Antimicrobial Agents and Chemotherapy, May 2010, p. 2023–2031 Vol. 54, No. 5
0066-4804/10/$12.00 doi:10.1128/AAC.01241-09
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In Vitro and *In Vivo* Activities of 1,3,4-Thiadiazole-2-Arylhydrazone Derivatives of Megazol against *Trypanosoma cruzi*[▽]

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Received 1 September 2009/Returned for modification 29 November 2009/Accepted 2 March 2010

From a series of 1,3,4-thiadiazole-2-arylhydrazone derivatives of megazol screened *in vitro* against *Trypanosoma cruzi*, eight (S1 to S8) were selected for *in vivo* screening by single-dose oral administration (200 mg/kg of body weight) to infected mice at 5 days postinfection (dpi). Based on significant decreases in both parasitemia levels and mortality rates, S2 and S3 were selected for further assays. Despite having no *in vivo* effect, S1 was included since it was 2-fold more potent against trypomastigotes than megazol *in vitro*. Trypomastigotes treated with S1, S2, or S3 showed alterations of the flagellar structure and of the nuclear envelope. When assayed on intracellular amastigotes, the selectivity index (SI) for macrophages was in the range of >27 to >63 and for cardiac cells was >32 for S1 and >48 for megazol. In noninfected mice, S1 did not alter the levels of glutamic oxalacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), or urea. S2 led to an increase in GOT, S3 to increases in GOT and GPT, and megazol to an increase in GOT. Infected mice were treated with each derivative at 50 and 100 mg/kg from dpi 6 to 15: S1 did not interfere with the course of infection or reduce the number of inflammatory foci in the cardiac tissue, S2 led to a significant decrease of parasitemia, and S3 decreased mortality. There was no direct correlation between the *in vitro* effect on trypomastigotes and amastigotes and the results of the treatment in experimental models, as S1 showed a high potency *in vitro* while, in two different schemes of *in vivo* treatment, no decrease of parasitemia or mortality was observed.

Trypanosoma cruzi is the etiologic agent of Chagas disease, an endemic parasitosis in Latin America with 12 to 14 million people infected (35). Acute infections are usually asymptomatic, but the ensuing chronic *T. cruzi* infections have been associated with high rates of morbidity and mortality. At present, the only accepted drugs for treatment of Chagas disease are nifurtimox (Lampit) and benznidazole (Rochagan), which present severe side effects. Further, their efficacy during the chronic phase is still controversial, with poor indices of apparent cure (19). Due to these inadequacies, an intense research program has been directed to finding alternative drugs for the treatment of chagasic patients (32).

The 5-nitroimidazole megazol was shown to be highly active against *T. cruzi*, including strains resistant to benznidazole, *in vitro* and *in vivo* (12, 17, 21) and has become a core structure for the design of new leads for the treatment of Chagas disease. Megazol has been described as a scavenger of trypanothione, the cofactor for trypanothione reductase (23, 42). Despite its noteworthy trypanocidal activity, megazol development was discontinued due to reports of *in vitro* mutagenic and genotoxic effects (16, 29, 30). In attempting to circumvent this undesired profile, megazol analogues were synthesized, but none have been shown to be more potent than the prototype (3, 6, 11).

Our group synthesized and assayed the trypanocidal activities of 34 new megazol derivatives (7, 8), exploring the hypothesis that the introduction of a radical scavenger arylhydrazone moiety in the heterocyclic framework of megazol could modulate the production of toxic nitro anion radical species, thus avoiding potentially mutagenic properties (22, 31). The derivatives were designed by molecular hybridization between megazol and guanylhydrazones, compounds previously shown by one of the authors to display a strong activity against trypomastigote forms of *T. cruzi* (26). Such a strategy led us to identify a new potent prototype trypanocide, 3-4-dihydroxybenzylidene [5-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,3,4-thiadiazol-2-yl]hydrazine [brazilizone A], hereinafter called S1, which was two times more potent than the prototype against the infective bloodstream form of the parasite. Seven other derivatives of the same nitroimidazole series also displayed interesting bioprofiles, with 50% inhibitory concentrations for 24 h of treatment (IC_{50}s/24 h) in the range of 11 to 63 μM: the 4-OH derivative (S2), the lipophilic para-bromo derivative (4-Br) (S3), the 4-NO_2 derivative (S4), the 3,5-ditertbutyl,4-OH derivative (S5), the 4-Cl derivative (S6), the 4-methoxy derivative (S7), and the 2-OH derivative (S8). In the present work, we further evaluate the *in vitro* and *in vivo* activities of these compounds.

MATERIALS AND METHODS

Parasites and cell cultures. We used the Y strain of *T. cruzi*. Bloodstream trypomastigotes were obtained from infected mice at peak parasitemia. Peritoneal macrophages were obtained from Swiss mice. Primary cultures of mouse embryo heart muscle cells (HMCs) were prepared as previously described (24).

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▽ Published ahead of print on 15 March 2010.

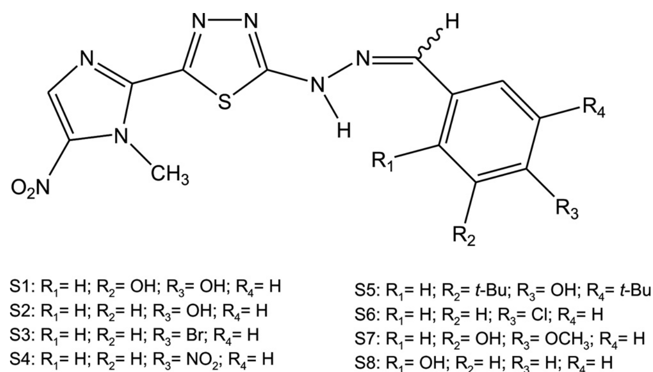

FIG. 1. Chemical structures of the 1,3,4-thiadiazole-2-arylhydrazone derivatives.

Briefly, hearts of 18-day-old mouse embryos were fragmented and dissociated with trypsin and collagenase in phosphate-buffered saline (PBS), pH 7.2. Thereafter, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with horse and fetal calf sera, chicken embryo extract, $CaCl_2$, and L-glutamine and then plated onto gelatin-coated glass coverslips and maintained at 37°C in a 5% CO_2 atmosphere.

Megazol derivatives. The 1,3,4-thiadiazole-2-arylhydrazone derivatives were obtained as previously described (7, 8). Briefly, megazol was converted in the corresponding chloride, followed by replacement of the chlorine atom using hydrazine hydrate to give the heterocyclic hydrazine which, by acid-catalyzed condensation with aromatic aldehydes in ethanol, generated derivatives S1 to S8. 3,4-Dihydroxybenzaldehyde was used for the synthesis of S1, 4-hydroxybenzaldehyde for S2, 4-bromobenzaldehyde for S3, 4-nitrobenzaldehyde for S4, 3,5-ditertbutyl-4-hydroxybenzaldehyde for S5, 4-chlorobenzaldehyde for S6, 3-hydroxy-4-methoxybenzaldehyde for S7, and 2-hydroxybenzaldehyde for S8 (Fig. 1). Stock solutions of the compounds were prepared in dimethyl sulfoxide (Merck, Darmstadt, Germany).

