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Original article

1,3,4-Thiadiazole derivatives of *R*-(+)-limonene benzaldehyde-thiosemicarbazones cause death in *Trypanosoma cruzi* through oxidative stress

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Abstract

This work evaluated the in vitro and in vivo activity of **TDZ 2** on *Trypanosoma cruzi* amastigotes and determined the possible mechanism of action of this compound on *T. cruzi* death. **TDZ 2** inhibited *T. cruzi* proliferation in vitro and had low haemolytic potential. It also induced morphological and ultrastructural alterations. We observed a reduction of cell volume, the depolarization of the mitochondrial membrane, an increase in ROS production, lipoperoxidation of the cell membrane, lipid bodies formation and production of nitric oxide, a decrease in reduced thiols levels and, presence of autophagic vacuoles. The in vivo study found a reduction of parasitemia in animals treated with **TDZ 2** alone or combined with benznidazole. Altogether, the alterations induced by **TDZ 2** point to an oxidative stress condition that lead to *T. cruzi* cell death.

Keywords: Chagas' disease; Thiadiazole; Mitochondrial dysfunction; Oxidative stress; Cell death

1. Introduction

Chagas' disease (American trypanosomiasis), caused by the parasite *Trypanosoma cruzi*, a hemoflagellate that belongs to the family *Trypanosomatidae,* is one of 17 illnesses that are considered neglected tropical diseases (NTDs) by the World Health Organization. An estimated of 6–7 million people are infected worldwide, mostly in 21 countries in Latin America where Chagas' disease is endemic [1].

Amastigotes (i.e., the infective and replicative form of *T. cruzi*) is the main form responsible for the chronicity of Chagas' disease. This form can persist for decades in host tissues, especially cardiac and skeletal muscle, often as asymptomatic infection. Up to 30% of infected individuals develop clinically evident chronic cardiomyopathy or gastrointestinal disease [2,3].

The available treatments for chagasic patients include two nitroheterocyclic compounds: nifurtimox and benznidazole (BZ). These drugs have many side effects, which may lead to immediate interruption of treatment [4]. Furthermore, they have limited effectiveness against the disease, with cure rates of 20% in the chronic phase of Chagas' disease [5]. Thus, there is an urgent need to develop new therapeutic options that have high therapeutic effectiveness and low toxicity.

Thiosemicarbazones are sulphur-donor Schiff-base ligands that have prompted considerable scientific interest because their important chemical and biological properties [6,7], such as antitumor, antioxidant, antibacterial, antifungal, antiviral,

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antimalarial, anti-*Leishmania*, and antitrypanosomal effects [6–10]. They have wide applications as precursors for many chemical compounds, including 1,3,4-thiadiazoles [11].

1,3,4-Thiadiazole is considered an important heterocyclic moiety that has versatile properties and special applications in pharmaceuticals, agrochemicals, and materials chemistry. It has a broad spectrum of biological activity, including antimicrobial, antituberculosis, antioxidant, antiinflammatory, anxiolytic, antihypertensive, anticancer, antifungal, and antitrypanosomal effects [12].

To discover new compounds that show effectiveness against *T. cruzi* with low toxicity, our research group evaluated the trypanocidal activity of a series of 1,3,4-thiadiazole derivatives of *R*-(+)-limonene benzaldehyde-thiosemicarbazones against epimastigotes and trypomastigotes of *T. cruzi* and interesting results were reported [13].

In the present study, we tested the in vitro and in vivo activity of *N*-{1-methyl-1-[(1*R*)-4-methylcyclohex-3-en-1-yl] ethyl}-5-(4-methylphenyl)-1,3,4-thiadiazol-2-amine (**TDZ 2**) against amastigote forms of *T. cruzi*, and suggested a possible mechanism of action involved in the cell death of this parasite.

2. Materials and methods

*2.1. Synthesis of N-{1-methyl-1-[(1*R*)-4-methylcyclohex-3-en-1-yl]ethyl}-5-(4-methylphenyl)-1,3,4-thiadiazol-2-amine*

The synthesis of *N*-{1-methyl-1-[(1*R*)-4-methylcyclohex-3-en-1-yl]ethyl}-5-(4-methylphenyl)-1,3,4-thiadiazol-2-amine, here denominated **TDZ 2**, was performed according to procedure reported in the literature [13].

