Buthionine Sulfoximine Increases the Toxicity of Nifurtimox and Benznidazole to *Trypanosoma cruzi*

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L-Buthionine (S,R)-sulfoximine (BSO) increased the toxicity of nifurtimox and benznidazole toward the epimastigote, trypomastigote, and amastigote forms of *Trypanosoma cruzi*. BSO at 500 μ M decreased total glutathione-derived thiols by 70 to 80% in 48 h. In epimastigotes, 500 μ M BSO decreased the concentration of nifurtimox needed to inhibit constant growth of the parasites by 50%, from 14.0 to 9.0 μ M, and decreased that of benznidazole from 43.6 to 24.1 μ M. The survival of epimastigotes or trypomastigotes treated with nifurtimox or benznidazole, as measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction, was significantly decreased by 500 μ M BSO. In Vero cells infected with amastigotes, 25 μ M BSO was able to potentiate the effect of nifurtimox and benznidazole as measured by the percentage of infected Vero cells multiplied by the average number of intracellular amastigotes (endocytic index). At 0.5 μ M nifurtimox, the proportion of Vero cells infected decreased from 27 to 20% and the endocytic index decreased from 2,500 to 980 when 25 μ M BSO was added. Similar results were obtained with benznidazole- and BSO-benznidazole-treated cells. This study indicates that potentiation of nifurtimox or benznidazole by BSO could decrease the clinical dose of both drugs and diminish the side effects or the length of therapy.

Chagas' disease, or American trypanosomiasis, is a serious parasitic ailment in Latin America (33). The World Bank estimated an annual loss of 2.74 million disability-adjusted life years, representing an economic loss to the countries in which the disease is endemic of equivalent to U.S. \$6.5 billion per year (32). Together with malaria, leishmaniasis, and African trypanosomiasis (sleeping sickness), Chagas' disease is a major parasitic cause of death and hardship, especially in the impoverished regions of the developing world. Chagas' disease is widely distributed throughout the Americas, and it is endemic in 21 countries, from Mexico in the north to Argentina and Chile in the south. According to the World Health Organization, there are 16 to 18 million people already infected and some 100 million (25% of the Latin American population) at risk of becoming infected, with more than 50,000 people dying every year (33).

Chagas' disease is caused by *Trypanosoma cruzi*, a flagellate protozoan transmitted to humans either by transfusion of infected blood, from an infected mother to her child, or by its most important vector, a blood-sucking insect (called vinchuca, chipo, barbeiro, kissing bug, cone nose, or assassin bug) which carries the parasite in its contaminated feces. Contagion usually occurs by contact of the insect's feces with the eyes, mouth, or open skin lesions. Most efforts to control the problem are being focused on insect control. However, even if the vectors could be immediately eradicated, as the disease evolves over decades and ends only with the host's death, many patients

would harbor the chronic variant and act as a real reservoir for the protozoa that would keep the door open for reinfection of the general population.

In about one-third of all acute cases, a chronic form develops around 10 to 20 years later, causing irreversible damage to the heart, esophagus, and/or colon, with severe disorders of nerve conduction in these organs. Patients with severe chronic disease become progressively more ill and ultimately die, usually from their heart condition.

At present, there is no effective treatment for chronic cases, and there is neither a vaccine nor preventive treatment. For acute, recent, or congenital disease, there are two drugs available for treatment: nifurtimox (manufactured by Bayer under the trade name Lampit), a nitrofurantoin derivative, and benznidazole (made by Roche under the trade name Radanil, Rochagan, or Roganil), a nitroimidazole derivative (6). Both compounds have low efficacy and severe side effects, particularly in adult patients. Unfortunately it was recently decided to discontinue the production of nifurtimox. Both drugs are believed to kill or inhibit the growth of the parasites by increasing their oxidative stress (8, 9, 10, 13, 19, 26).

