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Original article

Efficacy of the bisbenzylisoquinoline alkaloids in acute and chronic *Trypanosoma cruzi* murine model

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Original article

Efficacy of the bisbenzylisoquinoline alkaloids in acute and chronic *Trypanosoma cruzi* murine model

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Abstract

We have shown previously that daphnoline and cepharanthine are active against *Trypanosoma cruzi* and inhibited trypanothione reductase. The effects of oral treatments with daphnoline, cepharanthine and benznidazole were examined in Balb/c mice infected with *T. cruzi* acutely and chronically. In acute infections, parasitaemia was significantly reduced in the daphnoline-treated mice compared with controls and benznidazole-treated mice. The parasitological cure rate was increased in mice treated with daphnoline. Fifty days after infection, the negative serological response in both models was significantly different for the three tested drugs. Daphnoline showed the highest negative serological rate (48%). In chronically infected mice treated with daphnoline, we were unable to detect parasites in 70% of mice. The results obtained of oral treatment of daphnoline suggest that this bisbenzylisoquinoline may be useful in the treatment of acute and chronic Chagas' disease. This was not seen with cepharanthine, an excellent trypanothione reductase inhibitor. © 2000 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

Keywords: Chagas' disease; *Trypanosoma cruzi*; Bisbenzylisoquinoline; Daphnoline; Cepharanthine; Experimental treatment

1. Introduction

It is estimated that 16–20 million people in Latin America are infected with *Trypanosoma cruzi* [1], and concern has arisen lately that between 50,000 and 100,000 people are infected in the United States [2]. The absence of effective drugs to control Chagas' disease creates a need to find active chemotherapeutic agents as an urgent priority. [3]. The present drug, benznidazole is not only toxic, but of modest efficacy even in acute infections.

We have previously reported [4] the trypanocidal activity of bisbenzylisoquinoline (BBIQ) alkaloids against epimastigote forms of *T. cruzi* in vitro. Later, we confirmed their trypanocidal activity against bloodstream forms of *T. cruzi* [5]. BBIQ alkaloids have been described as antiprotozoal [6,7] and anti-inflammatory [8] compounds. These compounds have shown interesting activity in *T. cruzi*-infected BALB/c mice in the acute phase when administered orally [9]. In such experiments, we obtained parasitologic cure with curine, cycleanine, isotetrandrine, limacine and pheanthine. We also found a correlation between the inhibitory activity of trypanothione reductase (an enzyme which catalyses the elimination of reactive oxygen species within *T. cruzi*) and the lethal concentration for the parasites [10]. The trypanocidal action of BBIQ alkaloids can

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also be associated with blocking of calcium channels [11], an important mechanism for the penetration of the parasite into the host cell [12].

The present study was designed to compare the efficacies of cepharanthine and daphnoline (Fig. 1) with benznidazole in acute and chronic *T. cruzi*-infected mice. Can administration of these compounds suppress parasitaemia, prevent death and depress the serological response in acute and chronic infections?

2. Materials and methods

2.1. Compounds

Cepharanthine was extracted from the root bark of *Stephania cepharantha* (Menispermaceae) and daphnoline from *Albertisia papuana* (Menispermaceae) [13]. Physical and spectral data (proton magnetic resonance, ^1H - and ^{13}C -nuclear magnetic resonance, and mass spectrometry) were used to determine the chemical structure of the compounds, and compared against reference samples and literature values. Daphnoline and cepharanthine were used after conversion to hydrochloride form with solubility in phosphate-buffered saline (PBS). Benznidazole (*N*-benzyl-1,2-nitro-1-imidazoleacetamide) was purchased from Roche (Buenos Aires, Argentina) and used as a comparator drug.

2.2. Mice and parasites

Female and male BALB/c were bred at the Instituto de Investigaciones en Ciencias de la Salud (Asunción, Paraguay), and were 6–8 weeks of age when used.