2.2. Compound preparation

TDZ 2 was dissolved in dimethylsulfoxide (DMSO) and diluted in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen) at a final DMSO concentration of less than 1%.

2.3. In vitro assay

2.3.1. Parasites and cell cultures

$LLCMK_2$ cells (epithelial kidney cells [*Macaca mulatta*]; CCL-7; American Type Culture Collection, Rockville, MD, USA) were maintained in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated foetal bovine serum (FBS), 5000 U/ml penicillin, and 5 mg/ml streptomycin solution at 37 °C in a humidified 5% CO_2 atmosphere.

Trypomastigote and amastigote forms of the Y strain of *T. cruzi* [14] were obtained from the supernatant of $LLCMK_2$ cells that were preinfected by bloodstream trypomastigotes that were aseptically obtained from infected Swiss mice by heart puncture at the peak of parasitemia. The animal protocol was approved by the Ethics Committee of the Universidade Estadual de Maringá (no. 074/2011). Amastigotes and trypomastigotes that were present in the supernatant of infected $LLCMK_2$ cells were separated by differential centrifugation at 850 × *g* for 5 min. The trypomastigote forms were harvested from the supernatant, and amastigote forms were collected from the pellet.

2.3.2. Activity against intracellular amastigote forms

In a 24-well microplate that contained glass coverslips, a 1000 µl aliquot of $LLCMK_2$ cells (2.5×10^5 cells/ml) was seeded in each well and incubated for 24 h at 37 °C with 5% CO_2. The $LLCMK_2$ cell monolayer was infected with trypomastigotes (10:1) for 24 h, and fresh medium with different concentrations of **TDZ 2** (0.1, 0.5, 1.0, 5.0, and 10.0 µM) was added to the wells. The microplate was incubated for 96 h at 37 °C with 5% CO_2. The cells were stained with May-Grünwald-Giemsa (Gibco, Invitrogen Corporation, New York, NY, USA) for 20 min. The glass coverslips were then permanently prepared with Entellan (Merck, Darmstadt, Germany). The IC_{50} and IC_{90} values (concentrations that inhibited 50% and 90% of parasite growth, respectively) were determined by observing 200 cells with an Olympus CX31 light microscope (Olympus, Tokyo, Japan). The selectivity index (SI) between host cell $LLCMK_2$ and amastigote forms was then calculated: $SI = CC_{50}\ LLCMK_2/IC_{50}$ *amastigote forms*.

2.3.3. Haemolytic activity

Blood (type A+) from a healthy human donor was collected, defibrinated, and washed in glycosylated saline (0.9% NaCl, 1% glucose). Red blood cells were inoculated in 96-well plates at 3% in glycosylated saline with different concentrations of **TDZ 2** (10.0, 50.0, 100.0, 500.0, and 1000.0 µM). Triton X-100 (1%) was used as positive control. The plates were incubated for 3 h at 37 °C, and the supernatant was read at 550 nm. The percentage of haemolysis was then calculated: $Haemolysis\ (\%) = (A_s - A_c) \times 100/A_p$, where A_s, A_c, and A_p are the absorbances of the test sample, negative control, and positive control, respectively.

2.3.4. Electron microscopy

$LLCMK_2$ cell monolayers in DMEM supplemented with 2 mM L-glutamine, 10% FBS, 5000 U/ml penicillin, and 5 mg/ml streptomycin solution, buffered with sodium bicarbonate in a 5% CO_2 air mixture at 37 °C, were infected with trypomastigotes (10:1) for 24 h. Fresh medium with 10% FBS was then added. The bottles were incubated for 36 h at 37 °C in a 5% CO_2 atmosphere. After this period, the bottles that were treated with 1.3 µM (IC_{50}) and 9.0 µM (IC_{90}) **TDZ 2** were incubated for 24 h at 37 °C in 5% CO_2 and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C.

For scanning electron microscopy (SEM), the cultures were adhered on poly-L-lysine-coated coverslips. The samples were dehydrated in a graded series of ethanol, critical-point-dried with CO_2, and coated with gold. Intracellular amastigotes were observed in a Shimadzu SS-550 scanning electron microscope (Shimadzu Corporation, Kyoto, Japan).

For transmission electron microscopy (TEM), the cultures were postfixed in a solution that contained 1% OsO_4, 0.8% potassium ferrocyanide, and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in an increasing acetone gradient, and