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ORIGINAL PAPER

# Chemotherapy of chronic indeterminate Chagas disease: a novel approach to treatment

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**Abstract** Treatment of Chagas disease is a controversial issue because the available drugs are highly toxic. Clomipramine is a tricyclic antidepressant drug that inhibits *Trypanosoma cruzi*'s trypanothione reductase, provoking the death of the parasite and preventing the cardiac damage when used for the treatment of acutely infected mice. Here, we studied the effectiveness of clomipramine (5 mg/kg/day for one month) as chemotherapy for *T. cruzi*-infected mice in the chronic indeterminate stage of the infection. The animals were analyzed in the cardiac chronic phase. Survival of treated animals was 84% while for the untreated ones was 40%; most of the animals presented electrocardiographic abnormalities. Affinity and density of cardiac β receptors from infected and treated mice were similar to those in the indeterminate phase, showing that clomipramine treatment stopped the increment of functional alterations provoked by the infection, while untreated mice presented affinity and density significantly diminished. Hearts from infected and untreated mice in the chronic stage presented mononuclear cells, necrosis and fiber dissolution while hearts from treated animals showed only isolated inflammatory infiltrates. Present results demonstrate that clomipramine used in the chronic indeterminate phase of the *T. cruzi* infection modified the natural evolution of the chagasic cardiopathy.

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## Introduction

*Trypanosoma cruzi* is an hemoflagellate parasite that can infect people that live from Mexico to Argentina and Chile but, recent implementation of donor screening for *Trypanosoma cruzi* infection by the American Red Cross and other blood banks across the United States highlights the urgent need for clinicians, laboratorians and public health professionals to understand Chagas disease, its diagnosis and its treatment because it is estimated that more than 100,000 immigrants have Chagas disease and most of them are unaware of their infection (Schmunis 2007).

The course of the infection includes a short acute phase with symptoms that persist one or two months; a long silent period, the chronic indeterminate phase, that may last between 10 to 20 years in which patients present evidence of immunity without any symptom; and a cardiac chronic phase in which approximately 30% of the patients present evidence of heart disease or megavisceras (Rassi et al. 2000).

The fundamental purpose in using a drug for any infectious disease is that the drug will stop the infection without generating any important side effect in the human host. Additionally, one of the goals of the World Health Organization is to develop a treatment for infectious diseases produced by organisms in which there is a relatively good knowledge of their biology and their genome have been sequenced. These would provide the basis for the identification of metabolic pathways that can be used as drug targets present in the parasite but absent or modified in the mammalian host.

The mammalian redox defense system is based on oxidized glutathione and glutathione reductase. This system is replaced in trypanosomes by an analogous, but different, system based on trypanothione and trypanothione reductase (Fairlamb 1992). This system is indispensable for the parasite because of its antioxidant role. The enzyme trypanothione reductase has been widely recognized as a drug target (De Oliveira et al. 2006; Kahn 2007) and consequently, has been isolated, purified and studied by X-ray crystallography (Bond et al. 1999). Some tricyclic drugs used in psychiatric treatments, such as phenothiazines and related compounds, inhibit trypanothione reductase irreversibly by the peroxide/$H_2O_2$/phenothiazine system in a dose- and time-related manner (Gutierrez-Correa 2001).

Clomipramine is a tricyclic antidepressant drug that belongs to the above-mentioned compounds; besides its ability to inhibit trypanothione reductase, it also exerts an anticalmodulin action upon epimastigote and trypomastigote forms of *T. cruzi* (Barioglio et al. 1987). Previous works of our laboratory demonstrated that clomipramine was effective in preventing cardiac damage when used for the treatment of acutely infected mice (Rivarola et al. 2001) not only with the Tulahuen strain, but also with an isolate obtained from a chronic patient from an Argentinean endemic area (Rivarola et al. 2005).

However, the use of antiparasitary drugs in the chronic indeterminate or in the cardiac chronic phases of Chagas disease is still a controversial issue; for this, in the present work, we studied the effectiveness of clomipramine treatment upon *T. cruzi*-infected mice in the chronic indeterminate phase of the experimental infection.

