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Effects of artesunate against *Trypanosma cruzi*

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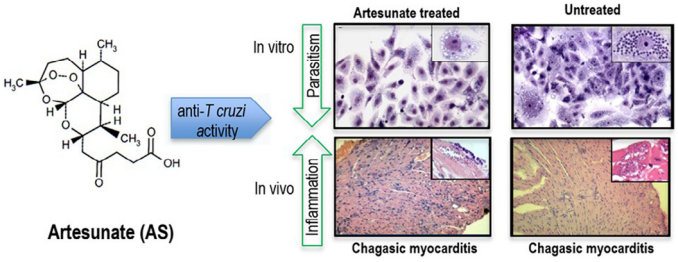
HIGHLIGHTS

- 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

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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://compdent.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

Table 1
Inhibitory activity of artesunate on *Trypanosoma cruzi* epimastigotes.

T. cruzi strain	Artesunate		Benznidazole	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
AR-SE23C	19.22* (50)	69.20* (180)	36.16† (140)	>500 (1.26 × 10^3)
Nicaragua	2.35 (6.10)	14.34 (37)	10.41 (40)	>500 (300)
Brazil	8.84 (23)	44 (100)	17.17 (60)	>500 (3 × 10^3)

Epimastigote cultures were treated with 10-fold dilutions (0.01–100 μg/ml) AS or BZ during 72 h. IC_{50} and IC_{90} data are expressed as μg/ml and (μM).
* $p < 0.05$ vs. AS-treated Nicaragua epimastigotes.
† $p < 0.05$ vs. BZ-treated Nicaragua and Brazil epimastigotes.

the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.6. Statistical analysis

All in vitro assays consisted of triplicate cultures per each experimental condition. The mean percentage of parasites obtained in 3 independent experiments was used to estimate the 50 and 90% percent inhibitory concentrations (IC_{50} and IC_{90}), applying sigmoidal fit nonlinear regression analysis in Origin software (OriginLab Corporation, Northampton, MA). Data were analyzed with ANOVA followed by Dunnet Multiple Comparisons or Bonferroni Test using InStat software (GraphPad Software, Inc. La Jolla, CA). Comparisons of the frequencies of mice with positive parasite nests were evaluated with the Fisher's exact test. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effect of artesunate against T. cruzi in vitro

The analysis of the data on epimastigote growth revealed that a significantly higher dose of AS was required to reach IC_{50} and IC_{90} for AR-SE23C strain compared to Nicaragua strain ($p < 0.05$), with intermediate values for Brazil strain (Table 1). In cultures treated with BZ, IC_{50} was significantly higher for AR-SE23C than for Nicaragua and Brazil epimastigotes ($p < 0.05$; Table 1).

The evaluation of the effect of treating infected Vero cell cultures with AS showed that IC_{50} for intracellular amastigotes was significantly higher for AR-SE23C strain than for Nicaragua strain ($p < 0.05$), with intermediate values for Brazil strain (Table 2). The study of AS cytotoxicity for uninfected Vero cells showed an IC_{50} of 3.6 mg/ml [9.3 mM], significantly lower than that for the parasites. The SI reflecting the inhibition of the particular parasite isolate with respect to Vero cells was highly variable among *T. cruzi* strains (Table 2).

In order to evaluate the activity of AS on trypomastigotes, we treated Brazil strain trypomastigotes released from Vero cell cultures and bloodstream forms with the compound and found that the effective IC_{50} were 56.9 μg/ml (148 μM) and 31.52 μg/ml (82 μM), respectively. The AS IC_{50} for trypomastigotes was significantly higher than IC_{50}s required to inhibit the growth of Brazil strain amastigotes and epimastigotes ($p < 0.05$). The trypanocidal SI of AS with respect to cytotoxicity for Vero cells were 62.84 and 113.40 for cell culture-derived trypomastigotes and blood forms, respectively.

Table 2
In vitro activity of artesunate on intracellular *Trypanosoma cruzi* amastigotes.

