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In vitro activity of *N*-benzenesulfonylbenzotriazole on *Trypanosoma cruzi* epimastigote and trypomastigote forms

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ABSTRACT

Chagas disease is still an important health problem in Central and South America. However, the only drugs currently available for specific treatment of this disease may induce toxic side effects in the host. The aim of this work was to determine the activity of *N*-benzenesulfonylbenzotriazole (BSBZT) against the protozoan parasite *Trypanosoma cruzi*. The effects of BSBZT and benzotriazole (BZT) were compared to those of benznidazole (BZL) on epimastigote and trypomastigote forms. BSBZT was found to have an *in vitro* growth inhibitory dose-dependent activity against epimastigotes, with flow cytometry analysis confirming that the treated parasites presented size reduction. BSBZT showed an IC_{50} of 21.56 μg/mL (81.07 μM) against epimastigotes at 72 h of incubation, whereas BZT did not affect the growth of this parasite form. Furthermore, the toxic effect of BSBZT, was stronger and appeared earlier (at 24 h) in trypomastigotes than in epimastigotes, with the LC_{50} of this compound being 28.40 μg/mL (106.79 μM) against trypomastigotes. The concentrations of BSBZT used in this study presented low hemolytic activity and cytotoxicity. Consequently, at concentrations near IC_{50} and LC_{50} (25 μg/mL), BSBZT caused only 2.4% hemolysis and 15% of RAW 264.7 cell cytotoxicity. These results reveal the potential of BSBZT as a prototype in drug design for developing new anti-*T. cruzi* compounds.

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is considered to be a major infectious heart disease through the world and is recognized by WHO as one of the world's 13 most neglected tropical diseases. It has been a scourge to humanity since antiquity, and continues to be a relevant social and economic problem in many Latin American countries (De Souza et al., 2011; Hotez et al., 2007; Moncayo and Silveira, 2009). The only available drugs for the specific treatment of this disease are the nitrofuran derivates, nifurtimox and 2-nitroimidazole benznidazole (BZL), but both have significant activities only in the acute or recent chronic form of the disease. These drugs also have severe limitations, such as high frequency of undesirable toxic effects (Urbina et al., 2003; Castro and Diaz de Toranzo, 1988), long treatment protocol and limited efficacy (Urbina and Docampo, 2003; Jannin and Villa, 2007). Consequently, the development of new, safer and more effective trypanocidal compounds is still a challenge since these drugs are not given high priority by the R&D-based pharmaceutical industry (Sülsen et al., 2006).

The "fragment-based drug design" for library generation is a strategy for making new discoveries. It makes the assumption that the combination of active structures could lead to molecules with better or new pharmacological responses (Erlanson, 2006). Based on this methodology, we designed and prepared a library of *N*-benzenesulfonyl derivatives of active heterocycles to combine two biologically active moieties: benzenesulfonyl (BS) and heterocycles. *N*-Benzenesulfonyl derivatives of 1,2,3,4-tetrahydroquinolines have already demonstrated antiparasitic activity (Pagliero et al., 2010a,b). In the present study, we designed and prepared a library of *N*-benzenesulfonyl derivatives of benzotriazole (BZT), by replacing the H at N_1 of the BZT by substituted-benzenesulfonyl groups (BS). As was previously reported (Hergert et al., 2008), the compounds were prepared by sulfonylation of BZT, with their structural characterization agreeing with previously reported data (Katritzky et al., 1992; Ramana and Kudav, 1985). It is also worth emphasizing that BZT is considered to have low toxicity (DECOS, 2000).

Some previous works have revealed that BSBZTs display bactericidal activity and induce oxidative stress against *Staphylococcus*

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aureus (Hergert et al., 2008). Taking into account these results, the purpose of this study was to investigate the *in vitro* activity of BZT and BSBZT against a representative protozoan parasite *T. cruzi* (Tulahuen strain).

2. Materials and methods

2.1. Drugs

Benznidazole (Radanil®), a product of Roche, Brazil, and chloroquine (Sigma Co., St. Louis, MO, USA) were used as the reference drugs. The synthesis of BSBZT has been described in a previous report (Hergert et al., 2008).