Ultrastructural analysis. Trypomastigotes (5×10^6 cells/ml) were treated with the compounds for 24 h at concentrations corresponding to their IC_{50}s (7). For scanning electron microscopy (SEM), the parasites were adhered to poly-L-lysine-coated coverslips, fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2) at room temperature for 40 min, and postfixed with a solution of 1% OsO_4, 0.8% potassium ferricyanide, and 2.5 mM $CaCl_2$ in the same buffer for 30 min. The cells were dehydrated in an ascending acetone series and dried by the critical point method with CO_2 (CPD 030; Balzers, Switzerland). The samples were mounted with silver cellotape on aluminum stubs, coated with a 20 nm-thick gold layer, and examined using a 940 DSM Zeiss microscope (Oberkochen, Germany). For transmission electron microscopy (TEM), after being washed in PBS, parasites were fixed, postfixed and dehydrated as described above, and embedded in epoxy resin. Ultrathin sections (Leica Ultracut; UCT; Leica, Vienna, Austria) were stained with uranyl acetate and lead citrate and then examined using an EM10C Zeiss microscope (Oberkochen, Germany).

Analysis of effects on intracellular amastigote proliferation. Macrophages were plated in 24-well plates (3×10^5 cells/well) for 24 h and were then infected with trypomastigotes (10:1 parasite/host cell) in DMEM supplemented with 10% fetal calf serum. After 3 h of incubation, the cultures were washed to remove noninternalized parasites and the selected compounds were added at final concentrations ranging from 2.5 to 30 μM. Alternatively, HMCs were plated onto gelatin-coated glass coverslips in 24-well plates (10^5 cells/well) and infected with parasites at the 10:1 ratio after 24 h. After 24 h of incubation, the cultures were washed and the compounds were added at a final concentration of 10 to 40 μM. At specified intervals, the cultures were fixed in Bouin's solution, stained with Giemsa stain, and counted, using the parameters percent infection and endocytic index (EI; refers to the number of parasites/100 cells), and the IC_{50} values for 1 to 4 days of treatment, corresponding to the concentration that led to 50% inhibition of each parameter, were calculated. To determine the possible toxic effects of the compounds on the host cells, uninfected macrophages and HMCs were incubated with the compounds at 37°C. After 2 days, the viability of the cells was measured using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] colorimetric assay (27). The absorbance was measured at 490 nm with a spectrophotometer (VERSAmax tunable; Molecular Devices, United States) for the determination of 50% lethal concentrations (LC_{50}s) against cellular viability.

***In vivo* studies.** Male albino Swiss mice (age, 6 to 8 weeks, and weight, 18 to 20 g) were maintained in our animal facilities under stable conditions of temperature and light with 12-h light/dark cycles with both food and water *ad libitum*. Mice were separated into the following groups, each with 8 to 10 animals: N (noninfected and nontreated), NS1-*x* to NS8-*x* and NMg-*x* (noninfected and treated with S1 to S8 or megazol [Mg] at various doses [*x* mg/kg of body weight] indicated below), Tc (infected and nontreated), and TcS1-*x* to TcS8-*x* and TcMg-*x* (infected and treated at various doses in mg/kg). Noninfected mice were treated by gavage with a single dose of 400 mg/kg or with 10 doses of 100 mg/kg of the compounds, and the levels of glutamic oxalacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), and urea were determined directly in the blood using the Reflotron system (Roche Diagnostics, F. Hoffmann-La Roche Ltd.; Basel, Switzerland). The Tc and TcS-*x* groups were infected with 10^4 bloodstream trypomastigotes via the intraperitoneal route (i.p.). The animals were treated by gavage with a single dose of 200 mg/kg at the fifth day postinfection (dpi) or for 10 consecutive days with 50 or 100 mg/kg beginning at the sixth dpi. Each experimental set used megazol as a positive treatment control. All eight compounds were dissolved in water containing 10% Tween-80, and preliminary experiments revealed that the vehicle did not alter the course of infection. Body weights were monitored weekly. The level of parasitemia was checked daily from 6 to 21 dpi by the Pizzi-Brener method (5). Mortality was noted daily, and the percent indices of cumulative mortality (%CM) at 40 dpi and the day when mortality attained 50% (M_{50}) were calculated (33). At 14 dpi, blood was collected and immediately subjected to analysis for the determination of GOT, GPT, and urea levels. All experiments were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (license 028/09).

Two animals each from the Tc, TcS1-100, TcS2-100, and TcS3-100 groups were euthanized at 14 dpi. The heart tissue was processed in paraffin-embedded sections and stained with hematoxylin and eosin. Quantification of the histopathological alterations was performed with an optical microscope (40× objective) by counting the number of nests of parasites (cells with amastigotes), and foci of inflammatory infiltrates (containing at least 10 mononuclear cells) per area (mm^2) in 30 fields.

Statistical analysis. Statistical significance ($P \leq 0.05$) was evaluated using Student's *t* test or analysis of variance for parasitemia and the log rank (Mantel-Cox) test for survival analysis. The Mann-Whitney test was used for comparison of GOT, GPT, and urea levels among the different experimental groups.

RESULTS AND DISCUSSION

Although discovered 100 years ago (10), Chagas disease still represents an important public health problem that is in need of new, effective, and nontoxic/low-toxicity drugs for its etiological treatment (38). A series of studies dealing with the antitrypanocidal activity of megazol have been reported (13, 21), including its use for the treatment of mice infected with the Colombiana strain of *T. cruzi*, recognized as resistant to nifurtimox and benznidazole (17). However, reports of the toxicity of megazol (29, 30) led to the abandonment of its study. In the late 1990s, with its resurgence as a new potential drug for African trypanosomes (2, 4), this nitroimidazole began to be explored as a template for the design of new analogues aimed at the treatment of Chagas disease (3, 6, 11).

From an *in vitro* screening using bloodstream trypomastigote forms of *T. cruzi*, eight 1,3,4-thiadiazole-2-arylhydrazones out of 34 synthesized derivatives were selected for further investigation based on their potent trypanocidal activities (7, 8). It is well known that some functionalized hydrazone-related derivatives (1, 9, 15) present trypanocidal activities; these activities could be correlated with action on essential enzymes of *T. cruzi*, such as cruzain cruzipain and trypanothione reductase, which are potential targets of new drugs for Chagas disease (38). Compounds S1 (brazilizone A) to S8 are from the nitroimidazole series and present different substituents on the phenyl group. For an *in vivo* screening, compounds S1 to S8 were administered to infected mice in a single dose of 200 mg/kg at

