Original article

In vivo studies of 5-arylethenylbenzofuroxans in acute murine models of Chagas' disease

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Abstract

5-Arylethenylbenzofuroxan derivatives with high *in vitro* anti-*Trypanosoma cruzi* activity were studied *in vivo* using acute murine models of Chagas' disease. The selected compounds, as pure isomeric forms, **1**, **2**, **3** and **4**, or as equimolecular mixture of geometric isomers, **1:2**, **3:4**, **5:6** were studied against different *T. cruzi* strains. Consequently, Tulahuen 2 strain, Colombiana strain (resistant to Nifurtimox and Benznidazole), and two different wild strains, one isolated from the wild reservoir *Didelphis marsupialis* and another one from Uruguayan patients, were selected. No relevant signs of *in vivo* toxicity were observed with the benzofuroxans orally administered. Compound **1** and the mixture of isomers **1:2** were the best for treating infection against the four studied strains.
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1. Introduction

Parasitic diseases represent a major health problem in Latin America. In particular, Chagas' disease or American Trypanosomiasis, caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), is the largest parasitic disease burden in the American continents. It affects approximately 20 million people from southern United States to southern Chile. The most important clinical manifestations of the chronic Chagas' disease are heart insufficiency (Chagasic cardiopathy and arrhythmias, 90% of cases approximately) and gastrointestinal syndromes (megaoesophagus and megacolon). Even though the enforcement of public health programs towards vector elimination in some Latin American countries has decreased the incidence of new infections, the disease is still endemic in many large areas. Every year, 21,000 people die from this parasitosis and over 200,000 new cases arise [1,2]. Despite this reality, pharmacological responses for this disease are still inadequate. Current treatment is based on old drugs (Nifurtimox, Nfx, and Benznidazole, Bnz) that, even though being able to wipe out parasitemia and reduce serological titers, do not guarantee complete cure and are associated with severe side effects [3]. For these reasons the search of new drugs against this parasitic disease is essential and urgent [4]. To this objective, our group has investigated and developed new agents derived from the benzofuroxan (benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide) heterocycle [5–10]. The developed compounds have been excellent *in vitro* anti-*T. cruzi* agents, against different strains of epimastigote form of the parasite. Moreover, the new benzofuroxan derivatives were less or as cytotoxic as the reference drugs (Nfx, Bnz, ketoconazole and terbinafine) [11,12]. From

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these studies lead compounds were identified, specifically 5-(2-phenylethenyl)benzofuroxan (isomers *E* and *Z*, **1** and **2**, respectively, Fig. 1) and its phenyl-substituted derivatives 5-[2-(3,4-methylendioxyphenyl)ethenyl]benzofuroxan (*E* and *Z* isomers, **3** and **4**, respectively, Fig. 1) and, 5-[2-4-chlorophenylethenyl]benzofuroxan (studied as the mixture of *E* and *Z* isomers, **5** and **6** Fig. 1). These compounds have been the subject of the following pre-clinical studies: *in vivo* efficacy, *in vitro* toxicity, safety and pharmaceutical feasibility. These studies were performed as part of a research project supported by DNDi (Drugs for Neglected Disease *initiative*) organization, which has as the primary goal the development of new and more effective drugs for people suffering from neglected diseases in developing countries [13].

In the present work, compounds **1**–**6** were tested *in vivo* on a murine model of *T. cruzi* infection, in order to complete the pre-clinical profile of these potential anti-Chagasic drugs. Four different strains of *T. cruzi* were used, and the efficacies of the studied compounds evaluated through parasitemia, level of anti-*T. cruzi* antibodies and histological studies. Furthermore, compound **1** was studied orally and intraperitoneally at higher doses, without signs of toxicity.

2. Chemistry

The benzofuroxan derivatives **1**–**6** were obtained as previously described [10,12]. Nfx (Lampit®, Bay 2502) was obtained from Bayer and Bnz (Rochagan®) was obtained from Roche. All compounds were identified by IR, MS, ^1H NMR, ^{13}C NMR, HSQC and HMBC experiments, and their purity established by TLC and microanalysis.

3. Pharmacology

In the *in vivo* studies four *T. cruzi* strains were employed including the *in vitro* studied Tulahuen 2 strain, the well known Nfx and Bnz resistant Colombiana strain [14,15], and two wild type strains from Argentine and Uruguay. Colombiana strain was defined initially, by Filardi and Brener [14], as
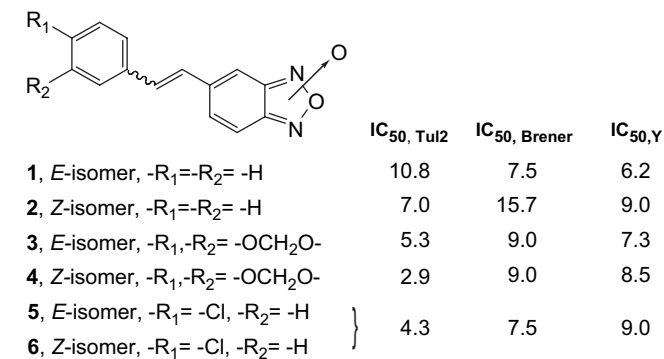
in vivo Nfx and Bnz resistant strain; however, recent studies, by Molina et al. [15], have described levels of *in vivo* parasitological cure in Bnz-treated animals higher than those reported by the former authors. On the other hand, the Argentinean strain was isolated from the wild reservoir *Didelphis marsupialis* (*D. marsupialis*) and the Uruguayan strain was isolated from Uruguayan patients.

