added to cell media and incubated for 48h at 34°C and 5% CO_2 after infection. Concentrations used for AmpB ranged from 0 to 20 µM (0, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM), while concentrations used for TCBZ ranged from 0 to 500 µM (0, 0.97, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 µM). Plates were washed twice with PBS, and cells were fixated with 4% PFA for 20 min, washed twice with ultrapure H_2O, stained with Hoechst for 20 min, and read at Operetta High Content Imaging System (Perkin Elmer). The number of intracellular amastigotes was evaluated to calculate half-maximum inhibitory concentration (IC_{50}). Non-treated wells were considered as having 100% viability of amastigotes.

2.5 Antiparasitic evaluation

Growth curve was performed using three-day-old promastigote cells culture of *L. amazonensis*. Parasites were harvested and had their concentration adjusted to $1x10^6$ parasites/mL and seeded in 24 well plates in 1.5 mL of M199 media. Non-treated parasites were used as control. Promastigotes were treated with increasing concentrations of AmpB (0.312, 0.625, 1.25, 2.5 and 5 µM) or TCBZ (3.125, 6.25, 12.5, 25 and 50 µM) and maintained in a BOD incubator at 25°C for 72h. Plates had absorbance measured daily for drug effect analysis at 600 nm (Kar et al., 2021).

For determination of promastigotes $IC_{50,}$ 3-day-old culture of promastigotes was seeded in 96 well plates at a concentration of 10^6 parasites/mL in the presence of AmpB (0, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 µM) or TCBZ (0, 0.097, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM) in M199 media. Plates were maintained at 25°C for 72h. After incubation, promastigotes viability was accessed by MTT assay (as described at item 2c).

2.6 Synergism of TCBZ and AmpB

Infected macrophages were treated with TCBZ and AmpB using a ratio of 10:1, 5:1, 2:1, and 1:1 in five serial dilutions (base 2) for 48h. The molar concentration for each ratio was designed as follows: 40 µM:4µM; 20µM:4 µM; 16µM:4µM and 4µM:4µM for TCBZ and AmpB respectively After the plates were analyzed using the Operetta CLS High Content Analysis System, Fractional Inhibitory Concentrations (FICs) were calculated for the IC_{50} of the drug alone and combined (Trinconi et al., 2014; Kar et al., 2021). Isobolograms were plotted using the sum of FICs (ΣFICs) of the ratio of each combined drug. The average of FICs sum of all ratios (χΣFICs) was calculated and the combinatory effect was determined according to Odds (2003), where χΣFICs ≤ 0,5 is synergic, χΣFICs > 0,5< 4 is indifferent, and χΣFICs > 4 is antagonist. The equation for the ΣFIC_{50} calculation is described below:

$$\Sigma FICs = \left[\frac{IC_{50}\ \text{drug A in combination}}{IC_{50}\ \text{drug A alone}}\right] + \left[\frac{IC_{50}\ \text{drug B in combination}}{IC_{50}\ \text{drug B alone}}\right]$$

To the present, there is no gold standard method established to test antimicrobial synergism. Although the method used in this work can be limited to only testing antimicrobials for a fixed incubation time, it was used because it is a well-established method.

2.7 Flow cytometry

Leishmania amazonensis (10^6 promastigotes/mL) were previously treated with TCBZ (IC_{50} for 72h). To assess the mitochondrial membrane potential (ΔΨm), the promastigotes were washed with PBS and incubated for 15 min at 26°C with 5 µg/mL of rhodamine 123 (Rh123) and immediately evaluated in a flow cytometer. For cell cycle analysis, promastigotes were resuspended in 500 µL of PBS, to which the same volume of permeabilizing solution and DNA staining solution was added (3.4 mM Tris-HCl, pH 7.4; 0.1% NP-40; 700 U/mL free RNase A-DNase, 10 mM NaCl and 75 µM propidium iodide). A fluorescence reading was taken after an incubation period of 10 min at room temperature. To evaluate cell death, promastigotes were resuspended in annexin binding buffer (140 mM NaCl, 5 mM $CaCl_{2,}$ and 10 mM HEPES-Na, pH 7.4) and labeled with Annexin V conjugated to Alexa Fluor 488 and 100 µg/mL of propidium iodide (PI). In all cases, events were read in a FACSCanto II flow cytometer (Becton – Dickinson, San Jose, CA, USA), and the data were analyzed using FlowJo v10 (Treestar Software, Ashland, OR, USA).

2.8 Reactive oxygen species

ROS quantification was performed as described by Bortoleti et al. (Bortoleti BT da et al., 2021). Promastigotes were cultured in 1 mL of M199 in presence of AmpB or TCBZ IC_{50} for 72h. In positive controls, 0.2 µL of 2 mM H_2O_2 was added to 1 mL of cell solution 60 min before withdrawing the treatment. The parasites were collected through centrifugation (2000 rpm for 7 min), the supernatant was discarded, and the pellet was resuspended in 500 µL of M199. Parasites were plated in black 96-well plates (99 µL per well). An initial reading was performed to assess cellular autofluorescence in a fluorimeter (Ex 495; Em 520). Subsequently, 1 µL of the H_2DCFDA solution was added (final concentration in the well of 20 µM). Plates were incubated for 1h in a 37°C incubator with an atmosphere of 5% $CO_{2,}$ and, after this time, the fluorescence was read in a fluorimeter.

2.9 Lipid quantification

Neutral lipids were detected as described in Bortoleti et al. (Bortoleti BT da et al., 2021). Promastigote forms previously treated with TCBZ or AmpB (IC_{50}) for 72h were collected and

washed twice with PBS. After that, 199.5 µL of M199 and 0.5 µL of Nile Red solution (1 mg/mL) were added and incubated for 30 min in a BOD. Cells were rewashed and resuspended in 500 µL PBS. Then, the cell suspension was plated in black 96-well plates (100 µL per well), and the fluorescence reading was performed in a fluorimeter (Ex 530; Em 635).

2.10 Electron microscopy

Parasites were treated with TCBZ or AmpB (CI_{50}) for 72h. The parasites were then harvested at a concentration of $1x10^6$ parasites/mL, washed in PBS, and fixed with Karnovsky fixation solution (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer) for 1h and washed with cacodylate buffer after incubation. Untreated parasites were also fixed and used as control. For scanning electron microscopy (SEM), cells were previously seeded in poly-L-lysin-coated coverslips. Cells were post-fixated with 1% osmium tetroxide for 1h in the dark and then dehydrated at growing ethanol concentrations (30 to 100%). Coverslips containing treated or untreated cells were submitted to critical point drying and then coated with a 20-nm-thick gold layer and observed under a JEOL JSM 6010 PLUS-LA (Akishima, Tokyo, Japan) scanning electron microscope. For transmission electron microscopy (TEM), treated and untreated cells were post-fixated with 1% osmium tetroxide for 1h in the dark, dehydrated using growing concentrations of acetone (30 to 100%) and infiltrated with increasing concentrations of Poly/Bed 812 resin and polymerized for 72h at 60°C. Ultrathin sections were contrasted for 30 min with uranyl acetate and 2 min with lead citrate. The ultrathin sections were observed in a JEOL JEM-1400 Plus transmission electron microscope operating at 80 kV.

2.11 Statistical analysis

Statistical analysis was performed in GraphPad Prism 8 (GraphPad Software, Inc. San Diego, CA, USA) using one or two-way ANOVA test followed by Dunnett's post-test. IC_{50} assay results were transformed in logarithm values and analyzed by dose-response inhibition (* p-value<0.04; ** p-value<0.009; *** p-value<0.0009; **** p-value<0.00009). Results were plotted, showing the standard error of the mean. All experiments were performed in biological triplicate.

3 Results

3.1 In silico predictions for AmpB and TCBZ

The physicochemical proprieties of AmpB and TCBZ were assessed to compare their predicted oral bioavailability using Lipinski's and Veber's criteria (Veber et al., 2002; Lipinski, 2004). As expected, AmpB presented higher violations than TCBZ (Table 1). This is in line with the high level of toxicity that has been described for it, even when using the intravenous route (Laniado-Laborín and Cabrales-Vargas, 2009). AmpB and TCBZ chemical structures are represented in Figures 1A, B, respectively.

For both drugs, pan assay interference compounds (PAINS) were not identified by the swissADME web tool (Daina et al., 2017). PAINS are substructural features of chemical compounds that are often related to false-positive results in high-throughput screens because they tend to react nonspecifically with numerous biological targets rather than specifically affecting one desired

TABLE 1 Molecular properties of Amphotericin B (AmpB) and Triclabendazole (TCBZ) on Lipinski's and Veber's criteria, the number of pan assay interference compounds according to SwissADME web tool, and the presence of drug interactions according to DrugBank.

	AmpB	TCBZ	Limits
Dug interactions	None		
MW	V	359.66 g/mol	≤ 500
Log $P_{o/w}$	-0.39	4.92	≤ 5
RB	3	3	≤ 10
H-Acc	18	2	≤ 10
H-Don	12	1	≤ 5
tPSA (Å²)	319.61	63.21 Å²	≤ 140
PAINS	0	0	-
Violations	4	0	-

MW, molecular weight; Log Po/w, Log of partition-coefficient (consensus LogP on swissADME); RB, number of rotatable bonds; H-Acc: number of Hydrogen bond acceptor. H-Don: number of Hydrogen bond donor; tPSA: molecular polar surface area; PAINS: pan assay interference compounds.

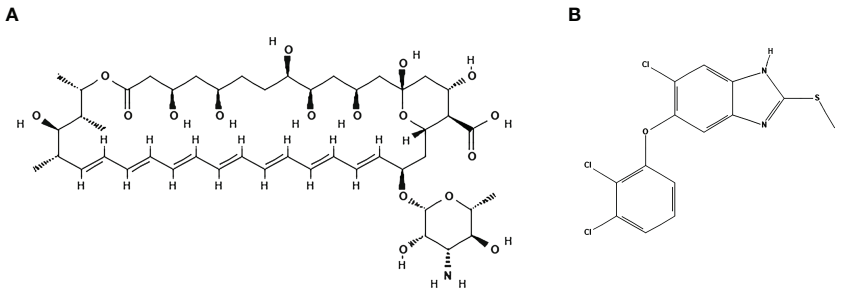


FIGURE 1
Structures of Amphotericin B and Triclabendazole. **(A)** Amphotericin B presents a molecular formula of $C_{47}H_{73}NO_{17}$ (PubChem CID: 5280965). **(B)** Triclabendazole presents a molecular formula of $C_{14}H_9C_{l3}N_2OS$ (PubChem CID: 50248). Structures were generated by PubChem (National Center for Biotechnology Information,2022).

target (Baell and Holloway, 2010). No drug interactions were found by the DrugBank tool for AmpB and TCBZ, suggesting that concomitant use of the drugs is safe.

3.2 TCBZ selectivity index is higher than AmpB for intracellular amastigotes

To evaluate the effects of TCBZ on parasite growth and determine which treatment time was the best for determining the effects of the drug on promastigote forms, parasites were seeded in 24-well plates, and their growth was estimated by absorbance for three consecutive days. AmpB was used as a comparative drug, as it is one of the most common drugs used to treat leishmaniasis worldwide. The results showed that AmpB affects *L. amazonensis* promastigote proliferation after 72h of treatment for all concentrations tested. On the other hand, TCBZ inhibited parasite growth at higher concentrations after 48h of treatment (Figure 2). Thus, promastigote assays were subsequently conducted only at 72h since this time point showed a statistically significant effect for both drugs.

The IC_{50} of TCBZ and AmpB for promastigotes were determined after 72h of treatment by MTT assay. TCBZ IC_{50} was 6 µM, while AmpB IC_{50} was 0.06 µM (Table 2).

Exploratory infection assays were performed for macrophages derived from THP-1 cells infected with intracellular amastigotes. Based on the results obtained (Supplementary Figure 1), it was decided that 48h would be the most accurate time-point for amastigote forms, since it presented a higher infection rate then 72h. Although 24h showed similar results when compared to 48h, based on the lack of statistically significant activity of AmpB and TCBZ in promastigotes proliferation analysis (Figure 2) this time-point was not considered for further evaluations. Infected macrophages were therefore treated for 48h with growing concentrations of AmpB and TCBZ, and the number of infected cells was analyzed using the Operetta High Content Imaging System. AmpB showed significant results at 5 µM and TCBZ at 3.1, 6.2, and 50 µM (Figure 3). The number of total amastigotes was used to determine the IC_{50} for both AmpB (2.02 µM) and TCBZ (45.67 µM).

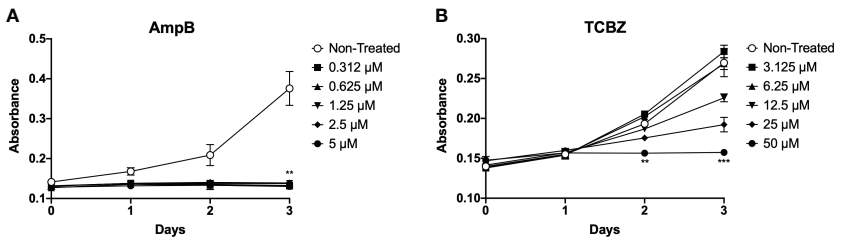


FIGURE 2
L. amazonensis promastigotes proliferation analysis. **(A)** Parasites treated with increasing concentrations of AmpB (0.312, 0.625, 1.25, 2.5 and 5 µM). For AmpB, statistically significant results were presented in all concentrations on the third day (72h) of treatment. **(B)** Parasites treated with increasing concentrations of TCBZ (3.12, 6.25, 12.5, 25 and 50 µM). For TCBZ, statistically significant results obtained in 50 µM concentrations from day 2 (48h) of the analysis. Absorbance (Abs) read at 600 nm. .

10.3389/fcimb.2022.1044665

TABLE 2 Half-maximum cytotoxicity concentration (CC_{50}) in macrophages, half-maximum inhibitory concentration (IC_{50}) in amastigotes from *L. amazonensis*, and Selectivity Index (SI, CC_{50}/IC_{50}) of AmpB and TCBZ.

	Macrophages	Amastigotes	
	CC_{50} 48h	IC_{50} 48h	SI 48h
Triclabendazole	276.6 ± 47	45.67 ± 7.69	6
Amphotericin B	8.1 ± 3.8	2.02 ± 0.26	4

CC_{50} and IC_{50} are shown in µM. Standard deviation is shown by ±.

Half-minimal cytotoxicity concentrations (CC_{50}) of TCBZ and AmpB were also determined by MTT assay in THP-1 derivate macrophages. Assays were conducted at 48 and 72h since these were the times evaluated in amastigote and promastigotes assays, respectively. TCBZ showed lower cytotoxicity than AmpB for both 48h (276.6 µM and 8.1 µM, respectively) and 72h (39.4 µM and 0.3 µM, respectively) (Figure 4). The selectivity index (SI) was determinate using CC_{50} and IC_{50} of amastigote forms being 4 and 6 for AmpB and TCBZ , respectively (Table 2).

3.3 Ultrastructural and morphological analysis of treated parasites

Treated parasites (IC_{50} for 72h) were observed by electron microscopy to analyze ultrastructural (Figure 5) and morphological (Figure 6) alterations induced by TCBZ or AmpB. In *L. amazonensis* promastigotes treated with AmpB IC_{50}, several cytoplasmatic electron-dense spherical bodies were observed, which probably contain lipids (Figure 5B). TCBZ IC_{50} treated parasites showed the same cytoplasmatic electron-dense

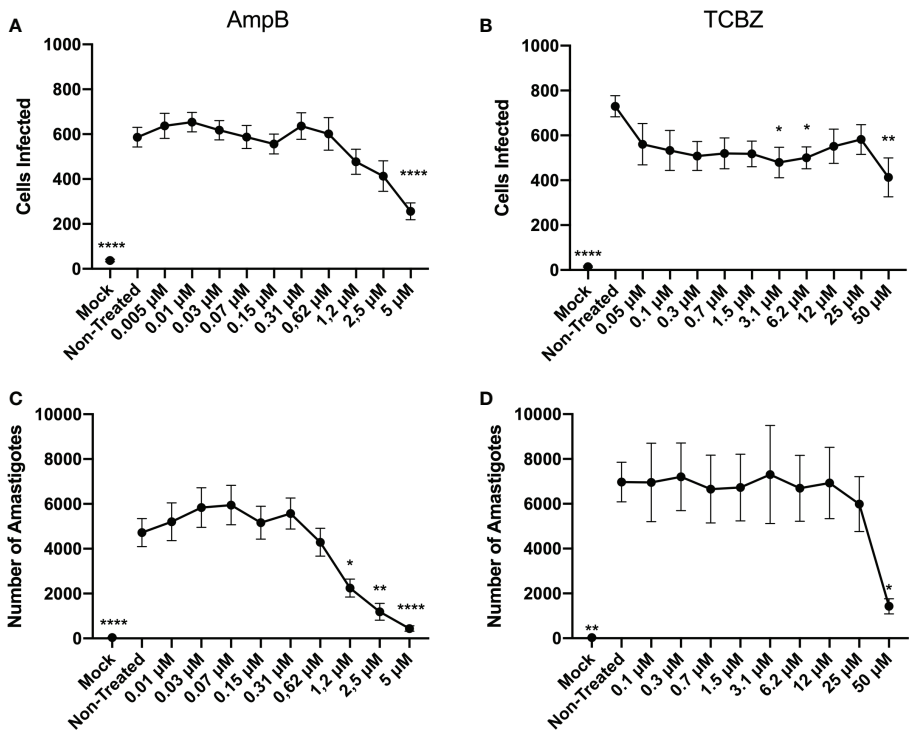


FIGURE 3
Anti-amastigote effect of AmpB and TCBZ at 48h of treatment. **(A)** Macrophages infected with *L. amazonensis* were treated for 48h in increasing concentrations of AmpB. Statistically significant results can be observed at 5 µM treatment. **(B)** Macrophages infected with *L. amazonensis* were treated for 48h in increasing concentrations of TCBZ. Statistically significant results can be observed at 3.1, 6.2, and 50 µM treatment. Mock cells (without parasite infection) were used as negative control in the Operetta System analysis. **(C)** Number of amastigotes in infected cells treated with AmpB for 48h. Statistically significant results can be observed from 1.2 µM. **(D)** Number of amastigotes in infected cells treated with TCBZ for 48h. Statistically significant results can be observed at 50 µM. * = $p < 0.0332$, ** = $p < 0.0021$, **** = $p < 0.0001$.

10.3389/fcimb.2022.1044665

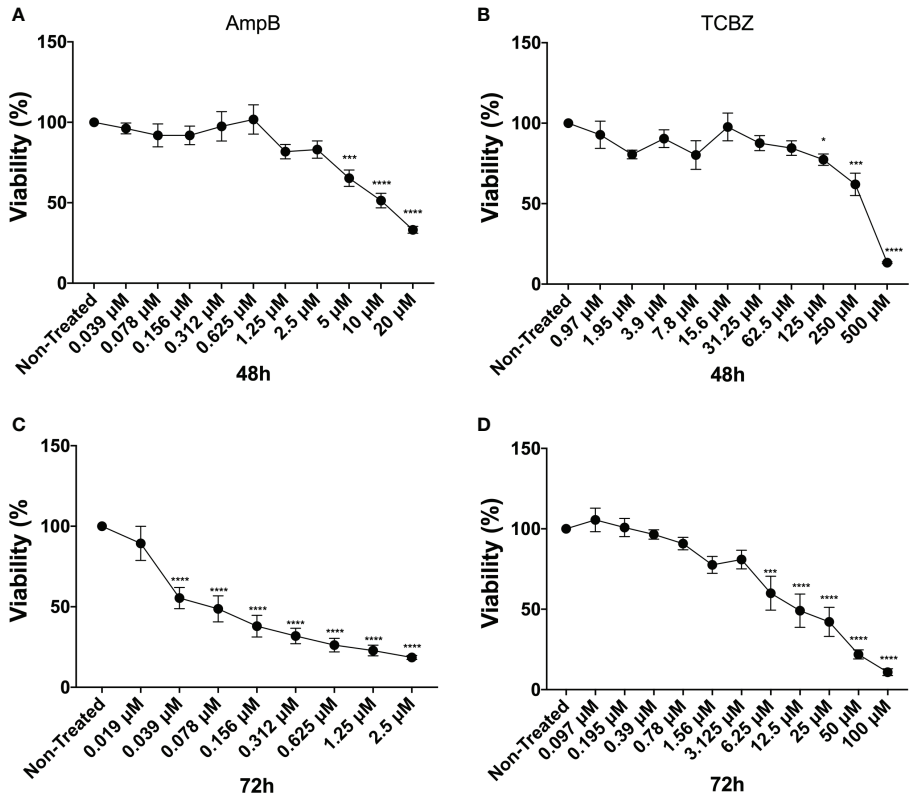


FIGURE 4
Cytotoxicity assay. Macrophages were treated with increasing concentrations of AmpB and TCBZ. Cytotoxicity evaluated by MTT assay after 48h and 72h of treatment with AmpB **(A)** and **(C)**, respectively. Cytotoxicity evaluated by MTT assay after 48h and 72h of treatment with TCBZ **(B)** and **(D)**, respectively. * = $p < 0.0332$, *** = $p < 0.0002$, **** = $p < 0.0001$.

spherical bodies observed after AmpB treatment (white arrowheads), and other ultrastructural alterations such as vesicles at the flagellar pocket were also frequently noticed (black arrows, Figures 5C, C'). Infected cells were also treated with the highest statistically relevant concentration of TCBZ (50 µM for 48h) to assess the drug's effects. Although the same electron-dense structures seen in promastigotes were not observed, treated parasites showed intense vesiculation, a feature not seen in untreated parasites (Figure 6B). Moreover, structures resembling cell debris inside the parasitophorous vacuole of treated samples were frequently observed in these cells (Figure 6B-B'', white arrowheads).

The morphology of treated promastigotes was observed by SEM. Untreated parasites were used as control and showed normal morphology (Figure 7, row 1). AmpB-treated parasites showed morphological alterations such as shrinkage and roundness of the parasite cell body (Figure 7, row 2, white arrowheads). *Leishmania amazonensis* promastigotes treated with TCBZ IC_{50} for 72h also showed morphological alterations like shrinkage and roundness in the parasite shape cell (Figure 7, row 3, white arrowheads). Moreover, dividing cells were frequently observed, indicating a possible alteration in the cytokinesis process (Figure 7, row 3, black arrows).

3.4 Cell cycle is affected by TCBZ treatment

To better understand TCBZ's mechanism of action, treated parasites were analyzed by flow cytometry for cell death, cell cycle, and mitochondrial membrane potential. Cell death analysis showed that treatment with AmpB increased the number of stained parasites using both Annexin V and PI (Figure 8A). Untreated promastigotes showed 99.4% of living cells, 0.29% of apoptosis, 0.14% of necrosis, and 0.17% of late apoptosis. Similarly, TCBZ-treated parasites showed 99.4% of living cells, 0.3% of apoptosis, 0.1% of necrosis, and 0.2% of late apoptosis. On the other hand, AmpB-treated parasites showed

10.3389/fcimb.2022.1044665

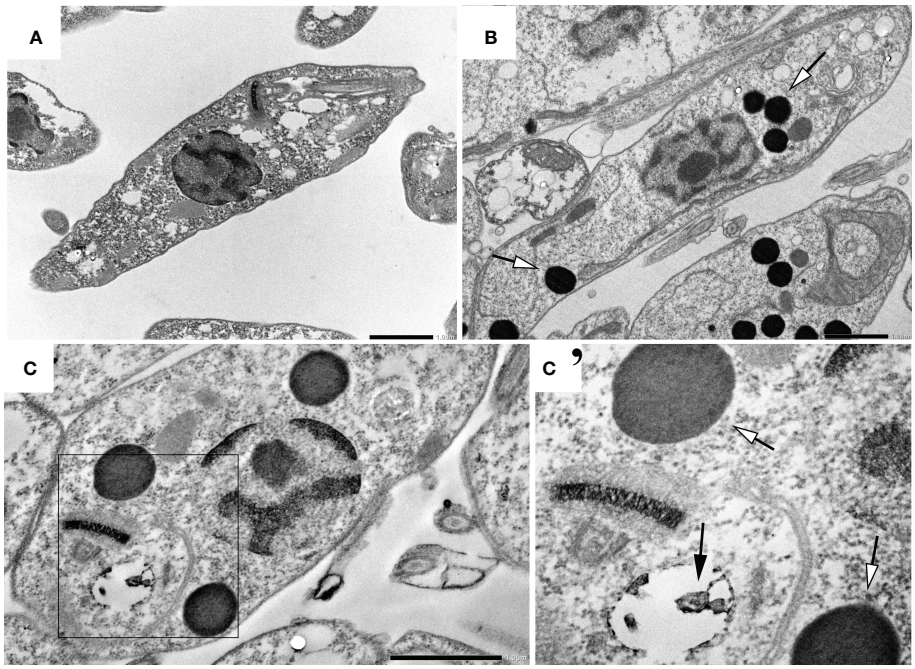


FIGURE 5
Ultrastructural analysis of *L. amazonensis* promastigotes treated with AmpB and TCBZ. **(A)** Non-treated parasites. **(B)** Parasites treated with AmpB IC_{50} showing large electrondense vesicles (pointed by white arrowheads). **(C)** Parasites treated with TCBZ IC_{50}. (C') Enlargement of the demarked area of Figure 3C where treated cells seem not only to present large electron-dense vesicles (white arrowheads) but also vesicles at the flagellar pocket, which were not found in control parasites (black arrows). Bars: 1 µm.

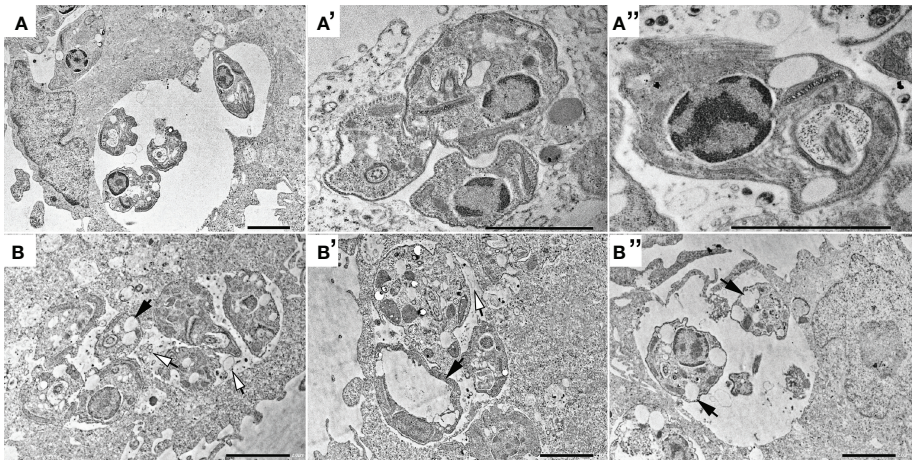


FIGURE 6
Transmission electron microscopy of non-treated intracellular amastigotes (**A**, **A'** and **A''**) and treated with 50 µM of TCBZ (**B**, **B'** and **B''**). Black arrows point to several large vesicles in treated parasites, both in AmpB and TCBZ treatment, absent in control. Also, treated samples seems to present possible cellular debris inside the parasitophorous vacuole (white arrowheads), indicating parasite death. Bars: 2 µm.

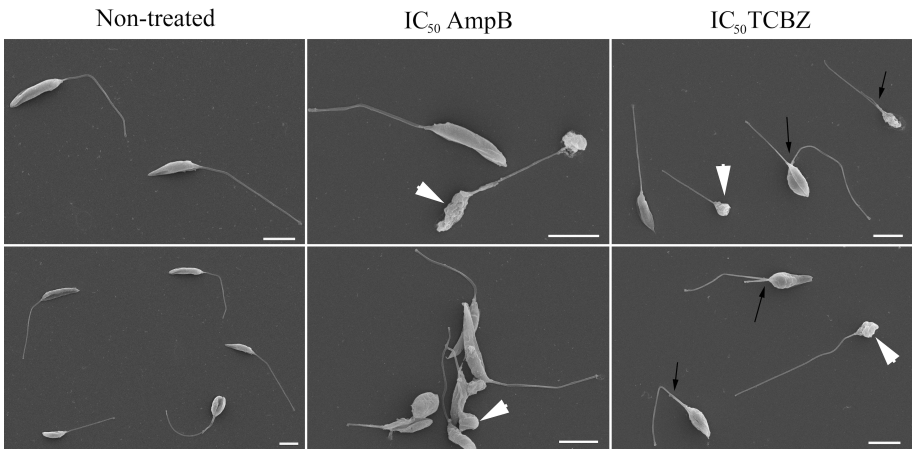


FIGURE 7
Morphological analysis of *L. amazonensis* promastigotes by scanning electron microscopy (SEM). The first column represents non-treated parasites, and the second and third columns represent parasites treated with AmpB IC_{50}/72h and TCBZ IC_{50}/72h, respectively. White arrowheads show shrinkage and roundness in the parasites that are presented in both AmpB and TCBZ treatment. Black arrows point to parasites in division, which was observed in a higher frequency in TCBZ treatment, when compared to control or AmpB treatment. Bars: 5 µm.

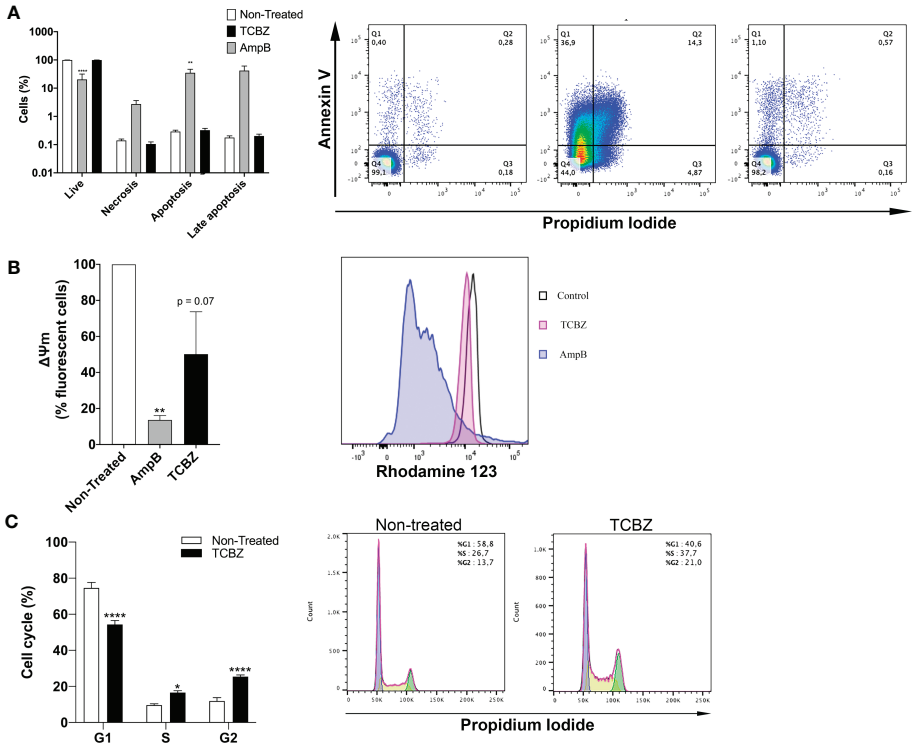


FIGURE 8
Flow cytometer assays in promastigotes treated with IC50 of AmpB and TCBZ for 72h. **(A)** Cell death evaluation with co-staining of Annexin V and PI of non-treated and TCBZ treated parasites. Typical dot plots of one representative experiment are shown. Q1: apoptosis, Q2: late apoptosis. Q3: living cells. Q4: necrosis. **(B)** Membrane potential evaluated with Rod123 assay. Typical histograms of one representative experiment are shown. **(C)** Cell cycle evaluation with PI staining. Typical histograms of one representative experiment are shown. Values are represented in percentage (%) of 10.000 evaluated events. * = $p < 0.0332$, ** = $p < 0.0021$, **** = $p < 0.0001$.

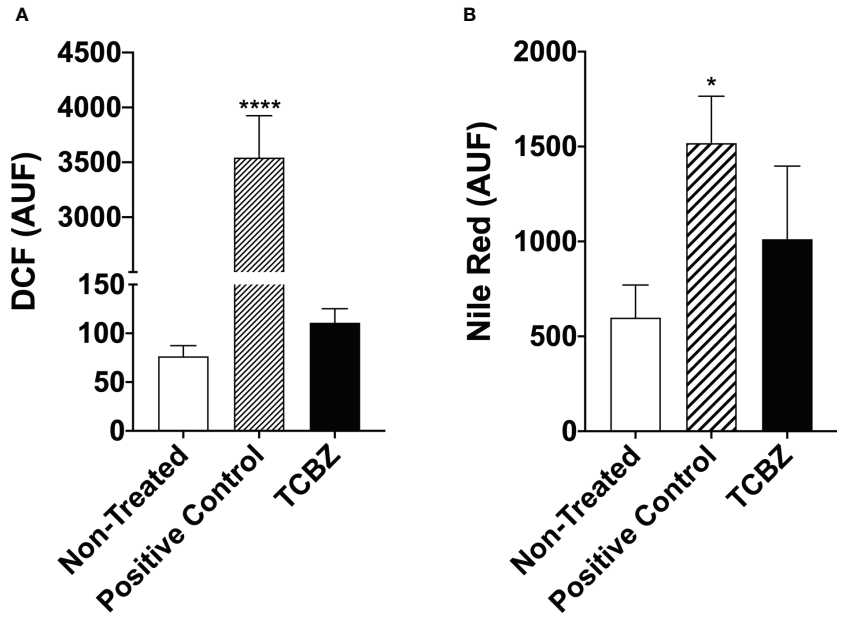


FIGURE 9
Reactive oxygen species evaluation and lipid quantification in *L. amazonensis* promastigotes treated with TCBZ. **(A)** ROS of *L. amazonensis* promastigotes treated with TCBZ IC_{50} for 72h. **(B)** Lipid quantification of *L. amazonensis* promastigotes treated with TCBZ IC_{50} for 72h. AUF – arbitrary units of fluorescence. * = $p < 0.0332$, ****= $p < 0.0001$.

20.4% of living cells, 34.9% of apoptosis, 2.7% of necrosis, and 42% of late apoptosis.

Mitochondrial membrane potential was evaluated in treated promastigotes. Parasites treated with AmpB showed a clear loss of fluorescence when compared to controls (93%), while TCBZ-treated parasites showed a decrease of 50% in fluorescence ($p = 0.07$, Figure 8B).

The cell cycle was analyzed with PI staining, and non-treated parasites were used as control. TCBZ-treated *L. amazonensis* promastigotes showed significant alterations in cell cycle phases, with a decrease in G1 from 74.6% to 54.3% and an increase in S and G2 from 9.7% to 16.6% and 11.9% to 25.4%, respectively (Figure 8C).

3.5 ROS evaluation and lipid quantification

ROS were quantified to access cell death pathway. For this, promastigote forms were treated with IC_{50} of both drugs for 72h. Results showed that treated cells present a ROS profile that is similar to non-treated parasites, indicating that the anti-*Leishmania* effect of TCBZ may not be using the ROS production pathway (Figure 9A).

Furthermore, to verify if electron-dense vesicles observed by TEM could be filled with neutral lipids, this lipid class was quantified in promastigotes after 72h of treatment with IC_{50}. We found a 69% tendency to increase the fluorescence intensity of parasites treated

TABLE 3 IC_{50} of intracellular amastigote forms in each proportion analyzed (1:1, 4:1, 5:1, 10:1 for TCBZ and AmpB, respectively).

Proportion	IC_{50} TCBZ (μM)	IC_{50} AmpB (μM)	FICs TCBZ	FICs AmpB	ΣFICs
01:01	0.38	0.38	0.01	0.19	0.20
04:01	0.72	0.37	0.02	0.19	0.20
05:01	2.30	0.40	0.05	0.20	0.25
10:01	4.97	0.49	0.11	0.25	0.36
00:01	**45.67**	**2.02**		**χΣ FICS**	**0.25**

0:1 proportion refers to the IC_{50} of each drug alone. All experiments were made in biological replicate; numbers represent average with R squared > 0.9. χΣFICS mean the average of the sum of all ΣFICs proportions.

with TCZB compared to untreated promastigotes. However, no statistical difference was found (Figure 9B).

3.6 Synergistic effect of TCBZ

The next step was to examine whether TCBZ and AmpB have a synergistic effect that lowers the IC_{50} of AmpB and, consequently, its toxicity, elevating the SI. In order to do this, combined proportions of 10:1, 5:1, 4:1, and 1:1 concentrations for TCBZ and AmpB were prepared, and infected macrophages were treated for 48h. These ratios were chosen based on the previous observations that TCBZ alone appears to have a comparable anti-*Leishmania* effect in doses 10x higher than AmpB. Also, as the IC_{50} of AmpB is around 2 µM, the focus of this assay was to evaluate if TCBZ was capable of lowering AmpB's IC_{50} through a synergistic effect. The results showed that at all ratios tested, the IC_{50} of the drug in combination ranged from three to 120 times lower than the drugs alone (Table 3). Cytotoxicity assay was also performed in macrophages derived from THP-1 cells. For 1:1 ratio, CC_{50} was 29.34 µM for both drugs. For 1:4, 1:5 and 1:10 ratios, AmpB CC_{50} was 14.73, 27.77 and 34.16 µM, respectively, while TCBZ CC_{50} was 33.60, 36.14 and 21.55 µM, respectively. Regarding SI, AmpB and TCBZ presented the value of 77.2 for 1:1 ratio. For ratios of 1:4, 1:5 and 1:10, AmpB presented values of 39.8, 74.4 and 69.7, respectively, while TCBZ presented values of 46.6, 15.7 and 5.5, respectively.

Once the IC_{50} for each drug in each combination was determined, the ΣFICs equation (see item 2e) was used to calculate the average of the sum FICs (χΣFICs). We found that the combination of TCBZ and AmpB resulted in a synergistic effect with a χΣFICs of 0.25. According to Odds (2003), the drug combination is considered synergic when χΣFICs ≤ 0.5. An isobologram plotted with every analyzed ratio also demonstrated FICs below the addictive line that represents theoretical addictive (or non-interactive) χΣFICs 1 value, corroborating with χΣFICs value found in this study (Figure 10).

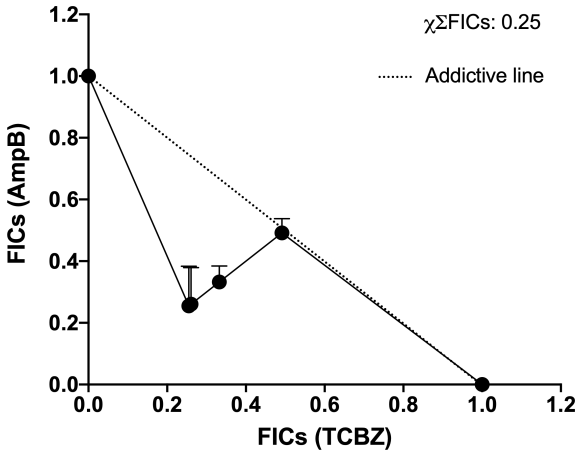


FIGURE 10
Isobologram of ΣFICs of TCBZ and AmpB. Each point represents FICs of each proportion (10:1, 5:1, 4:1, 1:1 for TCBZ and AmpB, respectively). The dotted line represents the addictive line. The result of χΣFICs of all ratios analyzed is at the upper right.

4 Discussion

Treatment for leishmaniasis has changed very little since the early 90s. This is not because available treatments are highly efficient. Rather, it is probably due to a very low level of investment in this neglected disease. We are far from having a vaccine for leishmaniasis and lack a less toxic, cheaper, and more effective drug. Drug repositioning may potentially be used to save time and money in this situation. In this study, we present the results of a benzimidazole derivate that could be a prospective treatment for leishmaniasis. Firstly, promastigote growth was evaluated in the presence of TCBZ and AmpB for 72h in increasing drug concentrations to determine IC_{50}. Intracellular amastigotes were treated for 48h for the same purpose. Regarding macrophage cytotoxicity, although tested concentrations were 10 times higher for TCBZ than for AmpB, CC_{50} values were comparable for both drugs. Furthermore, both drugs had similar SI for *L. amazonensis* amastigotes.

Ultrastructural analysis by transmission electron microscopy showed that treated parasites exhibited not only electron-dense structures compatible with lipid accumulation but also an alteration in flagellar pocket size, with the presence of small electron-dense vesicles. These alterations have already been shown in studies that tested possible sterol metabolism inhibitors in *L. amazonensis*, *L. chagasi*, and *L. braziliensis* (Rodrigues et al., 2008; Oliveira et al., 2009; Ceole et al., 2017). The inference about electrodense structures can be explained by osmium tetroxide post-fixation, which has a high lipid affinity and corroborates with sterol biosynthesis target drugs (de Souza and Rodrigues, 2009). So far, the mechanism of action of benzimidazole derivate drugs has only shown microtubule and protein inhibition in a variety of parasites (Robinson et al., 2002). To our knowledge, this is the first time TCBZ has shown any relation to lipid or sterol pathways.

Although cellular death and mitochondrial membrane potential analysis did not show substantial results in TCBZ-treated promastigotes, the cell cycle showed significant modification, with an increase in the number of parasites in the S and G2 phases. Indeed, it has been shown that some drugs that target the sterol biosynthesis pathway can alter the cell cycle, probably due to changes in the availability of essential sterols (Oliveira et al., 2009; Almeida-Souza et al., 2016), in line with TEM results. Another possible explanation is that it is a consequence of alterations in cytoskeleton organization due to direct influence on microtubule polymerization (Robinson et al., 2002) or nuclear membrane alteration, preventing complete cytokinesis (de Souza and Rodrigues, 2009). In addition, several cells in division were found in SEM when treated with TCBZ, in accordance with cell cycle results since cytokinesis appears to be

affected. Nevertheless, the quantification of ROS and neutral lipids did not show significant results.

When taken together, the results point to a possible anti-*Leishmania* effect of TCBZ, suggesting that this drug could be an interesting candidate to complement anti-leishmania therapy. Additionally, TCBZ may not only use microtubules as a pharmacological target but also interfere in lipid metabolism.

Since TCBZ showed a possible anti-*Leishmania* effect, we hypothesized that this drug could have a synergistic effect when combined with the anti-*Leishmania* effect of AmpB. So far, one of the few compounds containing imidazole in its composition tested in combination with AmpB for leishmaniasis treatment did not have a synergistic statistically significant effect (Rodriguez et al., 1995). However, because TCBZ has already shown successful repurposing as a bactericidal (Pi et al., 2021) and as a fasciolosis treatment in sheep, when used combined with other drugs, even in resistant parasites (Tabari et al., 2022), this specific combination may lead to desirable results. AmpB has already been used in successful treatments in combination with miltefosine, meglumine antimoniate, and pentamidine (Goswami et al., 2020; Ramesh et al., 2020; Vechi et al., 2020). Indeed, we found that TCBZ and AmpB have synergic effects *in vitro* when administrated in combination, and no interaction between these two drugs was found by the DrugBank prediction *in silico*, indicating that it is safe to combine them.

Patients often abandon treatment with AmpB due to its side effects and the fact that several trips to the hospital are required for parenteral administration (Neves et al., 2011). However, other ways to administer AmpB, such as through aerosol in local applications and oral administration, have already shown good results in combination with other drugs and the diminution of side effects (Basile et al., 2020; Parvez et al., 2020). The option of oral administration of AmpB added to the fact that TCBZ has demonstrated not only synergic effect when combined with AmpB, but it is also already an approved medication for oral administration, showing that this drug combination could be a prospective alternative treatment for leishmaniasis.

In summary, TCBZ appears to have an anti-*Leishmania in vitro* effect against *L. amazonensis* as it shows a decrease in parasite growth, cell cycle arrest in the S and G2 phases, and morphologically bigger cell body for several parasites in cell division. Also, TCBZ and AmpB have shown synergic effects on intracellular amastigotes when studied in combination. Although additional work is needed, including *in vivo* assays, these results show that TCBZ displays promising *in vitro* anti-*Leishmania* activity, especially in combination with AmpB, with the attractive characteristic that TCBZ would be a repurposed drug available in oral form.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

BB designed the study, performed experiments, analyzed the data, and wrote and prepared the manuscript. GB analyzed the data. FT-P performed experiments and analyzed the data. FF wrote and prepared the manuscript. LSM designed the study, analyzed the data, and wrote and prepared the manuscript. All authors contributed to the article and approved the submitted version.

Funding

BB was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). GB was supported by Instituto Carlos Chagas, Fiocruz-PR. FT-P was supported by National Council for Scientific and Technological Development (CNPq). FF holds a grant from CNPq for productivity in research (309862/2015-9). LM was supported by grants from FIOCRUZ (grants PEP ICC-008-FIO-21-2-13-30 and PROEP/ICC 442349/2019-0).

Acknowledgments

The authors would like to thank the Program for Technological Development in Tools for Health-RPT-FIOCRUZ for the use of the microscopy facility, RPT07C and cytometry facility (RPT08L), at the Carlos Chagas Institute, Fiocruz/Paraná-Brazil. Author also thanks "Escrita Lab – Laboratório de Escrita Científica" for editing the manuscript for the proper English language. The authors are also grateful to Dr. Marcio Rodrigues for the TCBZ powder.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1044665/full#supplementary-material

References

Alberca, L. N., Sbaraglini, M. L., Balcazar, D., Fraccaroli, L., Carrillo, C., Medeiros, A., et al. (2016). Discovery of novel polyamine analogs with anti-protozoal activity by computer guided drug repositioning. *J. Comput. Aided Mol. Des.* 30 (4), 305–321. doi: 10.1007/s10822-016-9903-6

Alcantara, C. L., Vidal, J. C., de Souza, W., and Cunha-e-Silva, N. L. (2014). The three-dimensional structure of the cytostome-cytopharynx complex of Trypanosoma cruzi epimastigotes. *J. Cell. Sci.* 127(Pt 10), 2227–2237. doi: 10.1242/jcs.135491

Almeida-Souza, F., Taniwaki, N. N., Amaral, A. C. F., De Souza, C. D. S. F., Calabrese, K. D. S., and Abreu-Silva, A. L. (2016). Ultrastructural changes and death of leishmania infantum promastigotes induced by morinda citrifolia linn. fruit (Noni) juice treatment. *Evidence-Based Complement Altern. Med.* 2016, 1–9. doi: 10.1155/2016/5063540

Baell, J. B., and Holloway, G. A. (2010). New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* 53 (7), 2719–2740. doi: 10.1021/jm901137j

Bansal, Y., and Silakari, O. (2012). The therapeutic journey of benzimidazoles: A review. *Bioorg. Med. Chem.* 20 (21), 6208–6236. doi: 10.1016/j.bmc.2012.09.013

Basile, G., Cristofaro, G., Locatello, L. G., Vellere, I., Piccica, M., Bresci, S., et al. (2020). Refractory mucocutaneous leishmaniasis resolved with combination treatment based on intravenous pentamidine, oral azole, aerosolized liposomal amphotericin b, and intralesional meglumine antimoniate. *Int. J. Infect. Dis.* 97, 204–207. doi: 10.1016/j.ijid.2020.06.003

Bortoleti, B. T. D. S., Gonçalves, M. D., Tomiotto-Pellissier, F., Contato, V. M., Silva, T. F., de Matos, R. L. N., et al. (2021). Solidagenone acts on promastigotes of l. amazonensis by inducing apoptosis-like processes on intracellular amastigotes by IL-12p70/ROS/NO pathway activation. *Phytomedicine* 85, 153536. doi: 10.1016/j.phymed.2021.153536

Burza, S., Croft, S. L., and Boelaert, M. (2018). Seminar leishmaniasis. *Lancet* 6736, 1–20. doi: 10.1016/S0140-6736(18)31204-2

Ceole, L. F., Cardoso, M. G., and Soares, M. J. (2017). Nerolidol, the main constituent of piper aduncum essential oil, has anti-leishmania braziliensis activity. *Parasitology* 2017), 1–12. doi: 10.1017/S0031182017000452

Croft, S. L., and Olliaro, P. (2011). Leishmaniasis chemotherapy-challenges and opportunities. *Clin. Microbiol. Infect.* 17 (10), 1478–1483. doi: 10.1111/j.1469-0691.2011.03630.x

Daina, A., Michielin, O., and Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci. Rep.* 7 (January), 1–13. doi: 10.1038/srep42717

Datta, A., Podder, I., Das, A., and Sil, A. (2020). Controversies in the management of cutaneous adverse drug reactions systemic corticosteroids in the management of SJS / TEN : Is it still. *Indian J. Dermatol.* 63 (2), 125–130. doi: 10.4103/ijd.IJD_585_17

de Souza, W., and Rodrigues, J. C. F. (2009). Sterol biosynthesis pathway as target for anti-trypanosomatid drugs. *Interdiscip. Perspect. Infect. Dis.* 2009, 1–19. doi: 10.1155/2009/642502

Escobar, P., Leal, S. M., Herrera, L. V., Martinez, J. R., and Stashenko, E. (2010). Chemical composition and antiprotozoal activities of Colombian lippia spp essential oils and their major components. *Mem. Inst Oswaldo Cruz* 105 (2), 184–190. doi: 10.1590/S0074-02762010000200013

Field, M. C., Horn, D., Fairlamb, A. H., Ferguson, M. A. J., Gray, D. W., Read, K. D., et al. (2017). Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. *Nat. Rev. Microbiol.* 15 (4), 217–231. doi: 10.1038/nrmicro.2016.193

Gandhi, P., Schmitt, E. K., Chen, C. W., Samantray, S., Venishetty, V. K., and Hughes, D. (2019). Triclabendazole in the treatment of human fascioliasis: A review. *Trans. R Soc. Trop. Med. Hyg.* 113 (12), 797–804. doi: 10.1093/trstmh/trz093

Goswami, R. P., Rahman, M., Das, S., Tripathi, S. K., and Goswami, R. P. (2020). Combination therapy against Indian visceral leishmaniasis with liposomal amphotericin b (FungisomeTM) and short-course miltefosine in comparison to miltefosine monotherapy. *Am. J. Trop. Med. Hyg.* 103 (1), 308–314. doi: 10.4269/ajtmh.19-0931

Ioset, J.-R., Brun, R., Wenzler, T., Kaiser, M., and Yardley, V. (2009). *Drug screening for kinetoplastid diseases: A training manual for screening in neglected diseases* Vol. 74 (DNDi Pan-Asian Screen Netw). Available at: http://www.dndi.org/images/stories/pdf_scientific_pub/2009/kinetoplastid_drug_screening_manual_final.pdf.

Kar, A., Jayaraman, A., Charan Raja, M. R., Srinivasan, S., Debnath, J., and Mahapatra, S. K. (2021). Synergic effect of eugenol oleate with amphotericin b augments anti-leishmanial immune response in experimental visceral leishmaniasis in vitro and *in vivo*. *Int. Immunopharmacol.* 91 (September 2020), 107291. doi: 10.1016/j.intimp.2020.107291

Klug, D. M., Gelb, M. H., and Pollastri, M. P. (2016). Repurposing strategies for tropical disease drug discovery. *Bioorg. Med. Chem. Lett.* 26 (11), 2569–2576. doi: 10.1016/j.bmcl.2016.03.103

Laniado-Laborín, R., and Cabrales-Vargas, M. N. (2009). Amphotericin b: side effects and toxicity. *Rev. Iberoam Micol.* 26 (4), 223–227. doi: 10.1016/j.riam.2009.06.003

Lipinski, C. A. (2004). Lead profiling lead- and drug-like compounds : the rule-of-five revolution. *Drug Discov Today Technol.* 1 (4), 337–341. doi: 10.1016/j.ddtec.2004.11.007

Neves, L. O., Talhari, A. C., Gadelha, E. P. N., da Silva, R. M., Guerra JA de, O., Ferreira LC de, L., et al. (2011). Estudo clínico randomizado comparando antimoniato de meglumina, pentamidina e anfotericina b para o tratamento da leishmaniose cutânea ocasionada por leishmania guyanensis. *Bras. Dermatol.* 86 (6), 1092–1101. doi: 10.1590/S0365-05962011000600005

Nosengo, N. (2016). New tricks for old drugs. *Nat. Rev. Microbiol.* 534 (16), 314–316. doi: 10.1016/S0140-6736(18)31204-2

Oliveira, V. C. S., Moura, D. M. S., Lopes, J. A. D., De Andrade, P. P., Da Silva, N. H., and Figueiredo, R. C. B. Q. (2009). Effects of essential oils from cymbopogon citratus (DC) stapf., lippia sidoides cham., and ocimum gratissimum l. @ on growth and ultrastructure of leishmania chagasi promastigotes. *Parasitol. Res.* 104 (5), 1053–1059. doi: 10.1258/td.2008.080369

Pace, D. (2014). Leishmaniasis. *J. Infect.* 69 (S1), S10–S18. doi: 10.1016/j.jinf.2014.07.016

Parvez, S., Yadagiri, G., Gedda, M. R., Singh, A., Singh, O. P., Verma, A., et al. (2020). Modified solid lipid nanoparticles encapsulated with amphotericin b and paromomycin: an effective oral combination against experimental murine visceral leishmaniasis. *Sci. Rep.* 10 (1), 1–14. doi: 10.1038/s41598-020-69276-5

Pi, H., Ogunniyi, A. D., Savaliya, B., Nguyen, H. T., Page, S. W., Lacey, E., et al. (2021). Repurposing of the fasciolicide triclabendazole to treat infections caused by *Staphylococcus* spp. and vancomycin-resistant Enterococci. *Microorganisms* 9 (8), 1697. doi: 10.3390/microorganisms9081697

Pires, M., Wright, B., Kaye, P. M., da Conceição, V., and Churchill, R. C. (2019). The impact of leishmaniasis on mental health and psychosocial well-being: A systematic review. *PloS One* 14 (10), 1–21. doi: 10.1371/journal.pone.0223313

Ramesh, V., Dixit, K. K., Sharma, N., Singh, R., and Salotra, P. (2020). Assessing the efficacy and safety of liposomal amphotericin b and miltefosine in combination for treatment of post kala-azar dermal leishmaniasis. *J. Infect. Dis.* 221 (4), 608–617. doi: 10.1093/infdis/jiz486

Robinson, M. W., Trudgett, A., Hoey, E. M., and Fairweather, I. (2002). Triclabendazole-resistant fasciola hepatica: β-tubulin and response to *in vitro* treatment with triclabendazole. *Parasitology* 124 (3), 325–338. doi: 10.1017/S003118200100124X

Rodrigues, J. C. F., Concepcion, J. L., Rodrigues, C., Caldera, A., Urbina, J. A., and De Souza, W. (2008). *In vitro* activities of ER-119884 and E5700, two potent squalene synthase inhibitors, against leishmania amazonensis: Antiproliferative, biochemical, and ultrastructural effects. *Antimicrob. Agents Chemother.* 52 (11), 4098–4114. doi: 10.1128/AAC.01616-07

Rodriguez, L. V., Dedet, J. P., Paredes, V., Mendoza, C., and Cardenas, F. (1995). A randomized trial of amphotericin b alone in combination with itraconazole in the treatment of mucocunateus leishmaniasis. *Nature* 388, 525–528. doi: 10.1590/S0074-02761995000400020

Santi, A. M. M., and Murta, S. M. F. (2022). Antioxidant defence system as a rational target for chagas disease and leishmaniasis chemoterapy. *Nature Mem. Inst Oswaldo Cruz* 117, 1–7. doi: 10.1590/0074-02760210401

Tabari, M. A., Vahdati, S. A. F., Samakkhah, S. A., Araghi, A., and Youssefi, M. R. (2022). Therapeutic efficacy of triclabendazole in comparison to combination of triclabendazole and levamisole in sheep naturally infected with fasciola sp. *J. Parasitic Dis.* 46, 80–86. doi: 10.1007/s12639-021-01422-w

Terashima, A., Canales, M., Maco, V., and Marcos, L. A. (2021). Observational study on the effectiveness and safety of multiple regimens of triclabendazole in human fascioliasis after failure to standard-of-care regimens. *J. Glob. Antimicrob. Resist.* 25, 264–267. doi: 10.1016/j.jgar.2021.03.023

Trinconi, C. T., Reimão, J. Q., Yokoyama-Yasunaka, J. K. U., Miguel, D. C., and Uliana, S. R. B. (2014). Combination therapy with tamoxifen and amphotericin b in experimental cutaneous leishmaniasis. *Antimicrob. Agents Chemother.* 58 (5), 2608–2613. doi: 10.1128/AAC.01315-13

Veber, D. F., Johnson, S. R., Cheng, H., Smith, B. R., Ward, K. W., and Kopple, K. D. (2002). Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* 45 (12), 2615–2623. doi: 10.1021/jm020017n

Vechi, H. T., De Sousa, A. S. V., Da Cunha, M. A., Shaw, J. J., and Luz, K. G. (2020). Case report: Combination therapy with liposomal amphotericin b, n-methyl meglumine antimoniate, and pentamidine isethionate for disseminated visceral leishmaniasis in a splenectomized adult patient. *Am. J. Trop. Med. Hyg.* 102 (2), 268–273. doi: 10.4269/ajtmh.18-0999

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 10 January 2023
DOI 10.3389/fcimb.2022.1045732

OPEN ACCESS

EDITED BY
Suzana Passos Chaves,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Rubem Figueiredo Sadok Menna-Barreto,
Oswaldo Cruz Foundation (Fiocruz), Brazil
Edson Roberto Silva,
University of São Paulo, Brazil
Camila Falcão,
IFRJ, Brazil

*CORRESPONDENCE
Valéria M. Borges
valeria.borges@fiocruz.br

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 16 September 2022
ACCEPTED 14 December 2022
PUBLISHED 10 January 2023

CITATION
Rebouças-Silva J, Santos GF, Filho JMB, Berretta AA, Marquele-Oliveira F and Borges VM (2023) *In vitro* leishmanicidal effect of Yangambin and Epi-yangambin lignans isolated from *Ocotea fasciculata* (Nees) Mez. *Front. Cell. Infect. Microbiol.* 12:1045732. doi: 10.3389/fcimb.2022.1045732

In vitro leishmanicidal effect of Yangambin and Epi-yangambin lignans isolated from *Ocotea fasciculata* (Nees) Mez

Jéssica Rebouças-Silva[1,2], Gabriel Farias Santos[1], José Maria Barbosa Filho[3], Andresa A. Berretta[4], Franciane Marquele-Oliveira[5] and Valéria M. Borges[1,2]*

[1]Laboratory of Inflammation and Biomarkers, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil, [2]Faculty of Medicine of Bahia, Federal University of Bahia (UFBA), Salvador, Bahia, Brazil, [3]Post-Graduate Program in Natural and Synthetic Bioactive Products, Federal University of Paraíba, João Pessoa, Paraíba, Brazil, [4]Laboratory of Research, Development and Innovation, Apis Flora Industrial e Comercial Ltda, Ribeirão Preto, São Paulo, Brazil, [5]Laboratory of Research, Development and Innovation, Eleve Science Research and Development, Ribeirão Preto, São Paulo, Brazil

Introduction: Yangambin and epi-yangambin are the main lignans found in Louro-de-Cheiro [*Ocotea fasciculata* (Nees) Mez], a tree native to the Atlantic forests of northeastern Brazil whose leaves and bark are widely used in folk medicine. The present study investigated the leishmanicidal and immunomodulatory effects of both lignans in *in vitro* models of infection by *Leishmania amazonensis* or *Leishmania braziliensis*, both etiological agents of Cutaneous Leishmaniasis in Brazil.

Methods: Bone marrow-derived mouse macrophages were infected with *L. amazonensis* or *L. braziliensis* and then treated for 48 h at varying concentrations of yangambin or epi-yangambin.

Results: Yangambin and epi-yangambin were found to reduce the intracellular viability of either *Leishmania* species in a concentration-dependent manner, with respective IC_{50} values of: 43.9 ± 5 and 22.6 ± 4.9 μM for *L. amazonensis*, compared to IC_{50} values of 76 ± 17 and 74.4 ± 9.8 μM for *L. braziliensis*. In this context, epi-yangambin proved more selective and effective against *in vitro* infection by *L. amazonensis*. However, both lignans were found to distinctly modulate the production of inflammatory mediators and other cytokines by macrophages infected by either of the *Leishmania* species evaluated. While yangambin increased the production of IL-10 by *L. braziliensis*-infected macrophages, both compounds were observed to lower the production of NO, PGE_2, IL-6 and TNF-α in both *Leishmania* species.

Discussion: The present results serve to encourage the development of novel studies aimed at screening natural bioactive compounds with the hope of discovering new therapeutic options for the treatment of Cutaneous Leishmaniasis.

KEYWORDS

yangambin, epi-yangambin, *Leishmania*, Leishmaniasis, natural compounds, treatment, lignan, *Ocotea fasciculata* (Nees) Mez

Introduction

Leishmaniasis a widespread group of neglected vector-borne tropical diseases caused by parasites of the genus *Leishmania*, which are transmitted to humans through phlebotomine vectors. Cutaneous Leishmaniasis (CL) is the most common clinical presentation of this disease. According to the WHO, in 2019 more than 87% of new CL cases were reported in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Iran (Islamic Republic of), Iraq, Libya, Pakistan, Syria and Tunisia (WHO, 2021). CL can produce multiple round or oval-shaped skin ulcers with prominent erythematous edges and a granular background; lesions may even compromise regions of the nasopharyngeal mucosa, which can lead to disfigurement (Brasil, 2017). In Brazil, *Leishmania amazonensis* and *Leishmania braziliensis* are the main etiological agents of CL (MINISTÉRIO DA SAÚDE/SVS/SINAM NET, 2021).

Since 1945, the first-choice drugs for leishmaniasis treatment have been based on pentavalent antimonials (Sb^{5+}) (Lima et al., 2007). In the event of therapeutic failure or inability to use antimonial therapy, a second-choice drug is employed, such as amphotericin B, pentamidine, paromomycin or miltefosine. However, these treatment options present serious disadvantages, such as high cost, toxic effects and increasing reports of therapeutic failure. Thus, the development of new, effective, safer and more accessible treatments is urgent.

The isomers yangambin and epi-yangambin are lignans found in *Ocotea fasciculata* (Nees) Mez (also known as *Ocotea duckei* Vatti-mo-Gil), a tree native to the Atlantic forests of northeastern Brazil, popularly known as Louro-de-Cheiro or Louro-canela. Yangambin has been described as being an antagonist of platelet activation receptor (Jesus-Morais et al., 2000; Castro-Faria-Neto et al., 1995), having antiallergic (Serra et al., 1997) and analgesic (Almeida et al., 1995) properties, exerting protective action against cardiovascular injury and anaphylactic shock (Ribeiro et al., 1996; Tibirica et al., 1996; Araújo et al., 2014), as well as antitumor activity in colorectal cells (Hausott et al., 2003) and in the KB tumor cell line (Bala et al., 2015). Studies have also reported the leishmanicidal effect of yangambin on promastigotes (Neto et al., 2007; Neto et al., 2011) and in intracellular amastigotes, as well as in an *in vivo* model of CL (Penha, 2010). However, few studies have evaluated the leishmanicidal and immunomodulatory properties of yangambin in an *in vitro* or *in vivo* model of infection. Moreover, to date, there are no known studies evaluating the leishmanicidal and immunomodulatory properties of epi-yangambin.

