Nitrotriazole-Based Compounds as Antichagasic Agents in a Long-Treatment *In Vivo* Assay

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ABSTRACT 3-Nitrotriazole-based compounds belonging to various chemical subclasses were found to be very effective against Chagas disease both *in vitro* and *in vivo* after a short administration schedule. In this study, five compounds with specific characteristics were selected to be administered for longer periods of time to mice infected with the virulent *Trypanosoma cruzi* Y strain to further evaluate their effectiveness as antichagasic agents and whether or not potential adverse effects occur. Benznidazole was included for comparison purposes. Complete parasitemia depletion, weight gain, 100% survival, and a lack of myocardial inflammation were observed with four of the compounds and benznidazole administered intraperitoneally at 15 or 20 mg/kg of body weight/day for 40 days. There was a significant reduction in the number of treatment days (number of doses) necessary to induce parasitemia suppression with all four compounds compared to that required with benznidazole. Partial cures were obtained with only one compound tested at 15 mg/kg/day and on the schedule mentioned above but not with benznidazole. Taken together, our data suggest that these compounds demonstrate potent trypanocidal activity comparable to or better than that of the reference drug, benznidazole, when they are administered at the same dose and on the same schedule.

KEYWORDS nitrotriazoles, benznidazole, Chagas disease, long treatment

American trypanosomiasis (Chagas disease) is a neglected tropical disease caused by infection with the parasitic protozoan *Trypanosoma cruzi*. This insect-transmitted disease is endemic in South and Central America (1); however, the number of cases at sites where it is not endemic (United States, Australia, Europe, and Japan) is rising, primarily due to human and vector migration and transfusions of contaminated blood (2). Approximately 8 million people are estimated to be infected worldwide, and over 25 million are at risk (1).

High levels of parasitemia and inflammation occur during the initial acute phase of the disease; however, most individuals can circumvent this acute phase of infection and enter an indeterminate chronic phase with low levels of parasitemia and no apparent pathology due to activation of the immune response. More problematic is the symptomatic chronic phase of Chagas disease, characterized by low levels of parasitemia but increased tissue injury, resulting in severe digestive and/or cardiac damage that can be lethal if the patient is untreated (3).

Despite dramatic advances in modern drug discovery tools, the treatment of Chagas disease is based on only two old drugs with serious limitations: benznidazole [Bzn; *N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide] and nifurtimox [4-(5-nitrofurfurylindenamino)-3-methylthio-morpholine-1,1-dioxide]. Both of these com-

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FIG 1 Preliminary *in vivo* evaluation of the antichagasic efficacy of tested compounds 1, 2, 3, and 5 using a murine model of acute infection with a Brazilian *T. cruzi* strain (10–13). The compounds were administered i.p. at 15 mg/kg/day (compounds 1 and 2) or 13 mg/kg/day (compounds 3 and 5) for 5 or 10 consecutive days. The level of parasitemia was calculated after 5 and 10 days of treatment by using a fast luminescence assay and is expressed as a percentage of that for the untreated controls. A Brazilian strain of transgenic *T. cruzi* parasite trypomastigotes expressing firefly luciferase was used for infection. Each group had 5 mice. Representative images of treated (compound 2) and untreated mice at 5 days of treatment are shown. The *P* values between each treated group and the control group were <0.001 (5-day treatment) and <0.0001 (10-day treatment).

pounds require prolonged treatment, display potentially severe toxicity (4), and have controversial efficacy in *T. cruzi*-infected adults with chronic indeterminate status, and there is some degree of variation in sensitivity among parasite populations (5, 6). There is, therefore, a critical need for new, effective, safer, and more affordable drugs for Chagas disease treatment.

We have demonstrated that various chemical classes of 3-nitro-1*H*-1,2,4-triazole-based compounds exhibit excellent antichagasic activity both *in vitro* and *in vivo* (7–15) and that this activity is in part due to their reductive activation by a type I nitroreductase (NTR) (7, 8, 10–15). Type I NTR is an oxygen-insensitive nitroreductase present in the mitochondrion of trypanosomatids and absent from most other eukaryotes (16–19). Moreover, 3-nitrotriazole-based compounds seem to be more potent and less toxic than their 2-nitroimidazole-based counterparts (7, 8, 10, 20). Since azoles play a significant role in the inhibition of CYP51 (sterol 14 α -demethylase, an enzyme crucial for the formation of viable membranes and the regulation of metabolic processes) not only in fungi but also in trypanosomatids (21–24), we have also developed subclasses of 3-nitrotriazole-based compounds that act as bifunctional antichagasic agents: they are substrates for type I NTR, in addition to being reversible inhibitors of *T. cruzi* CYP51 (12, 13). The idea of making bifunctional compounds was based on the observation that combination therapy with multiple targets offers advantages compared to monotherapy (25, 26). Indeed, such bifunctional compounds demonstrate remarkable antichagasic activity both *in vitro* and in a murine model of acute infection (12, 13).

