

Trypanothione reductase inhibitors: Overview of the action of thioridazine in different stages of Chagas disease

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ABSTRACT

Thioridazine (TDZ) is a phenothiazine that has been shown to be one of the most potent phenothiazines to inhibit trypanothione reductase irreversibly. Trypanothione reductase is an essential enzyme for the survival of *Trypanosoma cruzi* in the host. Here, we reviewed the use of this drug for the treatment of *T. cruzi* experimental infection. In our laboratory, we have studied the effect of TDZ for the treatment of mice infected with different strains of *T. cruzi* and treated in the acute or in the chronic phases of the experimental infection, using two different schedules: TDZ at a dose of 80 mg/kg/day, for 3 days starting 1 h after infection (acute phase), or TDZ 80 mg/kg/day for 12 days starting 180 days post infection (d.p.i.) (chronic phase). In our experience, the treatment of infected mice, in the acute or in the chronic phases of the infection, with TDZ led to a large reduction in the mortality rates and in the cardiac histological and electrocardiographical abnormalities, and modified the natural evolution of the experimental infection. These analyses reinforce the importance of treatment in the chronic phase to decrease, retard or stop the evolution to chagasic myocarditis. Other evidence leading to the use of this drug as a potential chemotherapeutic agent for Chagas disease treatment is also revised.

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1. Chagas disease

Chagas disease is a common cause of cardiomyopathy, resulting in premature or sudden death and disability across much of Latin America (Barrett et al., 2003), where *Trypanosoma cruzi*, the endemic protozoan parasite responsible for this disease, continues to represent a health threat for an estimated of 28 million people (Barrett et al., 2003; WHO, 2002, 2007).

T. cruzi infection in mammalian hosts has been divided into successive acute and chronic stages. The initial or acute phase is characterized by detectable circulating parasites and symptoms that persist for one or two months. These symptoms can include fever, edema, adenomegaly, hepatosplenomegaly, and myocarditis and/or meningoencephalitis.

This acute phase is followed by a long chronic stage that in most cases presents itself as the indeterminate form (asymptomatic), but may evolve into the cardiac or into the digestive forms, characterized by compromise of the heart and the digestive system respectively. In this phase the parasite cannot be directly detected in circulation. The chronic chagasic cardiomyopathy is the most expressive manifestation of the disease, both because of its frequency and because of its severity (Coura, 2003, 2007) and is a fatal form for which no effective treatments are available.

Despite advances made in recent years, the pathogenesis of chronic Chagas disease is still an unresolved and controversial issue (Teixeira et al., 2006; Manoel-Caetano and Silva, 2007; Haberland et al., 2013; Viotti et al., 2014). At present time, the disease is seen as the product of the interaction between two highly variable genomes: those of the parasite and the human host (Macedo et al., 2002; Higuchi et al., 2003). Questions related to the mechanisms by which tissue lesions are formed in the course of the infection however, have long been and still are a matter of intense debate. Several hypotheses have been raised to explain how cardiac pathology develops. Primary damage of the neuronal system, cell toxicity due to *T. cruzi* and/or *T. cruzi*-derived products, parasite-induced microvascular alterations, polyclonal B-cell activation, triggering of T-cell-mediated responses induced by persistent *T. cruzi* antigens and autoimmunity induced by *T. cruzi*-specific antigens or by the host antigens, are among the mechanisms that have been proposed to drive the pathogenesis of symptomatic chronic Chagas disease (Wood et al., 1982; Cossio et al., 1984; Morris et al., 1990; Kalil and Cunha-Neto, 1996; Tarleton and Zhang, 1999; Minoprio, 2001; Petkova et al., 2001; Bazán et al., 2012). A multiple background for the pathogenesis of chagasic cardiomyopathy can therefore not be ignored (Lescure et al., 2010; Haberland et al., 2013; Viotti et al., 2014). There is a growing consensus that the persistence of parasites, coupled with an unbalanced immune response in some individuals, leads to the sustained inflammatory responses that underlie the characteristic lesions of chronic Chagas disease (Urbina, 2010). An important point is that none of these alternative mechanisms is mutually exclusive. Without removing the parasite from the equation, it is impossible to attribute the inflammation to only one mechanism (Tarleton, 2001). These alternative hypothesis however, suggest different directions for the treatment of the disease: the autoimmunity hypothesis would advise treatment only during the acute phase when the parasite is present in circulation, while the parasite persistence one would advise it at any moment of the infection (Segura et al., 1994, 1996).

2. Chemotherapy

Drugs effective on *T. cruzi* as trypanocidal agents may be classified as (a) drugs of extensive clinical use: nifurtimox and benznidazole; (b) drugs of restricted clinical use: azoles (ketonazole, econazole, miconazole), amphotericin B, allopurinol,

allopurinol ribosides and primaquine; (c) drugs effective on *T. cruzi* and in experimental murine models: alkyllysophospholipids, 5-amino-imidazole-4-carboxamides, bisbenzyl-isoquinolines, cruzipain (crucein) inhibitors, gossipol and phenothiazines; and (d) drugs effective *in vitro* without other reported effects: acridines, actinomycin D, crystal violet, diterpenes, epoxidienthiol carbamates and Fe-chelators, among others (reviewed in Stoppani, 1999; Urbina, 2010; Bustamante and Tarleton, 2014).