The stock solutions were prepared at a concentration of 5 mg/mL solubilized in polyethyleneglycol (PEG) 400 and ethyl alcohol at a 7:3 ratio (Lamas et al., 2006) with the final concentration of the solvent being lower than 2% for all the experiments. The drug-free control medium contained similar concentrations of PEG and ethyl alcohol (parasites treated with solvent alone). All drugs were freshly diluted prior to use.

2.2. Parasite cultivation and incubation with drugs

Epimastigote forms were grown at 28 °C in brain heart infusion medium (BHI) (Becton Dickinson, France), supplemented with 10% (v/v) fetal bovine serum (FBS), 200 mg/mL hemin, 100 U/mL penicillin, and 100 mg/mL streptomycin. Parasites were harvested at the exponential growth phase, and centrifuged at 5000*g* for 10 min (Guiñazú et al., 2007), with cultures being initiated with a cell density of 2×10^6 parasites/mL. Epimastigotes were incubated in BHI supplemented with 10% (v/v) FBS with increasing doses of BSBZT or BZT (0–100 μg/mL), or without drugs in 24-well flat-bottom plates for 48, 72 and 96 h at 28 °C. BZL was used as the reference drug (Valdez et al., 2009).

T. cruzi growth was determined by counting the parasites with a haemocytometer chamber, and the IC_{50} values (50% inhibition concentration) were determined by using the Probit statistical program (Jorqueira et al., 2006).

Bloodstream forms were harvested by heart puncture from *T. cruzi*-infected BALB/c mice (purchased from Centro Nacional de Energía Atómica, Buenos Aires-Argentina). All mice were maintained according to the National Research Council's guide for the care and use of laboratory animals (Guiñazú et al., 2007), and protocols were approved by the Animals Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba. Parasites were maintained by serial passages from mouse to mouse (Carrera-Silva et al., 2010).

Trypomastigotes (10^6/mL) of Tulahuen strain were incubated at 37 °C for 24 h in triplicate, in the presence of increasing doses of BSBZT or BZT (0–100 μg/mL) diluted in Dulbecco's modified medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine (DMEM). Parasites were also incubated with BZL (3–30 μg/mL) and the assays conducted under the same conditions. To evaluate the anti-trypanosomal effect, direct quantification of live parasites after 24 h of treatment was determined using a Neubauer chamber (De Souza et al., 2011) and LC_{50} (the drug concentration that kills 50% of the parasites) was obtained by using Probit statistical program (Finney, 1971). Three independent experiments were performed in triplicate.

Images of the effects of BSBZT and BZT on trypomastigotes after 24 h of incubation were recorded for 10 s on NIKON ECLIPSE TE-2000 U Microscope equipped with UV and fluorescence broadband, and NIKON ACT-2U Imaging software was also employed.

2.3. Flow cytometry assay

Epimastigotes were grown for 48 and 72 h in BHI supplemented with 10% (v/v) FBS (untreated control) or treated with 25 or 50 μg/mL of BSBZT, BZT or BZL, and using PEG–ethanol as the solvent control. Then, parasites were washed twice with phosphate buffer saline (PBS), pH 7.4, suspended with ISOTON® II Diluent and analyzed in a flow cytometer (Ortho Diagnostic System, Raritan, NJ). The cellular parameters were collected and displayed using forward (size) light-scatter plots, and cells exhibiting size reduction were determined with a total of 40,000 events acquired. Data were analyzed using WinMDI software, and three independent experiments were performed (Deolindo et al., 2005).

2.4. Hemolytic activity assay

A hemolysis assay was used to determine the potential of the compound to hemolyze red blood cells from humans with heparinized red blood cells being suspended in PBS, pH 7.4 to 4% (v/v). Erythrocytes were incubated in the presence of 25–100 μg/mL of BSBZT or BZT, using chloroquine as the reference drug. Then, after 1 h of incubation at 37 °C, the blood samples were centrifuged at 1000*g* for 5 min, and the supernatants were separated from the pellet. The absorbance was measured at 540 nm. PBS and distilled water were used as negative and positive controls, respectively. The hemolytic activity was calculated as: Hemolytic activity (%) = $(A_{540\ nm}$ sample$-A_{540\ nm}$ saline$)/(A_{540\ nm}$ $H_2O-A_{540\ nm}$ saline$) \times 100$ (Barrett et al., 2001; Valdez et al., 2009).