The present study aimed to shed light on the leishmanicidal and immunomodulatory effects of yangambin and epi-yangambin using *in vitro* infection models of *L. amazonensis* and *L. braziliensis*, the main etiologic agents of CL in Brazil.

Materials and methods

Material and reagents

Schneider's insect medium, IFN-γ, Dimethylsulfoxide (DMSO) and Resazurin sodium salt were obtained from SIGMA-Aldrich (St Louis, MO, USA). A Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit and Nutridoma™-SP media supplement were obtained from Roche Diagnostics GmbH (Sandhofer Strasse, Mannheim, Germany). Inactivated fetal bovine serum (FBS), RPMI 1640 medium and RPMI 1640 medium without phenol red and penicillin were purchased from GIBCO (Carlsbad, CA, USA). Streptomycin and L-glutamine were obtained from Invitrogen (Carlsbad, CA, USA). A PGE_2 Elisa kit was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) and the MILLIPLEX® kit based on Luminex® xMAP® technology was obtained from Merck (Darmstadt, Germany).

Yangambin and epi-yangambin isolation & preparation

The dried leaves and stem bark of *Ocotea fasciculata* (commonly known as *Louro de Cheiro* in Portuguese) were collected in the state of Paraíba (northeastern Brazil). Both yangambin and epi-yangambin lignans were originally isolated

and purified by Martins et al. (2020), who also characterized these compounds using high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) approaches. The obtained yield was 17% and 29% for yangambin and epi-yangambin, respectively; the chemical formula identified was $C_{24}H_{30}O_{8,}$ with a corresponding molecular weight of 446 g/mol. Further, a negligible amount of impurities was detected given the quantity of compounds recovered (Martins et al., 2020). A representative illustration of both lignans is shown in Figure 1. Subsequently, both lignans were solubilized in DMSO to prepare stock solutions at a concentration of 5 mg/mL.

Ethics statement

Female BALB/c mice aged 6–8 weeks were obtained from the animal care facility at the Gonçalo Moniz Institute (IGM-FIOCRUZ), located in the city of Salvador, Bahia-Brazil. All animal experimentation was conducted in accordance with the Guidelines for Animal Experimentation established by the Brazilian Council for the Control of Animal Experimentation (CONCEA). The present study received approval from the local institutional review board (CEUA protocol no.: 015/2015, IGM-FIOCRUZ).

Parasites

Wild-type *L. amazonensis* (MHOM/Br88/Ba-125) and *L. braziliensis* (MHOM/BR/01/BA788) strains were cultured in Schneider's insect medium supplemented with 10% inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in 25 cm^2 flasks at 24°C.

Macrophage toxicity assay

Bone-marrow derived macrophages (BMDM) were obtained from BALB/c mice femurs and cultured at 37°C under 5% CO_2 for 7 days in RPMI medium supplemented with 20% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 30% L929 cell culture supernatant (a source of macrophage colony-stimulating factor). Next, differentiated BMDM were harvested using cold saline solution. BMDM (1×10^5/well) were plated on 96-well plates and cultured at 37°C under 5% CO_2 in RPMI medium without phenol red, supplemented with 1% Nutridoma™-SP (instead of FBS) overnight. BMDM were then treated with either yangambin or epi-yangambin at varying concentrations (15.6–1000 µM) at 37°C for 48h. Uninfected BMDM cultures supplemented with RPMI medium (Medium) or DMSO (~0.1% v/v) (Vehicle) were used as controls. Next, cells were reincubated for another 4 h with supplemented RPMI medium containing 10% Resazurin sodium salt. Absorbance was read at 570 nm and 600 nm using a spectrophotometer (SPECTRAMax 190). Quantification of cytoplasmic lactate dehydrogenase (LDH) enzyme activity was performed in cell-free supernatants following centrifugation at 250×g. Measured using a commercial LDH Cytotoxicity Detection Kit in accordance with the manufacturer's instructions, LDH absorbance was recorded at 490 nm using a spectrophotometer (SPECTRAMax 190). In addition, Total LDH activity was determined by lysing BMDM with 1%

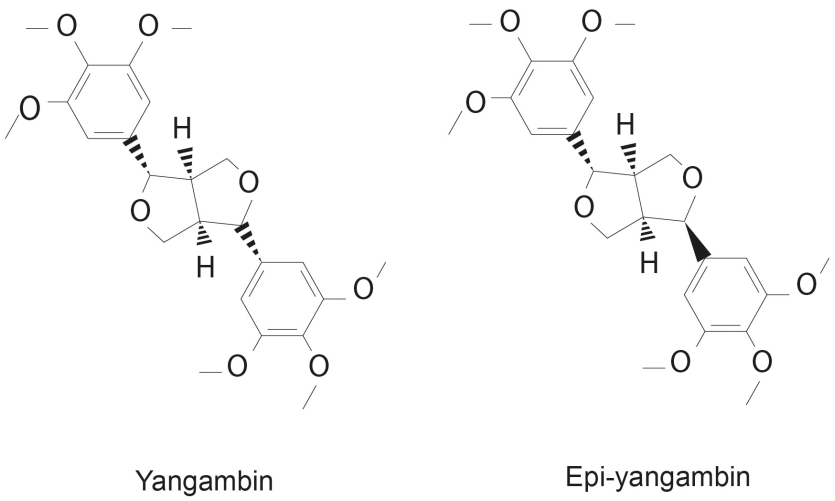


FIGURE 1
Chemical structures of yangambin and epi-yangambin lignans.

Triton X-100. The percentage of LDH release was calculated as follows: [(LDH sample – Blank LDH) × 100]/Total LDH.

Assessment of *Leishmania amazonensis* and *Leishmania braziliensis* intracellular viability

BMDM were isolated as described above; 10^5 cells/well were seeded on 96-well plates in supplemented RPMI medium. Macrophages were then infected (10:1) with stationary-phase *L. amazonensis* (MHOM/Br88/Ba-125) or *L. braziliensis* (MHOM/BR/01/BA788) promastigotes for 4 h or 24 h, respectively. Next, macrophage cultures were washed 3x with saline to remove any non-internalized parasites and treated with varying concentrations (50 - 200 µM) of yangambin or epi-yangambin for 48 h at 37°C under 5% CO_2. After replacing the medium with 0.2 mL of supplemented Schneider's insect medium, cells were cultured at 24°C for an additional 6 days, after which the number of viable parasites was determined by direct counting using a Neubauer chamber.

Quantification of inflammatory mediators

BMDM ($5x10^5$/well) were stimulated with IFN-γ (100 UI/mL) overnight and infected with stationary-phase *L. amazonensis* or *L. braziliensis* promastigotes for 4 h or 24 h, respectively. Macrophages were then washed thrice to remove any non-internalized parasites, the RPMI cell medium was replaced and IFN-γ stimulation was reapplied along with 100 µM of yamgambin or epi-yangambin for 48h. After collecting culture supernatants, the Griess reaction was used to measure nitric oxide (NO) production. Production levels were measured in culture supernatants using a commercially available MILLIPLEX® kit based on Luminex® xMAP® technology (Merck, Darmstadt, Germany) in accordance with the manufacturer's instructions.

Statistical analysis

Data are presented as means ± standard deviation (SD) from experiments performed in quintuplicate. Kruskal–Wallis nonparametric testing with Dunn's post-test was used for multiple comparisons. Comparisons between two groups were performed using the Mann–Whitney non-parametric test. Sigmoidal dose–response curves were used to determine mean inhibition concentration (IC_{50}) values relative to intracellular parasite viability, as well as cytotoxicity concentration implying 50% cell viability (CC_{50}). The selectivity index (SI), i.e., how selective a compound is to parasites versus macrophages, was determined by calculating CC_{50}:IC_{50} ratios. Mean values (Log10) of each mediator were measured in cell supernatants after 48h for each *in vitro* treatment condition. Hierarchical cluster analysis using Ward's method was performed to determine whether cytokine levels could differentiate between the two treatment conditions (yangambin or epi-yangambin) in the context of either *L. amazonensis* or *L. braziliensis* infection. Heatmaps were built using JMP Pro v.13.0.0. Analyses were performed using GraphPad Prism v.8.00 for Windows (GraphPad Software, San Diego California). Results were considered statistically significant when $p < 0.05$.

Results

Yangambin and epi-yangambin lignans exhibit low toxicity

The potential cytotoxic effects of yangambin and epi-yangambin were evaluated in uninfected BMDM *via* sodium resazurin and LDH assays. Under both techniques, only epi-yangambin was able to significantly affect cell viability at the highest concentrations used (1000 and 500 µM), as the highest concentration reduced cell viability by ~97% in sodium resazurin assays ($p<0.01$) (Figure 2A), and induced a ~77% release of LDH ($p<0.0001$) (Figure 2B). Thus, the CC_{50} for epi-yangambin was established at 534 ± 105 µM (Table 1). By contrast, treatment with yangambin produced no evident cytotoxic effect in our analysis.

Concentration-response effect of yangambin and epi-yangambin on *Leishmania amazonensis* and *Leishmania braziliensis* intracellular viability

The leishmanicidal effect of both lignans was evaluated in BMDM infected by *L. amazonensis* or *L. braziliensis* (Figure 3). Both lignans were found to reduce the intracellular viability of both *Leishmania* species evaluated in a concentration-dependent manner after 48h of treatment. However, epi-yangambin was shown to be more effective in reducing the intracellular viability of *L. amazonensis* compared to yangambin at similar concentrations (200 and 100 µM) ($p< 0.05$) (Figure 3A). Moreover, epi-yangambin also proved to be more potent against *L. amazonensis* infection compared to *L. braziliensis* infection ($p<0.05$), as evidenced by the respective IC_{50} values: 22.6 ± 4.9 versus 74.4 ± 9.8 (Table 1). Both lignans were found to present moderate and similar leishmanicidal effects against *L. braziliensis* infection (Figure 3B). Table 1 summarizes the CC_{50}, IC_{50} and Selectivity Index (SI) values calculated for each compound in accordance with either infection condition.

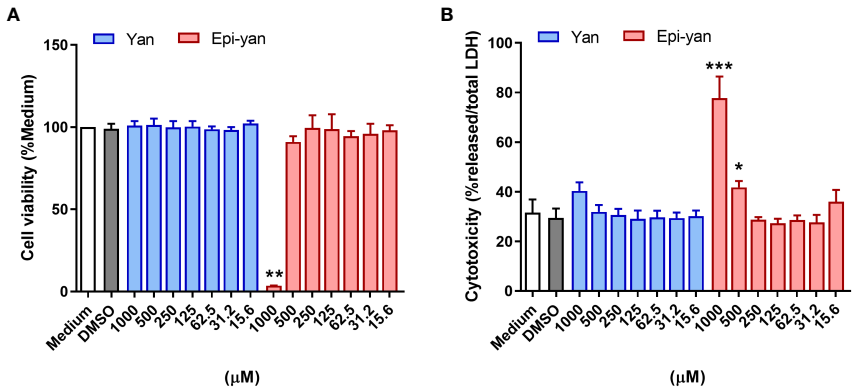


FIGURE 2
Cytotoxicity of yangambin and epi-yangambin. The cytotoxic potential of both lignans was assessed by **(A)** Sodium resazurin reduction assay and **(B)** total LDH release after 48 h of treatment at varying concentrations (1000 - 15.6 μM). Bars represent ± SD of one representative experiment, from three independent experiments performed in quintuplicate. The Kruskal-Wallis test, followed by Dunn's post-test, was used for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.0001). Yan, yangambin; Epi-yan, epi-yangambin.

Immunomodulatory effects of yangambin and epi-yangambin treatment

The immunomodulatory effects of the yangambin and epi-yangambin lignans were evaluated in the supernatants of macrophages previously stimulated with IFN-y, then infected with *L. amazonensis* or *L. braziliensis*, and finally treated with one of the lignans at 100 μM for 48 h, as described above in the *Materials and Methods* section. In *in vitro L. amazonensis* infection, treatment with either lignan similarly reduced the production of nitric oxide (NO) ($p<0.01$), TNF-α ($p<0.05$) and IL-6 ($p<0.05$) in infected cells, yet no statistical differences were observed in the production of IL-12p70 or IL-10 (Figure 4). Although both yangambin and epi-yangambin significantly reduced PGE_2 levels compared to untreated *L. amazonensis*-infected cells ($p<0.05$ and $p<0.01$, respectively), epi-yangambin exerted a more potent effect compared to yangambin ($p < 0.01$) (Figure 4B).

Regarding *in vitro L. braziliensis* infection, both lignans were observed to significantly reduce TNF-α levels ($p< 0.05$). However, only yangambin significantly reduced NO levels ($p<0.01$) and also increased IL-10 production ($p<0.05$) in infected macrophages. Again, epi-yangambin was shown to be more potent in reducing PGE_2 levels, both in relation to the infected control ($p<0.01$) and to yangambin ($p<0.05$) (Figure 5B). Neither of the lignans were shown to modulate the production of IL-6 or IL-12p70 (Figure 5).

A heatmap was generated to illustrate clustering in the modulation of inflammatory mediators produced by *L. amazonensis* or *L. braziliensis*-infected cells treated or not with yangambin or epi-yangambin (Figures 6A, B). This analysis revealed that treatment with either lignan attenuated the inflammatory profile of infected cells, more so in the context of *L. amazonensis* infection.

TABLE 1 IC_{50}, CC_{50} and Selective Index (SI) values of BMDM infected or not with *Leishmania amazonensis* or *Leishmania braziliensis* and treated for 48 h with yangambin or epi-yangambin.

Compound	*L. amazonensis* (intracellular amastigote) IC_{50}[a] ± SEM[b] (μM)	*L. braziliensis* (intracellular amastigote) IC_{50}[a] ± SEM[b] (μM)	Macrophage CC_{50}[c] ± SEM (μM)	SI[d] *L. amazonensis*	SI[d] *L. braziliensis*
Yangambin	43.9 ± 5 p=0.057	76 ± 17	ND[e]	ND[e]	ND[e]
Epi-Yangambin	22.6 ± 4.9*	74.4 ± 9.8	534 ± 105	23.6	7.1

[a]IC_{50}: half-inhibitory concentration, i.e., the concentration needed to inhibit a given biological process by half;
[b]SEM: standard error of the mean;
[c]CC_{50}: 50% cytotoxic concentration, the concentration needed reduce cell viability by 50%;
[d]SI: selectivity index, as calculated by the ratio: Macrophage CC_{50}/amastigote intracellular IC_{50};
[e]ND: not detected;
*Indicates statistically significant difference ($p <0.05$) between the IC_{50} of L. amazonensis and L. braziliensis after treatment with epi-yangambin.

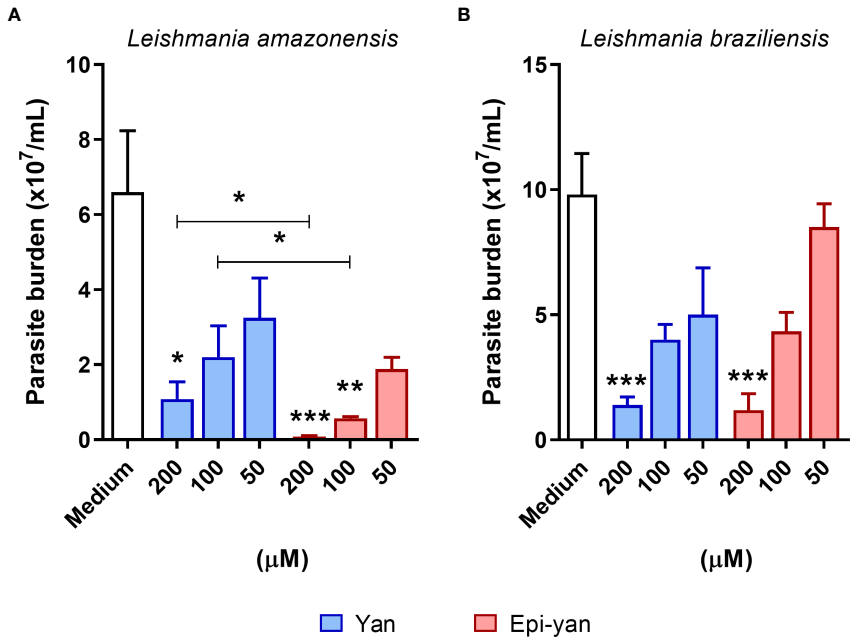


FIGURE 3
Leishmanicidal effect of yangambin and epi-yangambin on the intracellular viability of *L. amazonensis* and *L. braziliensis*. BMDM were infected with *L. amazonensis* or *L. braziliensis* and treated at varying concentrations of yangambin and epi-yangambin compounds (200 - 50 μM) for 48h. The counting of viable **(A)** *L. amazonensis* and **(B)** *L. braziliensis* promastigotes recovered from culture supernatants was performed six days after changing culture medium, as described in *Materials and Methods*. Bars represent ± SD one representative experiment from four independent experiments performed in quintuplicate. The Kruskal-Wallis test, followed by Dunn's post-test, was used for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.0001). La, *Leishmania amazonensis*; Lb, *Leishmania braziliensis*; Yan, yangambin; Epi-yan: epi-yangambin.

Discussion

The thalidomide tragedy in the 1950-60s shed light on the need for greater drug regulation. Introduced in the 1950s as a sedative, tranquilizer and antiemetic for morning sickness, thalidomide was a racemic mixture of two straight (R) and sinister (S) enantiomers, in which the R isomer acts as a sedative, while the S isomer serves as a teratogen; this in turn led to thousands of children being born with malformations in the limbs, ears, heart and internal organs (Moro and Invernizzi, 2017; Kesserwani, 2021). Thus, considering the importance of studying isomers to develop safe and highly effective drugs, the present work builds on previous reports by evaluating the leishmanicidal and immunomodulatory effects of the yangambin and epi-yangambin isomers.

In the present work, our results show that both lignans proved effective in controlling *in vitro* infection by both *Leishmania* species evaluated. Regarding cytotoxicity, epi-yangambin exhibited greater cytotoxicity than yangambin, which is consistent with data reported by Martins et al. (2020). Monte Neto et al. (2008), who evaluated the cytotoxicity of yangambin (24h incubation period) on murine macrophages using Trypan blue exclusion assays and an MTT colorimetric assay, described CC_{50} values of 187 μg/mL (383.8 μM) and 46.7 μg/mL (504.3 μM), respectively. In contrast, we observed no cytotoxic effects following *in vitro* treatment with yangambin in BALB/c macrophages. The discrepancies between the obtained results may be related to the cytotoxicity assay techniques used, specificities in cell types and the degree of purity of the compounds evaluated.

The leishmanicidal effects of the presently investigated lignans remains poorly studied in the scientific literature. Most publications have exclusively reported on the effect of yangambin on promastigote forms of *Leishmania* (Neto et al., 2007; Neto et al., 2011). Although efficacy data on promastigotes does shed light on this lignan's potential leishmanicidal effect, only by evaluating intracellular amastigote forms can this be confirmed. Herein, our results suggest that epi-yangambin was more potent in reducing the intracellular viability of *L. amazonensis* than yangambin, with respective IC_{50} values of 22.6 ± 4.9 μM and 43.9 ± 5 μM, respectively. Furthermore, epi-yangambin also demonstrated greater specificity for *L. amazonensis* compared to *L. braziliensis* infection, with respective IC_{50} values of 22.6 ± 4.9 μM and 74.4 ± 9.8 μM, respectively, and corresponding SI values of 23.6 and 7.1. According to Batista et al. (2009), compounds presenting IC_{50} values between 20-100 μM are considered to exert moderate activity. In addition, compounds with SI values >10 are

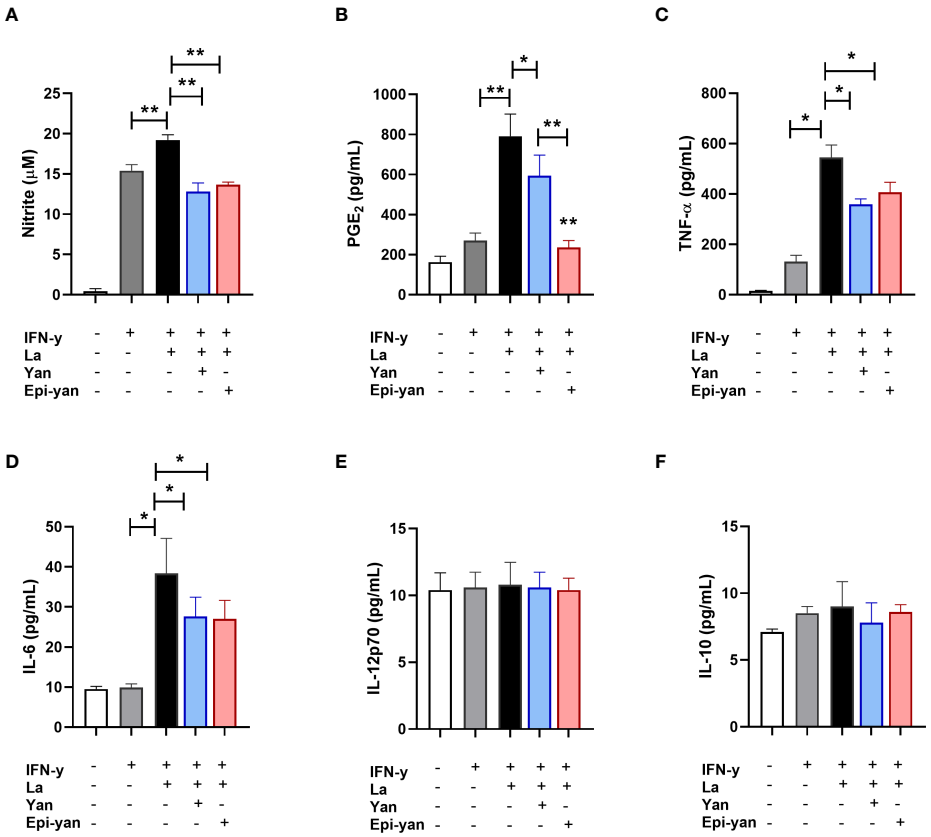


FIGURE 4
Immunomodulatory effects of yangambin and epi-yangambin on *L. amazonensis*-infected macrophages. Mediators were analyzed in the supernatants of cultured BMDM previously stimulated with IFN-γ, then infected with *L. amazonensis*, followed by treatment with yangambin or epi-yangambin (100 μM) for 48h, as described in *Materials and Methods*. **(A)** Nitrite, **(B)** PGE2, **(C)** TNF-α, **(D)** IL-6, **(E)** IL-12p70 and **(F)** IL-10. Bars represent ±SD of a single experiment performed in quintuplicate. The Kruskal-Wallis test, followed by Dunn's post-test, was used for multiple comparisons, while the Mann-Whitney test was used for comparisons between two groups (*p < 0.05 and **p < 0.01). La, *Leishmania amazonensis*; Yan, yangambin; Epi-yan, epi-yangambin.

considered bioactive, thus deserving further investigation (Indrayanto et al., 2021). Our data indicate that epi-yangambin is more selective and effective at reducing *L. amazonensis* intracellular viability in comparison to *L. braziliensis*. However, yangambin was shown to be less cytotoxic to host cells, which suggests greater selectivity of this compound in killing the intracellular forms of both *Leishmania* species evaluated. To the best of our knowledge, these results are novel in that they demonstrate the leishmanicidal effects of epi-yangambin on intracellular *Leishmania* parasite viability. Furthermore, this study also represents the first attempt to evaluate yangambin in the context of intracellular viability of *Leishmania* species related to CL.

The immunological spectrums of human infection by *L. amazonensis* or *L. braziliensis* species are distinct. *L. amazonensis* infection tends to induce clinical forms associated with the anergic pole of the host's cellular immune response (Diffuse Leishmaniasis), while *L. braziliensis* alternatively tends to induce cellular hypersensitivity (Mucocutaneous Leishmaniasis). In the middle of this immunological spectrum lies Localized Cutaneous Leishmaniasis (LCL) (Silveira et al., 2004; Silveira et al., 2009). Although the Th1 response (i.e., the production of pro-inflammatory mediators, such as IFN-*y*, TNF-α, ROS, NO, IL-6 and IL-12) contributes to the control of Leishmania infection, an exacerbated Th1 response can lead to severe tissue damage in CL. Thus, a pro-inflammatory response alone is insufficient for the control of infection, since patients with Mucocutaneous Leishmaniasis exhibit an exacerbated inflammatory response with reduced levels of the regulatory cytokine IL-10, yet they are unable achieve cure (Rodrigues et al., 2014).

In this context, drugs and therapeutic regimens exerting parasite control activity, while also balancing pro- and anti-

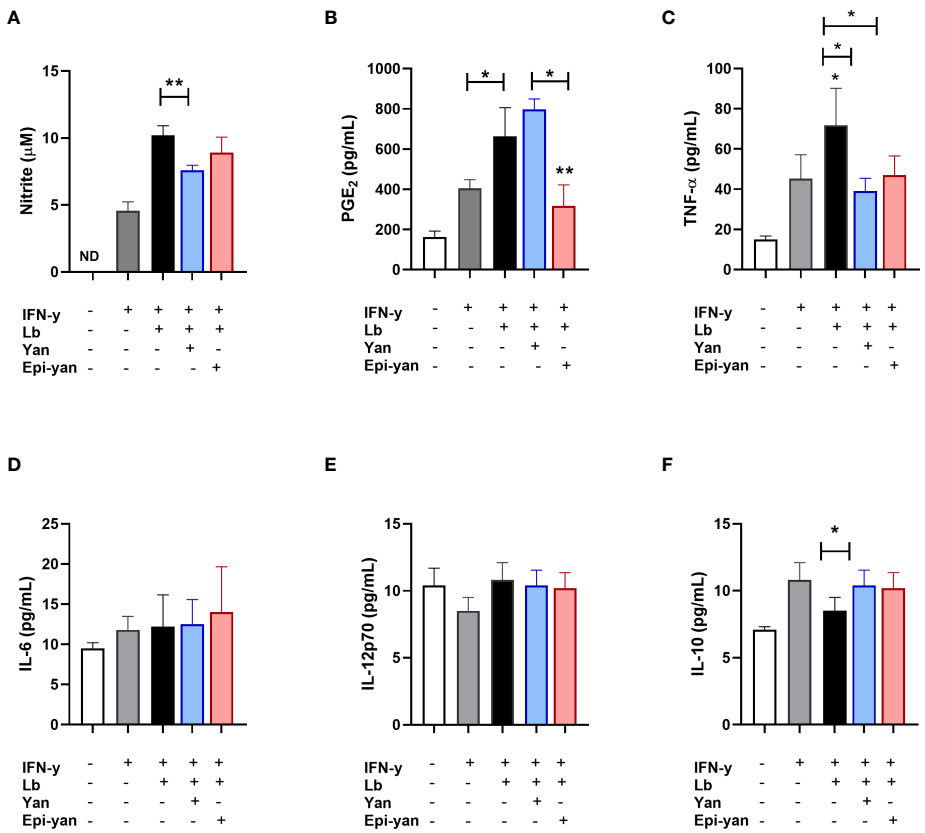


FIGURE 5
Immunomodulatory effects of yangambin and epi-yangambin on *L. braziliensis*-infected macrophages. Mediators were analyzed in the supernatants of cultured BMDM previously stimulated with IFN-γ, then infected with *L. braziliensis* and finally treated with yangambin or epi-yangambi (100 µM) for 48h, as described in *Materials and Methods*. **(A)** Nitrite, **(B)** PGE2, **(C)** TNF-α, **(D)** IL-6, **(E)** IL-12p70 and **(F)** IL-10. Bars represent ±SD of a single experiment performed in quintuplicate. The Kruskal-Wallis test, followed by Dunn's post-test, was used for multiple comparisons, while the Mann-Whitney test was used for comparisons between two groups (*p < 0.05 and **p < 0.01). Lb, *Leishmania braziliensis*; Yan, yangambin; Epi-yan, epi-yangambin.

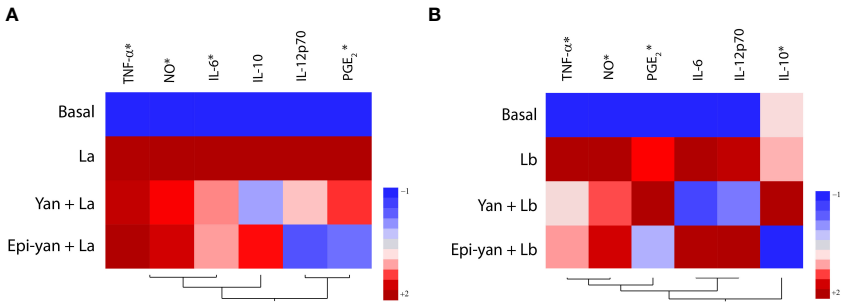


FIGURE 6
Inflammatory profile of yangambin- and epi-yangambin-treated macrophages infected with either *L. amazonensis* or *L. braziliensis*. Hierarchical clustering analysis (Ward's method) was used to assess whether cytokine production could differentiate between **(A)** *Leishmania amazonensis* or **(B)** *Leishmania braziliensis*, infection, in cells treated or not with yangambin or epi-yangambin, as described in Materials and Methods. La, *Leishmania amazonensis*; Lb, *Leishmania braziliensis*; Yan, yangambin; Epi-yan, epi-yangambin.

inflammatory responses, are desirable to control this condition. Accordingly, lignans yangambin and epi-yangambin have emerged as promising candidates for future evaluation in the development of new pharmaceutical options for CL treatment. Our data suggest that the leishmanicidal effects produced by both lignans are not related to macrophage activation. Despite the absence of a cellular activation pathway linked to the leishmanicidal effect presented by yangambin and epi-yangambin, our findings support the hypothesis that these compounds may exert a direct effect on intracellular parasite, which deserves further investigation.

Importantly, our results show that both lignans were found to reduce the levels of important pro-inflammatory mediators, e.g., NO, IL-6 and TNF-α, in cells infected by *L. amazonensis* or *L. braziliensis*, while also reducing intracellular viability. These data corroborate a report by Penha (2010) describing the ability of yangambin to reduce NO and TNF-alpha levels in *L. chagasi*-infected cells, a *Leishmania* species related to visceral leishmaniasis (Penha, 2010).

Although a range of pharmacological properties have already been attributed to yangambin and, to a lesser extent, epi-yangambin, the present study offers relevant insight into the immunomodulatory effects of both lignans in *in vitro* infection by *L. amazonensis* and *L. braziliensis*.

Conclusion

Lignans yangambin and epi-yangambin represent potential candidates for further evaluation in the development of novel treatments against CL. Both lignans demonstrated leishmanicidal effects *in vitro*, accompanied by an attenuated inflammatory response, in macrophages infected by *L. amazonensis* or *L. braziliensis*, both etiological agents of CL in Brazil. In addition, the study of yangambin and epi-yangambin lignans serves to highlight Brazilian biodiversity in the screening of bioactive compounds and molecules for therapeutic applications. It is important to study these compounds, not only for the possibility of discovering new, more affordable treatments, but also to encourage further investigation into the possible mechanisms of action involved in the biological effects identified herein, which open up new possibilities for the development of target-specific drugs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Brazilian Council for the Control of Animal Experimentation (CONCEA), IGM-FIOCRUZ.

Author contributions

All authors made substantial contributions to the study's conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This study was supported in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Financing Code 001. VB is a senior investigator from CNPq. JR-S has fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). GS has fellowship from Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB).

Acknowledgments

The authors thank Andris K. Walter for English Language revision and manuscript copyediting assistance.

Conflict of interest

Author AB was employed by Apis Flora Industrial e Comercial Ltda.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Almeida, R. N., Pachu, C., and Barbosa-Filho, J. (1995). Avaliação da possível atividade analgésica da iangambina obtida de ocotea duckei vatimo. *Cienc Cult Saúde* 14, 7–10.

Araújo, I. G. A., Silva, D. F., do Carmo de Alustau, M., Dias, K. L. G., Cavalcante, K. V. M., Veras, R. C., et al. (2014). Calcium influx inhibition is involved in the hypotensive and vasorelaxant effects induced by yangambin. *Molecules* 19, 6863–6876. doi: 10.3390/molecules19056863

Bala, M., Pratap, K., Verma, P. K., Singh, B., and Padwad, Y. (2015). Validation of ethnomedicinal potential of tinospora cordifolia for anticancer and immunomodulatory activities and quantification of bioactive molecules by HPTLC. *J. Ethnopharmacol* 175, 131–137. doi: 10.1016/j.jep.2015.08.001

Batista, R., de Jesus Silva Júnior, A., and de Oliveira, A. B. (2009). Plant-derived antimalarial agents: New leads and efficient phytomedicines. part II. non-alkaloidal natural products. *Molecules* 14, 3037–3072. doi: 10.3390/molecules14083037

Brasil, Ministério da Saúde (2017). *Manual De Vigilância Da Leishmaniose Tegumentar*. Brasília: Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância das Doenças Transmissíveis.

Castro-Faria-Neto, H. C., Araujo, C., Moreira, S., Bozza, P. T., Thomas, G., Barbosa-Filho, J. M., et al. (1995). Yangambin: A new naturally-occurring platelet-activating factor receptor antagonist: *In vivo* pharmacological studies. *Planta Med.* 61, 106–112. doi: 10.1055/s-2006-958026

Hausott, B., Greger, H., and Marian, B. (2003). Naturally occurring lignans efficiently induce apoptosis in colorectal tumor cells. *J. Cancer Res. Clin. Oncol.* 129, 569–576. doi: 10.1007/s00432-003-0461-7

Indrayanto, G., Putra, G. S., and Suhud, F. (2021). Validation of in-vitro bioassay methods: Application in herbal drug research. *Profiles Drug Subst. Excipients Relat. Methodol.* 46, 273–307. doi: 10.1016/bs.podrm.2020.07.005

Jesus-Morais, M., Assis, F., Cordeiro, S., Barbosa-Filho, M., Lima, T., Silva, L., et al. (2000). Yangambin, a lignan obtained fromOcoteaduckei, DifferentiatesPutative PAF receptor subtypes in the gastrointestina lTract of rats. *Planta Med.* 66, 211–216. doi: 10.1055/s-2000-8556.

Kesserwani, H. (2021). Death and rebirth of the thalidomide molecule: A case of thalidomide-induced sensory neuropathy. *Cureus* 13, 13140. doi: 10.7759/cureus.13140

Lima, E. B., Porto, C., da Motta, J. O. C., and Sampaio, R. N. R. (2007). Tratamento da leishmaniose tegumentar Americana *. *Bras. Dermatol.* 82, 111–124. doi: 10.1590/S0365-05962007000200002

Martins, J., Coelho, J., Tadini, M. C., de Souza, R. O., Figueiredo, S. A., Fonseca, M. J. V., et al. (2020). Yangambin and epi-yangambin isomers: New purification method from ocotea fasciculata and first cytotoxic aspects focusing on in vivo safety. *Planta Med.* 86, 415–424. doi: 10.1055/a-1118-3828

MINISTÉRIO DA SAÚDE/SVS/SINAM NET (2021) Leishmaniose tegumentar (LT)—Português (Brasil). Available at: https://www.gov.br/saude/pt-br/assuntos/saude-de-a-a-z/l/leishmaniose-tegumentar/leishmaniose-tegumentar [Accessed May 28, 2022].

Moro, A., and Invernizzi, N. (2017). A tragédia da talidomida: a luta pelos direitos das vítimas e por melhor regulação de medicamentos. *Hist Cienc Saude Manguinhos* 24, 603–622. doi: 10.1590/s0104-59702017000300004

Neto, R. L. M., Barbosa Filho, J. M., Sousa, L. M. A., Filho, F. A., Dias, C. S., Rcia, M., et al. (2007). Crude ethanolic extract, lignoid fraction and yangambin from ocotea duckei (Lauraceae) show antileishmanial activity. *Z. Naturforsch* 62, 348–352. doi: 10.1515/znc-2007-5-605.

Neto, R. L. M., Sousa, L. M. A., Dias, C. S., Barbosa Filho, J. M., Rcia, M., and Oliveira, R. (2008). Yangambin cytotoxicity: A pharmacologically active lignan obtained from ocotea duckei vattimo (Lauraceae). *Z. Naturforsch* 63, 681–686. doi: 10.1515/znc-2008-9-1012.

Neto, R. L. M., Sousa, L. M. A., Dias, C. S., Filho, J. M. B., Oliveira, M. R., and Figueiredo, R. C. B. Q. (2011). Morphological and physiological changes in leishmania promastigotes induced by yangambin, a lignan obtained from ocotea duckei. *Exp. Parasitol.* 127, 215–221. doi: 10.1016/j.exppara.2010.07.020

Penha, S. (2010). ESTUDO DA POTENCIALIDADE DA LIGNANA IANGAMBINA E DA QUITOSANA NO TRATAMENTO DA LEISHMANIOSE EXPERIMENTAL EM CAMUNDONGOS SUÍÇOS. *Programa Pôs-graduação em Prod. Nat. e Sintéticos Bioativos, Univ. Fed. da Paraíba*, 119.

Ribeiro, R., Carvalho, F. A. S., Barbosa-Filho, J. M., Cordeiro, R. S. B., and Tibiriçá, E. V. (1996). Protective effects of yangambin - a naturally occurring platelet-activating factor (PAF) receptor antagonist - on anaphylactic shock in rats. *Phytomedicine* 3, 249–256. doi: 10.1016/S0944-7113(96)80062-6

Rodrigues, F. M. D., Coelho Neto, G. T., Menezes, J. G. P. B., Gama, M. E. A., Gonçalves, E. G., Silva, A. R., et al. (2014). Expression of Foxp3, TGF-β and IL-10 in American cutaneous leishmaniasis lesions. *Arch. Dermatol. Res.* 306, 163–171. doi: 10.1007/s00403-013-1396-8

Serra, M. F., Diaz, B. L., Barreto, E., Paula, A., Pereira, B., Lima, M. C. R., et al. (1997). Anti-Allergic Properties of the Natural PAF Antagonist Yangambin. *Planta Med.* 63, 207–2012. doi: 10.1055/s-2006-957654.

Silveira, F. T., Lainson, R., and Corbett, C. E. P. (2004). Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil: a review. *Mem Inst Oswaldo Cruz* 99, 239–251. /S0074-02762004000300001

Silveira, F. T., Lainson, R., de Castro Gomes, C. M., Laurenti, M. D., and Corbett, C. E. P. (2009). Immunopathogenic competences of leishmania (V.) braziliensis and l. (L.) amazonensis in American cutaneous leishmaniasis. *Parasite Immunol.* 31, 423–431. doi: 10.1111/j.1365-3024.2009.01116.x

Tibirica, E. V., Mosquera, K., Abreu, M., Ribeiro, R., Carvalho, F. A., Barbosa-Filho, J. M., et al. (1996). Antagonistic effect of yangambin on platelet-activating factor (PAF)-induced cardiovascular collapse. *Phytomedicine* 2, 235–242. doi: 10.1016/S0944-7113(96)80048-1

WHO (2021) *Leishmaniasis*. Available at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (Accessed May 28, 2022).

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 13 January 2023
DOI 10.3389/fcimb.2022.1059168

OPEN ACCESS

EDITED BY
Suzana Passos Chaves,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Dirlei Nico,
Federal University of Rio de Janeiro, Brazil
Miria G. Pereira,
Federal University of Rio de Janeiro, Brazil

*CORRESPONDENCE
Silvia Amaral Gonçalves Da-Silva
silvasag@gmail.com

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 01 October 2022
ACCEPTED 12 December 2022
PUBLISHED 13 January 2023

CITATION
Santos RFd, Da Silva T, Brito ACdS, Inácio JD, Ventura BD, Mendes MAP, Azevedo BF, Siqueira LM, Almeida-Amaral EE, Dutra PML and Da-Silva SAG (2023) Therapeutic effect of oral quercetin in hamsters infected with *Leishmania Viannia braziliensis*. *Front. Cell. Infect. Microbiol.* 12:1059168. doi: 10.3389/fcimb.2022.1059168

Therapeutic effect of oral quercetin in hamsters infected with *Leishmania Viannia braziliensis*

Rosiane Freire dos Santos[1], Thayssa Da Silva[1], Andréia Carolinne de Souza Brito[1], Job Domingos Inácio[2], Bianca Domingues Ventura[1], Michely Aparecida Polido Mendes[1], Bruno Fonseca Azevedo[1], Larissa Moreira Siqueira[1], Elmo Eduardo Almeida-Amaral[2], Patrícia Maria Lourenço Dutra[3] and Silvia Amaral Gonçalves Da-Silva[1]*

[1]Laboratório de Imunofarmacologia Parasitária, Disciplina de Parasitologia/Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Laboratório de Bioquímica de Tripanossomatídeos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [3]Laboratório de Imunofisiologia do Exercício, Disciplina de Parasitologia/Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

Leishmaniasis is a parasitic disease caused by several species of intracellular protozoa of the genus *Leishmania* that present manifestations ranging from cutaneous ulcers to the fatal visceral form. *Leishmania Viannia braziliensis* is an important species associated with American tegumentary leishmaniasis and the main agent in Brazil, with variable sensitivity to available drugs. The search for new therapeutic alternatives to treat leishmaniasis is an urgent need, especially for endemic countries. Not only is quercetin well known for its antioxidant activity in radical scavenging but also several other biological effects are described, including anti-inflammatory, antimicrobial, and pro-oxidant activities. This study aimed to investigate the flavonoid quercetin's therapeutic potential in *L. (V.) braziliensis* infection. Quercetin showed antiamastigote (IC_{50} of 21 ± 2.5 μM) and antipromastigote (25 ± 0.7 μM) activities and a selectivity index of 22. The treatment of uninfected or *L. (V.) braziliensis*–infected macrophages with quercetin increased reactive oxygen species (ROS)/H_20_2 generation without altering Nitric Oxide (NO) production. Oral treatment with quercetin of infected hamsters, starting at 1 week of infection for 8 weeks, reduced the lesion thickness ($p > 0.01$) and parasite load ($p > 0.001$). The results of this study suggest that the antiamastigote activity of the flavonoid quercetin *in vitro* is associated, at least in part, with the modulation of ROS production by macrophages. The efficacy of oral quercetin treatment in hamsters infected with *L. (V.) braziliensis* was presented for the first time and shows its promising therapeutic potential.

KEYWORDS

quercetin, *Leishmania braziliensis*, hamsters, reactive species of oxygen, oral treatment

1 Introduction

Leishmaniasis is a worldwide parasitic disease caused by several species of intracellular protozoa of the genus *Leishmania* that present with manifestations ranging from skin ulcers to the fatal visceral form. This disease is endemic in approximately 100 countries and modern territories across Europe, Africa, Asia, and the Americas (World Health Organization, 2022). The drug options available for the treatment of the various clinical forms of leishmaniasis are limited, toxic, expensive, and even more critical due to the increasing resistance of the parasites (Amato et al., 2008; Ponte-Sucre et al., 2017; Mendes Roatt et al., 2020). Pentavalent antimonials (Pentostam™ and Glucantime™) are considered the first-choice treatments for leishmaniasis in most affected countries; however, this therapy is associated with a high incidence of adverse effects (Ponte-Sucre et al, 2017). In addition to pentavalent antimonials, other drugs used in the treatment of leishmaniasis as a second choice, such as amphotericin B and pentamidine, are also administered parenterally and cause serious adverse effects that limit and compromise adherence to treatment (Croft and Yardley, 2002; Pradhan et al., 2022). Miltefosine has the advantage of being an orally administered drug, but the sensitivity of *Leishmania* species is variable and its potential teratogenic effect restricts its use (Pradhan et al., 2022).

In addition to the differences in sensitivity naturally existing between the species of *Leishmania* to available drugs, there is also an increase in the reports of the emergence of resistance to these drugs (Ponte-Sucre et al., 2017; Uliana et al., 2018). *Leishmania* resistance mechanisms to conventional treatments involve a number of factors, such as molecular modifications of the parasite such as ATP-binding cassette (ABC) and aquaporin (AQP) transporters, changes in the lipid membrane, and oxidative stress (Ponte-Sucre et al., 2017; Horácio et al., 2021). *Leishmania Viannia braziliensis* is the main etiological agent of American tegumentary leishmaniasis (ATL) and associated with the cutaneous (CL) and mucocutaneous (ML) forms, including in Brazil, with frequent reports of refractoriness to treatment (Amato et al., 2008; Rugani et al., 2018; Anversa et al., 2018). In endemic regions of CL where *Leishmania (V.) braziliensis* is prevalent, the therapeutic failure rate is approximately 50% (Santos et al., 2004; de Prates et al., 2017)

This scenario demonstrates that the development of new drugs is indispensable to leishmaniasis control. The development of therapeutic alternatives for leishmaniasis that can be administered orally has been encouraged in order to facilitate logistics and improve patient adherence to treatment (CONITEC, 2018).

Quercetin is a polyphenolic flavonoid found in a wide variety of foods including citrus fruits, green leafy vegetables, and green tea. In addition to its well-documented antioxidant action (Behling et al., 2004; Gardi et al., 2015; Xu et al., 2019; Song et al., 2020), quercetin has pro-oxidative properties, depending on the used model cells, promoting cytotoxicity to malignant cell lines and embryonic stem cells and in injured neurons (Sak, 2014; Kim and Park, 2016; Bidian et al., 2020; Zubčić et al., 2020). Several therapeutic properties are described for quercetin such as antihypertensive (Marunaka et al., 2017; Elbarbry et al., 2020), anti-inflammatory (Lin et al., 2017; Sato and Mukai, 2020), antiallergic (Kahraman et al., 2003; Sakai-Kashiwabara et al., 2011; Mlcek et al., 2016; Jafarinia et al., 2020), antimicrobial (Giteru et al., 2015; Benjamin et al., 2017; Abbaszadeh et al., 2020), and antiviral activities, including SARS-CoV-2 (Colunga Biancatelli et al., 2020; Derosa et al., 2020). The *in vitro* antileishmanial action of quercetin was previously reported for *L. amazonensis* (Muzitano et al., 2006; Fonseca-Silva et al., 2013; Sousa-Batista et al., 2017) and *L. donovani* (Sen et al., 2008; Mehwish et al., 2021). The quercetin toxic effect on *L. amazonensis* was related to increased reactive oxygen species (ROS) production and mitochondrial dysfunction (Fonseca-Silva et al., 2011), and it is extended to the promastigotes of *L. (V.) braziliensis* (Cataneo et al., 2019). Quercetin also targets arginase in *L. amazonensis* (revised by Carter et al., 2021). However, the therapeutic activity of quercetin in animals infected with *L. (V). braziliensis* has not yet been demonstrated. In this study, our main objective was to evaluate the therapeutic potential of quercetin administered orally in hamsters infected with *L. (V.) braziliensis*.

2 Material and methods

2.1 Quercetin

The flavonoid quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) was commercially obtained (Sigma–Aldrich, St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich, St Louis, MO, USA). The final concentration of DMSO did not exceed 1% in the cell culture.

2.2 Parasites

L. (V.) braziliensis (MCAN/BR/98/R619) was routinely isolated from hamsters' skin lesions. The animals were infected on the dorsal hind paw with 5×10^6 promastigotes of *L. (V.) braziliensis* at the stationary phase. The infection was maintained for 30–40 days, and the skin of the lesion was surgically removed and homogenized with 1 ml of Phosphate-buffered saline (PBS) using a tissue grinder. The cell suspension was incubated with Schneider's medium (Sigma-Aldrich) containing 20% inactivated fetal bovine serum (FBS) at 27°C. Promastigotes were maintained with weekly passages in Schneider's medium with 20% FBS and 100 μg/ml gentamicin (Schering-Plough, Kenilworth, New Jersey, USA) at 27°C. Parasites were used for up to five passages in culture, at which time they were reisolated from infected hamsters.

2.3 Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation. This study protocol was approved by the Ethics Committee on Animal Use of the Instituto de Biologia Roberto Alcantara Gomes of the Universidade do Estado do Rio de Janeiro, by the number protocol 046/2017.

2.4 Animals

Female or male golden hamsters (*Mesocricetus auratus*) 6–8 weeks old were obtained from Centro de Criação de Animais de Laboratório (Fundação Oswaldo Cruz, Rio de Janeiro) and maintained under controlled temperature and food and water *ad libitum*.

2.5 Antipromastigote activity

The promastigotes of *L. (V.) braziliensis* (5×10^5 cells/well) were cultured in Schneider's medium supplemented with 20% FBS in the absence or presence of different quercetin concentrations in triplicate (5–320 μM) for 96 h at 27°C. The reference drug miltefosine was used as a positive control at 6 μM. The number of promastigotes was counted daily in a Neubauer chamber.

2.6 Macrophage toxicity

To assess the toxicity of quercetin on mammalian cells, resident macrophages were obtained from golden hamsters by peritoneal lavage with 10 ml of a cold RPMI 1640 medium. Cells were plated (4×10^6 in 200 μl) for 1 h at 37°C in the presence of 5% CO_2, and then, non-adherent cells were removed. Macrophage monolayers were treated in triplicate with quercetin (0–640 μM) for 48 h at 37°C/5% CO_2. Controls were macrophage monolayers treated with RPMI or 1% of vehicle DMSO (the major final concentration), and the positive control for reduced cellular viability (disrupted cells) was obtained by adding 1% Triton X-100. The viability of macrophages was then assessed by measuring the mitochondrial-dependent reduction of MTT [3-(4, 5-dimethyl- 2-thiazol)-2, 5-diphenyl-2H-tetrazolium bromide)] to formazan. MTT (10 μl to 10 mg/ml) was added to cell cultures and incubated at 37°C/5% CO_2 for 3 h. The medium was removed, and formazan crystals were dissolved in 180 μl of DMSO. The absorbance was read at 570 nm using a microplate spectrophotometer (μQuant, Biotek Instruments, Inc.). The 50% cytotoxic concentration (CC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 6 software.

2.7 Antiamastigote activity

The hamster peritoneal cells (2×10^6/ml), obtained as described in 2.6, were plated onto glass coverslips placed within the wells of a 24-well culture plate (0.5 ml/well) and incubated at 37°C/5% CO_2 for 24 h. After removing the non-adherent cells, the monolayers were infected with *L. (V.) braziliensis* promastigotes (5:1 ratio) for 4 h. The non-internalized parasites were removed, and the infected macrophage monolayers were incubated in triplicate with quercetin (0–320 μM) for 48 h. Controls were incubated with a medium or medium plus vehicle (DMSO 0.02%) or 3 μM miltefosine. After this time, the monolayers were stained with Giemsa, and at least 200 macrophages per sample were counted under optical microscopy. The results were shown as infection index (= % infected macrophages × number of amastigotes/total number of macrophages). The half-maximal inhibitory concentration (IC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 6 software.

2.8 Measurement of reactive oxygen species production by macrophage

Intracellular levels of ROS in uninfected macrophages or *L. (V.) braziliensis*–infected macrophages were performed using the cell-permeable dye H2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate). The monolayers of peritoneal macrophages were obtained as described in item 2.6 and plated in a 96-well plate (at 2×10^6/well) and infected with *L. (V.) braziliensis* promastigotes (5:1 ratio) for 4 h. Uninfected or *L. (V.) braziliensis*–infected macrophages were treated with 160 or 320 μM of quercetin for 48 h at 37°C/5% CO_2. The macrophage monolayers were washed twice with PBS and incubated with 20 mM of H2DCFDA for 30 min at 37°C. Fluorescence was measured in a fluorometer with an excitation wavelength of 507 nm and an emission wave of 530 nm. The positive control was obtained by the addition of 20 units/ml glucose oxidase + 60 mM glucose for 20 min. To evaluate hydrogen peroxide production, the Amplex Red probe (Invitrogen Molecular Probes, Leiden, the Netherlands) was used, following the manufacturer's recommendations. After quercetin treatment was completed, the wells were washed twice with PBS, the plate was incubated for 30 min with Amplex Red, and the reading was done on a fluorometer with an excitation wavelength of 560 nm and an emission wave of 590 nm. The results were expressed as folds relative to the control (macrophages treated with medium). Each test was performed in triplicate and repeated at least three times.

2.9 Evaluation of nitric oxide production

Nitric oxide was measured by detecting nitrite using the Griess reagent. After the respective treatments for 48 h, the supernatants of

macrophage monolayers were transferred to the plate where the Griess reagent [1% sulfanilamide added to 0.1% of N-1-naphthylethylenediamine dihydrochloride (Sigma-Aldrich) and 2.5% of phosphoric acid (Sigma-Aldrich)] was added at a ratio of 1:1 (v/v) and incubated for 10 min, at room temperature. Then, the plate was read in an ELISA reader, at 570 nm. The values of the readings were compared with a standard curve of $NaNO_2$ (Sigma-Aldrich).

2.10 Effect of treatment on infected hamsters

Female or male golden hamsters (8 weeks old) were infected on the dorsal hind paw with 5×10^6 promastigotes of *L. (V.) braziliensis* at the stationary phase. The animals were divided into groups (six-to-eight hamsters per group) 7 days after infection and treated for 8 weeks. The quercetin group (n = 8) was orally treated once a day for five consecutive days a week (2-day treatment-free interval between weeks) with 500 µl of quercetin (20 mg/kg) in an Ora-Plus suspension vehicle (Perrigo®, Paddock Laboratories, Minnesota, USA), using an 18G gavage needle (Kent Scientific, Torrington, Connecticut, USA). Control groups were constituted by the untreated group (n = 8) and by the group treated with the reference drug meglumine antimoniate (Glucantime, 80 mg/kg) (n = 6) intraperitoneally (100 µl) three times a week (every other day). The dose of 80 mg/kg of Glucantime was selected based on the range used in previously published studies (Sinagra et al., 2007; Costa et al., 2014; Kawakami et al., 2021) and which proved to be effective in our experimental model. The lesion thickness was measured weekly with a dial caliper (Mitutoyo, Brazil). The animals were euthanized by anesthetic overdose (association of 240 mg/kg of ketamine and 30 mg/kg of xylazine, corresponding to three times the usual anesthetic dose) followed by cardiac puncture for blood collection. Hepatotoxicity and nephrotoxicity were evaluated by the serum dosage of aspartate transaminase (AST), alanine transaminase (ALT), and creatinine, which was performed by the Animal Clinical Analysis Center of the Institute of Science and Technology in Biomodels (Fiocruz RJ) using the Vitros 250 equipment (Orthopedic Clinic—Johnson & Johnson). To determine the parasite load, limiting dilution analysis was used (Costa et al., 2014). The skin of the dorsal infected hind paw and draining lymph node were surgically removed, weighed, and individually homogenized with 1 ml of PBS using a tissue grinder. The cell suspension was serially diluted in quadruplicate (1:10) in Schneider's medium containing 20% FBS and 100 µg/ml gentamicin at 27°C. The presence of motile parasites was assessed, and the parasite load was determined by the highest dilution in which promastigotes grew after 7–10 days.

2.11 Statistical analyses

The data were analyzed by applying one-way analysis of variance with Tukey *post-test* using the GraphPad Prism 6 software program (San Diego, CA, USA). The difference between groups was considered significant when $p \leq 0.05$.

3 Results

3.1 *In vitro* activity of quercetin on *L. (V.) braziliensis* promastigotes and intracellular amastigotes

For the *in vitro* evaluation of quercetin activity against *L. (V.) braziliensis*, we performed analyses on the promastigotes and intracellular amastigotes. Promastigotes were cultured with quercetin (0–320 µM) or miltefosine (reference drug) for 96 h, and their growth was evaluated by daily counting. Quercetin was able to significantly reduce the growth of promastigotes in a dose-dependent manner (Figure 1), and the IC_{50} was estimated at 25 ± 0.68 µM (96 h). The activity against intracellular amastigotes was evaluated using macrophage monolayers infected with *L. (V.) braziliensis* and treated with quercetin (0–320 µM) or miltefosine (IC_{50}) for 48 h. In a dose-dependent manner, quercetin was able to significantly decrease the number of amastigotes in macrophages, with an IC_{50} estimated at 21 ± 2.5 µM (Figure 2A). As expected, the reference drug miltefosine, used at the concentration relative to the IC_{50}, was able to reduce the number of intracellular amastigotes.

The toxicity of quercetin to hamster macrophages was evaluated after 48 h of treatment. Cells showed a significant loss of viability only from 640 µM onward, with an estimated CC_{50} of 478 ± 89 µM (Figure 2B). The selectivity index (CC_{50}/IC_{50}) of quercetin was calculated to be 22, meaning that it is 22 times more toxic to intracellular amastigotes of *L.(V.) braziliensis* than to hamster macrophages.

3.2 Quercetin induces ROS without altering NO production by macrophages

To investigate whether quercetin anti-amastigote activity was associated with the ability to modulate host cells, we evaluated ROS and NO production by infected and uninfected macrophages. After 48 h of treatment with quercetin at 160 or 320 µM, intracellular ROS was evaluated by the H2DCFDA probe and NO was analyzed in the culture supernatants by the Griess method. In Figure 3A, we show a significant increase in ROS production by the infected macrophages treated with quercetin in both concentrations and in uninfected macrophages at the highest concentration of quercetin tested (320 µM). Using the Amplex Red® probe, it was possible to observe that treatment with quercetin significantly increased the production of H_2O_2 in infected and uninfected macrophages (Figure 3B). In uninfected macrophages, the increase in H_2O_2 was more than 70- and 100-fold with treatment at 160 and 320 µM of quercetin, respectively. In relation to NO, we observed no change in nitrite production in either *L. (V.)*

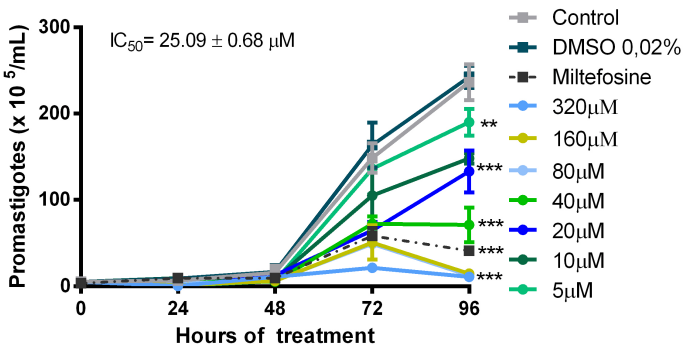


FIGURE 1
Activity of quercetin on the promastigote of *L. (V.) brazilliensis*. Promastigotes were cultivated in Schneider's medium supplemented with 20% fetal bovine serum (FBS) at 27°C for 96 h, in the absence or presence of quercetin (indicated concentrations). The number of parasites was determined daily by counting in a Neubauer chamber. Controls were promastigotes cultured with a vehicle [0.02% dimethylsulfoxide (DMSO)] or 6 µM miltefosine as a reference drug. The data presented are representative of three independent experiments performed in triplicate. Mean ± SD, n = 3. **p < 0.01, ***p < 0.001 (difference compared to DMSO or medium).

braziliensis–infected or –uninfected macrophages treated with quercetin for 48 h (Figure 3C).

3.3 Therapeutic activity of quercetin in hamsters infected with *L. (V.) braziliensis*

Using the susceptible experimental model for infection with *L. (V.) braziliensis*, the golden hamster, we evaluated the action of orally administered quercetin (20 mg/kg). Treatments with oral quercetin (five times a week) or intraperitoneal Glucantime (three times a week) were started from 7 days of infection (lesion average thickness = 0. 26 mm) and maintained for 8 weeks (9 weeks of infection). Quercetin treatment was able to significantly control lesion thickness from the fourth week of treatment (Figure 4) when compared to the untreated group. As expected, the reference drug Glucantime significantly controlled the lesion development. At the ninth week of infection, the lesions of the untreated animals were ulcerated, besides being smaller than untreated group, no ulceration was observed in the lesions of the animals treated with quercetin (Figure 4B). The results showed that the treatment with quercetin reduced significantly parasitic load in both the lesion (Figure 5A) and the draining lymph node (Figure 5B). Treatment with quercetin for 8 weeks did not alter the levels of renal (creatinine) and hepatic (ALT and AST) toxicity parameters, which were similar when compared to untreated animals (Figure 6).

4 Discussion

The treatment of CL continues to be a challenge, and there is an urgent need to discover new efficient and safe active drugs, particularly for local or oral use, that increase patient compliance. In the present study, for the first time, the therapeutic effect of the oral flavonoid quercetin was demonstrated in hamsters infected with *L. (V.) braziliensis*, the main species causing ATL, especially in Brazil.

In vitro, we showed that quercetin has activity against both promastigote (IC_{50} = 25 ± 0.7 µM/96 h) and intracellular-amastigote (IC_{50} = 21 ± 2.5 µM/48h) forms of *L. (V.) braziliensis*. Some studies have already reported *in vitro* the activity of quercetin for some species of Cataneo and collaborators (2021) showed which quercetin at 48 and 70 µM reduced the number of intracellular amastigotes of *L. (V.) braziliensis* within 24 h of treatment; however, they did not determine the IC_{50}. In another study, quercetin reduced the promastigote growth of *L. (V.) braziliensis* and *L. (V.) panamensis*, with IC_{50} estimated at 30 and 60 µM (72 h), respectively (Marín et al., 2009). Quercetin showed activity on *L. amazonensis* and IC_{50} determined at 3.4 µM (72 h) for intracellular amastigotes (Fonseca-Silva et al., 2013) and 31.4 µM (48 h) for promastigotes (Fonseca-Silva et al., 2011). Mehwish and collaborators (2021) demonstrated that quercetin has antiamastigote activity against *L. donovani* with an IC_{50} of 240 µM (72 h). The differences found in the IC_{50} of quercetin may reflect the variations in the sensitivity of each *Leishmania* species, as well as the experimental protocol, especially the duration of treatment. Although we have not investigated the antiparasitic mechanisms of quercetin directly in the parasite, it is possible that quercetin induces the generation of ROS and disrupts the parasite's mitochondria as seen for *L. amazonensis* (Fonseca-Silva et al., 2011).

