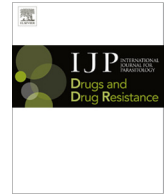




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Brief Report

Oral effectiveness of PMIC4, a novel hydroxyethylpiperazine analogue, in *Leishmania amazonensis*



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ABSTRACT

Pentavalent antimonials have saved the lives of thousands of *Leishmania*-infected patients more than seventy years but, unfortunately, they are highly toxic and require parenteral delivery. Therefore, the search for safer and orally delivered alternative is a need. This paper describes the antileishmanial properties of PMIC4, a novel hydroxyethylpiperazine analogue. PMIC4 showed potent activity against intracellular amastigotes of *Leishmania amazonensis*, with IC₅₀ of 1.8 μM and selectivity index higher than 100-fold, calculated in relation to the toxicity on the host cell. Following laboratory animal welfare policies, we analyzed the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties and calculated the Lipinski's rule of five of PMIC4 before proceeding to in vivo tests. PMIC4 satisfied Lipinski's rule of five and presented high probability of human intestinal absorption, suggesting a good chance of drug-likeness and oral bioavailability. For in vivo studies, PMIC4 was administered via intralesional injection (3.4 mg/kg/day, three times a week) or orally (34.0 mg/kg/day, five times a week) to *L. amazonensis*-infected BALB/c mice throughout the 98 day experiments. At the end of the treatment period, serum markers of toxicity were measured. When administered orally, PMIC4 controlled the lesions in *L. amazonensis*-infected BALB/c mice without altering serological markers of toxicity. These results demonstrate that PMIC4 is a promising molecular scaffold, orally effective against experimental leishmaniasis.

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1. Introduction

Leishmaniasis affects 2 million people each year in 98 countries or territories (Alvar et al., 2012). Although pentavalent antimonials have saved the lives of thousands of *Leishmania*-infected patients, they are expensive, require long-term parenteral administration and have serious side effects (Amato et al., 2008). Miltefosine, a new orally active compound, appears to be less active against New World leishmaniasis than it is in India (Dorlo et al., 2012). Therefore, the search for new treatment alternatives is a priority. Hydroxyethylamines are pharmacophore moieties present in a variety of biological active compounds, such as β-secretase inhibitors (Mondal et al., 2013), antimalarials (Jaudzems et al., 2014) and

anti-HIV (Bhattacharya et al., 2012). In recent years, several reports have described the antileishmanial activity of the hydroxyethylamines used in the highly active antiretroviral therapy (HAART) (Savoia et al., 2005; Santos et al., 2009, 2013b; Griensven et al., 2013). In a previous study, we described the synthesis and demonstrated the antimalarial activity of a new series of hydroxyethylpiperazines, analogues to HIV protease inhibitors (Cunico et al., 2009). Here, we show our results regarding the antileishmanial activity of PMIC4, the most promising hit from this series.

2. Materials and methods

2.1. Chemicals

The hydroxyethylpiperazine was synthesised in the Laboratory of Chemical Synthesis, FIOCRUZ. Meglumine antimoniate (Glucantime, Sanofi-Aventis) was a gift from the Instituto de Pesquisas Evandro Chagas, FIOCRUZ.

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2.2. Parasites

Promastigotes of *Leishmania amazonensis* (MHOM/BR/77/LTB0016) were maintained at 26 °C in Schneider's insect medium (Sigma–Aldrich, St Louis, MO, USA) with 10% serum, 100 µg/mL streptomycin and 100 U/mL penicillin until the 10th passage.

2.3. Antipromastigote activity

L. amazonensis promastigotes (1×10^6 /mL) were incubated with PMIC4 (up to 200 µM) for 72 h at 26 °C in Schneider's medium plus 10% serum in 96-well plates. Parasite viability was evaluated by adding 22 µL of MTT (5 mg/mL). After 2 h, 80 µL of DMSO was added to each well, and the optical density was measured at 570 nm.

2.4. Anti-amastigote activity

The infection of macrophages was performed as previously described (da Cunha-Júnior et al., 2011). Briefly, 2×10^6 /mL BALB/c peritoneal macrophages were infected with *L. amazonensis* promastigotes at a 3:1 parasite/macrophage ratio in Lab-Tek chambers (Nunc, Rochester, NY, USA). After 3 h of infection, PMIC4 in RPMI medium (Sigma–Aldrich) was added for further 72 h. The anti-amastigote activity was evaluated by counting at least 200 macrophages per sample under a microscope. The 50% inhibitory concentration (IC₅₀) was determined by logarithmic regression in GraphPad Prism.