Azoles achieve their *in vitro* and *in vivo* action upon *T. cruzi* growth through the inhibition of the ergosterol production: they have been found to reduce parasitemia and extend the survival of infected mice but do not produce parasitological cure (with a more suppressive rather than curative action) and their clinical effectiveness is questionable. Allopurinol, allopurinol ribosides and related compounds inhibit *T. cruzi* hypoxanthine-guanine ribosyl transferase, thus preventing the synthesis of adenylic and guanylic acids and tempering parasitic DNA synthesis: they reduce parasitemia and negativize xenodiagnosis but these effects may not be permanent, which hinders their clinical use. Cysteine-protease inhibitors in turn recognize *T. cruzi* protease (cruzipain, crucein) active site, thus allowing a covalent linkage with the inhibitor: these peptide inhibitors have been found to be effective in acute and chronic murine models. Finally, phenothiazines are promising trypanocidal agents for the treatment of Chagas' disease since they inhibit a parasite specific enzyme, as will be discussed in the following sections (Stoppani, 1999; Urbina, 2010; Bustamante and Tarleton, 2014).

Despite all these potential agents, the only two drugs approved by WHO for the treatment of Chagas disease are nifurtimox and benznidazole. They are both nitroheterocyclic compounds: nifurtimox is a nitrofuran and benznidazole is a nitroimidazole derivative, being the later the most available and widely used (Urbina and Docampo, 2003; Von et al., 2007). Nifurtimox acts via the reduction of the nitro group to destabilize nitroanion radicals, which in turn react to produce highly toxic, reduced oxygen metabolites (such as superoxide anion and hydrogen peroxide). It has been demonstrated that *T. cruzi* is deficient in detoxification mechanisms for oxygen metabolites, particularly hydrogen peroxide, and is thus more sensitive to oxidative stress than vertebrate cells (Docampo, 1990). Benznidazole acts via a different mechanism (reductive stress), which involves covalent modification of macromolecules by nitro-reduction intermediates (Docampo, 1990).

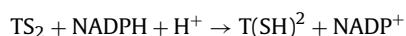
Both nifurtimox and benznidazole have significant activity in the acute phase, with 60–80% of parasitological cures in treated patients (Cançado, 1999; Urbina and Docampo, 2003; Von et al., 2007). However, chronic Chagas disease treatment is still an open field for investigation and remains as an unsolved problem, since these two currently available drugs are not entirely effective, are mutagenic and may produce adverse reactions and toxic effects (such as hypersensitivity reactions, bone marrow depression, neuropathies, and agranulocytosis) (Castro and Díaz de Toranzo, 1988; Morello, 1988; Rodrigues Coura and de Castro, 2002; Viotti et al., 2009). The effectiveness of chemotherapeutic treatment in reducing disease progression in long-term follow-up studies (Viotti et al., 2006) has been demonstrated, but these adverse side effects reported in patients treated with nifurtimox or benznidazole, together with the appearance of resistant parasite strains (Andrade et al., 1992; Cançado, 1999; Kirchhoff, 1999), has discouraged treatment in the chronic phase.

Given the significant limitations of the currently available drugs, particularly for the treatment of chronic patients, new approaches to the specific chemotherapy of Chagas disease have been advanced, particularly in the last two decades (reviewed in Urbina, 2010): as stated earlier, triazole derivatives (posaconazole, D0870, TAK-187, UR-9825 and revuconazole), squalene synthase inhibitors, cysteine protease (cruzipain) inhibitors (CRA-3316 or

K-777) and other compounds have been proposed as candidates for Chagas disease treatment. All these potential candidates achieve their anti-*T. cruzi* activity targeting different essential parasite metabolic pathways. The development of more-effective and safe drugs is a clear target to improve patient outcome and for clinical management (Viotti et al., 2014).

3. Trypanothione reductase

Experimental studies have identified several novel targets for chemotherapy, one of them being the parasite's enzyme trypanothione reductase (TR). TR is found in parasitic protozoa such as *Leishmania* and trypanosomes. Particularly, *T. cruzi* lacks the ubiquitous enzyme glutathione reductase (GR); thus to maintain a reducing intracellular redox environment, it relies on the flavoenzyme TR to keep their main thiols, bis-(glutathionyl) spermidine [trypanothione ($T(SH)_2$)] and mono (glutathionyl) spermidine (Gsp), in their thiol state (Meiering et al., 2005):



The presence of these endogenous antioxidant molecules in *T. cruzi* provides the ability to counteract the oxidizing environment generated by the cellular immune response of the host and by the action of trypanocidal redox cycling drugs (Thomson et al., 2003).

TR shares many mechanistic and structural properties with GR, the closest related mammalian enzyme (Meiering et al., 2005). While glutathione (γ -L-glutamyl-L-cysteinylglycine) is involved in the regulation of diverse aspects of metabolism, its thiol form (GSH) functions as a protective agent, maintaining a reduced intracellular environment. GSH can be oxidized to glutathione disulfide (GSSG), a reaction that generates potentially damaging radicals and oxidants. The enzyme GR ensures that high thiol levels are preserved by catalyzing the reduction of GSSG. *T. cruzi* uses TR instead of GR for this protective function. In contrast to GR, the active site of TR shows an overall negative charge and is much wider and more hydrophobic (Kuriyan et al., 1991; Hunter et al., 1992; Lantwin et al., 1994; Stoll et al., 1997). This opposed charge distribution forms the basis for the mutually exclusive specificity of these two enzymes toward their respective disulfide substrates (Faerman et al., 1996). Human GR and *T. cruzi*'s TR are both NADPH-dependent, flavin-containing disulfide oxidoreductases, sharing about 30% of their sequences. The key residues involved in their catalytic action are conserved, but each enzyme is specific for a particular substrate, despite their mechanistic and structural similarities (Henderson et al., 1987; Fairlamb and Cerami, 1992). This indicates that it should be possible to inhibit the parasite's TR without affecting the human GR, which makes this parasite-specific flavoenzyme an attractive target molecule for an anti-trypanosomal drug development. This is particularly important since genetic studies have revealed that TR is essential for the parasite, playing a key role against oxidative stress (Krieger et al., 2000).