In the following sections we will discuss the most relevant findings for each strain type. The 5-arylethenylbenzofuroxans **1**–**4** (Fig. 1) were studied as pure isomers and as equimolecular mixtures of *E* and *Z* geometric isomers, while compounds **5** and **6** were studied only as the equimolecular mixtures of *E* and *Z* geometric isomers.

3.1. In vivo activity against T. cruzi Tulahuen 2 strain

As a first approach, derivatives **1**, **1:2** (50:50) or **2** were administered orally at 60 mg/kg/day, according to the schedule (A) described in Section 5. Secondly, another experiment was performed, schedule (B), administering 120 mg/kg/day of **1**, **1:2** (50:50), **3**, **3:4** (50:50), **4** or **5:6** (50:50). In both regimens Bnz (200 mg/kg/day) and Nfx (100 mg/kg/day) were used as positive controls. For Bnz we have selected a dose that assures a complete suppression of parasitemia [16], in the case of Nfx we have selected an intermediate dose between both benzofuroxan studied doses. The best profiles in the parasitemia curves were observed for derivative **1** and the mixture **1:2** (data not shown). Remarkably, for the latter the number of circulating trypomastigotes was lower than in the untreated animals (infected animals treated only with vehicle, see Section 5) throughout almost all the studies. Besides, the level of anti-*T. cruzi* antibodies at 60th and 90th days post-infection (p.i.) with the parasite and the histological studies at the end of the experiment, were used to analyze the efficacy of these compounds. At 60 mg/kg/day, the mixture **1:2** reduced antibody levels, compared to those of untreated animals in both determination days (Fig. 2a). The same behaviour was observed with Bnz at 200 mg/kg/day. No improvement in the reduction of the antibody levels for animals treated with mixture **1:2** was observed in the 120 mg/kg/day schedule (Fig. 2b). In this assay, compound **3** was identified as an agent that reduces the level of antibodies in the animals infected with Tulahuen 2 strain. As we had already evidenced with Bnz and TAK-187 [16], negative serology was not observed with any of the studied benzofuroxans or controls, Bnz and Nfx. Since the experiment is an acute study negative serology was not reached at 90th day.

According to the results of histological studies, the treatment using the mixture **1:2** shows better results at 120 mg/kg/day than at 60 mg/kg/day (Fig. 3a). These results indicate that the mixture **1:2** was significantly superior to Bnz in preventing inflammatory infiltrates and tissue damage, particularly in the hearts and skeletal muscles of infected animals. However, compound **1** at 120 mg/kg/day showed neither better histological results than Bnz (Fig. 3b), nor lower antibody levels (data not shown). Similarly to compound **1**, derivative **4** and the equimolecular mixture **3:4**, showed a higher liver

|		IC$_{50, Tul2}$	IC$_{50, Brener}$	IC$_{50,Y}$
1, *E*-isomer, -R$_1$=-R$_2$= -H		10.8	7.5	6.2
2, *Z*-isomer, -R$_1$=-R$_2$= -H		7.0	15.7	9.0
3, *E*-isomer, -R$_1$,-R$_2$= -OCH$_2$O-		5.3	9.0	7.3
4, *Z*-isomer, -R$_1$,-R$_2$= -OCH$_2$O-		2.9	9.0	8.5
5, *E*-isomer, -R$_1$= -Cl, -R$_2$= -H		4.3	7.5	9.0
6, *Z*-isomer, -R$_1$= -Cl, -R$_2$= -H				

Fig. 1. Structures of the studied compounds. Tul2: epimastigotes from Tulahuen 2 strain; Brener: epimastigotes from CL Brener strain; Y: epimastigotes from Y strain. The IC$_{50}$, expressed in μM, are from *in vitro* studies [12].
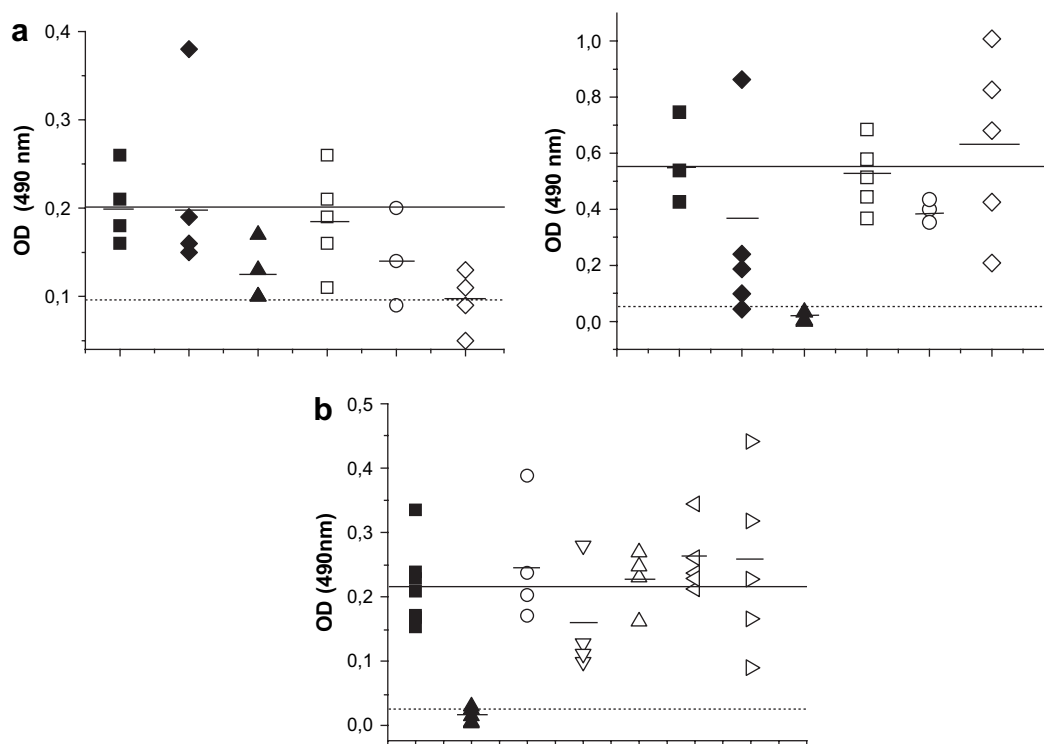
Fig. 2. (a) Dispersion diagrams of antibody levels in untreated animals (infected animals treated only with vehicle: see Section 5) (■) and those receiving, according to schedule (A), Nfx (◆), Bnz (▲), **1** (□), **1:2** (50:50) (○) or **2** (◇) treatments at 60 days p.i. (left) and 90 days p.i. (right). (b) Dispersion diagrams of antibody levels in untreated animals (■) and those receiving, according to schedule (B), Bnz (▲), **1:2** (50:50) (○), **3** (▽), **4** (△), **3:4** (50:50) (◁) or **5:6** (50:50) (▷) treatments at 90 days p.i.. The results are expressed as the absorbance of each serum sample at 490 nm. *Notes*: the cut-off for each reaction, dot-line, was the mean of the values determined for the negative controls (uninfected, normal mice) plus three times the standard deviation. The solid-line represents the mean values of antibody levels in untreated animals.

