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The effect of bisbenzylisoquinoline alkaloids on *Trypanosoma cruzi* infections in mice

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Abstract

Five bisbenzylisoquinoline (BBI) alkaloids, curine, cycleanine, isotetrandrine, limacine and pheanthine were tested for trypanocidal activity in C_3H/He mice infected with Y or CL strain of *Trypanosoma cruzi*. The activity was compared with the baseline drug, benznidazole. Oral treatment was more effective with curine at 10 mg/kg or with cycleanine at 2 mg/kg daily for 10 days in mice infected with Y or CL strain. In these groups, the parasitemias were negative after 5–7 weeks after inoculation and mortality time 50 (MT_{50}) was significantly higher than untreated mice. Benznidazole was effective in mice infected with CL strain but not in mice infected with Y strain. The other BBI showed a relative efficacy against both strains. The effect of BBI alkaloids could be due to a blocking of the Ca^{2+} channel for the regulation of *T. cruzi* infectivity to invade host cells or their selective immunosuppressive properties. © 1997 Elsevier Science B.V.

Keywords: Chagas' disease; *Trypanosoma cruzi*; Trypomastigotes; Bisbenzylisoquinoline; C_3H/He mice; Benznidazole; Curine; Cycleanine; Isotetrandrine; Limacine; Pheanthine

1. Introduction

Chagas' disease is a widespread infection in Central and South America. It is caused by the protozoan *Trypanosoma cruzi* and is naturally transmitted by Reduviidae bugs [1]. Blood transfusion is the second most-important mechanism of transmission in both endemic and non-endemic areas. This fact is of epidemiological importance due to migration of infected individuals from endemic rural environments to urban areas. Current treatment of Chagas' leaves much to be desired [2,3], two drugs are marked in endemic countries in Latin America, benznidazole and nifurtimox for treatment of both acute and chronic cases. We have described the in vitro trypanocidal activity of some bisbenzylisoquinoline (BBI) alkaloids against the epimastigotes forms [4] and against the bloodstream forms of *T. cruzi* [5] as well. BBI alkaloids have been described as anti-protozoal [6,7], anti-tumoral [8], anti-inflammatory and immunosuppressive compounds [9]. Nevertheless, no information about their trypanocidal properties has been published.

In this paper, we present further results of laboratory experiments concerning chemotherapeutic activity of five BBI alkaloids which have inhibited in vitro 100% of bloodstream forms of *T. cruzi*, namely, curine, cycleanine, isotetrandrine, limacine and pheanthine, in comparison with reference drug, benznidazole against acute

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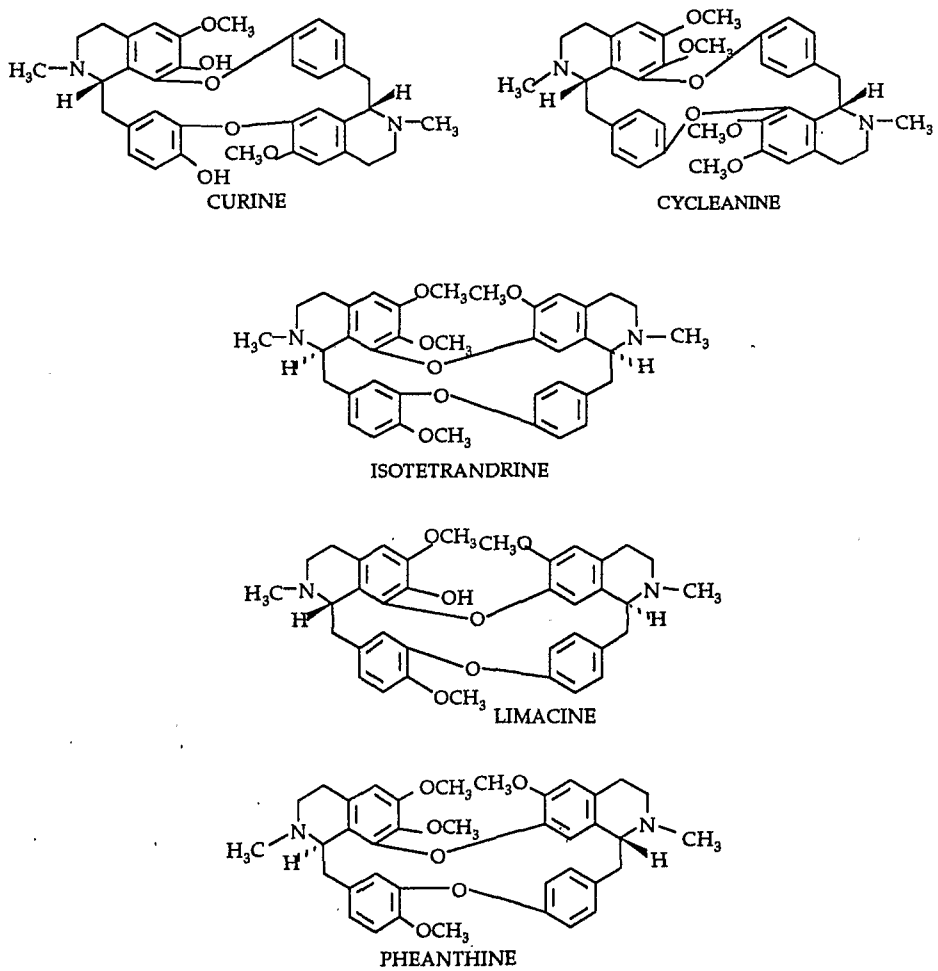