In the present study, we further explored the *in vivo* antichagasic properties of five 3-nitrotriazole-based analogs with specific characteristics by using mice infected with the virulent *T. cruzi* Y strain and longer periods of drug treatment. Benznidazole was used as the reference compound, and it was administered at the same dose and schedule as the tested compounds, in addition to a schedule of oral administration at 100 mg/kg of body weight/day for 20 days, a protocol that leads to a 55% cure rate in Y strain-infected mice (27). The intraperitoneal (i.p.) route of benznidazole administration was used in parallel for comparison purposes, since the tested compounds were administered intraperitoneally. We adopted this type of administration route as a result of our previously obtained *in vivo* data (Fig. 1) and due to a lack of oral bioavailability information for the tested compounds. The antichagasic activities of the tested compounds were evaluated by determination of the parasitemia reduction, the doses

TABLE 1 Chemical structures, *in vitro* antiparasitic activities, and host toxicities of the tested compounds

Compound	IC ₅₀ (μM) for <i>T. cruzi</i> ^a	SI ^b	IC ₅₀ (μM) for L6 cell cytotoxicity ^c	Chemical structure	Bnz/comp ^d	Reference(s)
Bnz	2.13 ± 0.11 ^e				1	11
1	0.462	278	128		4.4	10, 11
2	0.138	1,194	165		10.8	12
3	0.008	3,615	28.92		254	13
4	0.065	1,416	92.04		31.2	13
5	0.036	1,954	70.36		56.3	13

^a*T. cruzi* strain Tulahuen C4 amastigotes.^bSI, selectivity index, which is the ratio of the IC₅₀ for L6 cells/IC₅₀ for the parasite.^cIC₅₀s are the means of 2 or 3 measurements. The standard deviation was <5%.^dBnz/comp, ratio of the IC₅₀ of Bnz for *T. cruzi*/IC₅₀ of each compound for *T. cruzi*.^eThe IC₅₀ of Bnz is the mean from multiple measurements in parallel with the tested compounds.

required for complete parasitemia suppression, weight gain or loss, survival, parasitological cures by blood quantitative PCR (qPCR) assay, and the histopathology of the heart. The criteria used for selecting the five compounds (Table 1) were based on their previous *in vitro* and *in vivo* performance as antichagasic agents and the differences in their chemical structures. The *in vitro* antichagasic activities of the five compounds are summarized in Table 1. All compounds demonstrated a 50% inhibitory concentration (IC₅₀) against *T. cruzi* amastigotes at low to moderate nanomolar concentrations (8 to 462 nM), had high selectivity indices (278 to 3,615), and were 4.4- to 254-fold more potent against *T. cruzi* than benznidazole (Table 1). All compounds except compound 4 (which was not previously evaluated *in vivo*) demonstrated excellent antichagasic activity in a mouse model of infection with a Brazilian *T. cruzi* strain (10–13) when administered i.p. at 13 or 15 mg/kg/day, completely suppressing parasitemia after a 5- or 10-day treatment (Fig. 1). Compound 1, a 3-nitrotriazole-based chlorothiophenesulfonamide, is a type I NTR substrate but not a CYP51 inhibitor due to its flexible backbone core (11, 12). Compound 2, a 3-nitrotriazole-based amide, has been proven to be a substrate for type I NTR and a weak CYP51 reversible inhibitor due to its more rigid backbone core (12). Finally, compounds 3 to 5 are 3-nitrotriazole based aryloxyphenylamides activated by type I NTR and strong binders of *T. cruzi* CYP51 (TcCYP51), as has been demonstrated by docking studies (13).

RESULTS

The parasitemia levels and mortality rates of animals infected with the *T. cruzi* Y strain were assessed. All animals in the untreated control group demonstrated higher levels of parasitemia than treated animals, with peak values being 1.897×10^3

FIG 2 Parasitemia levels in peripheral blood of *Trypanosoma cruzi* (Y strain)-infected mice submitted to treatment with nitrotriazole compound 1 (A) or 2 (B) or benznidazole (A and B). The compounds were administered i.p. at 15 mg/kg/day for 20 days or at 20 mg/kg/day for 40 days. The treatment started on day 4 postinfection.

trypomastigotes/0.1 ml of blood on the 8th day postinfection (Fig. 2), and mortality occurred at 15 days postinfection, on average.