In our results, the quercetin CC_{50} for hamster macrophages was estimated at 478 ± 89 µM in 48 h of treatment. This result differs slightly from the findings of other studies with different

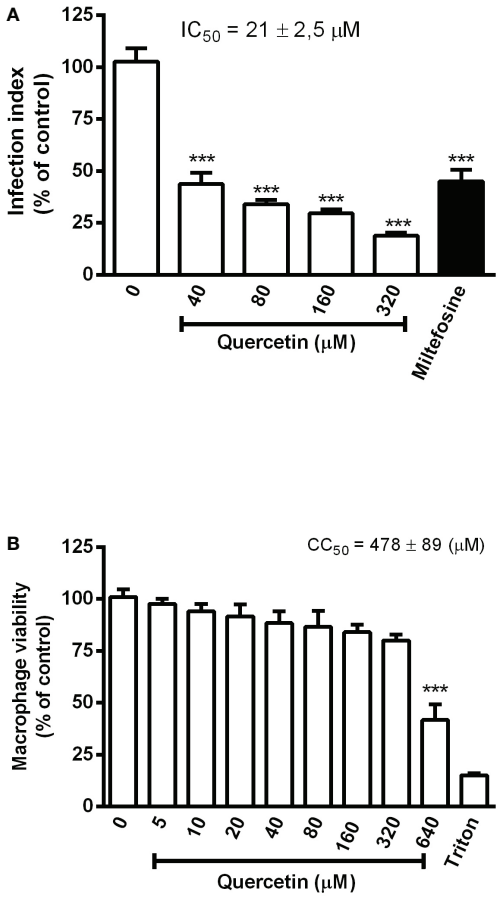


FIGURE 2
Effect of quercetin on the intracellular amastigotes of *L. (V.) braziliensis* and macrophage toxicity. **(A)** Monolayers of hamster peritoneal macrophages infected with *L. (V.) braziliensis* (5:1 ratio) were treated with the indicated concentrations of quercetin for 48 h. Controls were treated with a 0.02% DMSO vehicle or 3 μM miltefosine. After treatment, macrophage monolayers were stained with Giemsa and the infection index was established by counting at least 200 cells on each coverslip in triplicate. **(B)** Hamster peritoneal macrophage monolayers were incubated in triplicate with quercetin for 48 h, and cell viability was measured using the 3-(4, 5-dimethyl- 2-thiazol)-2, 5-diphenyl-2H-tetrazolium bromide) assay. Controls were vehicle (DMSO) or 0.1% Triton X-100 as a positive toxicity control for reduced cell viability. Values presented represent the mean ± SD of three independent experiments and are expressed as a percentage of control. ***$p < 0.001$ (difference compared to DMSO).

cell types and treatment times. For example, studies performed in J774 macrophages treated for 72 h with quercetin had the CC_{50} estimated at 125 μM (Marín et al., 2009), while, for mice peritoneal macrophages, the IC_{50} was 80 μM (Fonseca-Silva et al., 2013).

In order to determine whether the antiamastigote activity of quercetin would involve the modulation of host cell microbicidal activity, we evaluated the macrophage production of ROS and NO. In our results, we observed that quercetin did not induce changes in NO production; however, it increased ROS by both infected and uninfected macrophages. These results suggest, at least in part, which activity of quercetin against *L. (V.) braziliensis* involves the stimulation of ROS production by macrophages. Our findings are in agreement with studies on macrophages infected with *L. amazonensis* that demonstrated that quercetin induces an increase in ROS production by infected macrophages (Fonseca-Silva et al., 2013). In macrophages infected with *L. (V.) braziliensis*, quercetin reduced the number of amastigotes without modulating NO production, although it reduced alpha tumor necrosis factor-alfa (TNF-α) levels and increased IL-10 synthesis (Cataneo et al., 2019). The authors showed that treatment with quercetin was able to decrease labile iron in macrophages through the regulation of Nrf2/HO-1 expression, resulting in a decrease in the iron available to the parasite and consequently inducing its death (Cataneo et al., 2019).

10.3389/fcimb.2022.1059168

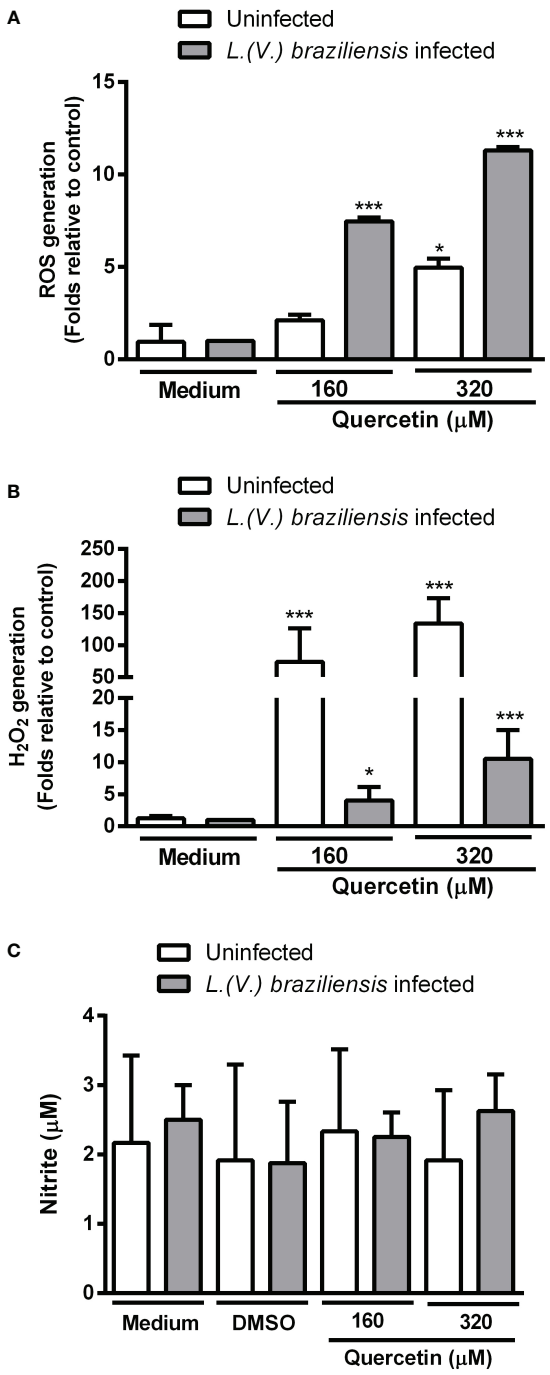


FIGURE 3
Production of toxic radicals by macrophages. Monolayers of peritoneal macrophages were infected or not with *L. (V.) braziliensis* and incubated in the presence or absence of quercetin for 48 h. **(A)** Reactive oxygen species (ROS) generation was measured using the fluorescent probe 2´, 7´-dichlorodihydrofluorescein diacetate, **(B)** H_2O_2 was measured by the *Amplexred* probe. Data were expressed as a fold increase in ROS production relative to control. **(C)** Nitric oxide production was evaluated by the Griess method and the results expressed as nitrite concentration. The values shown represent the mean ± SD of three independent experiments. *p < 0.05, ***p <0.001 (difference compared to DMSO or medium).

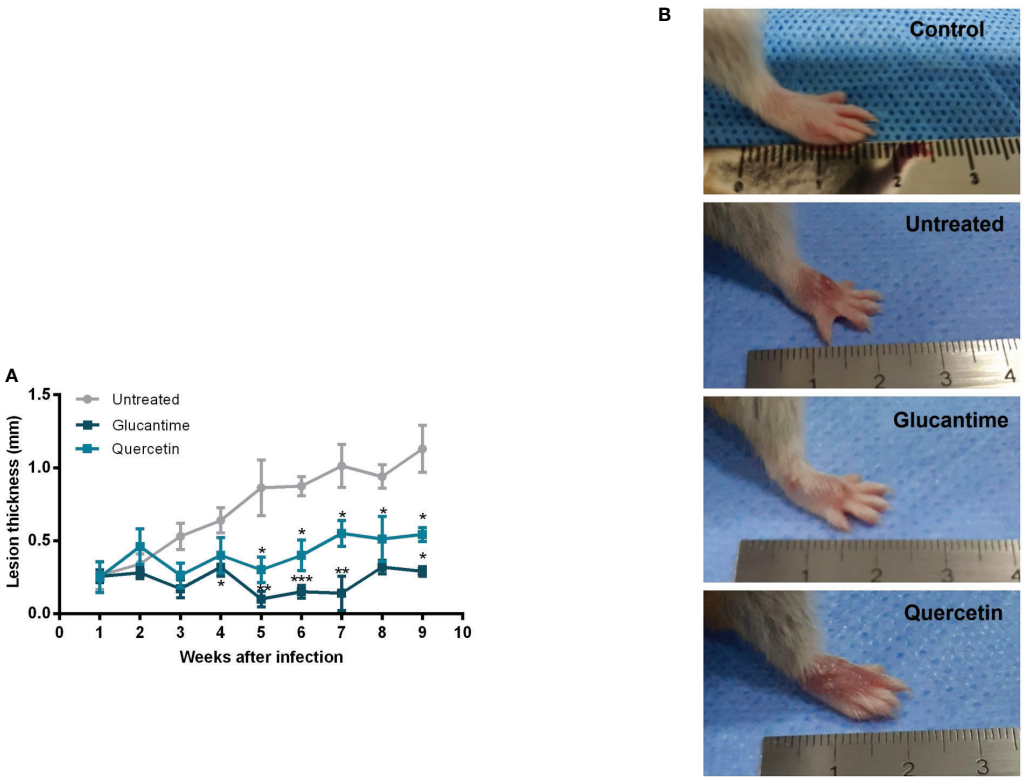


FIGURE 4
Therapeutic effect of quercetin by the oral route on hamster infected with *L. (V.) braziliensis*. Hamsters (six-to-eight animals per group) were infected in the dorsal hindpaw with 5×10^6 promastigotes of *L. (V.) braziliensis* and treated from the seventh day of infection for eight weeks with oral quercetin (20 mg/kg; five times a week) or glucantime (80 mg/kg; three times a week) intraperitoneally. **(A)** The thickness of the lesions was measured weekly and expressed as mean + standard error. **(B)** Representative images of the lesion of the animals of each experimental group and of an uninfected animal for reference. These results are representative of two independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 (difference compared to the untreated group).

Although the production of toxic radicals by macrophages (such as ROS and NO) is crucial for the control of infection by intracellular microorganisms, such as *Leishmania*, the excess of these mediators is also associated with tissue damage and pathogenesis (revised by Bogdan, 2020; Reverte et al., 2022) Several studies have demonstrated the anti-inflammatory effects of quercetin involving the inhibition of nitric oxide production as well as the production of proinflammatory cytokines (Kim et al., 2004; Tsai et al., 2022). In this sense, quercetin appears to be an interesting drug candidate for leishmaniasis since it has both direct antileishmanial activity and the potential to modulate the microbicidal and inflammatory activity of macrophages.

In the present study, we demonstrated the therapeutic effect of oral quercetin in hamsters infected with *L.(V.) braziliensis*. The hamster model is sensitive to infection by *L.(V.) braziliensis*, developing a pattern of cutaneous leishmaniasis that resembles the human disease; therefore, it is useful for therapeutic and vaccine trials (Gomes-Silva et al., 2013; Loría-Cervera and Andrade-Narváez, 2014; Ribeiro-romão et al., 2014, Mears et al., 2015).

We showed that the treatment with oral quercetin of hamsters infected with *L.(V.) braziliensis* from 7 days of infection, when the lesion was at the beginning (0.26 mm), significantly controlled the lesion size, as well as reduced the parasite load both in the lesion and in the draining lymph node. Despite the *in vitro* demonstration of the antileishmanial action of quercetin for several species of the parasite, there are relatively few studies conducted *in vivo* to investigate its therapeutic activity in experimental leishmaniasis. However, our *in vivo* findings demonstrating the potential of quercetin in experimental cutaneous leishmaniasis corroborate previous studies in both cutaneous and visceral leishmaniasis models. Quercetin administered orally (16 mg/kg of body weight) from 7

10.3389/fcimb.2022.1059168

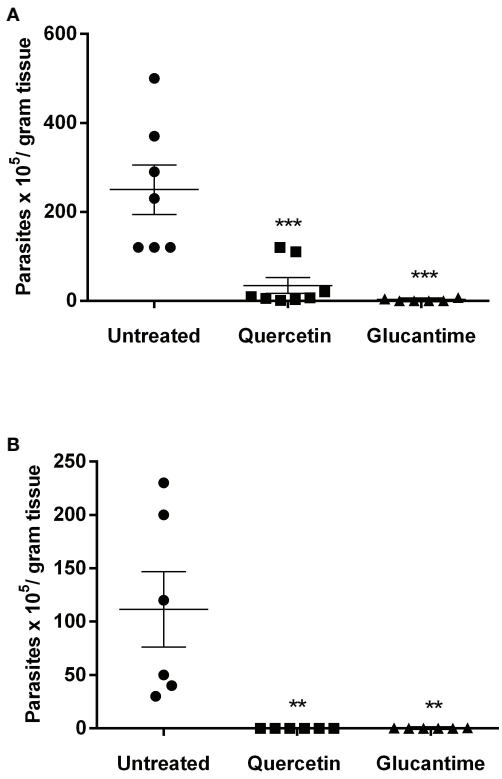


FIGURE 5
Parasitic load in the lesion and draining lymph nodes. The animals were euthanized, and the parasitic load was determined by the limiting dilution assay in the paw lesion **(A)** and the draining lymph nodes **(B)** 1 week after the end of treatment (9 weeks of infection). Each point represents one animal, and the horizontal bars express the mean values. The data are representative of two independent experiments. **p < 0.01, and ***p < 0.001 (difference compared to the untreated group).

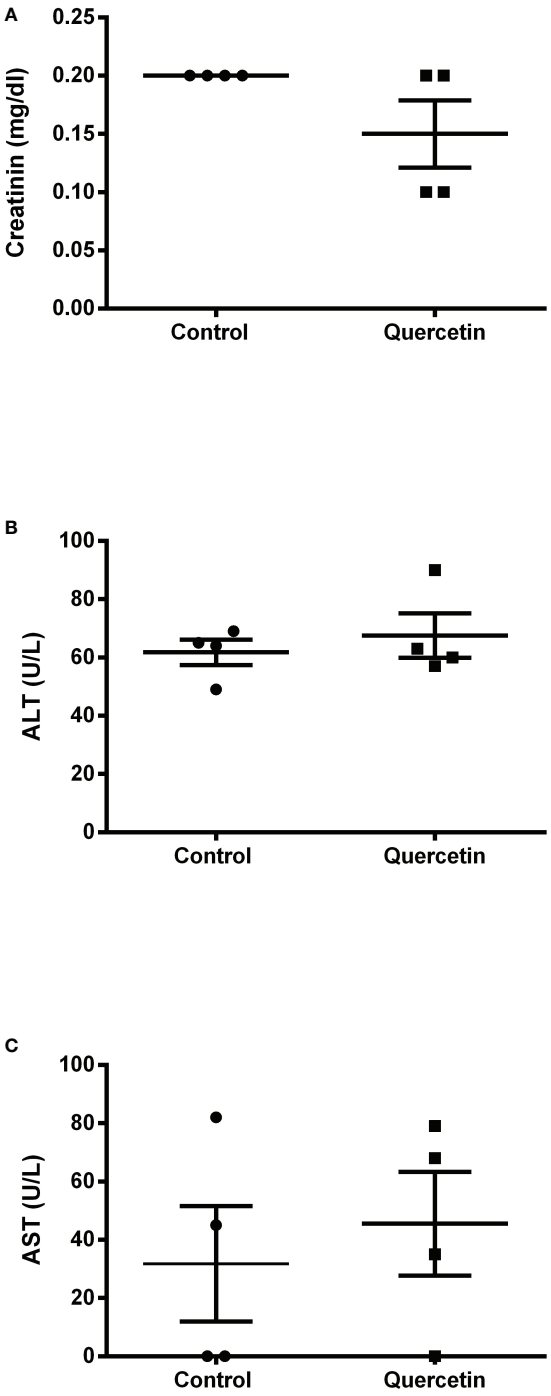


FIGURE 6
Serum toxicological analysis. At the end of treatment, serum samples were collected from the animals (n = 4) for the colorimetric determination of creatinine **(A)**, alanine transaminase **(B)** and aspartate transaminase **(C)** concentrations, as toxicity parameters for the liver and kidneys. Each point represents one animal, and the horizontal bars express the mean values.

days of infection for 30 days controlled the lesion and reduced the parasite load in BALB/c mice infected with *L. amazonensis* Muzitano et al., 2009). In a study conducted in hamsters infected with *L. donovani*, quercetin administered orally reduced the number of parasites in the spleen. Another study demonstrated that the encapsulation of quercetin in the lipid nanocapsules (LNCs) of poly(ϵ-caprolactone) was able to increase its efficacy in the treatment of BALB/c mice infected by *L. amazonensis* (Sousa-Batista et al., 2017).

In conclusion, the present study demonstrated for the first time the effect of quercetin in hamsters infected with *L. (V.) braziliensis*. Furthermore, the antileishmanial activity of quercetin may be associated with not only a direct action against the parasite but also the modulation of ROS production by macrophages.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethics Committee for the Care and Use of Experimental Animals-Instituto de Biologia Roberto Alcantara Gomes- Universidade do Estado do Rio de Janeiro.

Author contributions

RFS conducted experiments and contributed to the writing of the manuscript. TS, ACSB, JDI, BDV, MAPM, BFA, LMS contributed the experiments. EEAA and JDI contributed to the execution and the discussion of ROS analysis. PMLD contributed to data discussion and manuscript writing. SAGS supervised the experiments, analyzed the results and wrote the manuscript.

Funding

This study received funding from Brazilian Research Agencies: CAPES (Coordination for the Improvement of Higher Education Personnel), CNPq (National Council for Scientific and Technological Development) and FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro) through scholarships and financial support for the project (Proc. numbers E-26/010.001531/2014 and E-26/010.001815/2015).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abbaszadeh, S., Rashidipour, M., Khosravi, P., Shahryarhesami, S., Ashrafi, B., Kaviani, M., et al. (2020). Biocompatibility, cytotoxicity, antimicrobial and epigenetic effects of novel chitosan-based quercetin nanohydrogel in human cancer cells. *Int. J. Nanomed* 15, 5963–5975. doi: 10.2147/IJN.S263013

Amato, V. S., Tuon, F. F., Bacha, H. A., Neto, V. A., and Nicodemo, A. C. (2008). Mucosal leishmaniasis. current scenario and prospects for treatment. *Acta Trop.* 105, 1–9. doi: 10.1016/j.actatropica.2007.08.003

Anversa, L., Tiburcio, M. G. S., Richini-Pereira, V., and Ramirez, L. E. (2018). Human leishmaniasis in Brazil: A general review. *Rev. Assoc. Med. Bras.* 64, 281–289. doi: 10.1590/1806-9282.64.03.281

Behling, E. B., Sendão, M. C., Francescato, H. D. C., Antunes, L. M. G., and Bianchi, M. D. L. P. (2004). Flavonóide Quercetina : Aspectos gerais e. *Alimentos e Nutrição - Braz. J. Food Nutr.* 15, 285–292.

Benjamin, M., Rall, M.J.S., and Davis, Glen F. (2017). Activity of quercetin and kaemferol against streptococcus mutans biofilm yan. *Physiol. Behav.* 176, 139–148. doi: 10.1016/j.physbeh.2017.03.040

Bidian, C., Mitrea, D. R., Vasile, O. G., Filip, A., Cătoi, A. F., Moldovan, R., et al. (2020). Quercetin and curcumin effects in experimental pleural inflammation. *Med. Pharm. Rep.* 93 (3), 260–266. doi: 10.15386/mpr-1484

Bogdan, C. (2020). Macrophages as host, effector and immunoregulatory cells in leishmaniasis: Impact of tissue micro-environment and metabolism. *Cytokine X* 2, 100041. doi: 10.1016/j.cytox.2020.100041

Carter, N. S., Stamper, B. D., Elbarbry, F., Nguyen, V., Lopez, S., Kawasaki, Y., et al. (2021). Microorganisms natural products that target the arginase in leishmania parasites. *Hold Ther. Promise.* 9 (2), 267. doi: 10.3390/microorganisms902

Cataneo, A. H. D., Tomiotto-Pellissier, F., Miranda-Sapla, M. M., Assolini, J. P., Panis, C., Kian, D., et al. (2019). Quercetin promotes antipromastigote effect by increasing the ROS production and anti-amastigote by upregulating Nrf2/HO-1 expression, affecting iron availability. *Biomed Pharmacother* 113, 108745. doi: 10.1016/j.biopha.2019.108745

Colunga Biancatelli, R. M. L., Berrill, M., Catravas, J. D., and Marik, P. E. (2020). Quercetin and vitamin c: An experimental, synergistic therapy for the prevention and treatment of SARS-CoV-2 related disease (COVID-19). *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.01451

CONITEC (2018) *Miltefosina para o tratamento da leishmaniose tegumentar*. Available at: http://conitec.gov.br.

Costa, L., Pinheiro, R. O., Dutra, P. M. L., Santos, R. F., Cunha-Júnior, E. F., Torres-Santos, E. C., et al. (2014). Pterocarpanquinone LQB-118 induces apoptosis in leishmania (Viannia) braziliensis and controls lesions in infected hamsters. *PloS One* 9, 1–8. doi: 10.1371/journal.pone.0109672

Croft, S. L., and Yardley, V. (2002). Chemotherapy of leishmaniasis. *Current Pharmaceutic Design* 8, 319-342. doi: 10.2174/1381612023396258

de Prates, F. V. O., Dourado, M. E. F., Silva, S. C., Schriefer, A., Guimarães, L. H., Brito, M. D. G. O., et al. (2017). Fluconazole in the treatment of cutaneous leishmaniasis caused by leishmania braziliensis: A randomized controlled trial. *Clin. Infect. Dis.* 64, 67–71. doi: 10.1093/cid/ciw662

Derosa, G., Maffioli, P., D'Angelo, A., and Di Pierro, F. (2020). A role for quercetin in coronavirus disease 2019 (COVID-19). *Phytother Res.* 1–7, 1230–1236. doi: 10.1002/ptr.6887

Elbarbry, F., Abdelkawy, K., Moshirian, N., and Abdel-Megied, A. M. (2020). The antihypertensive effect of quercetin in young spontaneously hypertensive rats; role of arachidonic acid metabolism. *Int. J. Mol. Sci.* 21, 1–12. doi: 10.3390/ijms21186554

Fonseca-Silva, F., Inacio, J. D. F., Canto-Cavalheiro, M. M., and Almeida-Amaral, ,. E. E. (2011). Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in leishmania amazonensis. *PloS One* 6, 1–7. doi: 10.1371/journal.pone.0014666

Fonseca-Silva, F., Inacio, J. D. F., Canto-Cavalheiro, M. M., and Almeida-Amaral, E. E. (2013). Reactive oxygen species production by quercetin causes the death of *Leishmania amazonensis* intracellular amastigotes. *J. Nat. Prod.* 76, 1505–1508. doi: 10.1021/np400193m

Gardi, C., Bauerova, K., Stringa, B., Kuncirova, V., Slovak, L., Ponist, S., et al. (2015). Quercetin reduced inflammation and increased antioxidant defense in rat adjuvant arthritis. *Arch. Biochem. Biophys.* 583, 150–157. doi: 10.1016/j.abb.2015.08.008

Giteru, S. G., Coorey, R., Bertolatti, D., Watkin, E., Johnson, S., and Fang, Z. (2015). Physicochemical and antimicrobial properties of citral and quercetin incorporated kafirin-based bioactive films. *Food Chem.* 168, 341–347. doi: 10.1016/j.foodchem.2014.07.077

Gomes-Silva, A., Valverde, J. G., Ribeiro-Romão, R. P., Plácido-Pereira, R. M., and Da-Cruz, A. M. (2013). Golden hamster (Mesocricetus auratus) as an experimental model for leishmania (Viannia) braziliensis infection. *Parasitology* 140, 771–779. doi: 10.1017/S0031182012002156

Horácio, E. C. A., Hickson, J., Murta, S. M. F., Ruiz, J. C., and Nahum, L. A. (2021). Perspectives from systems biology to improve knowledge of leishmania drug resistance. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.653670

Jafarinia, M., Sadat Hosseini, M., Kasiri, N., Fazel, N., Fathi, F., Ganjalikhani Hakemi, M., et al. (2020). Quercetin with the potential effect on allergic diseases. *Allergy Asthma Clin. Immunol.* 16, 1–11. doi: 10.1186/s13223-020-00434-0

Kahraman, A., Köken, T., Aktepe, F., Erkasap, N., Erkasap, S., and Serteser, M. (2003). The antioxidative and antihistaminic properties of quercetin in ethanol-induced gastric lesions. *Toxicology* 183, 133–142. doi: 10.1016/s0300-483x(02)00514-0

Kawakami, M. Y. M., Zamora, L. O., Araújo, R. S., Fernandes, C. P., Ricotta, T. Q. N., de Oliveira, L. G., et al. (2021). Efficacy of nanoemulsion with pterodon emarginatus Vogel oleoresin for topical treatment of cutaneous leishmaniasis. *Biomed Pharmacother* 134, 1–12. doi: 10.1016/j.biopha.2020.111109

Kim, Y., and Park, W. (2016). Anti-inflammatory effect of quercetin on RAW 264.7 mouse macrophages induced with polyinosinic-polycytidylic acid. *Molecules* 21, 450. doi: 10.3390/molecules21040450

Kim, H. P., Son, K. H., Chang, H. W., and Kang, S. S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.* 96, 229–245. doi: 10.1254/jphs.CRJ04003X

Lin, X., Lin, C.-H., Zhao, T., Zuo, D., Ye, Z., Liu, L., et al. (2017). Quercetin protects against heat stroke-induced myocardial injury in male rats: Antioxidative and antiinflammatory mechanisms. *Chem. Biol. Interact.* 265, 47–54. doi: 10.1016/j.cbi.2017.01.006

Loría-Cervera, E. N., and Andrade-Narváez, F. J. (2014). Animal models for the study of leishmaniasis immunology. *Rev. Inst Med. Trop. Sao Paulo* 56, 1–11. doi: 10.1590/S0036-46652014000100001

Marín, C., Boutaleb-Charki, S., Díaz, J. G., Huertas, O., Rosales, M. J., Pérez-Cordon, G., et al. (2009). Antileishmaniasis activity of flavonoids from consolida oliveriana. *J. Natural Product* 72 (6), 1069–1074. doi: 10.1021/np8008122

Marunaka, Y., Marunaka, R., Sun, H., Yamamoto, T., Kanamura, N., Inui, T., et al. (2017). Actions of quercetin, a polyphenol, on blood pressure. *Molecules* 22, 209. doi: 10.3390/molecules22020209

Mears, E. R., Modabber, F., Don, R., and Johnson, G. E. (2015). A review: The current *in vivo* models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous leishmaniasis. *PloS Negl. Trop. Dis.* 9 (9), e0003889. doi: 10.1371/journal.pntd.0003889

Mehwish, S., Varikuti, S., Khan, M. A., Khan, T., Khan, I. U., Satoskar, A., et al. (2021). Bioflavonoid-induced apoptosis and dna damage in amastigotes and promastigotes of leishmania donovani: Deciphering the mode of action. *Molecules* 26, 1–16. doi: 10.3390/molecules26195843

Mendes Roatt, B., Mirelle De Oliveira Cardoso, J., Cristiane, R., de Brito, F., Coura-Vital, W., Dian, R., et al. (2020). MINI-REVIEW recent advances and new strategies on leishmaniasis treatment. *Appl. Microbiol. Biotechnol.* 104, 8965–8977. doi: 10.1007/s00253-020-10856-w/Published

Mlcek, J., Jurikova, T., Skrovankova, S., and Sochor, J. (2016). Quercetin and its anti-allergic immune response. *Molecules* 21, 1–15. doi: 10.3390/molecules21050623

Muzitano, M. F., Cruz, E., de Almeida, A. P., da Silva, S., Kaiser, C. R., Guette, C., et al. (2006). Quercitrin: An antileishmanial flavonoid glycoside from kalanchoe pinnata. *Planta Med.* 72, 81–83. doi: 10.1055/s-2005-873183

Muzitano, M. F., Falcão, C. A. B., Cruz, E. A., Bergonzi, M. C., Bilia, A. R., Vincieri, F. F., et al. (2009). Oral metabolism and efficacy of kalanchoe pinnata flavonoids in a murine model of cutaneous leishmaniasis. *Planta Med.* 75 (4) 307–311. doi: 10.1055/s-0028-1088382

Ponte-Sucre, A., Gamarro, F., Dujardin, J. C., Barrett, M. P., López-Vélez, R., García-Hernández, R., et al. (2017). Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PloS Negl. Trop. Dis.* 11, 1–24. doi: 10.1371/journal.pntd.0006052

Pradhan, S., Schwartz, R. A., Patil, A., Grabbe, S., and Goldust, M. (2022). Treatment options for leishmaniasis. *Clin. Exp. Dermatol.* 47, 516–521. doi: 10.1111/ced.14919

Reverte, M., Snäkä, T., and Fasel, N. (2022). The dangerous liaisons in the oxidative stress response to leishmania infection. *Pathogens* 11, 1–17. doi: 10.3390/pathogens11040409

Ribeiro-romão, R. P., Moreira, O. C., Osorio, Y., Cysne-finkelstein, L., Gomes-silva, A., Valverde, J. G., et al. (2014). Comparative evaluation of lesion development , tissue damage , and cytokine expression in golden hamsters (mesocricetus auratus) infected by inocula with different leishmania (viannia) braziliensis concentrations. *Parasites & Vectors* 82, 5203–5213. doi: 10.1128/IAI.02083-14

Rugani, J. N., Quaresma, P. F., Gontijo, C. F., Soares, R. P., and Monte-Neto, R. L. (2018). Intraspecies susceptibility of leishmania (Viannia) braziliensis to antileishmanial drugs: Antimony resistance in human isolates from atypical lesions. *Biomed Pharmacother* 108, 1170–1180. doi: 10.1016/j.biopha.2018.09.149

Sak, K. (2014). Site-specific anticancer effects of dietary flavonoid quercetin. *Nutr. Cancer* 66, 177–193. doi: 10.1080/01635581.2014.864418

Sakai-Kashiwabara, M., Abe, S., and Asano, K. (2011). Suppressive activity of quercetin on the production of eosinophil chemoattractants from eosinophils *In vitro*. *Arzneimittelforschung* 54, 436–443. doi: 10.1055/s-0031-1296996

Santos, J. B., Ribeiro De Jesus, L., Machado, P. R., Salgado, T., Carvalho, E. M., and Almeida, R. P. (2004). Antimony plus recombinant human granulocyte-macrophage colony-stimulating factor applied topically in low doses enhances healing of cutaneous leishmaniasis ulcers: A randomized, double-blind, placebo-controlled study. *J. Infect. Dis.* 190, 1793–1796. doi: 10.1086/424848

Sato, S., and Mukai, Y. (2020). Modulation of chronic inflammation by quercetin: The beneficial effects on obesity. *J. Inflamm. Res.* 13, 421–431. doi: 10.2147/JIR.S228361

Sen, G., Mukhopadhyay, S., Ray, M., and Biswas, T. (2008). Quercetin interferes with iron metabolism in leishmania donovani and targets ribonucleotide reductase to exert leishmanicidal activity. *J. Antimicrobial Chemother* 61, 1066–1075. doi: 10.1093/jac/dkn053

Sinagra, Àngel, Luna, C., Abraham, D., Iannela, M. d. C., Riarte, A., and Krolewieck, J.A. (2007). The activity of azithromycin against leishmania (Viannia) braziliensis and leishmania (Leishmania) amazonensis in the golden hamster model. *Revista da Sociedade Brasileira de Medicina Tropical* 40 (6), 627-630. doi: 10.1590/S0037-86822007000600005

Song, X., Wang, Y., and Gao, L. (2020). Mechanism of antioxidant properties of quercetin and quercetin-DNA complex. *J. Mol. Model.* 26, 1–8. doi: 10.1007/s00894-020-04356-x

Sousa-Batista, A. J., Poletto, F. S., Philipon, C. I. M. S., Guterres, S. S., Pohlmann, A. R., and Rossi-Bergmann, B. (2017). Lipid-core nanocapsules increase the oral efficacy of quercetin in cutaneous leishmaniasis. *Parasitology* 144, 1769–1774. doi: 10.1017/S003118201700097X

Tsai, C. F., Chen, G. W., Chen, Y. C., Shen, C. K., Lu, D. Y., Yang, L. Y., et al. (2022). Regulatory effects of quercetin on M1/M2 macrophage polarization and oxidative/antioxidative balance. *Nutrients* 14, 1–21. doi: 10.3390/nu14010067

Uliana, S. R. B., Trinconi, C. T., and Coelho, A. C. (2018). Chemotherapy of leishmaniasis: Present challenges. *Parasitology* 145, 464–480. doi: 10.1017/S0031182016002523

World Health Organization (2022). Fact sheets. leishmaniasis.

Xu, D., Hu, M. J., Wang, Y. Q., and Cui, Y. L. (2019). Antioxidant activities of quercetin and its complexes for medicinal application. *Molecules* 24, 1–15. doi: 10.3390/molecules24061123

Zubčić, K., Radovanović, V., Vlainić, J., Hof, P. R., Oršolić, N., Šimić, G., et al. (2020). PI3K/Akt and ERK1/2 signalling are involved in quercetin-mediated neuroprotection against copper-induced injury. *Oxid. Med. Cell Longev* 2020, 1–14. doi: 10.1155/2020/9834742

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 19 January 2023
DOI 10.3389/fcimb.2023.1025359

OPEN ACCESS

EDITED BY
Juliana Da Silva Pacheco,
University of Dundee, United Kingdom

REVIEWED BY
Rubens Monte Neto,
Fundação Oswaldo Cruz, Brazil
Iraj Sharifi,
Kerman University of Medical Sciences, Iran

*CORRESPONDENCE
Silvia Amaral Gonçalves Da-Silva
silvasag@gmail.com

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 22 August 2022
ACCEPTED 02 January 2023
PUBLISHED 19 January 2023

CITATION
Meira RdMV, Gomes SLdS, Schaeffer E, Da Silva T, Brito ACdS, Siqueira LM, Inácio JD, Almeida-Amaral EE, Da-Cruz AM, Bezerra-Paiva M, Neves RH, Rodrigues LS, Dutra PML, Costa PRR, da Silva AJM and Da-Silva SAG (2023) Low doses of 3-phenyl-lawsone or meglumine antimoniate delivery by tattooing route are successful in reducing parasite load in cutaneous lesions of *Leishmania* (*Viannia*) *braziliensis*-infected hamsters. *Front. Cell. Infect. Microbiol.* 13:1025359. doi: 10.3389/fcimb.2023.1025359

Low doses of 3-phenyl-lawsone or meglumine antimoniate delivery by tattooing route are successful in reducing parasite load in cutaneous lesions of *Leishmania* (*Viannia*) *braziliensis*-infected hamsters

Rafaella de Miranda Villarim Meira[1], Sara Lins da Silva Gomes[2], Edgar Schaeffer[2], Thayssa Da Silva[1], Andréia Carolinne de Souza Brito[1], Larissa Moreira Siqueira[1], Job Domingos Inácio[3], Elmo Eduardo Almeida-Amaral[3], Alda Maria Da-Cruz[4,5], Milla Bezerra-Paiva[5], Renata Heisler Neves[6], Luciana Silva Rodrigues[7], Patricia Maria Lourenço Dutra[8], Paulo Roberto Ribeiro Costa[9], Alcides José Monteiro da Silva[2] and Silvia Amaral Gonçalves Da-Silva[1]*

[1]Laboratório de Imunofarmacologia Parasitária, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Laboratório de Catálise Orgânica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [3]Laboratório de Bioquímica de Tripanosomatídeos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [4]Disciplina de Parasitologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [5]Laboratório Interdisciplinar de Pesquisas Médicas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [6]Laboratório de Helmintologia Romero Lascasas Porto, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [7]Laboratório de Imunopatologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [8]Laboratório de Imunologia do Exercício, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, [9]Laboratório de Química Bioorgânica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Current therapeutic ways adopted for the treatment of leishmaniasis are toxic and expensive including parasite resistance is a growing problem. Given this scenario, it is urgent to explore treatment alternatives for leishmaniasis. The aim of this study was to evaluate the effect of 3-phenyl-lawsone (3-PL) naphthoquinone on *Leishmania* (*Viannia*) *braziliensis* infection, both *in vitro* and *in vivo*, using two local routes of administration: subcutaneous (higher dose) and tattoo (lower dose). *In vitro* 3-PL showed low toxicity for macrophages (CC_{50} >3200 μM/48h) and activity against intracellular amastigotes (IC_{50} = 193 ± 19 μM/48h) and promastigotes (IC_{50} = 116 ± 26 μM/72h), in which induced increased ROS generation. Additionally, 3-PL up-regulated the production of cytokines such as tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein 1 (MCP-1), interleukin-6 (IL-6) and IL-10 in infected macrophages. However, the anti-amastigote action was independent of nitric oxide production. Treatment of hamsters infected with *L.* (*V.*) *braziliensis* from one week after infection with 3-PL by subcutaneous (25 μg/Kg) or tattooing (2.5 μg/Kg) route, during 3 weeks

(3 times/week) or 2 weeks (2 times/week) significantly decreased the parasite load ($p<0.001$) in the lesion. The reduction of parasite load by 3-PL treatment was comparable to reference drug meglumine antimoniate administered by the same routes (subcutaneous 1mg/Kg and tattoo 0.1mg/Kg). In addition, treatment started from five weeks after infection with 3-PL per tattoo also decreased the parasite load. These results show the anti-leishmanial effect of 3-PL against *L.* (*V.*) *braziliensis* and its efficacy by subcutaneous (higher dose) and tattoo (lower dose) routes. In addition, this study shows that drug delivery by tattooing the lesion allows the use of lower doses than the conventional subcutaneous route, which may support the development of a new therapeutic strategy that can be adopted for leishmaniasis.

KEYWORDS

***Leishmania (Viannia) braziliensis*, 3-phenyl-lawsone, chemotherapy, tattooing, subcutaneous, hamster-model**

1 Introduction

American Tegumentary Leishmaniasis (ATL) is the most common clinical form of leishmaniasis ranging from self-healing wounds to severe mucosal tissue damage of the infected individual. Of the total cases of ATL in 2020, 42% were reported by Brazil (Pan American Health Organization, 2021). The available therapies for various forms of leishmaniasis are based on systemic administration of antimonial pentavalent or amphotericin B and pentamidine presenting extensive toxicity, high costs, emerging drug resistance and varied efficacy depending upon the species, symptoms and geographical region (Berman, 2005; Santos et al., 2008; Pimentel et al., 2011; Ponte-Sucre et al., 2017). These limitations encourage the search for new therapeutic alternatives to treat leishmaniasis (Mudavath et al., 2014; Zulfiqar et al., 2017). In Brazil, ATL is caused mostly by species *Leishmania* (*Viannia*) *braziliensis* (Cupolillo et al., 2003; David and Craft, 2009; Ministério da Saúde, 2017) which cause both cutaneous (CL) and mucosal leishmaniasis (ML). Most studies related to this species are performed in humans since mice develop small, self-healing lesions that have limited application. In this sense, the hamster model demonstrates a more appropriate alternative, because when infected with *L.* (*V.*) *braziliensis* it mimics human CL (Gomes-Silva et al., 2013; Ribeiro-Romão et al., 2014; Dutra and da Silva, 2017) and proved to be a suitable model to evaluate antileishmania vaccines (da Silva-Couto et al., 2015) and drugs (Costa et al., 2014). In general, the drug of first choice for treating *L.* (*V.*) *braziliensis* infection is meglumine antimoniate (Glucantime®). However, this therapy has several limitations, as high parenteral doses are required, inducing moderate to severe side effects that lead to discontinuation of treatment and contribute to increased parasite resistance (Carvalho et al., 2019). Therefore, the development of new drugs and alternative drug delivery is necessary for the treatment of CL (Zulfiqar et al., 2017; Caridha et al., 2019). Intralesional (IL) pentavalent antimonials have been used for decades for the treatment of CL in the Old World (World Health Organization, 2022). However, in America, this use was recommended in 2013 for patients with single lesions, nursing mothers, and contraindications to systemic treatment (nephropathies, hepatopathies, cardiopathies). It is also contraindicated for lesions larger than 3 cm in diameter, or those located in the head or periarticular areas, and for immunosuppressed patients (Pan American Health Organization, 2013). More recently, Brazil adhered to this recommendation to treat lesions, up to 3 cm in the greatest diameter, at any location except the head and periarticular regions (Ministério da Saúde, 2017).

The use of a modified tattoo device for medical purposes has been utilized for various applications, such as in dermatological treatments (Sadeghinia and Sadeghinia, 2012; Arbache et al., 2018), implantation of glucose monitoring detectors (Yetisen et al., 2019), an indication of tumor location for surgery (Yigit et al., 2022) and DNA vaccines (Oosterhuis et al., 2012; Fotoran et al., 2020). New transdermal drug delivery methods, such as needle-free injectors (NFIs), microneedles, and tattoo devices have been developed and may have advantages over some traditional delivery methods, including the use of low doses and coverage of large areas of skin (Arbache et al., 2019; Mercuri and Rivas, 2021). Shio and collaborators (2014) used a tattoo device to administer a liposomal formulation of oleylphosphocholine to mice infected with *L.* (*L.*) *major* or *L.* (*L.*) *mexicana* and showed the efficacy of this approach.

Naphthoquinones are natural molecules with high biological activity and pharmacological potential due to its redox cycle, which promotes the production of reactive oxygen species (Ferreira et al., 2010; Qiu et al., 2018). There is a variety of natural and synthetic naphthoquinones with antimalarial, antihelminthic, anti-*Trypanosoma* and anti-*Leishmania* activities (Reimão et al., 2012; Hazra et al., 2013; Rezende et al., 2013; Rocha et al., 2013; Lorsuwannarat et al., 2014; Oliveira et al., 2018). Lapachol is a natural naphthoquinone isolated from several plants of the Bignoniaceae family, mainly *Tecoma* and *Tabebuia* species. Several studies demonstrate lapachol as a pharmacological agent (Hussain et al., 2007; Hussain and Green, 2017), with antitumor (Sunassee et al., 2013; Zu et al., 2019), antimicrobial (Oliveira et al., 2010; Souza et al., 2013) and antiparasitic activities (Teixeira et al., 2001; Lima et al., 2004; Salas et al., 2011). Therefore, the study of synthetic

lapachol derivates and analogues has been proposed as potential antiparasitic drugs (Lima et al., 2004; Rocha et al., 2013; Barbosa et al., 2014). Lawsone, a 2-hydroxy-1,4-naphthoquinone originally obtained from the henna plant (*Lawsonia inermis*), as well as derivatives molecules, have diverse biological properties such as antitumor, antimicrobial, and antiparasitic action (reviewed by Pradhan et al., 2012; al Nasr et al., 2019).

The aim of this study was to evaluate the activity of the synthetic lapachol derivative 3-phenyl-lawsone (3-PL) against *L.* (*V.*) *braziliensis in vitro*, as well as its therapeutic potential using local routes of administration subcutaneous (higher dose) and tattooing (lower dose) compared with the reference drug meglumine antimoniate, in experimentally infected hamsters.

2 Materials and methods

2.1 3-phenyl-lawsone

The lapachol derivative 3-phenyl-lawsone, 3-PL, (Figure 1A, insert) was synthesized in the Laboratory of Bioorganic Chemistry of the Federal University of Rio de Janeiro, Brazil by the Suzuki-Miyaura reaction as previously described (Gomes et al., 2017). For assays, the 3-PL was dissolved in DMSO (Sigma Aldrich, St Louis, MO, USA) whose final concentration did not exceed 1%.

2.2 Parasites culture

Leishmania (*Viannia*) *braziliensis* (MCAN/BR/98/R619) parasites were routinely isolated from hamster lesions and maintained as promastigotes in Schneider's insect medium (Sigma-Aldrich, St Louis, MO, USA) containing 20% heat-inactivated fetal bovine serum (FBS) (Cultilab, Brazil) and 10 µg/mL gentamicin (Schering-Plough, Kenilworth, New Jersey, USA). The medium was changed weekly and parasites were used until no more than six passages.

2.3 Activity on intracellular amastigotes

Peritoneal macrophages from BALB/c mice obtained as described above, were plated ($2x10^6$ cells/well) onto glass coverslips placed within the wells of a 24-well culture plate and infected with stationary phase promastigotes of *L.* (*V.*) *braziliensis* at a multiplicity of infection (MOI) of 5:1, in 5% atmosphere of CO_2 at 37°C, for 4h. The infected monolayers were washed to remove non-internalized parasites and incubated with RPMI 1640 medium supplemented with 10% of FBS in 5% atmosphere of CO_2 at 37°C, for 24h, to assure the differentiation into amastigotes forms. Then the infected macrophages were treated for 48h with 3-PL (0-800 µM), in 5% atmosphere of CO_2 at 37°C. The control cells were macrophages incubated with medium or vehicle (DMSO 0.2%) or reference drug miltefosine at 3µM (IC_{50}). Controls and 3-PL concentrations were performed in triplicate and the experiment was repeated at least three times. Then, the supernatants were collected and stored at -20°C for further measurement of nitric oxide or cytokine production. The monolayers were then dyed with a fast panoptic stain (Laborclin, Brazil) and amastigotes were quantified in, at least, 200 macrophages per sample. The results were expressed as an infection index (= % infected cells X number of amastigotes/total number of macrophages). The 50% inhibitory concentration (IC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 6.

To assess the viability of the remaining amastigotes after 48h of treatment, we investigated the ability of these amastigote to differentiate into promastigotes. Infected macrophage monolayers were washed twice with PBS (heated to 37°C) and incubated with Schneider's medium and 20% FBS at 28°C for an additional 48h, when promastigotes were counted in a Neubauer chamber.

2.4 Cytotoxicity assay to macrophages

The toxicity of 3-PL on mammalian cells was evaluated in peritoneal macrophages from BALB/c mice (6-8 weeks old) by MTT assay (Mosmann, 1983). Resident macrophages were obtained from peritoneal cells of BALB/c mice after intraperitoneal injection of 5 mL of cold RPMI 1640 medium without FBS (Cultilab, Brazil). The peritoneal cells of the mice (4-8 x 10^6 cells/animal) were pooled and plated in a 96-well culture plate ($8x10^5$ cells/well) and after 1h, non-adherent cells were removed. Monolayers of peritoneal macrophages were cultured in triplicate with RPMI 1640 medium supplemented with 10% of FBS and 3-PL over a wide concentration range (0-3200 µM) at 37°C in 5% atmosphere of CO_2 for 48h. MTT (5 mg/mL) was added (20 µL/well) and the plates were incubated for 3h. The supernatants were removed, formazan crystals were dissolved in DMSO and absorbance was determined in a spectrophotometer at 550 nm using a microplate reader (Epoch - Biotek). The cytotoxic concentration 50% (CC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 6.

2.5 Nitric oxide production assay

Macrophage production of nitric oxide was measured by nitrite detection using the Griess method (Green et al., 1982). Supernatants from macrophage monolayers were transferred to the plate where the Griess reagent [1% sulfanilamide, added to 0.1% of NEED and 2.5% of phosphoric acid (all purchased from Sigma-Aldrich, St. Louis, MO) was added at a ratio of 1:1 (v/v) and incubated for 10 min, at room temperature. Then, the plate was read in an ELISA reader, at 570 nm. Subsequently, reading values were compared to a standard curve of sodium nitrite ($NaNO_2$), and results were expressed in µM of nitrite.

2.6 Cytokine production

The evaluation of the production of cytokines in the macrophage supernatant was performed by the Cytometric Bead Array (CBA) method using the Mouse Inflammation kit (BD Bioscience), following the manufacturer's recommendations. Briefly, undiluted samples

10.3389/fcimb.2023.1025359

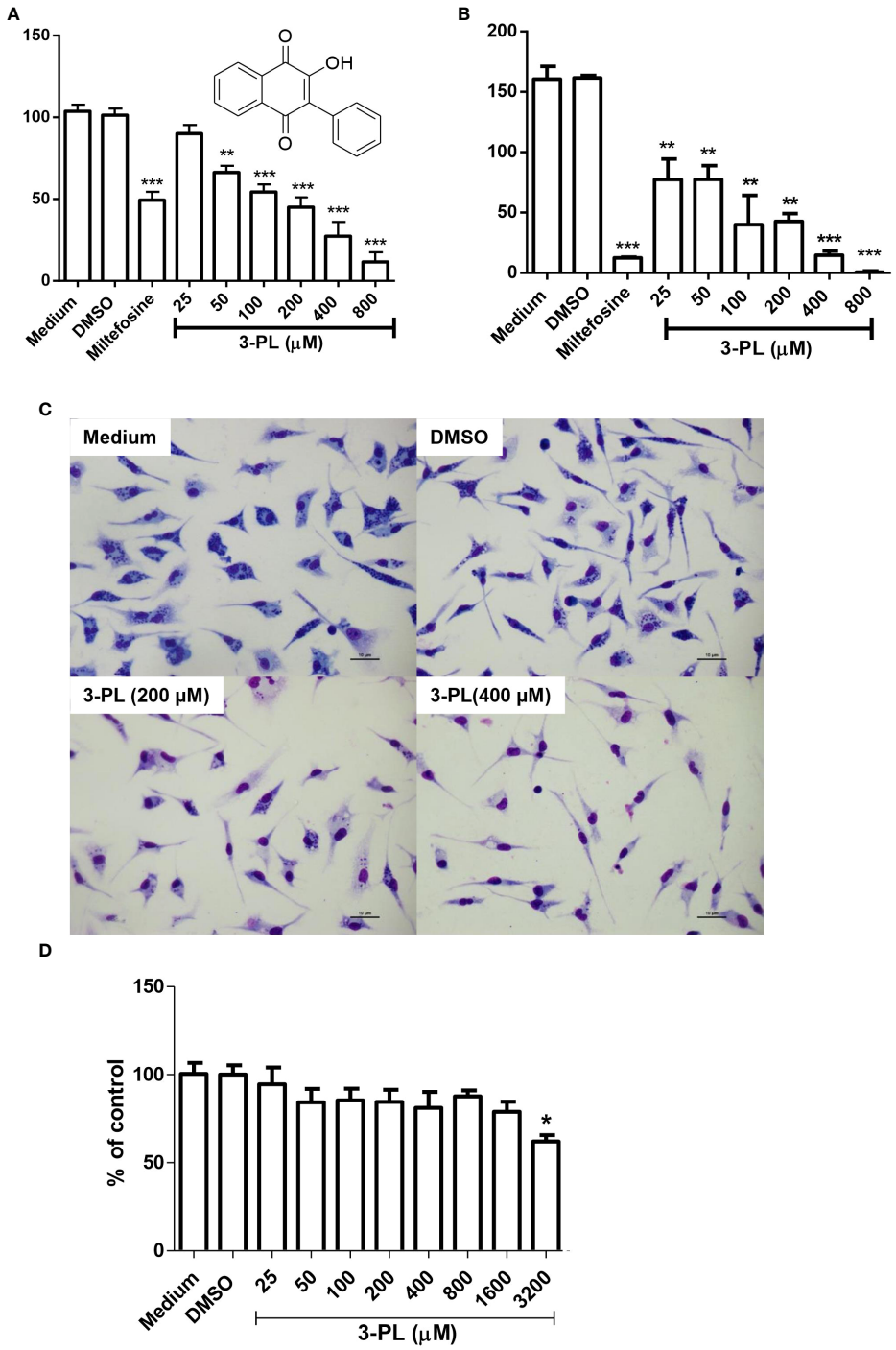


FIGURE 1
In vitro activity of 3-PL against *L. (Viannia) braziliensis* amastigotes and macrophage toxicity. **(A-C)** Murine macrophage monolayers were infected with stationary-phase promastigote of *L.* (*V.*) *braziliensis* in a ratio of 5:1 (parasites/macrophage). Infected macrophages were incubated in an atmosphere of 5% CO2 at 37°C, with RPMI 1640 medium supplemented with 10% FBS for 24h to differentiate amastigotes. Afterwards, the infected monolayers were submitted to treatment with indicated concentrations of 3-PL or 3 μM miltefosine for 48h. **(A)** The infection index was determined by counting at least 200 macrophages and expressed as % of control or **(B)** the treated infected macrophages were washed twice and incubated with Schneider's medium plus 20% FBS at 28°C for more than 48h and promastigotes were counted. **(C)** Representative light microscopy images of infected macrophage monolayers after 48h of treatment. **(D)** Toxicity on peritoneal murine macrophages treated during 48h at 5% CO_2/37°C. Results (A,B and D) were presented as means ± SD; n=3. *p < 0.05, **p < 0.01 and ***p < 0.001 in relation to control DMSO.

were incubated with capture beads labeled with distinct fluorescence intensity conjugated with cytokine-specific antibodies for about 3h in the dark at room temperature, followed by fluorescent detection antibody, and all unbound antibodies were washed away. Data were acquired on a BD fluorescence-activated cell sorting (FACS) FACSCanto II analyzer and results were analyzed using the FCAP Array Software program. Cytokine standard curves ranged from 0-5000 pg/mL.

2.7 Activity on promastigotes growth

Promastigotes forms of *L.* (*V.*) *braziliensis* were plated in a 24-well culture plate (2.5 x 10^5 cells/well) and incubated for 96h at 28°C with 3-PL (0-800 µM) diluted in Schneider's medium supplemented with 20% of FBS. Controls were promastigotes non-treated or treated with 0.2% DMSO (higher final concentration in this assay) or miltefosine. Controls and 3-PL concentrations were performed in triplicate and the experiment was repeated at least three times. The number of parasites was counted daily with a Neubauer chamber using an optic microscope.

2.8 Determination of ROS generation

The evaluation of ROS production was performed on promastigotes of *L.* (*V.*) *braziliensis* treated for 72h with 3-PL (100 and 50 µM, corresponding to IC_{50} and half of the IC_{50}), in 5% atmosphere of CO_2 at 37°C. The controls were incubated with culture medium or 0.025% vehicle DMSO (corresponding to higher final concentration in this assay). After treatment, promastigotes were washed twice in HBSS buffer, adjusted to the concentration of 2x10^6/mL and incubated in a dark 96-well plate (2 x 10^5/well) for 20 minutes, with 20 µM of the H2DCFDA probe (2'7'-dichlorodihydrofluorescein diacetate), which in the presence of ROS suffers deacetylation being converted to DCFDA (2'7'-dichlorofluorescein), which accumulated inside the cell was captured by fluorescence. The reading was made in a fluorimeter (Spectra Max M2 - Molecular Devices, Silicon Valley, USA) with wavelengths of 485 nm excitation and emission 530 nm. As a positive control, 2 mM H_2O_2 was used. Data was expressed as increased ROS production relative to control (medium).

2.9 Therapeutic activity on infected golden hamsters

The antiparasitic effect of 3-PL by local routes (subcutaneous or tattoo) were evaluated using the golden hamsters (*Mesocricetus auratus*) which is a model highly susceptible to infection (Gomes-Silva et al., 2013; Mears et al., 2015). The golden hamsters with 6 to 8 weeks old were infected with 10^6 promastigotes of *L.* (*V.*) *braziliensis* at stationary phase on the dorsal hind paw, and randomly divided into groups (4-6 animals per group). The treatments started 7 days after the infection and were applied to the infection site by subcutaneous or tattooing routes using two protocols.

Protocol I: Treatments were performed three times a week for three weeks. At the end of the treatment, the animals remained under observation for another three weeks (without treatment), after which they were euthanized. The animals were divided in 4 groups, as follows: group 3-PL sc (n = 6) treated with 3-PL (25 µg/Kg) given subcutaneously; group 3-PL tattoo (n = 6) treated with 3-PL per tattoo (± 2.5 µg/Kg, ranging from a minimum of 1.5 and a maximum of 2.5 µg/Kg); group DMSO sc (n = 6) consisted of animals treated with DMSO vehicle (0.05% in PBS) subcutaneously and group DMSO tattoo (n = 6) treated with DMSO vehicle (0.05% in PBS) per tattoo.

Protocol II: Treatments were performed with two administrations per week for two weeks. At the end of treatment, the animals remained under observation (without treatment) for another week, when they were euthanized. The animals were divided in 6 groups, as follows: group 3-PL sc (n = 4) treated with 3-PL (25 µg/Kg) given subcutaneously; group 3-PL tattoo (n = 4) treated with 3-PL per tattoo (± 2.5 µg/Kg, ranging from a minimum of 1.5 and a maximum of 2.5 µg/Kg); group DMSO sc (n = 4) treated with vehicle DMSO (0.05% in PBS) subcutaneously; group DMSO tattoo (n=4) treated with vehicle DMSO (0.05% in PBS) by tattoo; group Glucantime® sc (n=4) treated subcutaneously with the reference drug meglumine antimoniate (1 mg/Kg of Glucantime®, Sanofi-Avenetis Farmaceutica Ltda) and group Glucantime® tattoo (n=4) treated by tattoo (± 0.1 mg/Kg, ranging from a minimum of 0.06 and a maximum of 0.1 mg/Kg, of Glucantime®).

In addition, we evaluated the potential of lowest dose 3-PL treatment for tattooing initiated in more chronic stages of infection.

Protocol III: Hamsters were treated from 5 weeks of infection (lesion approximately 2 mm thickness) with 3-PL tattoo (n=3) twice a week for 2 weeks. Control group were DMSO tattoo animals (n=3). At the end of treatment, the animals remained under observation (without treatment) for another 2 weeks, when they were euthanized and parasite load evaluated.

Subcutaneous treatment was performed with 50 µL of the solution using a microsyringe with a 29G needle. Tattoo administration was performed using a professional commercial tattooing machine (White Head, Wujiang Kangtai Medical Instrument, China) and a magnum needle type (5 needs head/0.3 mm thickness each), following the protocol adapted from Shio and collaborators (2014). Each tattooing session consisted of twelve administrations lasting two seconds each, with the five needles oscillating at 60 Hz (60 perforations/second), with a total of 7200 punctures (5x12x2x60). The twelve applications of each session were distributed at the lesion site and we estimate that 3-5 µL of solution were injected per session. Both subcutaneous or tattooing administration were performed under anesthesia using 80 mg/kg Ketamine (Syntec, Brazil) plus 10 mg/kg Xylazine (Syntec, Brazil) by intraperitoneal route.

The lesion thickness were measured weekly with a dial caliper (Mitutoyo, Brazil) and expressed as the difference between the thickness of the infected and uninfected paws. At the end of the experiments, the animals were euthanized and the tissue from the lesions was aseptically removed, ground, and transferred to tubes containing Schneider's medium plus 20% fetal calf serum. The parasite load was evaluated by limiting dilution assay (Costa et al., 2014). The cell suspension was serially diluted in a 96-well plate, and the parasites were evaluated using limiting dilution analysis after 10 days of culture at 27°C.

2.10 Histophatological analysis

Skin fragments were fixed in 10% buffered formalin and processed for paraffin embedding Sections of 3 µm thickness were stained with hematoxylin-eosin (H&E) and observed under light microscopy (Nikon Eclipse 80i, Tokyo, Japan) and images captured in Nikon DS-Ri7 and edited by NIS-Elements AR 3.2 program. The inflammatory parameters were expressed by score, according to Yang and collaborators (2013), from a semi-quantitative analysis

according to the intensity of occurrence of each histological parameter. The score was defined as: not observed (score=0); slight observed (score=1); moderately observed (score=2) and full observed (score=3).

2.11 Statistical analysis

Each *in vitro* experiment was performed in triplicate in at least two independent experiments and the statistical analysis was based on Student's t-test. The statistical analysis of the *in vivo* experiments was performed using analysis of variance (ANOVA) and the Tukey *post hoc* test by software GraphPad Prism 6. Differences with a p-value <0.05 were considered as statistically significant. IC_{50} and CC_{50} values were calculated by non-linear regression.

3 Results

3.1 Anti-amastigote activity of 3-PL and toxicity to mammalian cells

In order to test the antileishmanial the *in vitro* effect of 3-PL against *L.* (*V.*) *braziliensis*, we utilized the intracellular amastigote form of the parasite. Treatment of infected macrophages with 3-PL for 48h showed a significant dose-dependent reduction of the infection index (Figures 1A, B). The IC_{50} values were estimated at 193 ± 19 μM. To evaluate the survival of the amastigotes remaining after 3-PL treatment, we investigated the ability of these amastigotes to differentiate into promastigotes. The monolayers of infected and treated macrophages were washed and reincubated with Schneider's medium plus 20% fetal bovine serum at 28°C for another 48h, and the promastigotes were then counted (Figure 1B). The results showed that after treatments with 3-PL or miltefosine the remaining amastigotes lost the capacity to differentiate into promastigotes in relation to untreated control. Although the treatment at 25 μM did not decrease the number of intracellular amastigotes (Figure 1A), their capacity to differentiate into promastigotes was compromised (Figure 1B). Furthermore, although after treatment with 800 μM 3-PL there was still a certain number of intracellular amastigotes in macrophages (Figure 1A) they totally lost the ability to differentiate into promastigotes (Figure 1B).

Figure 1C displays the appearance of the culture of infected macrophages under the conditions: untreated (medium), treated with vehicle (DMSO), and 3-PL in the concentration range IC_{50} (200 μM) and 2-fold IC_{50} (400 μM) for intracellular amastigote. In Figure 1D, we see that 3-PL has low toxicity to mice peritoneal macrophages, with a significant reduction in cell viability only from 3200 μM (38% inhibition). Therefore, the CC_{50} value is above 3200 μM and the estimated selectivity index (CC_{50}/IC_{50}) is greater than 16.

3.2 Nitric oxide and cytokine production by infected macrophage

To assess whether the anti-amastigote activity of 3-PL was associated with host cell activation, we measured nitric oxide and cytokine levels in supernatants from infected macrophages treated with IC_{50} range concentration (200 μM) for 48h. The results showed that nitric oxide was not altered by 3-PL treatment (Figure 2A), however, the production of cytokines TNF-α, MCP-1, IL-6, and IL-10 were significantly increased (Figures 2B–E).

3.3 Anti-promastigote toxicity

To evaluate the direct effect of 3-PL on parasites, we used the promastigote forms. The 3-PL reduced proliferation of *L.* (*V.*) *braziliensis* promastigote forms in a time- and dose-dependent manner, inhibiting significantly the growth of the parasite from a concentration of 100 μM. In the first 24h, the higher concentrations of 3-PL (400 and 800 μM) promoted the death of the parasites. The IC_{50} was estimated at 116 ± 26 μM for 72h (Figure 3A). In order to evaluate the cytotoxic effect of 3-PL on the parasite, we treated the promastigotes for 72h with a concentration in the IC_{50} range (100 μM) or with half the IC_{50} (50 μM) and analyzed the production of ROS by the H2DCFDA probe. Promastigotes treated with 3-PL significantly increased ROS generation (Figure 3B) compared to the controls (Medium or DMSO).

3.4 *In vivo* therapeutic effects of treatment with high (subcutaneous) and low (tattoing) doses 3-PL in *Leishmania* (*V.*) *braziliensis*-infected hamsters

The therapeutic effect of 3-PL in hamsters infected with *L.* (*V.*) *braziliensis* was evaluated subcutaneously (high doses) or tattooing (low doses) using two treatment protocols. A first approach, protocol I, we treated the animals for three weeks (3 times a week) and followed the lesion for more three weeks without treatment, when we then quantified the parasitic load (Figures 4A, B). Treatment with 3-PL subcutaneously or tattooing did not reduce the thickness of the lesion (Figure 4A). However, both treatment routes significantly reduced the parasite load (Figure 4B) compared to the control groups.

