The in vivo activity of 1,3,4-thiadiazolium-2-aminide compounds in the treatment of cutaneous and visceral leishmaniasis

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Objectives: Researchers have recently investigated the biological activities of mesoionic (MI) compounds, which have shown in vitro activity against many species of Leishmania, as well as Trypanosoma cruzi. The main goal of this study was to evaluate and compare the activity of three MI compounds against Leishmania amazonensis and Leishmania infantum infection in vivo.

Methods: The experiments were carried out using BALB/c mice infected with L. amazonensis or L. infantum as a highly sensitive murine model. The infected mice were treated with MI-HH, MI-4-OCH3, MI-4-NO2 or meglumine antimoniate by different routes (intralesional, topical or intraperitoneal).

Results: Treatment with MI-4-OCH3 and MI-4-NO2 efficiently contained the progression of cutaneous and visceral leishmaniasis in comparison with the control group or mice treated with meglumine antimoniate. Interestingly, these MI compounds did not produce toxicological effects after treatment. Furthermore, treatment with these compounds led to a modulation of the immune response that was correlated with disease control. In this study, MI compounds, and MI-4-NO2 in particular, exhibited high activity in the L. infantum murine model. In the L. amazonensis model, intralesional treatment with MI-4-OCH3 or MI-4-NO2 showed greater therapeutic efficacy than treatment with meglumine antimoniate, and the new topical formulations of these compounds also displayed great activity in the cutaneous leishmaniasis model.

Conclusions: Upon comparison of each MI compound, MI-4-NO2 was clearly the compound with the greatest activity in these two in vivo infection models by each administration route tested.

Keywords: mesoionic compounds, L. amazonensis, L. infantum

Introduction

Leishmaniasis represents a set of important human diseases that produce different clinical manifestations depending on the Leishmania species involved and the host immune response. The two general forms of the disease, which are caused by several species of Leishmania, are visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL).

Furthermore, these diseases constitute the third largest group of vector-transmitted infectious diseases, behind malaria and filariasis,1 and they belong to the group of neglected tropical diseases with research priority according to the WHO. Leishmaniasis affects approximately 12 million people worldwide; the annual incidence of new cases is approximately 2 million, and 350 million people are at risk for infection.2 There are 500,000 new cases of VL per year globally, although 90% of these new cases occur in just five countries (India, Bangladesh, Brazil, Nepal and Sudan). Tegumentary forms of the disease affect 150,000 people worldwide, and although TL is not a lethal disease like VL, disfigurement, disability, and social and psychological stigma are all severe consequences of TL.

In South American countries, Leishmania amazonensis causes a broad spectrum of clinical manifestations; these range from single to multiple cutaneous lesions and include disfiguring nodules associated with the mucosal form of the disease as well as visceral complications.8,9 This species is described as the unique aetiological agent of anergic diffuse cutaneous leishmaniasis in Brazil. This condition is associated with the specific impairment of the cell-mediated immune response at the early
stage of infection. Furthermore, diseases caused by this parasite display little to no tendency to self-cure.

However, human infections with Leishmania infantum (syn Leishmania chagasi), the protozoan causing South American VL, range from subclinical to progressive fatal disease. In southern Europe, VL is an opportunistic infectious disease mainly affecting patients treated with immunosuppressive agents or infected with HIV. The number of VL/HIV co-infection cases reported in Spain, France, Italy and Portugal has increased. Thus, improvements to the therapeutic regimen, the quality of diagnosis and disease control are needed.

In light of the current clinical scenario, the development of new drugs is desirable. Since the 1950s, pentavalent antimonials have been the recommended drugs for the treatment of leishmaniasis, although amphotericin B and its formulations, amos dine and pentamidine have also been used. These therapies are not ideal due to toxicity, the long duration of administration and the high cost. Furthermore, reports have indicated that a large proportion of cases have become unresponsive to traditional chemotherapy. In addition, the participation of the host immune response may negatively affect treatment and disease progression. Several new antileishmanial compounds are under development, although a drug with the capacity to completely cure this infection has yet to be discovered. Therefore, there is an urgent need to discover novel agents, as the efficacy of the currently available drugs is declining.