The parasite's life cycle presents three forms. The epimastigote forms are found in axenic cultures and in the insect intestine and are replicative but unable to produce infection. These epimastigotes are transformed into infective metacyclic trypomastigotes in the last portion of the insect intestine. The metacyclic forms are also found in old axenic cultures of epimastigotes. The nonreplicative but infective trypomastigote forms are found in circulating blood. The replicative amastigote forms are found inside the host's cells.

In previous communications, and using the epimastigote culture forms of several strains of *T. cruzi*, we reported that the

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TABLE 1. Effect of buthionine sulfoximine on thiol concentration in epimastigote, trypomastigote, and amastigote forms of *T. cruzi*

Thiol	Concn ^a in:								
	Epimastigotes			Trypomastigotes			Amastigotes		
	Control (nmol of GSH/mg of protein)	BSO nmol of GSH/ mg of protein	% of control	Control (nmol of GSH/mg of protein)	BSO nmol of GSH/ mg of protein	% of control	Control (nmol of GSH/mg of protein)	BSO ^a nmol of GSH/ mg of protein	% of control
GSH	2.7 ± 0.02	1.15 ± 0.31	42.6	1.74 ± 0.19	0.74 ± 0.01	42.5	1.50 ± 0.20	0.70 ± 0.05	46.6
GSH-spermidine	1.68 ± 0.36	0.19 ± 0.06	11.3	1.04 ± 0.20	0.12 ± 0.02	11.5	0.50 ± 0.12	0.12 ± 0.02	24
T(SH) ₂	7.41 ± 1.06	0.80 ± 0.28	10.8	2.38 ± 0.44	0.28 ± 0.08	11.8	2.90 ± 0.15	0.40 ± 0.01	13.8
Total	11.79	2.14	18.2	5.16	1.14	22.1	4.90	1.22	24.9

^a Values correspond to the mean ± SD from three independent experiments. For details, see Materials and Methods. BSO was added 48 h before thiol determination at a final concentration of 500 µM.

susceptibility of *T. cruzi* to nifurtimox and benznidazole is associated with the levels of free and conjugated glutathione (GSH) (20, 29). Buthionine sulfoximine (BSO) is an amino acid analog that inhibits the synthesis of glutathionylcysteine and thus of glutathione (Glu-Cys-Gly:GSH) and trypanothione [bis-glutathionyl spermidine:T(SH)₂]. BSO is an analog of the transition state or enzyme-bound intermediate formed in the reaction catalyzed by γ-glutamylcysteine synthetase. BSO is phosphorylated in the active site of this enzyme, and irreversible inhibition results from noncovalent but tight binding of buthionine sulfoximine phosphate. This inhibition occurs in a dose- and time-dependent manner (15, 16, 22, 23). When this drug is added to parasite cultures, the concentrations of intracellular glutathione and trypanothione are greatly decreased (21, 24).

Based on the above considerations, we undertook in this work to study how nifurtimox and benznidazole and their combination with buthionine sulfoximine affect the toxicity towards the trypomastigote, amastigote, and epimastigote forms of the parasite. The results in this communication show that buthionine sulfoximine increases the toxicity of nifurtimox and benznidazole in the parasite forms studied.

MATERIALS AND METHODS

Materials. Tryptose, fetal calf serum, yeast extract, and tryptone were obtained from Difco. Hemin, L-buthionine (S,R)-sulfoximine, N-2(hydroxyethyl)-

yl)piperazine-N'-(3-propanesulfonic acid) (HEPPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenazine metosulfate, and all other chemicals were purchased from Sigma Chemical Co.

***T. cruzi* forms.** (i) **Epimastigotes.** Epimastigotes of the MF strain were grown at 28°C in modified Diamond's medium as described previously (20). The cultures were initiated with a cell density of 3×10^6 epimastigotes per ml, and the drugs were added 24 h later. Cell densities were measured by direct counting with a hemocytometer. A total of 80×10^6 parasites are equivalent to 1 mg of protein or 12 mg of wet weight.