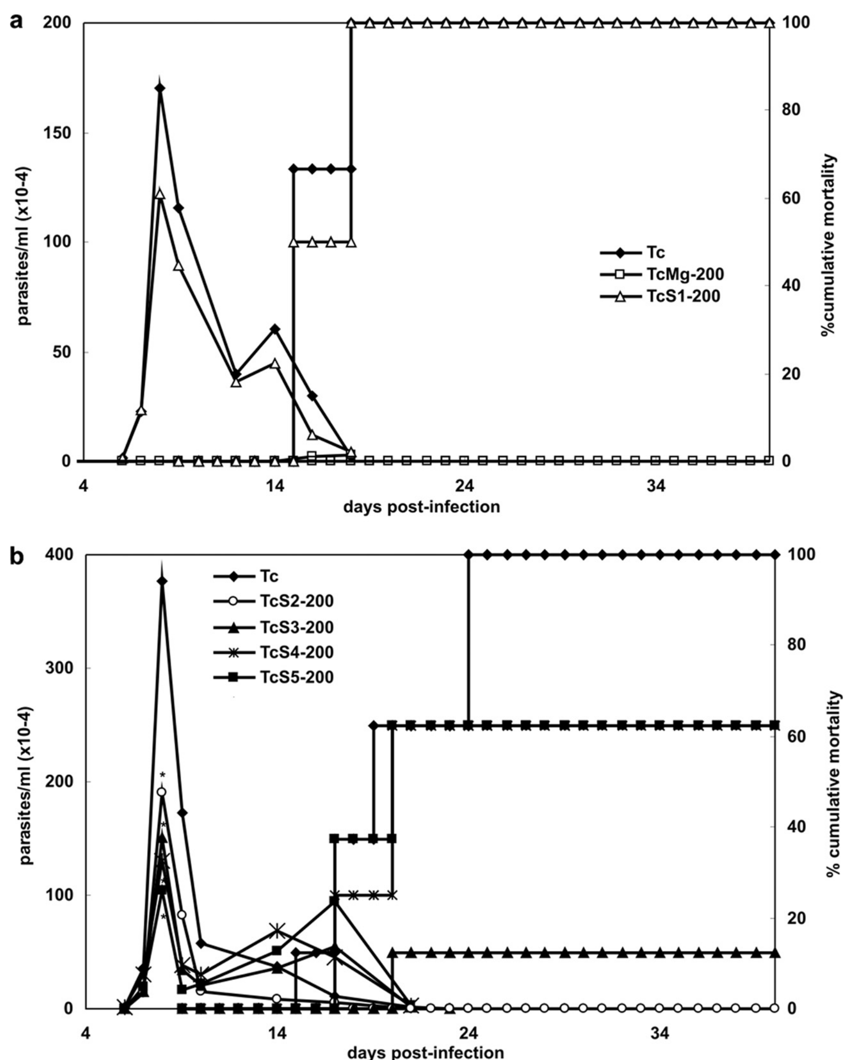

FIG. 2. Effects on parasitemia curves and cumulative mortality of oral treatment of *T. cruzi*-infected mice with a single dose on dpi 5 of 200 mg/kg of the compound indicated in the group name. (a) Results for the TcS1-200 and TcMg-200 groups. (b) Results for the TcS2-200, TcS3-200, TcS4-200, and TcS5-200 groups. Asterisks indicate *P* values of $\leq$0.05 for comparison of the results for each treated group with those for group Tc.

the onset of parasitemia, and the body weights, levels of parasitemia, and mortality rates of the mice were monitored up to 40 dpi. The treatment of infected mice with S1 (group TcS1-200 [200 mg/kg dose]) did not interfere with the course of infection, with the values of body weight, parasitemia, and mortality being similar to those of the control infected group (Tc) and 50%CM occurring at 16 dpi (Fig. 2a). For the TcS2-200 and TcS3-200 groups, significant decreases in parasitemia ($P = 0.004$ and $P = 0.006$, respectively) and mortality (log rank, $P = 0.0001$ and $P = 0.0007$, respectively) were observed (Fig. 2b). For the TcS4-200 and TcS5-200 groups, the treatment led to significant decreases in peak parasitemias ($P = 0.002$ and $P = 0.003$), while the levels of mortality were similar to that of the Tc group at 20 dpi (Fig. 2b). For the other derivatives (S6 to S8), no differences relative to the results for the infected and nontreated animals were found (data not shown). For the reference group TcMg-200, no parasites were detected in the blood by cell counting and 100% of the animals survived (Fig. 2a). Based on these *in vivo* results, three derivatives were selected: S2, which led to a 50% decrease of the parasitemia peak and all animals surviving at 40 dpi; S3, which inhibited the parasitemia peak in 60% of mice and led to 7/8 animals surviving; and S1, which, although it did not display an *in vivo* effect, was 2-fold more potent *in vitro* against the infective trypomastigote than the prototype megazol (4).

The ultrastructural and morphological alterations induced in trypomastigote forms by S1, S2, and S3 were analyzed by TEM (Fig. 3) and SEM (Fig. 4). The parasites were treated with the previously determined IC_{50}/24 h values (7), doses that caused no damage to mammalian cells. Control trypomastigotes displayed the characteristic morphology (Fig. 3a). Parasites treated with 5.3 μM S1 displayed alterations of the flagellar structure with expansion of its membrane, dilation of the nuclear envelope, the formation of autophagosome-like structures, and cellular disorganization (Fig. 3b). S3 at 17 μM led to an increase in the amount and size of vesicles near the flagellar pocket that corresponds to the endocytic site in trypomastigotes. Autophagosome-like structures were also observed after