congestion degree than the untreated animals (Fig. 4) probably as result of some toxic effects of these agents. At the highest dose, 120 mg/kg/day, compound **3** promoted the increase of the bloodstream form of Tulahuen 2 parasites (data not shown), probably due to an immunosuppressant mechanism. Additionally, derivative **4** and the mixture **3:4** significantly prevented lesions in the heart (Fig. 4). Finally, the equimolecular mixture **5:6** displayed a moderate biological profile according to the antibody levels and histological studies.

The animals tolerated the administration of these compounds during the 25-day treatments without any sign of toxicity, and 100% of the animals survived at the end of both regimens. In general, in these studies the compounds had solubility problems in the vehicles used at the highest analyzed doses. These problems were mainly evidenced with the *E* isomers, namely **1** and **3**.

According to these studies, the mixture **1:2** has the best *in vivo* biological profile against Tulahuen 2 strain decreasing the anti-*T. cruzi* antibody levels and preventing inflammatory infiltrates and tissue damage, particularly in the hearts and skeletal muscles of infected animals.

3.2. In vivo activity against T. cruzi Colombiana strain

Compounds **1**, **3**, **4**, and the equimolecular mixtures of **1:2** and **5:6** at 120 mg/kg/day were evaluated in the model of acute

infection by Colombiana strain, schedule (C). Treatment with the mixture **1:2** was modified in 15th day. At this day the dose was changed to 60 mg/kg/day due to the emergence of toxicity signs, namely decreasing movements and spoiling fur. After that, no more toxicity signs in the new **1:2** dosage, at 60 mg/kg/day, were observed. Bnz was included in this schedule at 120 mg/kg/day. The best profiles in the parasitemia curves were observed for Bnz. The treatment with the benzofuroxans did not produce any decrease in blood trypomastigotes in the first 44 days of the assay, the mixture **1:2** having the best behaviour (data not shown). Like untreated animals, since 45th day no trypomastigotes were observed in the blood of treated animals. After 65 days post-infection, animals treated with Bnz had an increase in blood trypomastigote levels while none of the benzofuroxans-treated animals showed this behaviour.