(ii) **Trypomastigotes.** Vero cells were infected with aged MF epimastigote cultures, which contain metacyclic trypomastigotes (7). Subsequently, the trypomastigotes harvested from this culture were used to reinfect further Vero cell cultures at a density of 10^6 parasites per 25 cm². Vero cell cultures infected with trypomastigotes were incubated at 37°C in humidified air and 5% CO₂ for 5 to 7 days. After that time, the culture medium was collected and centrifuged at $3,000 \times g$ for 5 min, and the trypomastigote-containing pellet was resuspended in RPMI supplemented with 5% fetal bovine serum and penicillin-streptomycin at a final density of 10^7 parasites/ml. A total of 210×10^6 trypomastigotes are equivalent to 1 mg of protein or 12 mg of wet weight.

(iii) **Amastigotes.** Amastigotes were obtained with the same technique used for trypomastigotes, but Vero cells were infected at a density of 6×10^6 parasites per 25 cm², which induces cell rupture and release of amastigotes into the medium. A total of 210×10^6 amastigotes are equivalent to 1 mg of protein or 12 mg of wet weight.

Epimastigote culture growth inhibition. Buthionine sulfoximine dissolved in distilled water and four to five different concentrations of nifurtimox or benznidazole dissolved in dimethyl sulfoxide were added to a suspension of 3×10^6 *T. cruzi* epimastigotes (MF strain) per ml. Final concentrations in the culture growth were between 1 and 30 µM for each drug. Parasite growth was monitored by nephelometry for 10 days. No toxic effect of dimethyl sulfoxide was observed (19).

The culture growth constant (k_c) for each drug concentration employed was calculated by using the epimastigote exponential growth curve (regression coefficient, >0.97 ; $P < 0.05$). The slope resulting from plotting the natural log of the nephelometry reading versus time corresponds to the k_c (days⁻¹). The drug concentration at which a 50% reduction of growth is obtained compared to control assays (IC_{k_c50}) is calculated by linear regression analysis from the k_c values obtained for each drug concentration studied (28).

Glutathione and trypanothione measurement by high-pressure liquid chromatography. Epimastigotes, trypomastigotes, amastigotes, or Vero cells equivalent to 1 mg of protein were suspended in 40 mM HEPPS–2 mM EDTA buffer (pH 8) with 2 mM monobromobimane and incubated at 70°C for 5 min. To precipitate proteins, 4 M methanesulfonic acid was added. After 10 min of incubation at 4°C, the sample was centrifuged at $10,000 \times g$ for 3 min. A 20-µl sample was applied to a Lichrospher 100 RP-1 8 (5 µm) reverse-phase column and eluted during 60 min at a flow rate of 1 ml/min. The eluting gradient was as follows: 0 to 20 min, 90% solvent A (0.25% [wt/vol] lithium α-camporsulfonate, pH 2.35) and 10% solvent B (25% [wt/vol] 1-propanol in solution A); 20 to 40 min, linear gradient of 10 to 50% solvent B; 40 to 60 min, 50% solvent B isocratic in solvent A, returning to the initial conditions in 30 min. All measurements were performed with a Merck-Hitachi L-6200 Intelligent Pump high-pressure chromatograph with an F-1050 fluorescence detector and a D-2500 integrator. Emis-

TABLE 2. Effects of nifurtimox, benznidazole, and buthionine sulfoximine on *T. cruzi* epimastigote growth

Treatment	IC _{k_c50} (µM) ^a
Nifurtimox.....	14.01 ± 1.75
Nifurtimox + 500 µM BSO.....	9.04 ± 0.03*
Benznidazole.....	43.64 ± 1.59
Benznidazole + 500 µM BSO.....	23.96 ± 0.77**
BSO.....	13,600 ± 520

^a IC_{k_c50} values correspond to the drug concentrations needed to reduce the growth constant by 50%. They were calculated by linear regression analysis of the plot of growth constant versus drug concentration. The control k_c value was 0.480 ± 0.02 day⁻¹ (r^2 , 0.97; $P < 0.005$). Values correspond to the means ± SD from three independent experiments. Nifurtimox and benznidazole IC_{k_c50}s were significantly lowered by the addition of 500 µM BSO ($P = 0.0385$ [*] and $P < 0.0001$ [**]) by two-way ANOVAs. See Materials and Methods for details.