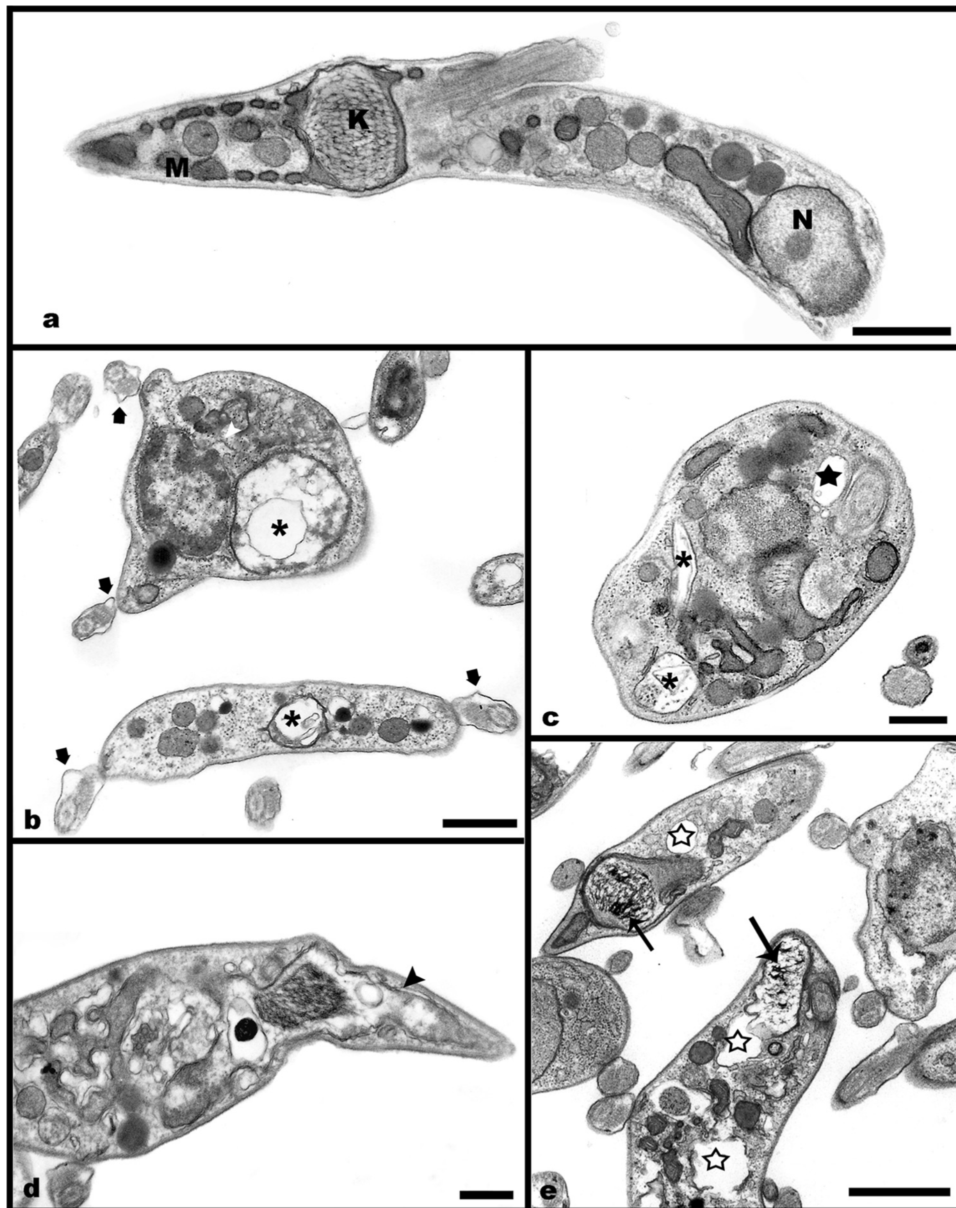

FIG. 3. Transmission electron microscopy images of *T. cruzi* trypomastigotes treated with S1, S3, or megazol for 24 h. (a) Control parasite showing the typical elongated body and normal morphology of the mitochondrion (M) and kinetoplast (K). N, nucleus. (b) A concentration of 5.3 μM S1 led to expansion of the flagellar membrane (thick arrows), dilation of the nuclear membrane (white arrowhead), and autophagosome-like structures (asterisks). (c) A concentration of 17 μM S3 induced increased vesiculation near the flagellar pocket (star) and formation of autophagosome-like structures (asterisk). (d and e) A concentration of 9.9 μM megazol induced alterations in the kDNA network (arrows) and in the mitochondrion (arrowhead) and also an intense vacuolization (white stars). Bars: 0.5 μm.

treatment with S3 (Fig. 3c). Normally, the endocytic activity in bloodstream trypomastigotes is very low or even nonexistent, but recently, intracellular compartments concentrated in the posterior region of this form that present lysosomal characteristics and show the presence of hydrolases have been described and named lysosome-related organelles (34). Their presence in the infective stages of *T. cruzi* may have significant implications for host cell invasion and may constitute a promising target of drugs. Megazol at 9.9 μM induced mitochondrial swelling with scarcity of the matrix and cristae and the presence of vesicles inside this organelle (Fig. 3d), disruption of the kDNA network, and an intense cytoplasmic vacuolization (Fig. 3e). This structure has been previously identified by our group as a target of this nitroimidazole, which led to ultrastructural alterations similar to those caused by nifurtimox and benznidazole (12). Kinetoplasts, the enlarged DNA-containing region of the single mitochondrion of members of the Kinetoplastida, account for over 20% of the total parasite genome. This structure represents a potential target in *T. cruzi* for different drugs (28, 41). Examples of such drugs are topoisomerase inhibitors (18, 20), aromatic diamidines and reversed amidines (37, 40), and the putrescine analogue 1,4-diamino-2-butanone (25).