Infected untreated mice presented high anti-*T. cruzi* antibody levels at both time points (Fig. 5). Treatment with the different benzofuroxans significantly reduced the circulating-antibody levels but these did not reach the basal levels of non-infected mice, even at the latter time point. The best findings in this study were observed for compound **1** and the mixture of isomers **1:2**. Compound **4** and the mixture of isomers **5:6** showed moderate antibody-reducing effects at 90th day. The histological findings indicated that all studied benzofuroxans, as pure isomeric forms or as mixtures, diminished
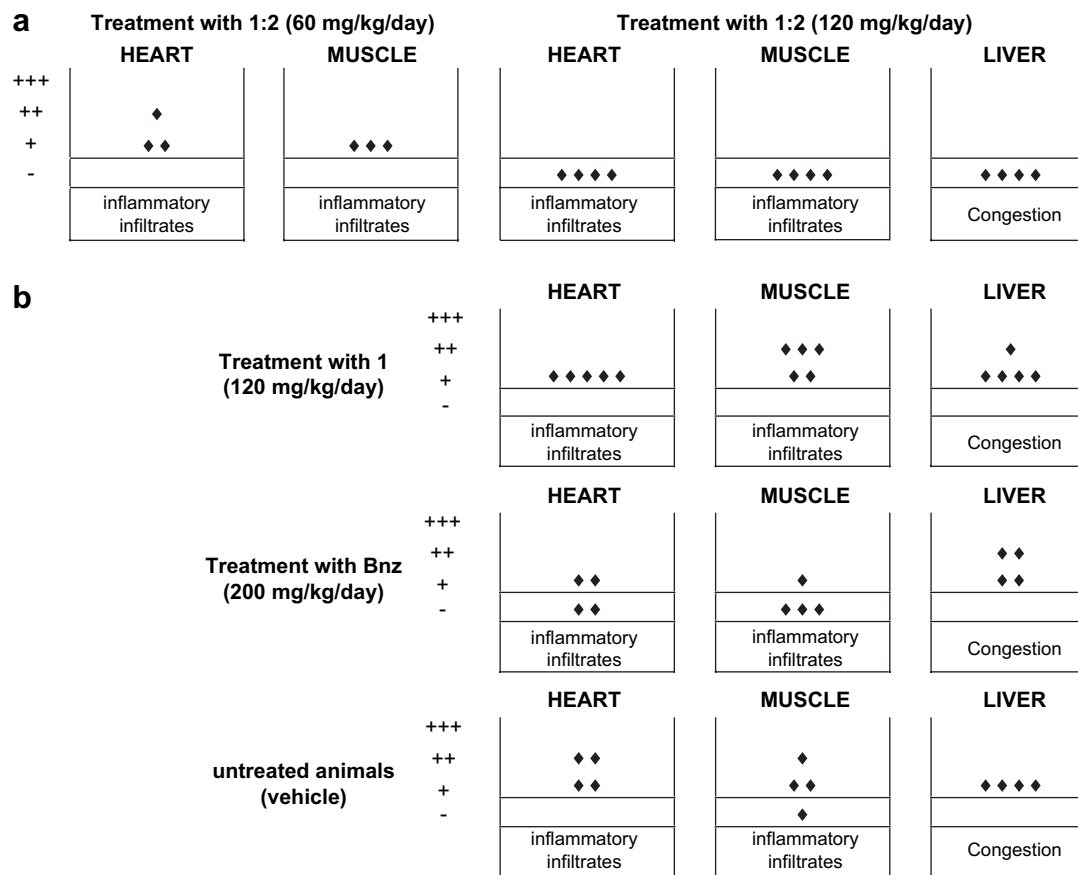
Fig. 3. (a) Dispersion diagrams indicating the intensities of inflammatory infiltrates in the two experimental conditions, schedules (A) and (B), for heart, muscle and liver of animals treated with mixture **1**:**2**. Findings for treatments with Bnz or untreated animals are gathered in part (b). (b) Dispersion diagrams indicating the histological findings in the animals' treatments with 120 mg/kg/day of **1**, Bnz or vehicle (untreated animals). The signs indicate the degree of the histopathological alterations, (+++) representing the maximum modification and (−) the absence of changes with respect to normal tissue.

the presence of inflammatory infiltrates and tissue damage compared to vehicle alone (Fig. 6). However, Bnz was better in preventing muscle inflammatory infiltrates and liver congestion than the studied benzofuroxans. Compounds **1** and **3** and the equimolecular mixture **1**:**2** were able to prevent heart inflammatory infiltrates, likewise the reference drug Bnz in the Colombiana infected animals.

Except for the mixture of **1**:**2**, at 120 mg/kg/day, the animals infected with Colombiana strain did not show toxicity signs after the administration of the benzofuroxans during the 25-day treatments. In all cases 100% of the animals survived at the end of the experiment.

According to these studies compound **1** and the mixture **1**:**2** have the best *in vivo* biological profile against Colombiana strain, decreasing the anti-*T. cruzi* antibody levels and preventing inflammatory infiltrates and tissue damage, particularly in the hearts of infected animals.

3.3. In vivo activity of compounds **1** and **2** and the mixture **1**:**2** against wild strains of T. cruzi

For the study with wild type strains of *T. cruzi* the compounds with best *in vivo* profiles in the previous experiments, **1**, **2**, and **1**:**2**, were selected and they were studied at the lowest

dose (60 mg/kg/day). Nfx was used as positive control. In the three treatments, with **1**, **2** or **1**:**2**, the animals infected with the Argentinean strain, schedule (D), showed lower parasitemias than the untreated animals from 25th day p.i. (Fig. 7a). Compound **1** showed the best behaviour in this study. When animals infected with the Uruguayan strain isolated from patients, schedule (E), were treated with compound **1**, a complete and permanent suppression of parasitemia at 17th day p.i. was observed (Fig. 7b).

The antibody-level findings showed that compound **1** has the best behaviour among the different benzofuroxans evaluated against the Argentinean strain isolated from *D. marsupialis* (data not shown). Additionally, the histological results showed that compound **1** had better behaviour than Nfx in the study with the Uruguayan strain isolated from patients (Fig. 8). Compound **1** diminished the presence of inflammatory infiltrates, amastigote nests and tissue damage comparing to both Nfx treatment and vehicle.