The reference treatment with benznidazole given orally at 100 mg/kg/day for 20 days was very effective in suppressing parasitemia after the 1st or 2nd day posttreatment in all animals, providing 100% survival during the treatment period, despite the fact that parasitemia rebounded in 4 out of 15 mice (Fig. 2 and Table 2). When benznidazole was administered i.p. at 15 mg/kg/day for 20 days, an initial dose that was used for two of the tested compounds (compounds 1 and 2), a reduction in the level of parasitemia but no complete parasitemia suppression occurred (Fig. 2 and Table 2). In this case, the rate of survival was 37.5% (3 out of 8 mice). Similar results were obtained when the tested sulfonamide (compound 1) with a flexible backbone core was administered i.p. at 15 mg/kg/day for 20 days (Fig. 2 and Table 2). The rigid amide (compound 2) was slightly better than the sulfonamide (compound 1) and benznidazole when given i.p. at 15 mg/kg/day for 20 days, providing parasite clearance in 3 out of 8 mice after 14.3 doses; however, it did not improve the rate of survival over the 37.5% achieved with benznidazole (Table 2).

Considering that these results indicated that the dose and treatment schedule were suboptimal, it was decided to increase the dose and treatment duration for the tested compounds in a second set of experiments. Thus, in these experiments mice were treated with compound 1 or 2 or benznidazole at 20 mg/kg/day for 40 days (Table 3). The more potent compounds (compounds 3, 4, and 5) (Table 1 and Fig. 1) were

TABLE 2 Efficacies of compounds 1 and 2 and benznidazole in a murine model of *Trypanosoma cruzi* infection after i.p. treatment for 20 days^a

Experimental group	No. of mice with parasitemia clearance/total no. of mice tested (mean no. of doses $\pm$ SD)	No. of mice with parasitemia rebound/total no. of mice tested		No. of mice with positive qPCR result/total no. of mice tested	Total no. of mice positive for parasitemia/total no. of mice tested	No. of mice that survived/total no. of mice tested
		Before IS	After IS			
Infected control		6/6	ND	ND	6/6	0/6
Uninfected control				0/12	0/12	12/12
Bnz at 100 mg/kg/day for 20 days orally	15/15 (1.4 $\pm$ 0.8)	3/15	4/15	6/15	6/15	15/15
Bnz at 15 mg/kg/day for 20 days i.p.	0/8	ND	ND	ND	8/8	3/8
Compound 1 at 15 mg/kg/day for 20 days i.p.	0/8	ND	ND	ND	8/8	3/8
Compound 2 at 15 mg/kg/day for 20 days i.p.	3/8 (14.3 $\pm$ 0.6)	8/8	ND	ND	8/8	3/8

^aFemale Swiss mice (6 to 8 mice/group; weight, 20 to 24 g) were inoculated with 5×10^3 *T. cruzi* (Y strain) trypomastigotes. Treatment was initiated at 4 days postinoculation and continued for 20 days. The compounds were administered intraperitoneally at 15 mg/kg/day or orally at 100 mg/kg/day (benznidazole). Parasite rebound was detected by examination of fresh blood before (until 30 days posttreatment) and after (after 30 days posttreatment) cyclophosphamide immunosuppression. A PCR assay was performed at 1 month posttreatment. IS, immunosuppression; ND, not determined.

TABLE 3 Efficacy of all tested compounds or benznidazole in a murine model of *Trypanosoma cruzi* infection after i.p. treatment for 40 days^a

Experimental group	No. of mice with parasitemia clearance/total no. of mice tested (mean no. of doses $\pm$ SD)	No. of mice with parasitemia rebound/total no. of mice tested		No. of mice with positive qPCR result/total no. of mice tested	Total no. of mice positive for parasitemia/total no. of mice tested	No. of mice that survived/total no. of mice tested
		Before IS	After IS			
Infected control		6/6	ND	ND	6/6	0/6
Uninfected control				0/12	0/12	12/12
Bnz at 100 mg/kg/day for 20 days orally	15/15 (1.4 ± 0.8)	3/15	4/15	6/15	6/15	15/15
Bnz at 15 mg/kg/day for 40 days i.p.	6/7 (13.8 ± 10.67)	7/7	7/7	7/7	7/7	7/7
Bnz at 20 mg/kg/day for 40 days i.p.	7/7 (12.6 ± 6.2)	1/7	5/7	7/7	7/7	7/7
Compound 1 at 20 mg/kg/day for 40 days i.p.	7/7 (6.1 ± 2.5)	0/7	7/7	7/7	7/7	7/7
Compound 2 at 20 mg/kg/day for 40 days i.p.	6/6 (10.5 ± 8.1)	1/6	6/6	6/6	6/6	6/6
Compound 3 at 15 mg/kg/day for 40 days i.p.	7/7 (2.14 ± 0.69)	5/7	7/7	7/7	7/7	7/7
Compound 4 at 15 mg/kg/day for 40 days i.p.	0/7	7/7	ND	ND	7/7	5/7
Compound 5 at 15 mg/kg/day for 40 days i.p.	6/6 (2.3 ± 0.8)	0/6	5/6	5/6	5/6	6/6