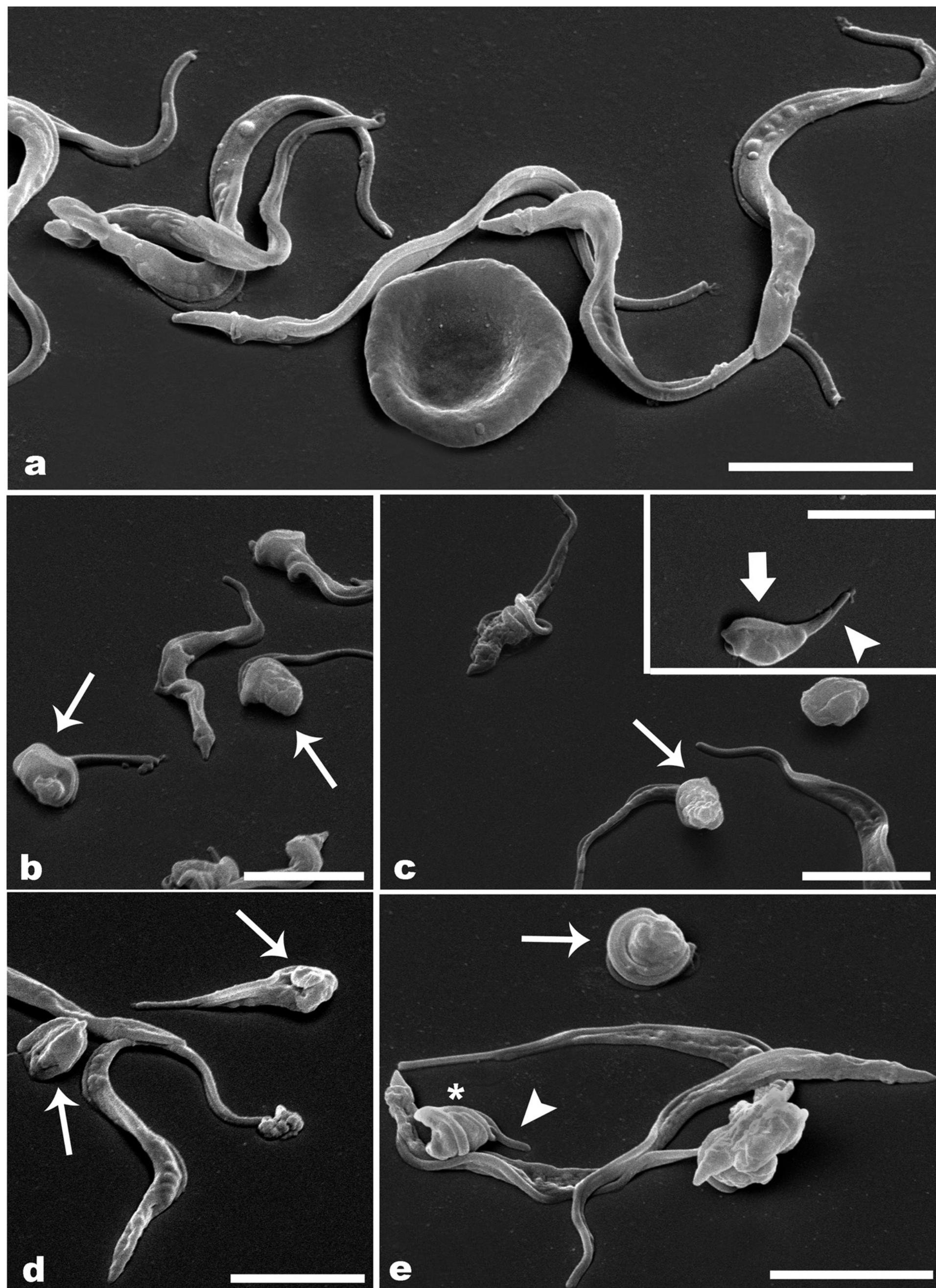

FIG. 4. Scanning electron microscopy images of *T. cruzi* trypomastigotes treated with S1, S2, S3, or megazol for 24 h. (a) Untreated parasites display normal morphology and surface topology. (b) S1 at 5.3 μM led to rounding of the parasite's body (arrows). (c) S2 at 11.6 μM induced shrinkage of the cell surface (arrow) and body (thick arrow) and shortening of the flagellum (arrowhead). (d) S3 at 17 μM caused rounding of parasites (arrows). (e) Megazol at 9.9 μM led to torsion (asterisk) and rounding (arrow) of the body of the parasites and shortening of the flagellum (arrowhead). Bars: 4 μm.

The SEM analysis revealed that the treatment induced severe morphological changes in trypomastigotes (Fig. 4). S1 led to rounding of the parasite's body and, in several instances, to shortening of the flagellum (Fig. 4b) compared to the morphology of control cells (Fig. 4a). Treatment with S2 induced a drastic morphological change, with torsion and shortening of the body (Fig. 4c), sometimes with the aspect of a tadpole (Fig. 4c, inset). S3 caused a pronounced reduction in the size of the parasite, while the region of the free flagellum was preserved (Fig. 4d). Megazol also led to rounding of the trypomastigotes, sometimes with retraction of the cell body and flagellum (Fig. 4e). These morphological alterations, such as surface shrinkage, induced by megazol and its analogues may indicate destabilization of cytoskeleton components or microtubule-associated proteins (39).

The *in vitro* effects of S1, S2, and S3 on intracellular amas-

TABLE 1. IC_{50} values[a] for inhibition of the indicated parameters by megazol and derivatives in *T. cruzi* amastigote-infected macrophages

Days of treatment	% Infection			Endocytic index (EI)		
	Megazol	S1	S2	Megazol	S1	S2
1	2.7 ± 1.3	2.1 ± 0.2	7.1 ± 1.6	2.0 ± 0.5	2.0 ± 0.3	5.0 ± 1.2
2	3.0 ± 1.7	4.4 ± 0.2	6.0 ± 0.5	1.6 ± 1.0 (>62)[b]	2.2 ± 0.8 (>27)	2.9 ± 0.6 (>41)
3	8.3 ± 0.8	2.6 ± 0.7	6.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.0	3.2 ± 0.3
4	1.7 ± 0.1	1.7 ± 0.0	1.7 ± 0.1	1.3 ± 0.0	1.9 ± 0.0	1.3 ± 0.1

[a] Values (μM) are the means ± standard deviations of the results of three independent experiments.
[b] SI = LC_{50}/IC_{50}; LC_{50}/2 day values were >100 μM for megazol and S2 and >60 μM for S1.

tigotes were analyzed using macrophages and HMCs. In macrophages, a dose-dependent inhibition of the infection was observed, with IC_{50}/2 day values for inhibition of the EI of 2.2 ± 0.8 (mean ± standard deviation), 2.9 ± 0.6, and 1.6 ± 1.0 μM for S1, S2, and megazol, respectively (Table 1), while S3 was active only at concentrations higher than 30 μM. When the experiments were performed with HMCs, a dose- and time-dependent inhibition of the infection was observed only with megazol and S1 (Fig. 5). In HMCs, the IC_{50}/2 day values for the inhibition of EI were 5.0 ± 0.1 μM for megazol and 7.6 ± 2.1 μM for S1, while for both S2 and S3, even at 40 μM, no effect on the course of the infection was observed (data not shown). Exposure of macrophages to a varied number of stimuli, such as interaction with microorganisms, may result in further macrophage activation with the generation of oxygen- and nitrogen-reactive intermediates (36). These species could act in synergism with the derivatives since intracellular amastigotes in macrophages appear to be more susceptible than the same developmental stage inside HMCs.

The cytotoxicities of the compounds in noninfected mammalian cells was determined after exposure of macrophages and HMCs for 48 h and quantification of viable cells by MTT