2.5. In vivo assays

BALB/c mice (5/group) were infected in the footpad with 2×10^6 *L. amazonensis* promastigotes and the treatment began 72 h after the infection. The animals were treated subcutaneously with 3.4 mg/kg PMIC4 diluted in PBS three days a week, orally through an orogastric tube with a suspension of 34.0 mg/kg PMIC4 diluted in PBS and 2% DMSO five days a week, or intraperitoneally with 17 mg Sb⁵⁺/kg/day of meglumine antimoniate five days a week; control mice remained untreated. The lesions were measured using a dial calliper every 3–4 days. At the end of the experiment (day 98), the animals were euthanised, and serum was collected for biochemical analysis. The data were analysed by two-way ANOVA with the Bonferroni post-test.

2.6. Ethics statement

Studies in *L. amazonensis*-infected BALB/c mice were performed in accordance with protocols approved by the Ethics Committee for Animal Use of the FIOCRUZ (LW07/2010).

3. Results and discussion

3.1. Selective antileishmanial activity of PMIC4

From a series of eight hydroxyethylpiperazines evaluated for antipromastigote activity, PMIC4 was the most potent, with IC₅₀ of 23.2 µM. We determined that PMIC4 has activity against intracellular amastigotes without affecting the host cells, with an IC₅₀ of 1.8 µM. Although comparisons are complicated by different methodologies, these results suggested that PMIC4 is more potent than the HIV protease inhibitors that have already been tested against *Leishmania*, as reviewed by Santos (Santos et al., 2013a). Uninfected macrophages remained unaffected by PMIC4 up to 300 µM, indicating a selectivity greater than 100-fold higher than the IC₅₀ on amastigotes.

3.2. In silico analysis

Before proceeding to in vivo assays, we performed some theoretical analysis of the druglikeness of PMIC4. The absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of PMIC4 were evaluated using the admetSAR tool (Cheng et al., 2012), and Lipinski's rule of five was calculated using Advanced Chemistry Development (ACD/Labs) Software V 11.02 (copyright 1994–2012 ACD/Labs). PMIC4 has seven hydrogen bond acceptors and two donors, molecular weight of 469.6 and logarithm of partition coefficient between n-octanol and water of 4.01, fulfilling the Lipinski rule of five (Table 1). The calculated ADMET properties indicated a good probability of PMIC4 be safe and orally absorbed (Table 1). We found that PMIC4 is predicted as a class III risk for acute toxicity, i.e., compounds with LD₅₀ greater than 500 mg/kg. The simulation also indicated that PMIC4 is not likely to act as inhibitor of CYP3A4, unlike most HIV protease inhibitors.

3.3. In vivo activity

Considering the in vitro and in silico results, we evaluated the activity of PMIC4 in a murine model of cutaneous leishmaniasis. Indeed, PMIC4 delivered orally was as effective as subcutaneously, and was more effective than pentavalent antimonial in controlling lesion development in mice (Fig. 1a). The observed therapeutic effect was similar to that previously reported with indinavir and ritonavir in *L. amazonensis*-infected BALB/c mice (Demarchi et al., 2012). No apparent signs of toxicity were observed, and there were no significant differences in serological markers of toxicity between experimental and control animals (Fig. 1b).

3.4. Conclusion

The selective activity in vitro, the theoretical predictions of druglikeness and the in vivo activity by oral administration suggested PMIC4 as a good scaffold for further studies aimed at developing a drug candidate for treating leishmaniasis.

Table 1
In silico analysis of druglikeness of PMIC4.

| Lipinski molecular descriptors | Result | Probability (%) |
|--------------------------------|-------------|-----------------|
| NHBA (≤ 10) | 7 | |
| NHBD (≤ 5) | 2 | |
| clogP (≤ 5) | 4.01 ± 0.69 | |
| MW (≤ 500) | 469.6 | |
| Absorption | | |
| Blood–brain barrier | – | 94.08 |
| Human intestinal absorption | + | 62.35 |
| Caco-2 | – | 70.92 |
| Metabolism | | |
| CYP450 2C9 substrate | NS | 81.15 |
| CYP450 2D6 substrate | NS | 72.71 |
| CYP450 3A4 substrate | S | 72.37 |
| CYP450 1A2 inhibitor | NI | 92.16 |
| CYP450 2C9 inhibitor | NI | 84.45 |
| CYP450 2D6 inhibitor | NI | 77.12 |
| CYP450 2C19 inhibitor | NI | 81.25 |
| CYP450 3A4 inhibitor | NI | 90.15 |
| Toxicity | | |
| AMES toxicity | – | 85.18 |
| Carcinogens | – | 92.12 |
| Acute oral toxicity | III | 62.31 |

I, inhibitor; NI, noninhibitor; NS, nonsubstrate; NHBA, number of hydrogen bond acceptors; NHBD, number of hydrogen bond donors; clogP, logarithm of compound partition coefficient between n-octanol and water; MW, molecular weight.

