The Leishmanicidal Effect of (3S)-16,17-Didehydrofalcarkinol, an Oxylipin Isolated from Tridax procumbens, is Independent of NO Production

Zhelmy Martín-Quintal1, María del Rosario García-Miss2, Mirza Mut-Martín3, Abril Matus-Moo2, Luis W. Torres-Tapia1, and Sergio R. Peraza-Sánchez1*

1Centro de Investigación Científica de Yucatán, Calle 43 #130, Col. Chuburná de Hidalgo, Mérida, Yucatán, México 97200 (CICY)
2Centro de Investigaciones Regionales ‘Dr Hideyo Noguchi’, Universidad Autónoma de Yucatán, Av. Itzáes # 490, Mérida, Yucatán, México 97000 (CIR-UADY)

The in vitro leishmanicidal effect of (3S)-16,17-didehydrofalcarkinol (1) isolated from Tridax procumbens whole plant against Leishmania mexicana, the causative agent of cutaneous leishmaniasis (chiclero’s ulcer) in the New World, was investigated. This oxylipin showed significant in vitro activity against promastigotes and intracellular amastigotes of L. mexicana. Its inhibitory effect on amastigotes was not due to activation of NO in recombinant γ-interferon-stimulated macrophages, since the production of NO was decreased in presence of the oxylipin. This is the first report on the leishmanicidal activity against the intracellular stage (amastigote) of the oxylipin (3S)-16,17-didehydrofalcarkinol. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Tridax procumbens; leishmaniasis; (3S)-16,17-didehydrofalcarkinol; amastigotes; nitric oxide; macrophages.

INTRODUCTION

Protozoal diseases continue to cause serious public health problems in developing regions of the world. Leishmaniasis is a group of diseases caused by trypanosomatid parasites of the genus Leishmania (Reithinger et al., 2007). Most forms of leishmaniasis infections originate in small mammals (reservoir hosts), which play a major role in the epidemiology of the disease. Leishmaniasis is a world-wide disease affecting 88 countries. Overall prevalence is 12 million people and the population at risk is 350 million. Recently, there has been an increase in the rate of Leishmania infections in human immunodeficiency virus-infected patients, together with the development of drug resistance by the parasites (Desjeux, 2004). In Mexico, cutaneous leishmaniasis (CL) was recorded for the first time in southeast Mexico by Seidelin (1912), who called it ‘chiclero’s ulcer’, because it was frequently found in workers in the chewing gum industry.

The drugs recommended for the treatment of leishmaniasis include the pentavalent antimonials sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), which constitute the first-line treatment. Pentavalent antimonials are potentially toxic and often ineffective, and the second-line drugs, such as amphotericin B and pentamidine, may be even more toxic (Berman, 1997; Croft and Coombs, 2003). Thus, there is an impelling need for new leishmanicidal drugs. One of the main sources for new leishmanicidal agents are secondary metabolites isolated from plants (Kayser et al., 2003).

Peraza-Sánchez et al. (2007) reported a screening done with extracts from native plants of the Yucatan Peninsula for potential leishmanicidal activity, and the results showed the methanol extract of the whole plant Tridax procumbens L. to have significant leishmanicidal activity. Tridax L. (Asteraceae) is a Central and South American genus with the greatest concentration of species in Mexico. One weedy species, T. procumbens, has been widely introduced in the tropical and subtropical regions of the world. T. procumbens is a perennial plant 15–40 cm high; leaves ovate to lanceolate, triplinerved, serrate or undulate-dentate to coarsely toothed (Powell, 1965). From this plant, saturated and unsaturated fatty acids (Gadre and Gabhe, 1988), lipid constituents (Verma and Gupta, 1988), flavonoids (Akbar et al., 2002), and polysaccharides (Raju and Davidson, 1994) have been isolated.

In the Central American and Caribbean pharmacopeia, T. procumbens is reported to be used against anemia, cold, inflammations, and hepatopathies (Germosén-Robineau, 1995), the last properties having been confirmed by Pathak et al. (1991) and Saraf and Dixit (1991). (3S)-16,17-Didehydrofalcarkinol (1) was isolated for the first time by Bohllmann et al. (1966) from Falcaria vulgaris (Umbelliferae) and by Bernat et al. (1996) from Dendropanax arboreus. (3S)-16,17-Didehydrofalcarkinol is an oxylipin (polycycleneyle), a class of plant-oxygenated lipid involved in responses to physical damage by animals or insects, stress, and attack by pathogens (Howe and Schilimiller, 2002). Polyacycetyl-
DOI: 10.1002/ptr

LEISHMANICIDAL EFFECT OF AN OXYLIPIN FROM TRIDAX PROCUMBENS

Copyright © 2009 John Wiley & Sons, Ltd.