According to the different findings, from both studies using wild type strains, benzofuroxan **1** is a good anti-*T. cruzi* agent. For this reason we performed, as preliminary toxicity study, oral and intraperitoneal treatment with compound **1** at doses 7.5 or 5 times superior to the schedule (E)-doses. The same study was performed for Nfx. After treatment, animals were
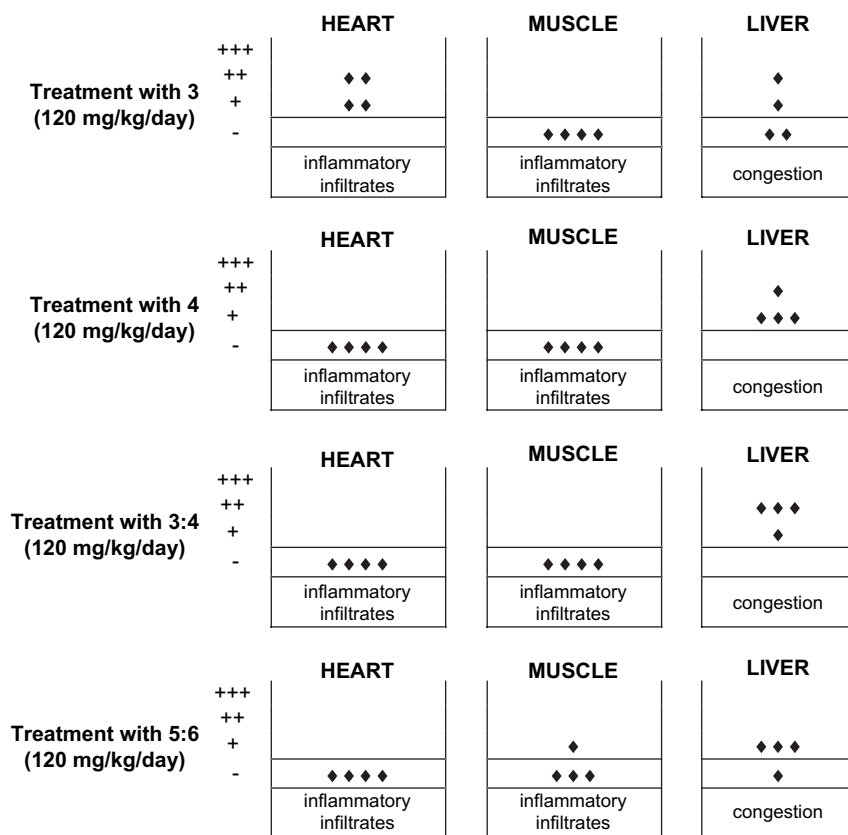
Fig. 4. Dispersion diagrams indicating the histological findings in the animals' treatments with 120 mg/kg/day of **3**, **4**, **3:4**, or **5:6**. The findings for treatments with Bnz or untreated animals are gathered in Fig. 3b.

weighed and monitored daily for vital signs; and at 3rd day post-administrations internal organs (lung, kidney, liver, spleen, brain, heart, adrenal gland, intestine, uteri and ovary) of surviving animals were submitted for further studies [17]. With both agents the animals' survival at 3rd day of trial was 100%. The changes in the body weight, macroscopic observations in the dissection process, clinic biochemistry and the histological studies of organs showed that there were no differences in the analyzed parameters between treated and healthy untreated alive animals (HUTA, Fig. 9).

4. Conclusions

In the present *in vivo* study, we confirmed that *in vitro*-active benzofuroxans could be employed as therapeutic alternative in the treatment of Chagas' disease after further studies. According to the different findings, compound **1** and the equimolecular mixture **1:2** were able to reduce the parasite loads of animals with fully established *T. cruzi* infections with, in some cases, comparable results to that obtained with both Nfx and Bnz. The results of histological studies indicated that
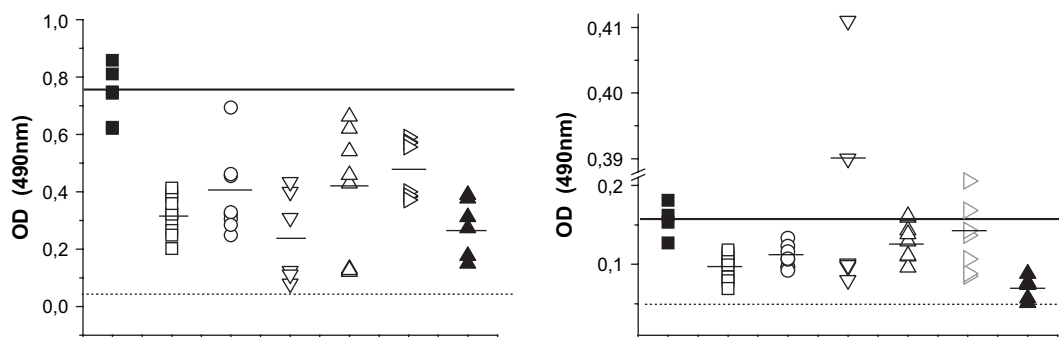

