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Renata Celi Carvalho de Souza Pietra, Federal University of Minas Gerais
Lucas Fonseca Rodrigues, Federal University of Minas Gerais
Eliane Teixeira, Fundacao Oswaldo Cruz
Levi Fried, Emory University
Benjamin Lefkove, Emory University
Ana Rabello Rabello, Fundacao Oswaldo Cruz
Jack Arbiser, Emory University
Lucas Antonio Miranda Ferreira, Federal University of Minas Gerais
Ana Paula Fernandes, Federal University of Minas Gerais

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Triphenylmethane Derivatives Have High *In Vitro* and *In Vivo* Activity against the Main Causative Agents of Cutaneous Leishmaniasis

Renata Celi Carvalho de Souza Pietra¹, Lucas Fonseca Rodrigues¹, Eliane Teixeira², Levi Fried³, Benjamin Lefkove³, Ana Rabello², Jack Arbiser³, Lucas Antônio Miranda Ferreira¹, Ana Paula Fernandes¹

¹Department of Clinical and Toxicology Analyses, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, ²Laboratory of Clinical Research, Instituto René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil, ³Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia, United States of America

**Abstract**

The current standard of care for cutaneous leishmaniasis (CL) is organic antimonial compounds, but the administration of these compounds is complicated by a low therapeutic - toxic index, as well as parenteral administration. Thus, there is an urgent need for the development of new and inexpensive therapies for the treatment of CL. In this study, we evaluate the activity of the triphenylmethane (TPM) class of compounds against three species of *Leishmania* which are pathogenic in humans. The TPM have a history of safe use in humans, dating back to the use of the original member of this class, gentian violet (GV), from the early 20th century. Initially, the *in vitro* efficacy against *Leishmania (Viannia) braziliensis*, *L. (Leishmania) amazonensis* and *L. (L.) major* of 9 newly synthesized TPM, in addition to GV, was tested. Inhibitory concentrations (IC) IC₅₀ of 0.025 to 0.84 μM had been found in promastigotes *in vitro* assays. The four most effective compounds were then tested in amastigote intracellular assays, resulting in IC₅₀ of 0.10 to 1.59 μM. A high degree of selectivity of antiparasitic activity over toxicity to mammalian cells was observed. Afterwards, GV and TPM 6 were tested in a topical formulation in mice infected with *L. (L.) amazonensis* leading to elimination of parasite burdens at the site of lesion/infection. These results demonstrated that TPM present significant anti-leishmanial activities and provide a rationale for human clinical trials of GV and other TPM. TPM are inexpensive and safe, thus using them for treatment of CL may have a major impact on public health.

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**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: anavi@uai.com.br

**Introduction**

The genus *Leishmania* spp. protozoa are pathogenic to a wide variety of hosts, including humans, and are most prevalent in tropical climates of developing countries. The major forms of leishmaniasis include cutaneous, mucosal and visceral leishmaniasis [1]. *Leishmania (Leishmania) major* is one of the main etiological agents of CL in the Old World, while *Leishmania (Viannia) braziliensis*, *Leishmania (L.) amazonensis* and *Leishmania (V.) guyanensis* are the main causative species of CL in the Americas. Lesions caused by these species frequently appear as ulcers at the site of infection and are commonly located in poorly-protected areas of the body, such as the face, arms and legs [2,3]. In addition, *L. (L.) amazonensis* may also lead to development diffuse cutaneous leishmaniasis in a few patients, which is characterized by nodular lesions, refractory to chemotherapy [4].

Current therapeutic alternatives for CL treatment are unsatisfactory. The conventional first-line therapy consists of pentavalent antimonials (sodium stibogluconate - Pentostan™ and meglumine antimoniate - Glucantime™). However, these drugs present inconvenient aspects that limit their use, such as the necessity of parenteral administration and a high incidence of toxic and adverse reactions [2]. Pentavalent antimonials have long been considered highly effective [3,5], however, there is a growing body of evidence of variable efficacy, depending on species, geographic region, presence of resistant strains, and therapeutic schemes [2,6–8]. Among the alternative therapeutic schemes, intralasional administration of pentavalent antimonials has been used to treat old world cutaneous leishmaniasis [9].

The second line therapies for leishmaniasis include amphotericin B (AmB), liposomal AmB, and pentamidine. AmB is a very powerful polyenic antibiotic against *Leishmania* but also presents significant adverse effects, including nephrotoxicity and infusion reactions. Liposomal AmB was developed to improve the tolerability profile of AmB deoxycholate [10]. In Brazil, liposomal AmB is recommended for CL treatment only upon failure of first line therapies. In addition, another limitation of liposomal AmB is...
its high cost [11]. Pentamidine is complicated by hypoglycemia and the requirement of intravenous administration.