Lipid peroxides are known to present several biological activities, such as antibiotic, antifungal, antimicrobial, antiprotozoal, insecticidal, among others (Hansen and Boll, 1986; Christensen and Brandt, 2006); however, to date, there is only one report on (3S)-16,17-didehydrofalcarinol as having great activity against human LOX melanoma in mice (Bernart et al., 1996).

In the present report we have demonstrated that (3S)-16,17-didehydrofalcarinol is active against promastigotes and intracellular amastigotes of L. mexicana, independent of nitric oxide (NO) production. In addition, the in vitro cytotoxicity of the isolated compound was studied in bone-marrow derived macrophages (BMM).

MATERIALS AND METHODS

Plant material. Samples of T. procumbens whole plant were collected in Merida, the capital of the state of Yucatan, Mexico, in the period February–July 2004; voucher specimen was authenticated by F. May-Pat and deposited at the herbarium of CICY under the code number FMay-1955.

Extraction and isolation. The whole plant was dried (1.5 kg), powdered, and then submitted to extraction with methanol (MeOH) at room temperature. The extract was evaporated to dryness in vacuo and tested for antipromastigote activity. The MeOH extract (A1, 150 g) was dissolved in 25% aqueous MeOH and partitioned with hexane (Hx, B1, 15 g), dichloromethane (CH2Cl2, B2, 7.5 g), and ethyl acetate (EtOAc, B3, 5.6 g). The resultant fractions were then screened for antileishmanial activity. The bioactive natural product (1, Fig. 1) was purified by chromatographic methods and characterized by RMN analyses, which have been described previously (Martín-Quintal et al., 2009).

Parasites. The membrane extract, fractions, and the pure compound 1 were tested first in an in vitro bioassay against the free promastigote form of L. mexicana [strain LV4 (MNYC/BZ62/M379)] for antiparasitic activity. Then, the active compound 1 was tested against intracellular amastigotes, which are the parasite forms found in the vertebrate hosts. Extracts, fractions, and compound 1 were re-dissolved with 0.5% dimethyl sulfoxide (DMSO) and the resultant solutions used for the bioassays.

Cultures of promastigotes of L. mexicana were maintained at 26 °C in 199 medium (GIBCO, Cat. 12350-039), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin/ml, 100 μg of streptomycin/ml, 20 mM sodium pyruvate, 10 mM L-glutamine, 50 μM 2-ME, and 1 g NaHCO3/liter as described by a modification of the method described by Hocquemiller et al. (1991). In all tests, 0.5% DMSO and medium alone were used as controls. Each experiment was performed in triplicate and repeated at least twice on separate occasions. Amphotericin B was used as the standard drug at IC50 = 1 μg/ml.

Leishmanicidal effect was expressed as the concentration at which an extract, fraction, or compound induces 50% reduction in parasite proliferation (IC50). IC50 values were calculated by Probit analysis.

Animals. BALB/c male mice (CIR Dr Hideyo Noguchi, Autonomous University of Yucatan, Mexico) were used at 7 weeks of age when bioassays were initiated. Food and water were available ad libitum. All efforts were made to minimize animal discomfort according to the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Murine bone marrow-derived macrophages (BMM). Bone marrow was removed from femur bones of BALB/c mice by perfusion with RPMI-1640 culture medium. BMM (5 × 10^7/10 ml) were grown in DMEM medium (GIBCO, Cat. 11965-092), supplemented with 15% FBS and M-CSF (macrophage colony-stimulating factor), and supernatant L929, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 20 μM sodium pyruvate, and 10 mM L-glutamine, and maintained at 37 °C for six days. The non-adherent cells were removed and fresh medium without M-CSF was added and maintained for two more days at 37 °C in 5% CO2.

Antiamastigote activity. BMM were suspended by vigorous pipetting, and cell viability was determined with trypan blue. Macrophages were adjusted to the necessary concentration and incubated for 2 h at 37 °C in 5% CO2, with RPMI-1640 medium supplemented with 10% FBS.