Fig. 1. Structure of BBI alkaloids.

mouse infection with a myotropic CL strain or a reticulotropic Y strain of *T. cruzi*.

2. Materials and methods

2.1. Compounds

Two types of compounds were used in these experiments (Fig. 1), they differ in the position of the linkages between the two monomeric benzylisoquinoline components and the nature and number of substituents on the aromatic rings. The five compounds used for this work were obtained from diverse sources. Curine and cycleanine were extracted from the root bark of *Isolona hexaloba* (Annonaceae, Congo) [10], isotetrandrine from the stem bark of Bolivian *Berberis boliviana* (Berberidaceae, Bolivia) [11], limacine from *Albertisia papuana* (Menispermaceae, New Caledonia) [12] and pheanthine from *Gyrocarpus americanus* (Hernandiaceae, New Caldonia) [13].

Physical and spectral data (proton magnetic resonance and mass spectrometry) were used to determine the chemical structure of the compounds and compared against reference samples and literature values. The BBI alkaloids were used after conversion to the hydrochloride form with solubility in phosphate buffered saline (PBS). Benznidazole (*N*-benzyl-1,2-nitro-1-imidazole-acetamide) was purchased from Roche, Buenos Aires, Argentina.

2.2. Mice and parasites

Female and male C_3H/HeN/JCL mice were supplied by CLEA, Tokyo, Japan and bred at Instituto de Investigaciones en Ciencias de la Salud (Asuncion, Paraguay). Mice weighed between 18 and 20 g and were 8 weeks old when experiments were initiated. Two strains of *T. cruzi* were used during these investigations. Y, a Brazilian strain isolated by Silva and Nussenzweig from a human case [14] and the clone CL Brener (from Dr Z. Brener, Brazil) [15]. Routine maintenance of the *T. cruzi* strains was carried out in C_3H/HeN/JCL mice inoculated by intraperitoneal route every 7–14 days according to strain, 10^2 bloodstream organisms of Y strain and 5×10^3 bloodstream forms of CL strain,