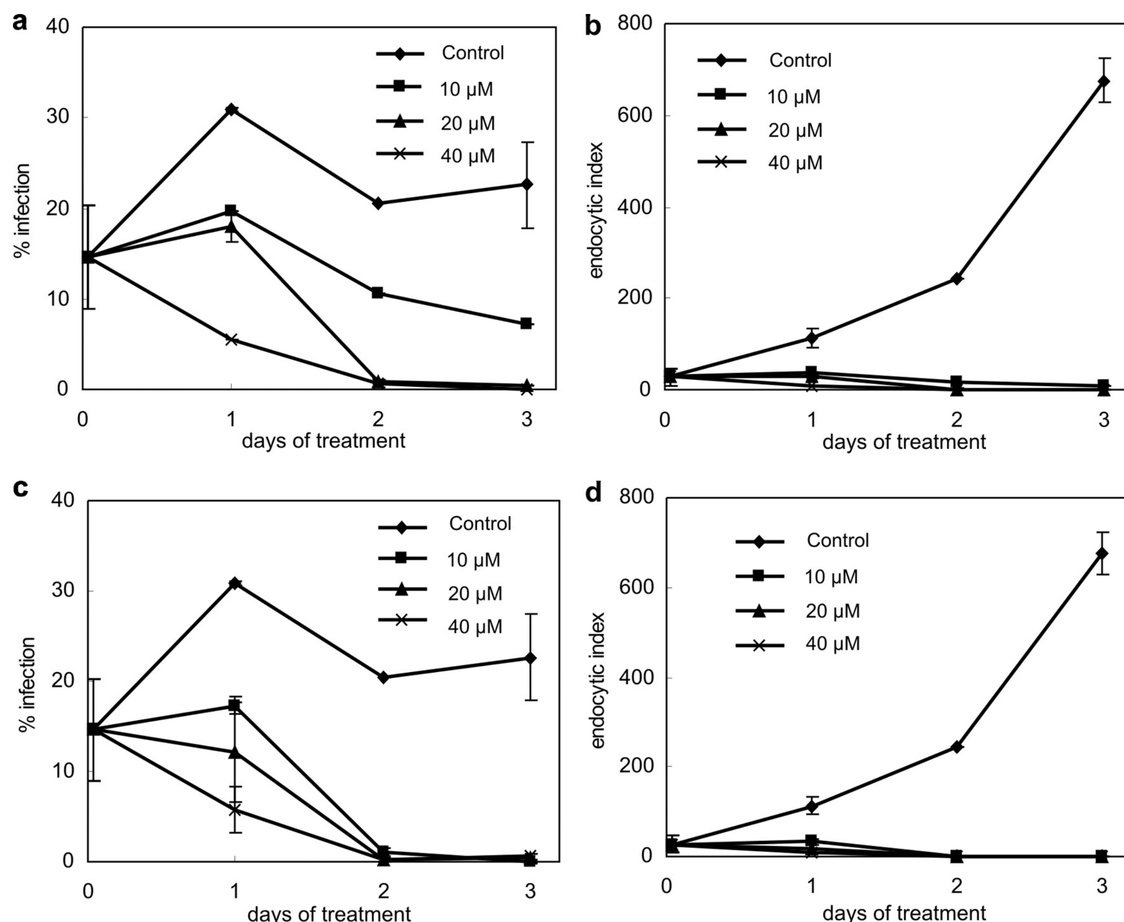

FIG. 5. Effects of various concentrations of S1 (a and b) and megazol (c and d) on *T. cruzi*-infected HMCs. The percent infection and the endocytic index were quantified; mean results ± standard deviations are shown. After 24 h of plating HMCs (10^5 cells/well) onto gelatin-coated glass coverslips, the cultures were infected at a 10:1 ratio (parasites/HMCs), and after 24 h of incubation, S1 or megazol was added as described in Materials and Methods.

TABLE 2. Body weights and GPT, GOT, and urea levels in uninfected mice 4 days after treatment with the compound indicated in the group name in a single dose of 400 mg/kg[a]

Group	Weight (g)	GOT (U/liter)	GPT (U/liter)	Urea (mg/dl)
N	25.9 ± 2.4	58.2 ± 13.1	21.6 ± 5.0	52.0 ± 9.0
NS1-400	25.8 ± 2.9	62.4 ± 9.1	22.7 ± 3.9	51.6 ± 5.4
NS2-400	25.5 ± 2.4	76.6 ± 14.8*	24.4 ± 7.3	58.6 ± 6.6
NS3-400	25.2 ± 1.6	118.0 ± 22.5*	27.9 ± 3.4*	62.7 ± 12.4
NMg-400	21.7 ± 1.8*	139.7 ± 30.2*	20.7 ± 7.2	49.5 ± 4.8

[a] Values are mean results ± standard deviations. *, significant difference in relation to the results for the control group (N) ($P \leq 0.05$).

assay, and the LC_{50}s were determined. For macrophages, the LC_{50} was >60 μM for S1 and S3 and >100 μM for S2 and megazol, while for HMCs, the LC_{50}s were >120 μM for S3 and >240 μM for S1, S2, and megazol. Next, based on the LC_{50} and IC_{50} of the EI, the selectivity index (SI) of the compounds was calculated. The results for macrophages are presented in Table 1 (>27 to >62), while for HMCs, the SI was >48 for megazol and >32 for S1.

The toxicity to noninfected mice of a single 400-mg/kg dose of S1, S2, or S3 was analyzed 4 days after the treatment, and the toxicity of 10 consecutive 100-mg/kg doses of S1 was analyzed. Treatment with S1 using both protocols (NS1-400 and NS1-100) did not alter the levels of GOT, GPT, and urea in comparison with the levels in the untreated group (N). For NS2-400, an increase of 1.3-fold in GOT was observed, and for NS3-400, increases of 2.0-fold in GOT and 1.3-fold in GPT were observed. In parallel experiments, the NMg-400 group showed an increase of 2.4-fold in GOT, and significant decreases in the body weights of mice were observed with this drug only (Table 2).

Infected mice were treated with each derivative at 50 and 100 mg/kg from dpi 6 to 15. As observed after the treatment with a single dose, S1 (group TcS1-100) did not decrease the parasitemia levels or mortality rate or reduce the number of inflammatory foci in the cardiac tissue (data not shown). For the TcS2-100 group, a significant decrease in the parasitemia (46%; $P = 0.005$) was observed; however, the mortality levels were similar to those of the Tc group. For the TcS3-100 group, the inverse occurred: the parasitemia was similar to and the mortality levels were lower than ($P = 0.0002$) those in the untreated group (Fig. 6). Although the analysis of heart tissues was limited by the reduced number of surviving mice ($n = 2$), a discrete reduction in the number of inflammatory loci was noticed in each treated group. Regarding the considerable *in vivo* activities of S2 and S3 while presenting limited *in vitro* effects on *T. cruzi*-infected heart muscle cells, it is important to note that this parasite can invade virtually all nucleated mammalian cells and the reduced parasitemia levels can be derived from the reduction in parasite burden in other noncardiac tissues. Besides, it is also possible that new metabolites derived from animal metabolism of S2 and S3 could exert even greater activities than the parental compounds. Megazol administered as a single dose of 200 mg/kg on dpi 5 or as 10 consecutive doses of 50 mg/kg led to undetectable levels of parasitemia, as observed by optical microscopy, and to survival of all the animals. It was previously reported that infection with *T. cruzi* led to significant increases in the levels of GPT, GOT, and urea at 14 dpi using the same experimental model and parasite inoculum as in this study (33). These data were confirmed in the present study using more animals, and treatment with the three megazol derivatives for 10 days did not alter the levels of these biochemical markers (data not shown).

Although intensive efforts have been directed to the identification of more active and less toxic megazol derivatives, the results are not encouraging. The design of different compounds with the 5-nitroimidazole moiety, such as 5-trifluoro-

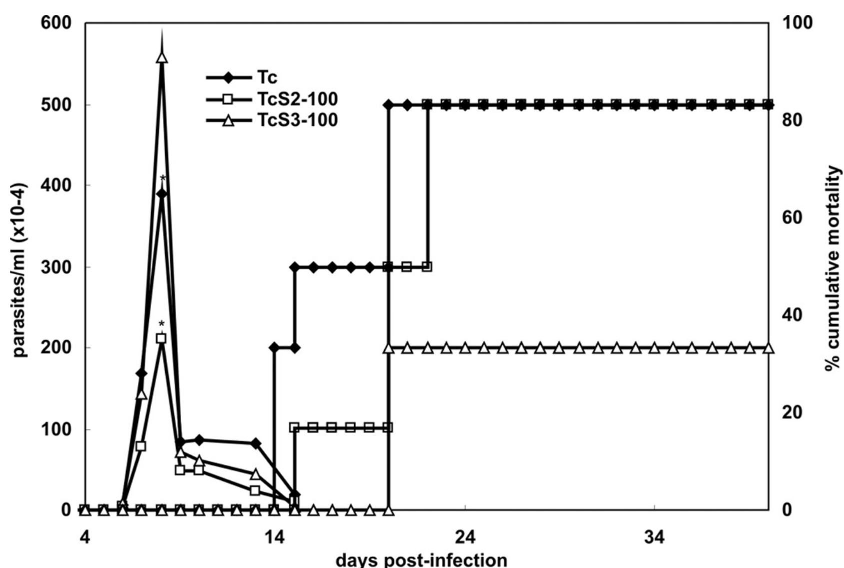

FIG. 6. Effects on parasitemia curves and cumulative mortality of oral treatment with 10 consecutive doses of 100 mg/kg of S2 (TcS2-100) and S3 (TcS3-100) from dpi 6 to 15. Asterisks indicate *P* values of ≤0.05 for comparison of the results for each treated group with those for the Tc group.

methyl and pyrazolyl derivatives (3), or of azaheterocycles (6) or semicarbazone derivatives (9), and also the replacement of the thiadiazole or of the nitroimidazole nucleus (11), allowed only the identification of S1 as a possible alternative to megazol (8). This thiadiazole derivative was 2-fold more potent against bloodstream infective forms than megazol *in vitro* but induced no decrease of parasitemia or mortality in infected mice even after 10 consecutive days of treatment. In fact, as already observed in the literature, there is not always a direct correlation between the *in vitro* effect on the clinically relevant trypomastigote and amastigote forms of *T. cruzi* and the results achieved during the treatment of experimental models (14, 32a), as occurred with S1 in the present study. However, our results demonstrate the promising activity of these derivatives, especially S2 and S3, on *T. cruzi*. This justifies further *in vivo* experiments with longer periods of treatment for thorough analysis of their toxicity (already under way), especially their potential genotoxic effect, and the continuation of the synthesis and screening of new analogues.