Fig. 5. Dispersion diagrams of antibody levels in untreated animals (■) and those receiving, according to schedule (C), Bnz (▲), **1** (□), **1:2** (50:50) (○), **3** (▽), **4** (△), or **5:6** (50:50) (▷) treatments at 60 days p.i. (left) and 90 days p.i. (right). The results are expressed as the absorbance of each serum sample at 490 nm. Notes: the cut-off for each reaction, dot-line, was the mean of the values determined for the negative controls (uninfected, normal mice) plus three times the standard deviation. The solid-line represents the mean values of antibody levels in untreated animals.
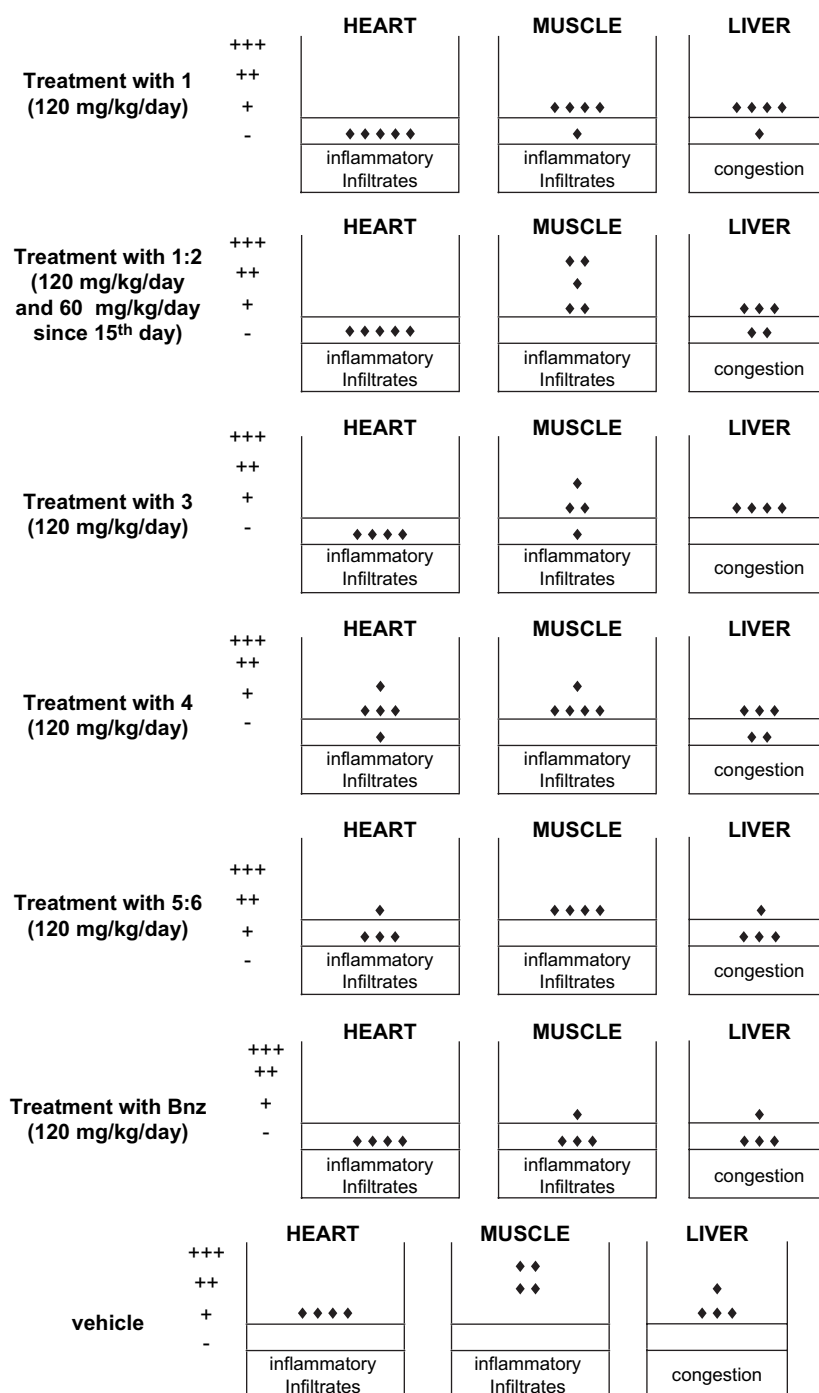
Fig. 6. Dispersion diagrams indicating the intensities of inflammatory infiltrates in the treatments of animals infected with Colombiana strain.

benzofuroxans could be adequate agents in preventing inflammatory infiltrates and tissue damage, particularly in heart of infected animals. The main problem found in our studies was the low compound solubility in the chosen vehicles. Observing this problem one could hypothesise that both benzofuroxans' *in vivo* biodisposal and *in vivo* activity could be compromised. Studies improving the *in vivo* biodisposal are currently in progress. Furthermore, according to our acute-toxicity study administration of compound **1**, at high doses did not produce remarkable damage on the animals. Taking into

account that severe mutagenic effects have been described for Nfx and Bnz [19,20], studies involving benzofuroxans' mutagenic and clastogenic effects are currently in progress.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. The
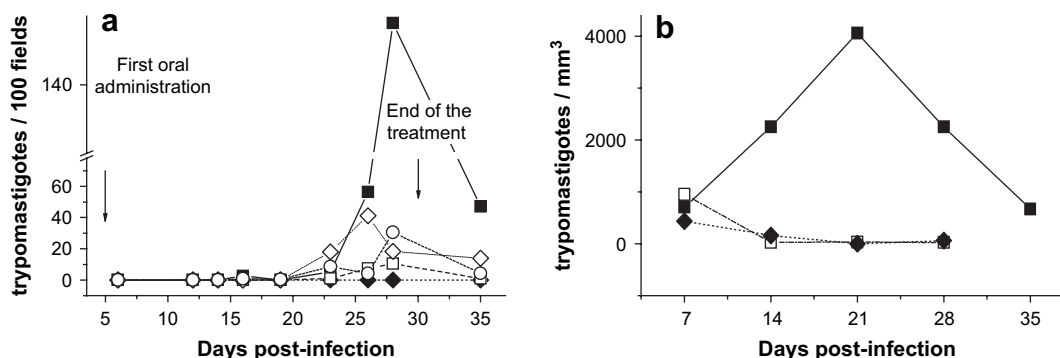
Fig. 7. Effects of studied benzofuroxans on parasitemia in the murine model of acute Chagas' disease: (a) with *T. cruzi* isolated from *D. marsupialis*; (b) with *T. cruzi* isolated from patients. Untreated animals (■) and those receiving Nfx (◆), **1** (□), **1:2** (50:50) (○) or **2** (◇).

benzofuroxan derivatives **1**–**6** were obtained as previously described [10,12]. The stereochemistry around the olefinic carbon–carbon bond was established using the corresponding ^{1}H NMR coupling constant. The compounds were isolated first by chromatographic column and then purified by successive crystallizations in the adequate solvents [21] until acceptable microanalyses (C,H,N).

5.2. Pharmacology

5.2.1. Formulation of drugs for in vivo trials

Schedule (A): the studied compounds were suspended in aqueous carboxymethylcellulose (vehicle solution) immediately prior to injection; *schedules (B)–(D)*: the studied compounds were suspended in corn oil (vehicle solution)

immediately prior to injection; *schedule (E)*: the studied compounds were suspended in sterile physiological saline: Tween 80 (4:1) (vehicle solution) immediately prior to injection. These preparations were made under aseptic conditions and in all cases homogeneous suspensions were obtained by shaking. Nfx (Lampit®, Bay 2502) was obtained from Bayer and Bnz (Rochagan®) was obtained from Roche.

