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The natural compounds piperovatine and piperlonguminine induce autophagic cell death on *Trypanosoma cruzi*

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ABSTRACT

The currently available treatments for Chagas disease show limited therapeutic potential and are associated with serious side effects. Our group has been attempting to find alternative drugs isolated from natural products as a potential source of pharmacological agents against *Trypanosoma cruzi*. Here, we demonstrate the antitrypanosomal activity of the amides piperovatine and piperlonguminine isolated from *Piper ovatum* against epimastigotes and intracellular amastigotes. We also investigated the mechanisms of action of these compounds on extracellular amastigote and epimastigote forms of *T. cruzi*. These amides showed low toxicity to $LLCMK_2$ mammalian cells. By using transmission and scanning electron microscopy, we observed that the compounds caused severe alterations in *T. cruzi*. These alterations were mainly located in plasma membrane and mitochondria. Furthermore, the study of treated parasites labeled with Rh123, PI and MDC corroborate with our TEM data. These mitochondrial dysfunctions induced by the amides might trigger biochemical alterations that lead to cell death. Altogether, our data evidence a possible autophagic process.

1. Introduction

Chagas' disease (American trypanosomiasis) is a neglected illness caused by the protozoan *Trypanosoma cruzi*. This illness continues to be an important public-health problem, endemic in South America with an overall prevalence estimated in 10 million cases word wide (WHO, 2010). The available drugs for the treatment of this infection show limited therapeutic potential and are associated with serious side effects, such as skin rashes, leucopoenia, neurotoxicity, fever, articular and muscular pain, peripheral neuropathy, lymphadenopathy, agranulocytosis, and thrombocytopenic purpura (Urbina and Docampo, 2003). Thus, there is an urgent need for the development of new antitrypanosomal agents with lower toxicity and greater activity, especially for the chronic phase of the disease.

In modern drug research, natural products have proved to be a rich source of therapies for a variety of human diseases and disorders. Advances in separation, purification, and high-throughput screening methods have also generated increased interest in drug discovery from natural sources. In addition, many plant-derived compounds have demonstrated activity at micromolar concentrations (Maya et al., 2007; Izumi et al., 2008; Menna-Barreto et al., 2008; Veiga-Santos et al., 2010). Among these are the various compounds isolated from members of the genus *Piper* (Martins et al., 2003; Luize et al., 2006; Lopes et al., 2008; Vendrametto et al., 2010). For example, *Piper ovatum* Vahl (Piperaceae), a herbaceous plant popularly known as "joão burandi" or "anesthetic" that occurs in tropical and subtropical regions, used in traditional medicine for the treatment of inflammation and as an analgesic (Correa, 1984). Additionally, a recent study demonstrated the activity of the amides piperovatine and piperlonguminine isolated from *P. ovatum* against amastigote and promastigote forms of *Leishmania amazonensis* (Rodrigues-Silva et al., 2009a). Furthermore, the mixture of these two substances showed antimicrobial and anti-inflammatory effects in *in vivo* assays (Rodrigues-Silva et al., 2008, 2009b).

In addition to that, several studies have been conducted to identify metabolic and selective pathways that might work as targets for new trypanocidal. In this context, mitochondria, redox metabolism, parasite life cycle and pathways of cell death are promising candidates (De Souza, 2002; Menna-Barreto et al., 2009; Chen et al., 2010; Zuma et al., 2011; Cabrera et al., 2011; Pelizzaro-Rocha et al., 2011).

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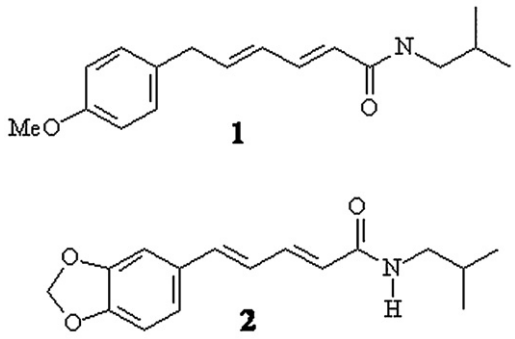

Fig. 1. Chemical structures of piperovatine and piperlonguminine isolated from *Piper ovatum*.

Based in this context and addressing the urgent necessity for the development of new trypanocidal drugs this paper describes the antitrypanosomal activity of the amides piperovatine and piperlonguminine obtained from *P. ovatum* against epimastigote and intracellular amastigote forms of *T. cruzi*. Both compounds demonstrated, using flow cytometry and electron microscopy techniques, potent activity, inhibiting the parasite proliferation and causing drastic ultrastructural alterations.