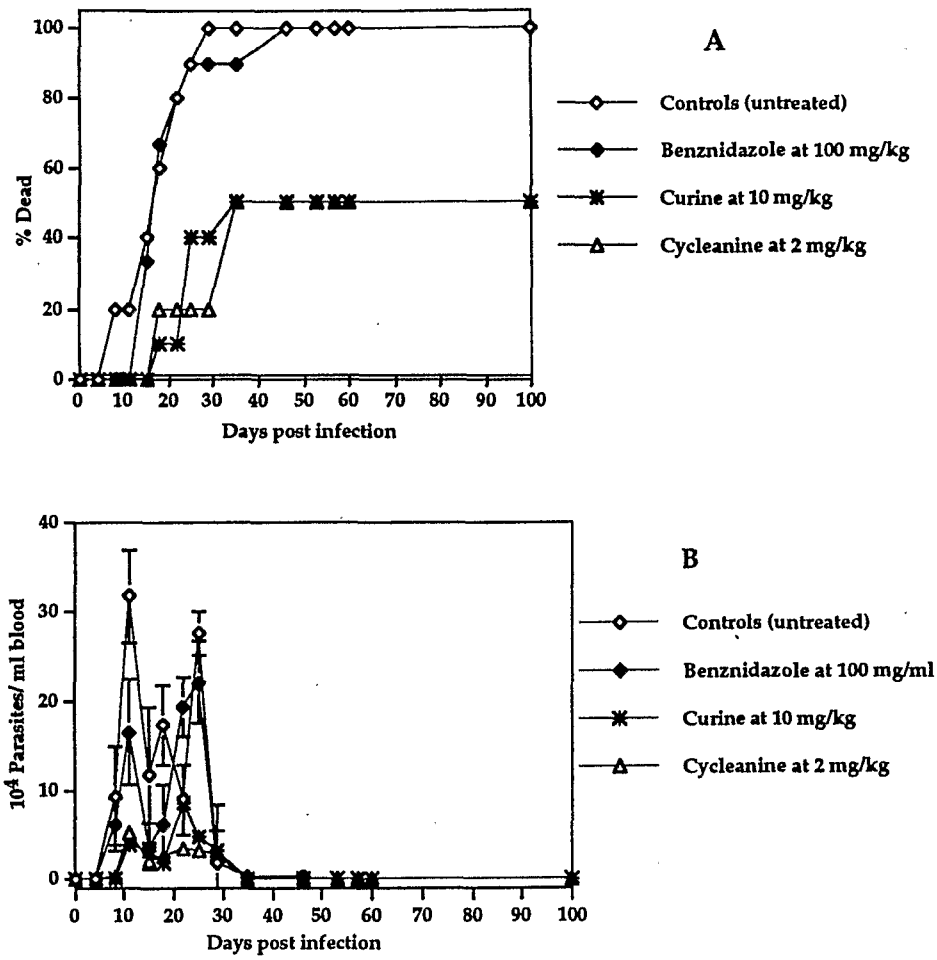

Fig. 2. Effects of benznidazole, curine and cycleanine on death of (A) or parasitemia (B) C3H/He mice infected intraperitoneally with 100 bloodstream forms of Y *T. cruzi* strain and treated orally for 4 days after the infection for a 10 day period.

respectively. Mice weighed between 18 and 20 g and were 8 weeks old when experiments were initiated.

Parasitemias in control and treated mice were determined twice weekly [16] for 200 days more by using tail blood. The mice were observed for time to death and total mortality and were considered to have passed into chronic stage of infection when parasitemia was subpatent or negative without mortality due to the infection, generally by 6–8 weeks of infection with the Y strain and by 8–10 weeks with the CL strain. Both males and females were tested and no differences have been found between the survival rates of the sexes of these mice after infection with *T. cruzi*.

2.3. Drug test

In all experiments, the treatments were initiated 4 or 5 days after the parasitic infection. The mice were ramdomly divided into groups of eight or ten. Benznidazole or salts of BBI alkaloids were dissolved in 50 μl of PBS and administered to C_3H/HeN/JCL mice in regimens of 100, 50 or 25 mg for benznidazole treatment, 2 or 5 mg (cycleanine), 10 (curine, limacine and pheanthine) and 60 mg (isotetrandrine). The applied doses were chosen between 1/5 and 1/6 of LD_{50} of each BBI alkaloid given in the literature [8]. Drugs were administered once daily by oral route for 10 days and the untreated group received 50 μl of PBS daily.

Data are presented as means $\pm$ standard deviations, unless indicated otherwise. Comparison of parasitemia and mortality of the untreated control group and drug-treated group were analyzed by the paired Student's t-test. Data were considered statistically significant at a P value of less than 0.05 (two tailed). Statistical analysis of the proportions were performed using the χ^2 for each time post-treatment. The death of 50% of mice was determined by data analysis program (Toxicologie®, G. Febvay, INSA 406, F-69621 Villeurbanne France) using the probit method of analysis.