In an effort to find new drugs for leishmaniasis, our research has focused on mesoionic (MI) compounds. In previous work we synthesized salts of MI derivatives of the 1,3,4-thiadiazolium-2-aminide class and assayed their activity against L. amazonensis, Leishmania braziliensis and L. chagasi and their effect on murine macrophage cytotoxicity. Certain MI derivatives were also tested for their activity against cutaneous leishmaniasis in vivo caused by L. amazonensis. The chemical structure of the MI compounds contains defined regions of positive and negative charge and is associated with a polyheteroatomic system, which enables these compounds to interact with biomolecules. Although the molecules are internally charged, they have a neutral overall charge and can therefore cross biological membranes. These characteristics have been highlighted by the interesting biological activities of these compounds, which include their anti-inflammatory, analgesic, antibacterial, antifungal and antitumour activities.

In addition, each class of MI compound has received considerable attention and has been extensively studied because of their unique structure, reaction behaviour and biological activity, as well as their potential medicinal properties as potent antiplatelet, fibrinolytic and broncholytic agents. These effects may be directly related to the presence of specific substituent groups on the molecular ring or to the ability of the structure to release nitric oxide (NO). In addition, high concentrations of NO in macrophages have been identified as a potent effector mechanism that targets the intracellular forms of Leishmania.

Based on this information on MI compounds, we assayed three MI derivatives (MI-HH, MI-4-OCH3 and MI-4-NO2) for their activity against L. amazonensis cutaneous leishmaniasis and L. infantum visceral leishmaniasis. These studies used BALB/c mice and alternative routes of administration depending on the infection. The cutaneous form was treated topically or intralesionally, and the visceral form was treated intraperitoneally.

Materials and methods

MI compounds

The preparation of the 4-phenyl-5-(4-H-, 4-OCH3- or 4-NO2-styryl)-1,3,4-thiadiazolium-2-phenylene chloride was carried out according to published procedures. These derivatives were fully characterized by infrared (IR), 1H, 13C NMR spectroscopy and mass spectrometry. The compounds were solubilized in DMSO (the highest concentration used was 1.6% w/v).

Mice

Inbred BALB/c mice at 6–8 weeks of age (25–30 g body weight) were provided by the mouse breeding facility at the CECEL (Centro de Criação de Animais de Laboratório) of FIOCRUZ (Rio de Janeiro, Brazil). All recommendations by national law (no. 6638,05/11/1979) for the scientific management of animals were respected, and the experiments were conducted using a protocol approved by the Comissão de Ética no Uso de Animais CEUA/Fiocruz (P0020-00).

Parasites

L. amazonensis (MHOM/BR/1LTBO016) and L. infantum (MHOM/MA677TIMAP263) were maintained by periodic passages in BALB/c mice. L. amazonensis promastigotes were grown at 26 °C in Schneider’s Drosophila medium (Sigma) supplemented with 10% fetal calf serum (FCS) at a pH of 7.2. L. infantum promastigotes were cultivated in Roswell Park Memorial Institute (RPMI) medium (Sigma) supplemented with 10% FCS and 2 mM l-glutamine at 26 °C. Parasites were harvested from the stationary phase and used to infect mice.

Infection and mouse treatment

L. amazonensis infection and treatment regimen

Mice were injected in the footpad with 1×106 promastigotes of L. amazonensis. After 4 weeks the mice received a single daily dose of the MI compound (MI-HH, MI-4-OCH3 or MI-4-NO2) subcutaneously in the footpad. The treatment lasted for 4 weeks, which consisted of 5 days of treatment followed by 2 days of rest. Animals in the control group received equivalent volumes of the vehicle as treatment animals (saline/ DMSO; Sigma). Meglumine antimoniate, a classic antileishmanial drug, was used as a reference. The concentrations of the drugs administered were as follows: 28 mg Sb(V)/kg/day for meglumine antimoniate, 24 mg/kg/day for MI-HH, 22 mg/kg/day for MI-4-OCH3, and 20 mg/kg/day for MI-4-NO2.

In the treatment regimen for L. amazonensis infection, the MI derivatives were used in a topical formulation at 1% of the concentrations listed above. This formulation was prepared using diadermine cream as an inert vehicle. Four weeks after infection the mice received topical cream on the entire infected footpad once a day, 5 days per week. During this application it was necessary to massage the cream into the footpad and to wait for the cream to be absorbed. Animals in the control group received the same volume of the vehicle (diadermine/DMSO).

