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An Effective Diaryl Derivative Against *Leishmania amazonensis* and its Influence on the Parasite X Macrophage Interaction

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The activity of several diarylheptanoid derivatives (curcuminoids) was previously evaluated against *Leishmania amazonensis* promastigotes and among them the most active compound was 5-hydroxy-7- (4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one. This study was carried out to investigate the influence of this diaryl derivative on the infective promastigotes and Balb/c mice peritoneal macrophage interaction. The potential *in vitro* toxicity was also evaluated. Promastigotes pretreated for 24 hours with the compound had their infective capacity significantly decreased. When the infection of Balb/c macrophage by *L. amazonensis* promastigotes was already installed, addition of the drug resulted in a diminishing of the infection rate. It was demonstrated that the compound was not toxic to the host macrophage in a concentration equivalent to the LD_{50}/24 h from the previous *in vitro* experiment.

Keywords: *Leishmania amazonensis*; Diaryl derivative; Curcuminoids; Infection; Inhibition

INTRODUCTION

Members of the genus *Leishmania* differentiate from proliferative promastigotes in the sandfly vector gut to infective metacyclic promastigotes. Parasites are inoculated by the vector as the flagellate metacyclic promastigotes into the mammalian host, where they infect macrophages differentiating to non-motile amastigotes and multiplying as such. *Leishmania* parasites cause a disease, which has been associated with different clinical forms, including cutaneous, hyperergic mucocutaneous, anergic diffuse cutaneous and visceral leishmaniasis.[1,2] The disease is endemic in some geographical areas of Brazil, where it constitutes a serious health problem. *L. amazonensis* has been isolated from patients with all the different clinical forms of the disease.[3–5] The lack of an effective and non-toxic anti-leishmanial drug has led an interest in the search for the development of new chemotherapeutical compounds with better activity and less toxic effects. We have been studying compounds belonging to the class of diarylheptanoids, which were derived from curcumin (diferuloyl methane), through several chemical modifications in the original structure, in order to potentially increase its activity against *Leishmania* sp.[6–8] The most effective derivative *in vitro* was chosen to be assayed for its influence on the parasite × macrophage interaction. In addition, we evaluated the toxicity of the compounds on the mice peritoneal macrophage.

MATERIALS AND METHODS

Parasite

L. amazonensis (MHOM/BR/77/LTB0016 strain) promastigotes were grown at 26°C in Schneider's medium (pH7.2) supplemented with 10% (v/v) of heat inactivated fetal calf serum (HIFCS), 100IU penicillin, 100 μg/mL streptomycin and 1mmol/L L-glutamine. To maintain infectivity of the parasite, Balb/c mice (highly susceptible to cutaneous infection by *L. amazonensis)* were inoculated every three months.

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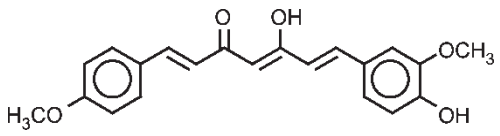

FIGURE 1 Chemical structure of the diaryl derivative 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one.

Cytotoxicity Assay

Murine resident peritoneal macrophages obtained from male (6–8 weeks old) BALB/c mice were collected in cold serum-free RPMI 1640 medium, seeded onto 12 mm glass cover slips (5×10^5 cells/well) and incubated for 2 h at 37°C in an atmosphere containing 5% CO_2. Non-adherent cells were removed by washing and RPMI 1640 with 1% of heat inactivated fetal calf serum (HIFCS) was added. 5-Hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one was added to the macrophage culture in a concentration corresponding to LD_{50}/24 h (514 nM) from the *in vitro* screening assay.[9,10] The cytotoxicity evaluation was conducted at 24 and 48 h. A macrophage culture with no drug added was used as a control. All experiments were done in triplicate. This project was submitted to and authorized by the Animal Ethics Commission of this Institution (CEUA-FIOCRUZ, Rio de Janeiro, Brazil).[11]

Interaction Parasite Macrophage

Using the same protocol as above, and to evaluate the effect of the diaryl derivative on the interaction of parasite × macrophage, an infection was performed for 24 and 48 h in the same medium, at a ratio of 10 parasites per macrophage. Experiments were conducted in triplicate as follows: 1) macrophage plus parasites, without drugs (infection control); 2) macrophage plus drugs, parasites were added 24 h later; 3) macrophages plus parasites, drugs were added 24 h later; 4) parasites treated for 24 h at 26°C were added to macrophages. The number of intracellular parasites/100 macrophages was monitored at 0, 24 and 48 h of culture.

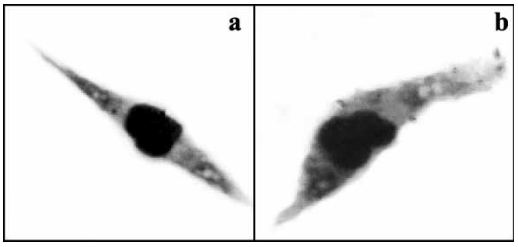

FIGURE 2 Evaluation of the cytotoxicity of the diaryl derivative 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one, where a) macrophage without parasite; b) macrophage treated with the drug.