T. cruzi strain	IC_{50}	IC_{90}	SI
AR-SE23C	6.63* (15)	19.25 (50)	620
Nicaragua	0.05 (0.12)	0.70 (1.8)	77500
Brazil	2.65 (6.9)	3.84 (10)	1347.8

T. cruzi-infected Vero cell cultures were treated with 10-fold dilution AS (0.001–100 μg/ml) during 72 h and the number of intracellular parasites evaluated in Giemsa-stained cells. IC_{50} and IC_{90} data are expressed as μg/ml and (μM).
* $p < 0.05$ vs. AS-treated Nicaragua amastigotes.
Selectivity index (SI) for *T. cruzi* is expressed as the ratio (IC_{50} Vero cells/IC_{50} *T. cruzi*).

3.2. Artesunate activity against T. cruzi in the acute infection of mice

The relatively broad safety window between inhibitory concentrations of AS against *T. cruzi* amastigotes and cytotoxicity for Vero cells prompted us to proceed studying the activity of the compound against *T. cruzi* in vivo. Balb/c mice were infected with Brazil strain blood forms and treated with 30 or 125 mg/kg/d AS, 50 or 100 mg/kg/d BZ, a combination of 75 mg/kg/d AS + 50 mg/kg/d BZ or 125 mg/kg/d AS + 100 mg/kg/d BZ. As shown in Fig. 1, the levels of parasitemia were variable in AS-treated as well as untreated infected mice. Although AS treatment resulted in earlier parasitemia peaks (11 and 16 dpi for mice treated with 125 and 30 mg/kg/d, respectively) compared to untreated infected controls (23 dpi), no differences in the number of parasites reached at the peak of parasitemia were found between AS-treated and untreated infected mice. As expected, 50 or 100 BZ mg/kg/d significantly reduced the levels of parasitemia ($p < 0.05$; Fig 1), and a few circulating parasites were occasionally seen after treatment began. Also, combined treatment with AS + BZ resulted in lower numbers of circulating parasites compared to AS-treated and untreated controls ($p < 0.05$; Fig. 1).

Histopathology of *T. cruzi* infected mice showed that treatment with 30 mg/kg/d AS 3 times a week for 3 weeks had no effect on the density of tissue parasites. However, the treatment with AS-30 significantly increased the extent of myocarditis compared to untreated mice ($p < 0.05$), without modifying skeletal muscle inflammation (Table 3; Supplementary Fig. S1). In contrast, the same scheme of treatment with 100 mg/kg/d BZ significantly reduced the density of tissue parasites and inflammation in both tissues ($p < 0.05$; Table 3; Supplementary Fig. S1).

The administration of 125 mg/kg/d AS during 6 consecutive days had no effect on the density of parasite nests or severity of inflammation in both the heart and skeletal muscles. Conversely, BZ administered during short periods, alone or in combination with AS, significantly reduced the density of heart parasites but had no effect on the extent of *T. cruzi*-induced myocarditis, regardless of the doses administrated ($p < 0.05$; Table 3). In the skeletal muscle, short-term administration of 50 and 100 mg/kg/d BZ or AS + BZ (125–100) resulted in a significant reduction of parasite density and inflammation compared with untreated infected controls ($p < 0.05$, Table 3). However, administration of 75 mg/kg/d AS + 50 mg/kg/d BZ reduced the density of parasite nests but did not modify skeletal muscle inflammation. There were no sex-related differences in the levels of parasitemia, tissue parasite density and inflammation within groups.

4. Discussion

In concert with intensive research to develop new efficient and safer drugs against *T. cruzi*, a growing number of unresponsiveness human infections to drugs currently available for Chagas disease have been identified (Pinto et al., 2009; Yun et al., 2009). It is suspected that with continuing use of BZ and NF, resistant parasites will be selected, and the number of treatment failures will increase over time. Thus, there is an imperative need for improved, easy to manage and less toxic anti-*T. cruzi* drugs that are inexpensive, and which can also be administered during pregnancy. In studies of drug susceptibility, a wide range of responses to trypanocidal drugs were documented for *T. cruzi* isolates circulating in different regions of Latin America (Andrade et al., 1985; Martins et al., 2007; Toledo et al., 2003). Herein, we investigated the in vitro action of AS against

