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Psilostachyin C: a natural compound with trypanocidal activity

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ABSTRACT

In this study, the antiprotozoal activity of the sesquiterpene lactone psilostachyin C was investigated. This natural compound was isolated from *Ambrosia scabra* by bioassay-guided fractionation and was identified by spectroscopic techniques. Psilostachyin C exerted in vitro trypanocidal activity against *Trypanosoma cruzi* epimastigotes, trypomastigotes and amastigotes, with 50% inhibitory concentration (IC_{50}) values of 0.6, 3.5 and 0.9 μg/mL, respectively, and displayed less cytotoxicity against mammalian cells, with a 50% cytotoxic concentration (CC_{50}) of 87.5 μg/mL. Interestingly, this compound induced ultrastructural alterations, as seen by transmission electron microscopy, in which vacuolisation and a structural appearance resembling multivesicular bodies were observed even at a concentration as low as 0.2 μg/mL. In an in vivo assay, a significant reduction in the number of circulating parasites was found in *T. cruzi*-infected mice treated with psilostachyin C for 5 days compared with untreated mice ($7.4 \pm 1.2 \times 10^5$ parasites/mL vs. $12.8 \pm 2.0 \times 10^5$ parasites/mL) at the peak of parasitaemia. According to these results, psilostachyin C may be considered a promising template for the design of novel trypanocidal agents. In addition, psilostachyin C inhibited the growth of *Leishmania mexicana* and *Leishmania amazonensis* promastigotes (IC_{50} = 1.2 μg/mL and 1.5 μg/mL, respectively).

1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is endemic in Latin America. Approximately 16–18 million people are infected in the Americas and ca. 100 million people are at risk of contracting the disease [1]. Prophylactic and therapeutic vaccines have been pursued but sterilising immunity has not yet been achieved [2,3]. Leishmaniasis is a group of infections caused by *Leishmania* spp. Annually, 1.5–2 million people around the world are infected by the parasites and 350 million are at risk of contracting the disease [4]. Chemotherapy for the treatment of these parasitoses, which are frequently found to co-infect patients in endemic areas [5–7], has limited efficacy and is not innocuous, mainly due to resistance phenomena and adverse effects. Consequently, new drugs are needed.

In previous work, we reported the in vitro trypanocidal activity of several Argentine medicinal plant species [8]. We have isolated two bioactive sesquiterpene lactones (STLs) from *Ambrosia tenuifolia* presenting in vitro activity against *T. cruzi* epimastigotes and *Leishmania* spp. promastigotes, one of which exerted a significant in vivo trypanocidal effect [9].

Ambrosia scabra Hook. & Arn. (Asteraceae) is a closely related species popularly known as 'ajenjo del campo' and traditionally used against intermittent fevers and worm infections [8,10]. Here we report the trypanocidal and leishmanicidal activities of the STL psilostachyin C isolated from *A. scabra* by bioassay-guided fractionation. In addition, the ultrastructural changes that this compound produced in *T. cruzi* epimastigotes were evaluated.

STLs are C-15 terpenoid compounds and represent an important and biogenetically homogeneous group of secondary metabolites present in higher plants [11]. They display great diversity and an enormously broad spectrum of biological activities, including antiprotozoal activity [12–15]. The discovery of artemisinin (an antimalarial STL isolated from the Chinese herb *Artemisia annua)* has been a major breakthrough in the field of parasitic diseases and has prompted the investigation of these kinds of compounds. In particular, psilostachyin C is a dilactone of the ambrosanolide type that was first isolated from *Ambrosia psilostachya* [16] and

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subsequently from other *Ambrosia* spp. [17]. It has been demonstrated to have molluscicidal activity [18] and inhibitory activity on the G_2 DNA damage checkpoint [19]. However, this is the first time that this compound has been found in *A. scabra* and the first report of its trypanocidal and leishmanicidal activities.

2. Methods

2.1. Plant material

Ambrosia scabra was collected in Buenos Aires, Argentina, in 2007 and was identified by Dr G. Giberti (Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina). A voucher specimen (BAF 650) was deposited at the Herbarium of the Museo de Farmacobotánica.

2.2. Bioassay-guided fractionation

Extraction of the aerial parts of *A. scabra* (500 g) was done by maceration with dichloromethane:methanol (1:1) at room temperature. The organic extract was subjected to open column chromatography over silica gel 60 and was eluted successively with cyclohexane, cyclohexane:ethyl acetate (1:1), ethyl acetate and methanol to give 23 fractions of 500 mL each. According to their profile in thin-layer chromatography, these fractions were combined into five final fractions (F_{1AS}–F_{5AS}) and were subsequently tested for trypanocidal activity against *T. cruzi* epimastigotes. Fraction F_{5AS} was chromatographed on a silica gel column eluted with a cyclohexane:CH_2Cl_2 gradient (100:0 to 0:100), CH_2Cl_2:ethyl acetate gradient (100:0 to 0:100) and 100% methanol to obtain 150 fractions ($F_{5AS\ 1–150}$) of 10 mL each. Of these fractions, $F_{5AS\ (75–77)}$ essentially contained one pure compound that crystallised from ethyl acetate.

