Antileishmanial activity of 1,3,4-thiadiazolium-2-aminide in mice infected with *Leishmania amazonensis*

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Efficacy of two mesoionic derivatives (MI-H-H and MI-4-OCH₃) was evaluated in CBA/J mice infected with *Leishmania amazonensis*. The treatment with these compounds demonstrated that MI-4-OCH₃ derivative and the reference drug Glucantime presented significant activity relative to untreated control. No apparent hepatic or renal toxicity was found for these mesoionic compounds.

Keywords: mesoionic derivatives; *in vivo* toxicity; antileishmanial activity

The World Health Organization (WHO) considers leishmaniasis to be one of the most serious diseases worldwide caused by protozoan parasites (24). However, the control of these diseases remains a problem, the available
antileishmanial drugs still relies on the highly toxic pentavalent antimonials (Glucantime and Pentostam), which cause serious side effects and require long-term treatment (5, 18). Second-line drugs include Pentamidine and Amphotericin B, but these drugs have not experienced widespread use due to toxicity and cost. Recently, the oral drug Miltefosine was approved for treatment of human visceral Leishmania infections and experimentally Fluconazole, taken orally, was also shown to be effective against cutaneous leishmaniasis (1). Nevertheless, extensive studies of new molecules with antileishmanial activities, including natural and synthetic compounds, have been undertaken (4) the problems with the side effects and drug resistance of the chemotherapies used at present have not yet been solved.

Our previous studies have proven that mesoionic derivatives of the 1,3,4-thiadiazolium-2-aminide class (figure 1) inhibits the in vitro growth of L. amazonensis, L. braziliensis and L. chagasi promastigotes (20, 22). The chemistry of mesoionic rings, especially their use as masked dipoles, has been a fruitful area of research since the late 1950s. Their structures having well separated regions of positive and negative charge, associated with a polyheteroatomic system, enable them to interact with biomolecules (13). These characteristics have been revealed by interesting biological activities including anti-inflammatory, analgesic, antibacterial, antifungal and antitumoral activities (21). In addition, this class of mesoionic compounds has known to have nitric oxide (NO) – releasing properties (10).

The present study was undertaken to investigate the in vivo efficacy of two mesoionic derivatives (MI-H-H and MI-4-OCH₃) on the L. amazonensis cutaneous infection in mice model. To examine the therapeutic efficacy of these mesoionic derivatives, CBA/J mice, 6-8 weeks of age, were each infected subcutaneously with 1.2 x 10⁶ promastigotes. In this experiment the MI-H-H (24mg/Kg/day), MI-4-OCH₃ (22mg/Kg/day) and reference drug Glucantime (100mg/Kg/day with 28mg Sb⁵⁺) (3, 21) were administered by subcutaneous route 27 days after the experimental infection, 5 doses/week during 4 weeks. Animals in the control group received the same volume of vehicle (DMSO/ phosphate-buffered saline).
Progression of the lesion was monitored until week 12 by measurement of footpad swelling.

At the end of drug administration (8th week), there was a slight difference between groups of treated mice with both the test compounds and the reference drug, and untreated infected mice (figure 2). However, on the 12th week post infection the animals treated daily with MI-4-OCH₃ or MI-H-H showed significantly reduced footpad thickness, as well as those treated with Glucantime, when compared with the control group. It is important to note that at that time, no significant differences in lesion size were observed in groups treated with mesoionic compounds or Glucantime.

In order to evaluate the toxicity of these compounds in mice, body weight and samples of blood were taken from the tail of the controls and infected animals, of both untreated and treated mice in different times during the compounds administration. The total number of leukocytes was estimated by counting in Neubauer’s chamber. The sera collected were assayed colorimetrically for alanine-aminotransferase (ALT), aspartate-aminotransferase (AST) and creatinine using commercial kits (Labtest Diagnostica, Brazil). No apparent signs of drug toxicity, weight loss, or lymphocytes, monocytes, neutrophils alterations were observed in any experiment and enzymes AST, ALT and creatinine concentration, showed no apparent hepatic or renal toxicity after the treatment with mesoionic compounds (table 1) when compared with uninfected mice, untreated or treated with these compounds (data not shown).