ACKNOWLEDGMENTS

We are grateful to Andreia Souza Meuser, Marcos Meuser Batista, and Patrícia Bernardino da Silva for their excellent technical assistance.

This work was supported by CNPq, FIOCRUZ, and FAPERJ.

REFERENCES

1. **Aguirre, G., M. Bolani, H. Cerecetto, A. Gerpe, M. Gonzalez, Y. F. Sainz, A. Denicola, C. O. Ocariz, J. J. Nogal, D. Montero, and J. A. Escario.** 2004. Novel antiprotozoal products: imidazole and benzimidazole N-oxide derivatives and related compounds. Arch. Pharm. **337:**259–270.
2. **Barrett, M. P., A. H. Fairlamb, B. Rousseau, G. Chauvière, and J. Périé.** 2000. Uptake of the nitroimidazole drug megazol by African trypanosomes. Biochem. Pharmacol. **59:**615–620.
3. **Boechat, N., A. S. Carvalho, E. Fernandez-Ferreira, R. O. Soares, A. S. Souza, D. Gibaldi, M. Bozza, and A. C. Pinto.** 2001. Novel nitroimidazoles with trypanocidal and cell growth inhibition activities. Cytobios **105:**83–90.
4. **Bouteille, B., A. Marie-Daragon, G. Chauvière, C. Albuquerque, B. Enanga, M. L. Darde, J. M. Vallat, J. Périé, and M. Dumas.** 1995. Effect of megazol on *Trypanosoma brucei brucei* acute and subacute infections in Swiss mice. Acta Trop. **60:**73–80.
5. **Brener, Z.** 1962. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. Rev. Inst. Med. Trop. São Paulo **4:**386–396.
6. **Carvalho, A. S., R. F. S. Menna-Barreto, N. C. Romeiro, S. L. De Castro, and N. Boechat.** 2007. Design, synthesis and activity against *Trypanosoma cruzi* of azaheterocyclic analogues of megazol. Med. Chem. **3:**460–465.
7. **Carvalho, S. A., F. A. S. Lopes, K. Salomão, N. C. Romeiro, S. M. V. S. Wardell, S. L. de Castro, E. F. Silva, and C. A. M. Fraga.** 2008. Studies toward the structural optimization of new brazilizone-related trypanocidal 1,3,4-thiadiazole-2-arylhydrazone derivatives. Bioorg. Med. Chem. **16:**413–421.
8. **Carvalho, S. A., E. F. Silva, R. M. Santa-Rita, S. L. de Castro, and C. A. M. Fraga.** 2004. Synthesis and antitrypanosomal profile of new functionalyzed 1,3,4-thiadiazole-2-arylhydrazone derivatives, designed as non-mutagenic megazol analogues. Bioorg. Med. Chem. Let. **14:**5967–5970.
9. **Cerecetto, H., R. Di Maio, M. González, M. Risso, G. Sagrera, G. Seoane, A. Denicola, G. Peluffo, C. Quijano, A. O. M. Stoppani, M. Paulino, C. Olea-Azar, and M. A. Basombrío.** 2000. Synthesis and antitrypanosomal evaluation of *E*-isomers of 5-nitro-2-furaldehyde and 5-nitrothiophene-2-carboxaldehyde semicarbazone derivatives. Structure-activity relationships. Eur. J. Med. Chem. **35:**343–350.
10. **Chagas, C.** 1909. Nova tripanosomíase humana—estudos sobre a morfologia e o ciclo evolutivo de *Schizotrypanum cruzi* gen. nov. sp. nov., agente etiológico de nova entidade mórbida do homem. Mem. Inst. Oswaldo Cruz **1:**159–218.
11. **Chauvière, G., B. Bouteille, B. Enanga, C. Albuquerque, S. L. Croft, M. Dumas, and J. Périé.** 2003. Synthesis and biological activity of nitro heterocycles analogs to megazol, a trypanocidal lead. J. Med. Chem. **46:**427–440.
12. **De Castro, S. L., and M. N. L. Meirelles.** 1987. Effect of drugs on *Trypanosoma cruzi* and on its interaction with heart muscle cell *in vitro*. Mem. Inst. Oswaldo Cruz **82:**209–218.
13. **de Castro, S. L., and M. N. L. Meirelles.** 1990. Mechanism of action of a nitroimidazole-thiadiazole derivative upon *Trypanosoma cruzi* tissue culture amastigotes. Mem. Inst. Oswaldo Cruz **85:**95–99.
14. **de Souza, E. M., G. Melo, D. W. Boykin, A. Kumar, Q. Hu, and M. N. C. Soeiro.** 2006. Trypanocidal activity of the phenyl-substituted analogue of furamidine DB569 against *Trypanosoma cruzi* infection *in vivo*. J. Antimicrob. Chemother. **58:**610–614.
15. **Du, X., C. Guo, E. Hansell, P. S. Doyle, C. R. Caffrey, T. P. Holler, J. H. McKerrow, and F. E. Cohen.** 2002. Synthesis and structure-activity relationship study of potent trypanocidal thio semicarbazone inhibitors of the trypanosomal cysteine protease cruzain. J. Med. Chem. **45:**2695–2707.
16. **Ferreira, R. C., and L. C. Ferreira.** 1986. Mutagenicity of CL 64855, a potent anti-*Trypanosoma cruzi* drug. Mut. Res. **171:**11–15.
17. **Filardi, L. S., and Z. Brener.** 1982. A nitroimidazole-thiadiazole derivative with curative action in experimental *Trypanosoma cruzi* infections. Ann. Trop. Med. Parasitol. **76:**293–297.
18. **Gonzales-Perdomo, M., S. L. de Castro, M. N. L. Meirelles, and S. Goldenberg.** 1990. *Trypanosoma cruzi* proliferation and differentiation are blocked by topoisomerase II inhibitors. Antimicrob. Agents Chemother. **34:**1707–1714.
19. **Jannin, J., and L. Villa.** 2007. An overview of Chagas disease treatment. Mem. Inst. Oswaldo Cruz **102:**95–97.
20. **Kerschmann, R. L., J. S. Wolfson, G. L. McHugh, G. R. Dickersin, D. C. Hooper, and M. N. Swartz.** 1989. Novobiocin-induced ultrastructural changes and antagonism of DNA synthesis in *Trypanosoma cruzi* amastigotes growing in cell-free medium. J. Protozool. **36:**14–20.
21. **Lages-Silva, E., L. S. Filardi, and Z. Brener.** 1990. Effect of the host specific treatment in the phagocytosis of *Trypanosoma cruzi* blood forms by mouse peritoneal macrophages. Mem. Inst. Oswaldo Cruz **85:**401–405.
22. **Mahy, J. P., S. Gaspard, and D. Mansuy.** 1993. Phenylhydrazones as new good substrates for the dioxygenase and peroxidase reactions of prostaglandin synthase: formation of iron(III)-sigma-phenyl complexes. Biochemistry **32:**4014–4021.
23. **Maya, J. D., S. Bollo, L. J. Nunez-Vergara, J. A. Squella, Y. Repetto, A. Morello, J. Périé, and G. Chauvière.** 2003. *Trypanosoma cruzi*: effect and mode of action of nitroimidazole and nitrofuran derivatives. Biochem. Pharmacol. **65:**999–1006.
24. **Meirelles, M. N. L., T. C. Araujo-Jorge, C. F. Miranda, W. de Souza, and H. S. Barbosa.** 1986. Interaction of *Trypanosoma cruzi* with heart muscle cells: ultrastructural and cytochemical analysis of endocytic vacuole formation and effect upon myogenesis *in vitro*. Eur. J. Cell Biol. **41:**198–206.
25. **Menezes, D., C. Valentim, M. F. Oliveira, and M. A. Vannier-Santos.** 2006. Putrescine analogue cytotoxicity against *Trypanosoma cruzi*. Parasitol. Res. **98:**99–105.
26. **Messeder, J. C., W. Tinoco, J. D. Figueroa-Villar, E. M. de Souza, R. M. Santa-Rita, and S. L. de Castro.** 1995. Aromatic guanylhydrazones: Synthesis, structural studies and *in vitro* activity against *Trypanosoma cruzi*. Bioorg. Med. Chem. Lett. **24:**3079–3084.
27. **Mossman, B. T.** 1983. *In vitro* approaches for determining mechanisms of toxicity and carcinogenicity by asbestos in the gastrointestinal and respiratory tracts. Environ. Health Perspect. **53:**155–161.
28. **Motta, M. C.** 2008. Kinetoplast as a potential chemotherapeutic target of trypanosomatids. Curr. Pharm. Des. **14:**847–854.
29. **Nesslany, F., S. Brugier, M. A. Mouries, F. Le Curieux, and D. Marzin.** 2004. *In vitro* and *in vivo* chromosomal aberrations induced by megazol. Mut. Res. **560:**147–158.
30. **Poli, P., M. A. Mello, A. Buschini, R. A. Mortara, C. Albuquerque, S. Silva, C. Rossi, and T. M. Zucchi.** 2002. Cytotoxic and genotoxic effects of megazol, an anti-Chagas disease drug, assessed by different short-term tests. Biochem. Pharmacol. **64:**1617–1627.
31. **Prusis, P., M. Dambrova, V. Andrianov, E. Rozhkov, V. Semenikhina, I. Piskunova, E. Ongwae, T. Lundstedt, I. Kalvinsh, and J. E. Wikberg.** 2004. Synthesis and quantitative structure-activity relationship of hydrazones of N-amino-N′-hydroxyguanidine as electron acceptors for xanthine oxidase. J. Med. Chem. **47:**3105–3110.
32. **Rodriques Coura, J. R., and S. L. De Castro.** 2002. A critical review on Chagas disease chemotherapy. Mem. Inst. Oswaldo Cruz **97:**3–24.