2.5. Mammalian cell toxicity assays

The cytotoxicity of the compounds was evaluated in mouse macrophage-derived RAW 264.7 cell line by microscopic examination of cell cultures after 72 h of incubation. All compounds were tested at the same concentrations used for screening antitrypanosomal activity.

The cell line was maintained in RPMI culture medium supplemented with 10% FBS in a 5% CO_2 atmosphere at 37 ± 0.5 °C. Toxicity assays were performed by incubating cells (10^5 cells/well) with different concentrations of BSBZT or BZL in 96-well culture plates. After 72 h, the culture medium was gently removed; 200 μL of a solution of 0.2% Trypan Blue was added, and cells were counted in an inverted microscope for the following 30 min. Each experiment was performed in triplicate, and cells unable to exclude Trypan blue were considered to be non-viable (Lamas et al., 2006).

2.6. Statistical analysis

The student's *t*-test was used to carry out the statistical analysis. A *P*-value < 0.05 was considered significant.

3. Results

3.1. In vitro evaluation of the drug effects on T. cruzi epimastigote

The addition of different concentrations of BSBZT to the epimastigotes resulted in growth arrest after 48, 72 or 96 h of incubation. A substantial decrease in the parasite number was observed after incubation at 72 h (Fig. 1A) and 96 h (data not shown) compared to epimastigotes treated with solvent (solvent control). Moreover, BSBZT presented a dose-dependent activity with a decrease of about 50% at 25 μg/mL and 64% at 50 μg/mL at 72 h of incubation, while BZT did not have this effect. These results imply that the substituted-benzenesulfonyl group contributed to