After 4 weeks of the end of treatment (12th week of infection) the animals were killed and the popliteal lymph nodes and spleen were aseptically removed, weighed, and then homogenized in Schneider’s medium supplemented with 10% of fetal calf serum (FCS). Briefly, under sterile conditions, eight serial dilutions (1:10) were prepared and distributed in 96-well microtiter plates in triplicate. After incubation at 26°C, the wells were examined using an inverted microscope. The final titer was the last dilution at which the well contained at least one parasite (figure 3) (2). The parasite loads in both the popliteal lymph nodes and spleens of animals treated with mesoionic derivatives or with Glucantime, were significantly
reduced, compared to untreated control animals (figure 3; \( p \leq 0.0001 \)). However, it was observed that the reduction of parasite load in both organs after mesoionic derivatives treatment was accentuated in comparison with Glucantime (\( p \leq 0.001 \)).

In order to elucidate possible NO induction in infected CBA/J mice, it was measured the concentration of nitrites present in the supernatant of the lymph node and spleen cells cultures, according to Green and collaborators (9). The results were expressed as \( \mu \text{M of NO}_2 \) based on a standard curve, from known concentrations of sodium nitrite (\( \text{NaNO}_2 \)) dissolved in cell culture medium. It was observed a significant increase of NO production on lymph node cell culture supernatants of infected mice, after the treatment with MI-4-OCH$_3$ comparing with untreated mice (figure 4). These results could suggest that mesoionic derivatives modulates infection by \textit{L. amazonensis} \textit{in vivo}, activating mechanisms that positively affect the host capacity to eliminate the parasites from the infected cells, thus controlling parasite dissemination. Further studies to evaluate this phenomenon would be interesting.

From studies concerning structure-activity relationships, especially those based on the nature of the group at the 4-position of phenyl ring (figure 1), it was selected the compounds with the best \textit{in vitro} activity as promising drug candidates (22). Between both selected compounds, the mesoionic with 4-OCH$_3$ substituent was more effective than the one with non substitution (MI-H-H). The MI-4-OCH$_3$ administered subcutaneously in mice for a 4 week period controlled the infection induced by \textit{L. amazonensis} in the paws, resulting in smaller lesions when compared to those of the reference drug (Glucantime) and to the control animals (\( p< 0.001 \)).

The study also demonstrates that both mesoionic derivatives treatment decreased parasite load (\( p< 0.0001 \)), in the regional popliteal lymph nodes and in the spleen, which suggests that there is a control of the infection progression and limiting dissemination. Due to the mesoionic treatment, the lymph node and spleen weights also decreased when compared to the untreated control group. This could be correlated with the decreasing parasite load in these groups. No renal or hepatic alterations occurred as evidenced by normal level of creatinine, AST and
ALT showed in treated mice infected with mesoionic compounds and Glucantime. The toxicity of Glucantime was also evaluated by Henao and collaborators (11) in cutaneous leishmaniasis hamster model and no hazard to the animals was observed (6). Moreover, it was observed an increase of NO production, on lymph node cell culture supernatants of infected mice, after treatment with MI-4-OCH$_3$ (p<0.01). The function of NO in the leishmanicidal activity of activated macrophages has been demonstrated both in vitro and in vivo (12, 14-17). Concerning the in vitro experiments from our group, it was clearly revealed that mesoionic derivatives can modulate macrophage infection by NO released by *L. amazonensis* (data not shown). In contrast, the in vitro production of NO by *L. amazonensis* alone (7, 8) was decreased by MI-4-OCH$_3$ and MI-3-OCH$_3$ addition (23).