In an attempt to minimize the contribution of probable inflammatory process induced by the local administration on the lesion thickness, a second protocol was performed with reduced time and number of administrations (protocol II). In this protocol, the parasite load determined one week after the end of treatment and included groups treated with the reference drug, meglumine antimoniate (Glucantime®). As in the protocol I, we did not observe a decreased thickness of the lesion in the groups treated with 3-PL (subcutaneous or tattoing routes) (Figure 4C). However, the parasite load significantly decreased in both 3-PL, subcutaneous or tattooing routes (Figure 4D). The treatment with Glucantime® by subcutaneous route, significantly reduces lesion thickness (Figure 4C) and parasite load (Figure 4D). On the other hand, the treatment of animals with Glucantime® by tattooing did not reduce the thickness of the lesion, however significantly decreased the parasitic load (Figures 4C, D). These data show that the administration of both 3-PL and Glucantime® by tattoo, at a dose approximately 10 times lower compared to the subcutaneous route, was able to significantly reduce the parasite load in the lesion, however without reducing the lesion size. Likewise, when we started treating infected animals from

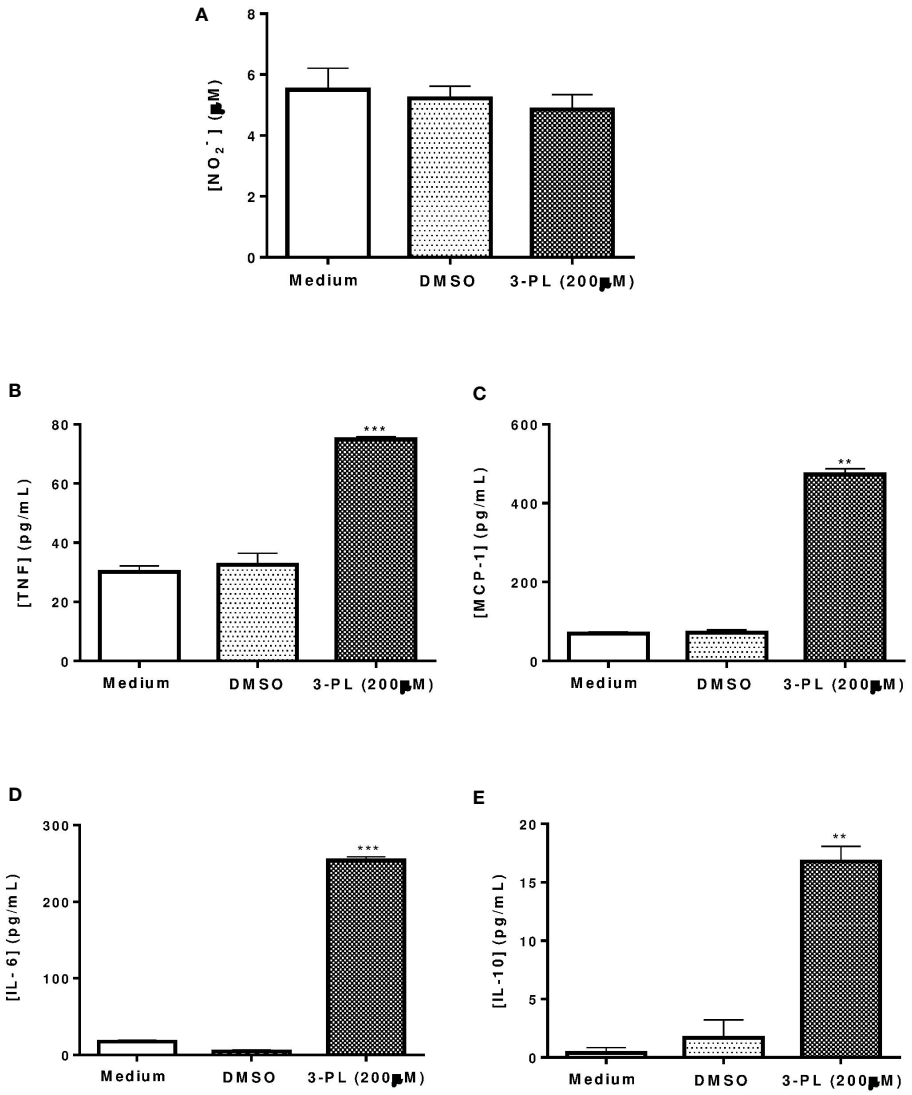


FIGURE 2
Production of nitric oxide and cytokines by infected macrophages treated with 3-PL. The mediators were measured in the supernatant of macrophages infected with *L.* (*V.*) *braziliensis* after 48h of treatment with 200 μM 3-PL. **(A)** Nitric oxide concentration was evaluated by the Griess method. **(B-E)** Cytokines TNF-α, MCP-1, IL-6 and IL-10 were measured by the Cytometric Bead Array (CBA) method. The data of nitrite (μM) and cytokines (pg/mL) concentration were expressed as the means ± SD, (n=3). **p < 0.01 and ***p < 0.001 in relation to control DMSO.

the fifth week of infection (lesions about 2 mm thickness) with 3-PL via tattooing for two weeks (Figure 4E), there was a significant reduction in the parasite load (Figure 4F), but without reducing the thickness of the lesions.

3.5 Histopathological analysis of the lesions

Histopathological analysis of the lesions was performed in the groups treated with protocol II (Figure 5). In Figure 5A, we show the normal appearance of the paw skin of an uninfected hamster. In Figure 5B, the infected hamster lesion presents the typical histopathological pattern with an inflammatory infiltrate composed of macrophages showing intracytoplasmic vacuoles containing amastigotes and rare neutrophils. In the lesion of animals treated with 3-PL, both, subcutaneous or tattooing (Figures 5E, F, respectively), we observed a predominantly mononuclear inflammatory infiltrate composed of macrophages, lymphocytes, and plasma cells, in addition to rare neutrophils and parasites. In lesions of animals that received 3-PL subcutaneously (Figure 5E), the inflammatory infiltrate is more discreet and more localized than in the tattooing route (Figure 5F), while amastigotes were not observed. The lesions of the animals treated with Glucantime® subcutaneously (Figure 5G) present epidermis, dermis, and a portion of muscle tissue similar in appearance to the histology of the paw tissue of an uninfected animal (Figure 5A). Animals treated with Glucantime® *via* tattooing (Figure 5H) present lesion tissue with a mixed inflammatory infiltrate (neutrophils, eosinophils, macrophages, and lymphocytes) and rare amastigotes. In Figure 6, we show a semi-quantitative analysis using a scoring system for the presence of amastigotes and inflammatory infiltrate in the skin lesion. In hamsters treated with 3-PL or reference drug (Glucantime®) by tattooing, amastigotes were rarely observed, while in subcutaneous treatment amastigotes were absent (Figure 6A). The inflammatory infiltrate was discreetly observed in animals treated subcutaneously with 3-PL, while in

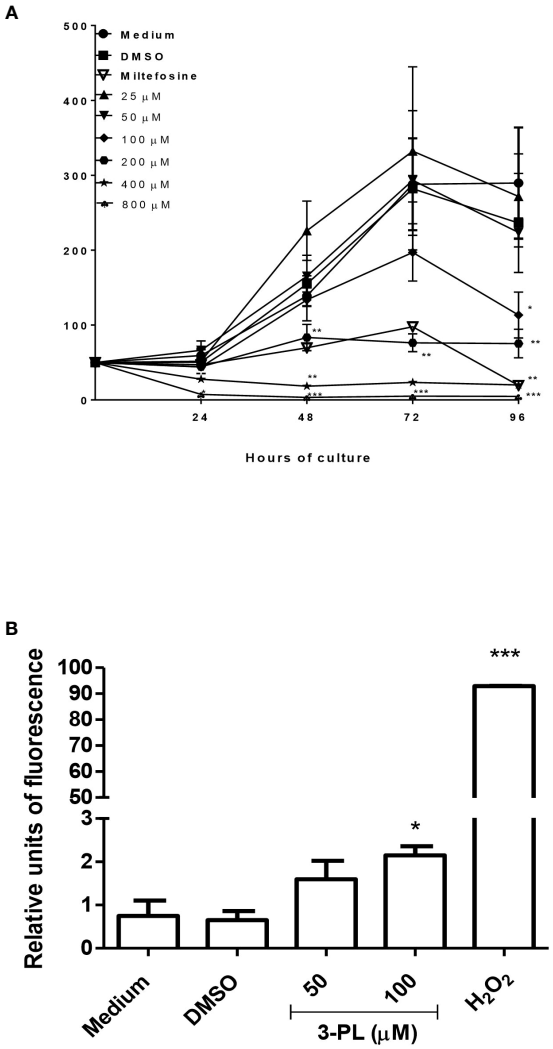


FIGURE 3
Evaluation of the direct toxic effect of 3-PL on the parasite. Promastigotes were cultured in the presence of indicated concentrations of 3-PL or 6µM miltefosine for 96h at 28°C. **(A)** Promastigote growth was assessed by daily counting in a Neubauer chamber. **(B)** Promastigote ROS generation detected by H2DCFDA probe after treatment with 100 µM 3-PL for 72h. Hydrogen peroxide (H_2O_2) was used as a positive control for ROS detection. Results were presented as means ± SD, n=3. *p < 0.05, **p < 0.01 and ***p < 0.001 in relation to control DMSO.

animals treated with Glucantime® by the same route it was not observed (Figure 6B). We observed frequent inflammatory infiltrate in the lesions of all groups of animals treated by the tattooing route. However, in the group treated with 3-PL this finding was significantly lower than in the controls groups, including the group treated with Glucantime® (Figure 6B).

4 Discussion

This study showed an *in vitro* and *in vivo* antileishmania effect of synthetic 3-PL against *L. (V.) braziliensis*. This naphthoquinone presented dose-dependent activity *in vitro*, as on promastigotes (IC_{50} = 116 ± 26 µM, 72h) as well intracellular amastigotes (IC_{50} = 193 ± 19 µM, 48h) forms of *L.* (*V.*) *braziliensis.* These data corroborate with the previous report from our group, that has shown the *in vitro* effect of 3-PL against *Leishmania* (*L.*) *amazonensis*, in both promastigotes (IC_{50} = 85 µM, 72h) and intracellular amastigotes (IC_{50} = 25 µM, 72h) forms (Gomes et al., 2017). The ability of 3-PL to have an effect against species of the different subgenus of the *Leishmania* (subgenus *Leishmania* and *Viannia*), makes it even more promising. The differences between the IC_{50} values obtained in this study for *L. (V.) braziliensis* and those reported for *L. (L.) amazonensis* is expected due to the susceptibility variations between different subgenera and species of *Leishmania*, as well as the experimental conditions used. We established the IC_{50} of 3-PL to *L.* (*V.*) *braziliensis* amastigotes at 48h, while IC_{50} to *L.* (*L.*) *amazonensis* was made at 72h (Gomes et al., 2017). The antileishmanial activity of lapachol, the naphthoquinone precursor molecule of 3-PL, presented wide values of IC_{50} dependent on *Leishmania* strain and experimental conditions. For example, the lapachol IC_{50} for intracellular amastigote of *L.* (*L.*) *amazonensis* was variable from 191 µM/48h (Araújo et al., 2019) to 250 µg/mL at 72h (corresponding to 1 mM/72h) (Costa et al., 2017).

The antileishmanial activity of naphthoquinones has been recognized (Croft et al., 1992; Murray and Hariprashad, 1996; Teixeira et al., 2001; Garnier et al., 2007; Reimão et al., 2012), including synthetic molecules derived from lapachol also show action on *L.* (*V.*) *braziliensis* (Costa

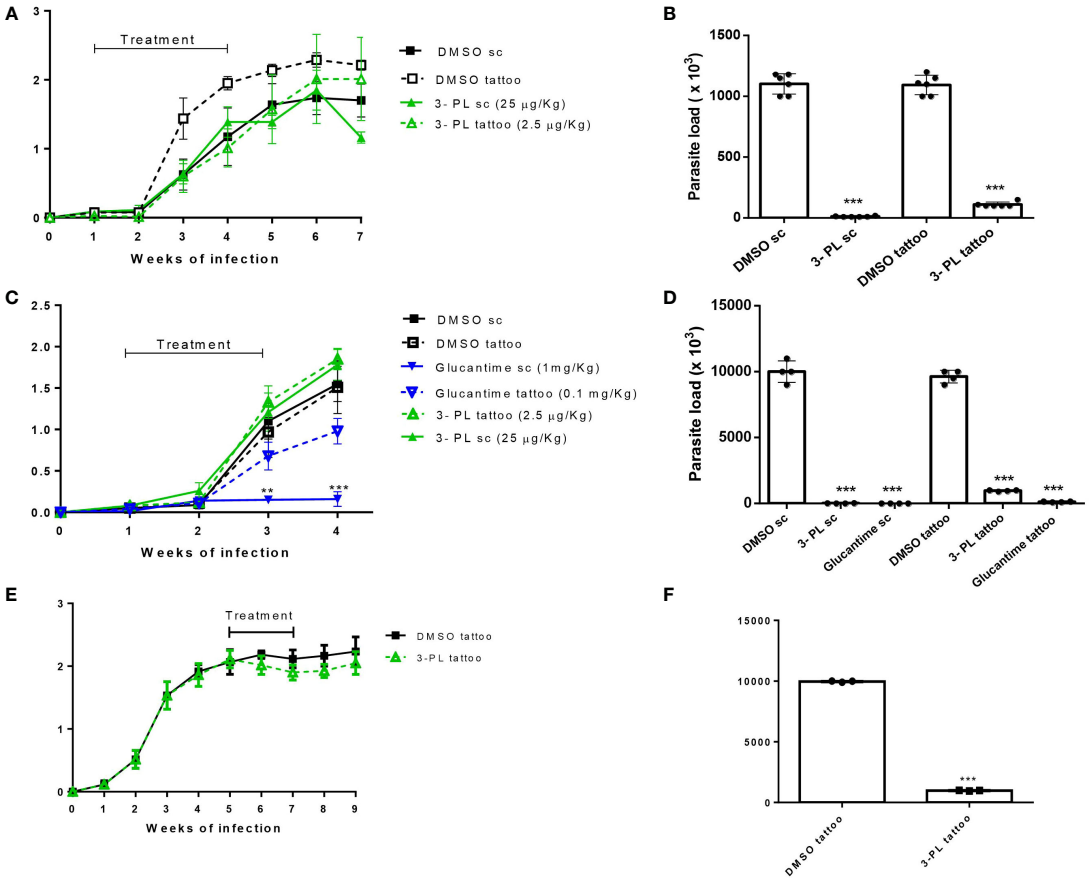


FIGURE 4
Therapeutic activity of 3-PL administered subcutaneously or tattooing in hamsters infected with *L.* (*V.*) *braziliensis*. Hamsters golden (3-6/group) were infected with 10^6 promastigotes of *L.* (*V.*) *braziliensis* in the dorsal hind paw and treated from 7 days **(A-D)** or from 5 weeks after infection **(E,F)** with vehicle DMSO 0.5% in PBS (DMSO sc or DMSO tattoo); 3-PL by subcutaneous (3-PL sc, 25 µg/Kg) or tattooing (3-PL tattoo, 2.5 µg/Kg); Glucantime® subcutaneous (Glucantime® sc, 1mg/Kg or tattooing (Glucantime® tattoo, 0.1 mg/Kg) routes. **(A)** Treatment during 3 weeks (3 times/week= 9 doses). **(C, E)** Treatment during 2 weeks (2 times/week= 4 doses). Lesion thickness **(A, C, E)**, expressed as the difference between the thickness of the infected and uninfected paws, were presented as means ± SD, and parasite load in skin paw was determined in the final experiment by limiting dilution analysis **(B, D, F)**; n=4-6; **p < 0.01 and ***p < 0.001 in relation to respective DMSO control (sc or tattoo route).

et al., 2014) and *L.* (*L.*) *amazonensis* (Cunha-Junior et al., 2011; Ribeiro et al., 2013). The generation of ROS and the modulation of redox signaling are properties of naphthoquinones related to the structural modification in the scaffold (Qiu et al., 2018). As to the mode of action, we observed that the antileishmanial effect of 3-PL was related to increased ROS production and DNA fragmentation (data not shown) of *L.* (*V.*) *braziliensis* promastigotes. Several naphthoquinones are capable of inducing apoptotic death in tumor cells (Wei et al., 2017; Liu et al., 2018; de Almeida et al., 2021) and protozoa (Corrêa et al., 2009; Anjos et al., 2016). Induction of ROS production and DNA fragmentation by quinones and naphthoquinones has also been described in *Leishmania* sp. (Ribeiro et al., 2013; Awasthi et al., 2016). Previous studies from our group showed that pterocarpanquinone LQB-118, a lapachol-pterocarpan derivative, induces ROS production, DNA fragmentation, and cellular death by apoptosis-like on both *L.* (*L.*) *amazonensis* (Ribeiro et al., 2013) and *L.* (*V.*) *braziliensis* (Costa et al., 2014). Furthermore, to induce oxidative stress by ROS, naphthoquinones can act to inhibit topoisomerase. This combination of effects contributes to its important antitumor action, which is part of the mechanism of some naphthoquinone derivatives approved and used clinically against cancer (Qiu et al., 2018). Molecular modeling studies have suggested that 1,4-naphthoquinones tethered to 1,2,3-1H-triazoles have a potential antitumor action mechanism related to inhibition of topoisomerase and/or hPKM2 activity leading to induced microtubule disorganization (Chipoline et al., 2020). Bis-lawsone analogues showed antileishmanal effect associeted with DNA topoisomerase-I inhibition of the parasite (Sharma et al., 2014). The new lawsone derivatives presented the anticancer activity associated with ROS formation and antiparasitic action on *Trypanosoma brucei brucei* related to deformation of the microtubule cytoskeleton (Mahal et al., 2017). Although the increase in ROS may be related to the induction of observed DNA fragmentation (data not shown), we cannot rule out the inhibition of the parasite's topoisomerase as part of the mechanism of action of 3-PL. Further investigations about the anti-leishmanial mechanism of 3-PL should be carried out. We observed that 3-PL inhibits the multiplication of intracellular amastigotes and also the ability of the remaining amastigotes to differentiate into promastigotes, suggesting an irreversible toxic effect. It is reasonable to assume that the 3-PL toxic mechanism demonstrated for the promastigote could extend to intracellular amastigote forms.

In addition to the direct effect on the parasite, antileishmanial drugs can activate the macrophage to kill intracellular amastigotes

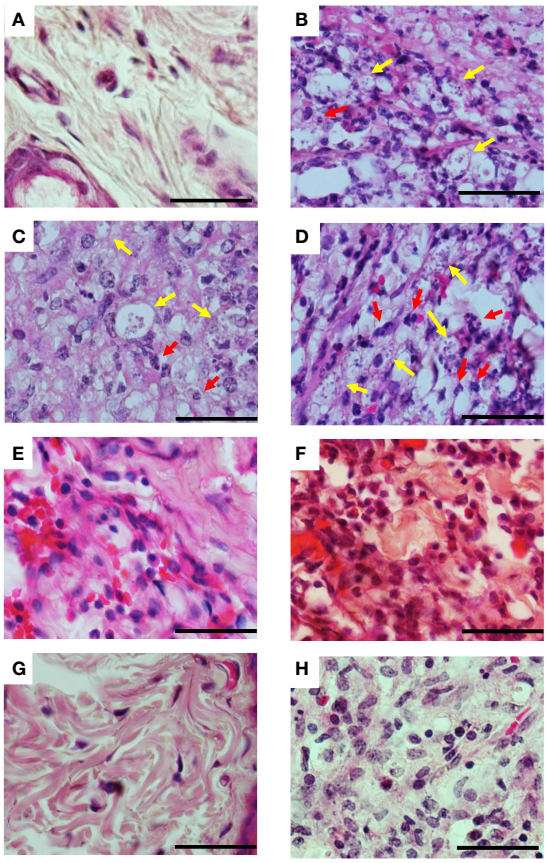


FIGURE 5
Histopathological aspects of lesions of infected hamsters treated with 3-PL. Golden hamsters (n = 4) infected in the dorsal hind paw with *L.* (*V.*) *braziliensis* were treated with 3-PL or reference drug Glucantime® from one week of infection for two weeks and histological analysis of the lesions was performed by H&E staining. **(A)** Uninfected; **(B)** Infected untreated; **(C)** DMSO sc; **(D)** DMSO tattoo; **(E)** 3-PL sc; **(F)** 3-PL tattoo; **(G)** Glucantime® sc; **(H)** Glucantime® tattoo. Data show representative pictures of each group. Yellow arrow: intracellular amastigotes; Red arrow: neutrophils. 1000x magnification, scale bar = 25 μm.

(Muniz-Junqueira and de Paula-Coelho, 2008; Ghosh et al., 2013). Our results show that 3-PL modulates infected macrophages by increasing their production of the cytokines TNF-α, IL-6, MCP-1, and IL-10. However, anti-amastigote activity was not related to nitric oxide production. Similar to our results with 3-PL, da Costa-Silva and collaborators (2017) observed that nanoliposomal buparvaquone, a hydroxynaphthoquinone, increases the production of cytokines TNF-α, MCP-1, IL-6 and IL-10 by *Leishmania*-infected macrophages without altering nitric oxide production. Some naphthoquinones have action to prevent the production of nitric oxide (Cheng et al., 2008; Pinho et al., 2011) by inhibition of iNOS protein expression through the downregulation of MAPKqNF-kappaB signaling (Cheng et al., 2008). Furthermore, the increased levels we observed of the anti-inflammatory cytokine IL-10, which downregulates inducible nitric oxide synthase (iNOS) in macrophages, may have contributed to preventing NO production.

The antileishmanial therapeutic potential of 3-PL was investigated for the first time in this study. The evaluation was performed by local routes in hamsters infected with *L.* (*V.*) *braziliensis*. When infected with *L.* (*V*). *braziliensis*, hamsters develop lesions very similar to those observed in humans, as well as the course of the infection, being considered a good model for therapeutic studies (Gomes-Silva et al., 2013; Mears et al., 2015; Dutra and da Silva, 2017). In the present study, treatment with 3-PL was performed in the lesion site by subcutaneous or tattooing routes. Using two treatment protocols where animals were treated from one week of infection for three or two weeks (Protocols I and II), treatment with 3-PL by both routes, subcutaneously or by tattooing, was able to significantly reduce the parasite load in the lesion. In the same way, when the treatment was initiated in the most chronic phase of the infection (5 weeks of infection, 2 mm thick lesion), protocol III, the administration of 3-PL tattoo for 2 weeks was able to significantly reduce the parasite load. Histopathological data also corroborate this finding and showed a reduction in the number of amastigotes in the tissue. It is important to emphasize that the dose of 3-PL administered in tattooing (± 2.5 μg/Kg) was approximately ten times lower compared to the subcutaneous (25 μg/Kg) route, and even so the molecule was able to reduce the parasite load. Subcutaneous treatment with the reference meglumine antimoniate, Glucantime®, has been applied in CL (Carvalho et al., 2019; Rodrigues et al., 2020) and our data confirm the effectiveness of this route of administration using 1mg/Kg. Interestingly, in addition to the well-documented subcutaneous route, lower dose Glucantime® administered by tattooing (± 0.1 mg/Kg) was also effective in reducing the parasite load. This first example of tattoo-mediated pentavalent antimonial delivery may open new therapeutic interventions in the treatment of CL.

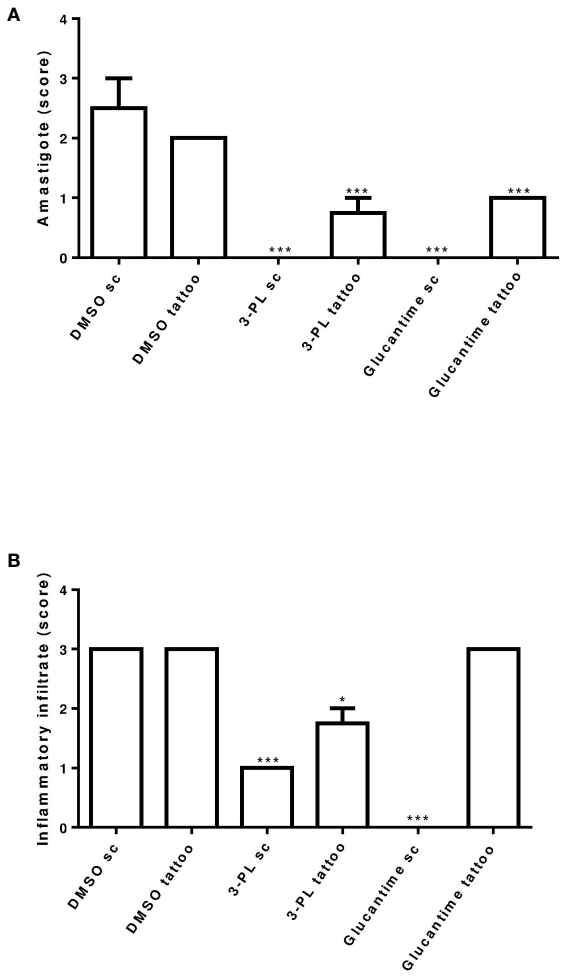


FIGURE 6
Semi-quantitative analysis of histopathological aspects of hamster skin lesions. Skin lesion sections of hamsters infected with *L.* (*V.*) *braziliensis* treated or not (as indicated) from 7 days of infection for two weeks were stained with H&E and analyzed under light microscopy. In **(A)** presence of amastigote and in **(B)** presence of inflammatory infiltrate. Results were expressed by scoring. The score value was determined as not observed (score = 0), little observed (score = 1), moderately observed (score = 2) and highly observed (score = 3). **p < 0.01 and ***p < 0.001 in relation to respective DMSO control group (sc or tattoo route). *p ≤ 0.05.

Treatment using the delivery drugs directly in skin lesions has been shown to be important in dermatological therapy (Arbache et al., 2018). Novel transdermal methods for drug delivery and vaccination provide a higher immunological response, drug dose sparing, and reduction in pain, which improves patient compliance (Mercuri and Rivas, 2021). Skin tattooing is a new approach to drug delivery (Arbache et al., 2018; Mercuri and Rivas, 2021), intradermal immunization, including DNA vaccination (van de Wall et al., 2015; Fotoran et al., 2020). In the only published study using tattooing as a drug administration route for experimental CL, the anti-*Leishmania* molecule, oleylphosphocholine, was injected in a liposomal formulation to treat mice infected by *L.* (*L.*) *major* or *L.* (*L.*) *mexicana* (Shio et al., 2014). Liposomal formulation increasing treatment efficacy since these particles are prone to ingestion by phagocytic cells such as macrophages (Bruni et al., 2017). In the present study, 3-PL was solubilized in DMSO and administered diluted in PBS and proved to be active. New formulations of 3-PL can be considered for further studies in order to reduce the number of administrations and local inflammation.

The local route of administration may have contributed to the maintenance of inflammation and thickness of the lesions, since it has been shown that there is an inflammatory response caused by tissue damage produced by the injection needle (Gomes-Silva et al., 2013; Mac-Daniel et al., 2014). Our histopathological data corroborate this data, showing the presence of inflammatory infiltrate in the tissue of the lesion in animals treated subcutaneously or tattooing, which probably contributes to not decreasing the thickness of the lesions. We used a total of 4 doses of treatment (twice a week/2 weeks), which must have provided more tissue damage and persistent inflammation. However, the inflammatory infiltrate was more intense in the lesions of the groups of animals treated with tattoos compared to those treated subcutaneously. A histological study evaluating inflammation from ink tattooing on mice skin showed that acute inflammation started at 12h, decreasing its incidence on day 14 (Gopee et al., 2005). In our study, the histopathological analysis was performed one week after the last tattoo session and showed the presence of an inflammatory process. On the other hand, the local inflammation promoted by tattooing may contribute to the induction of an important immune response mediated by T cells, as observed in studies with DNA tattoo vaccines (Platteel et al., 2017; Fotoran et al., 2020; Bakker et al., 2021). In studies of the hamster model infected with *L. (V.) braziliensis* carried out by

Ribeiro-Romão and collaborators (2014) and Paiva and collaborators (2021), the lower parasite load generates a more benign course of infection, without systemic involvement and associated with the expression of a more balanced cytokine network. Therefore, terapeutic intervention in the early stages of infection, when the immune response is being mounted by the host, may favor the best outcome of the disease. It is possible to assume that the reduction in the parasite load promoted by the treatment with 3-PL in the initial stages of infection (from seven days after infection) has allowed a more balanced production of cytokines and triggered a more benign course of the disease. Therefore, in addition to the antiparasitic effect of 3-PL, the possible induction of a cellular immune response by the tattoo could have contributed to the great reduction of the parasite load in the animals treated by this route of administration. Furthermore, even when the lesion was already well established, treatment with 3-PL tattooing was also able to reduce the parasite load, demonstrating the promising antileishmanial potential of this naphthoquinone.

In future studies, we may include a longer post-treatment follow-up with 3-PL, allowing the verification of inflammation regression, lesion evolution, and cytokine expression. In addition, we intend to further explore the effect of 3-PL treatment initiated in the more chronic phases of the infection, as well as to investigate the efficacy of combination therapy with reference drugs.

5 Conclusions

In this study, we demonstrate the antileishmanial activity of 3-PL against *L. (V.) braziliensis* associated with a direct toxic effect on the parasite involving induction of ROS production and modulation of macrophage cytokines. Using the hamster model of infection, 3-PL has shown efficacy in significantly reducing the parasite load when administered in low doses subcutaneously and by tattooing. Additionally, this study also showed, for the first time, the activity of the reference drug Glucantime® administered by tattooing. Drug administration by tattooing uses small volumes and can be useful in reducing the dose and toxicity of drugs. The dataset gathered in this study indicates that 3-PL has pronounced effects on *L.* (*V.*) *braziliensis* and deserves further preclinical investigations. Therefore, the data presented in this study may contribute to expanding treatment approaches for CL.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethics Committee on Animal Use (CEUA) of the Instituto de Biologia Roberto Alcantara Gomes of the Universidade do Estado do Rio de Janeiro-UERJ, by the number protocol CEUA/051/2017.

Author contributions

RM conducted experiments and contributed to the writing of the manuscript. SG, ES, TS, AB, LS, JI and MP conducted the experiments. EA-A, AC, RN, LR, PD, PC and AS contributed to the execution and discussion the experiments. SS conducted orientation, experimentation and conducted to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study received funding from Brazilian Research Agencies: CAPES (Coordination for the Improvement of Higher Education Personnel), CNPq (National Council for Scientific and Technological Development), and FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro) through scholarships and financial support for the project (Proc. numbers E-26/010.001531/2014 and E-26/010.001815/2015).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

al Nasr, I., Jentzsch, J., Winter, I., Schobert, R., Ersfeld, K., Koko, W. S., et al. (2019). Antiparasitic activities of new lawsone mannich bases. *Arch. Pharm. (Weinheim)* 352, 1900128. doi: 10.1002/ardp.201900128

Anjos, D.O.d., Alves, E. S. S., Gonçalves, V. T., Fontes, S. S., Nogueira, M. L., Suarez-Fontes, A. M., et al. (2016). Effects of a novel β–lapachone derivative on *Trypanosoma cruzi* : Parasite death involving apoptosis, autophagy and necrosis. *Int. J. Parasitol. Drugs Drug Resist.* 6, 207–219. doi: 10.1016/j.ijpddr.2016.10.003

Araújo, I. A. C., de Paula, R. C., Alves, C. L., Faria, K. F., de Oliveira, M. M., Mendes, G. G., et al. (2019). Efficacy of lapachol on treatment of cutaneous and visceral leishmaniasis. *Exp. Parasitol.* 199, 67–73. doi: 10.1016/j.exppara.2019.02.013

Arbache, S., Mattos, E., da, C., Diniz, M. F., Paiva, P. Y. A., Roth, D., et al. (2019). How much medication is delivered in a novel drug delivery technique that uses a tattoo machine? *Int. J. Dermatol.* 58, 750–755. doi: 10.1111/ijd.14408

Arbache, S., Roth, D., Steiner, D., Breunig, J., Michalany, N. S., Arbache, S. T., et al. (2018). Activation of melanocytes in idiopathic guttate hypomelanosis after 5-fluorouracil infusion using a tattoo machine: Preliminary analysis of a randomized, split-body, single blinded, placebo controlled clinical trial. *J. Am. Acad. Dermatol.* 78, 212–215. doi: 10.1016/j.jaad.2017.08.019

Awasthi, B. P., Kathuria, M., Pant, G., Kumari, N., and Mitra, K. (2016). Plumbagin, a plant-derived naphthoquinone metabolite induces mitochondria mediated apoptosis-like cell death in *Leishmania donovani*: an ultrastructural and physiological study. *Apoptosis* 21, 941–953. doi: 10.1007/s10495-016-1259-9

Bakker, N. A. M., Rotman, J., van Beurden, M., Zijlmans, H. J. M., van Ruiten, M., Samuels, S., et al. (2021). HPV-16 E6/E7 DNA tattoo vaccination using genetically optimized vaccines elicit clinical and immunological responses in patients with usual vulvar intraepithelial neoplasia (uVIN): a phase I/II clinical trial. *J. Immunother. Cancer* 9, 1–13. doi: 10.1136/jitc-2021-002547

Barbosa, M. I. F., Corrêa, R. S., de Oliveira, K. M., Rodrigues, C., Ellena, J., Nascimento, O. R., et al. (2014). Antiparasitic activities of novel ruthenium/lapachol complexes. *J. Inorg Biochem.* 136, 33–39. doi: 10.1016/j.jinorgbio.2014.03.009

Berman, J. (2005). Recent developments in leishmaniasis: Epidemiology, diagnosis, and treatment. *Curr. Infect. Dis. Rep.* 7, 33–38. doi: 10.1007/s11908-005-0021-1

Bruni, N., Stella, B., Giraudo, L., Pepa, C.d., Gastaldi, D., and Dosio, F. (2017). Nanostructured delivery systems with improved leishmanicidal activity: a critical review. *Int. J. Nanomed.* 12, 5289–5311. doi: 10.2147/IJN.S140363

Caridha, D., Vesely, B., van Bocxlaer, K., Arana, B., Mowbray, C. E., Rafati, S., et al. (2019). Route map for the discovery and pre-clinical development of new drugs and treatments for cutaneous leishmaniasis. *Int. J. Parasitol. Drugs Drug Resist.* 11, 106–117. doi: 10.1016/j.ijpddr.2019.06.003

Carvalho, S. H., Frézard, F., Pereira, N. P., Moura, A. S., Ramos, L. M. Q. C., Carvalho, G. B., et al. (2019). American Tegumentary leishmaniasis in Brazil: a critical review of the current therapeutic approach with systemic meglumine antimoniate and short-term possibilities for an alternative treatment. *Trop. Med. Int. Health* 24, 380–391. doi: 10.1111/tmi.13210

Cheng, Y. W., Chang, C. Y., Lin, K. L., Hu, C. M., Lin, C. H., and Kang, J. J. (2008). Shikonin derivatives inhibited LPS-induced NOS in RAW 264.7 cells *via* downregulation of MAPK/NF-κB signaling. *J. Ethnopharmacol.* 120, 264–271. doi: 10.1016/j.jep.2008.09.002

Chipoline, I. C., da Fonseca, A. C. C., da Costa, G. R. M., de Souza, M. P., Rabelo, V. W.-H., de Queiroz,, et al. (2020). Molecular mechanism of action of new 1,4-naphthoquinones tethered to 1,2,3-1H-triazoles with cytotoxic and selective effect against oral squamous cell carcinoma. *Bioorg. Chem.* 101, 103984. doi: 10.1016/j.bioorg.2020.103984

Corrêa, G., Vilela, R., Menna-Barreto, R. F. S., Midlej, V., and Benchimol, M. (2009). Cell death induction in *Giardia lamblia*: Effect of beta-lapachone and starvation. *Parasitol. Int.* 58, 424–437. doi: 10.1016/j.parint.2009.08.006

Costa, E. V. S., Brígido, H. P. C., Silva, J. V., Coelho-Ferreira, M. R., Brandão, G. C., and Dolabela, M. F. (2017). Antileishmanial activity of *Handroanthus serratifolius* (Vahl) s. grose (Bignoniaceae). *Evidence-Based Complementary Altern. Med.* 2017, 1–6. doi: 10.1155/2017/8074275

Costa, L., Pinheiro, R. O., Dutra, P. M. L., Santos, R. F., Cunha-Júnior, E. F., Torres-Santos, E. C., et al. (2014). Pterocarpanquinone LQB-118 induces apoptosis in *Leishmania* (*Viannia*) *braziliensis* and controls lesions in infected hamsters. *PloS One* 9, e109672. doi: 10.1371/journal.pone.0109672

Croft, S. L., Hogg, J., Gutteridge, W. E., Hudson, A. T., and Randall, A. W. (1992). The activity of hydroxynaphthoquinones against *Leishmania donovani*. *J. Antimicrobial Chemother.* 30, 827–832. doi: 10.1093/jac/30.6.827

Cunha-Junior, E. F., Pacienza-Lima, W., Ribeiro, G. A., Netto, C. D., Canto-Cavalheiro, M. M., da Silva,, et al. (2011). Effectiveness of the local or oral delivery of the novel naphthopterocarpanquinone LQB-118 against cutaneous leishmaniasis. *J. Antimicrobial Chemother.* 66, 1555–1559. doi: 10.1093/jac/dkr158

Cupolillo, E., Brahim, L. R., Toaldo, C. B., Paes de Oliveira-Neto, M., de Brito, M. E. F., Falqueto, A., et al. (2003). Genetic polymorphism and molecular epidemiology of *Leishmania* (*Viannia*) *braziliensis* from different hosts and geographic areas in Brazil. *J. Clin. Microbiol.* 41, 3126–3132. doi: 10.1128/JCM.41.7.3126-3132.2003

da Costa-Silva, T. A., Galisteo, A. J.Jr., Lindoso, J. A. L., Barbosa, L. R. S., and Tempone, A. G. (2017). Nanoliposomal buparvaquone immunomodulates *Leishmania infantum*-infected macrophages and is highly effective in a murine model. *Antimicrob. Agents Chemother.* 61, 1–15. doi: 10.1128/AAC.02297-16

da Silva-Couto, L., Ribeiro-Romão, R. P., Saavedra, A. F., da Silva Costa Souza, B. L., Moreira, O. C., Gomes-Silva, A., et al. (2015). Intranasal vaccination with leishmanial antigens protects golden hamsters (*Mesocricetus auratus*) against *Leishmania* (*Viannia*) *braziliensis* infection. *PloS Negl. Trop. Dis.* 9, 1–7. doi: 10.1371/journal.pntd.0003439

David, C. v., and Craft, N. (2009). Cutaneous and mucocutaneous leishmaniasis. *Dermatol. Ther.* 22, 491–502. doi: 10.1111/j.1529-8019.2009.01272.x

de Almeida, P. D. O., Jobim, G.d. S.B., Ferreira, C. C. d. S., Bernardes, L. R., Dias, R. B., Sales, C. B. S., et al. (2021). A new synthetic antitumor naphthoquinone induces ROS-mediated apoptosis with activation of the JNK and p38 signaling pathways. *Chem. Biol. Interact.* 343, 1–13. doi: 10.1016/j.cbi.2021.109444

Dutra, P. M. L., and da Silva, S. A. G. (2017). "Experimental models for trypanosomatids infection," in *Different aspects on chemotherapy of trypanosomatids*. Eds. L. Leon and E. C. Torres-Santos (New York: Nova Science Publishers, Inc), 39–57.

Ferreira, S. B., Gonzaga, D. T. G., Santos, W. C., Araújo, K. G., de, L., and Ferreira, V. F. (2010). ß-lapachone: Medicinal chemistry significance and structural modifications. *Rev. Virtual Química* 2, 140–160. doi: 10.5935/1984-6835.20100013

Fotoran, W. L., Kleiber, N., Glitz, C., and Wunderlich, G. (2020). A DNA vaccine encoding *Plasmodium falciparum* PfRH5 in cationic liposomes for dermal tattooing immunization. *Vaccines (Basel)* 8, 619. doi: 10.3390/vaccines8040619

Garnier, T., Mantyla, A., Jarvinen, T., Lawrence, J., Brown, M., and Croft, S. (2007). *In vivo* studies on the antileishmanial activity of buparvaquone and its prodrugs. *J. Antimicrobial Chemother.* 60, 802–810. doi: 10.1093/jac/dkm303

Ghosh, M., Roy, K., and Roy, S. (2013). Immunomodulatory effects of antileishmanial drugs. *J. Antimicrobial Chemother.* 68, 2834–2838. doi: 10.1093/jac/dkt262

Gomes, S. L. S., Militão, G. C. G., Costa, A. M., Pessoa, C. Ó., Costa-Lotufo, L., Cunha-Junior, E. F., et al. (2017). Suzuki-Miyaura coupling between 3-iodolawsone and arylboronic acids. synthesis of lapachol analogues with antineoplastic and antileishmanial activities. *J. Braz. Chem. Soc.* 28, 1573–1584. doi: 10.21577/0103-5053.20160326

Gomes-Silva, A., Valverde, J. G., Ribeiro-Romão, R. P., Plácido-Pereira, R. M., and Da-Cruz, A. M. (2013). Golden hamster (*Mesocricetus auratus*) as an experimental model for *Leishmania* (*Viannia*) braziliensis infection. *Parasitology* 140, 771–779. doi: 10.1017/S0031182012002156

Gopee, N., Cui, Y., Olson, G., Warbritton, A. R., Miller, B. J., Couch, L. H., et al. (2005). Response of mouse skin to tattooing: use of SKH-1 mice as a surrogate model for human tattooing. *Toxicol. Appl. Pharmacol.* 209, 145–158. doi: 10.1016/j.taap.2005.04.003

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and $[_{15}N]$nitrate in biological fluids. *Anal. Biochem.* 126, 131–138. doi: 10.1016/0003-2697(82)90118-X

Hazra, S., Ghosh, S., das Sarma, M., Sharma, S., Das, M., Saudagar, P., et al. (2013). Evaluation of a diospyrin derivative as antileishmanial agent and potential modulator of ornithine decarboxylase of *Leishmania donovani*. *Exp. Parasitol.* 135, 407–413. doi: 10.1016/j.exppara.2013.07.021

Hussain, H., and Green, I. R. (2017). Lapachol and lapachone analogs: a journey of two decades of patent research(1997-2016). *Expert Opin. Ther. Pat.* 27, 1111–1121. doi: 10.1080/13543776.2017.1339792

Hussain, H., Krohn, K., Ahmad, V. U., Miana, G. A., and Green, I. R. (2007). Lapachol: An overview. *Arkivoc* 2007, 145–171. doi: 10.3998/ark.5550190.0008.204

Lima, N. M., Correia, C. S., Leon, L. L., Machado, G. M., Madeira, M., de, F., et al. (2004). Antileishmanial activity of lapachol analogues. *Mem Inst Oswaldo Cruz* 99, 757–761. doi: 10.1590/S0074-02762004000700017

Liu, C., Shen, G.-N., Luo, Y.-H., Piao, X.-J., Jiang, X.-Y., Meng, L.-Q., et al. (2018). Novel 1,4-naphthoquinone derivatives induce apoptosis *via* ROS-mediated p38/MAPK, akt and STAT3 signaling in human hepatoma Hep3B cells. *Int. J. Biochem. Cell Biol.* 96, 9–19. doi: 10.1016/j.biocel.2018.01.004

Lorsuwannarat, N., Piedrafita, D., Chantree, P., Sansri, V., Songkoomkrong, S., Bantuchai, S., et al. (2014). The *in vitro* anthelmintic effects of plumbagin on newly excysted and 4-weeks-old juvenile parasites of *Fasciola gigantica*. *Exp. Parasitol.* 136, 5–13. doi: 10.1016/j.exppara.2013.10.004

Mac-Daniel, L., Buckwalter, M. R., Berthet, M., Virk, Y., Yui, K., Albert, M. L., et al. (2014). Local immune response to injection of *Plasmodium* sporozoites into the skin. *J. Immunol.* 193, 1246–1257. doi: 10.4049/jimmunol.1302669

Mahal, K., Ahmad, A., Schmitt, F., Lockhauserbäumer, J., Starz, K., Pradhan, R., et al. (2017). Improved anticancer and antiparasitic activity of new lawsone mannich bases. *Eur. J. Med. Chem.* 126, 421–431. doi: 10.1016/j.ejmech.2016.11.043

Mears, E. R., Modabber, F., Don, R., and Johnson, G. E. (2015). A review: The current *In vivo* models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous leishmaniasis. *PloS Negl. Trop. Dis.* 9, e0003889. doi: 10.1371/journal.pntd.0003889

Mercuri, M., and Rivas, D. F. (2021). Challenges and opportunities for small volumes delivery into the skin. *Biomicrofluidics* 15, 011301. doi: 10.1063/5.0030163

Ministério da Saúde (2017). *Manual de vigilância da leishmaniose tegumentar, manual de vigilância da leishmaniose tegumentar. 2nd ed* (Brasília: Ministério da Saúde).

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63. doi: 10.1016/0022-1759(83)90303-4

Mudavath, S. L., Talat, M., Rai, M., Srivastava, O. N., and Sundar, S. (2014). Characterization and evaluation of amine-modified graphene amphotericin b for the treatment of visceral leishmaniasis: *In vivo* and *in vitro* studies. *Drug Des. Devel Ther.* 8, 1235–1247. doi: 10.2147/DDDT.S63994

Muniz-Junqueira, M. I., and de Paula-Coelho, V. N. (2008). Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-α production, but only *via* TNF-α it indirectly increases nitric oxide production by phagocytes of healthy individuals, *in vitro*. *Int. Immunopharmacol.* 8, 1633–1638. doi: 10.1016/j.intimp.2008.07.011

Murray, H. W., and Hariprashad, J. (1996). Activity of oral atovaquone alone and in combination with antimony in experimental visceral leishmaniasis. *Antimicrob. Agents Chemother.* 40, 586–587. doi: 10.1128/AAC.40.3.586

Oliveira, R. A. S., Azevedo-Ximenes, E., Luzzati, R., and Garcia, R. C. (2010). The hydroxy-naphthoquinone lapachol arrests mycobacterial growth and immunomodulates host macrophages. *Int. Immunopharmacol.* 10, 1463–1473. doi: 10.1016/j.intimp.2010.08.023

Oliveira, L. F. G., Souza-Silva, F., de Côrtes, L. M., Cysne-Finkelstein, L., Pereira, M.C. de S., de Oliveira, J. F.O., et al. (2018). Antileishmanial activity of 2-Methoxy-4H-spiro-[naphthalene-1,2′-oxiran]-4-one (Epoxymethoxy-lawsone): A promising new drug candidate for leishmaniasis treatment. *Molecules* 23, 864. doi: 10.3390/molecules23040864

Oosterhuis, K., van den Berg, J. H., Schumacher, T. N., and Haanen, J. B. A. G. (2012). DNA Vaccines and Intradermal Vaccination by DNA Tattooing. *Curr. Top Microbiol. Immunol.* 351, 221–250. doi: 10.1007/82_2010_117

Paiva, M. B., Ribeiro-Romão, R. P., Resende-Vieira, L., Braga-Gomes, T., Oliveira, M. P., Saavedra, A. F., et al. (2021). A cytokine network balance influences the fate of *Leishmania* (*Viannia*) *braziliensis* infection in a cutaneous leishmaniasis hamster model. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.656919

Pan American Health Organization (2013). *Leishmaniasis en las Américas. Recomendaciones para el tratamiento*. 1st ed., ed. Pan American Health Organization (Washington, D.C.: Pan American Health Organization).

Pan American Health Organization (2021) *Leishmanioses: informe epidemiológico das américas. pan American health organization*. Available at: https://iris.paho.org/handle/10665.2/51742 (Accessed July 28, 2022).

Pimentel, M. I. F., Baptista, C., Rubin, É.F., Vasconcellos, É.de C.F., Lyra, M. R., de Matos Salgueiro, M., et al. (2011). Leishmaniose cutânea Americana causada pela *Leishmania* (*Viannia*) *braziliensis* resistente ao antimoniato de meglumina e com boa resposta terapêutica à pentamidina: Relato de um caso. *Rev. Soc. Bras. Med. Trop.* 44, 254–256. doi: 10.1590/S0037-86822011000200026

Pinho, B. R., Sousa, C., Valentão, P., and Andrade, P. B. (2011). Is nitric oxide decrease observed with naphthoquinones in LPS stimulated RAW 264.7 macrophages a beneficial property? *PloS One* 6, 1–9. doi: 10.1371/journal.pone.0024098

Platteel, A. C. M., Henri, S., Zaiss, D. M., and Sijts, A. J. A. M. (2017). Dissecting antigen processing and presentation routes in dermal vaccination strategies. *Vaccine* 35, 7057–7063. doi: 10.1016/j.vaccine.2017.10.044

Ponte-Sucre, A., Gamarro, F., Dujardin, J. C., Barrett, M. P., López-Vélez, R., García-Hernández, R., et al. (2017). Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PloS Negl. Trop. Dis.* 11, 1–24. doi: 10.1371/journal.pntd.0006052

Pradhan, R., Dandawate, P., Vyas, A., Padhye, S., Biersack, B., Schobert, R., et al. (2012). From body art to anticancer activities: perspectives on medicinal properties of henna. *Curr. Drug Targets* 13, 1777–1798. doi: 10.2174/138945012804545588

Qiu, H.-Y., Wang, P.-F., Lin, H.-Y., Tang, C.-Y., Zhu, H.-L., and Yang, Y.-H. (2018). Naphthoquinones: A continuing source for discovery of therapeutic antineoplastic agents. *Chem. Biol. Drug Des.* 91, 681–690. doi: 10.1111/cbdd.13141

Reimão, J. Q., Colombo, F. A., Pereira-Chioccola, V. L., and Tempone, A. G. (2012). Effectiveness of liposomal buparvaquone in an experimental hamster model of *Leishmania* (*L.*) *infantum chagasi*. *Exp. Parasitol.* 130, 195–199. doi: 10.1016/j.exppara.2012.01.010

Rezende, L. C. D., Fumagalli, F., Bortolin, M. S., de Oliveira, M. G., de Paula, M. H., de Andrade-Neto, V. F., et al. (2013). *In vivo* antimalarial activity of novel 2-hydroxy-3-anilino-1,4-naphthoquinones obtained by epoxide ring-opening reaction. *Bioorg. Med. Chem. Lett.* 23, 4583–4586. doi: 10.1016/j.bmcl.2013.06.033

Ribeiro, G. A., Cunha-Junior, E. F., Pinheiro, R. O., da-Silva, S. A. G., Canto-Cavalheiro, M. M., da Silva, A. J. M., et al. (2013). LQB-118, an orally active pterocarpanquinone, induces selective oxidative stress and apoptosis in *Leishmania amazonensis*. *J. Antimicrobial Chemother.* 68, 789–799. doi: 10.1093/jac/dks498

Ribeiro-Romão, R. P., Moreira, O. C., Osorio, E. Y., Cysne-Finkelstein, L., Gomes-Silva, A., Valverde, J. G., et al. (2014). Comparative evaluation of lesion development, tissue damage, and cytokine expression in golden hamsters (*Mesocricetus auratus*) infected by inocula with different *Leishmania* (*Viannia*) *braziliensis* concentrations. *Infect. Immun.* 82, 5203–5213. doi: 10.1128/IAI.02083-14

Rocha, M. N., Nogueira, P. M., Demicheli, C., de Oliveira, L. G., da Silva, M. M., Frézard, F., et al. (2013). Cytotoxicity and *In vitro* antileishmanial activity of antimony (V), bismuth (V), and tin (IV) complexes of lapachol. *Bioinorg Chem. Appl.* 2013, 1–7. doi: 10.1155/2013/961783

Rodrigues, B. C., Ferreira, M. F., Barroso, D. H., da Motta, J. O. C., de Paula, C. D. R., Porto, C., et al. (2020). A retrospective cohort study of the effectiveness and adverse events of intralesional pentavalent antimonials in the treatment of cutaneous leishmaniasis. *Int. J. Parasitol. Drugs Drug Resist.* 14, 257–263. doi: 10.1016/j.ijpddr.2020.11.002

Sadeghinia, A., and Sadeghinia, S. (2012). Comparison of the efficacy of intralesional triamcinolone acetonide and 5-fluorouracil tattooing for the treatment of keloids. *Dermatologic Surg.* 38, 104–109. doi: 10.1111/j.1524-4725.2011.02137.x

Salas, C. O., Faundez, M., Morello, A., Diego Maya, J., and XXXA. Tapia, R. (2011). Natural and synthetic naphthoquinones active against *Trypanosoma cruzi*: An initial step towards new drugs for chagas disease. *Curr. Med. Chem.* 18, 144–161. doi: 10.2174/092986711793979779

Santos, D. O., Coutinho, C. E. R., Madeira, M. F., Bottino, C. G., Vieira, R. T., Nascimento, S. B., et al. (2008). Leishmaniasis treatment - a challenge that remains: A review. *Parasitol. Res.* 103, 1–10. doi: 10.1007/s00436-008-0943-2

Sharma, G., Chowdhury, S., Sinha, S., Majumder, H. K., and Kumar, S. V. (2014). Antileishmanial activity evaluation of bis-lawsone analogs and DNA topoisomerase-I inhibition studies. *J. Enzyme Inhib Med. Chem.* 29, 185–189. doi: 10.3109/14756366.2013.765413

Shio, M. T., Paquet, M., Martel, C., Bosschaerts, T., Stienstra, S., Olivier, M., et al. (2014). Drug delivery by tattooing to treat cutaneous leishmaniasis. *Sci. Rep.* 4, 1–7. doi: 10.1038/srep04156

Souza, M. A., Johann, S., dos Santos Lima, L. A. R., Campos, F. F., Mendes, I. C., Beraldo, H., et al. (2013). The antimicrobial activity of lapachol and its thiosemicarbazone and semicarbazone derivatives. *Mem Inst Oswaldo Cruz* 108, 342–351. doi: 10.1590/S0074-02762013000300013

Sunassee, S. N., Veale, C. G. L., Shunmoogam-Gounden, N., Osoniyi, O., Hendricks, D. T., Caira, M. R., et al. (2013). Cytotoxicity of lapachol, β-lapachone and related synthetic 1,4-naphthoquinones against oesophageal cancer cells. *Eur. J. Med. Chem.* 62, 98–110. doi: 10.1016/j.ejmech.2012.12.048

Teixeira, M. J., de Almeida, Y. M., Viana, J. R., Holanda Filha, J. G., Rodrigues, T. P., Prata, J. R. C.Jr., et al. (2001). *In vitro* and *in vivo* leishmanicidal activity of 2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (Lapachol). *Phytother. Res.* 15, 44–48. doi: 10.1002/1099-1573(200102)15:1<44::AID-PTR685>3.0.CO;2-1

van de Wall, S., Walczak, M., van Rooij, N., Hoogeboom, B. N., Meijerhof, T., Nijman, H. W., et al. (2015). Tattoo delivery of a semliki forest virus-based vaccine encoding human papillomavirus E6 and E7. *Vaccines (Basel)* 3, 221–238. doi: 10.3390/vaccines3020221

Wei, X., Li, M., Ma, M., Jia, H., Zhang, Y., Kang, W., et al. (2017). Induction of apoptosis by FFJ-5, a novel naphthoquinone compound, occurs *via* downregulation of PKM2 in A549 and HepG2 cells. *Oncol. Lett.* 13, 791–799. doi: 10.3892/ol.2016.5522

World Health Organization (2022) *Leishmaniasis. world health organization*. Available at: http://www.who.int/leishmaniasis/en/.

Yang, X., Zhao, Y., Chen, X., Jiang, B., and Sun, D. (2013). The protective effect of recombinant *Lactococcus lactis oral* vaccine on a *Clostridium difficile*-infectedanimal model. *BMC Gastroenterologia* 13, 1–13. doi: 10.1186/2046-1682-4-13

Yetisen, A. K., Moreddu, R., Seifi, S., Jiang, N., Vega, K., Dong, X., et al. (2019). Dermal tattoo biosensors for colorimetric metabolite detection. *Angewandte Chemie - Int. Edition* 58, 10506–10513. doi: 10.1002/anie.201904416

Yigit, B., Kabul Gurbulak, E., and Ton Eryilmaz, O. (2022). Usefulness of endoscopic tattooing before neoadjuvant therapy in patients with clinical complete response in locally advanced rectal cancer for providing a safe distal surgical margin. *J. Laparoendoscopic Advanced Surg. Techniques* 32, 506–514. doi: 10.1089/lap.2021.0382

Zulfiqar, B., Shelper, T. B., and Avery, V. M. (2017). Leishmaniasis drug discovery: recent progress and challenges in assay development. *Drug Discovery Today* 22, 1516–1531. doi: 10.1016/j.drudis.2017.06.004

Zu, X., Xie, X., Zhang, Y., Liu, K., Bode, A. M., Dong, Z., et al. (2019). Lapachol is a novel ribosomal protein S6 kinase 2 inhibitor that suppresses growth and induces intrinsic apoptosis in esophageal squamous cell carcinoma cells. *Phytother. Res.* 33, 2337–2346. doi: 10.1002/ptr.6415

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 05 April 2023
DOI 10.3389/fcimb.2023.1066407

OPEN ACCESS

EDITED BY
Taís Fontoura de Almeida,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Daniela Melo Resende,
Oswaldo Cruz Foundation (Fiocruz), Brazil
Vítor Ennes-Vidal,
Oswaldo Cruz Institute (Fiocruz), Brazil
Patrícia Fampa Negreiros Lima,
Federal Rural University of Rio de Janeiro, Brazil
Paulo Pitasse-Santos,
University of Leicester, United Kingdom

*CORRESPONDENCE
Elmo E. Almeida-Amaral
elmo@ioc.fiocruz.br

SPECIALTY SECTION
This article was submitted to Parasite and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 10 October 2022
ACCEPTED 07 March 2023
PUBLISHED 05 April 2023

CITATION
Emiliano YSS and Almeida-Amaral EE (2023) Apigenin is a promising molecule for treatment of visceral leishmaniasis. *Front. Cell. Infect. Microbiol.* 13:1066407. doi: 10.3389/fcimb.2023.1066407

Apigenin is a promising molecule for treatment of visceral leishmaniasis

Yago S. S. Emiliano and Elmo E. Almeida-Amaral*

Laboratório de Bioquímica de Tripanosomatideos, Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brazil

Current treatment for visceral leishmaniasis is based on drugs such as pentavalent antimony and amphotericin B. However, this treatment remains mostly ineffective and expensive, resulting in several side effects and generating resistance. Apigenin, a flavonoid present in fruits and vegetables, has demonstrated several biological functions. In the present study, we observed a concentration-dependent inhibition of the *L. infantum* promastigote in the presence of apigenin, exhibiting an IC_{50} value of 29.9 μM. Its effect was also evaluated in *L. infantum*-infected murine peritoneal macrophages, presenting an C_{50} value against intracellular amastigotes of 2.3 μM and a selectivity index of 34.3. In a murine model of visceral leishmaniasis, the *in vivo* effect of apigenin was measured using short-term and long-term treatment schemes. Treatment with apigenin demonstrated 99.7% and 94% reductions in the liver parasite load in the short-term and long-term treatment schemes, respectively. Furthermore, no alterations in serological and hematological parameters were observed. Taken together, these results suggest that apigenin is a potential candidate for visceral leishmaniasis chemotherapy by oral administration.

KEYWORDS

natural products, oral treatment, flavonoid, short-term, long-term, *in vivo*, *in vitro*, leishmaniasis chemotherapy

Introduction

Leishmaniasis is a neglected tropical disease that is endemic in 98 countries and present in 200 territories, and 12 million people are affected worldwide. Visceral leishmaniasis is a form of this disease with a 95% fatality rate in untreated cases, and it is estimated that 30,000 new cases occur annually (Ruiz-Postigo et al., 2021). This disease has a high prevalence in Brazil, where 96% of cases are reported (Pan American Health Organization, 2018).

In endemic areas of visceral leishmaniasis, a rise in Leishmania–HIV coinfection has been observed. Visceral leishmaniasis accelerates HIV replication and progression, and HIV can influence the disease's epidemiology, clinical manifestations, and course. Additionally, *Leishmania*–HIV coinfected patients are more difficult to treat, and they do not respond well to standard treatments, facing more frequent and more severe side

effects and higher risks of disease recurrence and death (Alvar et al., 2008; van Griensven and Diro, 2012; DNDi, 2022).

Current treatment for visceral leishmaniasis is based on drugs such as pentavalent antimonials, which have been used as first-line agents for many years, and amphotericin B deoxycholate and lipid formulation amphotericin B (Uliana et al., 2017). These drugs are administered intramuscularly, leading to long-established administration, high cost, and high toxicity. Additionally, pentavalent antimonials are becoming increasingly ineffective due to resistance, and liposomal amphotericin B is a high-cost formulation, making this drug prohibitive in several endemic regions (Uliana et al., 2017; Zulfiqar et al., 2017). Miltefosine has emerged as an alternative treatment for visceral leishmaniasis; however, it is expensive and teratogenic, and its use is not licensed worldwide (Rijal et al., 2013). Therefore, it is necessary to search for new alternatives for the treatment of visceral leishmaniasis. In this context, several natural products have been demonstrated to have antileishmanial activities (Gervazoni et al., 2020).

We had previously demonstrated that apigenin, a flavonoid present in common fruits and vegetables, such as parsley, lemons and berries, has antiparasitic activity *in vitroin vitro* against *Leishmania amazonensis*-infected macrophages and can be used as an oral treatment in an experimental model of cutaneous leishmaniasis caused by *L. amazonensis* (Fonseca-Silva et al., 2016; Emiliano and Almeida-Amaral, 2018). In the present study, we investigated the *in vitro* activity of apigenin against promastigote and intracellular amastigotes of *Leishmania infantum*, the etiological agent of visceral leishmaniasis in the New World, and its *in vivo* activity in an experimental model of visceral leishmaniasis caused by *L. infantum*.

Materials and methods

Test compound and reagent

Apigenin (molecular formula: C15H10O5; molecular weight: 270.24 g/mol; purity ≥95%; lot WE445301/1) and other reagents were obtained from Merck KGaA. Apigenin was diluted in dimethyl sulfoxide (DMSO) such that the solvent concentration did not exceed 0.2% in the final solution and added to the culture medium. Endotoxin-free sterile disposables were used in all experiments. The chemical structure of apigenin is presented in Supplementary Figure 1.

Ethics statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto Oswaldo Cruz (CEUA-IOC, License Number: L-11/2017 A2).

Parasites and mice

In this study, we used a strain of *L. infantum* (MHOM/MA/67/ITMAP263). *L. infantum* promastigotes were cultivated at 26°C in Schneider's *Drosophila* medium (pH 6.9) supplemented with 20% fetal bovine serum (v/v), 100 µg/mL streptomycin, and 100 U/mL penicillin. Parasite maintenance was promoted by culture passage every 3 days. Female BALB/c mice (8-10 weeks; provided by the Instituto Ciencias e Tecnologia em Biomodelos, ICTB/FIOCRUZ) were used in this study. All animals were bred and maintained at the Instituto Oswaldo Cruz according to the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA).

Promastigote proliferation assay

L. infantum promastigotes (1.0 x 10^6 cell/mL) were incubated in the absence (DMSO 0.2%) or presence of different concentrations of apigenin (12 µM, 24 µM, 36 µM, 48 µM, 60 µM, 72 µM, 80 µM and 96 µM) for 72 h. Cellular proliferation was determined by the Alamar Blue assay. Fluorescence was monitored at excitation and emission wavelengths of 560 and 590 nm, respectively, using a spectrofluorometer. The 50% inhibitory concentration (IC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The experiments were performed in triplicate.