TABLE 3. Cytotoxic effects of nifurtimox, benznidazole, and buthionine sulfoximine on *T. cruzi* epimastigotes

Treatment	IC ₅₀ (μM) ^a
Nifurtimox.....	8.31 ± 1.01
Nifurtimox + 500 μM BSO.....	2.61 ± 0.31*
Benznidazole.....	14.26 ± 0.06
Benznidazole + 500 μM BSO.....	9.29 ± 0.70**
BSO.....	8,110 ± 320

^a IC₅₀ values correspond to the drug concentrations needed to decrease the reduction of MTT (absorbance) by 50%. They were calculated by linear regression analysis of the plot of survival constant (k_s) versus drug concentration. The k_s of the control was 0.231 day⁻¹ (r^2 , 0.96; P < 0.005). Values correspond to the means ± SD from three independent experiments. Nifurtimox and benznidazole IC₅₀s were significantly lowered by the addition of 500 μM BSO (P = 0.0001 [*] and P < 0.001 [**] by two-way ANOVAs). See Materials and Methods for details.

sion and excitation wavelengths were 480 and 385 nm, respectively. The method detects reduced free thiols but not disulfides (12, 18).

Cytotoxicity. Cytotoxicity assays were performed by using the MTT reduction method as described previously (25). Briefly, 3×10^6 epimastigotes/ml were incubated with different drug concentrations in Diamond's culture medium at 28°C; 1×10^7 trypomastigotes were incubated in fetal bovine serum-RPMI culture medium at 37°C for 48 h. An aliquot of the parasite suspension was extracted and incubated in a flat-bottom 96-well plate, and MTT was added at a final concentration of 0.5 mg/ml, incubated at 28°C for 4 h, and then solubilized with 10% sodium dodecyl sulfate–0.1 mM HCl and incubated overnight. Formazan formation was measured at 570 nm with the reference wavelength at 690 nm in a multiwell reader (Labsystems Multiskan MS).

The culture growth constant or survival constant, determined by MTT reduction per milliliter of parasite culture as monitored daily, was named k_s (s is for epimastigote survival). The k_s for each drug concentration employed was calculated by using the epimastigotes' exponential absorbance curve (regression coefficient, >0.96; P < 0.05). The slope resulting from plotting the natural logarithm of absorbance versus time corresponds to the k_s (days⁻¹). The drug concentration at which a 50% reduction of absorbance is obtained compared to control assays (IC₅₀) is calculated by linear regression analysis from the k_s values obtained for each drug concentration studied.

Endocytic index. Confluent Vero cells in 25-cm² Falcon flasks were infected with trypomastigotes (MF strain) and incubated in RPMI medium supplemented with 5% fetal bovine serum, penicillin, and streptomycin. Nifurtimox, benznidazole, and BSO were added as indicated in the tables. The effect on the amastigotes was monitored by direct visualization under a phase-contrast microscope (Zeiss Photomicroscope II) and microphotography. For each culture flask, total Vero cells, infected Vero cells, and the number of intracellular amastigotes per infected cell were determined in 10 random fields. The endocytic index (14) corresponds to the percentage of infected Vero cells multiplied by the average number of intracellular amastigotes.