Finally, paromomycin, an aminoglycoside antibiotic, is an antileishmanial drug that has been on the market since the 1960’s and has been used in several formulations for the topical treatment of CL, with inconclusive results [12–17]. Therefore, further research and studies based on new technologies aimed at improving the delivery and efficacies of topical treatments are still required, especially in regards to safety, efficacy, and cost [18].

Compounds with the triphenylmethane pharmacophore (TPM), such as gentian violet (GV), have a long history of human use as anti-bacterial and antifungal agents. In addition, GV has been shown to have antiparasitic activity against various human parasites and have been used in blood banks to circumvent Chagas’ disease transmission [19–24]. However, TPM have not been previously evaluated in vivo against CL.

We synthesized 9 novel TPM derivatives, as part of a structure-function study of TPM compounds and tested, in addition to GV, against 3 species of pathogenic Leishmania, including both Old World and New World Leishmania. These derivatives were tested against both promastigotes of L. (L.) amazonensis, L. (L.) major and L. (V.) braziliensis and intracellular amastigotes of L. (L.) amazonensis and L. (V.) braziliensis. Finally, we demonstrate that topical treatment with either GV or one of the novel TPM is highly effective in treating L. (L.) amazonensis infected mice.

**Materials and Methods**

**Ethics statement**

This study has been approved by Ethics Committee for Animal Experimentation from University Federal of Minas Gerais (CETEA/UFMG: 12/2009). The University Federal of Minas Gerais adheres to the standards as outlined by relevant national (CONCEA - Brazilian Government Council for Control of Animal Experimentation) and international guidelines for care and use of laboratory animals.

**Parasites**

Promastigotes of L. (L.) amazonensis (IFLA/BR/1967/PH-8), L. (V.) braziliensis (MHOM/BR/75/M2903), and L. (L.) major (MHOM/IL/80/Friedlin) were maintained at 23°C in Schneider’s Drosophila medium (Merck, Germany) supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gibco, Eggenstein, Germany), pH 7.2. The same strain of L. (L.) amazonensis was used for both in vitro and in vivo experiments.

**Triphenylmethane compounds (TPM)**

Novel TPM were synthesized by reacting aromatic substrates with 4,4’bis (diethylaminobenzophenone) in the presence of phosphorus oxychloride in a calorimeter bomb at 140°C under pressure. TPM formula and molecular weight are present in table 1 and structures in figure 1. All 124 reagents were obtained from Sigma-Aldrich, and were purified on silica column 125 chromatography. The molecular weight of TPM compounds were obtained by mass spectrometry. For *in vitro* assays, a stock solution was prepared in ethanol (EtOH) and maintained at −20°C. All subsequent dilutions were prepared in the respective fresh culture Schneider’s medium on the day of the assay, and the final maximum concentration of EtOH was 0.1%.

**Promastigotes assay**

Promastigotes of L. (L.) amazonensis, L. (V.) braziliensis or L. (L.) major with 3 or 4 days of growth were plated in 24 well plates at a plating density of 1×10⁶ parasites/mL in Schneider’s medium supplemented with 20% FCS, pH 7.2. Drugs were diluted at the same medium and added to a parasite suspension in different concentrations, in triplicate. After 48 h of incubation, the parasites were counted and compared to the controls containing parasites in the absence of drugs. The drug concentration corresponding to 50% of the parasite growth inhibition was expressed as IC₅₀.

Three independent experiments were performed to confirm the results. Data are presented as mean and 95% CI.

**Intracellular amastigote assay**

L. (L.) amazonensis and L. (V.) braziliensis promastigotes with 7–8 days and 5–6 days of growth, respectively, were harvested from cultures and added to a fresh Schneider’s medium supplemented with 5% FCS, pH 6.0, and incubated at 32°C for 7 days for L. (L.) amazonensis and 4–5 days for L. (V.) braziliensis until a complete transformation to amastigote-like.

Peritoneal macrophages from BALB/c mice were harvested by washing with ice-cold RPMI 1640 medium, 4 days after induction with a 3% thiglycollate solution. Macrophages were diluted in RPMI 1640 medium (Sigma, Poole, United Kingdom) plus 10% FCS and plated in 24 well plates with a circular cover glass at a plaque density of 2×10⁵ macrophages/well. Macrophages were allowed to adhere for 2 h at 37°C and 5% CO₂, when the medium was replaced by a fresh one and incubated overnight.