Leishmania-macrophage interaction assay

Peritoneal macrophages were collected from BALB/c mice in RPMI 1640 medium and plated at 2.0 x 10^6 macrophages/mL (0.4 mL/well) onto Lab-Tek eight-chamber slides for 1 h at 37°C in an atmosphere of 5% CO_2. Stationary-phase *L. infantum* promastigotes were washed with PBS, counted in a Neubauer chamber, and added to macrophages at a multiplicity of infection (MOI) of 5:1 for 5 h at 37°C in an atmosphere of 5% CO_2. Then, 2% heat-inactivated horse serum (v/v) was added for 18 hours (Inacio et al., 2019). After this, successive washes with RPMI 1640 medium were performed to remove free parasites. Then, *L. infantum*-infected macrophages were incubated in the absence (DMSO 0.2%) or presence of different concentrations of apigenin (3 µM, 6 µM, 12 µM and 24 µM) for 72 hours and stained using Instant Prov (Newprov, Curitiba/Brazil). The percentage of infected macrophages was determined by light microscopy, and at least 200 cells on each coverslip were randomly counted in duplicate. The results are expressed as the infection index (% infected macrophages x number of amastigotes/total number of macrophages). The IC_{50} was determined by logarithmic regression analysis using GraphPad Prism 7. The selectivity index (SI) was obtained as murine peritoneal macrophage CC_{50}/intracellular amastigote IC_{50}. The macrophage CC_{50} for apigenin is 78.7 µM (Fonseca-Silva et al., 2016).

In vivo infection using a murine model of visceral leishmaniasis and parasite load quantification

BALB/c mice (five animals per group) were maintained under specific pathogen-free conditions. The animals were infected *via* the peritoneum with 100 µL of stationary-phase *L. infantum* promastigotes at a concentration of 1.0 x 10^8 cells/ml as described (Cunha-Junior et al., 2016). At 7 days post-infection, the animals were separated into three different groups (control group, apigenin-treated group, and positive control group). The positive control group was treated using meglumine antimoniate. At the end of the treatment, mice were euthanized, and the livers were removed, weighed, and macerated in Schneider's medium with 20% FBS for analysis of the parasite load by limiting dilution assay (LDA). The number of viable parasites in each liver was estimated from the highest dilution at which promastigotes could be grown after 7 days of incubation at 26°C (Inacio et al., 2019).

Short-term therapeutic scheme

In the short-term therapeutic treatment scheme, the infected mice, divided into three groups, were treated with vehicle (DMSO; 0.2% v/v), which was incorporated in an oral suspension and administered orally by gavage twice per day (control group); apigenin (1 mg/kg diluted in DMSO 0.2% v/v), which was incorporated in an oral suspension and administered orally by gavage twice per day, reaching a maximum daily dose of 2 mg/kg/day (apigenin-treated group); and meglumine antimoniate, which was administered intramuscularly once per day at a dose of 200 mg Sb^{5+}/kg/day (positive control group). These treatments started 7 days post-infection and lasted 5 days. At the end of the experiment (Day 14), the animals were euthanized, and the liver was obtained for analysis of the parasite load by limiting dilution assay (LDA) as described above.

Long-term therapeutic scheme

In the long-term therapeutic treatment scheme, *L. infantum*-infected mice were divided into three groups and treated with vehicle (DMSO 0.2% v/v), which was incorporated in an oral suspension and administered orally by gavage twice per day (control group); apigenin (1 mg/kg diluted in DMSO 0.2% v/v), which was incorporated in an oral suspension and administered orally twice per day by gavage, reaching a maximum daily dose of 2 mg/kg/day (apigenin-treated group); and meglumine antimoniate, which was administered intramuscularly once per day at a dose of 100 mg Sb^{5+}/kg/day (positive control group). The animals were treated for 5 consecutive days beginning 7 days post-infection. At 18 days after treatment (Day 30), mice were euthanized, and the livers were obtained for analysis of the parasite load by limiting dilution assay (LDA) as described above.

Toxicology

Before euthanasia, BALB/c mice were anesthetized with a solution of ketamine (200 mg/kg) and xylazine (16 mg/kg) administered intraperitoneally. Blood was collected (1 mL) *via* cardiac puncture and distributed in EDTA-containing microtubes for hematological analysis or centrifuged to obtain serum. Both serum (toxicology markers) and total blood (hematological parameters) from the infected BALB/c mice treated as described above were measured by Technological Platforms Network -FIOCRUZ, Platforms of Clinical Analysis in Laboratory Animals - RPT12C.

Statistical analysis

The data were analyzed using the Mann–Whitney test or one-way analysis of variance (ANOVA) followed by Tukey's post-test in GraphPad Prism 7 (GraphPad Software). The results were considered significant at $p \leq 0.05$. The data are expressed as the means ± standard errors.

Results

To evaluate the *in vitro* effect of apigenin, promastigote forms and intracellular amastigote forms of *L. infantum* were used. Promastigotes were incubated in the absence or presence of different concentrations of apigenin (12-96 µM) for 72 h (Figure 1). Apigenin inhibited the cellular proliferation of *L. infantum* in a concentration-dependent manner, reaching 94.6% inhibition at the highest concentration (96 µM) with an IC_{50} value of 29.9 µM.

To determine the effect of apigenin on *L. infantum* intracellular amastigotes, peritoneal BALB/c mouse macrophages were infected with promastigotes of *L. infantum* for 5 h and then incubated with increasing concentrations of apigenin (3-24 µM) for 72 h. Apigenin was able to reduce the infection index in a concentration-dependent manner (Figure 2). The IC_{50} was 2.3 µM, reaching 88% inhibition at the highest concentration (24 µM). The reported CC_{50} value of apigenin is 78.7 µM (Fonseca-Silva et al., 2016), and the selectivity index (SI) was calculated to be 34.3.

According to the observed *in vitro* effect of apigenin, the efficacy of this molecule *in vivo* was evaluated using a murine model of visceral leishmaniasis using two therapeutic schemes. The first was short-term, where *L. infantum*-infected BALB/c mice were treated for 5 days beginning at 7 days post-infection and euthanized at 2 days post-treatment (Day 14). In the long-term therapeutic scheme, *L. infantum*-infected BALB/c mice were also treated for 5 days beginning at 7 days post-infection, but euthanization occurred at 18 days post-treatment (Day 30) (Le Fichoux et al., 1998).

As shown in Figure 3, during the short-term treatment, apigenin orally administered to *L. infantum*-infected BALB/c mice reduced the liver-parasite load, reaching 99.7% inhibition. However, we did not

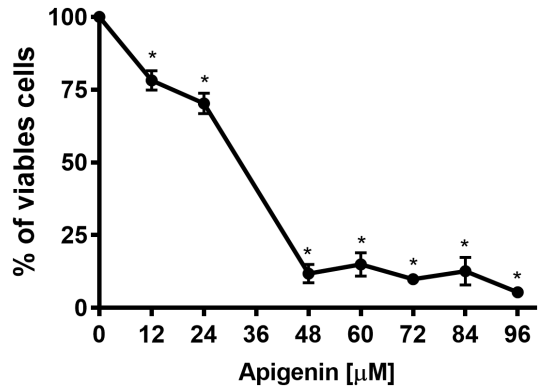


FIGURE 1
Effect of apigenin on *L. infantum* promastigotes. *L. infantum* promastigotes were incubated in Schneider's *Drosophila* medium in the absence or presence of increasing concentrations of apigenin (12-96 μM) for 72 h. Cellular proliferation was evaluated using the Alamar Blue assay. The values are presented as the mean ± standard error of three different experiments. * indicates a significant difference relative to the control ($p < 0.001$).

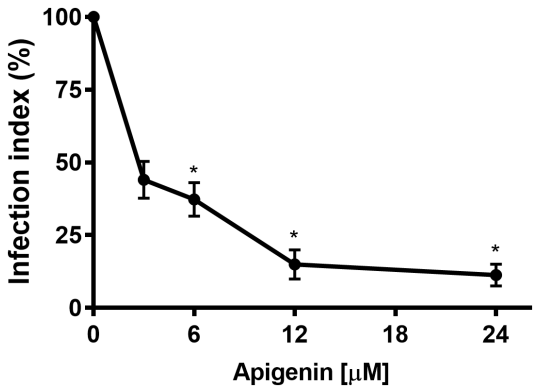


FIGURE 2
Effect of apigenin on *L. infantum*-infected macrophages. *L. infantum*-infected macrophages were incubated in the absence or presence of increasing concentrations of apigenin (3-24 μM) for 72 h. In control samples (absence of apigenin), a similar volume of vehicle (0.2% DMSO) was added to the cells. The infection index was determined using light microscopy to count at least 200 macrophages in each duplicated coverslip. The values presented refer to the mean ± standard error of three different experiments. * indicates a significant difference relative to the control ($p < 0.05$).

observe any significant differences between mice treated with apigenin and those treated with meglumine antimoniate.

Furthermore, serological toxicology parameters, such as total levels of albumin, creatinine kinase, urea, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, iron, calcium, sodium, and potassium (Supplementary Table 1), and several hematological parameters (Supplementary Table 2) were evaluated, and no significant alterations were observed, suggesting a lack of toxicity.

In the long-term therapeutic scheme (Figure 4), oral administration of apigenin to *L. infantum*-infected BALB/c mice resulted in a 94% reduction in the liver parasite load. Furthermore, differences between the infected mice treated with apigenin and meglumine antimoniate were observed in terms of parasite load, and meglumine antimoniate reduced the liver parasite load by only 55%.

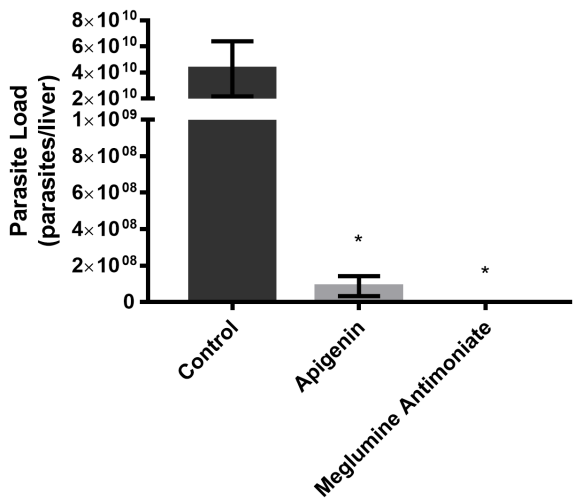


FIGURE 3
Short-term therapeutic efficacy of apigenin against *L. infantum*-infected BALB/c mice. Parasitic load of *L. infantum*-infected BALB/c mice (5 animals per group) untreated or treated with apigenin or meglumine antimoniate. Liver parasite load was estimated by a limiting dilution assay (LDA). These data represent one independent experiment with five mice per group (n = 5). * indicates a significant difference relative to the control group ($p < 0.01$).

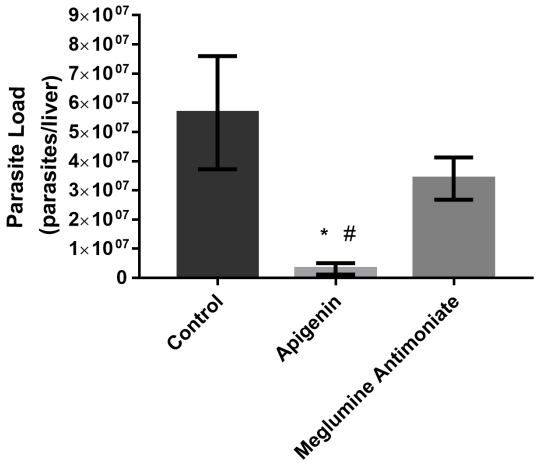


FIGURE 4
Long-term therapeutic efficacy of apigenin against *L. infantum*-infected BALB/c mice. Parasitic load of *L. infantum*-infected BALB/c mice (five animals per group) untreated or treated with apigenin or meglumine antimoniate. The liver parasite load was estimated by a limiting dilution assay (LDA). These data represent one independent experiment with five mice per group (n = 5). * indicates a significant difference relative to the control group ($p < 0.01$). # indicates a significant difference relative to the meglumine antimoniate group (positive control group) ($p < 0.01$).

Discussion

Current visceral leishmaniasis chemotherapy has presented many problems, such as several collateral effects, increased ineffectiveness due to resistance, and high cost making these drugs unaffordable in several endemic regions. Therefore, more research is necessary to improve this drug arsenal, giving priority to compounds with greater efficacy, reduced toxicity, and better affordability. Natural products have become an interesting alternative, and flavonoids have been studied as promising compounds for leishmaniasis treatment (Fonseca-Silva et al., 2011; Inacio et al., 2012; Fonseca-Silva et al., 2013; Inacio et al., 2013; Ndjonka et al., 2013; Inacio et al., 2014; Fonseca-Silva et al., 2015; Fonseca-Silva et al., 2016; Emiliano and Almeida-Amaral, 2018; Gervazoni et al., 2018; Inacio et al., 2019; Gervazoni et al., 2020). Apigenin has demonstrated a key role in the treatment of some health issues, such as diabetes, amnesia and Alzheimer's disease, depression, insomnia, and cancer (Salehi et al., 2019). Moreover, the activity of apigenin has been described against *L. donovani*, *T. brucei rhodesiense*, *T. cruzi*, *Encephalitozoon intestinalis* and *Cryptosporidium parvum* (Mead and McNair, 2006; Tasdemir et al., 2006).

Cellular proliferation of the promastigote forms of *L. infantum* in the presence of apigenin was inhibited in a concentration-dependent manner, presenting an IC_{50} of 29.9 μM. A similar effect of apigenin was observed in the promastigote forms of *L. amazonensis*, which showed an IC_{50} value of 23.7 μM (Fonseca-Silva et al., 2015).

Some conditions for the eligibility of a new drug for the treatment of visceral leishmaniasis have been described. The new drug should have an IC_{50} less than 10 μM against intracellular amastigotes; be, at a minimum, tenfold more active against intracellular amastigotes than against mammalian cells (SI ≥ 10); be active *in vivo*; and cause a reduction in liver parasite load more than 70% in a relevant small animal model after at most 5 doses at 50 mg/kg, preferably when administered by the oral route once or twice per day (Pink et al., 2005; Katsuno et al., 2015).

In the present study, we demonstrated that apigenin was active against intracellular amastigotes, with an IC_{50} of 2.3 μM and a selectivity index of 34.3. We also observed that the treatment of BALB/c mice infected with *L. infantum* with apigenin (1 mg/kg twice a day, reaching a maximum daily dose of 2 mg/kg/day, orally administered) was capable of reducing the liver parasite load by greater than 70% in both therapeutic schemes (short-term and long-term). It is important to point out that the treatment of apigenin did not compromise the overall health of the infected mice, satisfying all the criteria described above. A similar effect was observed with (-)-epigallocatechin 3-*O*-gallate, an abundant flavonoid constituent of green tea, that had an IC_{50} value of 2.6 μM for intracellular amastigotes and demonstrated a reduction of the liver parasite load, reaching 98.7% inhibition at 50 mg/kg/day (Inacio et al., 2019).

Taken together, the results indicate apigenin as a new compound for the treatment of visceral leishmaniasis and support further studies to determine the ideal therapeutic and optimal drug dose regimen.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by L-11/2017 A2.

Author contributions

Conceptualization, YE and EA-A; Methodology, YE and EA-A; Validation, YE and EA-A; Formal analysis, YE; Investigation, YE and EA-A; Data curation, YE and EA-A; Writing—original draft preparation, YE and EA-A; Writing—review and editing, EA-A; Supervision, EA-A. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Oswaldo Cruz (IOC), Coordenação de Aperfeiçoamento de Pessoal da Nivel Superior (CAPES) and Fundação Oswaldo Cruz (FIOCRUZ). YSSE received a scholarship from CAPES, and EEA-A is the recipient of a research scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We are grateful for the support given by Instituto Oswaldo Cruz (IOC), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Fundação Oswaldo Cruz (Fiocruz).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewers DR and VEV declared a shared affiliation with the authors to the handling editor at the time of review.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1066407/full#supplementary-material

References

Alvar, J., Aparicio, P., Aseffa, A., Den Boer, M., Canavate, C., Dedet, J. P., et al. (2008). The relationship between leishmaniasis and AIDS: The second 10 years. *Clin. Microbiol. Rev.* 21 (2), 334–359. doi: 10.1128/CMR.00061-07

Cunha-Junior, E. F., Martins, T. M., Canto-Cavalheiro, M. M., Marques, P. R., Portari, E. A., Coelho, M. G., et al. (2016). Preclinical studies evaluating subacute toxicity and therapeutic efficacy of LQB-118 in experimental visceral leishmaniasis. *Antimicrob. Agents Chemother.* 60 (6), 3794–3801. doi: 10.1128/AAC.01787-15

DNDi (2022). "DNDi 2021 annual report - unlocking the promise of medical innovation for all," in *Anual report*. (Geneva, Switzerland: Drugs for Neglected Diseases initiative)

Emiliano, Y. S. S., and Almeida-Amaral, E. E. (2018). Efficacy of apigenin and miltefosine combination therapy against experimental cutaneous leishmaniasis. *J. Nat. Prod.* 81 (8), 1910–1913. doi: 10.1021/acs.jnatprod.8b00356

Fonseca-Silva, F., Canto-Cavalheiro, M. M., Menna-Barreto, R. F., and Almeida-Amaral, E. E. (2015). Effect of apigenin on leishmania amazonensis is associated with reactive oxygen species production followed by mitochondrial dysfunction. *J. Nat. Prod.* 78 (4), 880–884. doi: 10.1021/acs.jnatprod.5b00011

Fonseca-Silva, F., Inacio, J. D., Canto-Cavalheiro, M. M., and Almeida-Amaral, E. E. (2011). Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in leishmania amazonensis. *PloS One* 6 (2), e14666. doi: 10.1371/journal.pone.0014666

Fonseca-Silva, F., Inacio, J. D., Canto-Cavalheiro, M. M., and Almeida-Amaral, E. E. (2013). Reactive oxygen species production by quercetin causes the death of leishmania amazonensis intracellular amastigotes. *J. Nat. Prod.* 76 (8), 1505–1508. doi: 10.1021/np400193m

Fonseca-Silva, F., Inacio, J. D., Canto-Cavalheiro, M. M., Menna-Barreto, R. F., and Almeida-Amaral, E. E. (2016). Oral efficacy of apigenin against cutaneous leishmaniasis: Involvement of reactive oxygen species and autophagy as a mechanism of action. *PloS Negl. Trop. Dis.* 10 (2), e0004442. doi: 10.1371/journal.pntd.0004442

Gervazoni, L. F. O., Barcellos, G. B., Ferreira-Paes, T., and Almeida-Amaral, E. E. (2020). Use of natural products in leishmaniasis chemotherapy: An overview. *Front. Chem.* 8 (1031). doi: 10.3389/fchem.2020.579891

Gervazoni, L. F. O., Goncalves-Ozorio, G., and Almeida-Amaral, E. E. (2018). 2'-hydroxyflavanone activity *in vitro* and *in vivo* against wild-type and antimony-resistant leishmania amazonensis. *PloS Negl. Trop. Dis.* 12 (12), e0006930. doi: 10.1371/journal.pntd.0006930

Inacio, J. D., Canto-Cavalheiro, M. M., and Almeida-Amaral, E. E. (2013). *In vitro* and *in vivo* effects of (-)-epigallocatechin 3-o-gallate on leishmania amazonensis. *J. Nat. Prod.* 76 (10), 1993–1996. doi: 10.1021/np400624d

Inacio, J. D., Canto-Cavalheiro, M. M., Menna-Barreto, R. F., and Almeida-Amaral, E. E. (2012). Mitochondrial damage contribute to epigallocatechin-3-gallate induced death in leishmania amazonensis. *Exp. Parasitol.* 132 (2), 151–155. doi: 10.1016/j.exppara.2012.06.008

Inacio, J. D. F., Fonseca, M. S., and Almeida-Amaral, E. E. (2019). (-)-Epigallocatechin 3-O-Gallate as a new approach for the treatment of visceral leishmaniasis. *J. Nat. Prod.* 82 (9), 2664–2667. doi: 10.1021/acs.jnatprod.9b00632

Inacio, J. D., Gervazoni, L., Canto-Cavalheiro, M. M., and Almeida-Amaral, E. E. (2014). The effect of (-)-epigallocatechin 3-O–gallate *in vitro* and *in vivo* in leishmania braziliensis: Involvement of reactive oxygen species as a mechanism of action. *PloS Negl. Trop. Dis.* 8 (8), e3093. doi: 10.1371/journal.pntd.0003093

Katsuno, K., Burrows, J. N., Duncan, K., van Huijsduijnen, R. H., Kaneko, T., Kita, K., et al. (2015). Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nat. Rev. Drug Discov.* 14, 751. doi: 10.1038/nrd4683

Le Fichoux, Y., Rousseau, D., Ferrua, B., Ruette, S., Lelievre, A., Grousson, D., et al. (1998). Short- and long-term efficacy of hexadecylphosphocholine against established leishmania infantum infection in BALB/c mice. *Antimicrob. Agents Chemother.* 42 (3), 654–658. doi: 10.1128/AAC.42.3.654

Mead, J., and McNair, N. (2006). Antiparasitic activity of flavonoids and isoflavones against cryptosporidium parvum and encephalitozoon intestinalis. *FEMS Microbiol. Lett.* 259 (1), 153–157. doi: 10.1111/j.1574-6968.2006.00263.x

Ndjonka, D., Rapado, L. N., Silber, A. M., Liebau, E., and Wrenger, C. (2013). Natural products as a source for treating neglected parasitic diseases. *Int. J. Mol. Sci.* 14 (2), 3395–3439. doi: 10.3390/ijms14023395

Pan American Health Organization (2018). *Leishmaniasis: Epidemiological report in the americas* (Washington: Pan American Health Organization).

Pink, R., Hudson, A., Mouries, M. A., and Bendig, M. (2005). Opportunities and challenges in antiparasitic drug discovery. *Nat. Rev. Drug Discov.* 4 (9), 727–740. doi: 10.1038/nrd1824

Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N. R., Dorlo, T. P., et al. (2013). Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin. Infect. Dis.* 56 (11), 1530–1538. doi: 10.1093/cid/cit102

Ruiz-Postigo, J. A., Jain, S., Mikhailov, A., Maia-Elkhoury, A. N., Valadas, S., Warusavithana, S., et al. (2021). "Global leishmaniasis surveillance: 2019–2020, a baseline for the 2030 roadmap," in *Weekly epidemiological record* (Geneva, Switzerland: WHO). C.o.N.T. Diseases.

Salehi, B., Venditti, A., Sharifi-Rad, M., Kregiel, D., Sharifi-Rad, J., Durazzo, A., et al. (2019). The therapeutic potential of apigenin. *Int. J. Mol. Sci.* 20 (6), 1–26. doi: 10.3390/ijms20061305

Tasdemir, D., Kaiser, M., Brun, R., Yardley, V., Schmidt, T. J., Tosun, F., et al. (2006). Antitrypanosomal and antileishmanial activities of flavonoids and their analogues: *In vitro*, *in vivo*, structure-activity relationship, and quantitative structure-activity relationship studies. *Antimicrob. Agents Chemother.* 50 (4), 1352–1364. doi: 10.1128/AAC.50.4.1352-1364.2006

Uliana, S. R., Trinconi, C. T., and Coelho, A. C. (2017). Chemotherapy of leishmaniasis: Present challenges. *Parasitology* 145, 1–17. doi: 10.1017/S0031182016002523

van Griensven, J., and Diro, E. (2012). Visceral leishmaniasis. *Infect. Dis. Clin. North Am.* 26 (2), 309–322. doi: 10.1016/j.idc.2012.03.005

Zulfiqar, B., Shelper, T. B., and Avery, V. M. (2017). Leishmaniasis drug discovery: Recent progress and challenges in assay development. *Drug Discov. Today* (22), 1516–1531. doi: 10.1016/j.drudis.2017.06.004

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 12 June 2023
DOI 10.3389/fcimb.2023.1192800

OPEN ACCESS

EDITED BY
Lucia Helena Pinto da Silva,
Federal Rural University of Rio de Janeiro, Brazil

REVIEWED BY
Patrícia Fampa Negreiros Lima,
Federal Rural University of Rio de Janeiro, Brazil
JanCarlo Delorenzi,
Mackenzie Presbyterian University, Brazil

*CORRESPONDENCE
Robson Coutinho-Silva
rcsilva@biof.ufrj.br

RECEIVED 23 March 2023
ACCEPTED 15 May 2023
PUBLISHED 12 June 2023

CITATION
Noronha LPT, Martins MDA, Castro-Junior AB, Thorstenberg ML, Costa-Soares L, Rangel TP, Carvalho-Gondim F, Rossi-Bergmann B, Savio LEB, Canetti CdA and Coutinho-Silva R (2023) Cysteinyl-leukotrienes promote cutaneous Leishmaniasis control. *Front. Cell. Infect. Microbiol.* 13:1192800. doi: 10.3389/fcimb.2023.1192800

Cysteinyl-leukotrienes promote cutaneous Leishmaniasis control

Letícia Paula Trajano Noronha[1], Monique Daiane Andrade Martins[1], Archimedes Barbosa Castro-Junior[1], Maria Luiza Thorstenberg[1], Laís Costa-Soares[1], Thuany Prado Rangel[1], Felipe Carvalho-Gondim[2], Bartira Rossi-Bergmann[2], Luiz Eduardo Baggio Savio[1], Claudio de Azevedo Canetti[3] and Robson Coutinho-Silva[1]*

[1]Laboratory of Immunophysiology, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Laboratory of Immunopharmacology, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [3]Laboratory of Inflammation, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Leishmaniasis is a neglected tropical parasitic disease with few approved medications. Cutaneous leishmaniasis (CL) is the most frequent form, responsible for 0.7 - 1.0 million new cases annually worldwide. Leukotrienes are lipid mediators of inflammation produced in response to cell damage or infection. They are subdivided into leukotriene B4 (LTB_4) and cysteinyl leukotrienes LTC4 and LTD4 (Cys-LTs), depending on the enzyme responsible for their production. Recently, we showed that LTB_4 could be a target for purinergic signaling controlling *Leishmania amazonensis* infection; however, the importance of Cys-LTs in the resolution of infection remained unknown. Mice infected with *L. amazonensis* are a model of CL infection and drug screening. We found that Cys-LTs control *L. amazonensis* infection in susceptible (BALB/c) and resistant (C57BL/6) mouse strains. *In vitro*, Cys-LTs significantly diminished the *L. amazonensis* infection index in peritoneal macrophages of BALB/c and C57BL/6 mice. *In vivo*, intralesional treatment with Cys-LTs reduced the lesion size and parasite loads in the infected footpads of C57BL/6 mice. The anti-leishmanial role of Cys-LTs depended on the purinergic P2X7 receptor, as infected cells lacking the receptor did not produce Cys-LTs in response to ATP. These findings suggest the therapeutic potential of LTB4 and Cys-LTs for CL treatment.

KEYWORDS

***Leishmania amazonensis*, cysteinyl-leukotrienes, cutaneous leishmaniasis (CL), LTC_4, LTD_4, P2X7**

1 Introduction

Leishmaniasis is a neglected tropical parasitic disease affecting more than 12 million people worldwide, with 0.7–1.0 million new cases yearly (Burza et al., 2018). *Leishmania* parasites come in two forms: promastigotes, which are flagellated and extracellular, and amastigotes, intracellular and with a retracted flagellum. Leishmania promastigotes enter hosts through the bite of sand fly vectors and infect macrophages, where they differentiate into amastigotes. Depending on the parasite species and the patient's immunological status, leishmaniasis can manifest as tegumentary leishmaniasis or visceral leishmaniasis. Tegumentary leishmaniasis can present as cutaneous leishmaniasis (CL), characterized by one or more high-edged wounds (Martins et al., 2014), mucosal leishmaniasis, characterized by mutilations in the oropharyngeal mucosa due to activation of the immune system and low parasitic load, diffuse cutaneous leishmaniasis, with non-ulcerated lesions spread across the skin, resulting from an anergic immune response and high parasitic load, and disseminated CL, caused by the spread of the parasite from the cutaneous lesion through the blood or lymph (Desjeux, 2004).

CL is the most common form, accounting for 90% of cases; all dermotropic species may cause it. After inoculation by sandflies, flagellated promastigotes infect skin phagocytes (neutrophils and macrophages), transforming and multiplying as amastigotes within phagolysosomes. Skin lesions develop slowly through macrophage infection and are often restricted to the bite site. Initially, a nodule forms and progresses chronically, eventually ulcerating as the inflammatory reaction intensifies. Many microbicidal intracellular innate mechanisms control infection by *Leishmania*, and macrophages are among the primary immune cells involved in parasite elimination through the degradation of phagolysosomal enzymes, the release of cytokines, and the production of reactive oxygen species and nitric oxide. The latter is the most critical control mechanism against phagocytosed intracellular microorganisms such as *Leishmania* (Moradin and Descoteaux, 2012).

During infection, nucleotides (well-recognized danger signals) are released into the extracellular environment and act by activating purinergic receptors under various pathological conditions (Virgilio et al., 2020). ATP nucleotides activate the P2X7 and $P2Y_2$ receptors and control *L. amazonensis* infection via leukotriene (LT) B_4 production (Chaves et al., 2014a; Chaves et al., 2016; Thorstenberg et al., 2018; Chaves et al., 2019). The first step for LTs synthesis is the release of arachidonic acid (AA) from membrane phospholipids hydrolyzed by the phospholipase A2. AA is oxygenated by 5-lipoxygenase (5-LO), which, with 5-LO activating protein, forms the unstable precursors of all other LTs, named LTA_4 (Murphy and Gijón, 2007). LTA_4 is converted by LTA_4 hydrolase to LTB_4, or it can be conjugated with reduced glutathione by LTC_4 synthase to yield LTC_4. LTB_4 and LTC_4 are exported to extracellular space by specific transporter proteins. The released LTC_4 is converted to LTD_4 and then to LTE_4 by sequential amino acid hydrolysis.

LTB_4 is an activator and chemotactic agent for leukocytes involved in pathophysiological conditions such as asthma and atherosclerosis. LTB_4 is also essential in controlling *L. amazonensis* infection through augmented nitric oxide production in infected macrophages (Serezani et al., 2006). Cys-LTs (LTC_4, LTD_4, and LTE_4) participate in the pathogenesis of allergic asthma inflammation by recruiting leukocytes and enhancing vascular permeability (Capra et al., 2007). However, the role of Cys-LTs in parasite infection is poorly understood. According to the literature, LTC_4 enhances the association of mouse peritoneal macrophages with *Trypanosoma cruzi*, increasing its uptake and intracellular destruction (Wirth and Kierszenbaum, 1985).

Leishmaniasis control in endemic regions is based on chemotherapy and the management of reservoirs or vectors. Chemotherapy has several shortcomings in terms of toxicity, development of resistance, stability, and cost. Furthermore, they are long-term treatments, have low tolerability, and are poorly adapted to remote areas, making them challenging to administer because their administration usually requires a hospital environment (DNDi Annual Report, 2016). These limitations drove researchers worldwide to identify new medications for leishmaniasis.

In this study, we assessed the role of Cys-LTs in controlling *L. amazonensis* infection in macrophages isolated from susceptible (BALB/c) and resistant (C57BL/6) mouse strains. We analyzed the *in vitro* effect of Cys-LTs on infected peritoneal macrophages and *in vivo* intralesional treatment in infected mouse footpads. We showed that Cys-LTs diminished the infection index in cultured macrophages and reduced lesion size and parasite load in footpads.

2 Material and methods

2.1 Chemicals

ATP was purchased from Sigma-Aldrich (St. Louis, MO, USA). LTB_4, LTC_4, and LTD_4 were obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.2 Animals

We used male and female 8–10-week-old BALB/c, wild-type C57BL/6, and P2X7 knockout mice ($P2X7^{-/-}$; Jackson Laboratory, USA). The experiments, maintenance, and care of mice were carried out according to the Brazilian College of Animal Experimentation guidelines. The mice were housed in a temperature-controlled room (26°C) with a light/dark cycle (12 h). Food and water were provided *ad libitum*. For *ex vivo* experiments, mice were anesthetized in a carbon dioxide (CO_2) chamber and sacrificed by cervical dislocation. The Ethics Committee on the Use of Animals (CEUA) approved all experimental protocols (IBCCF, UFRJ n° 077/15 and n° 152/21).

2.3 Parasites

The *L. amazonensis* (MHOM/BR/Josefa strain) was used for *in vitro* and *in vivo* experiments. Amastigotes isolated from mouse

lesions (from BALB/c mice) were allowed to transform into axenic promastigotes forms by growth at 24°C for 7 days in 199 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, MA), 2% male human urine, 1% L-glutamine, and 0.25% hemin. Promastigotes in the late stationary growth phase were used until the tenth passage to preserve parasite virulence.

2.4 Macrophage isolation, cell culture, and infection

Peritoneal macrophages were harvested from the peritoneal cavity by washes with cold phosphate-buffered saline (PBS). Cells were directly seeded on culture plates in RPMI 1640 medium, at 37°C, with 5% CO_2. After 1 h, cultures were washed gently with PBS (twice) to remove non-adherent cells. The cells were cultured for 24 h in RPMI 1640 supplemented medium (10% FBS and 100 units penicillin/streptomycin and 2 mM L-glutamine) at 37°C and 5% CO_2. Then cells were infected for 4 h with *L. amazonensis* promastigotes (MOI 10:1 - *Leishmania*:macrophage) at 37°C. The non-internalized parasites were removed by extensive washing with sterile PBS at 37°C. Then, infected cells were maintained in an incubator at 37°C and 5% CO_2 until stimulation.

2.5 Pharmacological treatments

Infected macrophages were treated with 100 nM LTB_4, LTC_4, or LTD_4 for 30 minutes at 37°C and 5% CO_2. Then, cell monolayers were washed with PBS and maintained in RPMI 1640 supplemented at 37°C and 5% CO_2 for 24 h.

2.6 Macrophages infection index

Intracellular parasite loads were analyzed as previously described (Thorstenberg et al., 2018). Briefly, mouse peritoneal cells were infected, treated with nucleotides, fixed onto slides, and stained with a panoptic stain (Laborclin ®, PR, Brazil). Parasites were counted using a Primo Star light microscope (Zeiss, Germany) with a 40X objective (100X for representative pictures). Images were acquired using a Bx51 camera (Olympus, Tokyo, Japan) using CellF software. We calculated the "infection index" representing the overall infection load, based on 100 cells in five fields to obtain the number of infected macrophages and the average number of parasites per macrophage. Individual amastigotes were visible in the cytoplasm of infected macrophages. The results were expressed as infection index II = (% infected macrophages) × (amastigotes/infected macrophages)/100.

2.7 *In vivo* infection and pharmacological treatments

Mice were infected in the footpad by intradermal injection of 10^6 *L. amazonensis* promastigotes in PBS. Intralesional treatment with 20 µL of 5 ng LTD_4 or vehicle for 3 weeks, twice a week, started 7 days post-infection (dpi). Lesion growth was calculated by evaluation of the "swelling" (i.e., the thickness of the infected footpad – thickness of the uninfected footpad from the same mouse) using a traditional caliper (Mitutoyo®). Forty-eight hours after the final injection (26 dpi), animals were euthanized. For parasitic load determination, the infected footpad and popliteal lymph nodes were removed and dissociated (in M199 supplemented culture medium).

2.8 Parasite load determination

The parasite load in the mice-infected tissues was determined using a limiting dilution assay, as previously described (Titus et al., 1985; Thorstenberg et al., 2018). Mice were euthanized in a CO_2 chamber, followed by cervical dislocation. Footpads and lymph nodes were collected and weighed, and cells from the whole footpad and draining lymph nodes were dissociated using a 40-µm cell strainer (BD®) in PBS. Large pieces of tissue debris were removed by centrifugation at 150 x *g*. The cells were then separated by centrifugation at 2,000 × *g* for 10 min and resuspended in supplemented M199. The samples were cultured in 96-well flat-bottom microtiter plates (BD®, USA) at 26–28°C. After a minimum of 7 days, wells were examined using phase-contrast microscopy in an inverted microscope (NIKON TMS, JP) and scored as "positive" or "negative" for the presence of parasites. Wells were scored "positive" when at least one parasite was observed per well.

2.9 Cys-LTs determination assay

Peritoneal macrophages (2 x 10^5 per well) from C57BL/6 and $P2X7^{-/-}$ mice were plated in 96-well plates in triplicate and infected or left untreated after twenty-four hours. At the end of the incubation period, the supernatants were removed, and a new medium with 5% FBS was added. The cultures were then treated with ATP for 30 min with subsequentially medium change. The culture supernatants were collected 30 min and 3 h after ATP treatment and stored at −80°C until analysis. To measure the Cys-LTs released in the supernatant of cells, enzyme immunoassays were performed using a Cys-LTs enzyme immunoassay kit (Cayman Chemical™) following the manufacturer's instructions. The cysteinyl leukotriene enzyme immunoassay kit does not distinguish among Cys-LTs. The cross-reactivity of the assay is 100% for LTC4 and LTD4 and 79% for LTE4. The cross-reactivity to LTB4 is 1.3% and 0.04% to 5(S)-HETE. The overall amount of LTC_4, LTD_4, and LTE_4 was measured in each sample.

2.10 Statistical analysis

Statistical analysis were performed using Student's t-test when comparing two groups. For more than two groups, data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparison *post hoc* test using Prism 8.0

software (GraphPad Software, La Jolla, CA). Differences between the experimental groups were considered statistically significant at $P < 0.05$.

3 Results

3.1 Cys-LTs (LTC_4 and LTD_4) decreased the parasite load of *L. amazonensis* in peritoneal macrophages from BALB/c and C57BL/6 mice

LTB_4 is essential to resistance to infection by *L. amazonensis*; therefore, it was used as a positive control for parasitic load assay (Figure 1B). Cysteinyl leukotrienes LTC_4 (Figure 1C) and LTD_4 (Figure 1D) treatment resulted in fewer *Leishmania* amastigotes in parasitophorous vacuoles than untreated macrophages from BALB/c mice (Figure 1A). A similar effect was observed with LTB_4. The data were quantified (Figure 1E), confirming that treatment with Cys-LTs reduces *L. amazonensis* infection by more than 60%.

As seen with BALB/c macrophages, C57BL/6 peritoneal macrophages treated with LTC_4 (Figure 2C) or LTD_4 (Figure 2D) showed fewer *Leishmania* in the parasitophorous vacuoles (marked with an asterisk) than the untreated group (Figure 2A). Similarly, we observed that LTB_4 treatment reduced the number of *Leishmania* in parasitophorous vacuoles from C57BL/6 macrophages (Figure 2B). The data were quantified (Figure 2E), and Cys-LTs reduced *L. amazonensis* infection by 50%.

3.2 Intralesional administration of cysteinyl leukotriene LTD_4 reduced cutaneous *Leishmania* lesion progression in footpads

Next, we analyzed the effect of Cys-LTs in modulating *Leishmania* skin lesions progression in C57BL/6 infected mice. We infected the right footpad with 10^6 *Leishmania* promastigotes. We administrated cysteinyl leukotriene LTD_4 intralesionally (twice a week for 3 weeks) seven days after infection. We compared the evolution of lesion size until the twenty-eighth day of infection (Figure 3). Lesion size progressed significantly slower in mice treated with LTD_4 than in those receiving the vehicle solution. From 14 dpi, the treated mice showed significative smaller lesion sizes, and on 28 dpi, lesions in LTD_4 treated mice were less than half the size of lesions in untreated lesions (Figure 3A). LTD_4 treatment induced a greater than 100-fold reduction in the number of parasites in the paw macerate (Figure 3B), while there was no change in the number of *Leishmania* in draining lymph nodes (Figure 3C).

3.3 ATP-induced Cys-LTs production depends on the P2X7 receptor

ATP is an important signaling molecule in the immune system that binds the P2X7 purinergic receptor (Savio et al., 2018). Our group showed that ATP-treated macrophages control *L. amazonensis* infection (Chaves et al., 2019). LTB_4 reduces the parasitic load of infected macrophages; additionally, the P2X7 receptor induces phospholipase A2 activation and AA mobilization. We also demonstrated that LTB_4 production depends on P2X7 receptor activation, as macrophages lacking the P2X7 receptor did not reduce parasite infection or produce LTB_4 in response to infection.

Therefore, we investigated whether macrophages would produce Cys-LTs in response to ATP in a P2X7-dependent way. The addition of ATP (500μM) to peritoneal macrophages induced the release of Cys-LTs compared to non-stimulated cells (Figure 4). This production appears to be partially dependent on P2X7 receptor as macrophages from $P2X7^{-/-}$ mice produced fewer Cys-LTs in response to ATP. Additionally, *Leishmania* infection decreased the production of Cys-LTs by macrophages, and P2X7-deficient macrophages had their production almost abolished.

4 Discussion

LTs, lipid mediators of inflammation, are well-described for participating in the inflammatory processes of chronic, allergic, and autoimmune diseases (Griffiths et al., 1995; Capra et al., 2007; Ohnishi et al., 2008). In this context, Cys-LTs are involved in events such as cell migration and leukocyte adhesion (Dahlén et al., 1981; Yuan et al., 2009; Dannull et al., 2012). In asthmatic patients, these molecules are essential in the respiratory system, participating in goblet cell hyperplasia, vascular permeability, and bronchoconstriction (Manning et al., 1990; Kim et al., 2006; Hashimoto et al., 2009; Kazani et al., 2011). However, their role in antimicrobial defense has been largely overlooked compared to cytokines and chemokines. This discrepancy likely reflects the common view that lipid mediators are exclusively pathogenic and the resultant idea of their pharmacological blockade as the primary objective of pharmacological research (Serezani et al., 2023).

Our group has demonstrated the interplay between the activation of purinergic receptors and LTB_4 in promoting the resolution of *Leishmania* infection in macrophages (Chaves et al., 2009; Marques-da-Silva et al., 2011; Chaves et al., 2014b; Marques-da-Silva et al., 2018). Our previous study demonstrated that P2X7 receptor activation releases leukotrienes, primarily LTB_4 (Chaves et al., 2014b). Serezani et al. (2006) demonstrated that LTB_4 induced the elimination of *L. amazonensis* in infected macrophages *in vitro* and that the 5-LO pathway of arachidonate is necessary for efficient parasite elimination. Cell-specific leukotriene profiles have been described with mast cells and eosinophils synthesizing primarily Cys-LTs, neutrophils and dendritic cells synthesizing primarily LTB_4, and macrophages producing a balance of both LT classes (Peters-Golden et al., 2005).

The effect of Cys-LTs in the modulation of intracellular parasite infection is much less clear. The role of Cys-LTs in intracellular parasite elimination was demonstrated almost 30 years ago when Wirth and Kierszenbaum (1985) reported that LTC_4 facilitated phagocytosis and elimination of *Trypanosoma cruzi* by peritoneal macrophages. The role of Cys-LTs in the modulation of *Leishmania*

10.3389/fcimb.2023.1192800

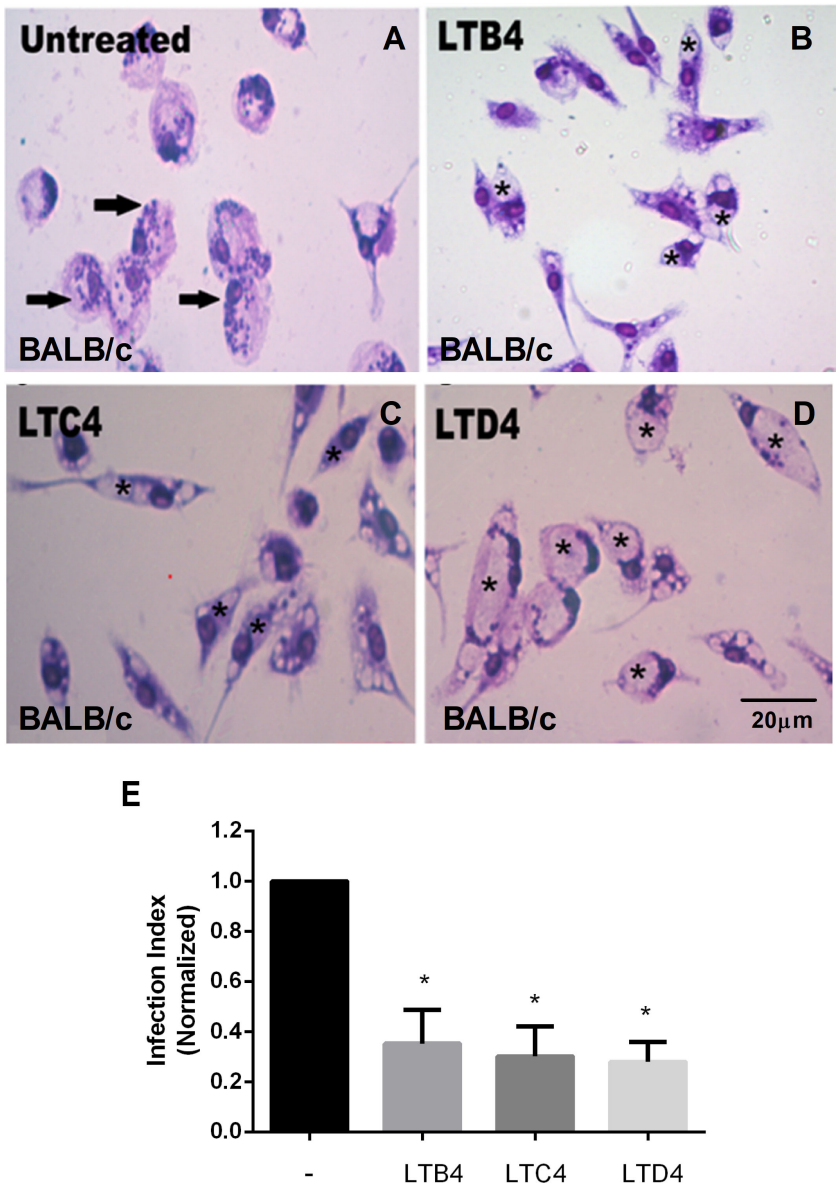


FIGURE 1
LTs reduce the parasite burden in infected BALB/c mouse macrophages. Peritoneal macrophages from BALB/c mice were infected with *L. amazonensis* promastigotes at 10:1 (*Leishmania*:macrophage). After 4 h, the free parasites were washed, and after 24 h, infected cells were treated **(B–D)** or not **(A)** with 100 nM of LTB4 **(B)**, cysteinyl leukotrienes LTC_4 **(C)**, and LTD_4 **(D)**. Twenty-four hours later, cells were stained with May Grunwald-Giemsa, and the infection index was determined by direct counting under light microscopy. Arrows indicate *Leishmania* amastigotes inside macrophages. Asterisks indicate parasitophorous vacuoles. Quantification of parasite load observed in macrophages is shown **(E)**. Normalized values represent means ± SEM of 3–4 independent experiments performed in triplicate. (*P < 0.05) compared to the control group (without treatment).

infection remains undescribed. This phenomenon was also observed for LTB_4 production by *Leishmania*-infected macrophages, which showed a reduction ability, reinforcing the idea that the parasite impairs LTs production/action to survive within macrophages.

The present study showed that Cys-LTs modulate *Leishmania* infection in peritoneal macrophages from susceptible and resistant mouse strains. The doses of Cys-LTs were based on the literature to activate high- and low-affinity receptors. Adding LTC_4 or LTD_4 reduced the parasite index in macrophages, similar to the already described effect of LTB_4. We detected Cys-LTs production by cultured macrophages responding to extracellular ATP (eATP). Nevertheless, *Leishmania*-infected macrophages appeared to reduce the amount of Cys-LTs produced, suggesting a possible adaptation of the parasite to evade the antiparasitic effect of Cys-LTs.

The P2X7 receptor appears to be partly necessary to Cys-LTs production, as P2X7-deficient macrophages exhibited nearly half the amount of Cys-LTs in response to eATP; *Leishmania* infection

10.3389/fcimb.2023.1192800

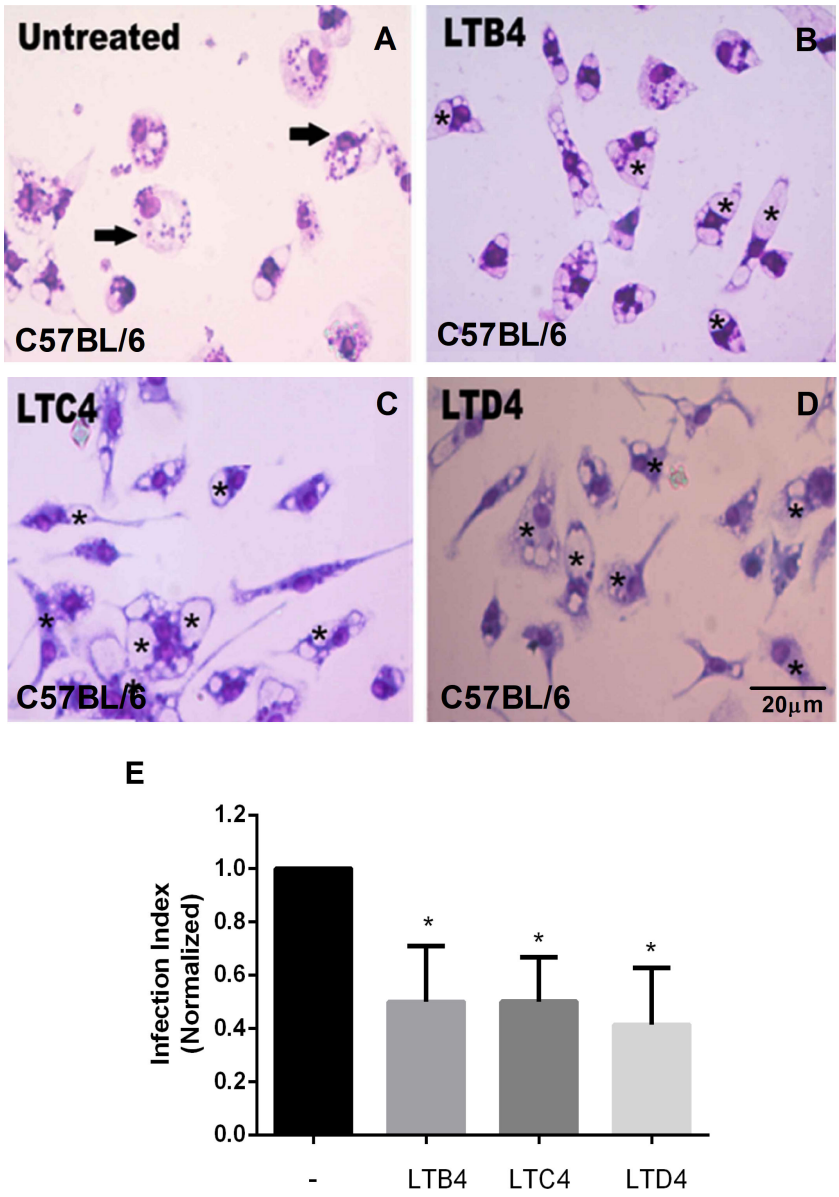


FIGURE 2
LTs reduce the parasite burden in infected C57BL/6 mouse macrophages. Peritoneal macrophages from C57BL/6 mice were infected with *L. amazonensis* promastigotes at 10:1 (*Leishmania*:macrophage). After 4 h, the free parasites were washed, and after 24 h, infected cells were treated **(B–D)** or not **(A)** with 100 nM of LTB4 **(B)**, LTC_4 **(C)**, and LTD_4 **(D)**. Twenty-four hours later, cells were stained with May Grunwald-Giemsa, and the infection index was determined by direct counting under light microscopy. Arrows indicate *Leishmania* amastigotes inside macrophages. Asterisks indicate parasitophorous vacuoles. Quantification of parasite load observed in macrophages is shown **(E)**. Normalized values represent means ± SEM of 3–4 independent experiments performed in triplicate. (*P < 0.05) compared to the control group (without treatment).

prevented Cys-LTs production by macrophages. These *in vitro* results demonstrate that exogenous Cys-LTs can interfere with parasite load in cultured cells, and macrophages can produce Cys-LTs in response to eATP. However, *Leishmania* appears to have developed a way to interfere with Cys-LTs production in infected cells, increasing survival. The need for P2X7 receptor activation by eATP reinforces the putative role of Cys-LTs in synergizing the antimicrobial events in infected macrophages. The mechanisms by which parasite infection decreases LTs synthetic capacity remain to be elucidated. Several possibilities are reasonable, including interference with the enzyme synthesis/activity, receptor expression, or signaling pathways.

Next, we tested the effect of exogenous Cys-LTs on wounds resulting from *L. amazonensis* infection in mice. Our results suggest that exogenous Cys-LTs (specifically LTD_4) induce a significant reduction in lesion size caused by *Leishmania* infection. The number of parasites in the wound was significantly reduced, suggesting a local effect of Cys-LTs on parasite load. These

10.3389/fcimb.2023.1192800

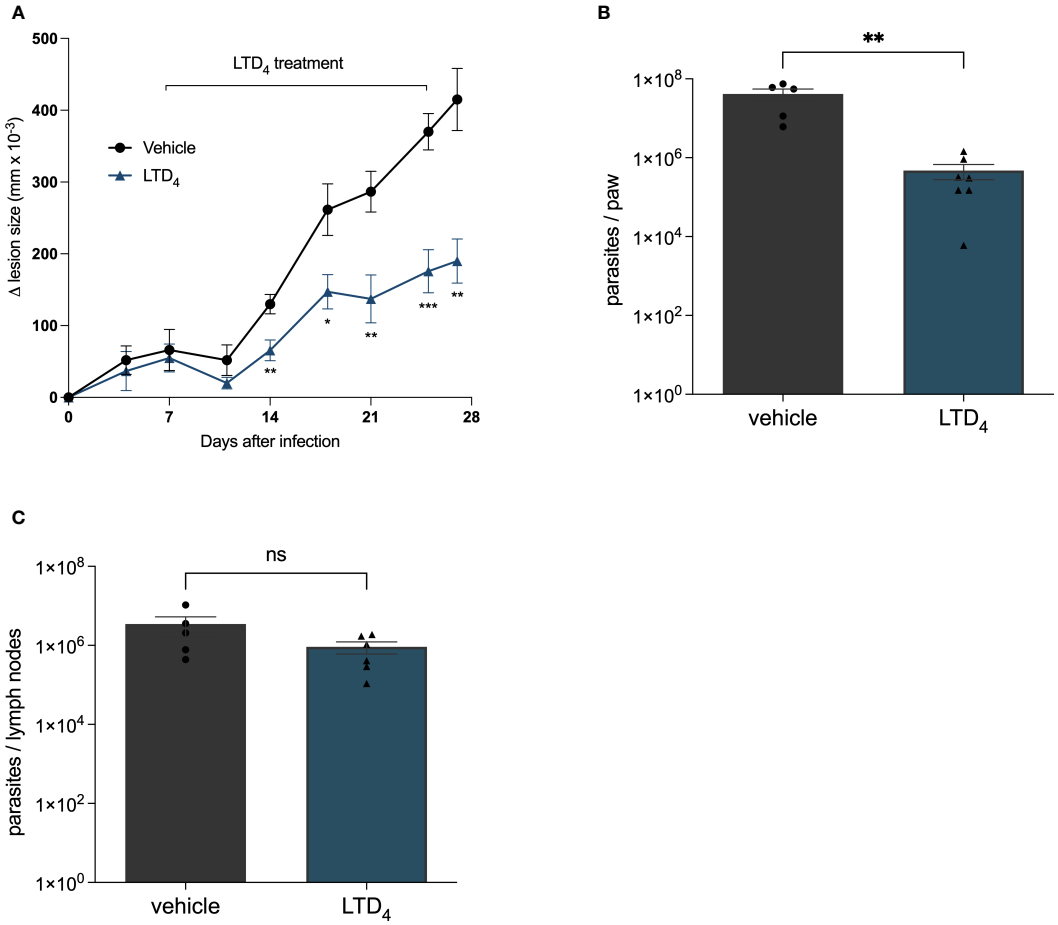


FIGURE 3
Intralesional treatment with LTD4 induced resistance to L. amazonensis infection in mice. C57BL/6 mice were subcutaneously injected into the footpad with 10^6 *L. amazonensis* promastigotes. Seven days post-infection (dpi), the mice were treated with 5 ng of LTD_4 in 20 µL PBS (six doses applied intralesionally twice a week) for 3 weeks (7–27 dpi). **(A)** Lesion size (edema) was determined using a traditional caliper (Mitutoyo®) and was expressed as 10^{-3} mm. Animals were euthanized at 29 dpi. The infected paw and popliteal lymph nodes were removed using a limiting dilution assay to measure parasite load in the paw **(B)** and lymph nodes **(C)**. The data represent the mean ± standard error of the mean of the group of animals (n = 5). ***p < 0.001, **p < 0.01 and *p < 0.05 compared to untreated control group. Data were analyzed using Student's T-test followed by the Mann-Whitney U-test. ns = non-significant.

findings indicate a previously undescribed role of Cys-LT in the leishmanicidal effect in macrophages. Although we used a short-term time of infection (1 week) to accelerate treatment evaluation, all living parasites were likely intracellular and represented active lesions by that time. Future experiments using lesions older than 3–4 weeks will provide insights into leukotriene effects in established infections.

Previous studies showed that inhibiting leukotriene production or its receptor using a 5-lipoxygenase-activating protein inhibitor (MK0591) or LTB_4 receptor antagonist (U75302) decreased leishmanicidal activity by infected macrophages. However, a CysLT1 receptor antagonist (MK571) did not affect the macrophage infection index (Serezani et al., 2006). These contradictory results indicate the need for further investigation into the molecular mechanisms underlying wound size reduction and the macrophage infection index, both of which were reduced in our experiments.

Cys-LTs bind to CysLT1R and CysLT2R. CysLT1R is the high-affinity receptor, primarily expressed in leukocytes (mostly eosinophils and monocytes/macrophages), basophils, vascular endothelial cells, mast cells, neutrophils, and subsets of B lymphocytes. By contrast, CysLT2R receptors are expressed in the heart, brain, and adrenal glands, despite some overlapping with CyslT1R-expressing cells. Moderate CysLT2R expression was observed in the spleen, lymph nodes, and peripheral blood leukocytes and was more highly expressed in eosinophils (Capra et al., 2007). Nevertheless, there may be an additional Cys-LT receptor because an antagonist of receptors (BAYu9773) failed to inhibit all Cys-LT functional responses (Walch et al., 2002). Therefore, the newly described leishmanicidal effect of Cys-LT may depend on an unknown receptor.

We conclude that the effect of Cys-LTs on *L. amazonensis* infection, either by reducing the parasite index in cultured infected

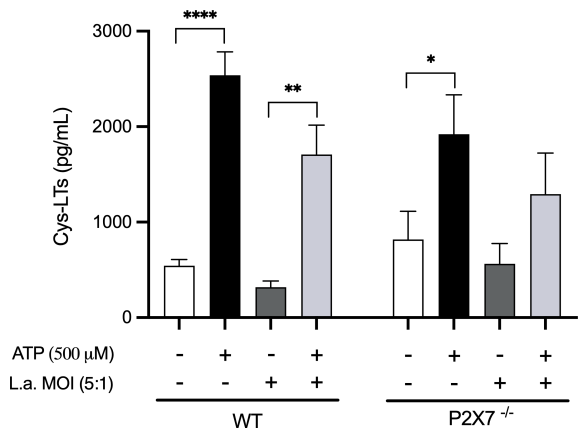


FIGURE 4
P2X7 receptors activation induces the production of Cys-LTs. Peritoneal macrophages (2 x 10^5) from wild-type and P2X7$^{-/-}$ mice were plated in 96-well culture plates. After 24 h, the cells were infected with *L. amazonensis* promastigotes at an MOI (5:1) for 4 h. After 24 h of infection, ATP (500 μM) was added to the cultures for 30 min. The supernatants were collected for the measurement of Cys-LTs by EIA. Data are presented as the mean ± standard error of the mean of three independent experiments performed in triplicates. Data were analyzed using analysis of variance followed by Tukey's Post-test. ****p < 0.0001, **p < 0.001 and *p < 0.05.

macrophages or hampering skin wound progression in intradermally infected mouse footpads, points to a promising new drug target for CL. Further mechanistic studies should be performed to elucidate its leishmanicidal effects.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The procedures were performed following the guidelines of the Brazilian College of Animal Experimentation (COBEA) and were approved by The Commission for Ethical Use of Research Animals (CEUA) from the Federal University of Rio de Janeiro (UFRJ) n° 077/15 and n° 152/21.

Author contributions

LN, MM, MT, and LC-S conducted *in vivo* and *in vitro* experiments. AC-J contributed to data analysis and wrote the paper. TR contributed to data analysis. FC-G and BR-B contributed to the *in vivo* experiments. LS contributed to the data analysis and article revision. CC conducted the cysteinyl leukotriene quantification and article revision. RC-S conceived and designed the experiment and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by funds from the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico do Brasil – CNPq (306839/2019-9) to RC-S and (305857/2020-7) to LS; and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ (E-26/010.002985/2014; E-26/201.086/2022) to RC-S.

Acknowledgments

We thank Sthefani Rodrigues for her technical assistance.

Conflict of interest

The authors declare that the research was conducted without commercial or financial relationships that could be construed as potential conflicts of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Burza, S., Croft, S. L., and Boelaert, M. (2018). Leishmaniasis. *Lancet* 392, 951–970. doi: 10.1016/s0140-6736(18)31204-2

Capra, V., Thompson, M. D., Sala, A., Cole, D. E., Folco, G., and Rovati, G. E. (2007). Cysteinyl-leukotrienes and their receptors in asthma and other inflammatory diseases: critical update and emerging trends. *Med. Res. Rev.* 27, 469–527. doi: 10.1002/med.20071

Chaves, M. M., Canetti, C., and Coutinho-Silva, R. (2016). Crosstalk between purinergic receptors and lipid mediators in leishmaniasis. *Parasites Vectors* 9, 489. doi: 10.1186/s13071-016-1781-1

Chaves, M. M., Marques-da-Silva, C., Monteiro, A. P. T., Canetti, C., and Coutinho-Silva, R. (2014a). Leukotriene B4 modulates P2X7 receptor-mediated leishmania amazonensis elimination in murine macrophages. *J. Immunol. (Baltimore Md.: 1950)* 192, 4765–4773. doi: 10.4049/jimmunol.1301058

Chaves, M. M., Marques-da-Silva, C., Monteiro, A. P. T., Canetti, C., and Coutinho-Silva, R. (2014b). Leukotriene B4 modulates P2X7 receptor–mediated leishmania amazonensis elimination in murine macrophages. *J. Immunol.* 192, 4765–4773. doi: 10.4049/jimmunol.1301058

Chaves, M. M., Sinflorio, D. A., Thorstenberg, M. L., Martins, M. D. A., Moreira-Souza, A. C. A., Rangel, T. P., et al. (2019). Non-canonical NLRP3 inflammasome activation and IL-1β signaling are necessary to l. amazonensis control mediated by P2X7 receptor and leukotriene B4. *PloS Pathog.* 15, e1007887. doi: 10.1371/journal.ppat.1007887

Chaves, S. P., Torres-Santos, E. C., Marques, C., Figliuolo, V. R., Persechini, P. M., Coutinho-Silva, R., et al. (2009). Modulation of P2X7 purinergic receptor in macrophages by leishmania amazonensis and its role in parasite elimination. *Microbes Infect.* 11, 842–849. doi: 10.1016/j.micinf.2009.05.001

Dahlén, S. E., Björk, J., Hedqvist, P., Arfors, K. E., Hammarström, S., Lindgren, J. A., et al. (1981). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *in vivo* effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci.* 78, 3887–3891. doi: 10.1073/pnas.78.6.3887

Dannull, J., Schneider, T., Lee, W. T., de Rosa, N., Tyler, D. S., and Pruitt, S. K. (2012). Leukotriene C4 induces migration of human monocyte–derived dendritic cells without loss of immunostimulatory function. *Blood* 119, 3113–3122. doi: 10.1182/blood-2011-10-385930

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27, 305–318. doi: 10.1016/j.cimid.2004.03.004

DNDi Annual Report. (2016). Available at: https://dndi.org/flippable/annualreport2016/mobile/index.html#p=8.