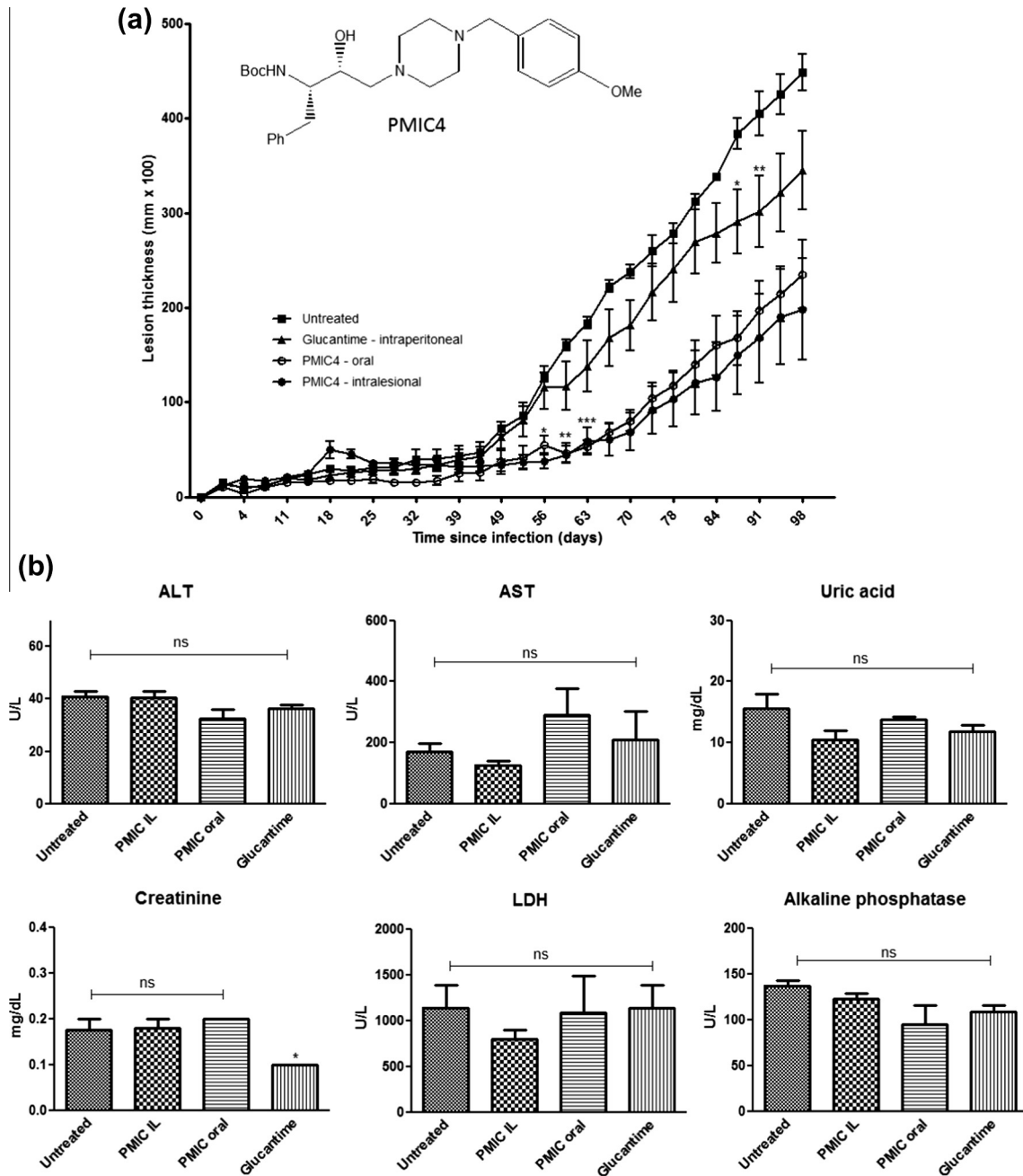


Fig. 1. PMIC4 was effective in vivo, delivered either locally or orally, without altering serological markers of toxicity. (a – insert) Schematic of the PMIC4 structure. (a) BALB/c mice were infected in the footpad with *L. amazonensis*. The animals were treated subcutaneously in the infected footpad with 3.4 mg/kg PMIC4 three days a week. The oral treatment consisted of 34.0 mg/kg PMIC4 five days a week. Control animals received either intraperitoneal injections of meglumine antimoniate (17 mg/kg Sb⁵⁺) five days a week or were untreated. The data are presented as the mean \pm standard error of 5 animals per group. * p <0.05, ** p <0.01 and *** p <0.001. Control versus Glucantime, day 91, ** p <0.01. Control versus PMIC4 oral and intralesional PMIC4, day 63, *** p <0.001. Two-way ANOVA with the Bonferroni post-test. (b) At the end of the treatment period, serum samples were collected, and serum toxicological markers were analysed. ns, not statistically significant; * p <0.05.

4. Conflict of interest

The authors declared that there is no conflict of interest.

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