RESULTS

In the present work, the effect was evaluated of a diaryl derivative 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one (Figure 1) on the interaction of parasite × macrophage and its toxicity to the host cell. It was observed for 24 and 48 hours that the compound caused no significant alteration on treated macrophages (Figure 2a/2b) in a concentration as higher as the LD_{50}/24 h from the previous *in vitro* screening experiments. This evaluation was realized through optical microscopy, using Trypan blue as a vital dye, when it was observed that the compound was not toxic to the host cell.

Our data showed that when parasites were previously treated (26°C/24 h) with the compound, most of the parasites were found surrounding the macrophage. It was also observed that few pretreated promastigotes were able to enter the phagocytic cell (Figure 3a). However, when macrophages were primarily treated with the compound, and later non-treated parasites were added to promote infection, a slightly difference was noted in comparison to the control group (macrophage and parasites), since treated macrophages were less infected (24%/48 h) (Figure 3c) in comparison to the infection control (53%/48 h) (Figure 3d).

Furthermore, the assayed compound was able to promote the clearance of amastigotes from macrophages, when added 24 h after the establishment of the infection. The addition of the diaryl derivative resulted in 6% of macrophages infected at 48 h (Figure 3b), in contrast with the control infection,

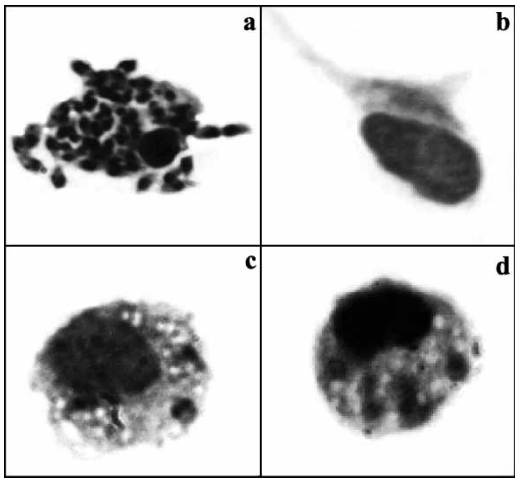

FIGURE 3 Evaluation of the effect of the diaryl derivative 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one on parasite × macrophage interaction, where: a) parasite treated with the drug 24 h before infection; b) macrophage infected with parasite and 24 h after infection was installed, the whole system was treated with the drug; c) macrophage treated with the drug, 24 h before infection; d) macrophage and parasite (control).

which presented at the same time 53% of macrophages infected (Figure 3d).

DISCUSSION

Studies on the efficacy of several drugs used against the different clinical forms of leishmaniasis have been performed in the last years. The search for new drugs for the disease requires the study of toxicity and recently, numerous *in vitro* assays using cultured mammalian cells have been developed, as an alternative to the use of laboratory animals.

Our *in vitro* toxicity assay showed that the diaryl derivative 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-1,4,6-heptatrien-3-one caused no significant alteration on treated murine peritoneal macrophages. This is important data, since a drug expected to be effective against parasites in the clinic should not be hazardous to the host cell.

A pre-requisite for the beginning of infection is the attachment of the parasite to the macrophage membrane, which occurs through a ligand-receptor mechanism. Our data showed that when parasites were previously treated with the compound, apparently their infective capacity was altered, since most of the parasites were found surrounding the macrophage.

It is already known that the survival of the parasite within the animal host depends on its capacity to become intracellular. It was observed that few pretreated promastigotes were able to enter the phagocytic cell, which would suggest that some kind of alteration was induced on the parasite's surface by the diaryl derivative, preventing or diminishing phagocytosis. However, when macrophages were primarily treated with the compound, and later non-treated parasites were added to promote infection, it was seen that the drug was not toxic to macrophages. Some changes occurred since treated macrophages were less infected compared to the infection control.

Furthermore, the assayed compound was able to promote the clearance of amastigotes from macrophages, since the addition of the diaryl derivative resulted in a lower degree of macrophage infection. Previous data from our group demonstrated that pentamidine isethionate, [$LD_{50}/24\,h = 0.3\,mg/mL$ (0.46 mmol/L)], a drug used in the clinic for leishmaniasis, and assayed under the same conditions was unable to interrupt the macrophage infection.[11]

In summary, this study demonstrates that the curcuminoid derivative that is highly effective against Leishmania, is not dangerous to the host macrophage and is effective in order to avoid the maintenance of in vitro Leishmania infection. As complementary data, it is important to relate the high effectiveness of this compound in previous *in vivo* experiment from our laboratory.[9,10]

Acknowledgements

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