The current thinking directed toward the discovery of a new drug involves the identification of the drug target, isolation and characterization of its molecular and kinetic properties, target validation by chemical or genetics means, identification of inhibitors and their optimization to improve pharmacological and toxicological properties (Fairlamb, 1999). TR has been widely identified as a drug target for Chagas disease treatment (Wang, 1995; Hunter, 1997) and has been isolated and its molecular and kinetic properties characterized in detail by X-ray crystallography (Hunter, 1997). The essential role of TR in the parasite thiol metabolism and its absence from the mammalian host render the enzyme a highly attractive target for structure based drug development against trypanosomatids. Many different classes of compounds have been found that show selective inhibition of the parasite compared to

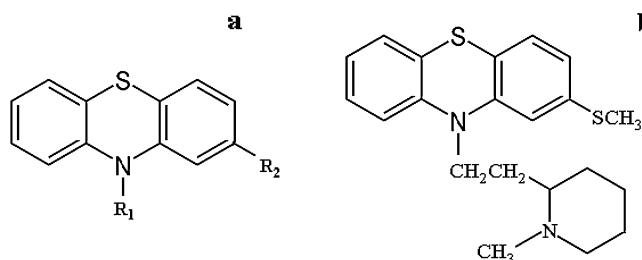


Fig. 1. (a) Chemical structure of phenothiazines and (b) chemical structure of thioridazine.

the host enzyme (Krauth-Siegel and Inhoff, 2003). Between them, competitive (tricyclic ring structures and related compounds, polyamine and peptide derivatives, dibenzazepines, etc.) and non-competitive (8-methoxy-naphtho[2,3-b] thiophen-4,9-quinone – Zani and Fairlamb, 2003), irreversible (2,2':6,2"-Terpyridine) platinum (II) complexes – Bonse et al., 2000) and turncoat (which transform the antioxidant enzyme into a pro-oxidant enzyme – Jockers-Scherübl et al., 1989; Krauth-Siegel and Schoneck, 1995) inhibitors has been tested.

4. Thioridazine: a trypanothione reductase inhibitor

Thioridazine (TDZ) is a phenothiazine that has been shown to be one of the most potent phenothiazines to inhibit TR irreversibly. Phenothiazines are currently used in psychiatry as antidepressant, anxiolytic and antipsychotic agents. They possess the ability to cross the blood-brain barrier and to accumulate in the brain, providing effective dopamine receptor blockade (Kebabian and Calne, 1979). They also interact with membranes or their compounds (Fernández et al., 1997; Thorsing et al., 2013), with cellular proteins (Paglini Oliva et al., 1987), with dopaminergic receptors (Scatton et al., 1976; Kebabian and Calne, 1979; Ggerfen et al., 1990), act to inhibit the activity of Mg²⁺-dependent ATPases, increase membrane fluidity and provide marked anti-calmodulin action (Bondy, 1995).

Phenothiazine drugs, in addition to their antipsychotic properties, have been shown to have significant antimicrobial activity against a variety of intracellular microorganisms (Amaral et al., 2006; Thanacoody, 2011). Phenothiazines are tricyclic drugs with three rings, two of them being benzenes joined by sulfur and nitrogen atoms, as can be observed in Fig. 1a. The substitutions in positions 2 and 10 (R₂ and R₁ in the figure, respectively) exhibit a number of interesting analytical properties due to their characteristic structure. In TDZ, these substitutions are a SCH₃ in position 2 and a 2-(1-methyl-2-piperidinyl)-ethyl in position 10 (Fig. 1b). The quantitative effects of phenothiazines are mediated by the substituents on the rings, especially at the 2-position (Keyzer, 1995); TDZ possesses a piperidine group as lateral chain; this structure would be responsible of the low side effects of this drug and the increment in its anti-muscarinic activity (Biel et al., 1978).

TDZ, extensively used for its antipsychotic properties, has also recently attracted attention as a potential candidate as an antimicrobial drug (reviewed in Amaral et al., 2001; Thanacoody, 2007; Amaral and Molnar, 2014; Hahn and Sohnle, 2014; Salie et al., 2014), since it is associated with the lowest risk of extrapyramidal side-effects of all phenothiazines (Yang et al., 2007). Gutierrez Correa and co-workers (2001), studied different phenothiazines as inhibitors of TR and showed that TDZ is one of the most potent phenothiazines to inhibit this enzyme irreversibly. More than 90% inhibition may be required to kill the parasites in absence of oxidant stress (Fairlamb, 1999); TDZ, at a concentration of 10 μ M attained 100% inhibition.