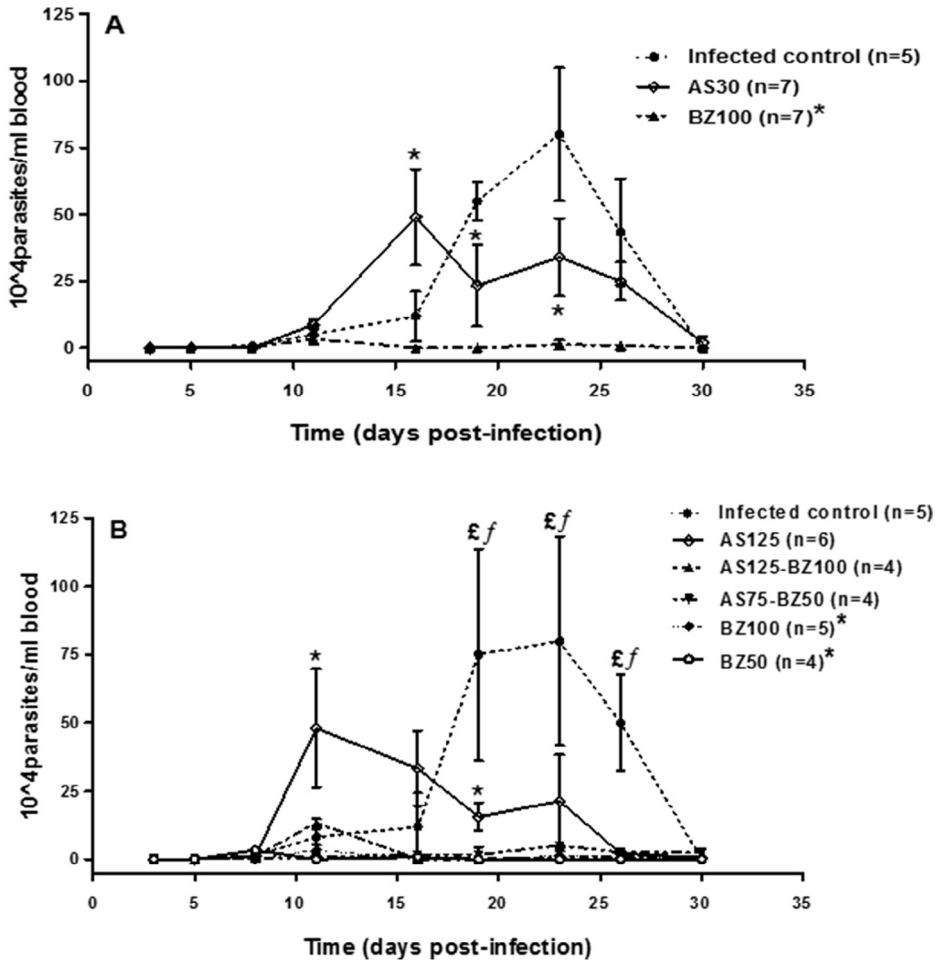

Fig. 1. Effect of AS on the levels of parasitemia in Balb/c mice infected with *Trypanosoma cruzi*. Mice were infected with 2×10^4 Brazil strain blood forms and treated during the acute stage of infection with (A) nine doses of 30 mg/kg/d AS (AS30) or 100 mg/kg/d BZ (BZ100) and (B) six doses of 125 mg/kg/d AS (AS125), 50 mg/kg/d BZ (BZ50), 100 mg/kg/d BZ (BZ100), 75 mg/kg/d AS + 50 mg/kg/d BZ (AS75-BZ50) or 125 mg/kg/d AS + 100 mg/kg/d BZ (AS125-BZ100); untreated infected mice were used as controls (infected controls; see Section 2). The data express the mean ± SD parasitemia levels evaluated in 4–7 animals per group. *$p < 0.05$ vs. infected controls; in the groups of mice treated with BZ100 and BZ50, parasitemia was statistically significant when compared to infected control after 16 days post-infection, but the corresponding symbols were omitted to avoid redundancy in the figure; $^{£}p < 0.05$ vs. BZ100 and AS125 -BZ100; $^{f}p < 0.05$ vs. BZ50 and AS75-BZ50.

T. cruzi strains originated in different endemic regions of Chagas disease and found that it is an effective compound in terms of inhibiting epimastigote and amastigote growth at low micromolar concentrations. Nevertheless, drug effectiveness varied according to the source of the parasites, as parasites derived from the Argentine strain were more resistant to AS as well as BZ than those derived from Nicaragua strain. Moreover, the cytotoxic activity of AS against culture-derived trypomastigotes and blood forms was less

Table 3
Effect of AS in Balb/c mice acutely infected with *T. cruzi* Brazil strain.