^aFemale Swiss mice (6 to 8 mice/group; weight, 20 to 24 g) were inoculated with 5×10^3 *T. cruzi* (Y strain) trypomastigotes. Treatment was initiated at 4 days postinoculation and continued for 40 days. The compounds were administered intraperitoneally at 15 or 20 mg/kg/day or orally at 100 mg/kg/day (benznidazole). Parasite rebound was detected by examination of fresh blood before (until 30 days posttreatment) and after (after 30 days posttreatment) cyclophosphamide immunosuppression. A PCR assay was performed at 1 month posttreatment. IS, immunosuppression; ND, not determined.

administered at 15 mg/kg/day for 40 days, and the results were compared to those obtained with a similar schedule of benznidazole administration (Table 3).

A dose-dependent effect was apparent. Therefore, complete parasitemia suppression was observed in all mice after 6.1 ± 2.5 , 10.5 ± 8.1 , and 12.6 ± 6.2 doses of compound 1, compound 2, and benznidazole, respectively, when each agent was administered i.p. at 20 mg/kg/day for 40 days (Table 3). The drugs were well tolerated by the animals without adverse effects or mortality. Therefore, survival was 100% by the end of the experiment in all the treated groups described above, whereas 100% mortality was observed in the untreated infected control group (Table 3). Parasitemia rebound occurred in one animal each in the benznidazole- and compound 2 (rigid amide)-treated groups but not in the flexible sulfonamide (compound 1)-treated group before immunosuppression (Table 3). However, treatment with compound 1 or 2 or benznidazole at 20 mg/kg/day for 40 days did not result in complete parasitological cures in mice infected with the Y strain, since a parasitemia rebound was detected in all treated mice after immunosuppression (Table 3).

The dose-dependent effect of treatment was also observed with regard to parasitemia levels (Fig. 2). The area under the curve (AUC) in the graph of the parasitemia level versus time for each animal in the groups treated with compound 1 or 2 or benznidazole at 15 mg/kg/day did not differ from that for each animal in the untreated control group ($P \geq 0.05$). On the other hand, a significant reduction of the AUC among the animals receiving 20 mg/kg/day of these compounds for 40 days compared to that among the animals in the infected control group could be verified. In addition, the AUC values in the compound 1-treated mice (20 mg/kg/day) were significantly less than those in mice treated with benznidazole at the same dose (Fig. 3).

The beneficial effect of the daily treatment with compound 1 or 2 at 20 mg/kg for 40 days could be seen in the significant weight gain of the animals during treatment. A similar effect was also observed in the group treated with the same dose of benznidazole (Fig. 4). On the contrary, there was no significant weight gain among animals treated with the same compounds at 15 mg/kg/day for 20 days (Fig. 4).

As mentioned earlier, in this set of experiments, compounds 3 to 5, which were more potent *in vitro* and against the Brazilian strain (Table 1 and Fig. 1), were administered i.p. at a lower dose than compounds 1 and 2, namely, 15 mg/kg/day for 40 days. The high antichagasic potencies of these compounds may be attributed to their bifunctional character, since they are substrates of type I NTR and might act as inhibitors of the TcCYP51 enzyme (13). Compound 4 was included in the study because

FIG 3 Analysis of the area under the curve of daily parasitemia for each animal infected with the Y strain of *Trypanosoma cruzi* and treated with 15 mg/kg/day or 20 mg/kg/day of compound 1 or 2 or benznidazole. IC, infected control group. The statistical significance of the difference among groups was determined by a nonparametric *t* test. a, significant difference in relation to the infected control group; b, significant difference in relation to benznidazole treatment at the same dose; c, significant difference between groups treated with 15 mg/kg/day or 20 mg/kg/day of the same compound.

it had an efficacy profile *in vitro* similar to the profiles of compounds 3 and 5 (Table 1), despite the fact that it was not previously evaluated in the fast *in vivo* luminescence assay. Benznidazole at 15 mg/kg/day for 40 days (i.p.) was also included in the study for comparison purposes. The efficacy data for these 3-nitrotriazole-based aryloxyphenylamides are also summarized in Table 3. The numbers of doses required to achieve complete parasitemia suppression were 2.14 ± 0.69 and 2.3 ± 0.8 for compounds 3 and 5, respectively, significantly lower than the number of doses of benznidazole given at the same i.p. daily dose required (13.8 ± 10.67) and comparable to the number of standard daily treatments with oral benznidazole at 100 mg/kg for 20 days required (1.4 ± 0.8) (Table 3). Both compounds 3 and 5 were well tolerated by the animals, and no mortality was detected. In contrast, compound 4 was not effective in significantly reducing the level of parasitemia compared to that in the infected and untreated control group (Table 3). Despite the inability of compound 4 to induce a parasitemia