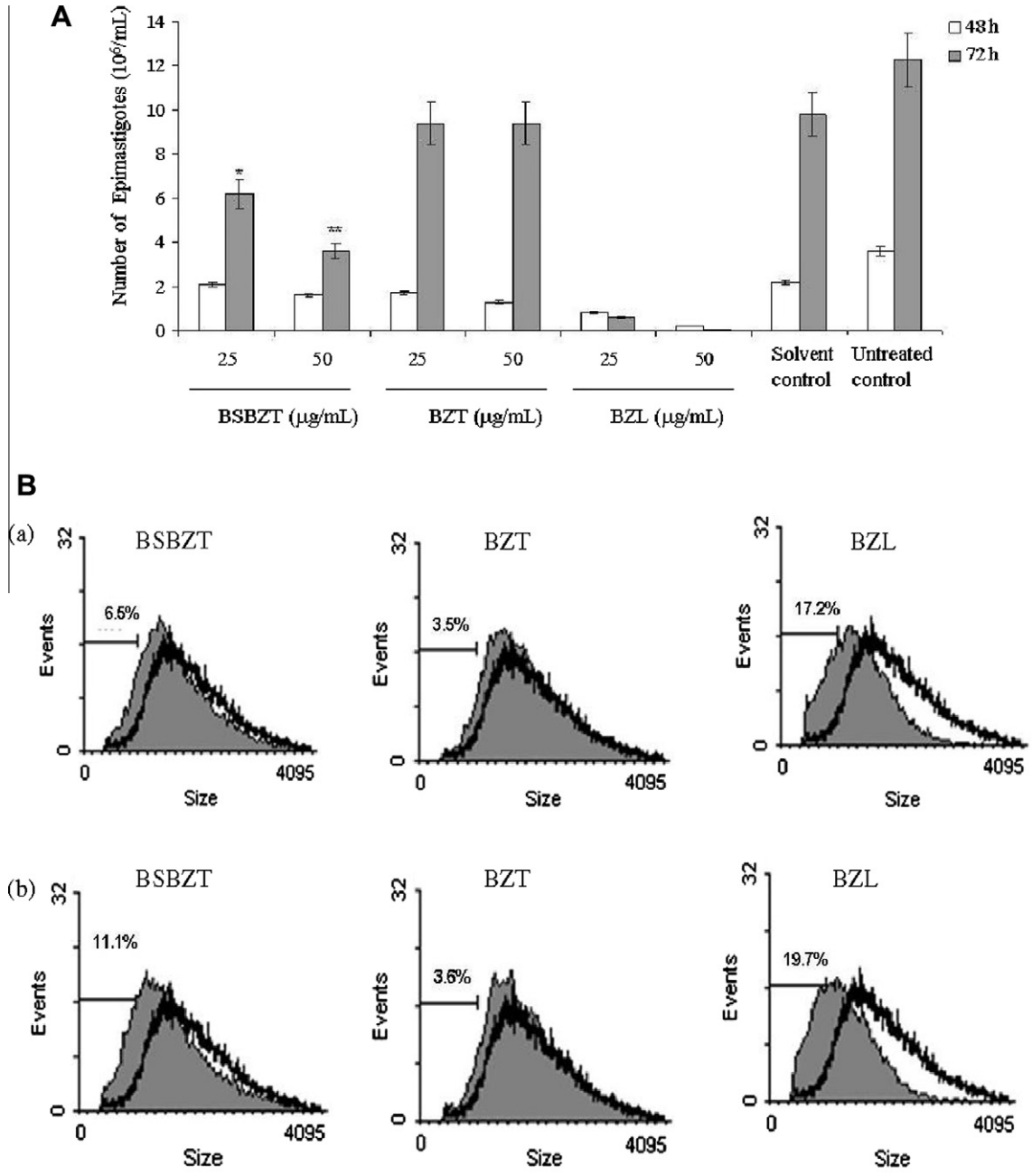

Fig. 1. *In vitro* evaluation of BSBZT and BZT effects on *Trypanosoma cruzi* epimastigotes. (A) Parasites were treated with 25 or 50 μg/mL of BSBZT, BZT or BZL for 48 and 72 h at 28 °C and the counting of epimastigotes was performed in a Neubauer chamber. Data are shown as means ± SD. Significant differences with respect to epimastigotes treated with solvent (solvent control) are indicated by *($p < 0.05$), **($p < 0.01$). (B) Effects of BSBZT, BZT or BZL on epimastigotes of *Trypanosoma cruzi* determined by flow cytometry analysis. Representative histograms show size decrease in epimastigotes treated with 25 μg/mL (a) or 50 μg/mL (b) of these drugs for 72 h. In each plot, the black line histogram represents the untreated control and the filled gray histogram represents results obtained with the drugs.

anti-trypanosomal activity. In addition, the IC_{50} obtained at 72 h of incubation for BSBZT was 21.56 μg/mL (81.07 μM) while the IC_{50} obtained for BZL (positive control) was 2.74 μg/mL (10.54 μM) (Table 1). The results show that the solvent did not affect the parasite number.

The activity of BSBZT on epimastigotes was also evaluated by flow cytometry at 48 h (Table 2) and at 72 h (Table 2 and Fig. 1B) of incubation. Parasite size reduction was observed with 25 and 50 μg/mL BSBZT compared to those treated with solvent alone. These values were lower than the percentages obtained with BZL. No effect was produced with BZT.

3.2. In vitro evaluation of the drug effects on T. cruzi trypomastigote forms

We also evaluated the potential effects of BSBZT and BZT on trypomastigotes, the infective form of the parasite. Interestingly, 50 μg/mL BSBZT was effective against trypomastigotes at an earlier time than on epimastigote forms. For this drug concentration, the percentages of dead trypomastigotes were higher than 95% at 24 h. By using 25 μg/mL, the percentages obtained were about 32% at 24 h of incubation (Fig. 2). It is important to note that a size reduction and a decrease in the parasite motility were observed in

Table 1
Percentage of live epimastigotes of *Trypanosoma cruzi* after 72 h of treatment with BSBZT and BZL.

	Concentration of drug (μg/mL)					
	7.5	15	25	50	100	IC_{50} (μg/mL)[a]
BSBZT	63 ± 8	59 ± 9	50 ± 5	40 ± 10	22 ± 5	21.56
BZL	20 ± 12	9 ± 10	6 ± 2	1 ± 3	0 ± 0	2.74

Results represent the means ± SD of triplicates assays.
[a] IC_{50} was calculated by using the Probit statistical program. Control corresponds to 100% of living cells with no drug treatment.

Table 2
Percentages of epimastigotes with size reduction determined by flow cytometry after incubating for 48 and 72 h with 25 or 50 μg/mL of BSBZT, BZT and BZL.