Macrophages were infected with amastigote-like parasites from L. (L.) amazonensis or L. (V.) braziliensis. The parasites were counted in a Neubauer’s chamber and adjusted to a macrophage-amastigote ratio of 1:8. Infected cultures were maintained at 37°C and 5% CO₂. After 4 h, extracellular parasites were removed by washing, and a fresh medium containing either TPM compounds, no drug, or the reference drug. AmB (Fungizone®-Bristol Meyers Squibb Pharmaceuticals Ltda, Bedford, USA) was the chosen reference drug and was used at 0.2 μg/mL. After 72 h, the cover glass was removed from the well, washed in RPMI 1640 medium, set in microscope slides, fixed with methanol, and stained with Giemsa for evaluation. Under the immersion microscope, infection indexes (number of amastigotes/100× percentage of infected macrophages) were determined by counting the numbers of intracellular amastigotes in 100 macrophages. The experiments were considered valid only when the control group (without drugs) displayed at least 80% of infection. Each point was tested in triplicate and three independent experiments were then performed. Results are presented as mean and 95% CI [25].

**Cytotoxicity**

To evaluate the toxicity of selected compounds, an *in vitro* cytotoxicity assay on macrophages from BABL/c mice was performed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, United Kingdom) assay. Briefly, peritoneal macrophages from BABL/c mice were harvested, as described previously and plated in 96-well flat-bottom microplates at a plating density of 1×10⁵ macrophages/well. Macrophages were allowed to adhere for 2 h at 37°C and 5% CO₂, at which time the medium was replaced by a fresh one and incubated over night. Then, the cells were exposed to ten points of serial dilution of TPM 1, 2, 6, 9 or GV (0.195 and 20 μL), which were used to obtain a curve to determine the IC₅₀. After 68 h of incubation, 10 μL of MTT (10 mg/mL) was added to each well and the plates were further incubated for 4 h. The enzymatic reaction was then stopped by addition of 100 μL of 50% isopropanol–10% sodium dodecyl sulfate solution. The optical density at 570 nm was quantified using an ELISA plate reader (BioSource, Inc., EUA). Three independent experiments, in
Figure 1. TPM structures.
doi:10.1371/journal.pone.0051864.g001
TPM 6 and GV, lesions were covered with 50 BALB/c mice were divided into three groups. For treatment with development of ulcerated lesions (average diameter of 7 to 9 mm), PLOS ONE | www.plosone.org 4 January 2013 | Volume 8 | Issue 1 | e51864

...was quantified by a limiting-dilution assay. Skin fragments from treatment, the number of viable parasites at the site of infection...
In vivo assay

The quantification of parasites within lesions was used to evaluate the efficacy of different treatments in BALB/c mice infected with L. (L.) amazonensis. First, the efficacy of GV and TPM 6 in a 1% gel was compared to a control group that received placebo. As seen in Figure 3A, treatment with TPM 6 gel led to a significant decrease in the parasite burdens at site of infection, from $1 \times 10^7$ (control group) to $1 \times 10^4$ (TPM 6 treated group), whereas, no parasites were found at lesion site in the GV treated group.

In a dose-effect assay, GV was tested in a gel either at 0.1, 0.5 or 1%. Five animals per group were treated twice a day for 20 days, as above described. As shown in Figure 3B, the number of parasites within the lesion decreased when gel concentration were increased, although a linear dose-response has been not observed. The number of parasites in the control group ($2.2 \times 10^5$) was higher than that observed in the groups treated with GV gel at 0.1% ($2.2 \times 10^3$), 0.5% ($2.62 \times 10^5$), or 1% (parasites were not detected). Statistical analysis showed a significant reduction in parasite numbers only in 1% GV treated group when compared with the control group ($p<0.05$).

Discussion

Given the worldwide prevalence of *Leishmania* infection in countries that have low budgets for health care, finding a safe and inexpensive treatment for leishmaniasis is still an unmet need. In this study, 10 novel TPM were evaluated against promastigotes and amastigotes from 3 species of *Leishmania*, recognized worldwide as major etiological agents of CL. The most effective compounds proved to be GV and TPM 6 for all the *Leishmania* species tested. Overall, there was no significant difference in the efficacy of the same compound against the promastigotes of three different species of *Leishmania*.

### Table 2. *In vitro* anti-leishmanial activity of TPM compounds expressed as IC\textsubscript{50} (µM) on promastigotes assay.