Furthermore, NO production by macrophages alone, does not fully explain the inhibitory effect of mesoionic compounds on lesions induced by *L. amazonensis* in vivo. Thus, while macrophages are one of the main sources of NO, this radical may also be released by other cells involved in the infectious process, including *Leishmania* parasites (7). Cytokines and other mediators released from activated cells, that modify macrophage functions, underscore the complexity of the process.

It is already know that Glucantime is usually parenterally administered and it irritate the intestinal mucosa, presenting a low absorption rate in the gastrointestinal tract, and for that reason this drug could not be used by oral route (19). However, the mesoionic derivatives would be tested orally in future research. These compounds are enable to interact with biomolecule, though the compounds are internally charged, they are neutral overall, and therefore can cross biological membranes in vivo (22). These properties could be allowing others routes to the treatment with mesoionic derivatives, meaning advantages in comparison with antimonials.

Given these considerations, further studies will be necessary to elucidate the mechanism of action of mesoionic compounds in the defense of the organism against infection, creating new perspectives for investigation of other mediators.
In conclusion, the lack of apparent toxicity of this compound, as attested by blood/serum pathology from treated mice, as well as its protective \textit{in vivo} effect during murine leishmaniasis encourage further studies of mesoionic derivatives, such as MI-OCH$_3$, as new anti-leishmanial drugs and as modifiers of the immunological response to combat infections with intracellular pathogens and histology of tissues to be carried out in future.

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References


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Figure 1. Chemical structures of 4-phenyl-5-(4-H- or 4-methoxy -cinnamoyl)-1,3,4-thiadiazolium-2- phenylamine mesoionic compounds

X= H, 4-OCH₃
Figure 2. Effects of different compounds on the development of *L. amazonensis* infection on several groups of CBA/J mice. Treatments were started on the 4\textsuperscript{th} week post infection and were continued for 4 weeks. Datum points represent the average measurements for groups of 7 mice each. Lesion diameter was expressed as the thickness of the infected footpad. Bars, standard errors of the means (* p< 0.01 and ** p ≤ 0.001).
Figure 3. Effect of treatment with mesoionic compounds (MI-4-OCH$_3$, MI-H-H) and Glucantime on lymph node (a) and on spleen (b) parasite number. CBA/J mice were inoculated with *L. amazonensis* promastigotes, and treated with the mesoionic compounds for 4 weeks. The popliteal lymph nodes and the spleens of 7 animals were then removed and parasite number was estimated using the limiting dilution technique (* p< 0.0001; ** p< 0.001).
Figure 4. NO production in suspensions of lymph node and spleen from four groups of CBA/J mice. NO production was assayed by measuring the nitrite concentration, present in the supernatant of the lymph node and spleen cells cultures of all four groups at the end of the experimental period. The results could be read in spectrophotometer (µQuant) at 550nm (* p ≤ 0.01).
Table 1. Hematological values and toxicological aspects for uninfected mice, untreated or treated with mesionic compounds, at 4th week of treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated mice</th>
<th>MI-4-OCH₃ treated mice</th>
<th>MI-H-H treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells, x 10³/µL</td>
<td>14</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Lymphocytes, %</td>
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<td>70</td>
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<td>Monocytes, %</td>
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<td>5</td>
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<tr>
<td>Neutrophils, %</td>
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<td>24</td>
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<td>Creatinine, dg/ml</td>
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<td>2.56 ± 0.48</td>
<td>2.84 ± 0.49</td>
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<tr>
<td>AST, U/ml</td>
<td>51 ± 6.8</td>
<td>53 ± 5.8</td>
<td>48 ± 5.6</td>
</tr>
<tr>
<td>ALT, U/ml</td>
<td>52 ± 6.2</td>
<td>57 ± 6.2</td>
<td>58 ± 8.5</td>
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<td>Body weight, g</td>
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<td>27</td>
<td>26</td>
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