L. infantum infection and treatment regimen

Mice were injected with 1×106 promastigotes of L. infantum intraperitoneally. Seven days after infection the mice received a single daily dose of the MI compound (MI-HH, MI-4-OCH3 or MI-4-NO2) intraperitoneally. The treatment lasted for 4 weeks, which consisted of 2 days of rest following 5 days of treatment. Animals in the control group received the same...
temperature. The reaction was then blocked with 3 N H2SO4, and the dase activity. The plates were incubated for 15 min in the dark at room

12 weeks post-infection), the footpad swelling was measured using a
dine (TMB; Zymed) substrate was added at 100
(1:4000) and added to the plate for 1 h at 37
immunoglobulins (BD Biosciences; IgM and IgG) were diluted in PBS
bated for 1 h at 37
8
PBS containing 10% FCS (1:50) and were added in triplicate and incu-
taining 10% FCS for 1 h at room temperature. The sera were diluted in
pooled into single cell suspensions as previously described.22 Cells were
for MI-4-OCH3,13 and 20 mg/kg/day for MI-4-NO2.

Measurement of cutaneous lesion size
At different times after L. amazonensis infection (4, 6, 8, 9, 10, 11 and
12 weeks post-infection), the footpad swelling was measured using a
dial caliper (Mitutoya). This value was expressed as the difference in
the thicknesses in millimetres between the inoculated footpad and the
median of the five footpads in the control group.

Parasite load in the lymph node and spleen
The parasite load was estimated using a parasite-limiting dilution assay.
To evaluate the parasite load in mice with cutaneous leishmaniasis
4 weeks after the end of treatment, the animals were euthanized in a
CO2 chamber, and the draining lymph nodes and spleen were aseptically
removed, weighed and homogenized in Schneider’s medium sup-
plemented with 10% FCS (Sigma). To evaluate the parasite load in
mice with the visceral form, the spleen and liver were prepared as
described above, but these mice were sacrificed directly following the
end of treatment. Briefly, 10-fold serial dilutions were prepared and
distributed to 96-well microtitre plates in triplicate and under sterile
conditions. During the 7 day incubation at 26 °C, the wells were examined
daily using an inverted microscope. The number of parasites per
milligram of tissue was estimated based on the tissue weight and the
parasite load from the culture dilutions, and this quantification followed
the method described by Taswell.20,21

Evaluation of MI compound toxicity in mice
At the end of the treatment period, blood samples were taken from the
tails of control and infected animals either treated or untreated with the
MI compounds. The white blood cell percentages were estimated by

High-binding ELISA plates (Falcon) were coated overnight at 4
\( ^\circ \)C with
50 \( \mu \)g/mL Leishmania sp. antigen in PBS. Plates were washed three
times with PBS containing 2% FCS (Cultilab) and blocked with PBS con-
taining 10% FCS for 1 h at room temperature. The sera were diluted in
PBS containing 10% FCS (1:50) and were added in triplicate and incu-
bated for 1 h at 37 °C. The peroxidase-conjugated goat anti-mouse
immunoglobulins (BD Biosciences; IgM and IgG) were diluted in PBS
(1:4000) and added to the plate for 1 h at 37 °C. The tetramethyl benzi-
dine (TMB; Zymed) substrate was added at 100 \( \mu \)L/well to detect peroxi-
dase activity. The plates were incubated for 15 min in the dark at room
temperature. The reaction was then blocked with 3 N H2SO4, and the
absorbance was read at 450 nm.

Antibody profile
Cytokine standards (IFN-\( \gamma \), IL-4 and IL-10; BD Pharmigen) or culture
supernatants were added to the designated wells (50 \( \mu \)L/well) and
incubated overnight at 4 °C. Binding was detected using the appropriate
biotinylated anti-cytokine capture antibodies (BD Pharmigen) and the
addition of streptavidin–horseradish peroxidase (BD Pharmigen) and the
substrate TMB. The reaction was blocked with 3 N H2SO4, and the
absorbance was measured at 450 nm.