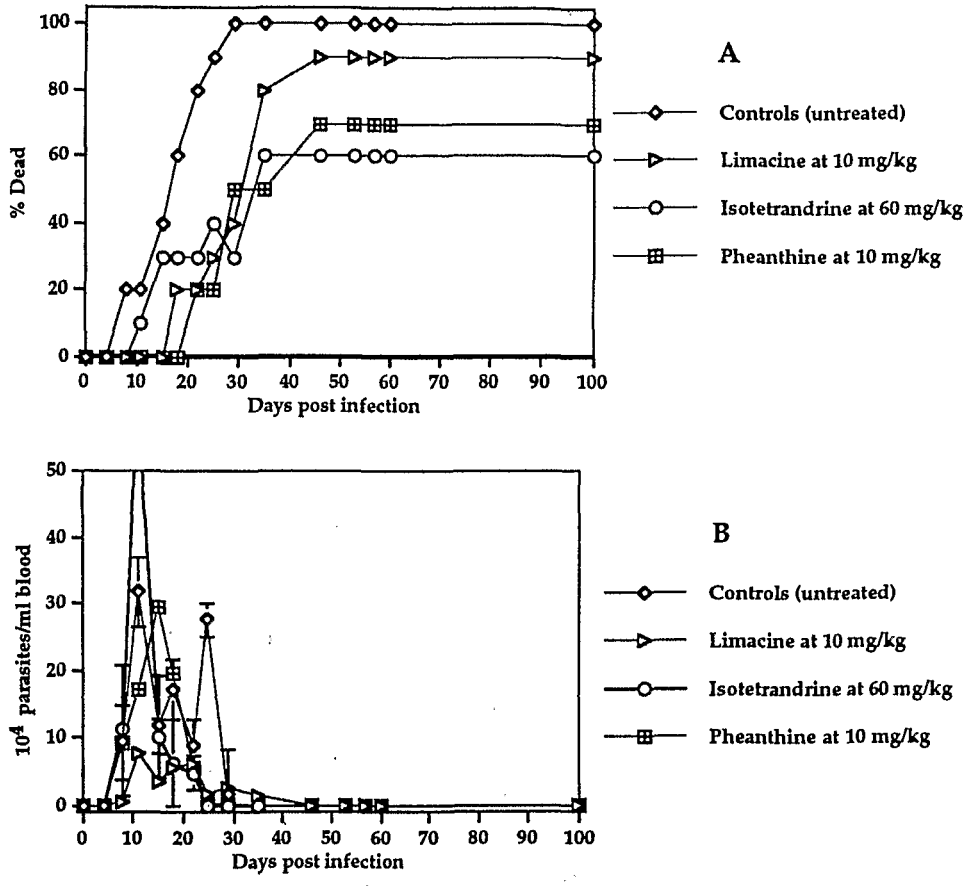

Fig. 3. Effects of limacine, isotetrandrine and pheanthine on death of (A) or parasitemia (B) C3H/He mice infected intraperitoneally with 100 bloodstream forms of Y *T. cruzi* strain and treated orally for 4 days after the infection for a 10 day period.

3. Results

Fig. 2A shows that all untreated control mice inoculated with the bloodstream forms of the Y strain died within 29 days after inoculation. At the same time the mice treated orally for 10 days with a daily dose of 100 mg/kg died within 46 days. In contrast, the mice treated for 10 days with curine or cycleanine at 10 mg/kg and 2 mg/kg respectively, had a mortality rate by 50% up to 7 months post inoculation. The parasitemia curves presented in Fig. 2B indicates that the survival mice treated with curine or cycleanine had a negative parasitemia by 35 days after inoculation. The untreated mice or benznidazole treated mice presented two parasitemia peaks at about day 11 and 25 postinoculation. In contrast, this second peak was not observed with the mice treated with curine or cycleanine. Negative parasitemia in the surviving curine or cycleanine treated mice was observed and was maintained during 180 days of experiment. We have also controlled the presence of circulating parasites in some surviving mice by hemocultures or subinoculations. Nevertheless, we have not detected circulating parasites in either of both procedures.