FIG 4 Body weight gain (in grams) in animals infected with *Trypanosoma cruzi* (strain Y) and treated intraperitoneally with compound 1 or 2 or benznidazole at 15 mg/kg/day for 20 days or at 20 mg/kg/day for 40 days. ○, body weight on the first day of treatment; ●, body weight on the last day of treatment. The weights of the animals in the uninfected control group were obtained after 20 and 40 days of treatment, while the weights of the infected and untreated animals were obtained on days 4 and 15 postinfection. Statistically significant differences were determined by the nonparametric Mann-Whitney test. *, untreated.

FIG 5 Parasitemia levels in peripheral blood of *Trypanosoma cruzi* (Y strain)-infected mice subjected to intraperitoneal treatment with nitrotriazole compounds 3 to 5 or benznidazole at 15 mg/kg for 40 days. The treatment started and finished on days 4 and 44 postinfection, respectively.

reduction, this compound was very effective in reducing mortality, providing 71.4% survival, whereas the rate of survival was 0% in the infected control group (Table 3).

Despite the parasitemia clearance and a lack of parasitemia rebound before immunosuppression seen in the group treated with compound 5, parasitological cure was only partially achieved. Thus, by examination of fresh blood, parasite presence was detected after immunosuppression in 5 of 6 compound 5-treated animals and in all compound 3- or benznidazole-treated ones. These data are in agreement with the positive results obtained by the blood qPCR assay (Table 3).

The potent anti-*T. cruzi* activities of the 3-nitrotriazole-based aryloxyphenylamides (compounds 3 and 5) were also demonstrated by the suppression of parasitemia during the treatment period. In contrast, mice receiving compound 4 had a parasitemia load similar to that of the mice in the infected and untreated control group (Fig. 5).

These observations were confirmed by analysis of the AUC of the daily parasitemia in each animal (Fig. 6). Our findings demonstrate that compounds 3 and 5 are more potent antichagasic agents than benznidazole when they are given under the same therapeutic regimen. In contrast, the parasitemia load of compound 4-treated animals was very similar to that of the animals in the infected control group (Fig. 6).

Once again, the results presented above were in line with the significant body weight gain observed in mice treated with the nitrotriazoles (compounds 3 and 5) but not mice treated with compound 4, as well as the body weight gain observed in mice treated with benznidazole at the same daily dose, compared to the body weights of the mice in the infected control group (Fig. 7).

To further evaluate the efficacy of the 40-day intraperitoneal treatment with 3-nitrotriazole-based compounds 1 to 5 or benznidazole at 15 or 20 mg/kg/day with regard to chronic myocardial lesion prevention in mice infected with the Y strain of *T. cruzi*, quantitative analysis of the inflammation of heart tissue was performed at 6 months posttreatment. Despite the fact that complete parasitemia elimination or cures were not achieved after the 40 days of intraperitoneal treatment with 3-nitrotriazole-based compounds at a 15- to 20-mg/kg daily dose, all compounds were shown to prevent or lessen the typical inflammation of heart tissue associated with the chronic phase of experimental Chagas disease (Fig. 8). As illustrated in Fig. 8, similar levels of

FIG 6 Analysis of the area under the curve of daily parasitemia in individual mice in each group. Animals were infected with the Y strain of *Trypanosoma cruzi* and treated intraperitoneally with compounds 3 to 5 or benznidazole at 15 mg/kg/day for 40 days. IC, infected control group. The statistical significance of the difference among groups was determined by a nonparametric *t* test. a, significant difference compared to the infected control group; b, significant difference compared to benznidazole treatment at the same dose.

inflammatory cells were observed in the heart tissue of healthy animals and animals treated intraperitoneally for 40 days with compound 1, 2, 3, or 5 or benznidazole (at 15 mg/kg/day). Similar results were also observed with the standard oral treatment with benznidazole (Fig. 8). In contrast, compound 4-treated mice presented a higher number of inflammatory cells in their heart tissue than healthy mice as well as mice that received the standard benznidazole treatment.

Taken together, our data show that 3-nitrotriazole-based compounds 1, 2, 3, and 5 demonstrate potent trypanocidal activity comparable to or better than that of the reference drug, benznidazole, when administered by the same route and dose. In addition, these compounds did not cause any adverse effects, prevented myocardial

FIG 7 Body weight gain (in grams) in animals infected with *Trypanosoma cruzi* (strain Y) and treated intraperitoneally with compounds 3 to 5 or benznidazole at 15 mg/kg/day for 40 days. ○, body weight on the first day of treatment; ●, body weight on the last day of treatment. The weights of the animals in the uninfected control group were obtained after 20 and 40 days of treatment, while the weights of the infected and untreated animals were obtained on days 4 and 15 postinfection. Statistically significant differences were determined by the nonparametric Mann-Whitney test. *, untreated.