		48 h	72 h
Control		2.0 ± 0.6	2.5 ± 0.8
Solvent		2.6 ± 0.4	2.9 ± 0.1
BSBZT	25 μg/mL	4.9 ± 0.5[a]	6.4 ± 0.1[a]
	50 μg/mL	6.9 ± 0.2[a]	10.5 ± 0.6[a]
BZT	25 μg/mL	2.5 ± 0.1	3.6 ± 0.2
	50 μg/mL	2.5 ± 0.1	3.5 ± 0.1
BZL	25 μg/mL	15.8 ± 0.7[a]	17.4 ± 0.3[a]
	50 μg/mL	16.4 ± 0.7[a]	20.0 ± 0.3[a]

Cells exhibiting size reduction were determined with a total of 40,000 events acquired.
The values shown are means ± standard deviation from three independent experiments.
[a] Represents significant differences with respect to epimastigotes treated with solvent ($p < 0.05$).

the remaining trypomastigotes (Supplemental movie 1) compared to the untreated parasites (Supplemental movie 2). The LC_{50} obtained for BSBZT was 28.40 μg/mL (106.79 μM) at 24 h of incubation.

BZT had activity against trypomastigotes, although to a lesser extent compared to BSBZT, causing only 14% of dead parasite at 25 μg/mL (Supplemental movie 3) and 21% at 50 μg/mL after 24 h of incubation. The results are presented in Fig. 2. It is important to remark that BZT at 50 and 100 μg/mL did not modify the size of the remaining trypomastigotes after 24 h of incubation, with only parasites without motility being observed.

Table 3
Hemolytic activity of BSBZT and BZT on human red blood cells after 1 h of incubation at 37 °C.

	Hemolytic activity[a] (%)	
Concentration (μg/mL)	BSBZT	BZT
25	2.4 ± 0.9	3.0 ± 1.0
50	3.9 ± 1.1	3.3 ± 0.9
100	6.0 ± 0.7	4.0 ± 1.1

[a] The values shown are means ± standard deviation from three independent experiments.

Table 4
Cytotoxicity activity of BSBZT on mouse macrophage-derived RAW 264.7 cell line after 72 h of incubation at 37 °C.

	Cellular viability[a] (% of cells alive)	
Concentration (μg/mL)	BSBZT	Benznidazole
12.5	98 ± 2	94 ± 3
25	84 ± 4	86 ± 2
50	76 ± 8	86 ± 3

Control corresponds to 100% of living cells with no drug treatment. Benznidazole was used as the reference drug.
[a] The values shown are means ± standard deviation from three independent experiments.

3.3. Hemolytic activity of BSBZT and BZT

Neither BSBZT nor BZT affected the red blood cell integrity at concentrations which inhibited the growth of epimastigote and trypomastigote forms of *T. cruzi*, with 25 μg/mL BSBZT or BZT only yielding about 2.4% hemolysis (Table 3). These percentages were low as in the case of the values obtained with chloroquine (1.7%).

3.4. Cytotoxicity assay of BSBZT in mammalian cells

In our experiments using 25 μg/mL BSBZT, the percentage of live macrophage cells was 85% at 72 h of culture. This concentration was selected because it approximately corresponded to the IC_{50} for epimastigotes and the LC_{50} for trypomastigotes. When cells were incubated with 50 μg/mL BSBZT, the percentage of cell survival was 76% (Table 4). In addition, no toxic effects of the solvent were observed (data not shown).