Griffiths, R. J., Pettipher, E. R., Koch, K., Farrell, C. A., Breslow, R., Conklyn, M. J., et al. (1995). Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis. *Proc. Natl. Acad. Sci.* 92, 517–521. doi: 10.1073/pnas.92.2.517

Hashimoto, K., Ichiyama, T., Hasegawa, M., Hasegawa, S., Matsubara, T., and Furukawa, S. (2009). Cysteinyl leukotrienes induce monocyte chemoattractant protein-1 in human Monocyte/Macrophages via mitogen-activated protein kinase and nuclear factor-κB pathways. *Int. Arch. Allergy Imm.* 149, 275–282. doi: 10.1159/000199724

Kazani, S., Sadeh, J., Bunga, S., Wechsler, M. E., and Israel, E. (2011). Cysteinyl leukotriene antagonism inhibits bronchoconstriction in response to hypertonic saline inhalation in asthma. *Resp. Med.* 105, 667–673. doi: 10.1016/j.rmed.2010.11.025

Kim, D. C., Hsu, F. I., Barrett, N. A., Friend, D. S., Grenningloh, R., Ho, I.-C., et al. (2006). Cysteinyl leukotrienes regulate Th2 cell-dependent pulmonary inflammation. *J. Immunol.* 176, 4440–4448. doi: 10.4049/jimmunol.176.7.4440

Manning, P. J., Watson, R. M., Margolskee, D. J., Williams, V. C., Schwartz, J. I., and O'Byrne, P. M. (1990). Inhibition of exercise-induced bronchoconstriction by MK-571, a potent leukotriene D4–receptor antagonist. *New Engl. J. Med.* 323, 1736–1739. doi: 10.1056/nejm199012203232504

Marques-da-Silva, C., Chaves, M. M., Chaves, S. P., Figliuolo, V. R., Meyer-Fernandes, J. R., Corte-Real, S., et al. (2011). Infection with leishmania amazonensis upregulates purinergic receptor expression and induces host-cell susceptibility to UTP-mediated apoptosis. *Cell Microbiol.* 13, 1410–1428. doi: 10.1111/j.1462-5822.2011.01630.x

Marques-da-Silva, C., Chaves, M. M., Thorstenberg, M. L., Figliuolo, V. R., Vieira, F. S., Chaves, S. P., et al. (2018). Intralesional uridine-5′-triphosphate (UTP) treatment induced resistance to leishmania amazonensis infection by boosting Th1 immune responses and reactive oxygen species production. *Purinerg. Signal* 14, 201–211. doi: 10.1007/s11302-018-9606-7

Martins, A. L. G. P., Barreto, J. A., Lauris, J. R. P., and Martins, A. C. G. P. (2014). American Tegumentary leishmaniasis: correlations among immunological, histopathological and clinical parameters. *Anais Brasileiros Dermatol.* 89, 52–58. doi: 10.1590/abd1806-4841.20142226

Moradin, N., and Descoteaux, A. (2012). Leishmania promastigotes: building a safe niche within macrophages. *Front. Cell. Infect. Microbiol.* 2. doi: 10.3389/fcimb.2012.00121

Murphy, R. C., and Gijón, M. A. (2007). Biosynthesis and metabolism of leukotrienes. *Biochem. J.* 405, 379–395. doi: 10.1042/bj20070289

Ohnishi, H., Miyahara, N., and Gelfand, E. W. (2008). The role of leukotriene B4 in allergic diseases. *Allergol. Int.* 57, 291–298. doi: 10.2332/allergolint.08-rai-0019

Peters-Golden, M., Canetti, C., Mancuso, P., and Coffey, M. J. (2005). Leukotrienes: underappreciated mediators of innate immune responses. *J. Immunol.* 174, 589–594. doi: 10.4049/jimmunol.174.2.589

Savio, L. E. B., de Mello, P. A., da Silva, C. G., and Coutinho-Silva, R. (2018). The P2X7 receptor in inflammatory diseases: angel or demon? *Front. Pharmacol.* 9. doi: 10.3389/fphar.2018.00052

Serezani, C. H., Divangahi, M., and Peters-Golden, M. (2023). Leukotrienes in innate immunity: still underappreciated after all these years? *J. Immunol.* 210, 221–227. doi: 10.4049/jimmunol.2200599

Serezani, C. H., Perrela, J. H., Russo, M., Peters-Golden, M., and Jancar, S. (2006). Leukotrienes are essential for the control of leishmania amazonensis infection and contribute to strain variation in Susceptibility1. *J. Immunol.* 177, 3201–3208. doi: 10.4049/jimmunol.177.5.3201

Thorstenberg, M. L., Ferreira, M. V. R., Amorim, N., Canetti, C., Morrone, F. B., Filho, J. C. A., et al. (2018). Purinergic cooperation between P2Y2 and P2X7 receptors promote cutaneous leishmaniasis control: involvement of pannexin-1 and leukotrienes. *Front. Immunol.* 9, 1531. doi: 10.3389/fimmu.2018.01531

Titus, R. G., Marchand, M., Boon, T., and Louis, J. A. (1985). A limiting dilution assay for quantifying leishmania major in tissues of infected mice. *Parasite Immunol.* 7, 545–555. doi: 10.1111/j.1365-3024.1985.tb00098.x

Virgilio, F. D., Sarti, A. C., and Coutinho-Silva, R. (2020). Purinergic signaling, DAMPs, and inflammation. *Am. J. Physiol-cell Ph.* 318, C832–C835. doi: 10.1152/ajpcell.00053.2020

Walch, L., Norel, X., Bäck, M., Gascard, J., Dahlén, S., and Brink, C. (2002). Pharmacological evidence for a novel cysteinyl-leukotriene receptor subtype in human pulmonary artery smooth muscle. *Brit. J. Pharmacol.* 137, 1339–1345. doi: 10.1038/sj.bjp.0704991

Wirth, J. J., and Kierszenbaum, F. (1985). Effects of leukotriene C4 on macrophage association with and intracellular fate of trypanosoma cruzi. *Mol. Biochem. Parasit.* 15, 1–10. doi: 10.1016/0166-6851(85)90024-6

Yuan, Y.-M., Fang, S.-H., Qian, X.-D., Liu, L.-Y., Xu, L.-H., Shi, W.-Z., et al. (2009). Leukotriene D4 stimulates the migration but not proliferation of endothelial cells mediated by the cysteinyl leukotriene CysLT1 receptor via the extracellular signal-regulated kinase pathway. *J. Pharmacol. Sci.* 109, 285–292. doi: 10.1254/jphs.08321fp

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 18 September 2023
DOI 10.3389/fcimb.2023.1253033

OPEN ACCESS

EDITED BY
Chaoqun Yao,
Ross University, United States

REVIEWED BY
Danilo Ciccone Miguel,
State University of Campinas, Brazil
Budhaditya Mukherjee,
Indian Institute of Technology Kharagpur, India

*CORRESPONDENCE
Marlene Jara
marlene.jara.p@gmail.com
Jean-Claude Dujardin
jcdujardin@itg.be

RECEIVED 04 July 2023
ACCEPTED 21 August 2023
PUBLISHED 18 September 2023

CITATION
Jara M, Arevalo J, Llanos-Cuentas A, Broeck FVd, Domagalska MA and Dujardin J-C (2023) Unveiling drug-tolerant and persister-like cells in *Leishmania braziliensis* lines derived from patients with cutaneous leishmaniasis. *Front. Cell. Infect. Microbiol.* 13:1253033. doi: 10.3389/fcimb.2023.1253033

Unveiling drug-tolerant and persister-like cells in *Leishmania braziliensis* lines derived from patients with cutaneous leishmaniasis

Marlene Jara[1*], Jorge Arevalo[2], Alejandro Llanos-Cuentas[2], Frederik Van den Broeck[1,3], Malgorzata Anna Domagalska[1] and Jean-Claude Dujardin[1*]

[1]Molecular Parasitology Unit, Institute of Tropical Medicine Antwerp, Antwerp, Belgium, [2]Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru, [3]Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium

Introduction: Resistance against anti-*Leishmania* drugs (DR) has been studied for years, giving important insights into long-term adaptations of these parasites to drugs, through genetic modifications. However, microorganisms can also survive lethal drug exposure by entering into temporary quiescence, a phenomenon called drug tolerance (DT), which is rather unexplored in *Leishmania*.

Methods: We studied a panel of nine *Leishmania braziliensis* strains highly susceptible to potassium antimonyl tartrate (PAT), exposed promastigotes to lethal PAT pressure, and compared several cellular and molecular parameters distinguishing DT from DR.

Results and discussion: We demonstrated *in vitro* that a variable proportion of cells remained viable, showing all the criteria of DT and not of DR: i) signatures of quiescence, under drug pressure: reduced proliferation and significant decrease of rDNA transcription; ii) reversibility of the phenotype: return to low IC_{50} after removal of drug pressure; and iii) absence of significant genetic differences between exposed and unexposed lineages of each strain and absence of reported markers of DR. We found different levels of quiescence and DT among the different *L. braziliensis* strains. We provide here a new *in-vitro* model of drug-induced quiescence and DT in *Leishmania*. Research should be extended *in vivo*, but the current model could be further exploited to support R&D, for instance, to guide the screening of compounds to overcome the quiescence resilience of the parasite, thereby improving the therapy of leishmaniasis.

KEYWORDS

***Leishmania*, antimonials, quiescence, drug tolerance, persisters**

1 Introduction

Chemotherapy is essential not only for the clinical management of infectious diseases but also for their control and elimination, especially if humans constitute the reservoir of respective pathogens (anthroponoses) and drugs can counter human- to-human transmission (WHO, n.d). Accordingly, treatment failure (TF) may have a major public health impact. TF is a clinical phenotype that can be expressed in different forms, like non-response, relapse, or recrudescence. It has a complex and multifactorial origin, essentially involving the drug (quality, compliance, dosage), the host (immune status, co-infections and concomitant morbidities, existence of sanctuary organs/cells), and the pathogen's biology (Barrett et al., 2019). From the microbial point of view, drug resistance (DR) is the usual suspect and culprit. Hence, DR is often and wrongly confounded with TF, while —in contrast with TF— drug resistance is a long-term parasite adaptive phenotype, more specifically a decreased drug susceptibility which is acquired through genetic modification of the microbe. As such, DR is heritable and the phenotypic adaptation can persist even in the absence of drug exposure (Balaban et al., 2019) until a mutation reverting or compensating the phenotype emerges and is selected.

However, other mechanisms can cause a reduction of drug susceptibility in pathogens. DT is one of them and it is intrinsically totally different from DR. Indeed, it refers to a short-term adaptive phenotype, consisting of decreased susceptibility of the pathogen to the drug, which is not due to an acquired genetic modification of the parasite. It is often associated with quiescence (syn. dormancy), a physiological state of the cell triggered by environmental insults and involving a reversible cell division arrest driven by a dynamic and regulated cell and metabolic remodeling program (Balaban et al., 2019). In microbiology, there are several examples of pathogens that enter in a transient quiescent state, in which they are refractory to one or more drugs, allowing them to persist in the host for long periods: *Mycobacterium tuberculosis*/streptomycin, isoniazid, ciprofloxacin, and rifampin (Keren et al., 2011); *Staphylococcus aureus*/aminoglycoside (Michiels et al., 2016); yeasts (Bojsen et al., 2017); and *Plasmodium falciparum*/artemisinin (Teuscher et al., 2012), among many others.

Leishmania are parasitic protozoa causing a spectrum of clinical forms in (sub-)tropical regions but also in Southern Europe. Leishmaniasis belongs to the category of most neglected diseases, which is reflected among others by the small chemotherapeutic arsenal to combat them. Failure of the few available drugs jeopardizes elimination programs, and some studies associated the TF of leishmaniasis with DR (Lira et al., 1999). However, in two epidemiological settings (Peru/*L. braziliensis* and Nepal/*L. donovani*), we documented a poor correlation between resistance to antimonials (as measured by *in-vitro* susceptibility assays) and TF (Yardley et al., 2006; Rijal et al., 2007). This motivated us to look for alternative mechanisms leading to reduced drug susceptibility in the parasite, more specifically, quiescence. Research on quiescence in *Leishmania* is still in its infancy. Quiescence was reported to occur *in vitro* and *in vivo*, and it is characterized by a reduced level of translational machinery (ribosomal RNA and proteins), low rates of RNA synthesis, protein turnover, and membrane lipid synthesis (Kloehn et al., 2015). Last but not least, parasite populations are found to be heterogeneous in terms of activity, with deeply and semi-quiescent subpopulations (Mandell and Beverley, 2017).

There are only two studies on *Leishmania* quiescence in the context of drug exposure. First, *in vivo*, following treatment with miltefosine, quiescent cells of *L. mexicana* were encountered in mesothelium-like tissues surrounding granulomas, in which the drug did not accumulate (Kloehn et al., 2021). Secondly, *in vitro*, we showed that a strain of *L. lainsoni* entered quiescence under a stationary phase or antimony pressure. Several transcriptional signatures were shared by the parasites surviving both environmental insults: among these, a series of transcripts were present in higher abundance, in a general background of transcriptional shift (Jara et al., 2022). These molecular similarities validated the quiescent character of *Leishmania* under antimony pressure. The objectives of the present study were to further explore *in vitro* the link between quiescence and DT in *Leishmania* and to address the diversity of the DT phenotype in genetically different strains of the same species. Therefore, we exposed nine strains of *L. braziliensis* to high doses of trivalent antimonials and compared several parameters distinguishing DT from DR in pre- and post-exposure lineages (inhibitory concentration, proliferation, cell viability, metabolic activity, lethal dose, and genome).

2 Materials and methods

2.1 Parasites

Clinical isolates were collected in Peru at the Institute of Tropical Medicine Alexander von Humboldt between 2001 and 2004 within the framework of the multiregional LeishNatDrug project (Yardley et al., 2006). Nine of them were selected for the present study, according to the following inclusion criteria: i) first episode of leishmaniasis, ii) cutaneous form of the disease, iii) no previous treatment before attending the clinic, iv) complete therapy with antimonial post-diagnosis, v) no concomitant disease, and vi) infecting species typed as *Leishmania braziliensis*. One strain of *Leishmania lainsoni* was included in the study as control: this is a line highly susceptible to antimonials, for which the existence of quiescence after PAT exposure was demonstrated (Jara et al., 2022). For each isolate, enhanced green fluorescent protein (EGFP) was integrated within the 18S ribosomal DNA locus, further called rEGFP with the use of the pLEXSY-hyg system (Jena Bioscience, Jena, Germany) as previously reported elsewhere (Bolhassani et al., 2011; Jara et al., 2022). After the generation of the transgenic rEGFP parasites, clones were obtained with the "micro-drop" method, as described elsewhere (Van Meirvenne et al., 1975; Jara et al., 2022). All the work presented here was made with these clones, further called rEGFP strains.

2.2 Cell culture of promastigotes and generation of axenic amastigotes

Promastigotes were maintained in complete M199 (M199 medium at pH 7.2 supplemented with 20% fetal bovine serum,

hemin 5 mg/L, 50 µg/mL of hygromycin Gold, 100 units/mL of penicillin, and 100 µg/mL of streptomycin) at 26°C with passages done twice per week. To obtain axenic amastigotes, 1 mL of stationary promastigotes was centrifuged (1, 500×*g*, 5 min), the pellet was resuspended in 5 mL of complete MAA (M199 at pH 5.5, supplemented with 20% fetal bovine serum, glucose 2.5 g/L, 5 g of tryptic soy broth, hemin 5 mg/L, 25 µg/mL of hygromycin Gold, 100 units/mL of penicillin, and 100 µg/mL of streptomycin), and parasites were incubated at 34°C. After observing morphological evidence of amastigogenesis on day 3, the lines were subcultured every 4 days, and growth was monitored microscopically over 3 weeks. If axenic amastigotes were not able to proliferate, three subsequent attempts for axenization were performed: the generation of axenic amastigotes was successful for three rEGFP strains (PER094, 122, and 362). Samples for further experimental procedures were prepared in complete M199 or MAA in the absence of hygromycin. The growth curves were monitored by daily counting of parasites, and the generation time was calculated with the following formula: $G = t/(\log b - \log B)/\log 2)$, where t = time interval in hours, B = number of parasites at the beginning of a time interval, and b = number of parasites at the end of the time interval.

2.3 Cell recovery and monitoring of survival after drug pressure

Exponentially growing promastigotes were exposed to 9 µg/mL of potassium antimonyl tartrate (PAT) for 48 h after which an aliquot of 50 µL was sub cultured in a medium without drug pressure (post-PAT). For each strain, a control without exposure to drug pressure and instead treated with PBS was maintained in parallel (post-PBS). The cell cultures were monitored microscopically, and if positive cellular growth was observed, additional subcultures for the evaluation of drug susceptibility and pellets for sequencing were prepared. Throughout the paper, lineages that were exposed to PAT were referred to as "post-PAT," while those that were not previously exposed to PAT were referred to as "post-PBS."

2.4 Drug susceptibility and estimation of the IC_{50}

The drug susceptibility was measured with the resazurin test. Briefly, exponentially growing parasites were plated into 96-well plates containing a serial dilution of PAT to reach final concentrations ranging from 83 µg/mL to 0.1 µg/mL. Each plate included controls without PAT and controls for monitoring proliferation and autofluorescence of the medium. Resazurin sodium salt (200 µg/mL, Sigma, Darmstadt, Germany) was added at two different time points: at the time of plating for the proliferation controls (T1) and 20 h post- drug pressure for the other wells (T2). After 4 h of incubation with resazurin, the fluorescence of its reduced form resorufin was recorded using the Victor X3 Multilabel Reader (Perkin Elmer, Waltham, USA) exciting at 560 nm and measuring emission at 590 nm. The test was considered valid for further analysis only if the fluorescence for the controls without drug pressure at T2 was at least 2- fold the fluorescence for the proliferation controls at T1. All experiments with the different lines were repeated three times with three technical replicates each. The blank-subtracted data expressed in relative fluorescence units (RFU) were exported to GraphPad Prism 8 to calculate the 50% inhibitory concentration (IC_{50}), using a sigmoidal dose–response model with variable slope. Statistical analysis and data visualization were performed in R Studio with built-in functions and ggplot2, respectively.

2.5 Flow cytometry assay for monitoring single-cell viability and quiescence

Exponentially proliferating cells (promastigotes and amastigotes) were exposed to PAT at concentrations ranging from 1 µg/mL to 83 µg/mL, and both the cell viability and the rEGFP expression were measured by flow cytometry at three time points: before drug exposure and after drug pressure at 24 h and 48 h. The rEGFP expression is a negative marker of quiescence: highly expressed in proliferative cells and downregulated during quiescence (Jara et al., 2019; Jara et al., 2022). The cell viability was evaluated by preincubating cells with the NucRed Dead 647 (Thermo Fisher Scientific, Waltham, USA) for the staining of dead cells and Vybrant Dye Cycle violet (Thermo Fisher Scientific) for the staining of DNA in all cells. The Vybrant Dye Cycle violet was used to select the subpopulation of cells having a healthy pattern for their DNA. A wild-type (non-rEGFP) strain and a non-stained sample were included as negative controls, together with a sample exposed to thermal shock as a positive control for cellular death. The samples were analyzed with a calibrated flow cytometer BD FACS Verse™ in the medium flow rate mode. In order to compare the rEGFP relative fluorescence units (RFU) among the samples, the acquisitions were made with the same settings during all the experiments. The FCS files were analyzed with the FCS 5 Express Plus Research edition. The subpopulation of cells with good cell viability was selected by sequential gating. Briefly, single cells were selected by pulse geometry gate; a first gate was selected by plotting the SSC-W vs. SSC-H, and a second gate was created by plotting the FCS-H and FCS-A. Among the single cells, a third gate was created by plotting the rEGFP vs. the NucRed and selecting the subpopulation NucRed-negative. Finally, a fourth gate was created by plotting the rEGFP vs. the Vybrant Dye Cycle violet and selecting the subpopulation having a healthy DNA pattern. The cell viability for each sample was estimated by multiplying the percentage of cells NucRed-negative and Vybrant DyeCycle-positive. For each developmental stage of *Leishmania* and for each time point post-exposure to PAT, the effect of the drug pressure over the rEGFP expression in the subpopulation of viable cells was evaluated with a two-way ANOVA considering the PAT drug pressure and strains as explanatory variables. Subsequently, a Tukey's HSD test was used to evaluate the statistical significance of differences between pairwise comparisons.

2.6 Estimating the lethal doses 10, 20, and 50

The measures of cell viability obtained after the flow cytometry assay over increasing concentrations of PAT were used to estimate the lethal doses of PAT that kill 10%, 25%, and 50% of the population (LD_{10}–LD_{50}). The LDs were estimated using the *drc* R package (Ritz et al., 2015). Briefly, the data were fitted using a log-logistic model with a lower limit at 0 and having as a formula the % of cell viability as explained by the concentration of PAT and the line of the parasite as the grouping factor. Finally, the desired LDs were estimated having as a reference the upper limit and setting the confidence interval to 0.95.

2.7 Whole genome sequencing

For each lineage (post-PBS and post-PAT), DNA was isolated from a sample maintained without drug pressure, and a sample recovered after 48 h of PAT drug pressure, using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). The DNA concentration was assessed with the Qubit DNA broad-range DNA quantification kit (Thermo Fisher). Library preparation and sequencing were performed at BGI with a DNBSEQ-WGS-PCR free library index and the DNBSEQ PE150 platform, respectively. The FASTQ files containing paired reads of 150 bp were subsequently analyzed for the assessment of sequence variants and ploidy. Briefly, the quality of the reads was evaluated with SAMtools and the multiqc command. Samples were aligned against the reference genome of *L. braziliensis* MHOM/BR/75/M2904 (Van den Broeck et al., 2020). The per position mean depth and the breadth of coverage were estimated with SAMtools. Variants (SNPs and INDELs) were called jointly for all 18 samples with BCFtools and the mpileup command (Danecek et al., 2021). Subsequently, the multisample vcf file was filtered according to the following criteria: overall variant quality (QUAL) higher than 200, the individual read depth per locus (FMT/DP) higher than 5, and the average mapping quality (MQ) and genotype quality (GQ) higher than 40. The annotation of the genomic location for the variants was done with SnpEff (Cingolani et al., 2012). Somy values were estimated by using the median read depth across each chromosome as reported elsewhere (Dumetz et al., 2017). A maximum likelihood phylogenetic tree was reconstructed with IQ-TREE v1.6.12 (Nguyen et al., 2015) reusing the HKY+G substitution model and 1,000 ultrafast bootstrap approximation (Hoang et al., 2018).

3 Results

3.1 *Leishmania* exposure to transient PAT drug pressure causes a reversible decrease of proliferation and does not select for parasites with lower drug susceptibility

For each of the nine rEGFP strains of *L. braziliensis* used, we measured the drug susceptibility of promastigotes of the corresponding post-PBS lineages with a resazurin test. Values ranged from 0.2 μg/mL to 7.48 μg/mL, with a median of 1.98 μg/mL (Figure 1A and Supplementary Figure 1). We then exposed all lines to the same concentration of PAT (9 μg/mL, representing ~ 4-fold the median IC_{50} of all nine strains) in order to evaluate the potential differences in their adaptation to survive. i) The potential cytostatic effect of PAT on *Leishmania* cells was monitored with the resazurin test by measuring the signal of its reduced form resorufin before the drug treatment and after 24 h of exposure to PAT or PBS. In the absence of the drug, the resorufin log2 FC signal ranged from 0.8 (PER206 EGFP Cl2) to ~ 3.2 relative fluorescence units (RFU, equivalent to 9- fold the initial signal, in PER122 EGFP Cl1), indicating that all strains were proliferating albeit at different rates. Under drug pressure, the resorufin log2 FC signal was significantly decreased compared with the one measured in the corresponding control without the drug in all strains suggesting that under our conditions of drug pressure, parasites have very diminished growth (Figure 1B). ii) After 48 h of exposure to 9 μg/mL of PAT, parasite death was observed in each lineage, but parasites were all able to resume their proliferation after subculturing in a fresh medium without a drug. This indicated that at least a proportion of the cells survived and maintained their capability to switch to a proliferative state once they were in optimal conditions. iii) We found that the susceptibility to the drug did not change in the post-PAT lineages (median IC_{50} = 2.35 μg/mL; interquartile range, IQR = 2.54) when compared with the corresponding post-PBS lineages (median IC_{50} = 2.00 μg/mL, IQR = 2.70, Mann–Whitney *U* test, $P = 0.9$, Figure 1A). Noteworthy, the capacity of *Leishmania* to survive concentrations of the drug beyond the IC_{50} of 9 μg/mL represents ~ 45 times the IC_{50} for the most susceptible line PER348 EGFP Cl1 (IC_{50} = 0.2 μg/mL).

3.2 *Leishmania* exposure to transient PAT drug pressure is accompanied by a decrease in the expression of rEGFP among viable cells

The expression of rEGFP is a negative biomarker of quiescence: it is high in proliferative cells, while it decreases or disappears in quiescent cells (Jara et al., 2022). i) In promastigotes, the exposure of proliferating cells to PAT (9.0 μg/mL) led to a significant reduction in the levels of rEGFP expression (RFU) among viable cells of each line when compared with the control treated with PBS at both 24 h and 48 h (Figure 2A). Furthermore, rEGFP expression was also different among the evaluated strains (two-way ANOVA: $P < 0.001$ for PAT treatment and $P < 0.001$ for strain). The same significant effect of the drug was observed in amastigotes. ii) We then explored if the reduction in rEGFP expression changes in response to increasing concentrations of PAT. The results showed that higher doses of PAT induced a more profound reduction of the rEGFP expression compared with the control without PAT treatment at both 24 h and 48 h post-PAT treatment in both promastigotes and amastigotes (Figure 2B). The reduction in rEGFP expression follows a dose–response model where there is a linear response that reaches a plateau approximately 9.0 μg/mL of PAT with an average relative rEGFP reduction of 44% and 40%

10.3389/fcimb.2023.1253033

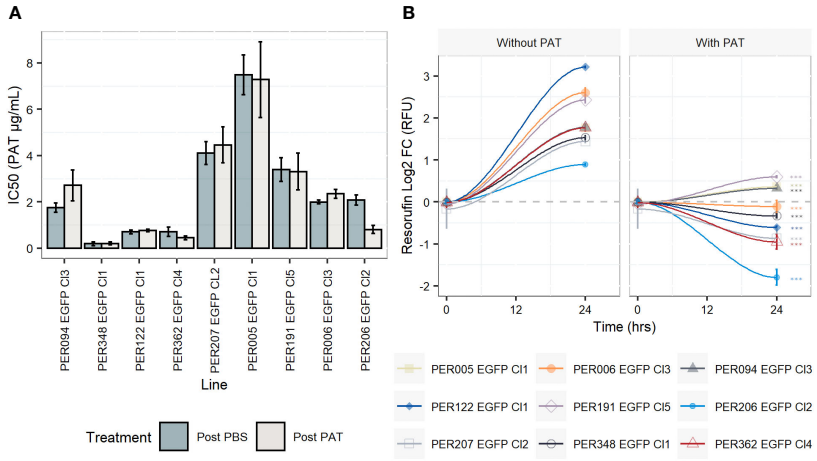


FIGURE 1
Drug susceptibility of *Leishmania braziliensis* promastigotes and their growth features under drug pressure. **(A)** Drug susceptibility to potassium antimonyl tartrate (PAT) in nine strains as estimated by the resazurin test after 24 h of drug pressure. For each strain, the IC_{50} was calculated in a lineage without prior exposure to the drug (post-PBS) and after the exposure to 9 µg/mL of PAT (~ 4 -fold the median IC_{50} considering all lineages, post-PAT). The results represent the mean ± SEM of three biological replicates. **(B)** Evaluation of the cytostatic effect of PAT at 9 µg/mL. The gray dashed line represents no change in the original resorufin signal compared with the day the cells were initially plated. A log2 FC above 1 indicates parasite duplication and a log2 FC below 0 indicates parasite arrest and/or cellular death. The asterisks represent statistically significant differences after Tukey's *post-hoc* test ($P < 0.001$) between cells after 24 h of plating without drug compared with cells under drug pressure.

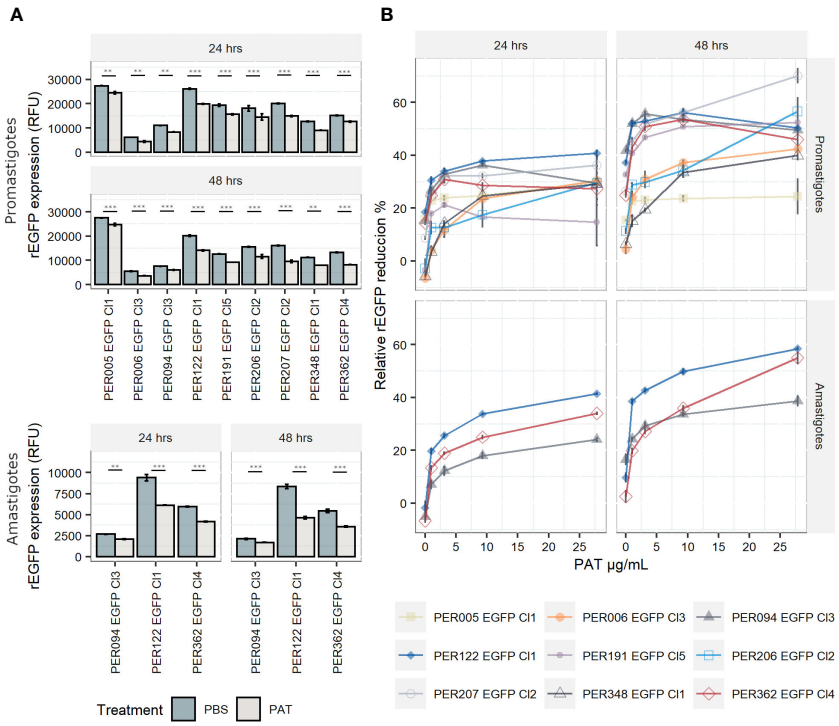


FIGURE 2
rEGFP expression in promastigotes (nine strains) and amastigotes (three strains) of *Leishmania braziliensis* under drug pressure; measures are made on viable cells. **(A)** rEGFP expression of *L. braziliensis* lineages after 24 and 48 h of exposure to 9 µg/mL of PAT. The results represent the mean ± SEM of three biological replicates. The asterisks represent statistically significant differences after Tukey's *post-hoc* test; ** $P < 0.01$, *** $P < 0.001$. **(B)** Relative rEGFP reduction (in %) in relation to increasing concentrations of PAT after 24 and 48 h of PAT exposure.

after 48 h of PAT in promastigotes and amastigotes, respectively. iii) The analysis of relative rEGFP reduction also shows differences among *L. braziliensis* strains (ranging between 23.7% and 56.2% in promastigotes and between 33.7% and 49.9% in amastigotes).

3.3 Survival of *Leishmania* to high lethal doses of PAT is accompanied by a decrease in rEGFP expression

When measuring the drug susceptibility with standard fluorometric procedures such as the resazurin test at a single point in time, it is not possible to address with certainty if the drug has cytostatic or cytotoxic effects. The decreased signal of resorufin in samples with the drug pressure compared with the control could result from cellular death, arrested growth, or likely, a mixture of both events. Therefore, we evaluated the cell viability at the single-cell level by flow cytometry and calculated the lethal doses 10, 25, and 50 (LD_{10}, LD_{25}, and LD_{50}), which are the concentrations of the drug that kill 10%, 25%, and 50% of the population, respectively. i) In promastigotes, the kinetics of the cell viability over different concentrations of PAT pressure suggests variable cytotoxic effects among the evaluated lines. The LD_{50} among all strains ranged from 5.98 µg/mL in PER206 EGFP Cl2 to 83.3 µg/mL in PER006 EGFP Cl3 (Figures 3A, B). The median LD_{50} among the nine strains was 28.2 µg/mL, which is approximately 10- fold the IC_{50} that was previously measured with the resazurin test. Noteworthy, four out of the nine lines had LD_{50} higher than 30 µg/mL of PAT, a non-physiological concentration. These strains may be considered highly tolerant to PAT compared with the other strains at the same parasite stage. ii) As promastigotes showed high variability in their rates of survival to increasing concentrations of PAT, we further evaluated the potential relationship between the capability to adopt a quiescent state as measured by the reduction in rEGFP expression and the success to overcome the drug pressure as measured by their cell viability. After 24 h of PAT treatment, there was a weak positive relationship at low concentrations of PAT (1 µg/mL to 3 µg/mL of PAT) that became strong at 9 µg/mL and 28 µg/mL of PAT (Figure 3C). iii) Amastigotes showed remarkably higher survival rates to PAT compared with the promastigotes of the respective strains. After twice the time of exposure to PAT (48 h), the highest concentration of the drug could not kill 50% of the population, and the estimation of the median LD_{25} for the three strains was 46.7 µg/mL (Figures 3D, E). Because these results with amastigotes of *L. braziliensis* were surprisingly high, we evaluated the LDs from a highly susceptible line of *L. lainsoni*. The same experimental setup estimated that in amastigotes, the LD_{25} and LD_{50} were 0.49 µg/mL and 0.97 µg/mL, respectively, ruling out that the *L. braziliensis* results would be an experimental artifact (Supplementary Figure 2). Accordingly, amastigotes of *L. braziliensis* are intrinsically more tolerant to PAT pressure compared with promastigotes. Further work is required to know if this could be due to a deeper quiescence state in amastigotes.

3.4 *Leishmania* lines exposed to PAT are genomically similar to their respective parental lines

We evaluated the genome stability between pre- and post-PAT lines by WGS, at the levels of somy, SNPs, and INDELs. i) At the somy level, we observed variability of karyotypes in a strain-specific manner. The number of aneuploid chromosomes/strain ranged from 1 to 10. Trisomy of chromosomes 5, 11, 25, and 29 and tetrasomy of chromosome 31 were among the most frequent occurrences being present in at least three out of the nine strains. For each strain, the original overall ploidy of the post-PAT lineage was identical to the corresponding post-PBS lineage (Figure 4A). ii) Compared with the *L. braziliensis* reference genome (MHOM/BR/75/M2904), a total of 163,823 high-quality SNPs and 17,055 high-quality INDELs were called across our panel of 18 lineages. A maximum likelihood phylogenetic tree based on SNPs shows that each post-PAT lineage clusters with its corresponding control, post-PBS lineage (Figure 4B), reflecting the high genomic similarity between post-PAT and post-PBS lineages. Indeed, each post-PAT lineage showed differences from its corresponding control lineage at only 11 to 208 SNP loci and 76 to 119 INDEL loci, the majority of which (73.4% SNPs and 93.1% INDELs) occurred in the non-coding region of the genome (Supplementary Data). None of these variants occurred in a homozygous state; in other words, all variants were either absent in the post-PBS lineage and heterozygous in the post-PAT lineage, or vice versa. For each strain, the genomic analysis indicates that PAT-exposed lines are genomically very similar to their parental line and that, although a limited number of mutations occur, they are not in genes known to be related to drug resistance in *Leishmania*.

3.5 After prolonged exposure to environmental stress, a small fraction of viable cells survive and show reduced rEGFP expression

Persisters represent another manifestation of quiescence and DT; in bacteriology, these correspond to a subpopulation of DT cells that can survive antibiotic treatment much better than the majority of the population (Barrett et al., 2019). We explored the occurrence of persister-like cells in promastigotes of *L. braziliensis* PER191 EGFP Cl5 under two conditions of prolonged stress (see Figure 5A for a description of the experimental design): i) by long-term stationary culture, where cells were maintained without subculturing and without PAT over 14 days (Sta D14), and ii) by exposing exponentially growing cells to PAT and maintaining the drug pressure (exposure 1) over 14 days (Log PAT D14). We reasoned that in these cultures majority of the cells would be dead, and only the ones with persister-like properties would remain. In both stress conditions, we tested the resilience of the survivors on day 14, by treating them (exposure 2) with PAT or PBS as untreated control, over 24 h; we measured viability, rEGFP expression, and reversibility to proliferative state.

10.3389/fcimb.2023.1253033

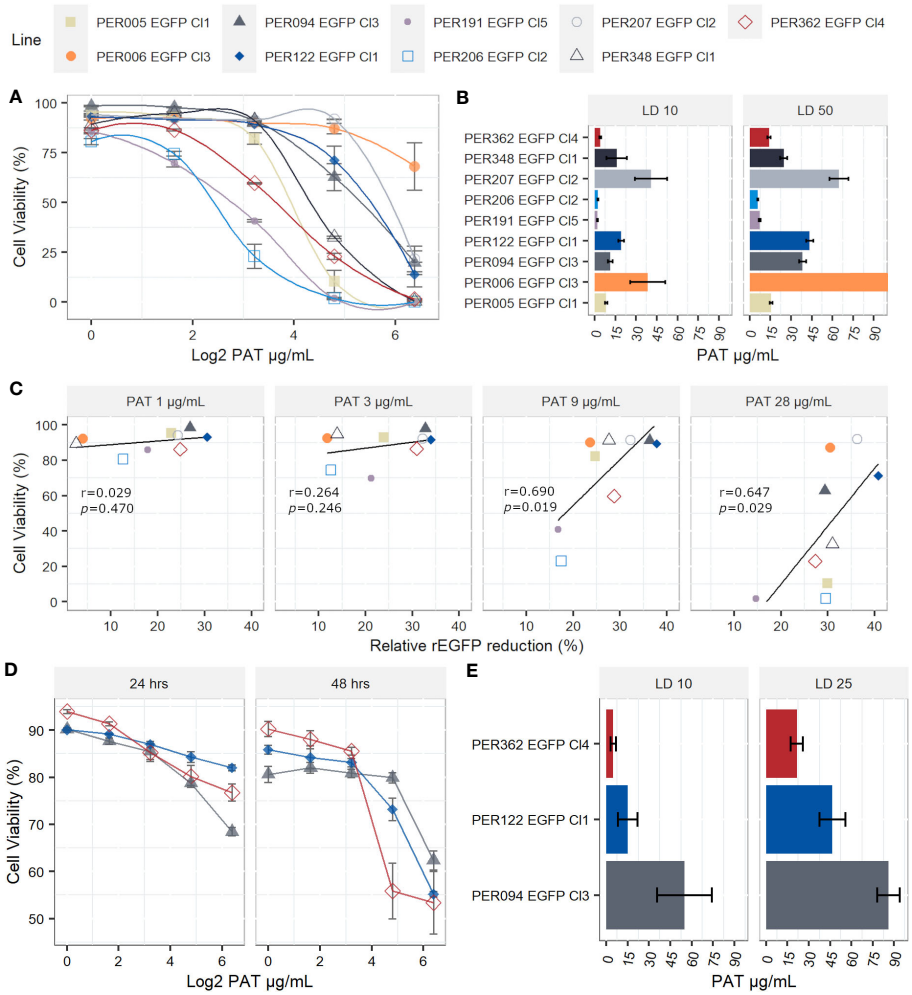


FIGURE 3
Lethal doses (LD) on *Leishmania braziliensis* promastigotes and amastigotes. **(A)** Viability of individual promastigotes after 24 h of exposure to increasing PAT concentration. **(B)** Lethal doses 10 and 50 on promastigotes. The results represent the mean ± SEM of three biological replicates. **(C)** The relationship between viability of promastigotes and reduction of rEGFP expression at different PAT concentrations. The Pearson's correlation coefficients and *P*-values are shown. **(D)** Viability of individual amastigotes after 24 and 48 h of exposure to increasing PAT concentration. **(E)** Lethal doses 10 and 25 on amastigotes, calculated after 48 h of PAT exposure. The results represent the mean ± SEM of three biological replicates.

3.5.1 Viability

First, the Sta D14 population receiving an exposure to PBS on day 14 (control) showed an average cell viability of 30.38% (three replicates, only one shown in Figure 5B), while Sta D14 cells treated with PAT on day 14 (exposure 2) showed a significant decrease of their average viability to 4.68% (*t*-test, $P < 0.001$). Secondly, the Log PAT D14 population showed a very small fraction of survivors (average of 0.60%) in the untreated control exposed to PBS on day 14, and after a second exposure to PAT, on day 14, there was no significant change in the proportion of survivors (average of 0.55%).

3.5.2 rEGFP expression

There was a significantly higher reduction of the levels of rEGFP expression compared with what was initially observed in cells exposed to 48 h of PAT (Figure 2): 87.5% vs. a maximum of 56.2%, respectively. There was no further rEGFP reduction when Sta D14 and Log PAT D14 samples received exposure 2 to PAT, suggesting that the minimum basal levels of expression required for survival were reached (Figure 5C). Aside from the analysis by flow cytometry, we also looked for the presence of survivors under a confocal microscope. It was possible to observe a very scarce number of cells that had very low levels of rEGFP expression and that, despite remaining at the same location, had a very clear motility of their flagellum.

3.5.3 Reversibility to proliferative state

Survivors from both conditions were able to reproliferate after passing them in a fresh medium. The new population reached 95% and 85% of cell viability by day 7 in Sta D14 and Log PAT D14, respectively.

4 Discussion

We studied here the resilience of *L. braziliensis* cells surviving high doses of PAT *in vitro*. Exposure to transient PAT pressure

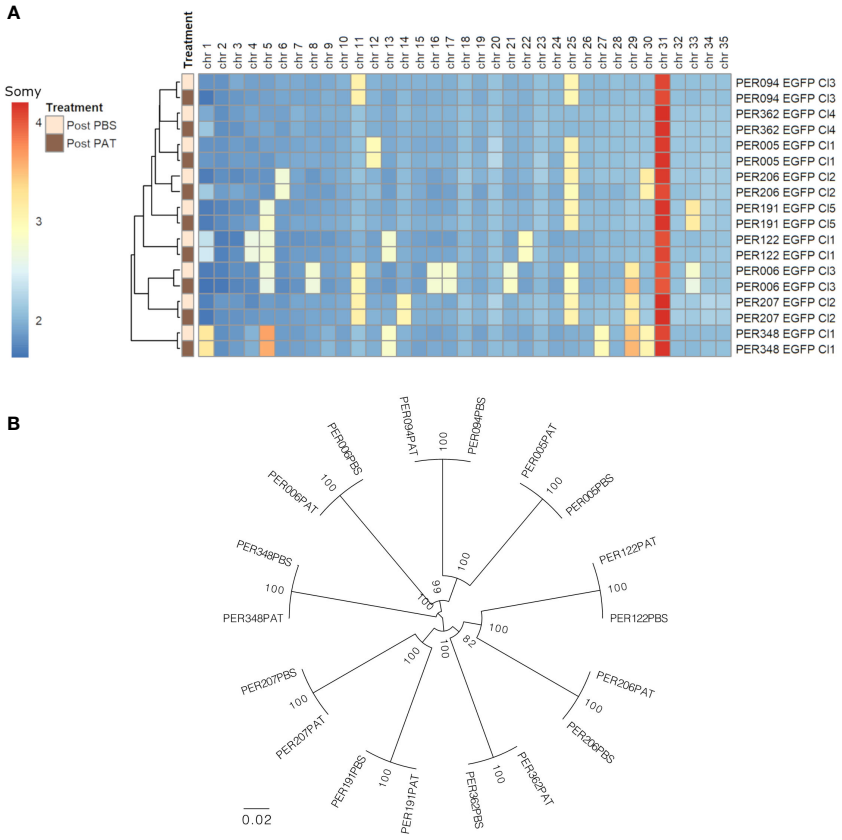


FIGURE 4
Genomic differences between the nine strains of *Leishmania braziliensis* and each lineage before (post-PBS) and after (post-PAT) drug pressure; bulk whole genome sequencing. **(A)** Ploidy. **(B)** Maximum likelihood phylogenetic tree based on 163,823 high-quality SNPs.

caused a reversible decrease in cell proliferation as well as a significant drop in rDNA transcription, measured here by a decrease in the rEGFP expression among viable cells. The coincidence of these two features is a hallmark of quiescence under stressful conditions (Jara et al., 2022), but this was also observed in an *L. lainsoni* strain exposed to PAT (Jara et al., 2022). We also found in the present study that PAT exposure did not select for parasites with lower drug susceptibility and that for each *L. braziliensis* strain, pre- and post-PAT lines were genomically similar, and no markers of DR were encountered. Altogether, these four observations suggest that surviving PAT exposure in our experimental conditions was not due to DR but to a form of quiescence-mediated DT.

Quiescence is a widespread adaptive strategy that allows cells to survive environmental insults. Pathogenic vector- borne parasites like *Leishmania* are "naturally" exposed to multiple stresses in the insect vector and the mammal host (Caljon et al., 2016), and quiescence could play a major adaptive role among other survival skills. Exposure to drugs is evolutionary recent and constitutes an "artificial" source of stress to which parasites can adapt by genetic variation (DR) or by the exploitation of pre-existing mechanisms of quiescence, through metabolic modulation (DT) as demonstrated here, with PAT. Under high concentrations of PAT (median LD_{50} of 28 μg/mL), a large proportion of cells remained viable, and this was significantly correlated with a decrease in metabolic activity as measured by the relative rEGFP reduction. As such, this is close to the definition of DT as given in bacteriology, i.e., the general ability of a population of cells to survive longer treatments, a.o. by having a lower killing rate (Balaban et al., 2019), here measured by the LD_{50} which was approximately 10 times the IC_{50}. Pushing our experimental conditions further —by longer exposure to environmental stress: long-term stationary phase and/or PAT exposure— showed that there were still survivors, albeit a smaller proportion than under short exposure to the drug. This was accompanied by a higher reduction of the rEGFP expression, indicating a deeper quiescent stage in those survivors. These highly resilient parasites are very similar to persisters, a subpopulation of tolerant cells in bacteria, which can survive antibiotic treatment much better than the majority of the population (Balaban et al., 2019).

The study was done with axenic promastigotes (model for the developmental life stage of the sand fly), and the results were confirmed in axenized amastigotes (models for the developmental life stage living in the mammal). Further work is required to extend and confirm these results in an intracellular context and *in vivo*, where stress factors can be different. Our collection of *L. braziliensis* strains with high and low capacity to adopt a quiescent state in the promastigote model could be further explored to set up an *in-vivo* quiescence model. The only report currently available on *in-vivo* quiescence in the context of chemotherapy revealed, in miltefosine-

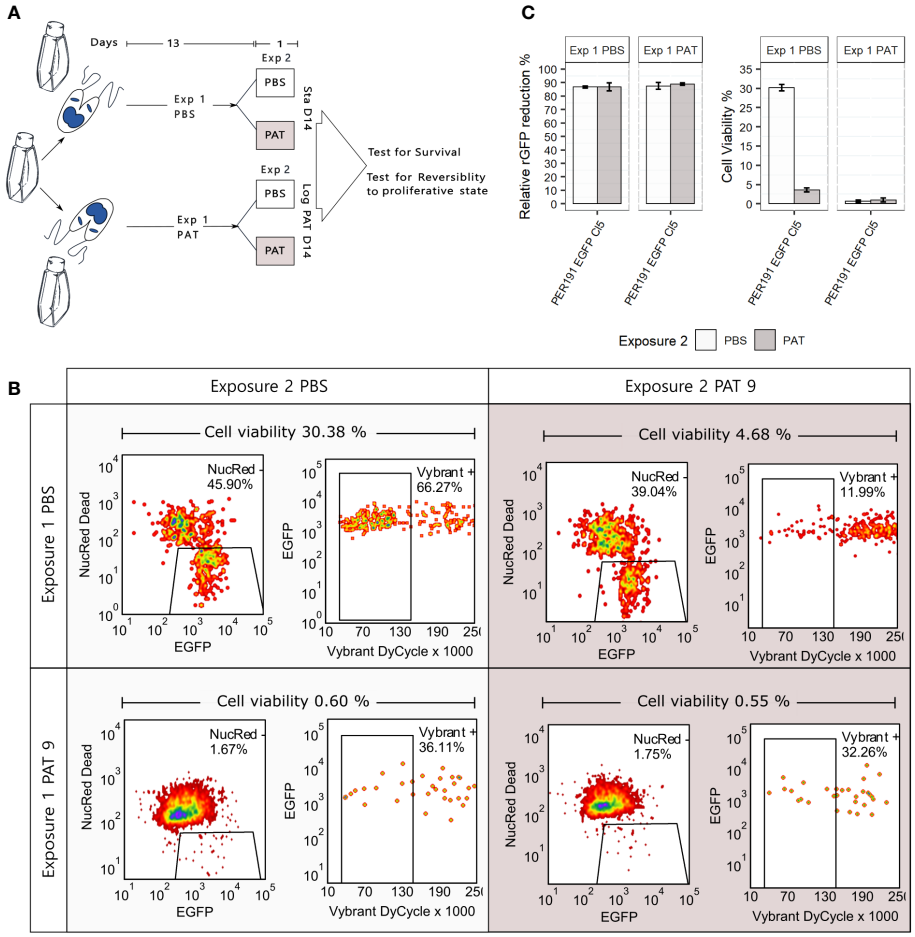


FIGURE 5
Promastigote (PER191 EGFP Cl5) viability under conditions of longer and stronger environmental stress. **(A)** Experimental outline. Cell viability as measured by flow cytometry in i) long-term stationary phase on day 14 (Sta D14) or ii) after exposing proliferating cells to 14 days of PAT pressure (log PAT D14); we tested the resilience of the survivors on day 14 after treating them with a second exposure to PAT or PBS as control over 24 h. The concentration of the first and second exposure to PAT was 9 µg/mL. See *Materials and methods* for details on sequential gating. **(B)** The density plots showing the percentage of survivors on day 14 in one representative biological replicate per experimental condition. **(C)** Cell viability and their levels of relative rEGFP reduction in long-term cultures as described in panel **(A)**. The bars represent the mean ± SEM of three biological replicates.

treated *L. mexicana*-infected BALB/c mice, the presence of quiescent amastigotes in collagen-rich, dermal mesothelium surrounding granulomas (Kloehn et al., 2021): the authors concluded that quiescence, together with the lack of miltefosine accumulation in the mesothelium, may contribute to drug failure and non-sterile cure. The potential for DT and persistence shown here by *L. braziliensis* strains and by *L. lainsoni* (Jara et al., 2022) might explain several (sub)clinical features associated with the species of subgenus *Viannia*, like i) the presence of parasites in 80% of scars, years after treatment with pentavalent antimony therapy (Schubach et al., 1998); ii) the mucosal metastases from healed primary cutaneous lesions (Jha et al., 2022); and iii) the treatment failure in the absence of DR (Yardley et al., 2006). Longitudinal studies of infected individuals and monitoring of quiescence are needed to test these hypotheses. Such studies are not likely to be easy given the paucity —by definition— of persister cells, and they require positive markers (and not negative markers, like the rEGFP that can be used in experimental studies like here). A first step in that direction was made by a transcriptomic study which indicated that in an overall context of transcriptional shift, some transcripts were relatively more abundant in quiescent cells, like leishmanolysin (GP63), amastin and amastin-like proteins, and autophagy-related genes (Jara et al., 2022).

Noteworthy, the nine *L. braziliensis* strains here studied showed different levels of quiescence and DT, which was not associated with the large genetic distances between each of the nine strains: for instance, the four strains with the highest LD_{50} (PER006, 094, 122, and 207) were spread over the phylogenetic tree shown in Figure 4B. There was also no apparent link with the treatment outcome of the patients from which the parasites were originating: for instance, PER006 and PER206, which showed respectively the highest and lowest values of LD_{50}, both came from unresponsive lesions. However, it is too premature to infer any impact of DT level measured *in vitro* on the treatment outcome in patients. Indeed, a huge selection bias is introduced when isolating and cultivating parasites. There is a strong bottleneck at isolation time, and during *in-vitro* maintenance, the parasites, which are the fittest in the culture medium, will be selected and will form a dominant

population; this is not necessarily the same as the dominant population present in the patient, a phenomenon that we observed in *L. donovani* (Domagalska et al., 2019). Accordingly, in the next step, quiescence and DT should —as previously recommended for DR (Domagalska and Dujardin, 2020)— also be studied directly in the patient to know its clinical impact and discriminatory molecular markers, and sensitive detection methods should be developed for this.

It is very likely that the results here observed are drug-specific and cannot be generalized to all antileishmanial drugs. Indeed, quiescence is expected to protect against drugs i) interfering with the parasite metabolism and ii) which can be countered by decreasing this metabolism. This is the case for three of the anti-leishmania drugs: pentavalent antimonials (Sb^V), miltefosine (MIL), and paromomycin (PMM). The reduced form of Sb^V (PAT) has a direct effect on the parasite by disturbing its redox potential (Wyllie et al., 2004), and a similar effect is expected with Sb^V itself which has an indirect effect through interfering with the signaling of the macrophage and triggering ROI/RNI production in the host cell (Basu et al., 2006). MIL interferes with the biosynthesis of lipids including sterols and sphingolipids (Armitage et al., 2018) and the metabolism of alkyl-lipids, also inducing mitochondrial depolarization and a decrease of intracellular levels of ATP (Ponte-Sucre et al., 2017). PMM targets the decoding A-site of the ribosomes' small subunit increasing misreading and translation inhibition (Chawla et al., 2011). In contrast, amphotericin B which binds to ergosterol-related sterols in the cell membrane, inducing the production of a pore and fatal exchange of ions (Ramos et al., 1996), is expected to induce less (if any) quiescence and DT.

Research on quiescence, DT, and persistence in *Leishmania* is still in its infancy. Aside from providing knowledge that might help better understand the pathophysiology of *Leishmania* infections, it might also support and guide further investigations on new chemotherapeutic interventions to counter the disease as well as the infection. Historically, research into new drugs screened replicative forms, creating a leishmaniasis intervention tool kit that neglects the impact of quiescent forms. We provide here a new model of quiescence and DT that could be further exploited for the *in-vitro* screening of compounds to overcome the quiescence resilience of the parasite, thereby improving the therapy of leishmaniasis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article. The raw data from the WGS for this study can be found in the Sequence Read Archive (SRA) submission: SUB12410472 (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB12410472/overview).

Author contributions

All authors made substantial contributions to the conception or design of the work; the acquisition, analysis, or interpretation of data; drafted the work or revised it critically for important intellectual content; agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

Funding

This study was financially supported by the Flemish Fund for Scientific Research (grant G075121N, postdoctoral grant to MJ, FWO-1223420N, and postdoctoral grant to FVdB, FWO-1226120N), the European Commission (contracts ICA4-CT-2001-10076 and INCO-CT2005-015407), and the Flemish Ministry of Science and Innovation (Secondary Research Funding ITM – SOFI, Grant MADLEI).

Acknowledgments

We acknowledge Ilse Maes for her logistic support in the lab.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1253033/full#supplementary-material

References

Armitage, E. G., Alqaisi, A. Q. I., Godzien, J., Peña, I., Mbekeani, A. J., Alonso-Herranz, V., et al. (2018). Complex interplay between sphingolipid and sterol metabolism revealed by perturbations to the leishmania metabolome caused by miltefosine. *Antimicrob. Agents Chemother.* 62, e02095-17. doi: 10.1128/AAC.02095-17

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17, 441–448. doi: 10.1038/S41579-019-0196-3

Barrett, M. P., Kyle, D. E., Sibley, L. D., Radke, J. B., and Tarleton, R. L. (2019). Protozoan persister-like cells and drug treatment failure. *Nat. Rev. Microbiol.* 17, 607–620. doi: 10.1038/s41579-019-0238-x

Basu, J. M., Mookerjee, A., Sen, P., Bhaumik, S., Sen, P., Banerjee, S., et al. (2006). Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in Leishmania donovani-infected macrophages. *Antimicrob. Agents Chemother.* 50, 1788–1797. doi: 10.1128/AAC.50.5.1788-1797.2006

Bojsen, R., Regenberg, B., and Folkesson, A. (2017). Persistence and drug tolerance in pathogenic yeast. *Curr. Genet.* 63, 19–22. doi: 10.1007/S00294-016-0613-3

Bolhassani, A., Taheri, T., Taslimi, Y., Zamanilui, S., Zahedifard, F., Seyed, N., et al. (2011). Fluorescent Leishmania species: Development of stable GFP expression and its application for in *vitro* and in *vivo* studies. *Exp. Parasitol.* 127, 637–645. doi: 10.1016/j.exppara.2010.12.006

Caljon, G., De Muylder, G., Durnez, L., Jennes, W., Vanaerschot, M., and Dujardin, J.C (2016). Alice in microbes' land: Adaptations and counter-adaptations of vector-borne parasitic protozoa and their hostsa. *FEMS Microbiol. Rev.* 40, 664–685. doi: 10.1093/femsre/fuw018

Chawla, B., Jhingran, A., Panigrahi, A., Stuart, K. D., and Madhubala, R. (2011). Paromomycin affects translation and vesicle-mediated trafficking as revealed by proteomics of paromomycin -susceptible -resistant Leishmania donovani. *PloS One* 6, e26660. doi: 10.1371/JOURNAL.PONE.0026660

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., et al. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin)* 6, 80–92. doi: 10.4161/fly.19695

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., et al. (2021). Twelve years of SAMtools and BCFtools. *Gigascience* 10, giab008. doi: 10.1093/GIGASCIENCE/GIAB008

Domagalska, M. A., and Dujardin, J.-C. (2020). Next-generation molecular surveillance of triTryp diseases. *Trends Parasitol.* 36, 356–367. doi: 10.1016/j.pt.2020.01.008

Domagalska, M. A., Imamura, H., Sanders, M., Van den Broeck, F., Bhattarai, N. R., Vanaerschot, M., et al. (2019). Genomes of Leishmania parasites directly sequenced from patients with visceral leishmaniasis in the Indian subcontinent. *PloS Negl. Trop. Dis.* 13, e0007900. doi: 10.1371/journal.pntd.0007900

Dumetz, F., Imamura, H., Sanders, M., Seblova, V., Myskova, J., and Pescher, P. (2017). Modulation of aneuploidy in *leishmania in vitro* and *in vivo* environments and its. *mBio* 8, e00599–e00517. doi: 10.1128/mBio.00599-17

Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q., and Vinh, L. S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Mol. Biol. Evol.* 35, 518–522. doi: 10.1093/MOLBEV/MSX281

Jara, M., Barrett, M., Maes, I., Regnault, C., Imamura, H., Domagalska, M. A., et al. (2022). Transcriptional shift and metabolic adaptations during leishmania quiescence using stationary phase and drug pressure as models. *Microorganisms* 10, 97. doi: 10.3390/microorganisms10010097

Jara, M., Maes, I., Imamura, H., Domagalska, M. A., Dujardin, J. C., and Arevalo, J. (2019). Tracking of quiescence in Leishmania by quantifying the expression of GFP in the ribosomal DNA locus. *Sci. Rep.* 9, 18951. doi: 10.1038/S41598-019-55486-Z

Jha, B., Reverte, M., Ronet, C., Prevel, F., Morgenthaler, F. D., Desponds, C., et al. (2022). In and out: Leishmania metastasis by hijacking lymphatic system and migrating immune cells. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/FCIMB.2022.941860

Keren, I., Minami, S., Rubin, E., and Lewis, K. (2011). Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. *mBio* 2, e00100-11. doi: 10.1128/MBIO.00100-11

Kloehn, J., Boughton, B. A., Saunders, E. C., O'callaghan, S., Binger, K. J., and McConville, M. J. (2021). Identification of metabolically quiescent leishmania mexicana parasites in peripheral and cured dermal granulomas using stable isotope tracing imaging mass spectrometry. *mBio* 12, e00129–e00121. doi: 10.1128/mBio.00129-21

Kloehn, J., Saunders, E. C., O'Callaghan, S., Dagley, M. J., and McConville, M. J. (2015). Characterization of metabolically quiescent leishmania parasites in murine lesions using heavy water labeling. *PloS Pathog.* 11, e1004683. doi: 10.1371/journal.ppat.1004683

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E., et al. (1999). Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of Leishmania donovani. *J. Infect. Dis.* 180, 564–567. doi: 10.1086/314896

Mandell, M. A., and Beverley, S. M. (2017). Continual renewal and replication of persistent Leishmania major parasites in concomitantly immune hosts. *Proc. Natl. Acad. Sci. U.S.A.* 114, E801–E810. doi: 10.1073/pnas.1619265114

Michiels, J. E., Van Den Bergh, B., Verstraeten, N., Fauvart, M., and Michiels, J. (2016). *In vitro* emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. *Antimicrob. Agents Chemother.* 60, 4630–4637. doi: 10.1128/AAC.00757-16

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., and Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. doi: 10.1093/MOLBEV/MSU300

Ponte-Sucre, A., Gamarro, F., Dujardin, J. C., Barrett, M. P., López-Vélez, R., García-Hernández, R., et al. (2017). Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PloS Negl. Trop. Dis.* 11, e0006052. doi: 10.1371/JOURNAL.PNTD.0006052

Ramos, H., Valdivieso, E., Gamargo, M., Dagger, F., and Cohen, B. E. (1996). Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. *J. Membr. Biol.* 152, 65–75. doi: 10.1007/S002329900086

Rijal, S., Yardley, V., Chappuis, F., Decuypere, S., Khanal, B., Singh, R., et al. (2007). Antimonial treatment of visceral leishmaniasis: are current in *vitro* susceptibility assays adequate for prognosis of in *vivo* therapy outcome? *Microbes Infect.* 9, 529–535. doi: 10.1016/J.MICINF.2007.01.009

Ritz, C., Baty, F., Streibig, J. C., and Gerhard, D. (2015). Dose-response analysis using R. *PloS One* 10, e0146021. doi: 10.1371/journal.pone.0146021

Schubach, A., Haddad, F., Neto, M. P. O., Degrave, W., Pirmez, C., Grimaldi, G., et al. (1998). Detection of Leishmania DNA by polymerase chain reaction in scars of treated human patients. *J. Infect. Dis.* 178, 911–914. doi: 10.1086/515355

Teuscher, F., Chen, N., Kyle, D. E., Gatton, M. L., and Cheng, Q. (2012). Phenotypic changes in artemisinin-resistant Plasmodium falciparum lines in *vitro*: evidence for decreased sensitivity to dormancy and growth inhibition. *Antimicrob. Agents Chemother.* 56, 428–431. doi: 10.1128/AAC.05456-11

Van den Broeck, F., Savill, N. J., Imamura, H., Sanders, M., Maes, I., Cooper, S., et al. (2020). Ecological divergence and hybridization of Neotropical Leishmania parasites. *Proc. Natl. Acad. Sci. U.S.A.* 117, 25159–25168. doi: 10.1073/pnas.1920136117

Van Meirvenne, N., Janssens, P. G., and Magnus, E. (1975)Antigenic variation in syringe passaged populations of Trypanosoma (Trypanozoon) brucei. 1. Rationalization of the experimental approach (Accessed June 3, 2020).