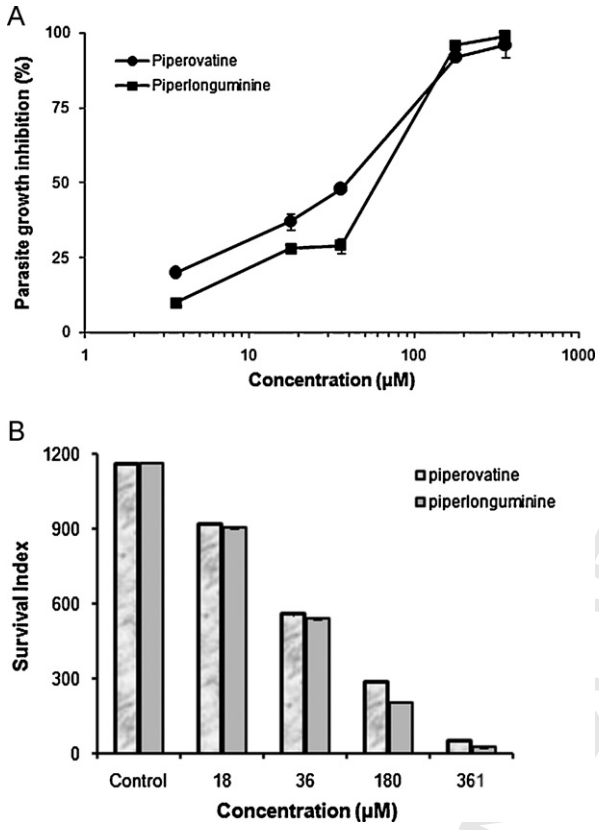

Fig. 2. Effects of piperovatine and piperlonguminine on *Trypanosoma cruzi*. (A) Epimastigote growth cultured for 96 h in the presence of these compounds at concentrations of 3.6, 18.0, 36.1, 180.4, and 360.0 μM. The results were analyzed as percentages of proliferation inhibition in relation to untreated parasites; (B) effects on intracellular amastigotes form. $LLCMK_2$ cell line was infected with trypomastigotes for 2 h, washed and then after 24 h were treated with different concentrations of compounds for 96 h. The drug activity was estimated by calculating the survival index (percentage of infected cells times the average number of intracellular amastigotes per infected host cell). Each experiment was conducted in duplicate and repeated at least three times. Bars represent standard errors. All results were significant at $p \leq 0.05$ as compared to the control group, by Student's *t*-test.

2. Materials and methods

2.1. Plant material

Plant materials were collected in November 2004, in Monte Formoso, state of Minas Gerais, Brazil. The samples were identified by Dr. Elsie Franklin Guimarães, and a voucher specimen was deposited in the herbarium of the Department of Biology, University of Maringá (HUM 10.621).

2.2. Extraction, fractionation, identification of the compounds and stock solutions

One thousand grams of plant material was sequentially extracted by maceration in ethanol:water (9:1) at room temperature, filtered, concentrated by rotary evaporator at 40 °C, and lyophilized, yielding 23 g. Subsequently, the hydroalcoholic extract (14 g) was chromatographed in a vacuum silica-gel column and eluted with gradients of hexane, dichloromethane-ethyl acetate (1:1, v/v), ethyl acetate, and methanol, which afforded fractions F1, F2, F3, and F4. The F2 fraction was rechromatographed on a silica gel 60 column (70–230 mesh, Merck) using hexane, dichloromethane ethylacetate, and methanol, which afforded 108 fractions. Fractions 23–38 were rechromatographed on a Sephadex LH 20, using ethyl acetate, obtaining 50 sub-fractions. Sub-fractions 15–32 and 42–50 were isolated and identified as piperovatine and piperlonguminine by analyses of spectral data of ^1H and ^{13}C and by comparison of data from the literature (McFerren and Rodriguez, 1998; Wu et al., 2004). Stock solutions of these compounds were prepared in DMSO at a concentration of 40 mM.

2.3. Parasites and cell cultures

All experiments were performed with "Y strain" of *T. cruzi*. Epimastigote forms were maintained axenically at 28 °C with weekly transfers in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at pH 7.4 (Camargo, 1964).

Trypomastigote and amastigote forms were obtained from the supernatants of previously infected $LLCMK_2$ cells monolayer (epithelial cells of monkey kidney – Macaca mulatta) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% FBS, 50 mg/l gentamicin and buffered with sodium bicarbonate in a 5% CO_2 air mixture at 37 °C.

2.4. Antitrypanosomal activity of piperovatine and piperlonguminine

Epimastigote forms were centrifuged at $2000 \times g$ for 10 min and 1×10^6 cells/ml were incubated for 96 h in the presence or absence of compounds (diluted in stock solution with 1% dimethyl sulfoxide, in concentrations of 3.6–361 μM). After that, the cells were counted in a Neubauer haemocytometer chamber (Improved Double Neubauer). The results were expressed as IC_{50} and IC_{90} (concentrations that inhibited 50% and 90% of the parasite growth). To evaluate the *in vitro* activity against intracellular amastigotes, $LLCMK_2$ cells were infected with trypomastigotes and incubated for 24 h. Infected $LLCMK_2$ was treated with different concentrations (18–361 μM) of the compounds and incubated for 96 h at 37 °C with 5% CO_2 atmosphere, following fixation in methanol and Giemsa staining. The drug activity was estimated by calculating the survival index (percentage of infected cells times the average number of intracellular amastigotes per infected host cell) in each condition used in this study. A total of 200 cells were counted using Neubauer chamber. The 50% inhibitory concentrations (IC_{50}) also were

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