## Materials and methods

*Experimental infection* Blood collected into heparinized tubes from mice infected with *Trypanosoma cruzi* Tulahuen strain was used as the inoculum for 76 Albino Swiss mice (each weighting 30±1 g) that previously had an electrocardiographic analysis. The parasite concentration in this blood was estimated in a Neubauer hemocytometer, so that each mouse was inoculated intraperitoneally with blood containing 50 blood stream trypomastigotes. This number of parasites is enough to produce the acute, chronic indeterminate and cardiac chronic phases of the experimental Chagas disease (Bustamante et al. 2003a; Paglini-Oliva et al. 1987).

In the chronic indeterminate phase of the infection (75 days post infection, dpi) and after an electrocardiographic study, the mice without any electrocardiographic alteration ($n$=58) were divided into two groups: (a) infected and left untreated ($n$=21) and (b) infected and treated intraperitoneally with clomipramine 5 mg/kg/day, daily, for 1 month ($n$=37). A non-infected group was also studied ($n$=20).

The effectiveness of clomipramine treatment was studied through parasitemia, electrocardiography, cardiac muscle histopathological studies, survival and cardiac β-adrenergic receptors in the cardiac chronic phase of the infection, 150 dpi.

*Parasitemias* in all groups were determined in a Neubauer hemocytometer using blood samples obtained from the tail of the mice, once a week, beginning 7 days after the infection.

*Survival* was monitored daily.

*Electrocardiograms* (ECG) were obtained with an electrocardiographic unit (CardioCom—Model CC12DER MCP) under Ketamine ClH (Ketalar®, Parke Davis, Warner Lambert Co, USA) anesthesia (10 mg/kg), before infection, in the chronic indeterminate phase of the experimental disease (75 dpi), before the beginning of the treatment and in the cardiac chronic phase, 150 dpi. The electrocardiographic tracings were obtained with six standard leads (dipolar leads DI, DII, DIII and unipolar leads aVR, aVL, aVF), recording at 50 mm/s with amplitude set to give 1 mV/10 mm.

*Histopathological studies* The hearts were removed, fixed in buffered 10% formaldehyde (pH 7.0), and embedded in paraffin. Each heart was cut horizontally into 5 μm sections from the apex to the auricles. All the sections were then stained with hematoxylin–eosin. A total of 50 slices from each group was analyzed and at least 30 areas from each slice were examined with a 40× objective.

*Cardiac β-adrenergic receptor binding* The density and affinity of cardiac β-receptors studies were performed in right and left ventricles obtained from the uninfected, infected and infected and treated with clomipramine groups. Both ventricles were homogenized in 10 volumes of ice-cold homogenization buffer (250 mM sucrose, 1 mM $MgCl_2$, and 20 mM Tris–HCl, pH 7.4). Homogenates were centrifuged at 2,000 ×$g$ for 10 min. Pellets were homogenized again and centrifuged at 40,000 ×$g$ for 30 min, washed with KCl 0.6 M and centrifuged twice with homogenization buffer only. The final pellet was suspended in incubation buffer (mM composition: 125 $MgCl_2$, 1.5 EDTA, 75 Tris–Cl, pH 7.65)

in a volume of 1 ml/g of wet tissue. $^{3}$H/dihydroalprenolol ($^{3}$H/DHA, specific activity $3.515\times10^{15}$ Bq/mol from NEN Life Science Products, Boston, MA, USA) was used as radioligand in β-adrenergic receptors' binding assays. Experiments were performed in triplicate with 100 μl of membrane suspension (480 mg protein) and $^{3}$H/DHA (2.4–11.5 nM). The suspension was incubated at 37°C for 10 min in a final volume of 1 ml. The incubation was concluded by adding 1 ml of cold incubation buffer to each tube and rapidly filtering the content under reduced pressure through Whatman GF/B filters. The filters were dried and transferred to vials with scintillation liquid (Aquasol Universal, NEN) for counting (Rivarola et al. 2001; Bustamante et al. 2003a,b; Rivarola et al. 2005).

Specific binding was defined as the difference in radioactivity bound in the absence or presence of 1 μM propranolol. Dissociation constant (Kd) and maximum $^{3}$H/DHA binding (Bmax) were determined by a saturation curve and Scatchard analysis using GraFit (Erithacus Software, Staines, UK).

*Immunofluorescence analysis* Cardiac β-adrenergic receptors in heart tissues from the different groups were visualized in 5 μm paraffin-embedded sections, using antibodies to $\beta_1$ adrenergic receptors. The sections were incubated with the primary antibody for 24 h, with the secondary antibody (fluorescein) for 30 min, and were examined with 10×, 20× and 40× objectives.