WHO *Ending the neglect to attain the Sustainable Development Goals: A road map for neglected tropical diseases 2021–2030*. Available at: https://www.who.int/publications/i/item/9789240010352 (Accessed June 23, 2021).

Wyllie, S., Cunningham, M. L., and Fairlamb, A. H. (2004). Dual action of antimonial drugs on thiol redox metabolism in the human pathogen Leishmania donovani. *J. Biol. Chem.* 279, 39925–39932. doi: 10.1074/JBC.M405635200

Yardley, V., Ortuño, N., Llanos-Cuentas, A., Chappuis, F., De Doncker, S., Ramirez, L., et al. (2006). American tegumentary leishmaniasis: Is antimonial treatment outcome related to parasite drug susceptibility? *J. Infect. Dis.* 194, 1168–1175. doi: 10.1086/507710

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 10 October 2023
DOI 10.3389/fmed.2023.1196063

OPEN ACCESS

EDITED BY
Taís Fontoura de Almeida,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Fatemeh Ghaffarifar,
Tarbiat Modares University, Iran
Rory Cristiane Fortes De Brito,
The Pirbright Institute, United Kingdom

*CORRESPONDENCE
Myrthe Pareyn
✉ myrthepareyn@itg.be

†These authors share first authorship

RECEIVED 29 March 2023
ACCEPTED 22 September 2023
PUBLISHED 10 October 2023

CITATION
van Henten S, Pareyn M, Tadesse D, Kassa M, Techane M, Kinfe E, Girma N, Demeke D, Mesay M, Kassa M, Temesgen R, Shewangizaw M, Massebo F, van Griensven J, Wegayehu T and Merdekios B (2023) Community-based treatment of cutaneous leishmaniasis using cryotherapy and miltefosine in Southwest Ethiopia: the way forward? *Front. Med.* 10:1196063.
doi: 10.3389/fmed.2023.1196063

Community-based treatment of cutaneous leishmaniasis using cryotherapy and miltefosine in Southwest Ethiopia: the way forward?

Saskia van Henten[1†], Myrthe Pareyn[1*†], Dagimawie Tadesse[2], Mekidim Kassa[3], Mehret Techane[4], Eyerusalem Kinfe[4], Nigatu Girma[5], Degnet Demeke[3], Mebratu Mesay[3], Mekibib Kassa[6], Rodas Temesgen[7], Misgun Shewangizaw[3], Fekadu Massebo[5], Johan van Griensven[1], Teklu Wegayehu[5] and Behailu Merdekios[3]

[1]Department of Clinical Sciences, Institute of Tropical Medicine Antwerp, Antwerp, Belgium, [2]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia, [3]Department of Public Health, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia, [4]Department of Dermatology, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia, [5]Department of Biology, College of Natural and Computational Sciences, Arba Minch University, Arba Minch, Ethiopia, [6]Leishmaniasis Research and Treatment Center, University of Gondar Hospital, Gondar, Ethiopia, [7]Department of Internal Medicine, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia

Background: Cutaneous leishmaniasis (CL) is a common, yet massively underreported skin morbidity in Ethiopia. Most patients never seek treatment, as this is offered only in specialized treatment centers. Early diagnosis and treatment through decentralization is crucial to decrease transmission and to reach the NTD roadmap goals. However, little information is available on outcomes and challenges of community-based treatment initiatives.

Methods: A community-based prospective cohort study was conducted in Ochollo. Patients with clinically or microscopy confirmed CL were included. Cryotherapy was (to be) given weekly with at least four sessions for uncomplicated lesions, and miltefosine was given for 4 weeks for complicated lesions. Miltefosine adherence was assessed by counting pill strips. Clinical and patient-reported outcomes (dermatological life quality index and patient-global assessment) were assessed at month 6 (M6).

Results: A total of 107 patients were included, with a median age of 6years. Two patients refused, and 15 could not be treated as they were too young (<4years) for miltefosine. Giving cryotherapy to patients weekly was not feasible due to long wound healing times and required use of topical antibiotics. Only 52.4% of miltefosine patients finished >90% of their tablets by M1. Among 46 patients treated with cryotherapy, 24 (52.2%) were cured at M6, and 9 (19.6%) had substantial improvement. The cure rate was 16/39 (41.0%) for miltefosine with 28.2% (11/39) substantial improvement. Before treatment, more than half (57.8%) of patients reported that CL did not negatively impact their life, which significantly increased to 95.2% at M6. At this time, 61.7% of patients said their lesion was clear, which was 1% before treatment.

Conclusion: Our study is the first to identify the challenges and opportunities of miltefosine and cryotherapy for community treatment of CL. Although overall cure rates were lower than expected, patient-reported outcomes were generally positive and quite some patients had good improvement.

KEYWORDS

decentralization, patient-reported outcomes, impavido, treatment outcomes, operational research

Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease (NTD) caused by *Leishmania* protozoa. In Ethiopia, the majority of the CL cases is caused by *Leishmania aethiopica*, with lesions generally severe, of long-standing duration and hard to treat compared to CL caused by other species (1).

Transmission of CL in Ethiopia is typically described to be zoonotic with hyraxes as its reservoir. However, studies by Mutinga et al. (2) and Pareyn and Kochora et al. (3) demonstrate that besides hyraxes, humans also seem to be an important reservoir that can fuel transmission of *L. aethiopica*. Therefore, early diagnosis and treatment of CL patients to tackle the human source of infection could be pivotal to decrease the disease burden (4).

Although the estimated yearly incidence of CL is 20,000 to 50,000, only 878 cases were reported to the WHO in 2018 (5, 6). These numbers show that there is severe underreporting of CL. This is primarily because CL diagnosis and care are only available in specialized treatment centers, often far from patients, impeding them to seek modern treatment. Decentralizing treatment closer to patients therefore seems crucial to obtain the NTD roadmap goal of detecting 85% of all cases and making sure 95% of them are treated (7).

However, the most widely available treatment, intramuscular or intralesional injections with sodium stibogluconate (SSG), is challenging to use in primary healthcare facilities. Cryotherapy (for smaller uncomplicated lesions) and miltefosine (for severe, complicated lesions) seem more suitable alternatives. Cryotherapy is used for the treatment of CL in Ethiopian referral hospitals (8, 9), although reports on its long term cure rate are scarce. It can be performed on an out-patient basis, has few side-effects, and is relatively cheap (10). Although liquid nitrogen comes with logistic challenges, projects in other countries have shown that administration of liquid nitrogen by nurses during field visits is possible, acceptable, and safe (11).

Miltefosine is the only available oral leishmaniasis treatment and is relatively safe with mostly mild gastro-intestinal adverse effects. Therefore, it has a good potential for outpatient treatment of CL patients who need systemic treatment. In a hospital-based study conducted in Northern Ethiopia, CL patients with severe, large, and long-standing lesions were treated with miltefosine in which it was found to be acceptable and safe. However, the effectiveness differed greatly between the two study sites (72.7% vs. 26.7% cure) (12). As we hypothesized that lesions in the community are more recent and less severe, community-based detection and earlier treatment of CL cases could improve the outcome of miltefosine treatment.

Most studies on CL treatment only consider clinical outcomes, while including patients perspectives by incorporating scar and quality of life evaluation is recommended (13). In this pilot project, we determined clinical and patient-reported outcomes of community treatment for CL with cryotherapy and miltefosine and also describe the challenges and opportunities that were encountered.

Methods

Ethics statement

This study was approved by the ethical review committees of the Institute of Tropical Medicine in Antwerp (1,513/21), the University Hospital of Antwerp (21/27/275), and Arba Minch University (IRB/1122/2021). Written informed consent was obtained from all participants or from the guardian/parent of patients below the age of 18, with additional assent collected for patients aged 12–17 years. All patients specifically provided consent for taking and using their lesion photographs, provided they could not be identified.

Setting

This study was carried out in Ochollo, a village in the southwest of Ethiopia, around 25 km north of Arba Minch. Ochollo is a rural, highly endemic, and well-known focus of CL. Previous research showed that 5.5% of the primary school children had active CL and 60% had scars due to a *Leishmania* infection (14). The village is inhabited by approximately 5,000 residents. Basic health service packages are provided to patients by health extension workers at the health post. More complicated medical cases are referred to Arba Minch General Hospital. CL treatment is not available at the health post or nearby district health center; rather, CL patients seek care from traditional healers living in Ochollo, who generally provide treatment for free.

Design

A prospective cohort study was conducted at the health post in Ochollo village from February until August 2022. Patients were treated with cryotherapy or miltefosine depending on the type of lesions with outcome assessment at 1, 3, and 6 months after starting treatment. The study procedures including recruitment, treatment, and follow-up visits at month 1 (M1), M3 and M6 are shown in Figure 1. This study follows the STROBE guidelines for reporting (15) (Supplementary material 1).

Population and recruitment

Patients with suspected CL (as identified by four experienced field workers, self-identified, referred by the health extension worker, or identified by the study team at the market or school) were advised to visit the health post for formal evaluation during the first week of the study. A dermatologist or general practitioner with 3 years dermatology work experience evaluated the patients, and classified them as follows: (1) clear clinical diagnosis of CL, (2) clinically

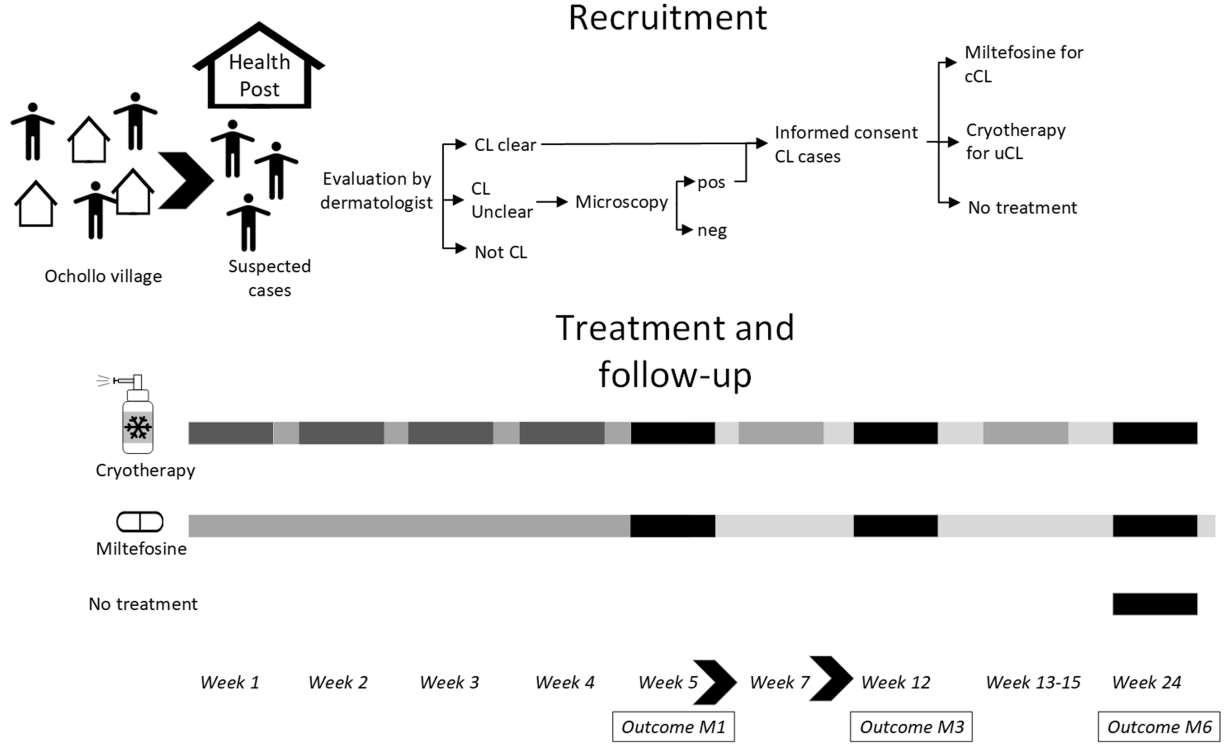


FIGURE 1
Study overview. All patients were enrolled in the first week of the study. Patients were screened by field assistants and asked to come to the health post. After evaluation by a dermatologists, patients were classified as clear CL, not CL or unclear CL, in which case a skin slit was taken for CL diagnosis by microscopy. Clear CL cases and those confirmed by microscopy were asked to give informed consent. Treatment allocation was done by a study physician: cryotherapy for uncomplicated CL (uCL) and miltefosine for complicated CL (cCL) patients. The next week, patients were started on treatment for four consecutive weeks, in which side-effects were also followed weekly. Treatment extension for cryotherapy could take place at week 5, 7, and 13–15 at the physicians' decision. Outcome assessment (clinical and patient-reported) was done at month 1 (M1), month 3 (M3), and month 6 (M6) after starting treatment.

suspected CL, but lab confirmation needed, (3) not CL. Patients with clinical diagnosis of CL were immediately eligible for inclusion, while for patients whose clinical diagnosis was not sufficiently clear without lab confirmation, a skin slit for microscopy was collected. Any patients who were unwilling to give a non-invasive D-Squame Skin Stripping Disc (Monaderm, Monaco) sample (intended to confirm CL diagnosis by PCR) were excluded.

The dermatologist allocated the treatment to patients by first classifying patients as uncomplicated CL (<4 lesions and < 4 cm in size and no involvement of the nose, mucosa, or joints) or complicated CL (>4 lesions or lesion size >4 cm or involvement of mucosa, nose, joints, or signs of dissemination). Patients with uncomplicated CL were assigned to cryotherapy and patients with complicated CL to miltefosine. Complicated patients who were less than 4 years old could not be treated, as the protocol specified miltefosine could only be given to those above age four. Similarly, complicated patients who were pregnant or breastfeeding were not treated. Additionally, no treatment was given to patients who declined treatment, or for whom the physician did not deem treatment to be beneficial. These three patient groups who did not receive treatment were still enrolled for the study population description and outcome assessment at M6. No sample size calculation was done as we intended to recruit all voluntary and eligible patients from the village into the study.

Sample collection and processing

A skin slit smear was collected from CL suspected patients for whom lab confirmation was needed by taking a sample with a scalpel from the border of the lesion. The skin slit was smeared on a microscopy slide, fixed with methanol, stained with Giemsa, and examined microscopically (1,000x magnification using oil immersion). Results were graded from negative to +6, based on WHO recommendations (16).

Two D-Squame Skin Stripping Discs were collected from every patient. The tape discs were placed one by one onto the border of the largest (index) lesion, pressed for approximately 10 s, and stored in 300 µL 1X DNA/RNA shield (Zymo Research, Baseclear, Netherlands) at −20°C.

An HIV test was performed for every patient using a fingerpick and HIV ½ STAT-PAK rapid test (Chembio diagnostic systems, inc., New York, United States).

For patients assigned to miltefosine treatment, blood was collected and checked the same day at Arba Minch General Hospital for creatinine, urea, AST, and ALT, as well as complete blood count. Results were discussed with an internist before patients were cleared for treatment. A urine dipstick pregnancy test was performed for every woman of child-bearing age and an intramuscular contraceptive was provided prior to and 3 months after starting miltefosine treatment.

Molecular confirmation of cutaneous leishmaniasis

The first D-Squame Skin Stripping disc in 300 µL 1X DNA/RNA shield was subjected to extraction with the Maxwell 16 LEV Blood DNA kit (Promega, Netherlands) as specified in the manufacturer's manual using the automated Maxwell 16 Instrument (AS1000, Promega). Nucleic acids were subsequently tested by qPCR targeting kinetoplast DNA (kDNA) as described by Merdekios et al. (17). The specific primers and probes used were LC-F (5'-TATTTTACACCAACCCCCAGT-3′), LC-R (5'-GGTAGGGGCGTTCTGC-3′) and a FAM-labeled LC-probe (5'-CAGAAAYCCCGTTCAAAAAATGGC-3′). If a sample was negative, the second D-Squame Skin Stripping disc was tested. If there was still no fluorescence, an HBB PCR was performed as described by Steinau et al. (18) to assess if there was sufficient tissue on the discs and whether the DNA extraction was conducted successfully.

Lesion assessment

Lesion size, number, and type of lesion [localized CL (LCL), muco-cutaneous CL (MCL) or diffuse CL (DCL); (19) and detailed lesion characterization] were recorded at baseline. The largest lesion was classified as the index lesion. Photographs were taken at all timepoints to allow for comparison for outcome assessment and external cross-checking of lesion types, characterization, and outcomes.

Patient reported outcomes and scar scale

Study staff administered the Dermatology Life Quality Index (DLQI) questionnaire to patients at baseline, M1, M3, and M6. For children up to 8, the questions were mainly asked to the parent/guardian, while for those aged 8–12, questions were asked both to the parent/guardian and the child. For children above 12, only the child's answers were considered. Questionnaires were scored and analyzed as previously reported (20). Only questionnaires with 8 or more answered questions were analyzed, others were invalid (21).

Patient-reported outcomes were assessed by asking patients to rate the severity of their lesion at baseline, M1, M3, and M6, ranking it as clear, almost clear, mild, moderate, or severe. A modified Vancouver scar scale (mVSS) according to (22) was used to grade scar appearance at all timepoints after treatment.

Treatment

Crusted lesions were first soaked with sterile saline to clean and remove the crust (if any). Cryotherapy was given using the CryoPro cryogun (Cortex Technology, Aalborg, Denmark), using at least 2 freeze–thaw cycles per application. Liquid nitrogen was applied on the lesion until the lesion and 1-2 mm margin of healthy skin was frozen, which took around 5–30 s, depending on the lesion size and thickness. The lesion was allowed to thaw, which took around 20–30 s, also depending on the freezing time and lesion size and thickness. After cryotherapy, patients were instructed to keep the lesion clean and all received 2% fusidic acid cream (Fusiderm, EVA-PHARMA, Egypt) to apply on the lesion daily. Cryotherapy was planned for 4 weeks with weekly application, but was withheld if lesions were still ulcerative, exudative, crusted, edematous or blistered after the previous application. Cryotherapy was extended if the physician considered it advantageous for the patient (Figure 1).

Miltefosine was given to patients to take at home, after instructing patients on their daily schedule, intake with food, and possible side-effects. Allometric dosing was used for children below 30 kg according to Dorlo et al. (23), 100 mg per day was given for patients of 30–44 kg, and 150 mg/day for patients of 45 kilos or above. Color-matched stickers were used on the pill strips and adherence monitoring forms to help patients take the correct dose in the morning and evening. For very young children, parents were advised to dissolve miltefosine in water mixed with sugar. Fusidic acid was given to patients allocated to miltefosine who had severe crusting or superinfection.

Follow-up visits, safety, and adherence

Patients were asked to come every week during the first 4 weeks for treatment follow-up. Side-effects were recorded and graded according to the principles of the common terminology criteria for adverse events (24) (CTCAE). Adherence was monitored for miltefosine treatment. Every week, pill count was done based on the used pill strips, the daily missed doses were checked on the adherence form, and patients were asked how they took the medication. Poor adherence was defined when patients took less than 90% of their total dose at the M1 visit.

Outcome assessment

Lesion outcomes were determined at M1, M3, and M6 based on the physician's assessment. Patients were categorized as cured if all lesions present at baseline had shown 100% flattening and reepithelization in case lesions were ulcerated. Patients were considered substantially improved if all treated lesions had at least 50–99% improvement compared to baseline in terms of flattening (and reepithelization if applicable), while minor improvement required all lesions to have at least 1–49% improvement. Worsening was used if any treated lesions was worse than at baseline, whereas worsening at M6 compared to M3 was also recorded. New lesions were separately assessed.

Data collection and analysis

Data was collected on paper-based forms, and double data entry was done in RedCap (25). Data analysis was done using R version 4.1.3 (26). Numbers, proportions, medians, and interquartile range (IQR) were used to describe the population. Treatment outcome was analyzed as a categorical variable with multinomial confidence intervals (CIs). Cure rate was also calculated as proportion (with 95% binomial CIs). Outcomes were calculated per treatment

category. Subgroup analyses of treatment outcomes were done for outcomes using the index lesion only, by dosing class (allometric vs. non-allometric), adherence to miltefosine (poor adherence defined as <90% of total dose at M1), age group (<5 and > 5) and number of cryotherapy sessions. Treatment outcomes of these different groups were compared using chi-square tests, or Fisher-exact tests if chi-square tests were inaccurate. McNemar's test was used to compare paired categorical data at different timepoints. DLQI-scores and patient-reported scores at different visits were compared with the Wilcoxon signed rank test for paired data, whereas the Mann–Whitney test was used to compared scores between different groups. Agreement between cure as assessed by the physician and whether the patient indicated the lesion to be clear was determined using kappa coefficient.

Results

Study population

A total of 147 patients were screened, of which 107 were included (Supplementary Figure 1). Of them, 95 (88.8%) were confirmed by PCR, three (2.8%) only by microscopy but not PCR, and nine (8.4%) clinically only. Forty-eight (44.9%) patients were allocated to cryotherapy, 42 (39.3%) to miltefosine, 15 (14.0%) could not be treated and two did not want to be treated. Thirteen of these patients could not be treated because they needed systemic treatment but were excluded from getting miltefosine as they were below 4 years old, one was pregnant, and for one the dermatologists decided not to treat.

The study population is described in Table 1, with some photographs of included patients in Figure 2. Patients were young, with a median age of 6 years old. Only 13 (12.1%) patients were adults. About 60% of the patients were male. Almost half had used traditional (mostly herbal) treatment previously, which was 64% in the miltefosine group. Only 3 patients had used modern treatment at the hospital (probably SSG). A quarter of included patients had at least another CL case in the house, and almost three quarters (72.9%) reported having someone with a CL scar living in their home.

The median lesion duration was around 1 year, but five patients (aged 1, 6, 8, 10 and 11), indicated that they had their lesions for their whole life. Most patients had a single lesion on the face, although more than a quarter had four or more lesions. Around 80% of patients were classified as LCL, 19 (17.8%) as MCL and only 2 (2.8%) as DCL. Overall, more than a third of patients had both an active lesion and concomitant scar. This was 50% among patients on miltefosine treatment, who also had slightly longer duration of their lesions. The most common lesion presentations were plaque (61/107, 57.0%), erythema (56/107, 52.3%), scaliness (49/107, 45.8%), and hyperpigmentation (40/107, 37.4%), whereas ulceration (8/107, 7.5%) and nodules (19/107, 17.8%) were uncommon. All patients were HIV negative.

For most patients, CL had only a minor impact on their life (Supplementary Table 1), with 37/64 (57.8%) with a valid DLQI questionnaire having a score indicating no effect, and 22/64 (34.4%) having a small effect. For two patients (3.1%), the DLQI score indicated CL had a very large effect on their life. DLQI scores were significantly higher in adults than in children ($p = 0.021$, Mann–Whitney test). There was no difference in DLQI scores between males and females ($p = 0.601$, Mann–Whitney test).

Cryotherapy treatment: follow-up and side-effects

Patients assigned to cryotherapy received from 1 up to 10 sessions, with a median of 4.5 (IQR 3.0–7.0). Cryotherapy was usually given in 2 or 3 freeze–thaw cycles, with a freeze–thaw duration of 5–30 s, depending on the lesion thickness, size, and patient cooperation. For many young children, the application of cryotherapy was challenging, requiring multiple short freeze–thaw cycles. At M1, only 9/48 (18.8%) patients had received four cycles, as was planned in the protocol. Cryotherapy was often postponed as lesions were still healing from the previous application, and patients were given fusidic acid as topical antibiotic daily for open wounds. Twenty-five patients who were not yet cured at M1 were extended on cryotherapy treatment at week 4 (M1) up to week 6, and week 13 (M3) up to 17.

Almost all patients treated with cryotherapy (43/48, 89.6%) experienced side-effects (Supplementary Table 2). Blistering (37/48, 77.1%), swelling (34/48, 70.8%) and infection (28/48, 58.3%) were common in the first week of treatment (Figure 3B), while pigmentation changes became more frequent after several cryotherapy applications (Figure 3C), but mostly recovered over time (Figure 3D). Most side-effects were mild (grade I), but several patients developed grade II events with infection (7/48, 14.6%) swelling (3/48, 6.3%) and blistering (4/48, 8.3%) for which three patients were given systemic antibiotics.

Miltefosine treatment: adherence and side-effects

Generally, adherence forms were completed poorly despite repeated explanation. By counting the pill strips, compliance to miltefosine was irregular and poor. Eleven patients (26.2%) finished their complete course at M1 while 20 patients (47.6%) had poor adherence as they had taken less than 90% of their treatment and were advised to continue.

Side effects were common in patients treated with miltefosine (see Supplementary Table 3), with three quarters (76.2%) of patients experiencing adverse events. Most common were vomiting (20/42, 47.6%), abdominal pain (14/42, 33.3%), diarrhea (9/42, 21.4%) and nausea (14/42, 33.3%). Most side-effects were of severity grade I, a few of grade II, and one patient experienced severe bloody diarrhea with vomiting, abdominal pain, headache, and weakness, graded as severity III. The patient was treated with antibiotics for acute bacterial diarrhea and responded well, but had to discontinue her miltefosine treatment after 3 weeks.

Treatment outcomes

Patient follow-up was good, with loss-to-follow-up below 10% in all treated patient groups at all visits (Supplementary Figure 1).

10.3389/fmed.2023.1196063

TABLE 1 Description of the study population.

Total	Total $N = 107$[a]	Cryotherapy $N = 48$ (44.9)	Miltefosine $N = 42$ (39.3)	No treatment $N = 17$ (15.9)
Age, median (years)	6.0 (3.0–11.0)	4.5 (2.0–10.0)	9.0 (6.0–12.0)	3.0 (1.5–3.0)
Sex, male	66 (61.7)	26 (54.2)	32 (76.2)	8 (47.1)
CL case in house	28 (26.2)	12 (25.0)	13 (31.0)	3 (17.6)
CL scar in house	78 (72.9)	34 (70.8)	30 (71.4)	14 (82.4)
Previous CL episode	17 (15.9)	11 (22.9)	5 (11.9)	1 (5.9)
Traditional treatment	53 (49.5)	14 (29.2)	27 (64.3)	12 (70.6)
Herbal[b]	27 (25.2)	3 (6.3)	16 (38.1)	8 (47.1)
Pressing	14 (13.1)	5 (10.4)	5 (11.9)	4 (23.5)
Burning	10 (9.3)	5 (10.4)	5 (11.9)	0 (0)
Other	3 (2.8)	1 (20.8)	2 (4.8)	0 (0)
Modern treatment[c]	7 (6.5)	1 (20.8)	6 (14.3)	0 (0)
Duration in months, median (IQR) ($n = 106$)	12.0 (6.0–24.0)	8.0 (4.0–24.0)	12.0 (12.0–45.0)	12.0 (4.0–12.0)
Type of lesion				
LCL	86 (80.4)	48 (100)	26 (61.9)	12 (70.6)
MCL	19 (17.8)	0 (0)	14 (33.3)	5 (29.4)
DCL	2 (1.9)	0 (0)	2 (0.5)	0 (0)
Number of active lesions				
1	50 (46.7)	34 (70.8)	11 (35.7)	5 (41.2)
2	17 (15.9)	6 (12.5)	7 (26.2)	4 (29.4)
3	13 (12.1)	3 (6.3)	9 (21.4)	1 (23.5)
≥4	27 (25.2)	5 (10.4)	15 (35.7)	7 (41.2)
Presence of concomitant CL scar	41 (38.3)	16 (33.3)	21 (50.0)	4 (23.5)
Size (largest diam), median IQR ($n = 100$)	2.2 (1.4–3.4)	1.6 (1.2–2.5)	3.5 (1.9–5.5)	2.1 (1.6–3.0)
Location index on face	97 (90.6)	44 (91.7)	37 (88.1)	16 (94.1)

CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; IQR, interquartile range; MCL, muco-cutaneous leishmaniasis; IQR, inter-quartile ranges. [a]If a different denominator is used, this is indicated behind the variables as ($n = x$), [b]For herbal medication, the traditional healers use different kinds of plant extracts [c]three patients took anti-leishmania treatment at the hospital, four used non-specific modern treatment (two ointments, one disinfectant, one antibiotic injection).

Outcomes for cryotherapy are shown in Table 2. Around a quarter of the patients were cured after 1 month, which increased to 45.8% (95% CI 33.3–61.8) at M3 and 52.2% (95% CI 39.1–67.6) at M6. An additional 19.6% had substantial improvement at M6. Around 20% (21.7%) showed worsening at M6 compared to before treatment. Analyzing only the index lesion (Supplementary Table 4) did not affect the cure rates. Outcomes in children below 5 years old treated with cryotherapy were significantly different ($p = 0.036$, Fisher-exact test) to those aged 5 years and above (Supplementary Table 5). Although the overall cure rate was similar, young children more frequently had worsening lesions and less substantial improvement.

Despite extending treatment sessions up to 10 times, many patients receiving cryotherapy did not reach cure (Figure 4). In fact, data shows that cure rates are significantly lower for patients getting more than four sessions of cryotherapy (37.5% for 5–6 sessions and 21.4% for 7–8 sessions) compared to those who were treated with cryotherapy less than four times ($p = 0.006$ for M3 and $p = 0.003$ for M6, chi-square test).

Treatment outcomes for miltefosine are shown in Table 3. The overall cure rate for patients receiving miltefosine was 36.8% (95% CI 23.7–55.3) at M3 and 41.0% (95% CI 25.6–57.0) at M6. An additional 28.2% (95% CI 12.8–44.2) had substantial improvement at M6. In contrast, 20.5% (5.1–36.5) of the patients had lesions classified as worse than baseline at M6. Looking only at the index lesion outcomes (Supplementary Table 6), results are slightly better, with 51.3% of patients cured, 25.6% substantially improved, and only 10% worsening. There was no significant difference ($p = 0.521$, Fisher-exact test) in treatment outcomes in children receiving allometric dosing (weight below 30 kg) compared to children on non-allometric dosing (Supplementary Table 7). Outcomes were not significantly different for those with good adherence compared to those with poor adherence ($p = 0.162$, Fisher-exact test).

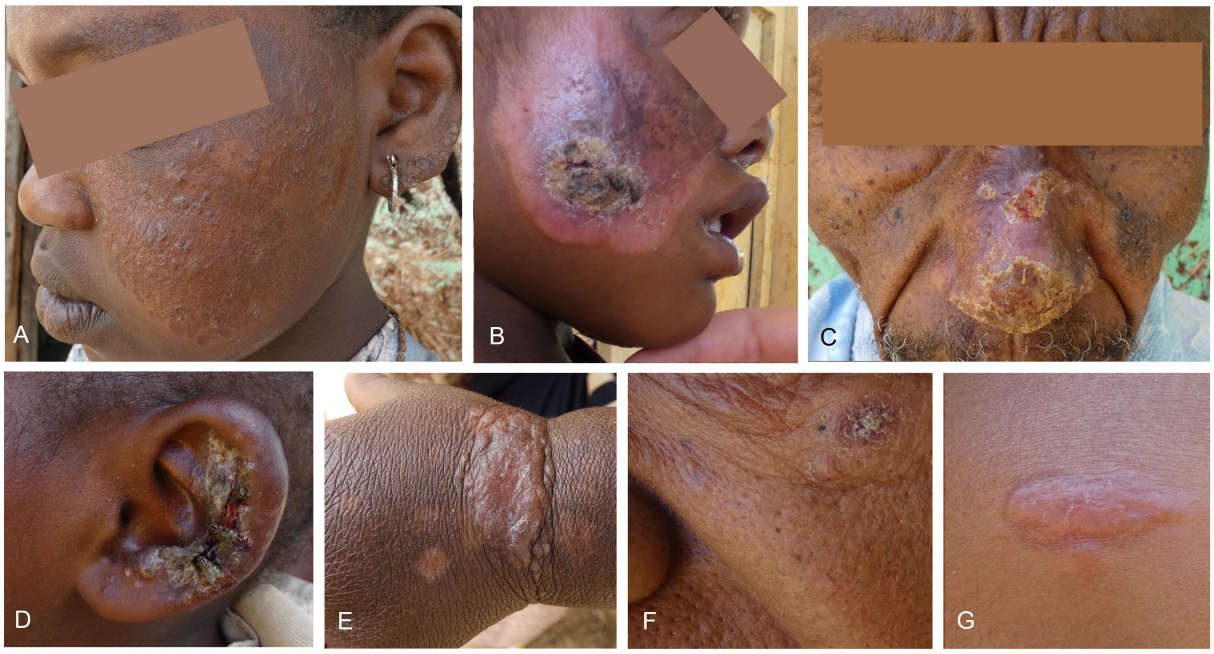


FIGURE 2
Lesion photographs of included patients. **(A)** An extensive lesion affecting the cheek and ear that the patient had as long as they can remember with active papules within a bigger scar, treated with miltefosine. **(B)** Superinfected lesion with scarring on the cheek, treated with miltefosine and systemic antibiotics. This patient had a contracture of the eye due to scar formation. **(C)** Crusted lesion on the nose treated with miltefosine. **(D)** Superinfected swollen lesion on the ear. No leishmaniasis treatment was given since the lesion was too big for cryotherapy and the patient was too young for miltefosine. Fusidic acid cream was given. **(E)** Plaque with papules on the wrist, treated with cryotherapy. **(F)** Small, crusted lesion underneath the eye treated with cryotherapy. **(G)** Nodular plaque lesion on the forehead treated with cryotherapy.

Almost one fifth (18/100, 18.0%) of all patients developed a new lesion by M6, which was more common in patients getting local treatment with cryotherapy (10/46, 21.7%), and in those not treated (5/15, 33.3%). Three patients (3/39, 7.7%) on miltefosine treatment developed new lesions at M6. Additionally, almost a quarter of patients had worsening of their index lesion at M6 when compared to M3, which was especially pronounced in the group treated with miltefosine, where more than 30% (11/36, 30.6%) had worsening at M6 compared to M3.

Fifteen out of 17 patients who were not treated could be found at M6 for outcome assessment (Table 4). Of these, eight (53.3%) were cured, although 95% CI were very large (95% CI 33.3–79.8%). Two showed substantial improvement (13.3, 95% CI 0–39.8) and three patients (20.0, 95% CI 0–46.5%) were classified as worsening. Three of the untreated patients had reported the use of traditional herbal medication in between the initial assessment and the M6 outcome visit, of which two were cured. At least eight received topical fusidic acid at the start of the study, as the lesions looked crusted or infected. Of these, four were cured, two had substantial improvement, one minor improvement and one worsening.

Scar assessment

At M6, 79.0% (79/100) of patients had a remaining scar, which was similar for those who had received cryotherapy (36/46, 78.3%), miltefosine (32/39, 82.0%), or no treatment at all (11/15, 73.3%), and the median overall modified Vancouver scar scale was also similar for the different groups at 1.0 (Supplementary Table 8). Most scars (52/79, 65.8%) had slight pigmentation issues while a subset of patients had moderate (17.7%) or severe (2.5%) hypo- or hyperpigmentation. Although more than half the patients had normal pliability and height of scars, a subset had supple, yielding, or firm scars, and around 45% of the patients with scars had a scar that was raised.

Patient reported outcomes

Patient reported outcomes over time are shown in Figure 5. Most patients rated their lesion as mild (39.3%), moderate (28.0%), or severe (20.6%) at the start of the study. Outcomes were significantly different at each follow-up visit (all $p < 0.001$, Wilcoxon Signed-rank -square test) compared to before treatment with 19 (17.8%) patients who said their lesion was cured at M1, 54 (50.5%) at M3, and 61.7% at M6.

DLQI scores significantly ($p < 0.001$, Wilcoxon Signed-rank Whitney test) changed over time with the median DLQI score rated 1 (IQR 0–3) before treatment and 0 (IQR 0-0)at M6. The proportion of patients who experienced negative effects on their quality of life due to their skin condition also decreased from 42.2% (27/64) before treatment to only 4.8% (3/63) at M6.

Overall, significantly less ($p < 0.001$, McNemar's test) patients experienced pain in the lesion before treatment (37.7%, 40/106) compared to M6 (3.0%,3/100; Supplementary Table 9). The change was significant for cryotherapy ($p < 0.001$) and miltefosine ($p = 0.004$),

10.3389/fmed.2023.1196063

but not for the patients not treated ($p = 0.845$,McNemar's test). Similar results were seen for itching, which was common before treatment at 42.5%, but which significantly decreased for the miltefosine ($p = 0.013$) and cryotherapy group ($p = 0.005$), but not for the patients who were not treated ($p = 0.307$).

Agreement between when patient saw the lesion as clear and physicians assessed the lesion to be cured was low with patients rating their response more positively than the physician (Supplementary Table 10), with kappa coefficient − 0.07 at M1, 0.37 at M3 and 0.52 at M6.

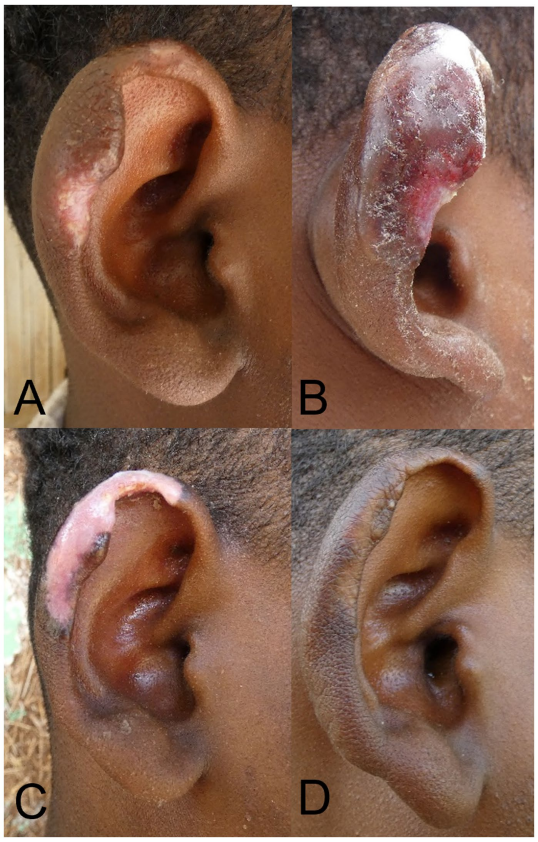


FIGURE 3
Patient with localized cutaneous leishmaniasis treated with cryotherapy. **(A)** Lesion before treatment. **(B)** Lesion 3 days post-cryotherapy with swelling and blistering. **(C)** Lesion at Month 1, with severe hypopigmentation. **(D)** Lesion at M6 with normal pigmentation and clinical cure.

Discussion

This is the first study to comprehensively report both clinical and patient-reported treatment outcomes for CL treatment. We also provide important information on challenges and opportunities of community treatment, which is scarce despite the emphasis placed by the WHO roadmap for NTDs (7) on community-based interventions.

CL is a common morbidity in Ochollo, which is reflected by the low age of the enrolled study patients, and the high number of patients who had previous or active CL cases in their household. These findings are in line with Bugssa et al. (14), who found that about 65% of primary school children had either active lesions or scars due to CL. Regardless of the high CL prevalence, only few patients had used modern treatment. Yet, half of them tried traditional medicine with a local healer in the village. This highlights that patients experience important barriers for seeking healthcare at Arba Minch General Hospital, about 25 km from Ochollo village. There barriers were further explored in a sub-study (manuscript in preparation).

While patients predominantly had lesions on their face, their quality of life was not much affected by CL. Most other studies that used the DLQI in CL patients found much higher impact (27–30), even though lesions were mostly on extremities. Some of the factors that could cause this discrepancy are age, since adults were impacted more, and the high endemicity of CL in the village. Others have shown that lack of knowledge about CL and its transmission can lead to fears related to contamination, causing rejection and isolation in communities (31–33). Researchers have been coming to Ochollo to study CL since the 1970's (14, 34–37), which probably contributed to good knowledge and accordingly less impact of CL on their quality of life, compared to other sites. This is further highlighted by the fact that 96% of people from Ochollo knew CL is caused by the bite of (sand) flies (36), which is much higher than in other CL-endemic areas in the country (4, 38, 39).

Overall, clinical outcomes of cryotherapy were lower than expected with only a bit more than half of the patients reaching cure at M6. However, an additional fifth of the patients reached substantial improvement. A few other studies done in Ethiopia had higher cure rates; in Silti Health Center, 80.5% of patients treated with cryotherapy were cured at three to 6 months (10). They used a cotton applicator and 3–4 times 10–30 s freeze thaw cycles in weekly sessions up to cure, with an average of 6.4 sessions needed. In ALERT hospital, cure rate with a similar protocol was 60.8% (8), but the majority of patients were treated for more than 13 sessions. A small report from Boru Meda Hospital showed that cryotherapy cure rate was 92.3% after three doses (9), although details on the treatment application and outcome assessment are not described. Generally, we used relatively

TABLE 2 Outcomes of cryotherapy for all lesions present at baseline.

	Month 1		Month 3		Month 6	
	N = 47 (97.9%)	95% CI	*N* = 48 (100%)	95% CI	*N* = 46 (95.8%)	95% CI
Cure	12 (25.5)	12.8–40.7	22 (45.8)	33.3–61.8	24 (52.2)	39.1–67.6
Substantial improvement	25 (53.2)	40.4–68.4	15 (31.3)	18.8–47.2	9 (19.6)	6.5–35.0
Minor improvement	6 (12.8)	0–28.0	4 (8.3)	0–24.3	2 (4.3)	0–19.8
No improvement	2 (4.3)	0–19.4	0 (0)	0–16.0	1 (2.2)	0–17.6
Worsening	2 (4.3)	0–19.4	7 (14.6)	2.1–30.6	10 (21.7)	8.7–37.2

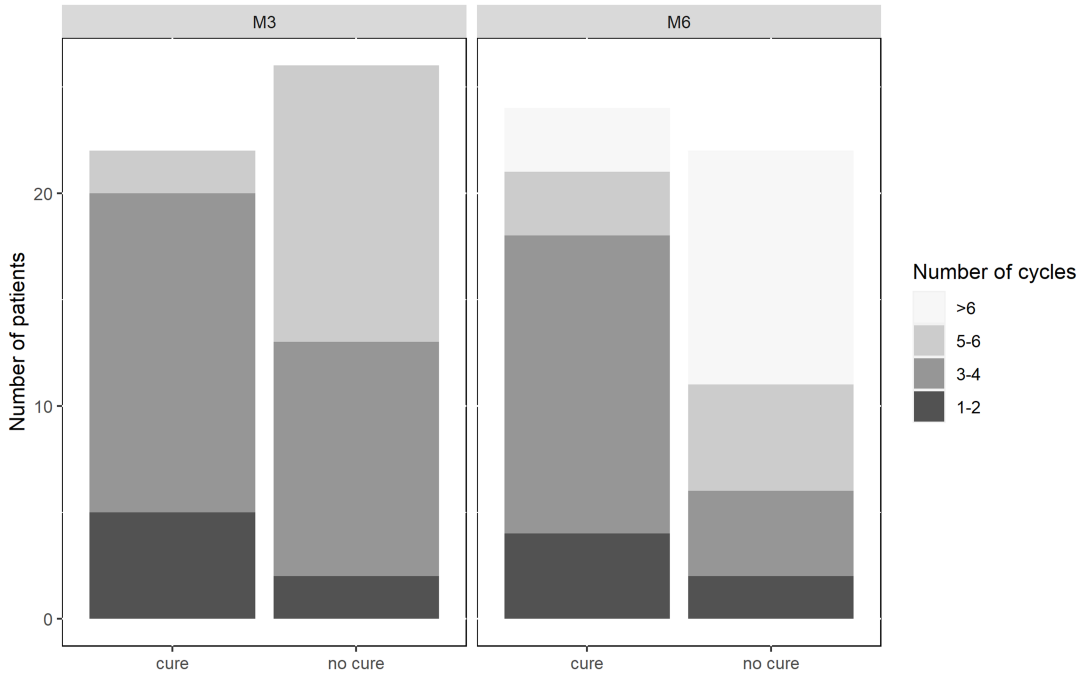


FIGURE 4
Cure rates of cryotherapy at Month 3 and Month 6 by number of cycles. Most patients (20/22) who were cured at M3 had received less than four cycles. At Month 6, three extra patients were cured with 5–6 cycles, and three who received more than six cycles. Around half (11/21) of the patients who were not cured at M6 had received more than six cycles of cryotherapy.

TABLE 3 Outcomes of miltefosine treatment for all lesions present at baseline.

	Month 1		Month 3		Month 6	
	N = 39 (92.9%)	95% CI	*N* = 38 (90.5%)	95% CI	*N* = 39 (92.9%)	95% CI
Cure	1 (2.6)	0–19.7	14 (36.8)	23.7–55.3	16 (41.0)	25.6–57.0
Substantial improvement	23 (59.0)	46.2–76.1	17 (44.7)	31.6–63.2	11 (28.2)	12.8–44.2
Minor improvement	15 (38.5)	25.6–55.6	4 (10.5)	0–28.9	3 (7.7)	0–23.7
No improvement	0 (0)	0–17.2	0 (0)	0–28.9	1 (2.6)	0–18.5
Worsening	0 (0)	0–17.2	3 (7.9)	0–26.3	8 (20.5)	5.1–36.5

TABLE 4 Outcome for patients not receiving treatment.

	Month 6	
	N = 15 (88.2%)	95% CI
Cure	8 (53.3)	33.3–79.8
Substantial improvement	2 (13.3)	0–39.8
Minor improvement	1 (6.7)	0–33.2
No improvement	1 (6.7)	0–33.2
Worsening	3 (20.0)	0–46.5

strict cure criteria, where all lesions had to have complete reepithelization and flattening to be cured at M6, which may not be the case for other studies. The young age of our patients could also play a role in low cure rates, as younger patients have been described to have poorer outcomes in several studies (12, 40–42).

Treatment with cryotherapy came with several challenges and observations. First, due to the very young patient population, Ochollo cryotherapy was applied mostly on small children. Especially the youngest were frightened by the sound of the cryogun, which made it difficult to apply the liquid nitrogen. Difficulties in application of cryotherapy on young patients could also be a cause of lower cure rates. We indeed showed that outcomes were significantly better in those above 5 years. Second, the patients reside in a rural area without access to running water. Although we advised patients to wash their lesion frequently to prevent infections, patients often came back with unclean or infected lesions. Consequently, we provided 2% topical fusidic to keep the wounds clean. Third, despite the aim for weekly cryotherapy application in our protocol, in practice this was almost never possible because lesions were not yet healed from the previous application. Therefore, we recommend application of cryotherapy every other week. Fourth, the number of cryotherapy sessions needed to heal a lesion were highly variable among patients. This is complicating standardization of treatment, which is needed to enable comparison of the effectiveness of cryotherapy with other treatments. Importantly, our results show that extending treatment does not necessarily lead to better outcomes. Therefore, clinicians should carefully assess whether there is sufficient improvement of the lesion after four sessions of cryotherapy before further treatment extension. Lastly, although severe hypopigmentation

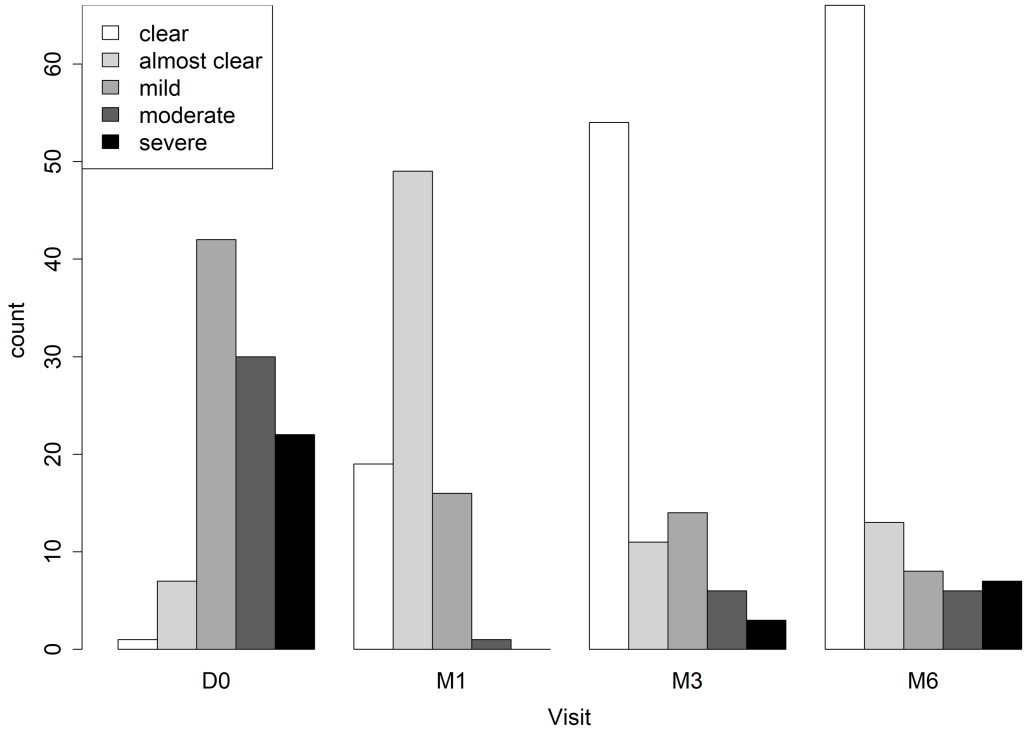


FIGURE 5
Patient reported lesion assessment at baseline and outcome visits. Patient-reported global assessment rated as clear, almost clear, mild, moderate, and severe is shown before treatment (D0), 1 month after starting treatment (M1), 3 months after starting treatment (M3), and 6 months after starting treatment (M6).

is often mentioned as a reason to avoid cryotherapy in the face on darker skin, our data showed that while pigmentation issues were common, they were mostly transient with good recovery at M6, similar to findings from Iran (43).

Outcomes of miltefosine treatment in our study were in line with a previous pilot study conducted in Ethiopia in a hospital population (12) where the corrected overall cure rate was 48.7% at M6. In short, patients showed good initial improvement but seemed to be unable to completely clear the infection, as a considerable proportion of patients had lesion worsening at M6 compared to M3. Based on this, we hypothesize that treatment extension or combination treatment could improve cure rates. We observed that adherence of miltefosine was poor, despite extensive efforts by the study team. This may have contributed to lower cure rates, even though our data did not show significantly different outcomes in this group. Closer follow-up by the village health extension worker through house-to-house visits and consulting patients may improve adherence and potentially treatment outcomes. The age limit for systemic treatment was set at age four in our study protocol because we feared patients would be too young to swallow the drug. In practice, this led to unnecessary exclusion of a lot of patients who needed systemic treatment but were below 4 years old. For the youngest patients, we opened the capsules and dissolved the miltefosine in a cup with water and sugar. We therefore recommend future studies to lower inclusion age to 2 years.

Results from our study highlight that the use of oral treatments, – even though deemed the future of leishmaniasis treatment (44) – comes with important challenges for decentralization. Many CL patients live in rural communities and are poorly educated. This requires extensive efforts to sensitize and instruct communities on drug adherence and prevent development of resistance. Directly observed therapy at the health post level, similar to what is done for tuberculosis, could be explored.

Patients who were not treated in our study because they were too young for systemic treatment or because they refused, showed similar cure rates as the treatment groups. Interestingly, especially among the patients who received topical fusidic acid, lesions seemed to improve quickly and had a response rate similar to the cryotherapy and miltefosine groups. It should be taken into account, however, that this non-treatment group only consisted of a few patients and therefore the evidence is anecdotal at best. Further investigation into the (natural) healing of lesions and the added effect of topical antibiotics on CL treatment is warranted as they are cheap, easy to apply and locally available.

Our findings indicate that many patients have an ineffective immune response to CL, shown by the long duration of lesions, high number of patients with new lesions, and a high proportion of patients who have both an active lesion and scar. The persistence of lesions also indicates patients could remain infectious for years and sustain transmission. Patients in Ochollo are presumably frequently exposed to infectious sand fly bites, 3.5% of sand flies were found infected with *Leishmania* in Ochollo (36). However, especially children seem to fail in raising protective immunity to prevent re-infection. Immunological studies should be performed to shed light on this phenomenon, and help determine whether early treatment of patients in a highly endemic setting is useful. In our view, it is more likely that treatment campaigns should be integrated with vector control measures to reduce the patients' exposure to infectious sand flies.

This is the first time that patient-reported outcomes and scar evaluation were done in conjunction with clinical assessment to give an integrated assessment of treatment outcomes, as recommended previously (13). Our findings show that the impact of CL after treatment measured by the DLQI decreased significantly after treatment, and lesion assessment was significantly better at M6 compared to baseline. Interestingly, patient-reported outcomes were much more positive than clinical outcomes, and did not correlate well with clinical findings. This highlights that it is vital to include patients' perspectives, as a pure clinical evaluation can underestimate the perceived effect of treatment, which is especially important for skin diseases which mainly have psychosocial impact. Other strengths of this study are good follow-up of patients due to close involvement of field assistants. Future community-based projects should closely involve village leaders and health extension workers in patient management and follow-up in order to increase local ownership and acceptance. Limitations of this study are the relatively low sample size of each treatment group, and potential social desirability bias in the patient-reported outcomes. Since this study provides results from a specific highly endemic locality, certain findings such as the patient population, impact of CL and number of new lesions are not generalizable to other settings. However, most lessons learned can be applied to all CL-endemic communities in Ethiopia.

Conclusion

Our study is the first to identify the challenges and opportunities of miltefosine and cryotherapy for community treatment of CL. We show that local engagement is crucial for the success of community studies. Application of cryotherapy should be spaced 2 weeks apart and topical antibiotics should be routinely supplied to avoid infection. Pigmentation problems were frequently encountered, but most improved after 6 months. Poor miltefosine adherence highlights that oral outpatient treatments for CL need more stringent follow-up. Cryotherapy and miltefosine are suboptimal in terms of cure-rate, although the majority of patients still experienced great improvement of their lesion. This indicates that patient-reported outcomes are very valuable, especially for skin NTDs. There currently seem to be no other treatments suitable for decentralization readily available. Therefore, integrated interventions aimed to reduce transmission in combination with early diagnosis and treatment should be explored.

Data availability statement

The datasets presented in this article are not readily available because data will not be made openly accessible due to ethical and privacy concerns. Data can however be made available after approval of a motivated and written request. Requests to access the datasets should be directed to ITM Research Data Access Committee, ITMresearchdataaccess@itg.be.

Ethics statement

The studies involving humans were approved by Arba Minch University Institutional Research Ethics Review Board Institute of Tropical Medicine Institutional Review Board University Hospital Antwerp Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

SvH, MP, JvG, FM, and BM contributed to conception and design of the study. DT, MdK, MT, EK, NG, DD, MM, MS, and RT were involved in field work and data collection. SvH, MP, DT, BM, and TW supervised and coordinated the study. DD and NG did data entry. MbK performed the molecular analyses while MP supervised. SvH organized the database and performed the statistical analysis. SvH and MP wrote the first draft of the manuscript. FM, JvG, and TW provided critical input to the writing of the manuscript. All authors contributed to the article, read, and approved the submitted version.

Funding

This project was funded by the Arba Minch University Grand project GOV/AMU/TH-NTD/CRTC/08/2020. Co-funding was provided by ITM internal funds.

Acknowledgments

We would like to thank all patients who participated in this study. We are grateful to the hard work of the field assistants of Ochollo, and to the Ochollo community in general for cooperating with this project. We thank the zonal and regional health bureau for their support, as well as the Collaborative Research and Training Center for Neglected Tropical Diseases of Arba Minch University. We are grateful to Wanzahun Godana for his support with importing study materials, and to Haruka Hayashi for help with data cleaning.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1196063/full#supplementary-material

References

1. van Henten S, Adriaensen W, Fikre H, Akuffo H, Diro E, Hailu A, et al. Cutaneous Leishmaniasis due to Leishmania aethiopica. *E Clini Med*. (2018) 6:69–81. doi: 10.1016/J.ECLINM.2018.12.009

2. Mutinga MJ, Odhiambo TR. Cutaneous leishmaniasis in Kenya.2. Studies on vector potential of phlebotomus-pedifer (diptera, phlebotomidae) in Kenya. *Insect Sci its Appl*. (1986) 7:171–4. doi: 10.1017/S1742758400008924

3. Pareyn M, Kochora A, van Rooy L, Eligo N, vanden Broecke B, Girma N, et al. Feeding behavior and activity of Phlebotomus pedifer and potential reservoir hosts of Leishmania aethiopica in southwestern Ethiopia. *PLoS Negl. Trop. Dis.* (2020) 14:e0007947. doi: 10.1371/journal.pntd.0007947

4. Merdekios B, Pareyn M, Tadesse D, Getu S, Admassu B, Girma N, et al. Detection of cutaneous leishmaniasis foci in south Ethiopia. *American J Trop Med Hygiene*. (2021) 105:156. doi: 10.4269/ajtmh.20-0708

5. World Health Organization. *Global health observatory data repository*. World Health Organization (2019).

6. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. (2012) 7:e35671. doi: 10.1371/journal.pone.0035671

7. World Health Organization. Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. (2020). Available at: https://www.who.int/publications/i/item/9789240010352 [Accessed October 25, 2021]

8. Leish-mapping team at AHRI in collaboration with WHO-Ethiopia. Proceedings of the *International Consultative Meeting on Cutaneous Leishmaniasis in Ethiopia*. Addis Ababa. (2011).

9. Seife T, Benecha AK, Zewdu FT, Ayal A, Misganaw M. Treatment patterns and Effectivness of anti-Leishmaniasis agents for patients with cutaneous Leishmaniasis at Boru Meda hospital, South Wollo, North East Ethiopia. *J Clin Exp Dermatol Res*. (2018) 9:1–6. doi: 10.4172/2155-9554.1000450

10. Negera E, Gadisa E, Hussein J, Engers H, Kuru T, Gedamu L, et al. Treatment response of cutaneous leishmaniasis due to Leishmania aethiopica to cryotherapy and generic sodium stibogluconate from patients in Silti, Ethiopia. *Trans. R. Soc. Trop. Med. Hyg.* (2012) 106:496–503. doi: 10.1016/j.trstmh.2012.02.006

11. Sankaranarayanan R, Rajkumar R, Esmy PO, Fayette JM, Shanthakumary S, Frappart L, et al. Effectiveness, safety and acceptability of "see and treat" with cryotherapy by nurses in a cervical screening study in India. *Br. J. Cancer*. (2007) 96:738–43. doi: 10.1038/sj.bjc.6603633

12. van Henten S, Tesfaye AB, Abdela SG, Tilahun F, Fikre H, Buyze J, et al. Miltefosine for the treatment of cutaneous leishmaniasis—a pilot study from Ethiopia. *PLoS Negl. Trop. Dis.* (2021) 15:e0009460. doi: 10.1371/journal.pntd.0009460

13. Erber AC, Arana B, Bennis I, Ben Salah A, Boukthir A, Castro Noriega MDM, et al. An international qualitative study exploring patients' experiences of cutaneous leishmaniasis: study set-up and protocol. *BMJ Open*. (2018) 8:e021372. doi: 10.1136/bmjopen-2017-021372

14. Bugssa G. The current status of cutaneous Leishmaniasis and the pattern of lesions in Ochollo primary school students, Ochollo. *Southwestern Ethiopia Sci J Clin Med*. (2014) 3:111. doi: 10.11648/j.sjcm.20140306.13

15. Von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. *Ann. Intern. Med.* (2007) 147:573–7. doi: 10.7326/0003-4819-147-8-200710160-00010

16. World Health Organization. *Control of the leishmaniases*. World Health Organization. (2010).

17. Merdekios B, Pareyn M, Tadesse D, Eligo N, Kassa M, Jacobs BK, et al. Evaluation of convential and four real-time PCR methods for the detection of Leismania in field-collected samples in Ethiopia. *PLoS Negl. Trop. Dis.* (2020) 15:e0008903. doi: 10.1371/journal.pntd.0008903

18. Steinau M, Rajeevan M, Unger E. DNA and RNA references for qRT-PCR assays in exfoliated cervical cells. *J Mol Diagn*. (2006) 8:113–8. doi: 10.2353/JMOLDX.2006.050088

19. Federal Ministry of Health E. *Guidelines for diagnosis, treatment and prevention of leishmaniasis in Ethiopia. 2nd* ed. Ethiopia: Addis Adaba (2013).

20. Finlay AY, Khan GK. Dermatology life quality index (DLQI)—a simple practical measure for routine clinical use. *Clin. Exp. Dermatol.* (1994) 19:210–6. doi: 10.1111/j.1365-2230.1994.tb01167.x

21. Finlay AY, Sampogna F. What do scores mean? Informed interpretation and clinical judgement are needed. *Br. J. Dermatol.* (2018) 179:1021–2. doi: 10.1111/BJD.17028

22. Nedelec B, Correa JA, Rachelska G, Armour A, Lasalle L. Quantitative measurement of hypertrophic scar: interrater reliability and concurrent validity. *J Burn Care Res*. (2008) 29:501–11. doi: 10.1097/BCR.0B013E3181710881

23. Dorlo TPC, Huitema ADR, Beijnen JH, De Vries PJ. Optimal dosing of miltefosine in children and adults with visceral leishmaniasis. *Antimicrob. Agents Chemother.* (2012) 56:3864–72. doi: 10.1128/AAC.00292-12

24. U.S. Department of Health and Human Services. Common terminology criteria for adverse events (CTCAE).V.5.0. Cancer Ther Eval Progr. (2017). 155. Available at: http://upen.terengganu.gov.my/index.php/2017

25. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J. Biomed. Inform.* (2009) 42:377–81. doi: 10.1016/J.JBI.2008.08.010

26. Team RC. *R: A language and environment for statistical computing*. Austria: R Found Stat Comput Vienna (2023).

27. Vares B, Mohseni M, Heshmatkhah A, Farjzadeh S, Safizadeh H, Shamsi-Meymandi S, et al. Quality of life in patients with cutaneous leishmaniasis. *Arch Iran Med*. (2013) 16:474–7.

28. Ahmed N, Naeem A, Zahid B, Tahir M, Bashir U, Kausar S, et al. Effect of cutaneous Leishmaniasis on quality of life of patients, a multicentric study in tertiary care hospitals in Pakistan using DLQI. *Int J Clin Experim Med Sci*. (2021) 7:103. doi: 10.11648/J.IJCEMS.20210704.16

29. de Castro Toledo AC, da Silva RE, Carmo RF, Amaral TA, Profeta Luz Z, Lia M, et al. Assessment of the quality of life of patients with cutaneous leishmaniasis in belo Horizonte, Brazil, 2009-2010. A pilot study. *Trans. R. Soc. Trop. Med. Hyg.* (2013) 107:335–6. doi: 10.1093/trstmh/trt021

30. Peleva E, Walker SL. Cutaneous leishmaniasis and health-related quality of life in returning travellers to the UK. *J Travel Med*. (2020) 27:1–2. doi: 10.1093/jtm/taaa188

31. Chahed MK, Bellali H, Ben Jemaa S, Bellaj T. Psychological and psychosocial consequences of zoonotic cutaneous Leishmaniasis among women in Tunisia: preliminary findings from an exploratory study. *PLoS Negl. Trop. Dis.* (2016) 10:e0005090. doi: 10.1371/JOURNAL.PNTD.0005090

32. Reyburn H, Koggel M, Sharifi AS. *Social and psychological consequences of cutaneous leishmaniasis in Kabul Afghanistan*. United States: University of Arizona Libraries (2000).

