Effect of Oral Treatment with Pyrazole Carbohydrazide Derivatives against Murine Infection by *Leishmania amazonensis*

Karen S. Charret, Raquel F. Rodrigues, Alice M. R. Bernardino, Adriana O. Gomes, Adriana V. Carvalho, Marilene M. Canto-Cavalheiro, Leonor Leon, and Veronica F. Amaral*

Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; Instituto de Química, Departamento de Química Orgânica, Universidade Federal Fluminense, Programa de Pós-graduação em Química Orgânica, Niterói, Brazil; Instituto de Biologia, Departamento de Imunobiologia, Universidade Federal Fluminense, Niterói, Brazil

Abstract. Newly synthesized pyrazole carbohydrazide derivatives with substituents X = Br/Y = NO$_2$ and X = NO$_2$/Y = Cl were independently investigated in the CBA mouse model of cutaneous leishmaniasis. Animals were infected with *Leishmania amazonensis* and treated two weeks after the parasitic infection with the pyrazole carbohydrazides for 45 days. Oral treatment with both compounds controlled evolution of footpad cutaneous lesions and dissemination of parasites to draining lymph nodes. Nitric oxide generation was observed in supernatants of lymph node cells from infected CBA mice that were treated with these compounds. The pyrazole carbohydrazide derivatives did not show any toxicity or cause alterations in body weight, plasma concentrations of alanine aminotransferase and aspartate aminotransferase, and urinary creatinine levels, but promoted a small decrease in blood neutrophils. These results provide new perspectives on the development of drugs with activities against leishmaniasis.

INTRODUCTION

Leishmaniasis is an important parasitic disease that affects approximately two million persons per year, with approximately 350 million persons at risk of infection. It is caused by protozoans of the genus *Leishmania* and the bite of phlebotomine sand flies. In Brazil, *Leishmania amazonensis* is responsible for most cases reported, which include cutaneous, mucosal, diffuse cutaneous, and visceral leishmaniasis, and is considered a species of epidemiologic importance.\(^2^-^4\)

Since 1960, chemotherapy for leishmaniasis has relied on administration of pentavalent antimonials compounds such as sodium stibogluconate (Pentostam; Glaxo Wellcome; Brentford, United Kingdom) and meglumine antimoniate (Glucantime; Aventis, Paris, France) as first-line agents.\(^5^-^6\) However, these compounds show serious toxic effects and resistance to these drugs is increasing. Amphotericin B, pentamidine, and nonparenteral miltefosine are alternative chemotherapy that has been introduced in recent decades. However, these drugs also show complications such as side effects and high costs.\(^7^-^8\) Thus, there is an urgency for development of affordable and less-toxic alternative drugs. Development of a single drug or formulation for the treatment of several clinical forms of leishmaniasis must address the issue that each infection imposes different pharmacokinetic requirements for the drugs to be used. Other aspects, such as variation in sensitivity of different *Leishmania* species and increased resistance of parasites to drugs, must also be considered.\(^9\) Successful treatment depends on compounds with activity against *Leishmania* species and an immunomodulatory effect.

Pyrazole carbohydrazides are compounds with anti-inflammatory, analgesic, and anti-thrombotic effects\(^10\) and anti-viral and anti-tumor activities.\(^11\) We have previously demonstrated the anti-*Leishmania in vitro* activity of 1-(4-X-phenyl)-N’-[4-Y-phenyl) methylene]-1H-pyrazole-4-carbohydrazide derivatives.\(^12\)

In the present study, we evaluated pyrazolic carbohydrazide (Figure 1) compounds with X = Br/Y = NO$_2$ (compound 1) and X = NO$_2$/Y = Cl (compound 2) substituents in experimental infection of CBA mice with *Leishmania amazonensis* and assessed drug effectiveness by lesion size and parasite burden. We also analyzed toxic effects in infected and non-infected CBA mice by body weight, leukocyte counts, and levels of aminotransferases and creatinine. Nitric oxide production by these compounds was also investigated in draining lymph nodes and spleen cells for an association with disease evolution.