Statistical analysis. Values are expressed as means ± standard deviations from three independent experiments. Linear regression analysis and the two-way

TABLE 4. *T. cruzi* trypomastigote survival when treated with nifurtimox and benznidazole alone or in combination with BSO

Drug	Concn (μM)	% of viable parasites ^a with:	
		Drug alone	Drug plus BSO (500 μM)
Nifurtimox	0	100 ± 7	100 ± 10
	2	97 ± 6	73 ± 8*
	4	88 ± 6	33 ± 18**
	6	73 ± 12	16 ± 12**
Benznidazole	0	100 ± 11	100 ± 5
	5	96 ± 11	44 ± 6**
	10	91 ± 13	22 ± 9**
	25	60 ± 7	13 ± 8**

^a One hundred percent of viable parasites corresponds to the absorbance value measured at 570 to 690 nm per milligram of protein in the MTT assays of the trypomastigotes not subjected to drug treatment. Values correspond to the means ± SD from three independent experiments. The BSO effect at all drug concentrations was statistically significant compared with the respective control (P > 0.001 in the two-way ANOVA) (*, P < 0.001; **, P < 0.0001 [Tukey post hoc test]). See Materials and Methods.

analysis of variance (ANOVA) test were performed when necessary with Prism GraphPad 2.01 software (GraphPad Software Inc.).

RESULTS

The effects of BSO on thiol content (Table 1), growth constant (Table 2), and parasite survival (Tables 3 and 4) in the epimastigote and trypomastigote forms were studied in the concentration range from 50 to 20,000 μM. BSO IC₅₀ and IC₅₀ values were several times greater than 500 μM. At BSO concentrations of higher than 2,000 μM for 48 h, the thiol concentration decreased to near zero and parasite growth and survival were decreased. Thus, 500 μM BSO was the concentration that produced a decrease in the thiol level of greater than 75% without affecting parasite growth or survival (Tables 1, 2, and 3).

Table 1 shows the effect of 500 μM BSO on the contents of glutathione and glutathione-derived thiols in the three forms of the *T. cruzi* parasite. A significant decrease in the concentration of thiols was observed, mainly at the expense of trypanothione.

The effects of nifurtimox and benznidazole alone and in combination with BSO upon epimastigote growth were studied

TABLE 5. Effects of buthionine sulfoximine on toxicity of nifurtimox and benznidazole against *T. cruzi* intracellular amastigotes^a

Drug concn (μM)	Nifurtimox				Benznidazole			
	Infected Vero cells (%)		Endocytic index		Infected Vero cells (%)		Endocytic index	
	Without BSO	With BSO (25 μM)	Without BSO	With BSO (25 μM)	Without BSO	With BSO (25 μM)	Without BSO	With BSO (25 μM)
0	32.4 ± 2.6	35.3 ± 2.8	5,505 ± 340	5,747 ± 359	29.0 ± 1.7	26.0 ± 1.6	5,787 ± 344	5,792 ± 347
0.5	27.0 ± 2.2	20.6 ± 1.7	2,494 ± 199	980 ± 78	17.7 ± 1.1	12.7 ± 0.8	4,015 ± 240	1,952 ± 117
1	24.3 ± 1.9	17.7 ± 1.4	802 ± 64	462 ± 69	15.8 ± 1.4	8.9 ± 0.5	2,356 ± 141	385 ± 23

^a Vero cells previously infected with MF trypomastigotes were incubated for 72 h in the presence or absence of benznidazole and nifurtimox alone or in combination with BSO. The culture medium with or without the drugs was changed every 24 h. At 72 h the total Vero cells, infected Vero cells, and number of amastigotes per Vero cell were determined by direct microscopic visualization, and the endocytic index was calculated as described in Materials and Methods. All values correspond to the means ± SD from three independent experiments. P values were <0.0001 from the two-way ANOVA for the percentage of infected Vero cells and the endocytic index with nifurtimox versus nifurtimox-BSO and benznidazole versus benznidazole-BSO treatments.

(Table 2). The addition of BSO at 500 μ M lowered the IC_{50} of nifurtimox by 65% and that of benznidazole by 55%.