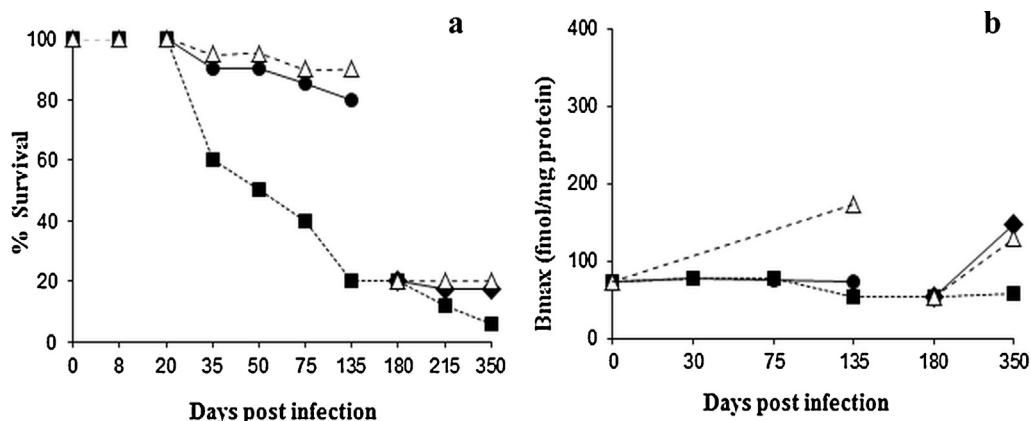


Fig. 2. Mice infected with the Tulahuen strain. (a) Survival and (b) cardiac β -adrenergic receptor density. (●) Mice treated with TDZ in the acute phase; (◆) mice treated with TDZ in the chronic phase. Results from mice treated with benznidazole (- - -) and mice infected and left untreated (---) are shown for comparison.

The results of exposing trypomastigotes and epimastigotes to TDZ *in vitro* confirms that the drug is trypanocidal, producing disruption of mitochondria and kinetoplasts in trypomastigotes and the condensation of these organelles close to the plasma membrane in epimastigotes (Paglini-Oliva et al., 1998).

5. Effect of thioridazine treatment upon *T. cruzi* infection in mice

In our laboratory we have studied the effect of TDZ for the treatment of mice infected with *T. cruzi* and treated in the acute (Lo Presti et al., 2004) or in the chronic phases (Bustamante et al., 2007) of the experimental infection, in order to determine if a reduction in the parasite levels at any moment of the infection (not just when the parasites are present in circulation – acute phase) would reduce the evolution of the cardiac damage. Since not all parasite strains show the same response to treatment, we studied mice inoculated with the Tulahuen strain or with the SGO-Z12 isolate, in order to compare the effectiveness of TDZ upon two different infection models (Lo Presti et al., 2004; Bustamante et al., 2007). When possible, the effect of TDZ was compared to that of benznidazole (Bustamante et al., 2007; Strauss et al., 2013).

The mice were infected with 50 trypomastigote forms from either the Tulahuen strain or the SGO-Z12 isolate and treated orally with TDZ. Two treatment schedules were used: TDZ at a dose of 80 mg/kg/day, for 3 days starting 1 h after infection (acute phase), or TDZ 80 mg/kg/day for 12 days starting 180 days post infection (d.p.i.) (chronic phase). The amount of parasites used was sufficient to reproduce the acute phase and the different forms (indeterminate and cardiac) of the chronic phase of the experimental *T. cruzi* infection (Bustamante et al., 2003a). In both cases, the effectiveness of the treatment was assayed through parasitemia, survival, electrocardiography, cardiac histopathology and cardiac β -adrenergic receptors (β -AR) density and affinity. The mice from the first group (treated in the acute phase) were studied 135 d.p.i. (Lo Presti et al., 2004) and the mice from the second group (treated in the chronic phase) were studied 350 d.p.i. (Bustamante et al., 2007). The first moment was chosen taking into consideration that the characteristics of the chronic phase of the infection (presence of inflammatory infiltrates, fibrosis and fiber fragmentation in the cardiac tissue, among others) would have appeared by this time (135 d.p.i.) (Bustamante et al., 2003b); the second one because the chronic alterations would have intensified by then (350 d.p.i.) (Lo Presti et al., 2009).

5.1. Effect of TDZ upon infection with the Tulahuen strain

TDZ assayed *in vivo* was effective for the treatment of mice infected with *T. cruzi*, Tulahuen strain, decreasing parasitemias levels when used in the acute phase of the infection. Treated mice cleared their parasitemias (no parasites were observed under the optical microscope using a Neubauer haemocytometer) in a shorter period of time (by day 15 post infection – p.i.) and 80% of them survived for at least one year after the infection (Fig. 2a). Untreated mice cleared their parasitemias by day 35 p.i., and only 20% of them were still alive 12 months later (Fig. 2a). When some of these untreated mice surviving 180 d.p.i. were treated with TDZ, survival rates showed that by day 350 p.i. only 30% of the mice that were left untreated were alive, while 88% of the treated ones survived.

Cardiac function was studied from two different points of view: by electrocardiography and by quantification of cardiac β -AR's affinity and density.