MATERIALS AND METHODS

Parasites. *Leishmania amazonensis* (MHOM/BR/77LTB 0016 strain) was maintained by animal passage and cryopreserved in liquid nitrogen. Promastigotes were cultured in Schneider’s *Drosophila* medium, pH 7.2 (Sigma, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal calf serum. Characterization of strains was made by using molecular techniques such as isoenzyme electrophoresis.\(^13\)

Animals. Mice (males, eight weeks of age) were acquired from the Nucleus for Laboratory Animals–Universidade Federal Fluminense and the Center for Biological Evaluation and Care of Research Animals–FIOCRUZ. Each experimental group contained eight animals. Experiments were conducted using a protocol (P0020-00) reviewed and approved by the Institutional Committee of CEUA/FIOCRUZ.

Chemicals. The 1-(4-X-phenyl)-N’-[4-Y-phenyl) methylene]-1H-pyrazole-4-carbohydrazides with substituents X = Br/Y = NO$_2$ and X = NO$_2$/Y = Cl were synthesized by using a molecular hybridization approach. Structures (Figure 1) of these stable crystalline compounds were characterized by standard methods (infrared spectroscopy, $^1$H analysis, $^{13}$C-nuclear magnetic resonance).\(^15\) Ketoconazole (Galena Química e Farmacêutica Ltda, Campinas, SP, Brazil) was used as a reference drug.

Evaluation of *in vivo* activity. Mice were inoculated in the left hind footpad with $1 \times 10^6$ promastigotes of *L. amazonensis*. Animals were treated orally with pyrazole carbohydrazide...
compounds (1.7 mg/kg/day for Br-0NO₂ and 1.5 mg/kg/day for NO₂-Cl) and ketoconazole (50 mg/kg/day) from the second week after infection continuously up to 45 days after infection. Lesion thickness was evaluated weekly by measuring diameters of both rear feet with a direct-reading dial caliper (Mitutoyo, Yokohama, Japan). Size of lesions in millimeters was calculated by subtracting the measurement of the uninfected foot from that of the infected foot.

Parasite quantification. Number of parasites in lymph nodes was estimated by a modified limiting-dilution assay. Popliteal lymph nodes of infected footpads were removed and used to prepare a cell suspension in phosphate-buffered saline. After centrifugation of the suspension at 1,500 rpm for 10 minutes, the pellet was resuspended in Schneider’s Drosophila medium, pH 7.2. The suspension was then serially diluted in eight-fold dilutions, incubated at 26°C for 7 days, and monitored in an inverted microscope for presence or absence of promastigotes. Analyses were by using the L-calc shortcut program (StemSoft Software, Inc., Vancouver, British Columbia, Canada).

Toxicologic study. Body weight, leukocyte counts, and levels of aminotransferases and creatinine were monitored in infected and non-infected and treated and non-treated groups. The treatment schedule for the non-infected groups was the same as that for the infected groups. To assay body weight, mice were weighed during and at the end of the experiment to compare the treated and non-treated mice. To assay aminotransferase levels, blood was drawn from the tail vein during and at the end of treatment. Plasma concentration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was detected by using a commercial assay (LabTest; Lagoa Santa, Minas Gerais, Brazil). To assay creatinine levels, urine was collected from mice during and at the end of treatment and tested by using a commercial assay (LabTest). To measure leukocyte counts, total blood leukocytes were counted in a hemocytometer after addition of Turk’s solution at a dilution of 1:50, and specific populations of leukocytes were identified by optical microscopy after samples were stained with May-Grünwald reagent.

Nitric oxide dosage. Cells were obtained from spleen or popliteal lymph nodes of infected and non-infected mice and cultured in RPMI 1640 medium (Sigma) without stimulation for 48 hours at 37°C in an atmosphere of 5% CO₂. The culture supernatant was analyzed for nitric oxide by using a Griess reaction on a 96-well plate and read at 510 nm in a spectrophotometer.  