A clone CL of *T. cruzi* supplied by Dr B. Zingales (Sao Paulo, Brazil) was used in all the experiments [14]. Maintenance of the *T. cruzi* strain was carried out in BALB/c mice inoculated by intraperitoneal route every 14 days. The mice were infected intraperitoneally with 5000 trypomastigotes to produce acute infections. To produce chronic infections, 1000 trypomastigotes of the CL strain were used. In chronic infections, there was a slow developing parasitaemia that attained a peak between 21 and 28 days, but was tolerated by the majority of infected mice; 70–80% of animals survive with slow deterioration of their general physical conditions together with a negative or inapparent parasitaemia. Each experiment was repeated four times.

2.3. Drug treatment

2.3.1. Acute infection

In the short-term infections, the treatments were initiated 4 days after inoculation of the parasite. The mice were randomly divided into groups of 10 or 15. A comparator drug, benznidazole (Roche), was selected for all experiments. Benznidazole and the BBIQ alkaloids, cepharanthine and daphnoline, in salt form (hydrochloride form), were dissolved in 50 μl PBS. The drugs were administered to BALB/c mice by the oral route in regimens of 25 mg/kg daily for 30 days. The applied doses of between one-fifth and one-seventh of LD_{50} were chosen for each BBIQ alkaloid as determined in our laboratory or as given in the literature.

2.3.2. Chronic infection

For the murine model of long-term infection, the treatment was started 60 days post-infection at a time when no circulating parasites are found in 50% of mice. The method of administration (dose and duration) was as for acute infection.

2.4. Outcome of treatment

The mortality rates were recorded. Parasitaemias in control and treated mice were determined weekly for 150 days from tail-vein blood.

Sera from acute or chronically infected mice were tested twice by immunoblotting, 100 and 150 days post-infection, and also by enzyme linked immunoassay (ELISA). Concordance between the ELISA test and immunoblotting was established using sera from non-infected and untreated, infected mice. A locally produced ELISA kit (Chagas test; IICS, Asunción, Paraguay) was used following the procedure recommended by the manufacturer. The optical density values were obtained using an ELISA plate reader (Titerek Unistan I).

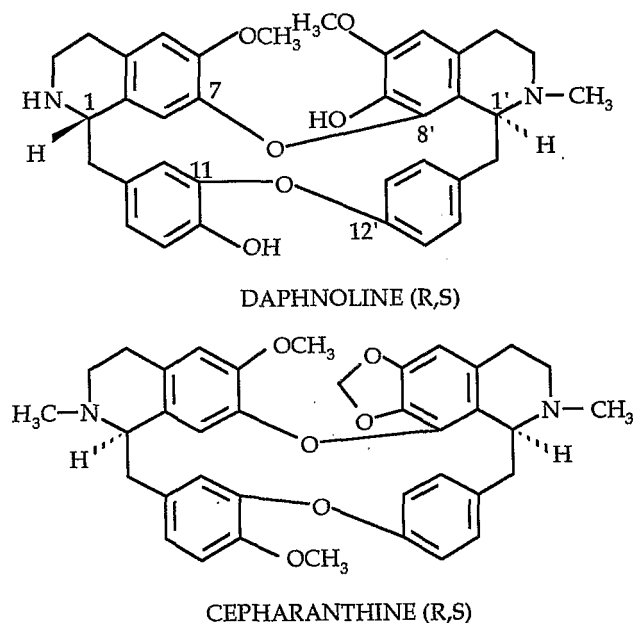


Fig. 1. Structures of daphnoline and cepharanthine.

Table 1
Effect of benznidazole, cepharanthine and daphnoline in mice with acute *T. cruzi* infection