5.2.2. Animals

The experiments were carried out on two month-old Swiss (Schedules (A)–(D)) and CD1 (Schedule (E)) female mice (20–22 g) bred under specific pathogen-free (SPF) conditions. Animals were housed in wire mesh cages at $20 \pm 2\,°C$ with artificial light–dark cycles. The animals were allowed to
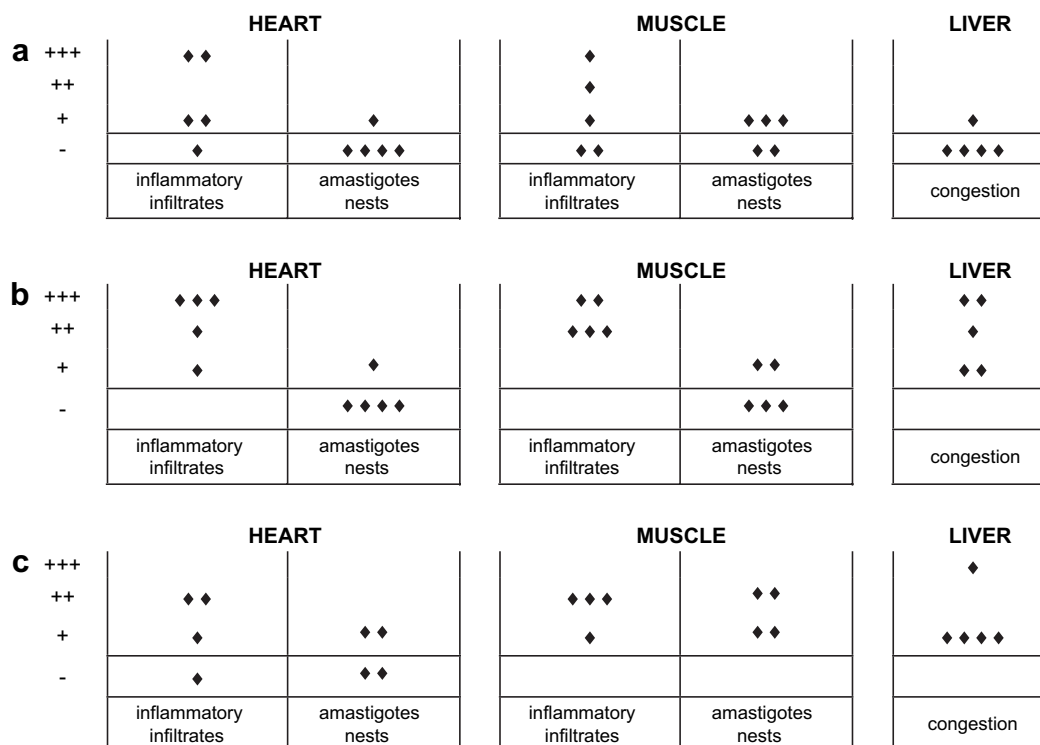

Fig. 8. Dispersion diagrams indicating the intensities of inflammatory infiltrates and amastigote nests in the treatments of animals infected with a wild strain isolated from patients. (a) Compound **1**. (b) Nfx. (c) Untreated animals.

a

b

Treatment	Organs [a,b,c]		
	liv	heart	kid
1, oral	+	−	+
1, intraperitoneal	+	−	+
Nfx, oral	+++	−	+++
Nfx, intraperitoneal	+++	−	−
vehicle, oral	+	−	−
vehicle, intraperitoneal	+	−	−

c

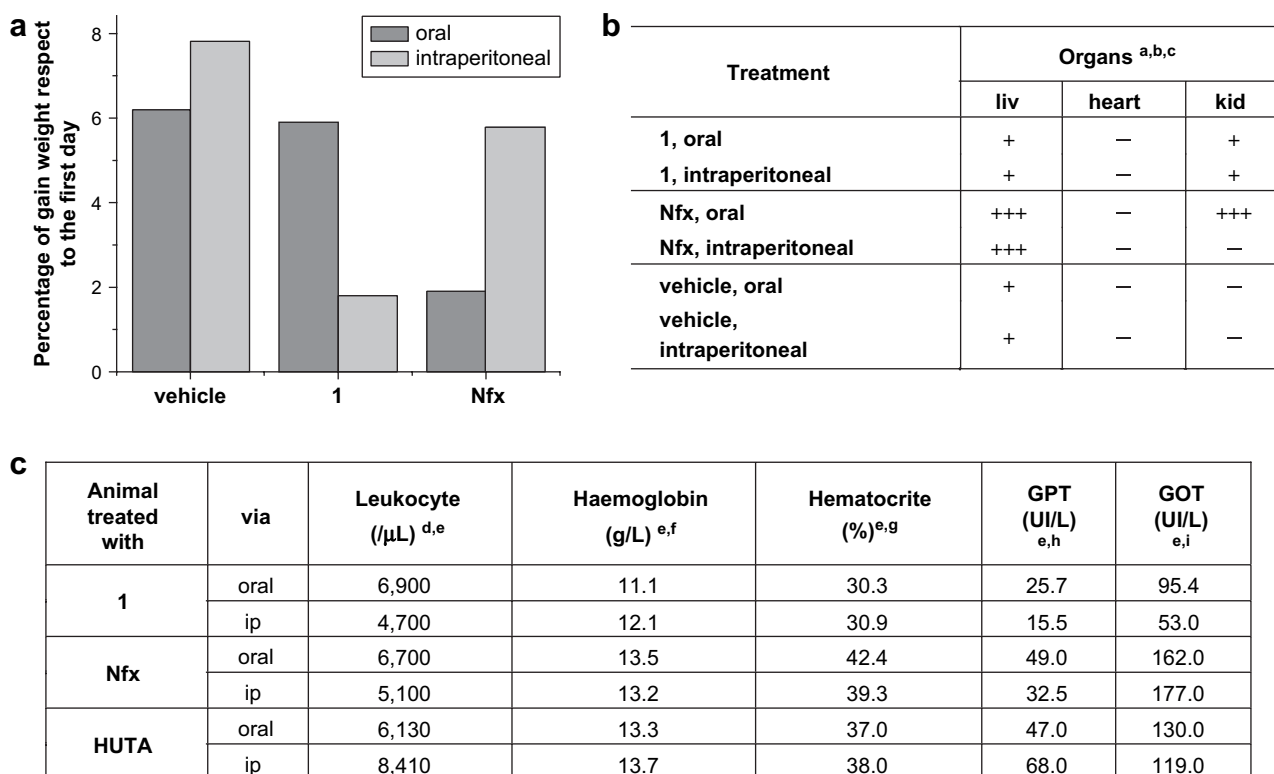| Animal treated with	via	Leukocyte (/μL) [d,e]	Haemoglobin (g/L) [e,f]	Hematocrite (%)[e,g]	GPT (UI/L) [e,h]	GOT (UI/L) [e,i]
1	oral	6,900	11.1	30.3	25.7	95.4
	ip	4,700	12.1	30.9	15.5	53.0
Nfx	oral	6,700	13.5	42.4	49.0	162.0
	ip	5,100	13.2	39.3	32.5	177.0
HUTA	oral	6,130	13.3	37.0	47.0	130.0
	ip	8,410	13.7	38.0	68.0	119.0