*Statistical analysis* Data were compared by ANOVA and multiple comparison by Fisher Test (β receptors' affinity and density), MANOVA (parasitemia studies), Kaplan Meier Survival test (survival) and Square Chi test (ECG and immunofluorescence analysis); significance level was set at 0.05.

## Results

### Parasitemias

Infected animals presented a peak in parasitemia levels 15 dpi (101.13±13.98 parasites per milliliter of blood), which became negative from day 56 post infection until the end of the experiments (data not shown).

### Survival

By day 150 post infection (cardiac chronic phase) 90% of the treated animals and only 48% of the untreated ones were alive, as can be observed in Fig. 1. No significant modifications were observed until 196 dpi that was the end of the experiments.

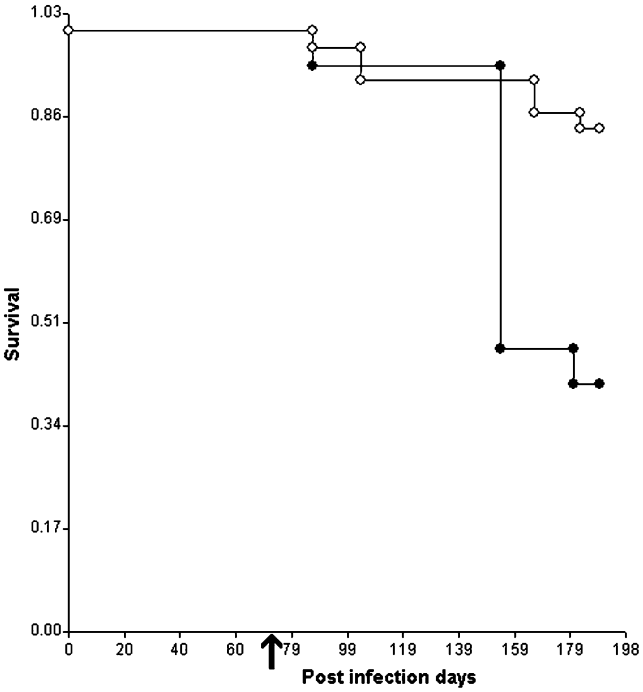


**Fig. 1** Survival of *Trypanosoma cruzi* (Tulahuen strain) infected mice and left untreated (*filled circle*, $n$=21) and treated with clomipramine 5 mg/kg/day for 1 month (*empty circle*, $n$=37) followed until the chronic phase of the experimental infection. This figure was performed using Kaplan Meier Survival test (*Y* axis shows proportions). *Arrow* shows the beginning of the treatment

### Electrocardiography

The abnormalities observed were in auricle–ventricle conduction (prolonged PR segment) and intra-ventricular conduction (prolonged QRST complex) and were more frequent in the treated group, because most of the untreated mice were dead by this moment. Table 1 summarizes these findings.

### Cardiac β-adrenergic receptors

By day 75 post-infection, chronic indeterminate phase, and before the beginning of clomipramine treatment, the affinity (Kd) of the cardiac β receptors from the infected animals was diminished ($p<0.01$) and their density (Bmax) was significantly higher as a compensatory mechanism, when compared to the uninfected controls ($p<0.01$), as can be observed in Table 2.

By day 150 post-infection the affinity and density of cardiac β receptors from the infected and treated mice were similar to those in the chronic indeterminate phase, showing that clomipramine treatment stopped the increment of the functional alterations provoked by the infection.

On the other hand infected untreated animals at this same moment (cardiac chronic phase) presented an affinity and density significantly diminished when compared to the

**Table 1** Electrocardiographic results from non-infected mice and infected with *Trypanosoma cruzi* Tulahuen strain and left untreated or treated with clomipramine 5 mg/kg/day

| Groups | | Parameters | | | |
|---|---|---|---|---|---|
| | | Heart frequency (bpm) | Axes (grades) | QRST complex (s) | PR segment (s) |
| Non-infected ($n$=20) | | 681±8.99 (a) | 67.1±3.68 (a) | 0.03±0 (a) | 0.02±0 (a) |
| 75 dpi, chronic indeterminate phase ($n$=58) | | 622±14.4 (b) | 44.4±1.8 (b) | 0.03±0 (a) | 0.03±0 (a) |
| 150 dpi, chronic cardiac phase | Treated ($n$=31) | 622±12 (b) | 53.2±4.12 (b) | 0.06±0.01 (b) | 0.03±0 (a) |
| | Untreated ($n$=7) | 668±27.4 (a, b) | 36.2±4.9 (b) | 0.04±0 (a, b) | 0.02±0 (a) |