Days post-infection	Mean parasitaemia (10^3 parasites/ml blood \pm S.D.)			
	Control	Benznidazole	Cepharanthine	Daphnoline
10	70.76 \pm 24.73	23.58 \pm 6.12	50.93 \pm 6.42	13.52 \pm 3.00 ^a
20	226.65 \pm 67.28	14.90 \pm 4.14 ^b	331.66 \pm 119.86	126.50 \pm 23.71
30	292.45 \pm 65.50	13.60 \pm 3.87 ^c	488.44 \pm 115.52	251.72 \pm 98.39
50	126.07 \pm 60.12	3.05 \pm 0.67	136.28 \pm 41.90	96.94 \pm 39.84
75	2.68 \pm 1.38	3.22 \pm 1.27	13.73 \pm 4.77	2.88 \pm 0.68
100	0.65 \pm 0.26	2.52 \pm 1.01	3.86 \pm 1.89	0.41 \pm 0.13
150	0.42 \pm 0.17	0.64 \pm 0.25	2.85 \pm 0.96	0.13 \pm 0.06 ^a

^a $P < 0.02$ compared with control.

^b $P < 0.01$ compared with control; $P < 0.001$ compared with daphnoline treatment.

^c $P < 0.001$ compared with control; $P < 0.02$ compared with daphnoline treatment.

2.5. Immunoblotting

T. cruzi epimastigotes were harvested, washed in phosphate buffered saline, pelleted and then lysed in 0.5 ml sodium dodecyl sulfate (SDS) sample buffer (1% SDS, 1% 2-mercaptoethanol, 10 mM Tris, pH 6.8, and 20% glycerol) containing 1 mM phenylmethylsulfonyl fluoride. The lysate was then heated at 100°C for 3 min before electrophoresis was performed in 12.5% polyacrylamide gels using Laemmli buffer system [15].

Gel-separated polypeptides were transferred to nitrocellulose membranes following procedures described previously [16]. Non-specific sites were blocked with 5% skim milk in Tris buffer saline (TBS) for 1 h at room temperature (RT) with constant agitation. Membranes were then cut and treated with 1:50 diluted sera in 3% skim milk in TBS for 1 h at RT. These strips were then washed with TBS and incubated with rabbit antiserum to mice immunoglobulins conjugated to horseradish peroxidase (Dako, Denmark) diluted 1:1000 for 30 min at RT. After the strips were washed with 0.05% Tween-20 in TBS, they were developed with 0.05% 3,3-diaminobenzidine made in TBS with 0.03% hydrogen peroxide. The reaction was stopped by washing the strips with distilled water.

The means and standard deviations were then calculated. The differences between groups were determined by using Student's *t*-test or the Kruskal–Wallis non-parametric analysis of variance test for comparing two groups. Significance was established for $P < 0.05$.

3. Results

3.1. Acute infections treated 4 days after infection

The administration of cepharanthine did not modify the course of infection; parasitaemia levels of animals receiving this treatment were similar to the control

group (Table 1). The mean value of maximum parasitaemia obtained at the 30th day post-infection was $292.45 \pm 65.50 \times 10^3$ parasites per ml blood in controls and $488.44 \pm 115.52 \times 10^3$ in cepharanthine-treated animals. The mean parasitaemia at day 10 for the daphnoline-treated mice was significantly reduced ($P < 0.02$ compared with controls). Increased parasitaemia was observed between 15 and 30 days post-infection. Mice receiving daphnoline treatment had maximum parasitaemia at the 30th day post-infection, a mean value similar to the control group. Parasitaemia counts between days 20 and 30 confirmed that benznidazole treatment significantly decreased parasitaemia ($P < 0.001$ and $P < 0.02$ compared with daphnoline). At day 50, there was no significant difference in parasitaemia values between controls and mice treated with benznidazole ($P = 0.075$). Prior to sacrifice at the 150th day post-infection, the parasitaemia was significantly reduced in mice treated with daphnoline compared with controls and benznidazole groups ($P < 0.02$).

The results of parasitologic cure are presented in Table 2. Significant parasitologic cures were obtained in mice treated with daphnoline (60%) compared with controls (23.3%) ($P < 0.05$). A mortality rate of 60%, however, was seen in mice treated with cepharanthine compared with controls. Sera taken at 100 days from mice treated with benznidazole or with daphnoline showed a significant serological cure ($P < 0.05$) of 34.4 and 35.5%, respectively (Table 2). These data were confirmed at 150 days post-infection in the daphnoline-treated mice ($P < 0.002$) with a serological cure of 44.0%, and a cure of 31.0% in the benznidazole-treated mice ($P < 0.05$).