Electrocardiographic results showed that TDZ significantly reduced the number and the quality of the conduction alterations when used either in the acute or in the chronic phases of the experimental infection. Atrio-ventricular blockades (prolonged PR segment) and intra-ventricular blockades (prolonged QT interval) were the most common alterations presented by 60% of the untreated infected animals 135 d.p.i.; 37% of the mice treated in the acute phase presented less intense alterations at this point. These abnormalities were also more frequent among the untreated infected group 350 d.p.i (66.6% of the animals), while TDZ treatment in the chronic phase decreased the percentage of mice showing conduction disturbances (43.3% of the treated mice). Abnormalities that could be due to the effects of TDZ on cardiac electrical conduction were not detected and treated uninfected mice presented PR segment and QT interval values similar to those presented by uninfected controls.

By day 135 p.i., untreated infected mice presented a lower β -AR's density and a 3-fold decrease in the receptor's affinity. The animals treated in the acute phase also had lower receptor affinity (1.52-fold decrease) but a density that remained similar to that of the uninfected controls. TDZ treatment in the chronic phase did not reverse this reduction in the affinity values but significantly increased the receptor's density as an important compensatory mechanism for the loss of affinity (2-fold increase) (Fig. 2b).

The hearts from the untreated infected animals presented disperse lympho-monocitary infiltrates, fiber dissolution and isolated necrosis when studied 135 d.p.i. (Fig. 3a) and similar, but more intense cardiac alterations with inflammatory infiltrates, especially in the right ventricle, thickening of the epicardium, fibrosis and necrosis in the inter-ventricular septum when observed 350 d.p.i.

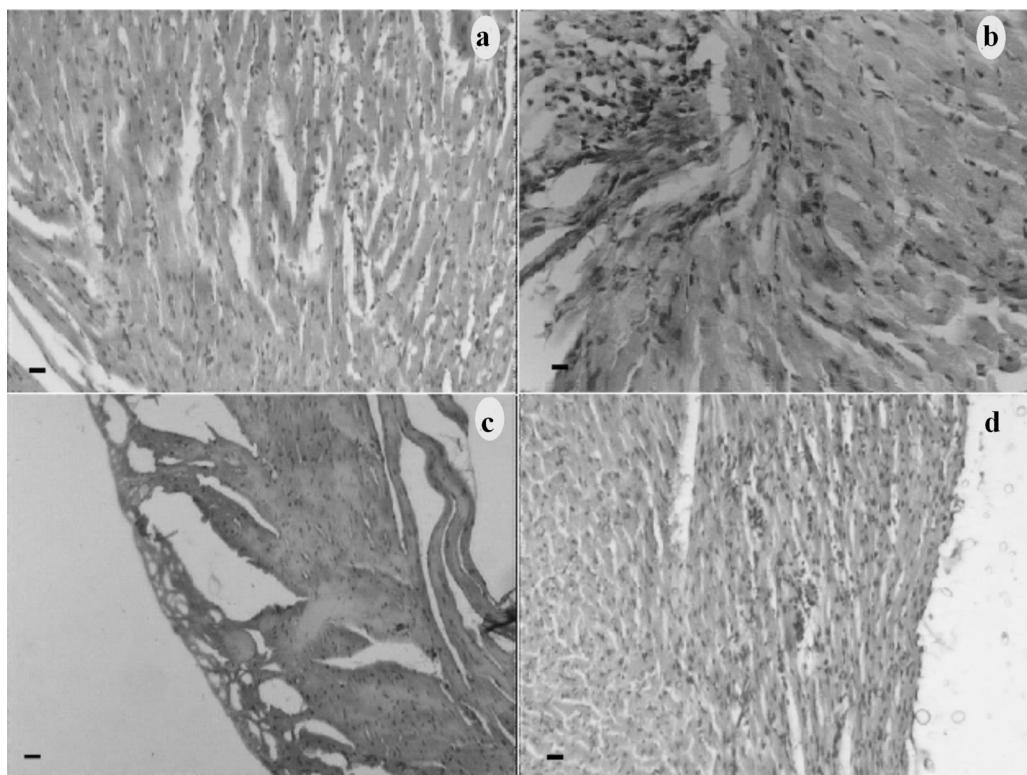


Fig. 3. Cardiac histological sections from the different groups infected with the Tulahuen strain: (a) infected and left untreated, 35 d.p.i.; (b) infected and treated with TDZ during the acute phase; (c) infected and left untreated, 350 d.p.i. and (d) infected and treated with TDZ during the chronic phase. Bar: 50 μm .

(Fig. 3c). TDZ treatment in the acute phase prevented the appearance of the typical histological alterations of the chronic infection since no structural modifications were observed in the treated hearts (Fig. 3b); TDZ treatment in the chronic phase prevented the evolution to a higher degree of fiber fragmentation, necrosis and fibrosis and significantly reduced the inflammatory infiltrates (Fig. 3d).

These results suggest that both treatment schedules (TDZ in the acute or in the chronic phase) were able to prevent the evolution of the cardiopathy produced by the infection with *T. cruzi* Tulahuen strain. Heart histology, electrocardiographic studies and β -AR's density were similar to the ones presented by uninfected controls when the drug was used in the acute phase of the infection, thus preventing the acute phase evolving into the chronic phase and the typical cardiopathy in most of the treated mice. Treatment with TDZ in the chronic phase of infection prevented the evolution of the histological and electrocardiographic alterations typical of the disease and compensated the modifications in the β -ARs, thus preventing greater damage to the cardiac function.