Another way of studying the toxic effects of nifurtimox, benznidazole, and BSO during parasite growth is to monitor the mitochondrial reduction of MTT to formazan (25). Table 3 shows that the toxicities of nifurtimox and benznidazole were increased about twofold by the addition of BSO. When the experiments were done with the nonreplicative trypomastigote forms, the toxicity increased significantly (between three- and fourfold) by the addition of BSO to both drugs (Table 4). Thus, 500 μ M BSO lowered the IC_{50} s of nifurtimox and benznidazole to approximately 6 and 25 μ M, respectively. Similar results were obtained with the *T. cruzi* amastigotes (data not shown). Also, by direct microscopic observation, there was a significant decrease in the motility of the BSO- and drug-treated trypomastigotes compared with those treated with nifurtimox or benznidazole alone.

The intracellular antiparasitic activities of nifurtimox, benznidazole, or the combinations with BSO are shown in Table 5. BSO alone at 25 μ M did not significantly affect either the infection of Vero cells or the endocytic index. With 0.5 μ M nifurtimox, the percentage of infected Vero cells decreased by 22% and the endocytic index decreased by 61% compared with the control when BSO was added. The increased toxicity due to BSO was greater when 1 μ M benznidazole was used. The percentage of infected Vero cells decreased by 43%, and the endocytic index decreased by 84% (Table 5). The endocytic index reductions with nifurtimox and benznidazole at concentrations higher than 1 μ M did not show any significant potentiation with BSO, since both drugs alone accounted for almost 100% of the toxicity.

DISCUSSION

Mice infected with *Trypanosoma brucei brucei* have been cured with BSO alone (2). Similar approaches with an in vitro model with megazol in *T. brucei* (11) and with *Leishmania* (17) have been reported. Nevertheless, in the *Leishmania* model BSO was barely effective (17).

In humans, the BSO-glutathione synthesis inhibition strategy used to overcome the resistance (5) and to potentiate the effect of antineoplastic agents such as doxorubicin (31), melphalan (1), or cyclophosphamide (30) has been successful. In phase I trials (3, 4, 27), the concentration of BSO in blood reached 0.5 to 1 mM, reducing the glutathione level in white blood cells by 80%.

In this report we show that 500 μ M BSO significantly decreased all thiol concentrations (Table 1) and that BSO potentiated the effects of both nifurtimox and benznidazole in the epimastigote and trypomastigote forms (Tables 2, 3, and 4). The effects of nifurtimox and benznidazole upon amastigotes in Vero cells were substantially potentiated by 25 μ M BSO (Table 5). BSO potentiated the effect of nifurtimox and benznidazole in two ways: first, by lowering the percentage of infected Vero cells, which might imply a Vero cell cure; and second, by an important decrease in the number of amastigotes per infected Vero cell.

In this report, we show that the infective trypomastigote form (Table 4) and the replicative amastigote form (Table 5) were more sensitive to the potentiation of nifurtimox and ben-

znidazole by BSO than the epimastigote form (Tables 2 and 3). A possible explanation for this observation is found in Table 1, which shows that the trypomastigote and amastigote forms contain less than half of the total thiol concentration in the epimastigote form.

Nifurtimox or benznidazole and buthionine sulfoximine decrease the total free thiol content by two different mechanisms (18, 20). BSO inhibits the synthesis of glutathione (Table 1), and the reductive metabolism of nifurtimox and benznidazole produces electrophilic metabolites or free radicals that conjugate with the free thiols. In both situations, the concentrations of reduced GSH and $T(SH)_2$ are decreased, rendering nifurtimox or benznidazole more toxic.

At present, no new drugs are being used to treat Chagas' disease. Apparently, the new drugs tested clinically showed no clear advantage over nifurtimox or benznidazole. Our studies suggest that the use of BSO in combination with nifurtimox or benznidazole could lower the doses of both drugs needed to obtain the same clinical effect and consequently should diminish the side effects and/or the duration of therapy.

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