FIG 8 Myocardial inflammatory cell count in cardiac tissue of mice infected with the Y strain of *Trypanosoma cruzi* obtained at 6 months posttreatment for 40 days with a daily intraperitoneal dose of 20 mg/kg of compound 1 or 2 or 15 mg/kg of compounds 3 to 5 or benznidazole. Infected and untreated (IC) and uninfected (NIC) groups as well as a group receiving a standard benznidazole regimen (100 mg/kg/day for 20 days orally) were used as controls. *, significant difference in relation to the infected group; ♦, significant difference in relation to the uninfected group; α, significant difference in relation to the group treated with the standard benznidazole regimen (100 mg/kg/day for 20 days orally); ***, infected animals were necropsied at 15 days after infection (this strain induces 100% mortality at about 18 to 20 days of infection).

inflammation, and provided 100% survival, despite the lack of complete cures in treated animals.

DISCUSSION

As mentioned earlier, in the present work we further investigated the *in vivo* antichagasic properties of five 3-nitrotriazole-based analogs with specific characteristics by using mice infected with the virulent *T. cruzi* Y strain and treatment periods longer than those used in our initial studies (10–13). In the absence of long-term toxicity and pharmacokinetic studies with any of these compounds, doses previously effective *in vivo* in the short-treatment studies were used (Fig. 1). Starting with a conservative daily dose of 15 mg/kg i.p. and a 20-day treatment period for the flexible sulfonamide (compound 1) and the bifunctional rigid amide (compound 2), we were able to reduce the level of parasitemia and improve the rate of survival by 37.5% compared to the level of parasitemia and the rate of survival for the untreated and infected control group; however, no complete parasitemia clearance was observed with either nitrotriazole or benznidazole at the same dose and route (Table 2). By slightly increasing the daily dose to 20 mg/kg and doubling the treatment duration to 40 days, we were able to achieve 100% survival (versus 0% survival for the untreated control group) and complete parasitemia clearance (Table 3), confirming the dose-dependent anti-*T. cruzi* activities of 3-nitrotriazole-based agents 1 and 2. Interestingly, by use of the same administration protocol, a lower dose of both compounds compared to the dose of benznidazole was needed for complete parasitemia clearance. This could be attributed to a 4-fold better activation of compound 1 than of benznidazole by NTR (11), whereas for the rigid amide (compound 2), the lower effective dose could be attributed both to the better activation of the compound than that of benznidazole (1.3-fold) by *T. cruzi* NTR (TcNTR) and to the ability of compound 2 to reversibly inhibit the *T. cruzi* CYP51 enzyme (12). Noticeably, bifunctional compound 2 resulted in 37.5% parasitemia clearance even at 15 mg/kg/day, something that was not achieved with the sulfonamide (compound 1) or benznidazole by use of the same administration regimen (Table 2). Since the antichagasic activities of nitrotriazole compounds 1 and 2 were dose dependent, the lack of parasitological cures could be attributed to the use of a suboptimal dose. In the present study, conservative i.p. doses were applied due to the absence of long-term toxicity studies and because of the high *in vitro* potency of 3-nitrotriazoles compared to that of benznidazole (Table 1). Both compounds demon-

strated good metabolic stability and good stability in plasma (10, 12); however, their intraperitoneal bioavailabilities have not yet been determined. Thus, when each compound was incubated with mouse microsomal protein in the presence of NADPH for 1 h at 37°C, the proportions of parent compound remaining were 96.6 and 79.4% for compounds 1 and 2, respectively, whereas their stability in mouse plasma was about 89% (10, 12). The increased animal weight, the lack of myocardial inflammation, and the 100% survival provided verification that both compounds 1 and 2 are safe and quite effective antichagasic agents even at this suboptimal dose.

With regard to the aryloxyphenylamides (compounds 3 to 5), unsubstituted compound 4 was the only one of this subclass without significant antichagasic activity in this *in vivo* model, despite its excellent anti-*T. cruzi* potency in infected amastigotes (Table 1). Compound 4 was activated by *T. cruzi* NTR to a slightly higher degree than compounds 3 and 5 and benznidazole (1.30-, 1.58-, and 1.20-fold, respectively), but it demonstrated a lower docking score in the ligand binding pocket of sterol-14 α demethylase (PDB accession number 4H6O) (13). Therefore, its lack of activity in the present study may be strain related. The *T. cruzi* Y strain used in this study was previously characterized to be partially resistant to benznidazole (27) and most likely to other nitrocompounds that are activated by NTRs. In addition, it has been suggested that drug uptake played a more important role than drug metabolism in modulating resistance to nifurtimox in *T. cruzi* strains (28). This may be the case for compound 4 as well, since it is less lipophilic than compounds 3 and 5 (it had a lower value of the calculated logarithm of the partition coefficient between *n*-octanol and water [clogP]) (13).