Treatment protocol[a]	Tissue parasitism[b]		Inflammation index (mean ± SD)	
	Heart	S. muscle	Heart	S. muscle
AS30[c] (n = 7)	57.14 (170–400)	71.43 (3.85–780)	2.21 ± 0.7*	3.5 ± 0.41
BZ100[c] (n = 7)	0*	0*	0.27 ± 0.23*	0.08 ± 0.13*
AS125[d] (n = 6)	33.33 (97–397)	83.35 (0.185–170)	1.28 ± 0.61	2.67 ± 0.56
BZ100[d] (n = 5)	0*	20* (0–0.67)	1.53 ± 0.27	1.25 ± 0.50*
BZ50[d] (n = 4)	0	25* (0–0.91)	1.33 ± 0.28	1.50 ± 0.90*
AS125-BZ100[d] (n = 4)	0*	0*	1.51 ± 0.09	1.50 ± 0.43*
AS75-BZ50[d] (n = 4)	0	25* (0–1.56)	1.33 ± 0.40	1.92 ± 0.14
Infected controls (n = 7)	42.85 (106–420)	57.15 (0.7–780)	1.29 ± 0.61	3.11 ± 0.75
Untreated controls (n = 5)	0	0	0	0
Uninfected AS-125 treated (n = 5)	0	0	0	0

[a] Drug treatment, mg/kg/day.
[b] Percentage of infected mice with tissue parasites and (range) of parasite nests/0.5 cm^2.
[c] Protocol 1:3 doses/week during 3 weeks.
[d] Protocol 2:6 doses on consecutive days.
* $p < 0.05$ vs. untreated infected controls.

significant than that for epimastigotes and amastigotes, indicating that its trypanocidal action is stage-specific.

In our work, the cytotoxicity assay was designed in the context of testing the action of AS against intracellular parasites in Vero cell cultures (72 h exposure), and the compound proved low toxic for uninfected Vero cells in these conditions. Other authors have reported substantially lower IC_{50} values of AS for human neoplastic and non-neoplastic liver cell lines treated during 48 h (Hou et al., 2008) and for RAW264.7 cells and peritoneal macrophages treated during 4 and 24 h (Li et al., 2008), indicating that AS cytotoxicity depends on the time of exposure and the particular cell line tested.

The selectiveness (SI > 50) of AS toward different strains and developmental forms of *T. cruzi* in vitro was found initially very encouraging (Nwaka et al., 2009), and prompted us to proceed studying the activity of the compound against *T. cruzi* infections in vivo. Different authors have established the role of parasite and host genetics on the outcome of a *T. cruzi* infection (Andersson et al., 2003; Trischmann and Bloom, 1982; Wrightsman et al., 1982). The convenience of using the Balb/c model system for studies of trypanocidal drugs is that these mice develop an acute disease characterized by transient parasitemia and a high rate of mortality after infection with *T. cruzi* (Martins et al., 2008; Toledo et al., 2004). Unpredictably, treatment with AS alone was inefficient to reduce parasite load and inflammation in cardiac and skeletal muscles in Balb/c mouse model of acute infection independently of the scheme, at doses reportedly effective for *P. berghei* and other protozoan parasites (Dhingra et al., 2000; Posner et al., 2008).