The in vitro sensitivity of amastigotes to oxylipin 1 was determined in BMM. In order to evaluate the effect of 1 on intracellular amastigotes, 10^6 macrophages were plated onto 13-mm diameter coverslips in 24-well plates for 2 h at 37 °C in a 5% CO2 atmosphere.

L. mexicana LV4 promastigotes were centrifuged at 800 × g for 10 min at room temperature, and the pellet was resuspended with RPMI-1640 supplemented with 20% FBS, counted in a Thoma chamber and diluted to a density of 3 × 10^7 parasites/ml. Adhered macrophages were infected with promastigotes at a parasite/macrophage ratio of 30:1 and incubated for 2 h at 37 °C in a 5% CO2 chamber. Next, free promastigotes were removed by extensive washing with phosphate buffered saline (PBS). The infected macrophages were treated with oxylipin 1 at different concentrations (3, 1, 0.3, 0.1, and 0.03 μM). The cells were incubated, and after 24 h, the coverslips were washed with PBS and fixed with MeOH, dried, and stained with Giemsa (Merck, Darmstadt, Germany). Medium alone was used as control. The macrophages were also treated with 0.5% DMSO.

At least 200 cells per experiment were inspected by bright-field microscopy. The macrophages were counted to calculate the percentage of infection by the following...
formula: \% \text{ of infection} = \frac{\text{number of cells infected}}{\text{total number cells}} \times 100. \text{ The data were normalized respective to negative control (medium). Experiments were carried out in triplicate. Data were analyzed by one-way ANOVA for more than two groups, and, when significant, by Dunnet’s post hoc test for comparison with the negative control (culture medium), using the GraphPad Prism 5 Program. P values of less than 0.05 were considered significant.}

**Cytotoxicity to BMM.** Colorimetric assay of cell survival was done by the method of Mosmann (1983). The percentage of viable cell was determined by the colorimetric method using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide]. Cytotoxicity was evaluated by exposing BMM to different concentrations (10, 3, 1, 0.3, 0.1, and 0.03 \mu M) of oxylipin \textbf{I}. The cells were seeded at 10^6/ml in 96-well microplates with different concentrations of \textbf{I}, previously dissolved in DMSO and diluted in medium. After incubating for 48 h at 37°C in 5% CO_2, MTT (5 mg/mL) was added to the plate and incubated for 4 h at 37°C. The enzymatic reaction was then stopped adding 100 \mu L of 20% SDS (water-insoluble formazan crystals were dissolved), and absorbance was read at 540 nm. The cytotoxicity of \textbf{I} was calculated by the following equation: 100 – (OD treatment/OD control) \times 100. Controls containing only non-treated BMM were also included. Each experiment was performed five times in triplicate. Data were analyzed by one-way ANOVA for more than two groups, using the GraphPad Prism 5 Program. P values of less than 0.05 were considered significant.

**Nitric oxide production.** BMM were plated and incubated at 10^3 cells/well in 24-well plates with five concentrations of oxylipin \textbf{I} either in the presence or absence of 10 U/ml \gamma-IFN. Alternatively, macrophages were incubated with different concentrations of \textbf{I} in addition to the activator \gamma-IFN. Nitrite concentrations in 48 h culture supernatants were determined by the Griess method. The reaction was read at 540 nm, and the concentration of NO_2^- was determined with reference to a standard curve using sodium nitrite. Results are expressed as micromolar concentrations of nitrite. Experiments were carried out in triplicate. Data were analyzed by Student’s t-test when comparing two groups, using the GraphPad Prism 5 Program. P values of less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

The MeOH crude extract (\textbf{A1}) obtained from \textit{T. procumbens} whole plant was tested in the antileishmanial assay. This extract inhibited promastigotes growth of \textit{L. mexicana} LV4 with a 50% inhibitory concentration (IC_{50}) of 16.52 \mu g/mL. Partitioning of \textbf{A1} with organic solvents yielded a hexane extract, which showed a significant antileishmanial effect, better than the MeOH crude extract, with an IC_{50} = 9.42 \mu g/mL. Antipromastigote activity-guided purification of this extract led to the isolation of compound \textbf{I}.