Statistical analysis. Data were analyzed by the Student’s t-test. P values < 0.05 were considered significant. The InStat program (Graph Pad Software, San Diego, CA) was used for these tests. All experiments were repeated at least three times.

RESULTS

Therapeutic effects of pyrazole carbohydrazide derivatives on a murine model of infection by L. amazonensis. In preliminary studies, leishmanicidal activity and cytotoxicity screening tests were used in vitro assays with pyrazole carbohydrazide derivatives. Compounds 1 (X = Br, Y = NO₂) and 2 (X = NO₂, Y = Cl) showed better leishmanicidal activity, as demonstrated by mild toxicity in mammalian cells. This study confirmed the marked leishmanicidal activity of new pyrazole carbohydrazides on L. amazonensis. Thus, these derivatives were selected for chemotherapeutic experiments in the murine model of leishmaniasis.

The CBA mice were subcutaneously infected with 1 × 10⁶ L. amazonensis promastigotes and orally treated with the compounds 1 and 2 (Figure 1) two weeks after infection for 45 days continuously. All animals had skin lesions of an erythematous papular nature by the fifth week after infection. Treated animals showed a significant reduction in the size of skin lesions (P < 0.05) during the sixth and 23rd weeks post-infection (Figure 2). Results with these new compounds were similar to those with ketoconazole. Although cutaneous lesions in treated animals were smaller than those in untreated animals, the presence of edema and erythema indicated establishment of an inflammatory process with reduced severity of lesions. None of the lesions healed completely.

To better understand the effects of compounds 1 and 2 on the spread of the parasite in the host, we analyzed parasite burden in the spleen and lymph nodes of infected animals. These compounds reduced approximately 98% of the parasite burden from popliteal lymph nodes. The reference drug ketoconazole eliminated 99% of the parasites. Thus, at the 16th week post-infection, treatment with pyrazole carbohydrazides showed a significant decrease in parasite burden compared with the untreated control (P ≤ 0.05) (Figure 3). At 16th week post-infection, parasites were not isolated from spleens of all groups of animals. Additionally, weights of the lymph node and spleen measured at the 16th week post-infection showed weights comparable to those in non-treated infected mice.
Toxicologic effects of pyrazole carbohydrazide derivatives in the murine model. During the treatment period with the two pyrazole carbohydrazide derivatives, we conducted a toxicologic evaluation of healthy animals and those infected with *L. amazonensis*. Body weight was used as an indicator of systemic toxicity. Compounds 1 or 2 did not show an effect on body weight in infected and uninfected animals (Table 1). Possible alterations of renal and hepatic functions in healthy animals or those infected with *L. amazonensis* and treated with these compounds were detected by analysis of urine creatinine and hepatic aminotransferase levels. No significant increase in plasma ALT and AST levels was observed in non-infected and infected mice (Table 1) after 42 days of treatment compared with the levels in healthy animals that did not receive treatment. When compared with control animals, non-infected animals and those infected with *L. amazonensis* did not show any changes in urine creatinine levels 14 and 42 days after treatment (Table 1).

Potential hematologic changes were also used as a measure of toxicity. During the period of infection with *L. amazonensis*, there was a moderate variation in the leukocyte count of total peripheral blood. Mice treated with the compound 1 showed a slight decrease in total number of leukocytes during the second week of treatment. However, there was no significant change in leukocyte counts in treated animals relative to those in untreated animals in the absence of infection. At the end of treatment with both compounds, we observed a decrease in neutrophils in mice infected with *L. amazonensis* compared with infected animals that were not treated (Figure 4).