Values show mean±standard error. The statistical comparison was made within the values from each column. Different letter in brackets show significant difference between the values of each parameter ($p$<0.05).

uninfected controls, the infected before the treatment and the infected and treated with clomipramine ($p$<0.01).

### Immunofluorescence studies

The immunofluorescence studies showed that the heart from treated mice in the chronic stage of the infection, presented a similar cardiac β receptors density than in the indeterminate phase. On the other hand, the β receptors density from the untreated mice, in this same stage, was significantly diminished (see Fig. 2). These results are in accordance with the Bmax obtained for the different groups.

### Cardiac histology

The hearts from infected and untreated mice presented moderate inflammatory infiltrates by day 75 post-infection, and mononuclear cells, isolated necrosis and fiber dissolution in the cardiac chronic stage (150 dpi). By day 150 post-infection hearts from the treated animals, on the other hand, showed only isolated inflammatory infiltrates, less than the ones described for the untreated mice in the chronic indeterminate stage (Fig. 3).

## Discussion

The mechanisms responsible for the chagasic cardiopathy are not clearly understood and, for years, the presence of chronic myocardial damage in the absence of parasitemia suggested that this was an autoimmune disease (Girones et al. 2005). However, the use of more sensitive techniques and the recrudescence of the infection in immunosuppressed patients (Schijman et al. 2004) induced that even the most optimistic authors of autoimmunity studies as the main cause of disease in *T. cruzi* infection, have accepted that parasite persistence is a co-requisite, if not a pre-requisite for pathology (Hyland et al. 2007; Kierszenbaum 2005).

The main available drug for Chagas disease treatment is benznidazole. It is likely that the reduced metabolites of benznidazole are involved in its trypanocidal effects by covalent binding to macromolecules (Díaz de Toranzo et al. 1988). It has also been shown that benznidazole improves phagocytosis and increases trypanosomal death through IFN-γ (Romanha et al. 2002) and is trypanocidal to all forms of the parasite (Coura and Castro 2002). However, they can cause systemic toxicity and adverse effects such as anorexia, nausea, central nervous system depression, peripheral polyneuropathies, and dermatitis. The major problems are described in adults.

However, cardiac disease can be arrested and sometimes reversed, in patients with or without mild cardiac damage with benznidazole treatment (Fabbro et al. 2001; Sosa Estani and Segura 2006; Viotti and Vigliano 2007) and a decrease in the cardiac lesions has been attained to this drug (Segura et al. 1994; Andrade et al. 1989). Nevertheless, a long-term treatment with benznidazole at a very low dose in chronic infected mice can be effective to decrease the

**Table 2** β-Adrenergic receptor's affinity and density of non-infected and infected with *Trypanosoma cruzi* Tulahuen strain and left untreated or treated with clomipramine 5 mg/kg/day

| | | Bmax (fmol/mg protein) | Kd (nM) |
|---|---|---|---|
| Non-infected ($n$=12) | | 71.9±0.36 (a) | 3.61±0.05 (a) |
| Infected | Before the treatment (75 dpi; $n$=5) | 77.3±0.91 (b) | 6.86±0.2 (b) |
| | Treated (150 dpi; $n$=5) | 77.2±1.08 (b) | 6.27±0.23 (b) |
| | Untreated (150 dpi; $n$=5) | 53.3±0.71 (c) | 11.2±0.26 (c) |

Values show mean±standard error. The statistical comparison was made within the values from each column. Different letter in brackets show significant difference between the values of each parameter ($p$<0.01).