- NO dosage
  - NO is quickly degraded into nitrite and nitrate, therefore the nitrite con-
  concentration was determined using the Griess reaction. NO production
  was assayed, after 48 h of incubation, by measuring the concentration of
  nitrite present in the supernatants of the lymph node and spleen
cell cultures by the Green method.23 Fifty microlitres of cell culture
supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride and 2.5%
orthophosphoric acid) for 10 min at room temperature. The absorbance
was measured spectrophotometrically at a wavelength of 540 nm
using a blank that consisted of medium plus Griess reagent (v/v). The
results were expressed as millimoles NO2 based on the standard curve
of known concentrations of sodium nitrite (NaNO2) dissolved in medium.

Statistical analysis
Statistical analysis was performed using a one-way and two-way analysis
of variance (ANOVA) to test for statistical significance (GraphPad Prism;
GraphPad Software, La Jolla, CA, USA). A P value of \( \leq 0.05 \) was considered
statistically significant. Three separate experiments were performed.

Results
Differences in the efficacy of topical and intranasional treatment with MI compounds on the experimental
infection with L. amazonensis
This study utilized a high-sensitivity murine model of L. amazo-
nensis infection to evaluate the therapeutic activity of MI com-
ounds, the subsequent alterations of the host immune
response and the toxicity of this treatment in mice.

The results found that the surface area of the cutaneous leish-
maniasis (CL) lesions increased in the 4 weeks after infection. Intra-
asional or topical treatment with the MI compounds MI-4-OCH3
and MI-4-NO2 efficiently controlled the growth of the lesions. Meglumine antimonitate (intranasional) also demonstrated thera-
peutic activity in this model. Four weeks after the end of treatment,
there was a statistically significant reduction in lesion size in the
treated mice (Figure 1a and b), while the lesion growth continued
in the control group (P < 0.05). The examinations found that the
use of topical formulations of MI-4-OCH3 and MI-4-NO2 showed
greater efficacy in the regulation of the surface area of the cutaneous lesions (P < 0.005). Intralesional treatment with the MI compounds MI-4-OCH₃ and MI-4-NO₂ was more effective than meglumine antimoniate treatment at 12 weeks post-infection (P < 0.05). The intralesional or topical treatment with MI-HH did not show therapeutic activity for improving the lesion size.

Many mice given intralesional treatments were found to have ulcers (control or not), while only control animals or those in the MI-HH group given topical treatments were found to have ulcers in the footpad.

Table 1 shows the parasitic load from the different tissues (footpad, draining lymph nodes, and spleen) used by Leishmania spp. to infect and disseminate within the host animal. Each of the treatments (MI-HH, MI-4-OCH₃, MI-4-NO₂ or meglumine antimoniate) was found to decrease the parasitic load following administration by either the intralesional or topical route. However, there were differences in their antileishmanial efficacies in different organs.

In the footpad, meglumine antimoniate was most effective in decreasing the parasite load in the lesion (P ≤ 0.005), although the MI-4-OCH₃ and MI-4-NO₂ treatments reduced the parasite load more effectively than the control treatment (P ≤ 0.005) and both routes of administration were equally effective. In the draining lymph nodes, the intralesional treatment with meglumine antimoniate or MI-4-NO₂ had similar efficacy as the topical treatment with MI-4-OCH₃ and MI-4-NO₂. In the spleen, MI-4-OCH₃ and MI-4-NO₂ treatments were found to completely interrupt parasite dissemination, as no parasites were isolated from this organ. Interestingly, meglumine antimoniate treatment was not as effective at blocking this migration, as parasites were isolated from spleen. However, this treatment was significantly more effective (P ≤ 0.005) than the control treatment. In addition, both routes of treatment with MI-HH were able to reduce the parasite load in all tissues, but this reduction was less than for the other compounds tested.

Based on the toxicity parameters analysed in this study, there were no indications of any alterations (Table 2). The haematological white blood cells counts were not altered by treatment, as noted in Table 2. Lymphocytes, neutrophils and monocytes were differentially analysed and counted, and there were no statistically significant differences observed following treatment (data not shown).

The hepatic enzymes AST and ALT and creatinine levels were analysed from the sera of the mice at the end of the treatment period (8 weeks post-infection). Intralesional treatment with meglumine antimoniate correlated with greater levels of AST and creatinine than did the control treatment (P ≤ 0.05). Treatment with the MI compounds did not result in alterations of these levels. In addition, no behavioural changes or changes in weight were noted in mice infected and treated.