Effect of pyrazole carbohydrazide derivatives on production of nitric oxide. Compounds 1 and 2 were further examined for their effects on generation of nitric oxide by spleen and lymph node cells of non-infected mice after 42 days of treatment. Administration of compound 1 clearly increased production of nitric oxide 24 hours after cell culture in lymph node cells, but not in spleen cells. In addition, treatment with compound 2 did affect production of nitric oxide 24 hours after cell culture in lymph node cells or spleen cells. There was no significant difference in production of nitric oxide after 48 hours of lymph node and spleen cell culture of cells from mice treated with both compounds. Treatment with compounds 1 or 2 induced production of nitric oxide 48 hours after cell culture in lymph node cells, but not in spleen cells, from animals at 16 weeks post-infection (Figure 5).

**DISCUSSION**

Our data highlight new insights for therapy of murine experimental cutaneous leishmaniasis and make use of oral administration of two independent pyrazole carbohydrazides derivatives. Results verify their capacity to control cutaneous lesion evolution in CBA mice infected with *L. amazonensis*.

In an earlier report, we evaluated the *in vitro* leishmanicidal activity and cytotoxic effect of these compounds. This study showed that these compounds had strong activities against promastigotes of *L. amazonensis* and lower activities against those of *L. braziliensis* and *L. chagasi*. Additionally, the *in vitro* assay with murine peritoneal macrophage showed a low cytotoxic effect of these compounds. When compared with reference drugs such as pentamidine or ketoconazole, *in vitro* results showed that these reference drugs were more toxic to cells than pyrazole carbohydrazide derivatives.

In this study, CBA mice infected with *L. amazonensis* and orally treated with pyrazole carbohydrazide derivatives (Figure 1) controlled development of skin lesions. The CBA mice were chosen because they are susceptible to infection with *L. amazonensis* and develop cutaneous lesions. In a previous anti-inflammatory assay with acylhydrazone compounds. In our study, treatment with these compounds was started in the second week after *L. amazonensis* infection and continued for 45 days without interruption. Effects of treatment on progression of skin injury were observed between the sixth and 23rd weeks after infection (Figure 2). The dosing scheme of compounds used in this investigation was lower than that used with ketoconazole. Thus, it may be possible to increase the dose of pyrazole carbohydrazides to induce a better therapeutic effect without risks to the animals because these compounds showed less cytotoxicity when tested in murine macrophages.

A key goal in pharmaceutical development is a good understanding of *in vitro* and *in vivo* performance. In a previous *in vitro* report, positions of substituents on compound 1...
(Figure 1) showed that $X = \text{Br, Y = NO}_2$ was more leishmanicidal than that observed for molecule 2 ($X = \text{NO}_2, Y = \text{Cl}$). However, in our in vivo experiment with the new pyrazole carbohydrazides, compounds 1 and 2 showed similar inhibition of the progression of cutaneous lesions in CBA mice infected with L. amazonensis. Thus, in the in vivo system, it appeared that different positions of substituents on molecules 1 or 2 did not interfere with therapeutic effectiveness.

Currently, the recommended drugs for the treatment of leishmaniasis are administrated parenterally, which complicates their use. Solubilization of pyrazole carbohydrazides is a prerequisite for drug absorption and in vivo effectiveness. Oral treatment with pyrazole carbohydrazide derivatives showed apparently good absorption in the gastrointestinal tract because these compounds effected the evolution of lesions. Recently, miltefosine, an orally used drug that has shown parasite resistance and teratogenicity,19 has been used for treatment of cutaneous20 and visceral leishmaniasis.21 Moreover, ketoconazole has been used as an alternative oral treatment for cutaneous leishmaniasis, and has shown effectiveness comparable to that of pentavalent antimonials.22,23 Ketoconazole treatment outcome could be influenced by Leishmania species.24 Azoles can inhibit a key enzyme of sterol synthesis.25 However, the mechanism of action of pyrazole carbohydrazide is not known.