AS is a potent water-soluble anti-malarial drug and has been widely indicated for the treatment of severe malaria. Beyond its antiparasitic activity, AS has been reported to inhibit the expression of TLR4 and TLR9 (Li et al., 2008) and exhibit an anti-inflammatory effect by suppressing Th-1 IFN-γ and TNF-α production via inhibition of NF-κB activities (Ho et al., 2014; Miranda et al., 2013; Xu et al., 2007). In cerebral malaria (CM), the accumulation of proinflammatory cytokines (TNF-α, IL1b) in the brain has been associated with the pathogenesis of damage in the central nervous system in humans and in experimental models (Armah et al., 2005; Jennings et al., 1997). An in vivo study conducted by Miranda et al. (2013) demonstrated that a single dose of AS decreased the levels of pro-inflammatory cytokines in the brain and improved survival and clinical signs of CM in *P. berghei*-infected mice. In the acute infection of mice with *T. cruzi*, activation of TLR4 and TLR9 and a potent IFN-γ and TNF-α Th1 immune response are known to be crucial for host resistance (Abrahamsohn, 1998; Antúnez and Cardoni, 2000; Oliveira et al., 2010). Furthermore, in chronic human Chagas disease, the frequency of IFN-γ-producing T-cells was demonstrated to be inversely correlated with disease severity, suggesting that the severe disease is prevented by an immune response dominated by type-1 cytokines (Laucella et al., 2004). Thus, the inhibitory action of AS on key mechanisms of host resistance might be held responsible for the failure of the compound to control *T. cruzi* infection in vivo. However, we cannot ignore that there are differences among mouse strains in the mechanisms controlling the onset and fate of the Th1 response triggered by the parasite, and the important role of parasite genetics in the outcome of mouse *T. cruzi* infection (World Health Organization, 2014). Besides, we should consider the systemic bioavailability of AS after oral dosing, which depends on a variety of factors such as drug solubility, absorption, distribution, metabolism and excretion. Pharmacokinetic and pharmacodynamics studies have shown that AS is rapidly absorbed and hydrolyzed to the active metabolite dihydroartemisinin (DHA), primarily by plasma or tissue choline esterase. Following oral administration of AS, the tmax of DHA is very close to that of AS, indicating the rapid conversion of AS to DHA (Navaratnam et al., 1998). Furthermore, the stability of AS has been shown to be dependent on the pH, being rather short lived in the acid environment of the stomach and more stable at neutral pH (Olliaro et al., 2001). During the acute phase of infection with *T. cruzi*, the parasite experiences repeated cycles of multiplication inside infected cells prior to the development of a protective immune response; it is possible that prolonged exposition to the drug by more frequent dosing is required for a better anti-*T. cruzi* effect in vivo.

In our mouse model of infection, the effectiveness of BZ to reduce *T. cruzi*-induced inflammation was dependent on the scheme of treatment; the administration of nine doses of 100 mg/kg/d BZ decreased heart and skeletal muscle inflammation, whereas six doses of 50 and 100 mg/kg/d BZ were effective to reduce the extent of skeletal muscle, but not heart inflammatory cell infiltration. Interestingly, the beneficial action of the lower dose of BZ on skeletal muscle pathology was reverted by the addition of 75 mg/kg/d AS to the treatment protocol. However, the administration of high doses of BZ in combination with high doses of AS did not modify the action of BZ on skeletal muscles, suggesting that the potent trypanocidal action of BZ is not affected by the immunomodulatory action of AS.

Considering the data described earlier and that AS exhibited significant inhibition on the amastigote forms, the administration of AS might restrict its usefulness to the treatment of chronic *T. cruzi* infections with low parasitemia.

In *P. falciparum*, artemisinin was proposed to generate oxidative free radicals capable of intracellular damage through its interaction with heme, leading to cleavage of the peroxide bridge and formation of transient radical intermediates (Balint, 2001). The radicals bind specifically to the sarco-endoplasmic reticulum Ca2 + ATPase (SERCA) of the parasite, inhibiting its growth (Eckstein-Ludwig et al., 2003). Several membrane-associated Ca2+ ATPases have been described in *T. cruzi* which were suggested to be responsible, at least in part, for the inhibitory effect of artemisinins on this parasite (Mishina et al., 2007). Interestingly, BZ and NF exert their biological activity through the formation of free radicals (ROS) and/or electrophilic metabolites, but acting on *T. cruzi* cytochrome P450-related nitroreductases and inhibiting the synthesis of intracellular macromolecules (Maya et al., 2007). *T. cruzi* is deficient in detoxification mechanisms, making it particularly susceptible to oxidative stress (Moreno et al., 1982). It is possible that the high levels of free radicals provoked by BZ might render the parasite incapable to further increase ROS levels in response to AS. It will be interesting to study combinations of AS with trypanocidal drugs targeting at mechanisms other than the release of free radicals.

In summary, we demonstrate herein that AS is effective against the epimastigote and amastigote *T. cruzi* forms in vitro, acting on parasite strains from different geographical regions and developmental stages, at doses devoid of toxicity for normal human cells. However, the compound resulted unsuitable against the acute infection in vivo, probably related to the immune suppression properties of AS besides its anti-parasitic activity. Understanding AS structure-trypanocidal and -immune suppression activity–relationship might lead to the development of new drugs capable of eradicating in vitro as well as in vivo infections with the parasite.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.exppara.2015.05.014.

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