<table>
<thead>
<tr>
<th>TPM</th>
<th>L. (L.) amazonensis</th>
<th>L. (L.) major</th>
<th>L. (V.) braziliensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM 1</td>
<td>0.436 (0.375; 0.497)</td>
<td>0.567 (0.521; 0.613)</td>
<td>0.492 (0.445; 0.539)</td>
</tr>
<tr>
<td>TPM 2</td>
<td>0.546 (0.462; 0.630)</td>
<td>0.764 (0.677; 0.851)</td>
<td>0.551 (0.476; 0.626)</td>
</tr>
<tr>
<td>TPM 6</td>
<td>0.031* (0.026; 0.036)</td>
<td>0.045± (0.041; 0.049)</td>
<td>0.063± (0.054; 0.072)</td>
</tr>
<tr>
<td>TPM 7</td>
<td>&gt;5.0 (n.d.) (n.d.)</td>
<td>&gt;5.0 (n.d.) (n.d.)</td>
<td>&gt;5.0 (n.d.) (n.d.)</td>
</tr>
<tr>
<td>TPM 9</td>
<td>0.769 (0.614; 0.924)</td>
<td>0.734 (0.658; 0.810)</td>
<td>0.839 (0.745; 0.933)</td>
</tr>
<tr>
<td>TPM 10</td>
<td>&gt;4.0 (n.d.) (n.d.)</td>
<td>&gt;4.0 (n.d.) (n.d.)</td>
<td>&gt;4.0 (n.d.) (n.d.)</td>
</tr>
<tr>
<td>GV</td>
<td>0.025 (0.016; 0.034)</td>
<td>0.034 (0.029; 0.039)</td>
<td>n.d. (n.d.) (n.d.)</td>
</tr>
</tbody>
</table>

### Table 3. Cytotoxicity, anti-leishmanial *in vitro* activity and selectivity index (SI) of TPM compounds.

<table>
<thead>
<tr>
<th>TPM</th>
<th>Cytotoxicity</th>
<th>L. (L.) amazonensis</th>
<th>L. (L.) major</th>
<th>L. (V.) braziliensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} (µM)</td>
<td>IC\textsubscript{50} (µM)</td>
<td>SI</td>
<td>IC\textsubscript{50} (µM)</td>
<td>SI</td>
</tr>
<tr>
<td>TPM 1</td>
<td>8.21</td>
<td>0.76</td>
<td>10.80</td>
<td>0.52</td>
</tr>
<tr>
<td>TPM 2</td>
<td>9.49</td>
<td>1.59</td>
<td>5.97</td>
<td>1.53</td>
</tr>
<tr>
<td>TPM 6</td>
<td>4.16</td>
<td>0.10</td>
<td>41.60</td>
<td>0.10</td>
</tr>
<tr>
<td>TPM 9</td>
<td>7.03</td>
<td>0.34</td>
<td>20.68</td>
<td>0.17</td>
</tr>
<tr>
<td>GV</td>
<td>4.03</td>
<td>0.17</td>
<td>23.71</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values correspond to mean and 95% CI of results obtained from triplicates; n.d., not determined; data obtained for linear regression on MiniTab\textsuperscript{®} 15.1 software, a,b p<0.05 compared IC\textsubscript{50} determined for L. (L.) amazonensis, L. (L.) major and L. (V.) braziliensis. doi:10.1371/journal.pone.0051864.t002

IC\textsubscript{50} values correspond to mean and 95% CI of results obtained from triplicates; n.d., not determined; data obtained for linear regression on MiniTab\textsuperscript{®} 15.1 software; mean value of parasite growth inhibition observed for control drug (0.2 µg/ml AmB) was 98% for L. (V.) braziliensis and 99.5% for L. (L.) amazonensis. doi:10.1371/journal.pone.0051864.t003