Parasite burden was investigated in CBA mice infected and treated with these compounds. In draining lymph nodes, parasite burden showed a consistent decrease in both groups of treated mice. Oral administration of compounds 1 and 2 was effective in reducing the number of parasites (98%). Both compounds were as effective as ketoconazole in reducing parasite burden. In draining lymph nodes (Figure 3), parasite burden was significantly higher in non-treated mice 16 weeks post-infection. Furthermore, BALB/c mice treated with indomethacin, an inhibitor of prostaglandin E2 synthesis, developed smaller cutaneous lesions and lower parasitic burdens compared with the control group.26 However, other studies showed that footpad thickness does not always reflect parasite burden and may be influenced by leukocyte infiltration at the infection site.27,28 The anti-inflammatory effects on evolution of skin lesions and anti-Leishmania activity of pyrazole carbohydrazide derivatives should be investigated.

CBA mice infected with L. amazonensis had an increase in levels of neutrophils in peripheral blood, which was not observed when animals were infected and treated with pyrazole carbohydrazide derivatives. Levels in treated animals were similar to those in healthy animals. Studies with pyrazole carbohydrazide derivatives have shown several biological activities for these compounds.29,30 Some of these pharmacologic properties may be caused by the hydrazone group in this molecule, which determines analgesic, anti-inflammatory, and anti-thrombotic activities.10,31,32 Moreover, pyrazole carbohydrazide derivatives also have the pyrazole group and show a variety of pharmacologic properties. In experimental studies, the pyrazoles have shown antimicrobial,33,34 antiviral,35 and antitumor activities.36,37 The pyrazole ring present in some

### Table 1

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>AST (U/mL)</th>
<th>ALT (U/mL)</th>
<th>Creatinine (mg/dL)</th>
<th>Body weight (g)</th>
<th>Leukocytes, $\times 10^3$/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>145 ± 26</td>
<td>100 ± 11</td>
<td>3.2 ± 0.8</td>
<td>25.2 ± 2.1</td>
<td>9.3 ± 2.02</td>
</tr>
<tr>
<td>Compound 1</td>
<td>100 ± 24</td>
<td>145 ± 42</td>
<td>2.58 ± 0.55</td>
<td>25.2 ± 1.5</td>
<td>12.5 ± 2.3</td>
</tr>
<tr>
<td>Compound 2</td>
<td>133 ± 32</td>
<td>80 ± 36</td>
<td>2.86 ± 0.6</td>
<td>25.9 ± 0.7</td>
<td>10.5 ± 1.6</td>
</tr>
<tr>
<td>Non-infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>105 ± 30</td>
<td>114 ± 16</td>
<td>3.4 ± 0.9</td>
<td>25.58 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>Compound 1</td>
<td>110.5 ± 21</td>
<td>82 ± 10</td>
<td>2.7 ± 1.4</td>
<td>26.6 ± 2.8</td>
<td>ND</td>
</tr>
<tr>
<td>Compound 2</td>
<td>106.66 ± 24</td>
<td>78 ± 46</td>
<td>3.4 ± 0.5</td>
<td>27 ± 1.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

* All values correspond to the 42nd day of treatment. AST = aspartate aminotransferase; ALT = alanine aminotransferase; ND = not determined.

* $P < 0.05$. 

**Figure 4.** Percentage of specific blood leukocyte populations from CBA mice infected with Leishmania amazonensis and treated with pyrazole carbohydrazides for 45 days. All data correspond to the 42nd day of treatment (n = 8 per group). Cells were counted in by microscopy (1,000×) after staining with May-Grünwald reagent. Significance levels comparing treatment compounds and infected mice are indicated. * $P \leq 0.05$.

**Figure 5.** Nitric oxide production in mice 16 weeks post-infection with Leishmania amazonensis and treated with pyrazole carbohydrazide derivatives. Nitric oxide was measured in supernatants from unstimulated lymph node cells after culture for 48 hours (n = 8 per group). Significance levels comparing treatment compounds and infected mice are indicated, * $P \leq 0.05$. 