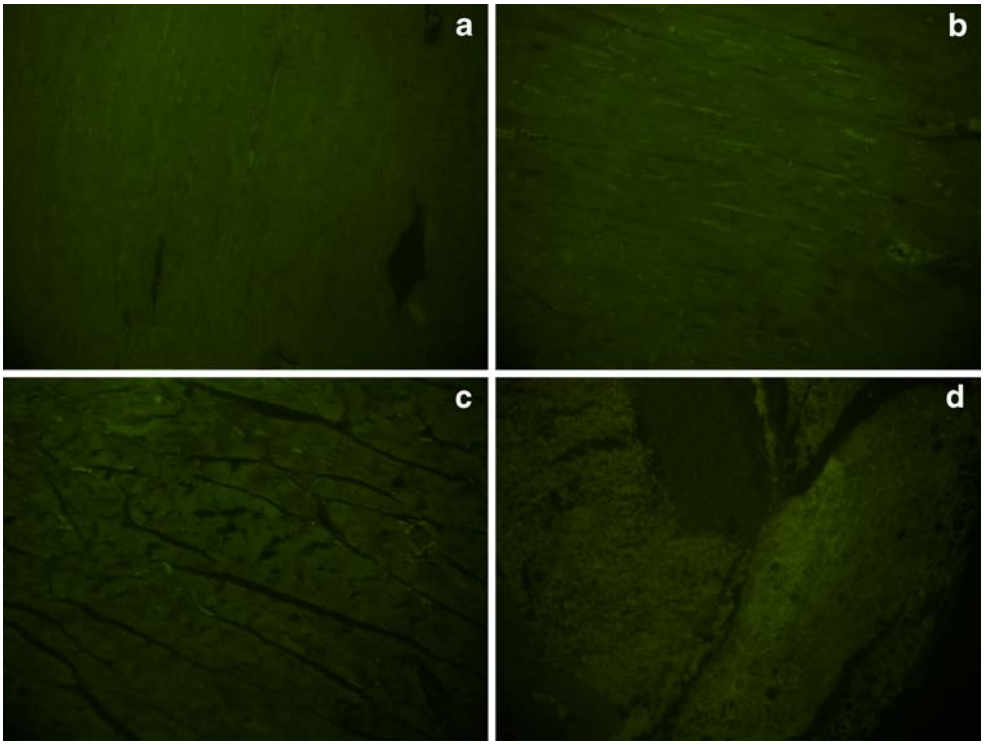


**Fig. 2** Immunofluorescence heart sections using antibodies to $\beta_1$ adrenergic receptors ($\beta_1$-AR). **a** Section from an uninfected mice showing the $\beta_1$-AR along the myocardial cell membranes. 400×. **b** Section from a *Trypanosoma cruzi* (Tulahuen strain) infected mice, 75 dpi. The $\beta_1$-AR can be observed along the myocardial cell membranes. 400×. **c** Section from a *T. cruzi* (Tulahuen strain) infected mice, 135 dpi., showing less density of $\beta_1$-AR along the myocardial cell membranes than **b**. 400×. **d)** Section from a *T. cruzi* (Tulahuen strain) infected mice and treated with clomipramine 5 mg/kg/day, 135 dpi. The $\beta_1$-AR can be observed along the myocardial cell membranes showing a receptor density similar to that found in **b**. 400×

myocarditis and cardiac dysfunction, but not to eradicate the parasite (Garcia et al. 2005).

All these studies and previous works from our laboratory (Bustamante et al. 2003a,b, 2004), point out to the important role of parasite persistence in the pathogenesis of chagasic myocardiopathy and in its evolution to greater tissue damage, and highlight the importance of treatment at any time of the infection.

In this work, we demonstrated, in a murine model infected with *T. cruzi* Tulahuen strain, two different points: the importance of using trypanocidal drugs at any moment of the infection and the effectiveness of clomipramine for the treatment of Chagas disease. Clomipramine was administered to infected mice without any electrocardiographic alteration 75 dpi (chronic indeterminate phase) and its effects upon parasitemia, electrocardiography, cardiac muscle histopathological studies, survival and cardiac β-adrenergic receptors were analyzed in the cardiac chronic phase of the infection, 150 dpi.

Parasitemia of infected mice became negative by day 56 post infection and remained the same way until the end of the experiments in treated and untreated animals. Survival of the treated group was 84% in the cardiac chronic stage whereas the untreated one presented a survival of only 40% at this stage. Similar results were obtained when treating infected groups in the acute phase of the infection (Rivarola et al. 2001).