This study evaluated certain aspects of the murine immune response, including the concentrations of NO, IFN-γ, IL-4, IL-10 and IgG and IgM antibodies.
For this infection, the therapeutic activity was evaluated by comparing the parasite load in the spleen and liver. As seen in Table 1, intraperitoneal treatment with MI-4-NO2 efficiently controlled the disease; no parasites were isolated from spleens or livers of mice infected with L. infantum after 4 weeks of treatment. Treatment with MI-4-OCH3 or meglumine antimoniate decreased the parasite load in the spleen in comparison with the control (P ≤ 0.005), and no parasites were isolated from the liver. In addition, MI-HH treatment decreased the parasite load in the spleen as effectively as meglumine antimoniate, although parasites were isolated from the liver following treatment.

As demonstrated for L. amazonensis infection, toxicological aspects were evaluated at the end of treatment in mice infected with L. infantum (Table 2). Meglumine antimoniate treatment resulted in changes in the serum AST concentration and creatinine levels (P ≤ 0.05). However, treatment with MI-4-NO2 also resulted in greater AST concentrations than did the control treatment (P ≤ 0.05). During the 5 weeks of the experiment, there were no behavioural or body weight changes observed in the mice.

Changes in the immune response, including alterations in NO level and cytokine release, were analysed following the stimulation of spleen cells with Con A or L. infantum antigens (as shown in Figure 2c). Following treatment, the NO levels did not change (data not shown), but the levels of IFN-γ, IL-4 and IL-10 produced following stimulation were altered. Treatment with MI-4-OCH3 or MI-4-NO2 increased the IFN-γ concentration (P ≤ 0.005) and decreased the IL-4 and IL-10 concentrations in comparison with the controls (P ≤ 0.005 and P ≤ 0.05, respectively). Treatment with meglumine antimoniate decreased the levels of IL-10 produced (P ≤ 0.05), and treatment with MI-HH did not alter any of these values.

The IgG and IgM levels from the sera of mice infected with L. infantum are shown in Figure 3(c). There was no fluctuation in IgG levels in any of the treatment or control groups (1 and 5 weeks post-infection). However, lower IgM levels were noted at 5 weeks post-infection in the mice treated with MI-4-OCH3, MI-4-NO2 and meglumine antimoniate compared with the controls (P ≤ 0.05).

### Discussion

The cutaneous and visceral forms of leishmaniasis remain therapeutic challenges and serious public health problems. Pentavalent antimonials are the most commonly used drugs for the treatment of leishmaniasis, although the existence of drug-resistant infections and the fact that these drugs need to be administered parenterally represent major limitations for leishmaniasis chemotherapy. Unfortunately, ideal therapies for leishmaniasis have yet to be identified.24

The first topical drugs used against cutaneous leishmaniasis, imiquimod25 and paromomycin,26 are at the same phase of clinical testing. The most significant result from this study, in terms of the cutaneous form of the disease, was that topical treatment with MI compounds was effective in a highly susceptible murine model. Furthermore, intraperitoneal treatment with MI-4-NO2 was shown to effectively control visceral leishmaniasis. The previously described in vitro and in vivo activities of these MI

### Table 2. Haematological values and a toxicological analysis for mice infected with the given Leishmania species after treatment with the MI compounds or meglumine antimoniate