3.2. Chronic infection treated 60 days post-infection

Treatment was given for 30 days. At the start of treatment, 25% of mice presented with negative parasitaemia (Table 3). After 20 days of benznidazole,

Table 2
Cure rates in mice with *T. cruzi* acute infection treated with benznidazole, cepharanthine and daphnoline, as judged by absence of parasitaemia or by negative serology

Treatment	n	Absent parasitaemia/number of survivors					Negative serology/number of survivors	
		20 days post-infection	50 days post-infection	75 days post-infection	100 days post-infection	150 days post-infection	100 days post-infection	150 days post-infection
None (control)	45	3/44	6/42	9/38	13/38	7/30	5/38	2/30
Benznidazole	35	7/34	9/33	6/33	12/32	12/29	11/32 ^a	9/29 ^a
Cepharanthine	35	2/34	1/22	0/20	5/20	5/20	2/20	3/20
Daphnoline	45	8/45	10/34	9/33	10/31	15/25 ^a	11/31 ^a	11/25 ^b

^a $P < 0.05$ compared with control.

^b $P < 0.002$ compared with control.

Table 3

Cure rates in mice with *T. cruzi* chronic infection treated with benznidazole, cepharanthine and daphnoline, as judged by absence of parasitaemia or by negative serology

Treatment	Absent parasitaemia/number of survivors				Negative serology/number of survivors	
	0 days post-treatment	20 days post-treatment	40 days post-treatment	90 days post-treatment	100 days post-infection	150 days post-infection
None (control)	8/34	14/33	11/33	11/30	1/33	2/30
Benznidazole	8/33	20/28 ^a	18/27 ^b	13/24	9/27 ^b	8/24 ^b
Cepharanthine	9/35	12/32	19/32	17/27	9/32 ^a	10/27 ^a
Daphnoline	8/34	19/30	18/30 ^a	21/30 ^c	10/30 ^b	14/30 ^b

^a $P < 0.05$ compared with control.

^b $P < 0.01$ compared with control.

^c $P < 0.005$ compared with control.

cepharanthine and daphnoline, the numbers of mice without parasitaemia increased significantly to 71.4% ($P < 0.05$) with the benznidazole-treated mice and by 63.3%, but not significantly, with the daphnoline-treated mice. Forty days post-treatment, these differences of parasitaemia were more accentuated between the untreated-infected mice and the mice treated with benznidazole and daphnoline ($P < 0.01$ and $P < 0.005$, respectively). Prior to sacrifice at 150 days post-infection, the mice treated with daphnoline were also parasite-negative by 70.0% ($P < 0.005$). Over 50% of the treated mice did not present with parasites in blood. During these experiments, we observed that a higher rate of mortality occurred with benznidazole-treated mice than with other treated groups.

3.3. Serological response

The serological response was assessed by immunoblotting at 100 and 150 days post-infection (Table 3). At 100 days post-infection, the serological response rates of mice treated with benznidazole and daphnoline were significantly reduced compared with controls ($P < 0.01$) by 32.1 and 33.3%, respectively. At 150 days post-infection, the serological response increased for daphnoline-treated mice to 48.3% ($P < 0.01$).

A 100% concordance was obtained between ELISA and immunoblotting assay from both acute and chronic experimental *T. cruzi* infection protocols (Table 4).