Fig. 3A also shows that after treatment schedules with isotetrandrine at 60 mg/kg body weight for 10 days and pheanthine at 10 mg/kg body weight for days, 40 and 30% of mice survived about 35 and 45 days, respectively. The treatment with limacine at 10 mg/kg body weight for 10 days was not effective, alone 10% of mice survived and were cured about 45 days postinfection. In all cases, 100% of surviving mice presented a negative parasitemia which was confirmed by control circulating level twice weekly for 180 days. Also, Fig. 3B shows the suppression of the second parasitemia peak in the mice treated with the BBI alkaloids, but the level of circulating parasites in the isotetrandrine treated mice was more important than in the untreated mice during the parasitemia peak by 6.4×10^5 and 3.2×10^5 cells/ml, respectively.

Fig. 4A shows the mortality curves obtained in mice infected with the CL *T. cruzi* strain and treated with two different doses of benznidazole, at 50 and 25 mg/kg body weight or with curine at 10 mg/kg or with cycleanine at 5 mg/kg body weight for 10 days. The mice treated with the reference drug, benznidazole, were protected of mortality by 100 and 90% of survival after a 200 day-period of observation. The curine treatment for 10 days led to 70% of surviving mice after the 200

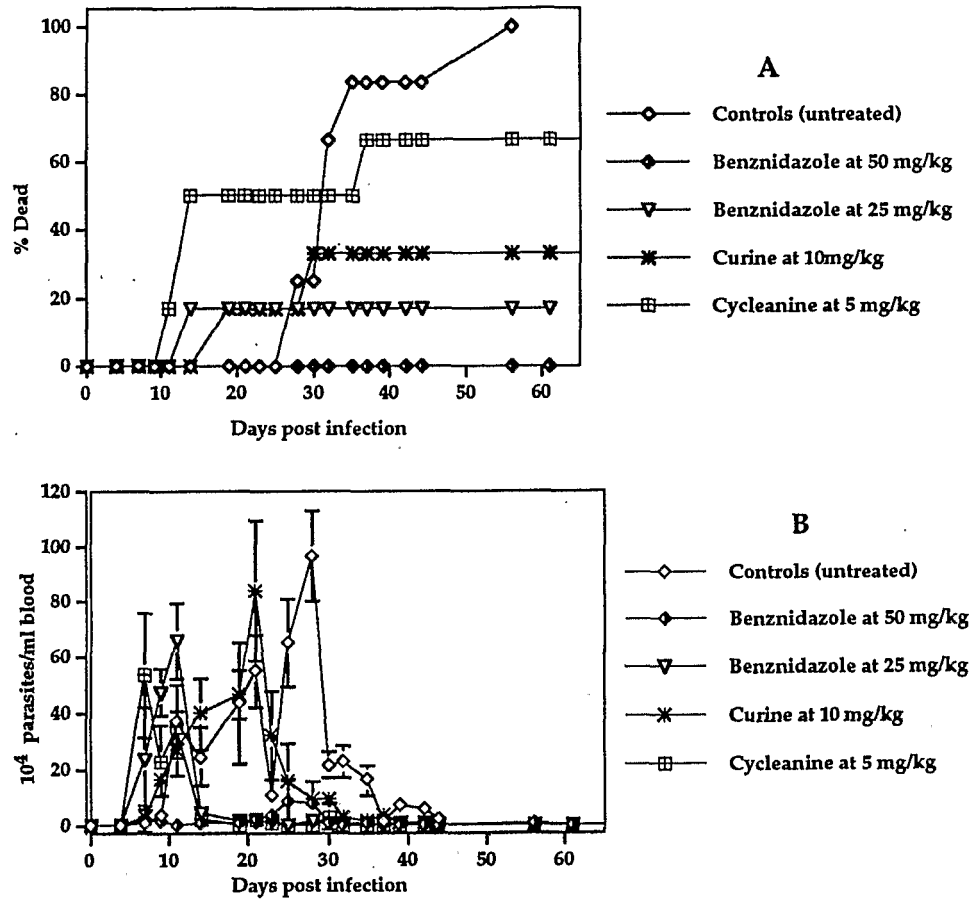

Fig. 4. Effects of benznidazole, curine and cycleanine on death of (A) or parasitemia (B) C3H/He mice infected intraperitoneally with 5000 bloodstream forms of clone CL *T. cruzi* strain and treated orally for 4 days after the infection for a 10 day period.

day-period, but the cycleanine treatment at 5 mg/kg body weight was not too effective producing mortality by 70% of mice in the same period. Fig. 4B presents the level of circulating parasites in mice infected with the CL strain. In untreated infected mice, two parasitemia peaks at 14 days and an important level of parasitemia 30 days after inoculation were observed. In contrast, in mice treated with benznidazole or with BBI alkaloids (curine or cycleanine), the last parasitemia peak did not record. Sixty percent of mice treated with benznidazole at 50 mg/kg body weight presented a negative parasitemia 15 days after inoculation.