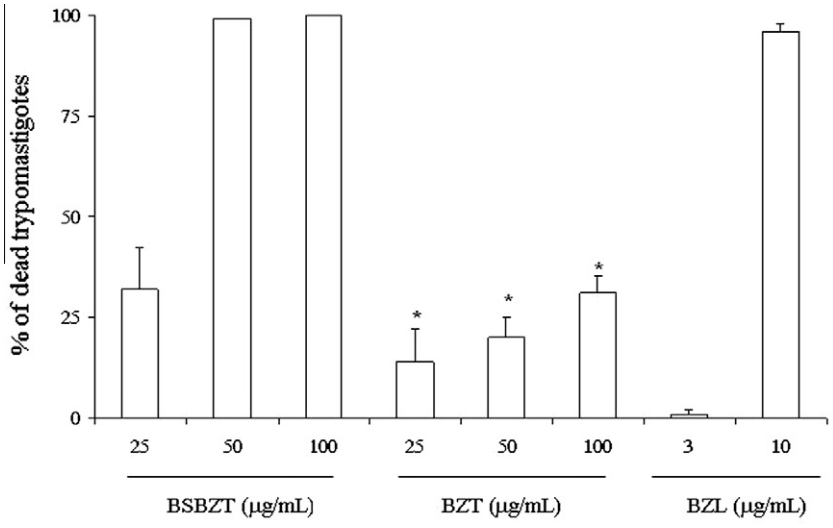

Fig. 2. *In vitro* evaluation of BSBZT and BZT effects on *Trypanosoma cruzi* trypomastigotes. Parasites were incubated with different concentrations of BSBZT and BZT for 24 h at 37 °C. BZL treatment was used as a positive control. The results are expressed as a percentage of dead trypomastigotes. Data are shown as means ± SD. *Represents significant differences for trypomastigotes treated with BSBZT with respect to BZT for the same concentrations ($p < 0.05$).

4. Discussion

Many BZT derivatives have been reported to be active pharmacological compounds (Carta et al., 2007; Dixit et al., 2006; Nanjunda Swamt et al., 2006), but none of these have a BS moiety bound to the nitrogen. As BS moiety is frequently present in molecules with biological activity, the combination of this substituent and a biologically active heterocycle may help in designing new derivatives of anti-parasite drugs, as previously reported (Pagliero et al., 2010a,b).

In the present study, we explored for the first time the effect of BSBZT against the *T. cruzi* protozoan parasite. Incubation of epimastigotes and trypomastigotes with this drug resulted in a decrease in the number of parasites, which may have been be related to a lower epimastigote proliferation capacity as well as to the induction of parasite death. By flow cytometry, an applied method to determine the effects of different compounds on *T. cruzi* epimastigotes (Cardoso et al., 2008; Villarreal et al., 2004; Yong et al., 2000), a size reduction of epimastigotes treated with BSBZT was also observed. In previous investigations, the reduction in parasites was reported to be the result of the generation of parasite apoptosis (Deolindo et al., 2005), whilst others researchers observed growth arrest and subsequent cell lysis (Urbina et al., 2000). However, considering the diverse mechanisms reported as being responsible for growth inhibition, more studies are still needed to understand how BSBZT toxicity affects the parasite. Since the exact mechanism of the action of BSBZT is still unknown, the elucidation of the parasite target molecules involved in the inhibition of parasite growth should be the aim of future research.

The present study has shown the useful ability of BSBZT to produce inhibition of epimastigotes growth. Moreover, a greater and faster inhibitory activity of BSBZT on trypomastigotes compared to the epimastigote forms was observed. This decrease in parasite motility and size reduction provoked by this drug is very important in anti-parasite activity, since trypomastigote motility is necessary for cell invasion and consequently for parasite multiplication (Sibley, 2011).

It is worthwhile pointing out that we were able to perform the experiments by using PEG 400 and ethanol (Li and Zhao, 2007) to dissolve BZT and BSBZT with successful results. The solubilization of BSBZT presents a difficult challenge owing to its low solubility in aqueous solutions, and other solvents have been frequently utilized in biological assays. Nevertheless, in our case, using a mixture of PEG 400 and ethanol, the compound was both soluble and stable. Furthermore, this solvent did not affect the viability of the parasites as determined by counting and by flow cytometry. Lamas et al. (2006) also previously used PEG 400 and ethanol as the solvent of choice, due to its good solubilization properties and overall acceptability in terms of its side-effect profile. They prepared several systems based on PEG 400, and the solubility of BZL increased when the cosolvents PEG 400 and ethanol were mixed under physiological pH conditions.

An important criterion in the search for active compounds with potential therapeutic use against *T. cruzi* is to determine whether they produce toxic effects on mammalian host cells (Luize et al., 2006). Our results revealed that BSBZT has activity against the epimastigotes and trypomastigotes, although less than that of the reference drug, BZL. In addition, the BSBZT compound had a low cytotoxic effect on RAW cells and on hemolytic activity in human erythrocytes.

In conclusion, this is the first report showing the anti-*T. cruzi* effect of BSBZT. Using the easy synthetic approach of "fragment-based drug design" and *in vitro* results, we found the new scaffold *N*-benzenesulfonylbenzotriazole to be a useful anti-*T. cruzi* entity. Future research should examine the potential applicability of BSBZT in further *in vivo* studies in experimental models. This compound may prove helpful in the further exploration of the structural requirements for anti-parasitic activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2012.02.028.

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