32a. **Romanha, A. J., M. N. C. Soeiro, J. Lannes-Vieira, S. L. de Castro, I. Ribeiro, A. Talvani, B. Bourdin, B. Blum, B. Olivieri, C. Zani, C. Spadafora, E. Chiari, E. Chatelain, G. Chaves, J. E. Calzada, J. Bustamante, L. J. Freitas, L. Romero, M. T. Bahia, M. Lotrowska, M. Soares, S. G. Andrade, T. Armstrong, W. Degrave, and Z. A. Andrade.** Experimental *in vitro* and *in vivo* models for drug screening and development for Chagas disease. Mem. Inst. Oswaldo Cruz, in press.

33. **Salomão K., E. M. de Souza, A. Henriques-Pons, H. S. Barbosa, and S. L. De Castro.** 11 March 2009. Brazilian green propolis: effects *in vitro* and *in vivo* on *Trypanosoma cruzi.* Evid. Based Complement. Alternat. Med. doi: 10.1093/ecam/nep014.
34. **Sant'Anna, C., F. Parussini, D. Lourenço, W. de Souza, J. J. Cazzulo, and N. L. Cunha-e-Silva.** 2008. All *Trypanosoma cruzi* developmental forms present lysosome-related organelles. Histochem. Cell Biol. **130:**1187–1198.

35. **Schmunis, G. A.** 2007. Epidemiology of Chagas disease in non endemic countries: the role of international migration. Mem. Inst. Oswaldo Cruz **102**(Suppl. 1)**:**75–85.
36. **Sforcin, J. M.** 2007. Propolis and the immune system: a review. J. Ethnopharmacol. **113:**1–14.
37. **Silva, C. F., M. B. Meuser, E. M. de Souza, M. N. L. Meirelles, C. E. Stephens, P. Som, D. W. Boykin, and M. N. C. Soeiro.** 2007. Cellular effects of reversed amidines on *Trypanosoma cruzi*. Antimicrob. Agents Chemother. **51:**3803–3809.
38. **Soeiro, M. N. C., and S. L. de Castro.** 2009. *Trypanosoma cruzi* targets for new chemotherapeutic approaches. Expert Opin. Ther. Targets **13:**105–121.
39. **Souto-Padrón, T., W. de Souza, and J. E. Heuser.** 1984. Quick-freeze, deep etched rotary replication of *Trypanosoma cruzi* and *Herpetomonas megaseliae*. J. Cell Sci. **69:**167–178.
40. **Souza, E. M., A. Lansiaux, C. Bailly, W. D. Wilson, Q. Hu, D. W. Boykin, M. M. Batista, T. C. Araújo-Jorge, and M. N. C. Soeiro.** 2004. Phenyl substitution of furamidine markedly potentiates its anti-parasitic activity against *Trypanosoma cruzi* and *Leishmania amazonensis*. Biochem. Pharmacol. **133:**593–600.
41. **Vannier-Santos, M. A., and S. L. de Castro.** 2009. Electron microscopy in antiparasitic chemotherapy: a (close) view to a kill. Curr. Drug Targets **10:**246–260.
42. **Viodé, C., N. Bettache, N. Cenas, R. L. Krauth-Siegel, G. Chauvière, N. Bakalara, and J. Périé.** 1999. Enzymatic reduction studies of nitroheterocycles. Biochem. Pharmacol. **57:**549–557.