5.2. Effect of TDZ upon infection with the SGO-Z12 isolate

T. cruzi's population comprises a set of strains and isolates that circulate between vectors, humans and domestic and sylvatic reservoirs. The different parasite isolates have demonstrated intraspecific variation in their morphology, virulence, ability to induce lesions, antigenic constitution, infective capacity and treatment susceptibility, among other characteristics (Manoel-Caetano and Silva, 2007). Therefore, we also studied the effectiveness of TDZ treatment upon the infection produced by an isolate obtained from an endemic area of Argentina (SGO-Z12) (Lo Presti et al., 2004). The electrophoretic pattern of this isolate has shown that it belongs to zymodeme 12 from Argentina (Bustamante et al., 2003a,b);

patients infected with zymodeme 12 parasites have demonstrated to present higher risk of cardiac lesions (Montamat et al., 1996).

Parasite levels were significantly lower in this group than in the Tulahuen group from the beginning of the infection until 35 d.p.i. when circulating parasite started to decrease in both infected groups. Similarly to what was observed with the Tulahuen strain, parasitemias in the mice infected with the SGO-Z12 isolate and treated with TDZ in the acute phase were significantly less intense than those observed in the untreated infected animals and lasted for a shorter period (56 vs. 63 d.p.i.). By day 40 p.i. survival of the treated animals began to be higher than the untreated ones and by day 135 p.i. 80% of the untreated infected but only 50% of treated mice were dead (Fig. 4a). As can be observed, despite the lower parasite levels observed in the SGO-Z12 group, the Tulahuen infected mice (treated and non-treated) showed a higher survival rate in the acute phase of the infection. Survival rates were similar in either infected group (Tulahuen or SGO-Z12) however, by day 135 p.i. and they showed similar results when treated either with TDZ or with benznidazole in the chronic phase.

In the SGO-Z12 group, electrocardiographic abnormalities were more frequent among the untreated infected mice than the treated ones; the treated group showed a cardiac electric conduction similar to the non-infected controls 135 days after the inoculation of the parasites. Similar results to the Tulahuen group were observed with the treatment in the chronic phase: TDZ decreased the percentage of mice showing conduction disturbances (33% of the treated mice vs. 63% of the untreated ones) by day 350 p.i.

The infection with the SGO-Z12 isolate seems to affect differently the cardiac β -AR: while the affinity of the β -ARs diminished with the evolution of the infection, as found in the hearts infected with the Tulahuen strain, the receptors' density was significantly higher in the hearts infected with this isolate throughout all the stages of the experimental infection (until 350 d.p.i. when it started to decrease) (Lo Presti et al., 2006, 2008, 2009). The mice treated

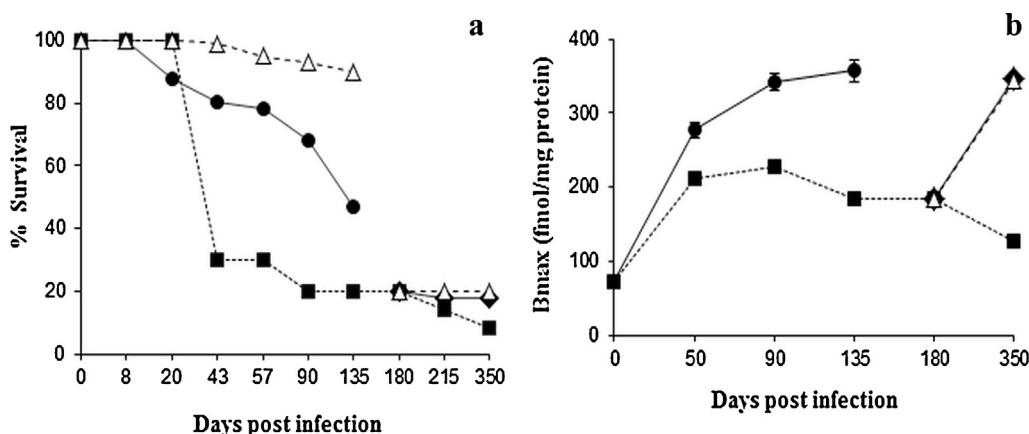


Fig. 4. Mice infected with the SGO-Z12 isolate. (a) Survival; (b) cardiac β -adrenergic receptor density. (●) Mice treated with TDZ in the acute phase; (▲) mice treated with TDZ in the chronic phase. Results from mice treated with benznidazole (—△—) and mice infected and left untreated (—■—) are shown for comparison.

in the acute phase presented an even higher density than the untreated ones (5-fold vs. 2.5-fold increase) by day 135 p.i. Similar results were obtained with the treatment in the chronic phase studied 350 d.p.i. (4.8-fold increase in the density of the treated hearts; 1.77-increase in the untreated ones) and with benznidazole (Fig. 4b).

The hearts from the SGO-Z12 infected group also showed the lympho-monocitary infiltrates and fiber disorganization characteristic of the chronic infection 135 days after the inoculation of the parasites (Fig. 5a). In this stage no detectable histopathological abnormalities were detected in 67% of hearts from the mice treated with TDZ in the acute phase and 33% of them presented only mild inflammatory infiltrates (Fig. 5b). Similar results as the ones obtained with the Tulahuen strain were observed with the treatment in the chronic phase: TDZ treatment prevented the evolution to a higher degree of fiber fragmentation, necrosis and fibrosis and reduced the characteristic inflammatory infiltrates (Fig. 5d).