With regard to the substituted aryloxyphenylamides (compounds 3 and 5), we have shown that both compounds are able to completely eliminate parasitemia at doses 6.4- and 6.0-fold lower than the dose of benznidazole (given at a similar i.p. daily dose), respectively, to provide 100% long-term survival, weight gain, and the absence of myocardial lesions, while fluoro-substituted compound 5 also resulted in some parasitological cures (16.7%). Both compounds 3 and 5 were more lipophilic than the unsubstituted aryloxyphenylamide (compound 4), a fact that may have resulted in better cell uptake, and both compounds 3 and 5 might have stronger binding to *T. cruzi* CYP51 than compound 4 (13). Therefore, better *in vivo* antichagasic activity was achieved.

In conclusion, our data demonstrate that 3-nitrotriazole-based compounds, especially the bifunctional ones, can act as efficacious antichagasic agents *in vivo* and their activity is dose dependent. All but one of the tested compounds behaved similarly to or better than benznidazole given at the same dose and by the same route. Additional experiments with increased doses of each compound or the compounds in combination should be performed to see if parasitological cures can be achieved. However, pharmacokinetic and toxicity studies should precede such experiments to better understand and elucidate the *in vivo* behaviors of these promising compounds.

MATERIALS AND METHODS

Ethics statements. All procedures and experimental protocols were conducted in accordance with Brazilian School of Animal Experimentation (COBEA) guidelines for the use of animals in research and were approved by the Ethics Committee in Animal Research at the Federal University of Ouro Preto (UFOP), Minas Gerais, Brazil (approval number 2014/15).

Compounds. The tested compounds were synthesized in-house as previously described (11–13) and included 5-chloro-*N*-[4-(3-nitro-1*H*-1,2,4-triazol-1-yl)butyl]thiophene-2-sulfonamide (compound 1), *N*-[4-(4-cyanophenyl)phenyl]-2-(3-nitro-1*H*-1,2,4-triazol-1-yl)acetamide (compound 2), *N*-[4-(4-chlorophenoxy)phenyl]-2-(3-nitro-1*H*-1,2,4-triazol-1-yl)acetamide (compound 3), 2-(3-nitro-1*H*-1,2,4-triazol-1-yl)-*N*-(4-phenoxyphenyl)acetamide (compound 4), and *N*-[4-(4-fluorophenoxy)phenyl]-2-(3-nitro-1*H*-1,2,4-triazol-1-yl)acetamide (compound 5).

The following drugs were commercially available and were obtained from their respective pharmaceutical companies: benznidazole [*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide; Lafepe, Brazil] and cyclophosphamide {2-[bis(2-chloroethyl)amino]-1,3,2-oxazaphosphinane 2-oxide; Genuxal; Asta Medica Oncologica}.

Parasite strain. The *Trypanosoma cruzi* Y (DTU II) strain was used in the present study (27). The Y strain is partially resistant to benznidazole and induces high levels of parasitemia, accompanied by 100% mortality rates, in Swiss mice. Mortality is generally observed 10 to 19 days postinfection in the absence of treatment. The original isolates were preserved in the trypomastigote form in liquid nitrogen, periodically transferred to mice, and then refrozen, with full retention of the biological and drug susceptibility characteristics being achieved.

In vivo efficacy studies. Female Swiss mice (weight, 20 to 24 g) were obtained from the Animal Facility at the Federal University of Ouro Preto, Minas Gerais, Brazil, and maintained in a temperature-controlled room with access to water and commercial food *ad libitum*. The animals were inoculated intraperitoneally with 5,000 blood-stage trypomastigotes of the *T. cruzi* Y strain. After 4 days, tail blood was examined microscopically for the presence of parasites.

Groups of mice (6 to 8 animals/group) were treated i.p. with each compound at 15 or 20 mg/kg/day for 20 or 40 days, beginning at the time of detection of parasitemia, which generally occurred at 4 days postinfection. A group of 15 mice was treated orally with benznidazole at 100 mg/kg/day for 20 days, which was used as the reference treatment (29), since such treatment provides a rate of cure of 55% in mice infected with the *T. cruzi* Y strain (27); 6 mice were maintained infected and used as untreated controls, whereas another 12 mice were maintained uninfected and used as untreated controls.