4. Discussion

This work compared benznidazole, a recommended drug for the treatment of acute and congenital *T. cruzi* infection [17], and two bisbenzylisoquinoline alkaloids, cepharanthine and daphnoline, in the therapy of experimental Chagas' disease. Recently, we reported [10] that cepharanthine possessed an excellent ratio trypanocidal activity and inhibitory activity against trypanothione

reductase, a target for a specific chemotherapy against Chagas' disease. Daphnoline presented satisfactory activity against this target and has an interesting activity against *T. cruzi* in the bloodstream [5]. For this evaluation, we chose a non-lethal *T. cruzi* strain, clone CL Brener, and a non-lethal inoculum of parasites, an option that resembles, in many aspects, the chronic behaviour of the human trypanosomal disease. It is difficult to define the criteria of cure in acute phase. It is generally recognized that persistent negative results for parasite detection and for antibodies are sufficient [18]. In the acute model, when results were compared with infected and untreated animals, the tested drugs showed little impact on the level of parasitaemia after day 50. However, the immune response in this model was more evident in those animals treated with benznidazole and the experimental drug daphnoline. For chronic phase, there is no consensus, but it seems that immunoblot assay is a useful method for the immunodiagnosis of acute and chronic Chagas' disease [19,20].

The study showed that daphnoline treatment of mice with acute *T. cruzi* infection increased parasitologic and serological cure compared with benznidazole treatment. Assessment of the effects of daphnoline treatment in

Table 4

Concordance between the ELISA and the immunoblotting assay from both, acute and chronic experimental *T. cruzi* infection protocols

Experimental protocol	Serological test	
	ELISA ^a	Positive immunoblotting ^b
Negative control	0.182 ± 0.027	0/33 (0)
Acute <i>T. cruzi</i> -infected mice	0.932 ± 0.752	10/10 (100)
Chronic <i>T. cruzi</i> -infected mice	1.590 ± 0.739	16/16 (100)

^a Optical density mean ± standard deviation. Cut-off, 0.264.

^b Number of positive infected mice/total of infected mice (percentage).

chronic *T. cruzi* infection was more important than in acute infection. The effectiveness of cepharanthine treatment in acute *T. cruzi* infection was limited, but in the case of chronic infection, the serological cure rate was similar to the benznidazole treatment. In chronic *T. cruzi* infection, we observed a higher rate of mortality in benznidazole-treated mice than in other groups.

The use of a quantitative test makes the serological diagnosis more objective. Criteria of *T. cruzi* serological cure after treatment is currently used, incorporating a decrease in titre as a parameter. In a qualitative test such as immunoblotting, a diminution of antibodies is more subjective. In further studies, the use of ELISA tests will allow us to incorporate the criteria of cure in treatments on experimental *T. cruzi* infections when the search for parasites is persistently negative. Excellent concordance had been found with these assays, and the incorporation of the ELISA test will be very useful due to the simple technical procedure and reduced time and cost involved. Our results of benznidazole treatment on *T. cruzi*-infected mice confirm recent studies on the therapeutic efficacy of benznidazole. This drug is active in chronic *T. cruzi* infection [17]. The chronic model presents low inapparent parasitaemia and an immune response produced by internal cycles of the parasites or by their fractions in tissues. The negative serology, when parasites cannot be detected, is considered as a parameter of cure in treatment with benznidazole [17,21]. Prompt disappearance of parasites is not necessarily followed by serological parameters. Sgambati et al. [22] described the persistence of parasite antigens captured by dendritic spleen cells in infected and treated animals. Therefore, immune response can be attributed to these antigens, without detectable parasites [23].

The mechanism of action of BBIQ alkaloids is not well known, but the interesting results obtained with daphnoline against trypanothione reductase [10] confirm, in part, its inhibitor activity against this target in acute or chronically *T. cruzi*-infected mice. This finding, however, is not seen in treatment with cepharanthine. Different studies with BBIQ have shown their capacity to block calcium channels [11,24,25]. Recent studies demonstrated Ca^{2+} changes in tissue to be an important factor in the penetration of the trypomastigote forms of *T. cruzi* into the host cell [12,26]. Other possible explanations of the biological properties of BBIQ alkaloids are the inhibitory effect on nitric oxide production [27], or on tumour necrosis factor- α [28]. It is generally accepted that nitric oxide and tumour necrosis factor- α [29–31] produced by activated macrophages are cytostatic or cytotoxic for *T. cruzi*. The results obtained with the oral treatment of daphnoline suggest the possible value of such a BBIQ alkaloid in the treatment of acute and chronic Chagas' disease.

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