<table>
<thead>
<tr>
<th>Leishmania sp., treatment</th>
<th>White blood cell count (10^3/μL)</th>
<th>AST (U/mL)</th>
<th>ALT (U/mL)</th>
<th>Creatinine level (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. amazonensis (intranasal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>15 ± 2.36</td>
<td>13 ± 2.6</td>
<td>21 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>MI-HH</td>
<td>15 ± 3.6</td>
<td>14 ± 3.1</td>
<td>2.2 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>MI-4-OCH3</td>
<td>18 ± 2.6</td>
<td>15 ± 3.5</td>
<td>2.3 ± 0.68</td>
<td></td>
</tr>
<tr>
<td>MI-4-NO2</td>
<td>19 ± 3.4</td>
<td>16 ± 2.6</td>
<td>2.5 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>meglumine antimoniate</td>
<td>23 ± 4.2*</td>
<td>16 ± 3.5</td>
<td>3.8 ± 0.45*</td>
<td></td>
</tr>
<tr>
<td>L. amazonensis (topical)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>16 ± 2.5</td>
<td>13 ± 4.1</td>
<td>2.6 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>MI-HH</td>
<td>14 ± 3.5</td>
<td>12 ± 3.6</td>
<td>2.7 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>MI-4-OCH3</td>
<td>19 ± 4.2</td>
<td>16 ± 2.7</td>
<td>2.3 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>MI-4-NO2</td>
<td>18 ± 3.2</td>
<td>17 ± 2.5</td>
<td>2.2 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>L. infantum (intraperitoneal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>20 ± 4.1</td>
<td>14 ± 4.5</td>
<td>2.3 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>MI-HH</td>
<td>22 ± 2.8</td>
<td>13 ± 2.6</td>
<td>2.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>MI-4-OCH3</td>
<td>28 ± 3.01</td>
<td>15 ± 3.9</td>
<td>2.9 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>MI-4-NO2</td>
<td>30 ± 3.02*</td>
<td>16 ± 2.1</td>
<td>3.5 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>meglumine antimoniate</td>
<td>35 ± 3.12*</td>
<td>18 ± 5.1</td>
<td>4.2 ± 0.26*</td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.05.

The efficacy of intraperitoneal treatment with MI compounds on experimental infection with L. infantum

This is the first report on the therapeutic efficacy of MI compounds in the treatment of visceral leishmaniasis. The factors controlling this disease are different from those controlling CL.

Figure 2(a and b) shows the profiles of cytokine levels from the supernatants of the draining lymph node cells stimulated with either ConA or L. amazonensis antigen. The production of NO was not altered by the treatments (data not shown). The concentration of IFN-γ was greater following the MI-4-OCH3, MI-4-NO2 or meglumine antimoniate treatments (both routes) in comparison with the control (P ≤ 0.05).

Interestingly, intranasal treatment with MI-4-NO2 was found to increase the level of IL-4 and decrease the level of IL-10 (P ≤ 0.05), but topical treatment was not found to change these levels. In addition, intranasal treatment with MI-4-OCH3 decreased IL-10 levels and did not change IL-4 levels, which is the opposite of the results seen after topical treatment (decreased IL-4 and no changes in IL-10).

The serum antibody levels (IgG and IgM) are shown in Figure 3(a and b). There were increased IgG levels in the groups treated with MI-4-OCH3, MI-4-NO2 (both routes) and meglumine antimoniate compared with controls (P ≤ 0.05) at the end of treatment (8 weeks post-infection). Low IgM levels were found in all groups at 4 and 8 weeks post-infection.

Rodrigues et al.
derivatives in the growth inhibition of Leishmania sp. support these results and strengthen the potential of these MI compounds for clinical treatment. Although many compounds have shown activity in in vitro experiments, few have succeeded in rodent models of infection. However, several studies on MI derivatives have demonstrated an in vivo anti-tumour activity in BALB/c mice. Previous work from our group demonstrated that MI derivatives inhibit the in vitro growth of L. amazonensis promastigotes and axenic amastigotes. Consequently, MI-HH and MI-4-OCH3 were evaluated for efficacy in L. amazonensis infection in CBA/J mice. In the present study we have shown the effects of three substituted MI compounds (MI-HH, MI-4-OCH3 and MI-4-NO2) used to treat susceptible BALB/c mice infected with L. amazonensis or L. infantum.

The murine model of leishmaniasis has been extensively studied, and it is generally accepted that cell-mediated immunity plays a causal role in host resistance to infection. Accordingly, this murine system provides a convenient and biologically relevant model to investigate the immunological parameters influenced by the application of diverse compounds. In general, the involvement of T helper 1 (Th1) and T helper 2 (Th2) subsets in the protection or exacerbation of disease has been demonstrated in murine TL. However, the Leishmania species may influence the immune response, as L. amazonensis infection causes a mixed Th1/Th2

Figure 2. An analysis of cytokine profiles of cells (lymph node cells and spleen) from BALB/c mice infected with Leishmania species following treatment with MI compounds or meglumine antimoniate. These cell cultures were stimulated with concanavalin A and Leishmania antigens. (a) Analysis of lymph node cells from BALB/c mice infected with L. amazonensis at the end of the intraleisonal treatment period. (b) Analysis of lymph node cells from BALB/c mice infected with L. amazonensis at the end of the topical treatment period. (c) Analysis of spleen cells from BALB/c mice infected with L. infantum at the end of the intraperitoneal treatment period. *P ≤ 0.05; **P ≤ 0.005.