Table 1 shows mortality values obtained in mice treated with BBI alkaloids and infected with Y or CL *T. cruzi* strains. Mortality was recorded daily and the number of days required for the death of 50% of the mice in experiment (MT_{50} = mortality time 50%) was calculated using the probit method. The Y strain was more virulent than the CL strain producing high mortality rates in untreated mice, 20.35 and 33.08 days, respectively. The MT_{50} in the mice treated with benznidazole were different, 18.25 days with the Y strain and more than 200 days with the CL strain. The mice infected with Y or CL *T. cruzi* strains and treated with curine presented MT_{50} by 129.37 ($P < 0.001$) and 79.41 days ($P < 0.01$), respectively. The oral treatments with cycleanine showed efficacy when the mice were infected with Y or Cl strains, in this case the MT_{50} calculated were similar 134.16 ($P < 0.001$) and 135.29 days ($P < 0.001$). Subcutaneous administration of cycleanine at 5 mg/kg body weight for 10 days produced the MT_{50} of 100.21 days ($P < 0.01$) and a negative parasitemia by 83% of surviving mice after 200 days of experiment. The oral or subcutaneous isotetrandrine treatment at 60 mg/kg for 10 days resulted in an MT_{50} of 69.11 ($P < 0.05$) and 91.86 days ($P < 0.01$) with the Y strain infection. The results obtained with the pheanthine treatments were different when the mice were infected with the Y or CL strain, with the MT_{50} of 86.33 ($P < 0.01$) and 29.93 days (insignificant in untreated mice). In this case, no mice survived after 50 days after inoculation. Oral treatment with limacine resulted in an MT_{50} of 88.91 days ($P < 0.01$) and a negative parasitemia in surviving infected mice with the Y *T. cruzi* strain, no parasites were observed in the blood of these mice during 180 days.

Table 1
Mortality values and efficacy of benznidazol and BBI alkaloids-treated C3H/He mice infected with Y and CL *T. cruzi* strains

Compounds	Route of administration	Dose (mg/kg)	*T. cruzi* strain	Mice (*n*)	Negative parasitemia on days following infection (mortality: no. dead/total)				Mortality time 50% (days)
					15	50	100	200	
Untreated mice			Y	10	0 (0/10)	ND (10/10)	ND (10/10)	ND (10/10)	20.35
			CL	12	0 (0/10)	0 (9/10)	ND (10/10)	ND (10/10)	33.08
Benznidazole	Oral	100	Y	10	0 (0/10)	ND (10/10)	ND (10/10)	ND (10/10)	18.25
		100	CL	10	0 (0/10)	0 (0/10)	0 (0/10)	40 (0/10)	ND
		50	CL	10	60 (0/10)	10 (0/10)	20 (0/10)	0 (0/10)	ND
		25	CL	10	0 (0/10)	20 (1/10)	0 (1/10)	10 (1/10)	ND
Curine	Oral	10	Y		0 (0/10)	100 (5/10)	100 (5/10)	100 (5/10)	129.37*
	Subcuta neous	10	CL	10	0 (0/20)	20 (3/10)	83 (4/10)	50 (4/10)	210.09*
	Oral	10	CL	10	0 (0/10)	0 (7/10)	33 (7/10)	33 (7/10)	79.41***
Cycleanine	Oral	2	Y	10	0 (0/10)	100 (5/10)	100 (5/10)	100 (5/10)	134.16*
	Oral	5	CL	10	0 (0/10)	20 (5/10)	20 (5/10)	20 (5/10)	135.29*
	Subcuta neous	5	CL	10	0 (0/10)	50 (6/10)	50 (6/10)	83 (6/100)	100.21*
Isotetrandrine	Oral	60	Y	10	0 (0/10)	100 (8/10)	100 (8/10)	100 (8/10)	69.11**
	Subcuta neous	60	Y	10	0 (0/10)	100 (6/10)	100 (6/10)	100 (6/10)	91.86***
Limacine	Oral	10	Y	10	0 (0/10)	100 (7/10)	100 (7/10)	100 (7/10)	88.91***
Pheanthine	Oral	10	Y	10	0 (0/10)	100 (7/10)	100 (7/10)	100 (7/10)	86.33***
	Oral	10	CL	10	0 (0/10)	ND (10/10)	ND (10/10)	ND (10/10)	29.93

ND, not done.
* $P<0.001$; ** $P<0.05$; *** $P<0.01$.