Again, both treatment schedules (TDZ in the acute or in the chronic phase) were able to prevent the evolution of the cardiopathy produced by the infection with *T. cruzi* SGO-Z12 isolate. In this case, treatment in the acute phase modified the course of the infection and prevented the cardiopathy; treatment in the chronic phase prevented the evolution of the histological and electrocardiographic alterations, reducing the evolution of the cardiac disease.

6. Discussion

The use of anti-parasitic drugs in the chronic phase of Chagas disease is still a controversial issue. Those authors in favor of the treatment propose that it will prevent and reduce the cardiopathy and stop its evolution, thus reducing morbi-mortality in patients with chronic infection (Segura et al., 1996; Viotti and Vigliano, 2007). Scientific evidence regarding the role of the parasite as a stimulus and trigger for tissue damage has accumulated over the last two decades, providing a solid basis to consider antiparasitic treatment for chronic adult patients (Marin-Neto et al., 2008, 2009; Viotti et al., 2014). Those who discourage treatment with antiparasitic drugs during the chronic phase of the cardiopathy refer to the remote possibility of parasitological and serological cure and to the side effects of the currently available drugs (Manussur and Barbieri, 2002). Aiming to clarify this issue, a large-scale, randomized and placebo-controlled clinical trial is currently underway to assess the effects of etiologic treatment with Benznidazole in chronic chagasic patients (BENEFIT – Benznidazole Evaluation for Interrupting Trypanosomiasis – trial) (Marin-Neto et al., 2008, 2009). Other authors have emphasized that the clinical assessment of the side effects

must take into account the short time of the treatment and the fact that the side effects are reversible and not life threatening (Viotti et al., 2009).

Additionally, the suggestion that autoimmune processes could underlie the pathogenesis of chagasic myocarditis may have had a negative impact on the efforts to develop a vaccine against *T. cruzi* infection and to discover more effective and less toxic drugs for its chemotherapy (Zhang and Tarleton, 1999). The persistence of the parasite in the host tissues, on the other hand, has been demonstrated as a determining factor in inducing chronic Chagas disease (Zhang and Tarleton, 1999; Braga et al., 2000; Apt et al., 2005; Garcia et al., 2005; Bazán et al., 2012). This idea has been supported by the finding of *T. cruzi* DNA in hearts of patients with chagasic cardiomyopathy (Jones et al., 1992). This can also be illustrated by the recrudescence of the infection in many patients with heart failure due to Chagas disease who undergo cardiac transplantation; when these patients are immunosuppressed, myocarditis usually develops in the transplanted heart and *T. cruzi* parasites are detected in blood and tissues (Stolf et al., 1987).

If the immune response directed against *T. cruzi* is responsible for the cardiac damage, treatment after the acute phase of the disease would not be reasonable; however, if parasite levels are a significant issue in the progression to a severe disease with greater cardiac damage, treatment during the chronic phase would prevent or decrease the evolution of cardiac disease.

In our experience, the treatment of infected mice with different parasite strains, in the acute or in the chronic phases of the infection, with TDZ led to a large reduction in the mortality rates and in the cardiac histological and electrocardiographical abnormalities, and modified the natural evolution of the experimental infection (Rivarola et al., 1999; Lo Presti et al., 2004; Bustamante et al., 2007). Similar results were obtained with benznidazole used in the chronic phase of the experimental infection (Bustamante et al., 2007). These results reinforce the importance of treatment in the chronic phase to decrease, retard or stop the evolution to chagasic myocarditis. Other studies with mice treated with benznidazole during chronic *T. cruzi* infection have demonstrated that although parasite eradication could not be achieved with the treatment in the chronic phase, a decrease in the parasite load could be observed, showing that the drug also produced a decrease in the cardiac lesions and dysfunction (Garcia et al., 2005).

Our results using TDZ are probably related to the effect of the drug upon the parasite's enzyme TR. The absence of this enzyme in the mammalian cells and its essential role in the antioxidant defense of the parasite render TR as an attractive target molecule for rational drug design. TDZ and other phenothiazines are drugs