The effect of mesionic compounds against leishmaniasis
Figure 3. An analysis of the antibody levels (IgM and IgG) in BALB/c mice infected with the given Leishmania species at the start and end of the treatment period. (a) Serum analysis from mice infected with L. amazonensis and given intralesional treatment with MI compound or meglumine antimoniate. (b) Serum analysis from mice infected with L. amazonensis and given topical treatment. (c) Serum analysis from mice infected with L. infantum and given intraperitoneal treatment. wpi, weeks post-infection. *P ≤ 0.05.
response. In this study, high concentrations of IFN-γ were observed following treatment with MI-4-OCH3, MI-4-NO2 or meglumine antimoniate for *L. amazonensis* infection and these values were correlated with decreased lesion size and parasite load. However, the IL-4 concentrations varied according to MI compound and the routes of administration and therefore do not explain these effects. Low levels of IL-10 were observed in the groups treated with MI compounds and that contained the lesion growth and had high production of IFN-γ.

A similar pattern of Th cell subset-associated cytokines has been shown for VL, and this is most likely due to the ability of IL-4 to regulate macrophage function. Unexpectedly, some studies in animal models have shown that protection against VL is associated with the production of both Th1 and Th2 cytokines. However, in the models used in this study, increased levels of Th1 cytokines were associated with a positive therapeutic response following treatment with the MI compounds MI-4-OCH3 and MI-4-NO2 and meglumine antimoniate. Cytokines and other mediators released from activated cells, which modify macrophage functions, underscore the complexity of this process.

In general, high levels of antibodies are related to disease progression, as these are involved in the Th2 response and increased B cell function. B cell-derived immunoglobulins (IgM and IgG) have been found to play a critical role in the progression of leishmaniasis, particularly for VL. The persistence of high levels of IgM in the control group until 5 weeks post-infection was an indication of parasite survival and was correlated with the high levels of IL-10 in this group.

The function of NO in the leishmanicidal activity of activated macrophages has been demonstrated. However, the NO production by macrophages does not fully explain the inhibitory effects of the MI compounds on lesion size or parasite load during *Leishmania* infection. In support of this, only treatment with MI-4-OCH3 was found to increase the NO concentration, but this increase was not statistically significant. However, these levels were only examined at 12 weeks post-infection, and other timepoints, such as 8 weeks post-infection, at the end of the treatment may better demonstrate this effect.

Given these considerations, further studies will be necessary to elucidate the role of administered MI compounds in the defence against infection. These studies will create new perspectives for the investigation into other mediators and/or cytokines in this process.

This study has shown that MI compounds exhibit significant activity in the treatment of *L. infantum* infection in mice. Furthermore, MI-4-NO2 was the most effective compound tested in this model. Whereas intrasplemal treatment with MI-4-OCH3 and MI-4-NO2 had greater therapeutic efficacy than meglumine antimoniate in the *L. amazonensis* model, the new topological formulations of these compounds also displayed significant activity in the CL model. Upon comparison of these molecules, MI-4-NO2 was the compound with satisfactory activity in both in vivo models and for all routes of administration tested. However, new topological formulations could be prepared using other vehicles, such as an ethanolic aqueous solution, gentamicin or urea to increase the permeability of these compounds.

In conclusion, the lack of apparent toxicity, as demonstrated by serum analyses, as well as the protective in vivo effect on murine leishmaniasis (CL and VL) encourage further studies of these MI derivatives, including MI-4-OCH3 and MI-4-NO2, as new antileishmanial drugs. Studies on the pharmacokinetics, dosage optimization and mechanism of action of these compounds in relation to their antileishmanial activity should be carried out to determine an adequate dosing regimen and administration routes for therapeutic use.

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**Transparency declarations**

None to declare.

**References**