The electrocardiographic studies showed that treated and untreated animals presented electrical conduction distur-

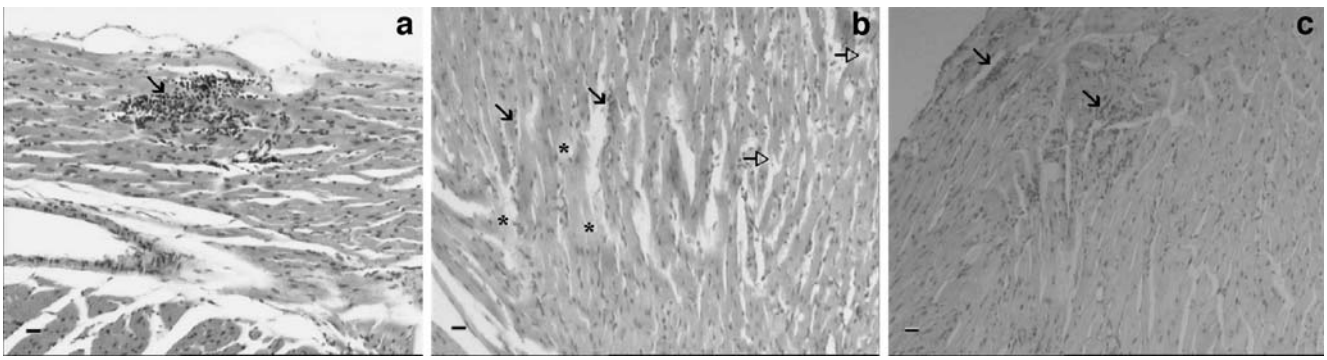


**Fig. 3** Histological sections from hearts from *Trypanosoma cruzi* (Tulahuen strain) infected mice **a** 75 dpi., a lympho-monocytary infiltrate (*arrow*) can be observed; **b** untreated, 135 dpi., lympho-monocytary infiltrates (*black arrows*), fiber dissolution (*hollow arrows*) and necrosis (*asterisk*) can be observed; and **c** treated with clomipramine 5 mg/kg/day, 135 dpi, small lympho-monocytary infiltrates (*arrows*) can be observed. The *bars* correspond to 50 μm. 100×

bances during the cardiac chronic stage. These results would show that clomipramine was not effective to prevent electrocardiographic alterations, but if one takes into account that most of the treated animals ($n=37$) were alive at this moment, whereas the untreated ones ($n=7$) were mostly dead, this difference could explain our findings. Treatment with this drug in the acute phase (Rivarola et al. 2001, 2005) clearly prevented the electrical damage of the heart. Even though clomipramine and other antidepressant drugs are known to prolong the electrical heart conduction, provoking reversible changes in the QT segment (Coupland et al. 1997), these side effects were not detected in our results because the electrocardiographic studies were performed 50 days after the end of the treatment and none of the animals died during clomipramine administration.

Cardiac β receptors affinity and density are modified in different manner in each *T. cruzi* infection stage (Enders et al. 1995) indicating different degrees in cardiac function alteration. For this reason clomipramine efficiency was also evaluated studying β receptors behavior. These results demonstrated that in the cardiac chronic stage, the affinity and density of cardiac β receptors in the treated group presented similar values to the ones obtained 75 dpi (chronic indeterminate phase) and significantly different from those obtained in the cardiac chronic stage ($p<0.01$), indicating that clomipramine stopped any further the damage to the receptors function.

Cardiac histopathological studies demonstrated that, 150 dpi, the untreated mice presented the characteristic lesions described for Chagas disease (necrosis and fibrosis) whereas myocardium from the treated animals only showed few inflammatory infiltrates, indicating that clomipramine also stopped any further the damage to the cardiac structure.

Clomipramine is currently used in clinical treatments for its antidepressant effect and possess the ability to cross the blood–brain barrier providing dopamine receptors blockade. The therapeutic effect verified here in clomipramine treated animals, could be achieved to the ability of the drug to selectively inhibit trypanothione reductase and to its anticalmodulin action. Our results demonstrate the importance of clomipramine treatment in the chronic indeterminate phase of *T. cruzi* infection in order to decrease, retard or modify the natural evolution of the chagasic cardiopathy, reinforcing the idea that parasite persistence plays a determinant role in the evolution of the disease. They also highlight the possibility of trypanothione reductase inhibitors to become new drug candidates for the treatment of this infection.

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