33. Ramdas S. *Perceptions and treatment of cutaneous leishmaniasis in Suriname: a medical-anthropological perspective*. Netherlands: University of Amsterdam (2015).

34. Ashford RW, Bray MA, Hutchinson MP, Bray RS. The epidemiology of cutaneous leishmaniasis in Ethiopia. *Trans. R. Soc. Trop. Med. Hyg.* (1973) 67:568–601. doi: 10.1080/00034983.1973.11811669

35. Mengistu G, Laskay T, Gemetchu T, Humber D, Ersamo M, Evans D, et al. Cutaneous leishmaniasis in South-Western Ethiopia: Ocholo revisited. *Trans. R. Soc. Trop. Med. Hyg.* (1992) 86:149–53. doi: 10.1016/0035-9203(92)90546-O

36. Pareyn M, van den Bosch E, Girma N, van Houtte N, van Dongen S, van der Auwera G, et al. Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochollo, a hotspot in southern Ethiopia. *PLoS Negl. Trop. Dis.* (2019) 13:e0007667. doi: 10.1371/journal.pntd.0007667

37. Kebede N, Worku A, Ali A, Animut A, Negash Y, Gebreyes WA, et al. Community knowledge, attitude and practice towards cutaneous leishmaniasis endemic area Ochello, Gamo Gofa zone, South Ethiopia. *Asian Pac. J. Trop. Biomed.* (2016) 6:562–7. doi: 10.1016/J.APJTB.2016.01.018

38. Tamiru HF, Mashalla YJ, Mohammed R, Tshweneagae GT. Cutaneous leishmaniasis a neglected tropical disease: community knowledge, attitude and practices in an endemic area. *Northwest Ethiopia BMC Infect Dis*. (2019) 19:1–10. doi: 10.1186/s12879-019-4506-1

39. Tesfay K, Mardu F, Berhe B, Negash H, Legese H, Adhanom G, et al. Household knowledge, practice and treatment seeking behaviors towards cutaneous leishmaniasis in the endemic rural communities of Ganta- afeshum district, Tigrai, northern Ethiopia, 2019: a cross-sectional study. *Trop Dis Travel Med Vaccines*. (2021) 7:1–10. doi: 10.1186/S40794-021-00144-4/TABLES/4

40. Layegh P, Rahsepar S, Rahsepar AA. Systemic Meglumine Antimoniate in acute cutaneous Leishmaniasis: children versus adults. *Am J Trop Med Hyg*. (2011) 84:539–42. doi: 10.4269/AJTMH.2011.10-0002

41. Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, Miranda-Verastegui C, Santamaria-Castrellon G, Ramirez L, et al. Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous Leishmaniasis in Peru. *Clin. Infect. Dis.* (2008) 46:223–31. doi: 10.1086/524042

42. Castro M del M, Cossio A, Velasco C, Osorio L. Risk factors for therapeutic failure to meglumine antimoniate and miltefosine in adults and children with cutaneous leishmaniasis in Colombia: a cohort study. *PLoS Negl. Trop. Dis.* (2017) 11:e0005515. doi: 10.1371/journal.pntd.0005515

43. Layegh P, Pezeshkpoor F, Soruri AH, Naviafar P, Moghiman T. Efficacy of cryotherapy versus intralesional meglumine antimoniate (glucantime) for treatment of cutaneous leishmaniasis in children. *Am J Trop Med Hyg*. (2009) 80:172–5. doi: 10.4269/ajtmh.2009.80.172

44. Drugs for Neglected Diseases Initiative. (2023). Target product profile for cutaneous leishmaniasis | DNDi. Available at: https://dndi.org/diseases/cutaneous-leishmaniasis/target-product-profile/ [Accessed February 21, 2023]

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 16 November 2023
DOI 10.3389/fcimb.2023.1221246

OPEN ACCESS

EDITED BY
Harry P. De Koning,
University of Glasgow, United Kingdom

REVIEWED BY
Sébastien Pomel,
Université Paris-Sud, France
Sudharshan S. J.,
Indian Institute of Science (IISc), India

*CORRESPONDENCE
Eduardo Caio Torres-Santos
ects@ioc.fiocruz.br

RECEIVED 12 May 2023
ACCEPTED 26 October 2023
PUBLISHED 16 November 2023

CITATION
Pinheiro LS, Andrade-Neto VV, Mantuano-Barradas M, Pereira EC, Barbosa RCF, de Oliveira MCC, Menna-Barreto RFS, Cunha-Júnior EF and Torres-Santos EC (2023) Biological effects of *trans, trans*-farnesol in *Leishmania amazonensis*. *Front. Cell. Infect. Microbiol.* 13:1221246. doi: 10.3389/fcimb.2023.1221246

Biological effects of *trans, trans*-farnesol in *Leishmania amazonensis*

Liliane Sena Pinheiro[1,2], Valter Viana Andrade-Neto[1], Marcio Mantuano-Barradas[1], Elisa Cavalcante Pereira[1], Rodrigo Cesar Fernandes Barbosa[3], Marcia Cristina Campos de Oliveira[3], Rubem Figueiredo Sadok Menna-Barreto[4], Edézio Ferreira Cunha-Júnior[5] and Eduardo Caio Torres-Santos[1]*

[1]Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brazil, [2]Universidade Federal dos Vales do Jequitinhonha e Mucuri, Teófilo Otoni, MG, Brazil, [3]Instituto de Química, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil, [4]Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brazil, [5]Laboratório de Imunoparasitologia, Unidade Integrada de Pesquisa em Produtos Bioativos e Biociências, Centro Multidisciplinar UFRJ-Macaé, Universidade Federal do Rio de Janeiro, Macaé, Brazil

Introduction: Farnesol, derived from farnesyl pyrophosphate in the sterols biosynthetic pathway, is a molecule with three unsaturations and four possible isomers. *Candida albicans* predominantly secretes the *trans*, *trans*-farnesol (*t*, *t*-FOH) isomer, known for its role in regulating the virulence of various fungi species and modulating morphological transition processes. Notably, the evolutionary divergence in sterol biosynthesis between fungi, including *Candida albicans*, and trypanosomatids resulted in the synthesis of sterols with the ergostane skeleton, distinct from cholesterol. This study aims to assess the impact of exogenously added *trans*, *trans*-farnesol on the proliferative ability of *Leishmania amazonensis* and to identify its presence in the lipid secretome of the parasite.

Methods: The study involved the addition of exogenous *trans*, *trans*-farnesol to evaluate its interference with the proliferation of *L. amazonensis* promastigotes. Proliferation, cell cycle, DNA fragmentation, and mitochondrial functionality were assessed as indicators of the effects of *trans*, *trans*-farnesol. Additionally, lipid secretome analysis was conducted, focusing on the detection of *trans*, *trans*-farnesol and related products derived from the precursor, farnesyl pyrophosphate. *In silico* analysis was employed to identify the sequence for the farnesene synthase gene responsible for producing these isoprenoids in the *Leishmania* genome.

Results: Exogenously added *trans*, *trans*-farnesol was found to interfere with the proliferation of *L. amazonensis* promastigotes, inhibiting the cell cycle without causing DNA fragmentation or loss of mitochondrial functionality. Despite the absence of *trans*, *trans*-farnesol in the culture supernatant, other products derived from farnesyl pyrophosphate, specifically α-farnesene and β-farnesene, were detected starting on the fourth day of culture, continuing to increase until the tenth day. Furthermore, the identification of the farnesene synthase gene in the

Leishmania genome through in silico analysis provided insights into the enzymatic basis of isoprenoid production.

Discussion: The findings collectively offer the first insights into the mechanism of action of farnesol on *L. amazonensis*. While *trans, trans*-farnesol was not detected in the lipid secretome, the presence of α-farnesene and β-farnesene suggests alternative pathways or modifications in the isoprenoid metabolism of the parasite. The inhibitory effects on proliferation and cell cycle without inducing DNA fragmentation or mitochondrial dysfunction raise questions about the specific targets and pathways affected by exogenous *trans, trans*-farnesol. The identification of the farnesene synthase gene provides a molecular basis for understanding the synthesis of related isoprenoids in *Leishmania*. Further exploration of these mechanisms may contribute to the development of novel therapeutic strategies against *Leishmania* infections.

KEYWORDS

***Leishmania*, farnesol, farnesene, isoprenoid, sesquiterpenoid, lipid secretome**

Introduction

The mevalonate pathway is a biosynthetic route that originates cholesterol in mammals and ergosterol in fungi and trypanosomes. (Goldstein and Brown, 1990; Roberts et al., 2003; Nickerson et al., 2006). This pathway also generates precursors of essential isoprenoids, including farnesyl pyrophosphate (FPP), which serves as the substrate for the synthesis of farnesol (FOH), α-farnesene, and β-farnesene through dephosphorylation These metabolites play crucial roles in cell signaling processes that are essential for survival, growth, differentiation, and proliferation of eukaryotic cells. Moreover, certain enzymes involved in isoprenoid metabolism were identified in the pheromone gland of *Lutzomyia longipalpis*, the primary vector of the protozoan parasite *Leishmania infantum* in Latin America (González-Caballero et al., 2013; Spiegel et al., 2016). Both forms of farnesene are odorant components of several plants, included some used in human alimentation, such as chamomile (Rafieiolhossaini et al., 2012). Furthermore, β-farnesene is well known as an aphid alarm pheromone (Bowers et al., 1972; Vandermoten et al., 2012), and a study has shown that it is a feeding stimulant for *Lutzomyia longipalpis* (Tesh et al., 1992).

Besides the metabolic importance of FOH within the cells, it has been shown that in particular concentrations in microenvironment, FOH inhibits cell proliferation and induces apoptosis in a broad range of cell types (Semighini et al., 2008; Joo and Jetten, 2010).

It has been demonstrated that *Candida albicans*, a medically significant polymorphic fungus, synthesizes and secretes FOH. Through *quorum sensing*-type signaling, this isoprenoid modulates morphological transition processes and regulates the fungus's virulence. While FOH can exist in four isomeric forms, only the *trans, trans*- FOH (*t, t*- FOH) has been implicated in signaling activity within *C. albicans* cultures (Hornby et al., 2001). Furthermore, studies indicate that FOH's effect may vary depending on the culture conditions. Therefore, in richer nutrient medium, the fungus has a proportionally greater ability to tolerate higher concentrations of FOH. In this context, *t, t*- FOH can exhibit a toxic effect on the fungi, inducing apoptosis, or it can act as a signaling molecule (Langford et al., 2010).

Trypanosomatids and fungi have their own sterols synthesis machinery that differs in some steps from the mammalian pathway. Due to evolutionary divergence, fungi and trypanosomatids do not synthesize cholesterol; instead, their sterols have an ergostane skeleton (Roberts et al., 2003). However, it is still unknown which evolutionary pressures led fungi and trypanosomatids to differentiate their sterol metabolism from that other eukaryotes, especially considering their phylogenetic distance. Thanks to the discovery of this biochemical convergence, the activity of clinically used antifungal drugs targeting the ergosterol biosynthetic pathway has been extensively investigated in trypanosomatids. It has been observed that several drugs effective against fungi also demonstrate activity against *Leishmania* spp. and *Trypanosoma cruzi* (Goad et al., 1984; Vannier-Santos et al., 1995; Urbina, 1997; Yao and Wilson, 2016).

Considering the similarities observed between fungi and trypanosomatids and the reports that FOH influences the development of *C. albicans*, the interference of the isoprenoid FOH on the proliferative ability of *Leishmania amazonensis* promastigotes and its presence in the lipid secretome of the parasite were investigated.

Materials and methods

Maintenance and cultivation of parasites

L. amazonensis promastigotes (strain MHOM/BR/77/LTB0016) were maintained at 26°C in Schneider's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 5% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin. Alternatively, the parasites were grown in a nutritionally restricted medium with defined chemical composition, characterized by the absence of lipids and

FBS. This medium consisted of a mixture of RPMI media (Sigma-Aldrich, St. Louis, USA) and DMEM (Invitrogen Corporation, GIBCO, Leiden, Netherlands) in the proportion of 1:1 (v/v). The composition of the nutritionally restricted medium was adapted from the protocol elaborated by Merlen et al. (Merlen et al., 1999), which has been described as suitable for the cultivation of several strains of *Leishmania* spp. and also of *Trypanosoma cruzi* epimastigotes. The main modification concerning the original protocol consisted of withdrawal of cholesterol supplementation. Regarding the other supplements used, the concentrations were maintained similar to those established by the authors.

Acquisition and storage of *t, t*-FOH

The isoprenoid *t, t*-FOH (96% purity) was purchased from Sigma-Aldrich. After the bottle was opened, all contents were aliquoted, in the dark environment, for small bottles of amber glass. Immediately before sealing the flasks, the internal atmosphere was saturated with nitrogen gas (N_2). The aliquots' vials were stored in a container with silica at low temperatures (-20°C) until use.

Analysis of *t, t*-FOH activity on the culture growth of *L. amazonensis* promastigotes

To analyze the activity of *t, t*-FOH on the growth of *L. amazonensis*, promastigotes were cultured in Schneider's medium (5% FBS) or nutritionally restricted medium containing different concentrations of *t, t*-FOH. The initial inoculum was $5x10^5$ parasites/mL, and the concentration of promastigotes in the culture was determined after 24, 48, and 72 h by counting the parasites diluted in formalin (4%) using a hemocytometer. Graphs and IC_{50} values were obtained from the GraphPad Prism software (version 7; GraphPad Software, Inc., La Jolla, CA, USA).

Evaluation of *t, t*-FOH effects on the cell division of *L. amazonensis* promastigotes

To assess whether *t, t*-FOH interfere with the cell division of *L. amazonensis*, carboxyfluorescein succinimidyl ester (CFSE) labeled promastigotes were cultured in Schneider's medium or in the nutritionally restricted medium in the presence of *t, t*-FOH. Labeling of the parasites was performed by incubating a suspension of 2.5 x 10^7 parasites/ml in PBS (0.1% bovine albumin) with 10 µM CFSE (Kit Cell Trace, Molecular Probes/ Life Technologies) for 15 minutes at 26° C and protected from light. After this time, the reaction was stopped using cold Schneider's medium (5% FBS). After labeling, the promastigotes were incubated with 37 µM in Schneider's medium or 7.5 µM in the nutritionally restricted medium of *t, t*-FOH. The fluorescence intensity of the CFSE was analyzed 24, 48, and 72 h after labeling with CFSE. The data were acquired using a FACSCalibur flow cytometer equipped with the Cell Quest program. The obtained data were analyzed using the Summit v4.3 computer program.

Evaluation of cell cycle

Promastigotes treated with 46 µM of *t, t*-FOH, corresponding to the IC_{50}, were fixed with 70% ethanol, and maintained at -20° C for one hour. Next, the cells were centrifuged, and the pellet was resuspended and incubated for one hour at 26° C in 500µl of RNAse (200 µg/mL) and solubilized in PBS. Subsequently, 20 µL of propidium iodide (PI, 40µg/mL) were added, and the cells were incubated in the dark for 20 minutes at room temperature (Sen et al., 2007). The data were acquired using a FACSCalibur flow cytometer equipped with the Cell Quest program. The data were analyzed using the Summit v4.3 computer program.

Evaluation of DNA fragmentation

DNA fragmentation in promastigotes was analyzed using a terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) apoptosis detection system (Promega, Madison, WI, USA) to the manufacturer's recommendations. Briefly, $5×10^6$ promastigotes were collected after a 48 h treatment with 46 µM of *t, t*-FOH, washed twice with PBS, and fixed with fixation/permeabilization solution (eBioscience, San Diego, CA, USA) for 10 minutes at 25°C. The fixed cells were incubated with a TdT reaction mixture containing FITC-labelled dUTP for 1 h at 26°C. Cells were washed and resuspended in 0.5 mL of PBS (pH 7.4) containing 0.5 mg/mL PI (BD Biosciences) before analyzing using a FACSCalibur flow cytometer. DNAse was utilized as a positive control.

Evaluation of mitochondrial membrane potential (ΔΨm)

The mitochondrial functionality of *L. amazonensis* promastigotes were evaluated by flow cytometry using the rhodamine 123 fluorescent probe (Rho 123). In these analyzes, the promastigotes ($1x10^6$ parasites/mL) treated with 46 µM of *t, t*-FOH for 24 hours were incubated with 10 µg/mL of Rho 123 (Sigma-Aldrich, St. Louis, USA) for 20 minutes at room temperature and protected from light. In addition, promastigotes incubated under the same conditions with miltefosine (20 µM) were used as a positive control of the assay. The data were acquired using a FACSCalibur flow cytometer equipped with the Cell Quest program. The analysis of the data obtained by the cytometer was performed using the Summit v4.3 computer program.

Extraction and identification of isoprenoids in secretome of *L. amazonensis* promastigotes

L. amazonensis promastigotes were cultivated in the nutritionally restricted medium using glass bottles and subjected to slight agitation. Initially, the promastigotes in the culture were removed by centrifugation (3000 rpm/15 minutes), followed by sterilization by a filtration membrane filter of 0.22 µM pore size

(Merck Millipore, Brazil). Next, an aliquot of 0.5 µg of progesterone was added to the sterile supernatant, which was used as the internal control of the extraction technique. For lipid extraction, we used ethyl acetate (99.9% purity, Sigma-Aldrich). Subsequently, the ethyl acetate in the sample was evaporated using a rotary evaporator, and to remove residual solvent, nitrogen gas (N_2) was used. Finally, the dried samples were stored at -20°C until analysis of the compounds.

Lipids extracted from the secretome were analyzed by chromatography gas and mass spectrometry (GC/MS). The dried samples were resuspended in 100 µL of ethyl acetate immediately before being injected into the equipment GCMS-QP2010 Ultra (Shimadzu Scientific Instruments, Tokyo, Japan). After injection, the column temperature was maintained at 50° C for 1 minute and then increased to 270°C in a ratio of 10° C/min and finally to 300°C in a ratio of 1° C/min. The helium gas flow was kept constant at 1.1 ml/min. The injector and detector temperatures were 250°C and 280°C, respectively (Torres-Santos et al., 2009).

In silico analysis

To perform *in silico* analysis and identify putative farnesene synthase sequences, we gathered annotated sequences of the enzyme. A total of 109 protein sequences from plants and bacteria were obtained from RefSeq (NCBI). Given the need to focus on distant homologs, we emplyed the Hidden Markov Model (HMM profile) approach (Eddy, 1996). Initially, the sequences were aligned using MAFFT 7 software (Katoh and Standley, 2013). Subsequently, we constructed an HMM protein model using hmmbuild from HMMER 3.2.1 (Finn et al., 2011) and utilized hmmsearch to search the profile against the predicted proteins of *Leishmania amazonensis*, obtained from the Laboratory of Computational Biology and Systems. Concurrently, an OrthoMCL analysis (Li et al., 2003) was conducted, involving seven species of the *Leishmania* genus (*L. panamensis, L. infantum, L. braziliensis, L. amazonensis, L. major, L. donovani*, and *L. mexicana*), to identify orthologs within these species. Following this, with the validation of InertPro (Mitchell et al., 2019), we successfully identified putative farnesene synthase protein sequences in each *Leishmania* species.

Results

Evaluation of the effect of *t, t*-farnesol on the growth of *L. amazonensis* promastigotes

The effect of *t, t*-FOH on *L. amazonensis* promastigotes growth was evaluated using a nutritionally rich medium composed of Schneider´s medium supplemented with 5% FBS (Figure 1A) or a nutritionally restricted medium (Figure 1B) containing different concentrations of *t, t*-FOH. The results indicate that the concentration of *t, t*-FOH required to inhibit 50% of the growth of *L. amazonensis* is significantly higher in a nutritionally rich medium compared to a nutritionally restricted medium, with the respective IC_{50} values (µM) as follows: 24 h (46.2 ± 2.3 vs 7.2 ± 0.6), 48 h (33.0 ± 1.2 vs 4.5 ± 0.3) and 72 h (36.4 ± 1.4 vs 5.7 ± 0.7).

Evaluation of *t, t*-FOH effects on cell division of *L. amazonensis* promastigotes

To evaluate the cell division of *L. amazonensis* promastigotes, the parasites were labeled with CFSE and then cultured in a nutritionally rich or restricted medium containing the IC_{50} of *t, t*-FOH, and the fluorescence was measured after 24, 48, and 72 hours.

Figures 2A-D show the cell division of *L. amazonensis* promastigotes grown in a nutritionally rich medium containing 37 µM of *t, t*-FOH. By analyzing the histogram obtained after 48 hours of incubation, it is observed that there was a decrease in the number of cell divisions of the parasite.

Figures 2E-H show the cell division profile of the parasites grown in a nutritionally restricted medium with 7.5 µM of *t, t*-FOH. Interestingly, *t, t*-FOH had a very distinct effect on cell division of the promastigotes in the poor medium. We can observe that the

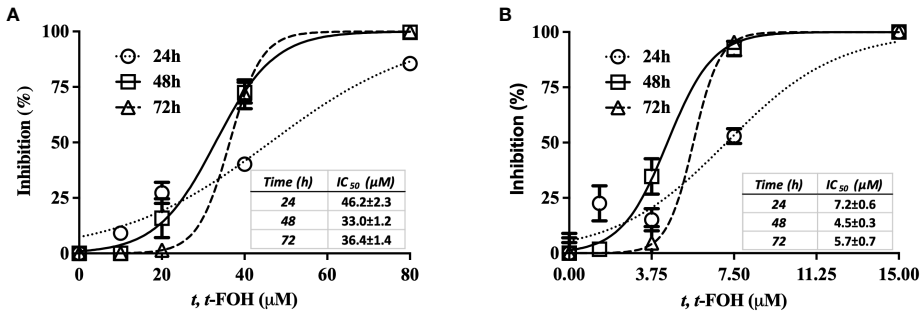


FIGURE 1
Effect of *t, t*-FOH on the parasite growth. *L. amazonensis* promastigotes were cultured in Schneider´s medium supplemented with 5% FBS (nutritionally rich medium) **(A)** or in a nutritionally restricted medium **(B)** containing different concentrations of *t, t*-farnesol (*t, t*-FOH).

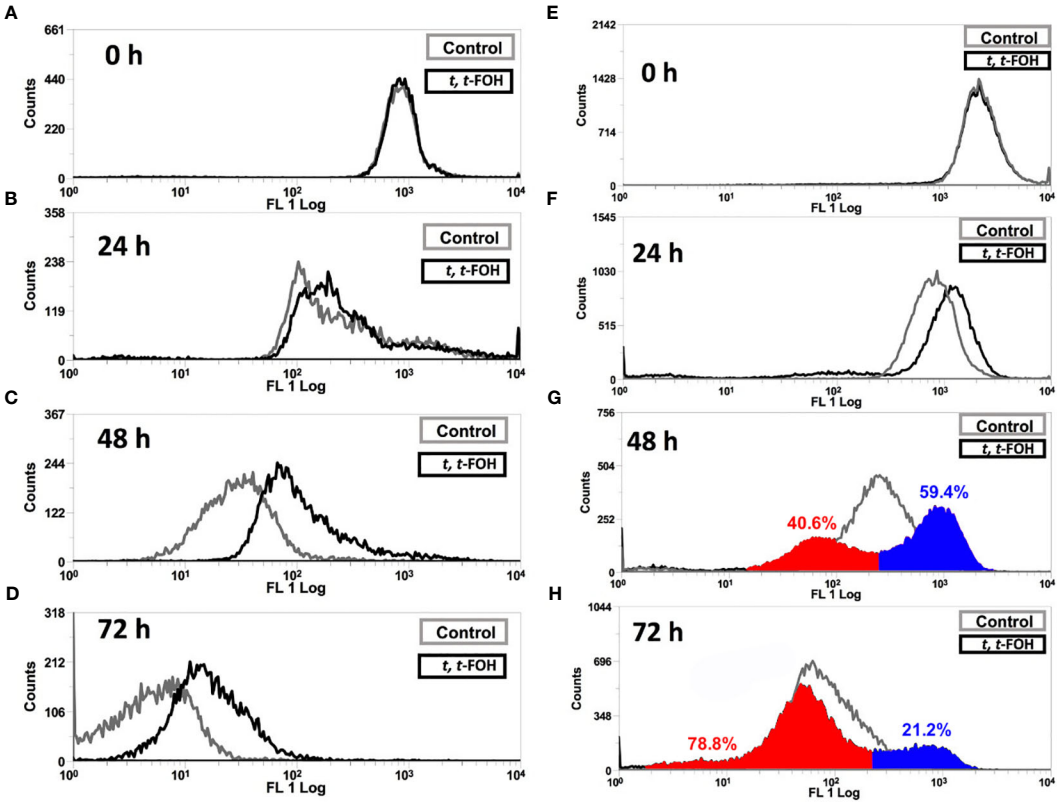


FIGURE 2
Effect on cell division of *L. amazonensis* promastigotes. Promastigotes of *L. amazonensis* labeled with CFSE were grown in a medium nutritionally rich (Schneider´s medium, 5% FBS) **(A-D)** containing 37 μM or nutritionally restricted medium **(E-H)** containing 7.5 μM of *t, t*-FOH. After 24, 48, and 72 h, the proliferative capacity of the parasites was analyzed using an FACSCalibur flow cytometer.

parasites are distributed in two different populations, with a particular pattern of cell division. One group divides faster than the untreated control, while the other divides slower. It is more evident in 48 hours of culture when 40.6% of the treated cells divided faster than the median of the untreated parasites, while the remaining 59.4% divided slower. This is partially reversed in 72 h, possibly because the faster group reached the stationary phase, and the control continued to divide.

Evaluation of *t, t*-FOH effects on the cell cycle of *L. amazonensis* promastigotes

In this assay, we evaluated whether *t, t*-FOH reduces the proliferative capacity of the promastigotes by interfering in the cell cycle of the parasite. The promastigotes were cultured in the nutritionally rich medium with the concentration of *t, t*-FOH corresponding to the IC_{50} (46 μM). After 24 hours, a culture sample was labeled with PI and analyzed by flow cytometry. Figure 3 shows that after the promastigotes remained incubated with the IC_{50} value of *t, t*-FOH, there was a significant decrease in cells in the G_2 phase, compatible with a stop in the cell cycle. At the same time, we also observed an increase in cells in the region corresponding to Sub-G1, that is, with less DNA than G1. The occurrence of these hypodiploid cells may be suggestive of apoptosis.

Evaluation of *t, t*-FOH effects on DNA fragmentation in promastigotes of *L. amazonensis*

To investigate the increase of promastigotes in the sub-G_0/G_1 phase after incubation with *t, t*-FOH, we looked for DNA fragmentation in the parasites. Analysis of the effect of *t, t*-FOH on the promastigotes cultured in a nutritionally rich medium was done after 48 hours of incubation, and the concentration of *t, t*-FOH used was equal to the IC_{50} (46 μM). However, despite the hypodiploidy observed in the cell cycle analysis, there was no DNA fragmentation in parasites incubated with *t, t*-FOH (Figure 4).

Determination of *t,t*-FOH effects on mitochondrial membrane potential ($\Delta\Psi_m$) of L. amazonensis promastigotes

Mitochondrial membrane potential ($\Delta\Psi_m$), another hallmark of apoptosis, was also investigated in cells incubated with *t, t*-FOH. Promastigotes cultured in a nutritionally rich medium (Schneider´s medium supplemented with 5% FBS) were treated with *t, t*-FOH corresponding to the IC_{50} (46 μM). After 24 hours, the $\Delta\Psi_m$ was assessed by flow cytometry. Figure 5 shows that *t, t*-FOH does not interfere with the mitochondrial membrane potential of the parasites.

10.3389/fcimb.2023.1221246

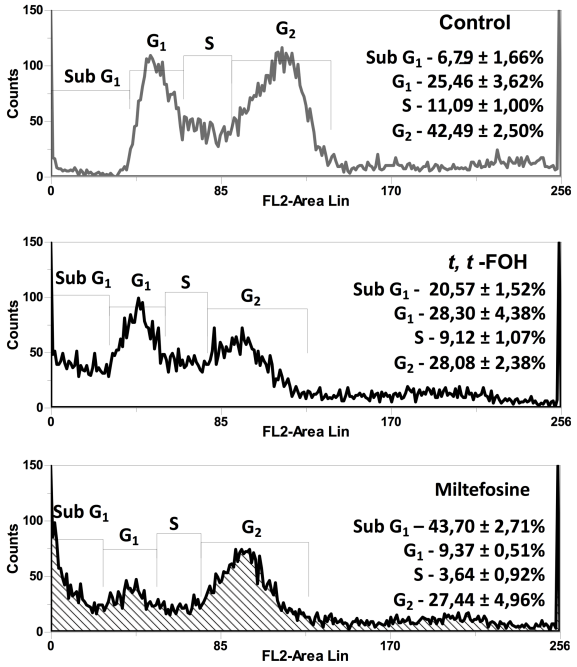


FIGURE 3
Effect of *t, t*-FOH on the cell cycle on *L. amazonensis* promastigotes. The parasites were grown in a nutritionally rich medium (Schneider, 5% FBS) containing IC_{50} of *t, t*-FOH, or 20 μM miltefosine. After 24h, a culture sample was labeled with propidium iodide (PI) and analyzed using a FACSCalibur flow cytometer. The percentage of cells in each cell cycle phase is described in the histograms.

Identification of isoprenoid derivatives in the secretome of promastigotes of *L. amazonensis*

To extract lipophilic metabolites in the culture supernatant, promastigotes of *L. amazonensis* were cultivated for ten days. The lipids were extracted on days 1, 4, 7, and 10 of the culture supernatants. The days 1 and 4 represent the logarithmic phase, and the days 7 and 10 illustrate the stationary phase of the cultures.

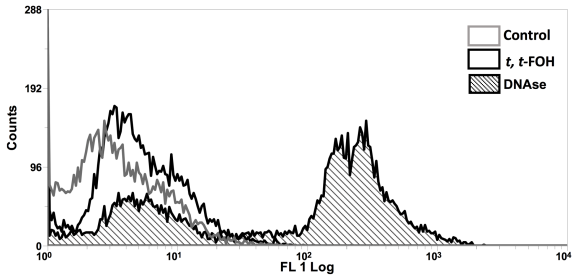


FIGURE 4
Effect of *t, t* -farnesol on DNA fragmentation in promastigotes of *L. amazonensis*. Promastigotes were incubated with IC_{50} or DNAse for 48 h, and then a TUNEL assay was performed. The fluorescence was analyzed by flow cytometry. DNAse was utilized as a positive control. The histogram is representative of three independent experiments.

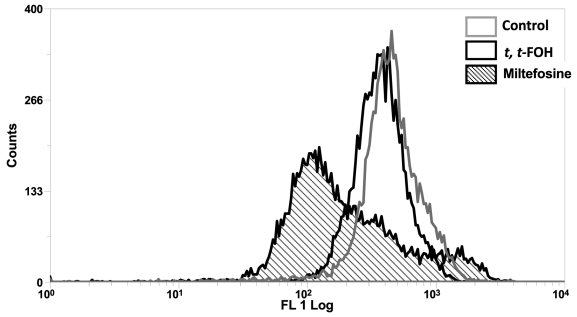


FIGURE 5
Effect of *t, t* FOH on the mitochondrial membrane potential ($\Delta\Psi_m$) of promastigotes of *L. amazonensis* grown in a nutritionally rich medium. Promastigotes of *L. amazonensis* were cultured in a schneider medium (5% of PBS) containing IC_{50} *t, t*-FOH. After 24h, the mitochondrial membrane potential ($\Delta\Psi_m$) was analyzed by flow cytometry using Rhodamine 123 (Rho123). Miltefosine was used as a positive control of the test. The histogram is representative of three independent experiments.

GC-MS analysis was conducted to examine the neutral lipids in the supernatant, and the representative chromatograms are depicted in Figure 6. The compound β-farnesene was detected starting from day four, while α-farnesene was observed in increasing concentrations on days 7 and 10 (Table 1).

The analysis of the chromatograms and mass spectra obtained by GC-MS, with assistance from the NIST11 spectral library, revealed the presence of both α-farnesene and β-farnesene isomers. Although the spectra are highly similar, a notable distinction lies in the intensity of the peak at *m/z* 81, which constitutes 50% of the total intensity in the mass spectrum of α-farnesene. This distinction can be explained through the proposed fragmentation mechanisms, which indicate potential differences in the stability of the *m/z* 81 fragments between the two isomers (Figure 7). Specifically, the fragmentation of β-farnesene, through sigma cleavage between the C5-C6 carbons, leads to a fragment at *m/z* 81 with resonance structures alternating between a primary and a secondary carbocation. In contrast, the same fragmentation mechanism for α-farnesene results in resonance structures alternating between a primary and a tertiary carbocation, with the latter offering greater stability to this fragment. This accounts for higher intensity of the *m/z* 81 peaks in the mass spectrum of α-farnesene. Another contributing factor to the lower stability of the *m/z* 81 fragments obtained from β-farnesene is the ring tension within the proposed fragment structure.

Identification of farnesene synthase in *Leishmania* spp.

α- and β-farnesene were found in the secretome of *L. amazonensis*, prompting an investigation into the enzyme responsible for this reaction, which is interconnected with the mevalonate pathway. This enzyme, previously described in plants (Pechous and Whitaker, 2004) and annotated in RefSeq-NCBI as farnesene synthase, was sought after. Using these sequences, we

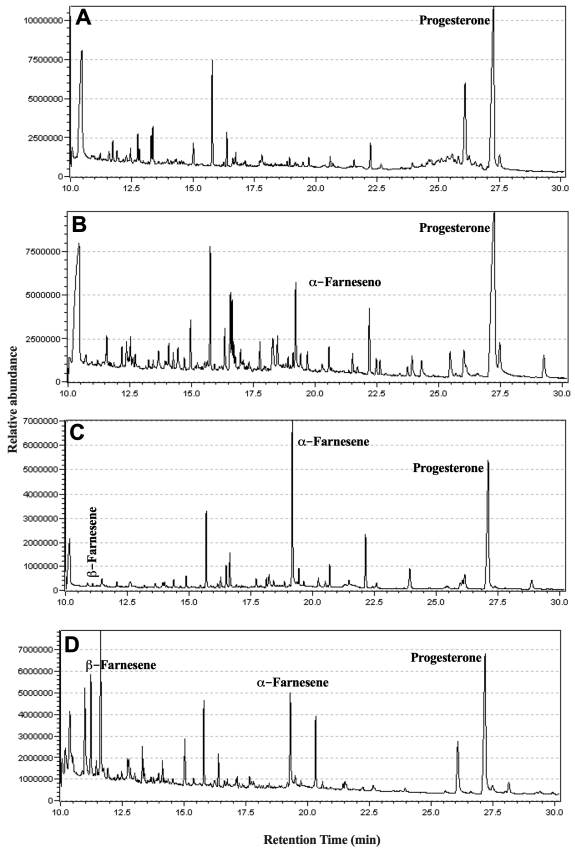


FIGURE 6
Identification of isoprenoids in the culture supernatant of promastigotes of *L. amazonensis*. Promastigotes were cultured in medium culture nutritionally restricted in the absence of lipids and fetal bovine serum. On days 1 **(A)**, 4 **(B)**, 7 **(C)**, and 10 **(D)**, the lipophilic metabolites present in the supernatant were extracted using ethyl acetate and identified by GC-MS. Data representative of a total of two experiments.

initiated a search for distant homologs to identify a corresponding sequence in *Leishmania* spp. Through this *in silico* analysis, we identified a putative farnesene synthase for *Leishmania* spp. (Figure 8). This search allowed us to identify a putative homologous sequence of farnesene synthase in *L. amazonensis*, based on a profile constructed using previously annotated sequences from different species. Utilizing the OrthoMCL software, which included seven other species of *Leishmania*, including *L. amazonensis*, we determined the homologous group to which it belonged based on its sequence identifier. Subsequently, we verified the classification of the enzymes in *Leishmania* and the bacteria used to construct the HMM profile with InterPro, aiming to validate the putative homolog. In this *in silico* validation, we found that all sequences exhibited similar characteristics to the farnesene synthase found in bacteria. These characteristics include protein family membership, involvement in the same biological processes, and molecular functions (Table 2).

Discussion

The first step of the work consisted in evaluating the effects of *t, t*-FOH on *L. amazonensis* growth. We observed a different pattern between parasites grown in the rich or poor medium in the presence of *t, t*-FOH. A higher FOH concentration was required to inhibit the growth of the parasites in the rich medium (Figure 1). Similar results were also described by Langford et al. (2010). The authors analyzed the culture conditions in that FOH promotes toxicity or differentiation in *C. albicans*. They explained that cultures of *C. albicans* maintained in a nutritionally rich environment were more tolerant to the treatment with FOH in comparison to the cells maintained in PBS. From this observation, the authors suggested that the mechanism of tolerance of *C. albicans* to FOH consists of physiological adaptation depending on energy availability. Thus, the richer in nutrients is the culture medium in which the fungus grows, the proportionally higher will also be the cell's ability to tolerate higher concentrations of FOH in the medium. Another aspect that has also been described favoring the mechanism of tolerance of *C. albicans* to FOH consists of the albumin present in the FBS. Mosel et al. suggested that serum albumin can bind to FOH, which would result in FOH blockade in the culture and consequently in greater tolerance of the fungus to the compound (Mosel et al., 2005).

We then evaluated the effect of *t, t*-FOH on the cell division of *L. amazonensis* promastigotes cultured in nutritionally rich or restricted medium containing the IC_{50} of *t, t*-FOH (Figure 2). The results showed that *t, t*-FOH decreased the number of cell divisions of *L. amazonensis* promastigotes grown in rich medium, mainly after 48 e 72 hours of incubation, suggesting a delay in cell cycle. Intriguingly, the impact of *t, t*-FOH in the nutrient-poor medium exhibited a dual effect. Some cells divided faster than the control, while others divided more slowly. This outcome suggests the presence of subpopulations of promastigotes that respond differently to *t, t*-FOH under nutritional stress. It's important to note that although we often treat promastigotes as a homogeneous population, they are known to differentiate into at least four distinct developmental stages inside the sand fly midgut or in culture

TABLE 1 Identification of isoprenoids in the culture supernatant of promastigote of *L. amazonensis*.

Compound	MW	Relative amount (%) *			
		D1	D4	D7	D10
α–Farnesene	204.357	–	11.53	37.13	19.43
β–Farnesene	204.357	-	-	0.9	19.03

* The relative abundance was calculated using progesterone (internal standard). -, not detected.

10.3389/fcimb.2023.1221246

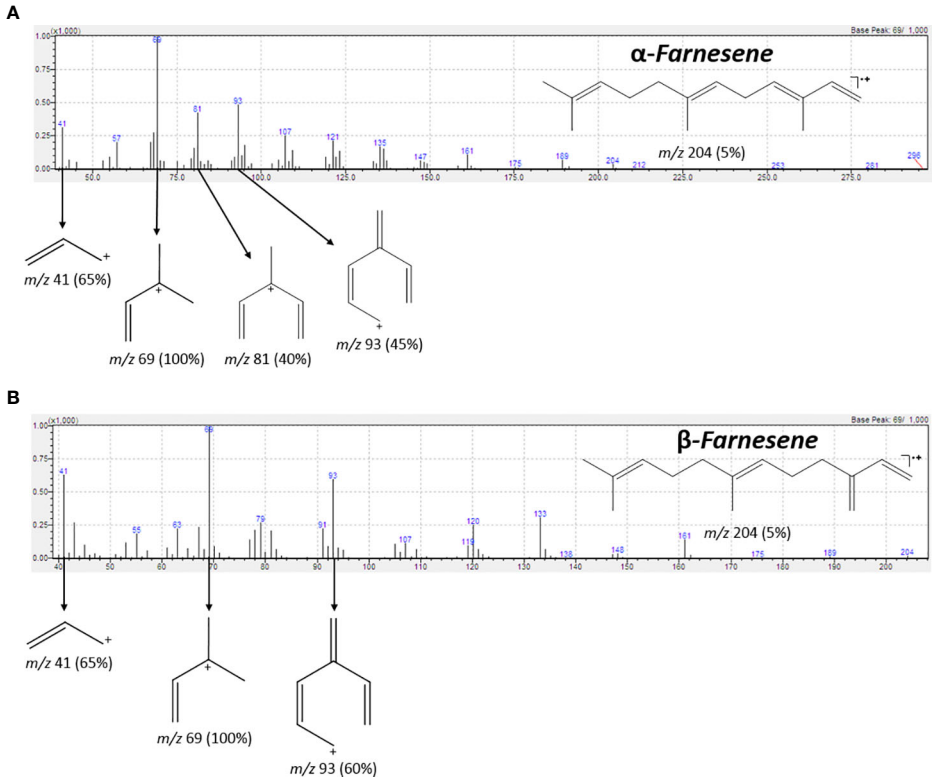


FIGURE 7
IE-MS spectrum and the fragmentation pathway of α-farnesene **(A)** and β-farnesene **(B)**. Highlight for the fragment m/z 81 for **(A, B)**. The difference in intensity of peaks m/z 81 in **(A, B)** can be explained in terms of the stability of the carbocations formed, and the ring stresses in the structures in **(B)**.

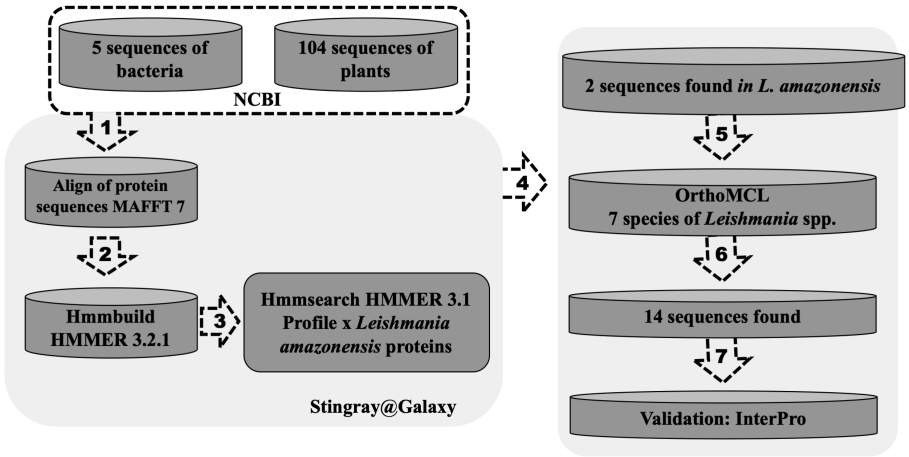


FIGURE 8
In silico prospection of a farnesene synthase putative protein sequence in *Leishmania* spp. One hundred four protein sequences of farnesene synthase from plants and bacteria were obtained from RefSeq (NCBI) and (1) aligned using software MAFFT. After that, (2) the HMM profile was built with hmmbuild from HMMER 3.2.1, and then using (3) hmmsearch to search the profile against the predicted proteins of *Leishmania infantum* obtained from TriTrypDB. Using this approach, two sequences were obtained in *L. infantum*. Meanwhile (4), an OrthoMCL analysis was performed using seven species of the *Leishmania* genus (*L. panamensis, L. infantum, L. brazilensis, L. amazonensis, L. major, L. donovani* and *L. mexicana*) to identify orthologs of these species. After that, it was possible to (5) find the orthologs groups of these two sequences found in *L. infantum* (6), totalizing 14 sequences from 7 species separated into two orthologs groups. After the validation using InertPro (7), it was possible to identify a farnesene synthase putative protein sequence in each species of *Leishmania*.

TABLE 2 Identification of farnesene synthase putative protein sequence in each species of *Leishmania*.

Specie	Sequence ID	Database	InterPro
L. infantum	LinJ.34.3110:mRNA	TriTryp	Cytochrome P450, E-class, group I (*[1]IPR002401) Biological Process *[2]GO:0055114 oxidation-reduction process Molecular Function *[2]GO:0005506 iron ion binding *[2]GO:0016705 oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen *[2]GO:0020037 heme binding
L. panamensis	XP_010698508.1	RefSeq	
L. amazonensis	LAJMNGS018C06.b.3162	*[3]LBCS	
L. braziliensis	LbrM.20.2920:mRNA	TriTryp	
L. mexicana	LmxM.33.3330.1	TriTryp	
L. major	LmjF.34.3330:mRNA	TriTryp	
L. donovani	LdBPK_343110.1.1	TriTryp	
Planktotalea frisia	OJI93009.1	RefSeq	

*[1] IPR InterPro identifier *[2] GO Gene Onthology identifier *[3] Laboratory of Computational Biology and Systems

(procyclic, nectomonad, leptomonad, and metacyclic), influenced by nutrient availability and other environmental factors. Recent work by Coutinho-Abreu and colleagues demonstrated that these forms, when directly collected from the invertebrate host, exhibit differential gene expression (Coutinho-Abreu et al., 2020). As the first three forms are replicative, while the infective metacyclic form is not, it is possible that *t, t*-FOH serves as a signal for the proliferation of a specific differentiation stage under nutritional stress. Over time, this population may reach a stationary phase and transform into non-replicative metacyclic promastigotes, as suggested by the data in 72 h (Figure 2H). Further experiments are needed to investigate this hypothesis.

Then, we assessed whether *t, t*-FOH reduces proliferative ability by interfering with the cell cycle of the parasite (Figure 3). After the promastigotes remained incubated for 24 hours with the IC_{50} of *t, t*-FOH occurred a significant decrease in the percentage of cells in the G_2 phase and an increase of cells in sub G_0/G_1 phase. However, no significant difference was observed in the percentage of promastigotes distributed in the other cell cycle stages (G1 and S phases). These results show that the growth inhibition seen after treatment with FOH is partly due to a reduction in the number of cells cycling. Furthermore, some toxicity was also observed once a hypodiploid population was noticed.

To evaluate whether the observed toxicity was due to apoptosis, we analyzed the action of the isoprenoid on DNA integrity and the mitochondrial functionality of the parasites (Figures 4, 5). Interestingly, the promastigotes treated with the IC_{50} of *t, t*-FOH showed preserved mitochondrial membrane potential and DNA integrity. The functionality of mitochondria is an important indicator of cell viability since this organelle is the main metabolic energy-generating center. So, the permeabilization of the mitochondrial membrane and consequent dysfunction of the organelle is often a decisive event to determine cell death (Kroemer et al., 2007). These results showed that the parasites were not in the process of apoptosis after 48 hours of incubation with *t, t*-FOH, considering that loss of mitochondrial membrane potential and DNA fragmentation are two of the main characteristics of this kind of death (Henry et al., 2013).

A possible explanation for the emergence of the hypodiploid population in the treated culture could be the performance of *t, t*-FOH as a detergent, causing the death of a small number of parasites due to necrosis. Indeed, it has already been demonstrated in bacterial and fungal cultures that the hydrophobic nature of FOH promotes its action as a detergent due to accumulation in membranes, resulting in cell rupture immediately afterward (Shirtliff et al., 2009).

After studying the effects of *t, t*-FOH on cultures of *L. amazonensis* promastigotes, we analyzed the lipophilic content in the secretome of *L. amazonensis* (Figure 6). To guarantee that the parasite released the lipids present in the culture supernatant, we used a nutritionally restricted culture medium whose composition is known and characterized by the absence of lipids and FBS. Thus, the possibility that the lipids identified were from an exogenous source of FBS was discarded. Some authors have also used similar strategies to attend to the need to grow parasites without FBS because it could interfere with the results of studies involving biochemical and immunological analyzes, due to the lack of knowledge of the exact composition of the product, besides the heterogeneity from batch to batch that would reflect in variation in the results (Merlen et al., 1999; Santarém et al., 2014).

The search for isoprenoid derivatives in the secretome of *L. amazonensis* resulted in identifying α-farnesene and β-farnesene in the culture (Table 1) and *in silico* analysis indicated that the parasite has the DNA sequence for the farnesene synthase enzyme. The time interval between days 7 and 10 is representative of the stationary phase of the growth curve of the culture. It has already been shown that isoprenoids can perform signaling activity in cultures of *C. albicans* (Shchepin et al., 2003).

Interestingly, a set of sesquiterpenoids described in the literature are referred to as juvenile hormones (HJs). Juvenile hormones have been extensively studied because of their importance to the physiology of insects, playing central roles in the embryonic development, metamorphosis, and reproduction of these arthropods. Among the HJs, juvenile hormone III (HJ III) is the most commonly expressed among insects (Sperry and Sen, 2001; Cao et al., 2009). The mevalonate biosynthetic pathway synthesizes HJ III, and FOH production is critical for the endogenous control of the synthesis of this hormone (Cao et al., 2009). In addition, it has been reported that the presence of precursors is the limiting factor for the production of HJs (Bloch et al., 2013). According to these data, the presence of FOH is fundamental for the flow of isoprenoids along pathways that are compromised with the production of these hormones.

Thus, the data in the literature describing sesquiterpenes as essential molecules for insect physiology and the identification of isoprenoid derivatives in the culture supernatant of *L. amazonensis* promastigotes suggest that these compounds can act as members of some type of interaction established between the parasite and its invertebrate host. *Leishmania* and sand flies constitute one of the

oldest pairs of parasite-vector. Throughout this co-evolutionary process, the parasite developed varied adaptive mechanisms to guarantee success in establishing infection (Bates, 2008; Ramalho-Ortigao et al., 2010). It has been described that trypanosomatids have a secretory system adapted to the parasitic lifestyle (McConville et al., 2002). *Leishmania* secretes compounds such as chitinases, neuropeptides, and different types of glycoconjugates. All these products are supposed to act to affect the phlebotomine physiology or behavior to guarantee the establishment of the infection and the continuation of the cycle with the parasite for the vertebrate host (Kamhawi, 2006). Intriguingly, it has been reported that β-farnesene serves as a feeding stimulant for *Lutzomyia longipalpis* (Tesh et al., 1992). If the promastigotes secrete this isoprenoid in the insect midgut, as observed in culture in this study, the physiological effects are yet to be elucidated. As mentioned earlier, *t, t*-FOH is involved in the biosynthesis of molecules that alert the physiology of sand flies. However, further studies are needed to state that *L. amazonensis* promastigotes secrete some of these sesquiterpenes to interact with its invertebrate host.

In conclusion, *trans*, *trans*-farnesol added exogenously interferes in the proliferation of promastigotes and inhibits the cell cycle without causing DNA fragmentation or loss of mitochondrial functionality. Subsequently, the lipid secretome was analyzed, and α-farnesene and β-farnesene isomers were detected starting on the fourth day of culture, increasing until the tenth. Together, these results demonstrate for the first time the biological activity of farnesol on *L. amazonensis* and the identification of α-farnesene and β-farnesene in the lipid secretome of the parasite.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was performed following the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (COBEA) and had the approval of the Animal Ethics Committee of Oswaldo Cruz Foundation (license number L-02/2022).

Author contributions

Conceptualization, ET-S; Data curation, VA-N, MB, RM-B, and EC-J; Formal analysis, VA-N, RM-B, and EC-J; Funding acquisition, ET-S; Investigation, LP, VA-N, and EC-J; Methodology, LP, VA-N, MB, and RM-B; Supervision, ET-S; Writing – original draft, LP; Writing – review and editing, ET-S. All authors contributed to the article and approved the submitted version.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Fundação de Apoio à Pesquisa do Estado do Rio de Janeiro Carlos Chagas Filho (FAPERJ).

Acknowledgments

The authors would like to thank Pedro Ripper and Bruna Portella for the flow cytometry calculations.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Bates, P. A. (2008). Leishmania sand fly interaction: progress and challenges. *Curr. Opin. Microbiol.* 11, 340–344. doi: 10.1016/j.mib.2008.06.003

Bloch, G., Hazan, E., and Rafaeli, A. (2013). Circadian rhythms and endocrine functions in adult insects. *J. Insect Physiol.* 59, 56–69. doi: 10.1016/j.jinsphys.2012.10.012

Bowers, W. S., Nault, L. R., Webb, R. E., and Dutky, S. R. (1972). Aphid alarm pheromone: isolation, identification, synthesis. *Science* 177, 1121–1122. doi: 10.1126/science.177.4054.1121

Cao, L., Zhang, P., and Grant, D. F. (2009). An insect farnesyl phosphatase homologous to the N-terminal domain of soluble epoxide hydrolase. *Biochem. Biophys. Res. Commun.* 380, 188–192. doi: 10.1016/j.bbrc.2009.01.079

Coutinho-Abreu, I. V., Serafim, T. D., Meneses, C., Kamhawi, S., Oliveira, F., and Valenzuela, J. G. (2020). Distinct gene expression patterns in vector-residing Leishmania infantum identify parasite stage-enriched markers. *PloS Negl. Trop. Dis.* 14, e0008014. doi: 10.1371/journal.pntd.0008014

Eddy, S. R. (1996). Hidden markov models. *Curr. Opin. Struct. Biol.* 6, 361–365. doi: 10.1016/S0959-440X(96)80056-X

Finn, R. D., Clements, J., and Eddy, S. R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res.* 39, W29–W37. doi: 10.1093/nar/gkr367

Goad, L. J., Holz, G. G., and Beach, D. H. (1984). Sterols of Leishmania species. Implications for biosynthesis. *Mol. Biochem. Parasitol.* 10, 161–170. doi: 10.1016/0166-6851(84)90004-5

Goldstein, J. L., and Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature* 343, 425–430. doi: 10.1038/343425a0

González-Caballero, N., Valenzuela, J. G., Ribeiro, J. M. C., Cuervo, P., and Brazil, R. P. (2013). Transcriptome exploration of the sex pheromone gland of Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae). *Parasit Vectors* 6, 56. doi: 10.1186/1756-3305-6-56

Henry, C. M., Hollville, E., and Martin, S. J. (2013). Measuring apoptosis by microscopy and flow cytometry. *Methods* 61, 90–97. doi: 10.1016/j.ymeth.2013.01.008

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., et al. (2001). Quorum sensing in the dimorphic fungus candida albicans is mediated by farnesol. *Appl. Environ. Microbiol.* 67, 2982–2992. doi: 10.1128/AEM.67.7.2982-2992.2001

Joo, J. H., and Jetten, A. M. (2010). Molecular mechanisms involved in farnesol-induced apoptosis. *Cancer Lett.* 287, 123–135. doi: 10.1016/j.canlet.2009.05.015

Kamhawi, S. (2006). Phlebotomine sand flies and Leishmania parasites: friends or foes? *Trends Parasitol.* 22, 439–445. doi: 10.1016/j.pt.2006.06.012

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 87, 99–163. doi: 10.1152/physrev.00013.2006

Langford, M. L., Hasim, S., Nickerson, K. W., and Atkin, A. L. (2010). Activity and Toxicity of Farnesol towards Candida albicans Are Dependent on Growth Conditions. *Antimicrob. Agents Chemother.* 54, 940–942. doi: 10.1128/AAC.01214-09

Li, L., Stoeckert, C. J., and Roos, D. S. (2003). OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* 13, 2178–2189. doi: 10.1101/gr.1224503

McConville, M. J., Mullin, K. A., Ilgoutz, S. C., and Teasdale, R. D. (2002). Secretory pathway of trypanosomatid parasites. *Microbiol. Mol. Biol. Rev.* 66, 122–154. doi: 10.1128/mmbr.66.1.122-154.2002

Merlen, T., Sereno, D., Brajon, N., Rostand, F., and Lemesre, J. L. (1999). Leishmania spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous *in vitro* cultivation of infective promastigote forms. *Am. J. Trop. Med. Hyg.* 60, 41–50. doi: 10.4269/ajtmh.1999.60.41

Mitchell, A. L., Attwood, T. K., Babbitt, P. C., Blum, M., Bork, P., Bridge, A., et al. (2019). InterPro in 2019: improving coverage, classification and access to protein sequence annotations. *Nucleic Acids Res.* 47, D351–D360. doi: 10.1093/nar/gky1100

Mosel, D. D., Dumitru, R., Hornby, J. M., Atkin, A. L., and Nickerson, K. W. (2005). Farnesol concentrations required to block germ tube formation in Candida albicans in the presence and absence of serum. *Appl. Environ. Microbiol.* 71, 4938–4940. doi: 10.1128/AEM.71.8.4938-4940.2005

Nickerson, K. W., Atkin, A. L., and Hornby, J. M. (2006). Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl. Environ. Microbiol.* 72, 3805–3813. doi: 10.1128/AEM.02765-05

Pechous, S. W., and Whitaker, B. D. (2004). Cloning and functional expression of an (E, E)-alpha-farnesene synthase cDNA from peel tissue of apple fruit. *Planta* 219, 84–94. doi: 10.1007/s00425-003-1191-4

Rafieiolhossaini, M., Adams, A., Sodaeizadeh, H., Van Damme, P., and De Kimpe, N. (2012). Fast quality assessment of German chamomile (*Matricaria chamomilla* L.) by headspace solid-phase microextraction: influence of flower development stage. *Nat. Prod Commun.* 7, 97–100. doi: 10.1177/1934578X1200700133

Ramalho-Ortigao, M., Saraiva, E. M., and Traub-Csekö, Y. M. (2010). Sand fly-Leishmania interactions: long relationships are not necessarily easy. *Open Parasitol. J.* 4, 195–204. doi: 10.2174/1874421401004010195

Roberts, C. W., McLeod, R., Rice, D. W., Ginger, M., Chance, M. L., and Goad, L. J. (2003). Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Mol. Biochem. Parasitol.* 126, 129–142. doi: 10.1016/S0166-6851(02)00280-3

Santarém, N., Cunha, J., Silvestre, R., Silva, C., Moreira, D., Ouellette, M., et al. (2014). The impact of distinct culture media in Leishmania infantum biology and infectivity. *Parasitology* 141, 192–205. doi: 10.1017/S0031182013001388

Semighini, C. P., Murray, N., and Harris, S. D. (2008). Inhibition of Fusarium graminearum growth and development by farnesol. *FEMS Microbiol. Lett.* 279, 259–264. doi: 10.1111/j.1574-6968.2007.01042.x

Sen, N., Banerjee, B., Das, B. B., Ganguly, A., Sen, T., Pramanik, S., et al. (2007). Apoptosis is induced in leishmanial cells by a novel protein kinase inhibitor withaferin A and is facilitated by apoptotic topoisomerase I-DNA complex. *Cell Death Differ.* 14, 358–367. doi: 10.1038/sj.cdd.4402002

Shchepin, R., Hornby, J. M., Burger, E., Niessen, T., Dussault, P., and Nickerson, K. W. (2003). Quorum sensing in Candida albicans: probing farnesol's mode of action with 40 natural and synthetic farnesol analogs. *Chem. Biol.* 10, 743–750. doi: 10.1016/S1074-5521(03)00158-3

Shirtliff, M. E., Krom, B. P., Meijering, R. A. M., Peters, B. M., Zhu, J., Scheper, M. A., et al. (2009). Farnesol-induced apoptosis in Candida albicans. *Antimicrob. Agents Chemother.* 53, 2392–2401. doi: 10.1128/AAC.01551-08

Sperry, A. E., and Sen, S. E. (2001). Farnesol oxidation in insects: evidence that the biosynthesis of insect juvenile hormone is mediated by a specific alcohol oxidase. *Insect Biochem. Mol. Biol.* 31, 171–178. doi: 10.1016/S0965-1748(00)00115-6

Spiegel, C. N., Dias, D. B. D. S., Araki, A. S., Hamilton, J. G. C., Brazil, R. P., and Jones, T. M. (2016). The Lutzomyia longipalpis complex: a brief natural history of aggregation-sex pheromone communication. *Parasit Vectors* 9, 580. doi: 10.1186/s13071-016-1866-x

Tesh, R. B., Guzman, H., and Wilson, M. L. (1992). *Trans*-beta-farnesene as a feeding stimulant for the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.* 29, 226–231. doi: 10.1093/jmedent/29.2.226

Torres-Santos, E. C., Sampaio-Santos, M. I., Buckner, F. S., Yokoyama, K., Gelb, M., Urbina, J. A., et al. (2009). Altered sterol profile induced in Leishmania amazonensis by a natural dihydroxymethoxylated chalcone. *J. Antimicrob. Chemother.* 63, 469–472. doi: 10.1093/jac/dkn546

Urbina, J. A. (1997). Lipid biosynthesis pathways as chemotherapeutic targets in kinetoplastid parasites. *Parasitology* 114 Suppl, S91–S99. doi: 10.1017/S0031182097001194

Vandermoten, S., Mescher, M. C., Francis, F., Haubruge, E., and Verheggen, F. J. (2012). Aphid alarm pheromone: an overview of current knowledge on biosynthesis and functions. *Insect Biochem. Mol. Biol.* 42, 155–163. doi: 10.1016/j.ibmb.2011.11.008

Vannier-Santos, M. A., Urbina, J. A., Martiny, A., Neves, A., and Souza, W. D. (1995). Alterations induced by the antifungal compounds ketoconazole and terbinafine in leishmania. *J. Eukaryotic Microbiol.* 42, 337–346. doi: 10.1111/j.1550-7408.1995.tb01591.x

Yao, C., and Wilson, M. E. (2016). Dynamics of sterol synthesis during development of Leishmania spp. parasites to their virulent form. *Parasit Vectors* 9, 200. doi: 10.1186/s13071-016-1470-0

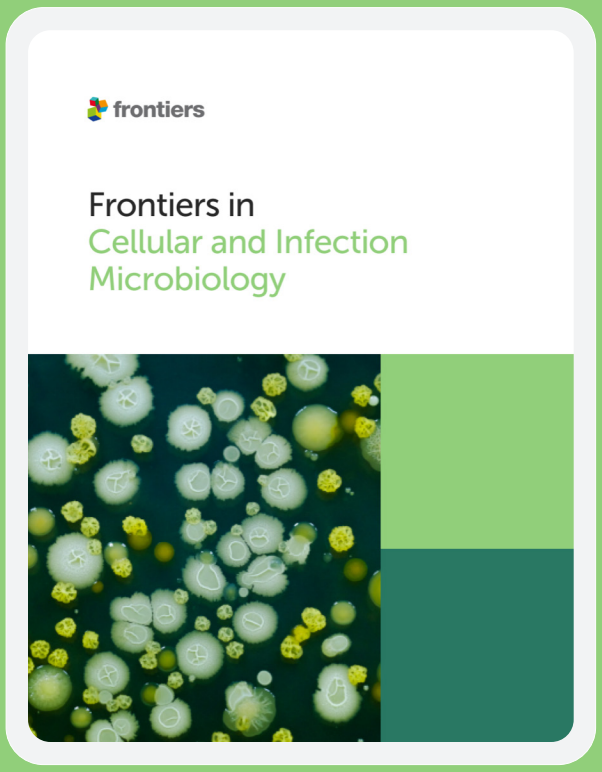
Frontiers in
Cellular and Infection Microbiology

Investigates how microorganisms interact with their hosts

Explores bacteria, fungi, parasites, viruses, endosymbionts, prions and all microbial pathogens as well as the microbiota and its effect on health and disease in various hosts.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact