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Effects of artesunate against *Trypanosma cruzi*

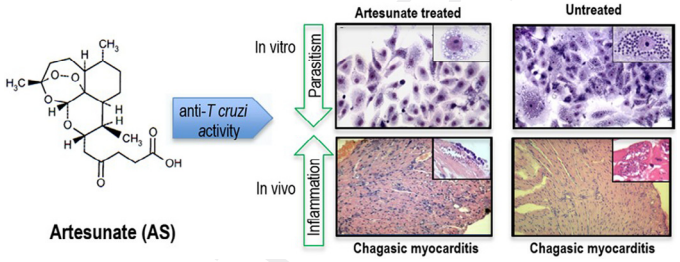
Gabriela Carina Olivera [a,b,1], Miriam Postan [a,b], Mariela Natacha González [a,b,2,*]

[a] Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", Ave. Paseo Colón 568, Ciudad Autónoma, Buenos Aires ZC 1063, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

HIGHLIGHTS

- We examined the action of artesunate on *T. cruzi* strains from different regions.
- Artesunate is highly effective against epimastigotes and amastigotes in vitro.
- Artesunate resulted inefficient against acute *T. cruzi* infection in mice.

GRAPHICAL ABSTRACT



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ABSTRACT

Therapy against *Trypanosma cruzi* relies on only two chemically related nitro-derivative drugs, benznidazole and nifurtimox, both limited by poor efficacy and toxicity. It is suspected that with prolonged usage of these drugs, resistant parasites will be selected, which results in risk for treatment failure over the time. Herein, we studied the in vitro activity of artesunate, the most effective drug to treat severe *P. falciparum* and chloroquine-resistant *P. vivax*, on three strains of *T. cruzi* originated in different regions of Latin America (Argentina, Nicaragua and Brazil). The results of these assays showed that artesunate inhibits multiplication of epimastigotes (IC_{50} = 50, 6.10 and 23 μM, respectively) and intracellular amastigotes (IC_{50} = 15, 0.12 and 6.90 μM, respectively), indicating that it represents a potent anti-*T. cruzi* compound in terms of inhibiting parasite multiplication in vitro. We then tested the effect of artesunate in Balb/c mice infected with Brazil strain and found that it failed to cure the infection, suggesting that the drug may be unsuitable for in vivo treatment. When infected mice were treated with high doses AS + BZ, the outcome of infection was similar to that observed in mice treated with BZ alone. Nevertheless, understanding of structure–activity relationship of artesunate might lead to the development of new and effective drugs against *T. cruzi*.

1. Introduction

It is estimated that 7–8 million people are infected with *Trypanosma cruzi*, the causative agent of Chagas disease, with a further 100 million persons remaining at risk in Latin America (World Health Organization, 2014). Approximately 30% of chronically infected individuals develop cardiac and/or digestive symptoms and megaviscera leading to irreversible disease. It has been long suspected that the spectrum of clinical forms of human Chagas disease is at least in part related to the genetic characteristics of the infecting parasite (Andrade et al., 1992; Filardi and Brener, 1987; Schlemper et al., 1983). So far, therapy against *T. cruzi* has relied on only two chemically related nitro-derivative drugs, a 2-nitroimidazole (benznidazole, BZ) and a nitrofurane (nifurtimox, NF), both limited by poor efficacy and toxicity (Jackson et al., 2010; Yun et al., 2009). The development of parasite strains resistant to these drugs, the

* Corresponding author. Fax: +54 11 4331 7142.
E-mail address: marnatch@yahoo.com.ar (M.N. González).
[1] Current address: Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden.
[2] Current address: Laboratório de Pesquisas sobre o Timo, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil.

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difficulties in eradicating the vector from households in endemic areas and insecticide-resistance vector, constitute major causes behind the persistence of the Chagas disease problem.

In spite of the enormous health and economic burden caused by Chagas disease, economic incentives for drug development are lacking. The scarcity of a sufficient portfolio of drugs is particularly perceived in the treatment of chronic infections and infected pregnant women. There is, thus, an imperative need for improved, simple to use and less toxic anti-*T. cruzi* drugs that are inexpensive and which can be administered during pregnancy. Drugs available for other human pathologies such as allopurinol, itraconazole and posaconazole among others have been tested against *T. cruzi* with variable success (Coronado et al., 2006; Molina et al., 2000; Toledo et al., 2003). Artemisinin, a sesquiterpene lactone extracted from the leaves of the plant *Artemisia annua* used to treat *Plasmodium falciparum* and chloroquine-resistant *P. vivax* (Dhingra et al., 2000, World Health Organization, 2010), and artemisinin-derivatives artemisone, 4-fluorophenyl artemisinin, and dihydroartemisinin were all described to inhibit the growth of *T. cruzi* epimastigotes in vitro (Mishina et al., 2007). Semisynthetic derivatives of artemisinin have been developed to improve the poor solubility and low bioavailability that has artemisinin itself (Haynes and Krishna, 2004). Artesunate (AS), a sodium salt of the hemisuccinate ester of artemisinin, has been shown to exhibit a wide spectrum of activity against protozoan parasites such as *T. gondii*, *L. donovani* and *Plasmodium spp.* (El Zawawy, 2008; Gomes et al., 2012; LaCrue et al., 2011; Mutiso et al., 2011) and it is currently recommended for severe *P. falciparum* and *vivax* malaria (World Health Organization, 2010; Rosenthal, 2008). This knowledge prompted us to explore the trypanocidal action of AS, and we established that AS exerts an inhibitory effect on epimastigotes and amastigotes of *T. cruzi* strains from different geographical regions in vitro. Despite their anti-parasitic properties on *T. cruzi* in vitro this compound was ineffective to modify parasite load in a mouse model of acute Chagas disease. These results indicate the complexity involved in the development of new therapeutic tools for Chagas disease.

2. Materials and methods

2.1. Parasites

Three strains of *T. cruzi* were used in this study as follows: (1) AR-SE23C strain (DTU V; Bua J and Perrone AE, personal communication), recovered in our laboratory from a chronic human infection by hemoculture in 2009 in Province Santiago del Estero, Argentina, (2) Nicaragua strain (DTU I, Grosso et al., 2010), isolated in our laboratory from the feces of an infected *Triatoma dimidiata* captured in Nicaragua in 1987 (Dr. Teresa Rivera Bucardo, UNAN-León, Nicaragua) and (3) Brazil strain (DTU IIc, Brisse et al., 2000) originated from a Brazilian human case of Chagas disease.

2.2. Epimastigotes growth inhibition assay

Axenic cultures of *T. cruzi* strains AR-SE23C, Nicaragua and Brazil were set up in 15 ml culture tubes containing liver infusion tryptose medium supplemented with 20 µg/ml hemin, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, at an initial concentration of 10^6 epimastigotes/ml. Cultures were incubated with serial 10-fold dilutions (0.01–100 µg/ml) artesunate (AS; Fundación Mundo Sano, Buenos Aires, Argentina and Sigma-Aldrich, St. Louis, USA) or beznidazole (BZ; Radanil®, Roche, Rio de Janeiro, Brazil) during 72 h at 27 °C. Untreated cultures were used as controls.

2.3. Amastigote growth inhibition assay

Vero cell (African green monkey kidney) cultures were set up with 10% FBS-RPMI media onto coverslips contained in 24 well plates (2×10^4 cells/well), incubated overnight with cell culture-derived AR-SE23C, Nicaragua or Brazil trypomastigotes at a 10:1 parasite/cell ratio, and treated with 10-fold dilutions AS (0.001–100 µg/ml) at 37 °C in a 5% CO_2 incubator during 72 h. Afterward, cells were fixed with methanol, stained with Giemsa and mounted on microscope slides. The number of intracellular amastigotes per infected cell was quantified on microphotographs of randomly selected 400× microscopic fields of Giemsa-stained smears (Leica CTR Mic, Germany), using Image Tool software (http://ddsdx.uthscsa.edu/dig/itdesc.html). A minimum of 100 infected cells/experimental condition were analyzed.

The effect of AS on the viability of Vero cells was measured in uninfected cell cultures using the resazurin cell viability assay (Sigma-Aldrich, St. Louis, MO). Briefly, Vero cells were plated in 96-well plates (2×10^3 cells/well) and incubated with 10-fold dilutions AS (0.001–100 µg/ml) at a final volume of 100 µl/well during 72 h at 37 °C in a 5% CO_2 incubator. Then, 10 µl of resazurin solution (0.01% in PBS 1×) was added to each well and returned to the incubator for additional 4 h prior to recording of absorbance at 578 and 630 nm.

Selectivity index (SI) was expressed as the ratio (IC_{50} for Vero cells/IC_{50} for *T. cruzi* parasites).

2.4. Activity of AS against cell culture-derived trypomastigotes

Brazil strain trypomastigotes liberated from infected Vero cell cultures and blood forms were re-suspended at a concentration of 10^6 trypomastigotes/ml in complete RPMI-1640 media and treated with 10-fold serial dilutions of AS (0.1–100 µg/ml) during 24 h at 4 °C. Afterwards, cultures were allowed to attain room temperature and the number of moving trypomastigotes quantified in a Neubauer chamber.

2.5. Activity of AS against T. cruzi in vivo

Four-week-old male and female Balb/c mice, obtained from the Animal Breeding Facility of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén", were inoculated intraperitoneally with 2×10^4 Brazil strain blood forms/mouse and evaluated twice a week for the presence of circulating parasites by light microscope examination of fresh blood samples obtained from the tail. In this model system, parasitemia is a constant finding during the acute phase of infection, and treatment with AS and/or BZ begun when parasites were first detected in the blood. A total of 47 mice with positive parasitemia were randomly selected and treated as follows: Protocol 1 – Oral administration of AS (30 mg/kg/d), BZ (100 mg/kg/d) or saline for 3 weeks (3 days/week), beginning at 7 dpi. Protocol 2 – Oral administration of AS (125 mg/kg/d), AS (125 mg/kg/d) + BZ (100 mg/kg/d), AS (75 mg/kg/d) + BZ (50 mg/kg/d), BZ (100 mg/kg/d), BZ (50 mg/kg/d) or saline for 6 consecutive days, beginning at 10 dpi (Bustamante et al., 2008; Posner et al., 2008). Uninfected treated and untreated mice were included as controls. All experimental and control groups included mice of both sexes. Parasitemia levels were scored as described by Brener (1962). Heart and skeletal muscle samples obtained from mice harvested at 35 dpi were fixed in 10% formalin and embedded in paraffin. Hematoxylin/eosin-stained sections were coded and blindly evaluated by two independent observers as described previously (González et al., 2013). The presence and number of parasitized cells per section were recorded, and tissue parasite density was estimated by the number of parasite nests over the total section area, measured with Image Tool software and standardized to 0.5 cm^2. All animals received humane care and study protocols comply with the Guide for

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