Compound \textbf{I} exhibited the highest relative toxicity for promastigotes of \textit{L. mexicana} LV4, having an IC_{50} = 0.55 \mu M, compared to the leishmanicidal effect of amphotericin B with an IC_{50} = 1.08 \mu M. In the mammalian host, promastigotes transform into amastigotes after invading macrophages. Therefore, amastigotes represent the parasite form responsible for the disease. Treatment with \textbf{I} of macrophages infected with the amastigote forms showed that \textbf{I} influenced the growth of the parasites. The effect of \textbf{I} on amastigotes-infected macrophages incubated 24 h are shown in Fig. 2. Inhibition of infected macrophages using 3 \mu M of \textbf{I} and incubated 24 h was 60%. Thus, \textbf{I} showed good activity against amastigote forms, with an IC_{50} = 0.48 \mu M after 24 h. Cell toxicity was not observed after treating BMM with 3 \mu M of \textbf{I}, the same concentration that induced a potent leishmanicidal effect (data not included). The highest concentration tested of \textbf{I} (10 \mu M) caused cytotoxicity to BMM. Although the inhibition of infected macrophages by amphotericin B at 0.8 \mu M is higher than that of \textbf{I}, it is known that amphotericin B is more cytotoxic. However, when comparing amphotericin B at 0.27 \mu M with \textbf{I} at the leishmanicidal concentrations, differences are not observed (Fig. 2).

(3S)-16,17-Didehydrofalcarkinol has shown to have great activity against human LOX melanoma in mice (Bernart et al., 1996). NO plays an important role in the regulation of tumor evolution. Because of its toxicity to cancer cells, NO inhibits tumor growth (Wink et al., 1998; Gauthier et al., 2004). Therefore the activity of this compound could be due to the activation of NO. It appears that high levels of NO (for example, generated by activated macrophages) may be cytostatic or cytotoxic for tumor cells. When macrophages are activated, they become competent to lyse tumor cells and to kill several pathogens.

In general, a drug may act directly against the parasite or indirectly by activating macrophage mitochondrial mechanism, such as nitric oxide (NO) production, which

**Figure 2.** Effect of oxylipin \textbf{I} and amphotericin B against intracellular amastigotes. Macrophages were infected with promastigotes of \textit{L. mexicana} LV4, treated with different concentrations of \textbf{I}, and then incubated 24 h. Percentage of infected macrophages was calculated. Data are means ± standard error (SE) (n = 3). Asterisks indicate significant differences between control (medium) and treatment (*P < 0.05). Significant differences were not observed among concentrations 0.2, 1.0 and 2.0 \mu M (P > 0.05). Analyzed by Tukey post hoc.

**Inhibition of infected macrophages (%)**

<table>
<thead>
<tr>
<th>Oxylin</th>
<th>Amphotericin B (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Copyright © 2009 John Wiley & Sons, Ltd.


DOI: 10.1002/ptr
has been shown to be the most effective antileishmanial mechanism. NO is the principal effector molecule mediating intracellular killing of *Leishmania* both in *vitro* and *in vivo* (Assreuy *et al*., 1994; Holzmuller *et al*., 2006). In the literature, there are several secondary metabolites that stimulate NO production in macrophages (Delorenzi *et al*., 2001; Ueda-Nakamura *et al*., 2006).

To determine whether the inhibition of intracellular parasite growth was due to a general activation of macrophage microbicidal mechanisms, we measured NO production. However, when macrophages were incubated for 48 h in the presence of increasing concentrations of 1, a steady decrease in NO production was observed (Fig. 3). The capacity of macrophages to produce NO was reduced by 1. In fact, 1 was able to diminish NO production induced by stimulus caused by the presence of γ-IFN in BMM. Similar results were observed for the polyacetylenes falcarinol and falcarindiol isolated from the roots of *Angelica furcijuga*, which substantially inhibited NO production in mouse peritoneal macrophages (Yoshikawa *et al*., 2006). This result suggested that the antiamastigote activity of 1 is not due to activation of this leishmanicidal mechanism evolved by the macrophages, but may be due to the direct effect of 1 on the parasites. In this study, we report the biological activity of the metabolite (3S)-16,17-didehydrofalcarinol (1), isolated from *T. procumbens*, on the amastigote form of *L. mexicana*, with no effect upon mammalian cells, coinciding this result with those obtained against two cellular lines (MDCK and KB) and peripheral blood mononuclear human cells (PBMC) in which no cytotoxic activity was observed (Martín-Quintal *et al*., 2009). This effect represents an important advance in the search for novel antileishmanial agents from natural products, since a significant activity against the intracellular stage (amastigote) of the protozoo was demonstrated.

**Acknowledgement**

This work was supported by FOMIX-Yucatan, Mexico (Grant No. 66262).

**REFERENCES**