For intraperitoneal administration, each nitrotriazole-based compound or benznidazole was first dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and then in saline at a final volume of 100 μ l/animal. The intraperitoneal route was used due to a lack of oral bioavailability data for the tested compounds. Although intraperitoneal delivery is considered a parenteral route of administration, the pharmacokinetics of substances administered intraperitoneally are comparable to those seen after oral administration, because the primary route of absorption is into the mesenteric vessels and a first-pass metabolism in the liver is observed, as is the case with oral administration (30). For oral administration, benznidazole was administered as a suspension in polysorbate 80 containing carboxymethyl cellulose 0.5% (wt/vol) (Sigma-Aldrich, St. Louis, MO, USA).

The parasitological cure was determined by using two independent tests: examination of fresh blood before and after cyclophosphamide immunosuppression (Cyl), followed by PCR assays performed on blood samples from mice negative for parasitemia. Animals showing negative results by the two tests were considered cured.

Examination of fresh blood and cyclophosphamide immunosuppression. The level of parasitemia of the animals was evaluated during and up to the 30th day posttreatment to determine the natural reactivation of infection. Animals that did not present with reactivation of parasitemia after treatment were submitted to Cyl, which consisted of three cycles of treatment with 50 mg of cyclophosphamide per kg of body weight for four consecutive days with intervals of 3 days between each cycle. The level of parasitemia of these animals was evaluated during the Cyl cycles, as well as for the 10 days after immunosuppression.

Real-time PCR (qPCR). For the qPCR assays, animals were bled from the orbital venous sinus, and 200 μ l of blood was collected 30 days after the end of treatment. PCR was performed only with samples from animals with negative results by examination of fresh blood. The collected blood was mixed with 35 μ l of sodium citrate solution at 129 mM (Dolles, Brazil) and used for DNA extraction. DNA extraction was performed using a Wizard genomic DNA purification kit (Promega). qPCR assays were performed using a SYBR green system (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and the primers TCZ-F (5'-GCTCTTGCCACAMGGGTGC-3', where M is A or C) and TCZ-R (5'-CCAAGCAGCGATAGTTCAGG-3') (Invitrogen) for *T. cruzi* DNA amplification. The internal control (corresponding to a segment of the murine tumor necrosis factor alpha gene) was amplified using the primers TNF-5241 (5'-TCCTCTCATCATGTTCTATGGCCCA-3') and TNF-5411 (5'-CAGCAAGCATCTATGCACTTAGACCCC-3') (Invitrogen). Cycles of amplification were carried out in a StepOnePlus real-time PCR system (Applied Biosystems). The cycles consisted of an initial denaturation hold of 10 min at 95°C, followed by 40 cycles of 15 s at 94°C and 1 min at 64.3°C with fluorescence acquisition. Amplification was immediately followed by a melt program with an initial denaturation for 15 s at 95°C, cooling to 60°C for 1 min, and then a stepwise temperature increase from 60 to 95°C at 0.3°C/s. The limits of detection of the parasites were verified by the use of 6 serial dilutions (1:10) of 100 parasite equivalents (from blood standards) in DNA (25 ng/liter) from the blood of healthy mice. All samples were analyzed in duplicate, negative samples and reagent controls were processed in parallel in each assay, and all experiments were conducted under controlled conditions.

Myocardial tissue assessment. For histopathological analysis of myocardial tissues, mice were sacrificed at 180 days after treatment by cervical dislocation, and heart tissues were fixed with 10% formalin and embedded in paraffin. Blocks were cut into 4- μ m-thick sections and stained with hematoxylin and eosin (H&E) for inflammation assessment. Twenty fields of stained slides were randomly chosen under a $\times 40$ magnification for analysis of a total area of myocardium of 1.49×10^6 μ m². Images were captured using a microscope equipped with a Leica DM 5000 B microcamera (model 2.4.0R1; Leica Application Suite), and the images were processed with Leica Qwin (v3) image analyzer software.

Statistical analysis. To evaluate the dose-dependent effect of the compounds in reducing the level of parasitemia, the data were converted using a logarithmic transformation and tested using Pearson's correlation coefficient. The variations in the levels of parasitemia among the animals treated with each drug were evaluated using analysis of variance, and comparisons among the groups were performed using Tukey's multiple-comparison test. The results of the morphometric analysis were expressed as the

average $\pm$ standard deviation and analyzed using the Mann-Whitney test. Differences were considered significant when P was <0.05 .

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M. V. Papadopoulou was responsible for the synthesis of the compounds and participated in the design of the experiments, the analysis and interpretation of the data, and the preparation of the manuscript; W. D. Bloomer and H. S. Rosenzweig participated in the analysis and interpretation of the data; A. L. Mazzeti, K. R. Gonçalves, and P. F. Mendes performed the experimental *in vivo* work; and M. T. Bahia was responsible for the design and execution of the study as well as the analysis, interpretation, and presentation of the data.

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