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anti-inflammatory drugs could be responsible for selective inhibition of the cyclooxygenase 2. The proposed mechanism of action for the biological effects of pyrazole carboxyhydrazide derivatives may be inhibition of the enzyme 5-lipoxygenase. This enzyme is involved in the arachidonic acid pathway that induces leukotrienes formation. Leukotrienes have several functions, including chemotaxis for neutrophils. Therefore, in this study, the decrease in neutrophils in peripheral blood of treated animals may be caused by inhibition of 5-lipoxygenase.

During evaluation of new synthetic compounds against experimental leishmaniasis in the murine model, it is necessary to evaluate possible toxic effects. These data will enable study and application of the new drugs in the future. The obvious question was to evaluate toxicity in treated animals. This evaluation was performed by assessing body weight, leukocyte counts, hepatic enzymes, and urine creatinine. Systemic toxicity is defined as a reduction in body weight in experimental animals. In addition to reduction of body weight, systemic toxicity could be manifested through alterations of organ weights. In the present study, there was no alteration in body weights and spleen weights. The effect of the test compounds on leukocyte counts, hepatic enzymes, and urine creatinine was assessed by blood and serum analysis. No alterations in levels of ALT and AST were found. These enzymes have been extensively used as markers for toxicologic study of hepatic functions.

Production of nitric oxide by macrophages is essential for controlling growth of L. major or L. amazonensis. In treated animals, production of nitric oxide was induced by compound 1 when measured 24 hours after culture of unstimulated lymph node cells. However, when treated animals were infected with L. amazonensis, compounds 1 and 2 induced production of nitric oxide in the lymph node cell culture when measured after 48 hours (Figure 5). This result may be an important factor in controlling infection. In the present study, cells from CBA mice treated with pyrazole carboxyhydrazide derivatives and infected with L. amazonensis produced more nitric oxide than did cells from untreated susceptible mice.

Conflicting studies have reported a correlation of the capacity of CBA mice infected with L. amazonensis to induce production of nitric oxide with resistance or susceptibility to L. amazonensis. Additionally, when nitric oxide was used as an inhibitor of nitric oxide synthase in L. major-infected mice, a considerable increase in parasite burden and development of larger skin lesions was observed, which corroborated the importance of this mechanism in regulating parasite growth in vivo.

Treatment of CBA mice infected with L. amazonensis with pyrazole carboxyhydrazide derivatives controlled progression of cutaneous lesions and dissemination of parasite in draining lymph nodes. No toxicity was observed (no variations in AST, ALT, creatinine, or body weight) in treated mice. Thus, the therapeutic effect of these compounds may be comparable with that of ketoconazole. Another feature observed was the capacity to decrease the number of neutrophils in both groups treated with compounds than in infected controls. It has often been suggested that these compounds, in addition to showing leishmanicidal activity reflected in reductions in parasitic burden, may also control evolution of the inflammatory process.

In conclusion, further experiments on dosage optimization and mechanism of action will be carried out. These experiments will involve possible anti-inflammatory effects and anti-Leishmania activity to determine an adequate dosing regimen for therapeutic use.

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Authors’ addresses: Karen S. Charret, Raquel F. Rodrigues, Marilene M. Canto-Cavalcheiro, and Leonor Leon, Laboratório de Bioquímica de Tripanosomatideos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil, 4.365 pav. 26 sl. 405, Manguinhos, CEP 21045-900, Rio de Janeiro, RJ, Brazil. Alice M. R. Bernardino and Adriana O. Gomes, Instituto de Química, Departamento de Química Orgânica, Universidade Federal Fluminense, Programa de Pós-graduação em Química Orgânica, Outeiro de São João Baptist, CEP 24020-150, Niterói, RJ, Brazil. Adriana V. Carvalho and Verónica F. Amaral, Instituto Biologia, Departamento de Imunobiologia, Universidade Federal Fluminense, Outeiro de São João Baptist, Valonguinho, CEP 24020-150, Niterói, RJ, Brazil.

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