2.3. Spectrometric analyses

The isolated compound was identified by proton nuclear magnetic resonance (^{1}H NMR) and carbon NMR (^{13}C NMR) (Inova NMR spectrometer; Varian, Palo Alto, CA) (500 MHz in $CDCl_3$), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY), electron impact-mass spectrometry (EI-MS) (Agilent 5973) and infrared spectroscopy (Bruker FT-IR IFS25).

2.4. Cell cultures

Trypanosoma cruzi epimastigotes (RA strain) were grown in biphasic medium. *Leishmania mexicana* promastigotes (MNYC/BZ/62/M strain) and *Leishmania amazonensis* promastigotes (IFLA/BR67/PH8 strain) were grown in liver infusion tryptose (LIT) medium. *Trypanosoma cruzi* and *Leishmania* spp. cultures were routinely maintained by weekly passage at 28 °C and 26 °C, respectively.

Trypanosoma cruzi bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture at the peak of parasitaemia on Day 15 post infection. Trypomastigotes were routinely maintained by infecting 21-day-old CF1 mice.

2.5. Animals

Inbred male CF1 and female C3H/HeN mice were nursed at the Departamento de Microbiología, Facultad de Medicina (Universidad de Buenos Aires). Mice were housed in groups of five per cage. Mice were kept in a conventional room at 24 ± 1 °C with free access to a standard commercial diet and water ad libitum under a 12 h light/12 h dark cycle. All procedures were approved by the Ethics Review Board of the Instituto de Estudios de la Inmunidad Humoral (IDEHU-CONICET) and were conducted in accordance with the guidelines established by the National Research Council [20].

2.6. In vitro evaluation of antiprotozoal activity

Growth inhibition of *T. cruzi* epimastigotes as well as *L. mexicana* and *L. amazonensis* promastigotes was evaluated by a [^{3}H] thymidine uptake assay according to Sülsen et al. [21]. Fractions F_{1AS}–F_{5AS} were tested at 10 μg/mL and 100 μg/mL, and the pure compound and fraction F_{5AS} were tested at concentrations ranging from 0.3 μg/mL to 100 μg/mL. Cell density was adjusted to 1.5×10^6 parasites/mL and cells were cultivated in the presence of each fraction or the pure compound for 72 h or 120 h. Benznidazole (1.3–20.8 μg/mL) (Roche, Rio de Janeiro, Brazil) and amphotericin B (0.025–0.8 μg/mL) (ICN, Costa Mesa, CA) were used as controls for *T. cruzi* and *Leishmania* spp. growth inhibition, respectively. Percentage inhibition was calculated as 100–{[(cpm of treated parasites)/(cpm of untreated parasites)] × 100}, and 50% inhibitory concentration (IC_{50}) values were estimated by the Alexander method [22].

To determine whether the parasites could recover after treatment, *T. cruzi* epimastigotes were incubated with the isolated compound (0.2–2.5 μg/mL) for 24 h. Parasites were then centrifuged at 6000 rpm for 10 min, washed once with phosphate-buffered saline (PBS) (NaCl 0.15 M, NaH_2PO_4 0.02 M, NaOH 0.017 M, pH 7.2) and were incubated in fresh medium for 6 days.

The pure compound was also tested on bloodstream trypomastigotes as previously described [9]. Parasite concentration was adjusted to 1.5×10^6 parasites/mL by diluting mouse blood containing trypomastigotes in complete LIT medium. Parasites were seeded (150 μL/well) in duplicate in a 96-well microplate and 2 μL of the compound (0.1–100 μg/mL) or control drug (benznidazole) (0.4–900 μg/mL) was added per well. Plates were incubated for 24 h and the remaining live parasites were counted in a haemocytometer. Percentage inhibition was calculated as 100–{[(live parasites in wells after compound treatment)/(live parasites in untreated wells)] × 100}.

To evaluate the effect of the isolated compound on intracellular forms of *T. cruzi*, 96-well plates were seeded with murine peritoneal macrophages at 5×10^3 per well in 100 μL of culture medium and were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. Cells were washed and infected with transfected blood trypomastigotes expressing β-galactosidase [23] at a parasite:cell ratio of 10:1. After 2 h of co-culture, plates were washed twice with PBS to remove unbound parasites and the pure compound was added at 0.01–10 μg/mL per well in 150 μL of fresh complete RPMI medium without phenol red (Gibco, Rockville, MD). Controls included infected non-treated cells (100% infection control) and uninfected cells (0% infection control). The assay was developed by addition of chlorophenolred-β-D-galactopyranoside (CPRG) (100 μM) and 1% Nonidet P40, 48 h later. Plates were incubated for 4–6 h at 37 °C. Wells with galactosidase activity turned the media from yellow to red and this reaction was quantified at 570 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). Percentage inhibition was calculated as 100–{[(absorbance of treated infected cells)/(absorbance of untreated infected cells)] × 100} and the IC_{50} value was estimated.

2.7. Cytotoxicity assay

Murine peritoneal macrophages were assayed for determination of cell viability by the MTT method. Cells (5×10^5) were settled at a final volume of 150 μL in a flat-bottom 96-well microtitre plate and were cultured at 37 °C in a 5% CO_2 atmosphere