4. Discussion

The above results demonstrate and confirm [5] the trypanocidal properties of the BBI alkaloids against two different *T. cruzi* strains (Y and Cl). The protection is significant but not complete, the determination of the criterias of cure depends upon the results of hemoculture, tissue inoculation or serologic studies which were not systematically carried out in this study. Nevertheless, we have observed high differences in the sensitivity of Y and CL *T. cruzi* strains towards the reference drug, benznidazole. These findings have been also described in the literature [17,18]. Two out of five BBI alkaloids tested (curine and cycleanine) showed high efficacy, against *T. cruzi* infection of C3H/He mice. These compounds belong to the same structural classification, the bisbenzylisoquinoline alkaloids with two diphenylether linkages head to tail [19]. Limacine, isotetrandrine and its isomer, pheanthine, three BBI alkaloids with two diphenyl ether linkages head to head and tail to tail, did not show the same efficacy although we did not detect circulating parasites in surviving mice infected with Y strain. The effective molecular doses between these compounds and the baseline drug benznidazole differed significantly, for instance cycleanine was administered at 2 mg/kg or 0.002 mMol/kg body weight and benznidazole at 100 mg/kg or 0.38 mMol/kg. Satisfactory bioavailabilities of BBI alkaloids were observed and their oral administration did not cause any obvious toxicity in the mice at therapeutic doses when assessed by appearance, weight change, blood aspect and organ histology of treated mice. In similar previous assays with longer periods of treatment (20 days) were tested. Nevertheless, in this condition we did not observe either enhance of the drug efficacy nor apparent toxicity effect (unpublished results). Although the mechanism of action of BBI alkaloids is not understood, recent studies showed the capacity for these compounds (isotetrandrine, curine and pheanthine) to block Ca^{2+} uptake through the L-type Ca^{2+} channel and modulate binding of ligands to distinct sites in a variety of tissue, including cardiac and smooth muscle [20]. In effect, Yabuku [21–23] described a possible role of the intracellular Ca^{2+} level in the regulation of *T. cruzi* infectivity to invade host cells. BBI alkaloids could act as regulators or inhibitors of invasive capacity of trypomastigotes. Another hypothesis, BBI alkaloids have been described as possible new drugs for therapy of chronic inflammatory and autoimmune diseases [24] showing that BBI alkaloids, particularly isotetrandrine or cycleanine, had inhibitory effects on synthesis of interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) by human monocytes-macrophages. Although the monomeric benzylisoquinoline, *N*-methylcoclaurine and dimeric compounds had a similar activity. In an in vitro assay (results unpublished), we have found a different activity of monomeric and dimeric compounds against circulating parasites in vitro (unpublished results) where monomeric compounds did not present any effect. However, it is not known whether BBI alkaloids transform biologically into monomeric benzylisoquinolines. The cleavage of BBI alkaloids with two diphenyl ether linkages is not easy and needs strong reducing conditions (metallic sodium in liquid ammonia). A recent study [25] described the significant influence of BBI alkaloids on the production of TNF-α and the level of nitric oxide (N=O) in murine peritoneal macrophages, two important factors involved in the mechanisms of invasion and replication of *T. cruzi* [26].

Many BBI alkaloids have been described as antiprotozoal drugs against *Plasmodium falciparum*, *Entomoeba hystolitica* [6] and *Leishmania* spp. [7]. To our knowledge, this study is the first to show the activities of BBI alkaloids for treating experimental Chagas' disease. Following these studies, we have initiated (in collaboration) an evaluation of the inhibitory activity of BBI alkaloids towards trypanothione–glutathione thioltransferase a target for a specific chemotherapy Chagas' disease [27,28], in order to elucidate its immunological activity.

Acknowledgements

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