Fig. 9. (a) Percentages of gain weight at 3rd day with respect to 1st day. (b) Histological results for selected organs in healthy animals treated with compounds **1**, Nfx, and vehicle. [a]Oral doses = 450 mg/kg at 1st day, intraperitoneal doses = 300 mg/kg at 1st day. [b]The histological results were summarized as, −: without changes with respect to normal tissue; +: the organ did not present relevant changes with respect to normal tissue; ++: the organ presented moderate changes with respect to normal tissue; +++: the organ presented a great number of changes with respect to normal tissue. [c]liv = liver, kid = kidneys. (c) Mean values of the biochemical and the haematological findings in healthy animals treated orally and intraperitoneally (ip) with single doses of compounds **1**, Nfx and healthy untreated animals (HUTA). [d]Normal value: 5000–13,700/μL. [e]Normal values from Ref. [18]. [f]Normal value: 11.0–14.5 g/L. [g]Normal value: 35.0–45.0%. [h]Normal value: 28.0–184.0. [i]Normal value: 55.0–251.0.

feed "ad libitum" to standard pellet diet and water and were used after a minimum of 3 days' acclimation to the housing conditions [22]. Infected and treated with vehicle, untreated animals, and experimental benzofuroxans-treated group consisted of 5–7 animals. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care [23]. Animals were evaluated by supervision of international protocols and they were sacrificed in accordance with recognized guidelines on experimentation. At the end of experiments they were anaesthetised with ethyl ether and sacrificed by cervical dislocation.

5.2.3. Biological samples

For the *in vivo* studies two sets of biological samples were obtained: (1) blood for parasitemia, biochemical and haematological studies and serum for antibody level determination were drawn by sectioning the subclavian artery and studied immediately or maintained in EDTA or heparin anticoagulant at 0 °C. The biochemical, haematological and antibody level determinations were carried out no more than 24 h post extraction. (2) Organs (lung, kidney, liver, spleen, heart, and intestine) were obtained by autopsy and maintained in aqueous formalin solution (10%) for further histological studies.

5.2.4. In vivo generation of Chagas' disease

T. cruzi was isolated from different origins, and mice were infected by intradermal inoculation of $10–150 \times 10^6$ cells. *T. cruzi* of lineage I was isolated from the wild reservoir *D. marsupialis*.

5.2.5. Treatment of healthy animals with higher doses than the established posological dose

The animals were treated orally with a unique dose of 450 mg/kg and intraperitoneally (ip) with one dose of 300 mg/kg. The oral administration was carried out via intragastric syringe (1.0 mL) and the ip administration via injection (0.5 mL). Also, healthy animals treated only with vehicle (healthy untreated animals, HUTA) were included for negative controls. During the 3-day long experiments the animals were daily weighed and observed for alterations in skin, physical appearance, activity and faeces aspect, as well as their microenvironment. At the end of the experiments the animals were sacrificed and dissected and the organs and blood were submitted for further studies.

5.2.6. In vivo anti-Chagasic trials

At day five post-infection with the parasite, the animals were randomly assigned to different groups of 5–7 animals and the derivatives were administered orally using intragastric

syringe (1.0 mL) daily during 25 days (schedules (A)–(D)) and 14 days (schedule (E)). Suitable controls, infected animals treated only with vehicle, Bnz-treated and Nfx-treated animals were also included. The effect of each drug was weekly evaluated by determining the blood trypomastigote levels. At the end of the studies, all surviving animals were sacrificed and autopsied, and samples of the organs were taken and fixed for histological studies.

5.2.7. Determinations

Parasitemia determinations and haemocultures were carried out as described previously [24,25]. Quantitative evaluation of circulating anti-*T. cruzi* antibodies was carried out by the use of an enzyme-linked immunospot assay method in a microwell plate format. A soluble homogenate of *T. cruzi* epimastigotes was used as plate antigen, which was reacted with sera diluted 1:100 [16].

Acknowledgements

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