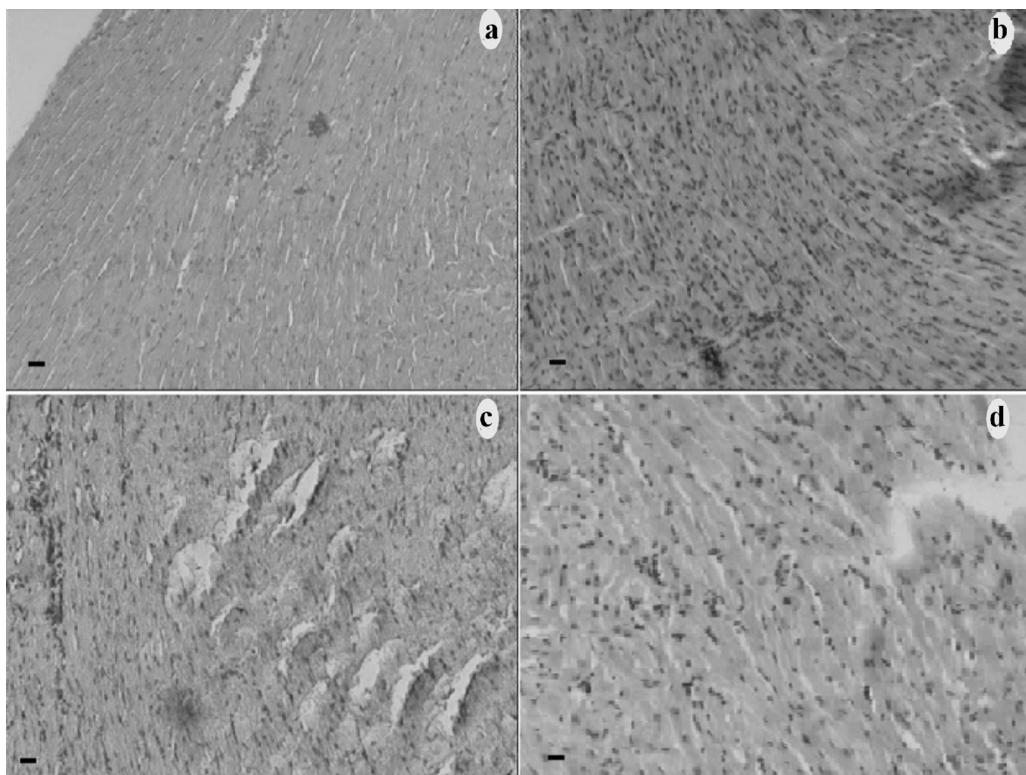


Fig. 5. Cardiac histological sections from the different groups infected with the SGO-Z12 isolate: (a) infected and left untreated, 35 d.p.i.; (b) infected and treated with TDZ during the acute phase; (c) infected and left untreated, 350 d.p.i.; (d) infected and treated with TDZ during the chronic phase. Bar: 50 μ m.

in clinical use that have demonstrated to be potent TR inhibitors, inducing the death of the parasites in *in vitro* and *in vivo* experiments, modifying the evolution of experimental *T. cruzi* infection. The inhibition of this enzyme results in the accumulation of oxidizing agents that ultimately lead to the death of the parasites and the consequent reduction in the parasite load in the host.

Other mechanism by which phenothiazines, and therefore TDZ, may exert their action, is by binding with sufficient strength to the calcium-binding protein calmodulin to prevent the influx of calcium into the cells (Hidaka and Naito, 1998). Additionally, it has been proposed that phenothiazines may inhibit different bacterial efflux pumps that are associated with resistance to antibiotics, thus enhancing the activity of such antibiotics when used in combination with them (Kaatz et al., 2003; Kristiansen et al., 2003). Efflux-related multidrug resistance as such, has become appreciated as a significant complicating factor in the chemotherapy of bacterial infections (Kaatz et al., 1993; Neyfakh et al., 1993; Poole and Srikumar, 2001) and inhibitors of these pumps have shown to be promising agents to overcome such resistance (Couto et al., 2008). TDZ particularly has been found to reduce resistance of methicillin-resistant *Staphylococcus aureus* by inhibiting a reserpine-sensitive efflux pump (Kristiansen et al., 2006). Such mechanism (although not yet studied) could be taking place here, which would encourage the use of TDZ in combination with other trypanocidal agents to potentiate their action. TDZ in combination with different antibiotics has been described for tuberculosis (reviewed in Amaral and Molnar, 2014) and benznidazole in combination with a related tricyclic compound (clomipramine) for the treatment of Chagas disease (Strauss et al., 2013).

Another important point to highlight is that TDZ is already in clinical use, although the advent of newer antipsychotic drugs has decline its use in psychiatry in developed countries (Thanacoody, 2007); TDZ potential as an antimicrobial agent however can no longer be ignored: in experimental *T. cruzi* infection, TDZ has

reduced parasite levels when used in the acute phase, has improved survival of the infected animals, has reduced electrocardiographic and histopathological alterations produced by the evolution of the infection and has permitted compensatory mechanisms in the cardiac β -ARs, all of which lead us to conclude that the cardiac function was re-established in the treated animals. Despite the fact that different results were observed in the mice infected with the Tulahuen strain or with the SGO-Z12 isolate, both infected groups showed some degree of improvement in the different parameters when treated with TDZ at either moment (acute or chronic phase). The antimicrobial properties of this drug and its potential use for the treatment of other microbiological infections have already been suggested by other authors (reviewed in Thanacoody, 2007).

7. Final comments

Present results reinforce the idea that parasite persistence is a determining factor in inducing chronic alterations characteristic of Chagas disease and this evidence would encourage the treatment of chronic patients, as well as chronic experimental models, with less toxic drugs. They also clearly demonstrate that if the parasite load is reduced, cardiac damage diminishes significantly; the survival of treated animals and the compensatory mechanism of cardiac β -receptors demonstrate that cardiac function is reestablished by either TDZ treatment (in the acute or the chronic phases).

Therefore, TDZ should be considered as a potential agent for the treatment of Chagas disease, with no apparent toxic effects at the used dose and treatment schedule. As this drug is clinically used as a neuroleptic, these results are of great interest because they may be the basis for new agents for the treatment or prevention of this disease.

Taking into consideration the distinct mechanism of action of all the available drugs and the differences described for the several parasite strains/isolates, this drug could be used in combination

with others to improve the results and the evolution of the treated individuals.

Competing interests

None declared.

Ethical approval

All the experimental procedures reviewed here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health, publication N° 85-23 (revised 1996) and had been approved by the Institutional Committee for the Care and Use of Laboratory Animals from the Faculty of Medicine, National University of Córdoba, Argentina.

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