*Figure 2. Dose-effect analysis of TPM 6 against *L. (L.*) amazonensis*. Promastigotes of *L. (L.) amazonensis* were plated in 24 well plates at a plating density of $1 \times 10^6$ parasites/mL in Schneider’s medium supplemented with 20% FCS, pH 7.2. TPM 6 was diluted in the same medium and added to parasites suspension at 0.001; 0.005; 0.01 and 0.05 µM, in triplicate. After 48 h, the parasites were counted and compared to the controls containing parasites in absence of drugs. Three independent experiments were done and the results were analyzed with MiniTab\textsuperscript{®} Program. Data are the mean ± SD. doi:10.1371/journal.pone.0051864.g002
Tests against intracellular amastigotes are more relevant to infer the sensitivity of anti-leishmanial drugs, since this is the parasite stage found in the vertebrate host [26]. In the present study, no significant differences were observed regarding the sensitivity of the different Leishmania species to each tested compound. In intracellular amastigote assays, the TPM compounds presented an IC₅₀ between 0.10 µM and 1.59 µM for L. (L.) amazonensis and between 0.10 µM and 1.53 µM for L. (V.) braziliensis. Morais-Teixeira et al. [27], using the same methodology for intracellular amastigote assays described for Glucantime™, the first choice drug in CL treatment, an IC₅₀ of 22.9 µg/mL (188.07 µM) for L. (L.) amazonensis and 24.2 µg/mL (198.75 µM) for L. (V.) braziliensis. Thus, the TPM compounds were between 10–100 fold more potent against Leishmania than the current standard of care. Moreover, based on the cytotoxicity data, the selectivity index could also be estimated. It is generally considered that biological efficacy is not due to in vitro cytotoxicity, when this index is ≈10 [28], as it was observed for TPM 1, TPM 6, TPM 9 and GV against L. (L.) amazonensis and TPM 1, TPM 6 and TPM 9 against L. (V.) braziliensis.

Two mechanisms of action have been ascribed to TPM. An initial study from our laboratory has shown that TPM are potent inhibitors of NADPH oxidase [29]. A second study of ours demonstrated that TPM, but not other NADPH oxidases, also localize in mitochondria and form covalent adducts with thioredoxin 2 (Trx2) [30]. We believe that the first mechanism can be excluded for direct activity against Leishmania, as compounds that were inactive against Leishmania species show potent NADPH oxidase inhibitory activity, namely the compounds with larger aromatic substituent. The possible role of Trx2 as a target for Leishmania is particularly attractive given a recent report of the role of Trx2 in malaria parasite survival, and may provide an explanation of the activity of TPM against both promastigotes and amastigotes of Leishmania [31].

Among the TPM compounds, TPM6, TPM 9 and GV demonstrated the highest selectivity indexes (Table 3). In agreement to the findings observed on the in vitro assays, both TPM 6 and GV showed high activity against parasites in vivo. TPM 6 decreased the parasite burdens by three orders of magnitude, while the 1% GV gel promoted complete elimination of parasites in treated animals. In a dose-response experiment with GV gel, a linear dose dependent response was not observed, but again complete elimination of parasite burden was observed in animals treated with the GV gel at 1%. Similar results were observed in preliminary experiments in L. (V.) braziliensis infected hamsters (data not shown).

Limitations of this study may include the use of an extreme susceptible experimental model and the lack of an extended period to follow up lesions healing. It is noteworthy that previous data have indicated that intramuscular administration of Glucantime, at doses equivalent to those used in human chemotherapy, to either hamsters infected L. (V.) braziliensis or BALB/c mice infected with L. (L.) amazonensis did not lead to significant reductions in lesions size [15]. Similar findings were observed when testing topical administration of PA to BALB/c mice infected with either L. (L.) amazonensis or L. (V.) braziliensis [15,32,33].

Thus, for the first time, we have demonstrated that GV and other members of the TPM class are effective against Leishmania species in vitro and in vivo. Humans have a long history of topical GV use as an antibacterial and antifungal agent, and we extend the potential use of GV as a potent antileishmanial agent. In addition, humans have tolerated systemic exposure of GV as well, given that GV is routinely added to transfused blood in areas where Chagas disease is prevalent [19–24].

This study indicates that some TPM derivatives have in vitro anti-leishmanial activity and that this activity is not limited to a single species. In addition, we have demonstrated that topical GV is highly effective against the usually refractory species L. (L.) amazonensis in vivo, perhaps making it an alternative treatment agent where species diagnosis is not possible. The findings described herein are of public health relevance for the following reasons. First, these drugs are inexpensive and stable at room temperature,
making them ideal for use in areas where *Leishmania* is endemic. Second, GV is readily accessible and has an established safety record, making clinical trials rapidly feasible. Finally, gentian violet has anti-angiogenic properties, which might lead to an enhanced host response, in addition to direct anti-parasitic activity.

References


Author Contributions

Conceived and designed the experiments: RCCdSP LAMF APF. Performed the experiments: RCCdSP LF LR ET LF BL. Analyzed the data: RCCdSP JA LAMF APF. Contributed reagents/materials/analysis tools: LF BL AR JA LAMF APF. Wrote the paper: RCCdSP JA APF.