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Short Communication

Novel cruzipain inhibitors for the chemotherapy of chronic Chagas disease

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ABSTRACT

Despite current efforts worldwide to develop new medications against Chagas disease, only two drugs are available, nifurtimox and benznidazole. Both drugs require prolonged treatment and have multiple side effects and limited efficacy on adult patients chronically infected with *Trypanosoma cruzi*. Recently, computer-guided drug repositioning led to the discovery of the trypanocidal effects of clofazimine and benidipine. These compounds showed inhibitory effects on cruzipain, the major cysteine protease of *T. cruzi*, of different parasite stages and in a murine model of acute Chagas disease. The aim of this work was to determine the efficacy of these novel cruzipain inhibitors when administered in a murine model of chronic Chagas disease. Benidipine and clofazimine were able to reduce the parasite burden in cardiac and skeletal muscles of chronically infected mice compared with untreated mice as well as diminish the inflammatory process in these tissues. Further studies should be performed to study the synergism with benznidazole and nifurtimox in view of combined therapies.

1. Introduction

Chagas disease is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi*. Almost 7 million people are infected worldwide, mostly in Latin America. However, due to human migration, infected patients have become increasingly detected in the USA and Canada as well as in many European and some Western Pacific countries [1].

Chagas disease presents in two phases: an acute phase and a chronic phase. Years to decades after the onset of the infection, 30% of chronically infected patients will suffer from cardiac disorders and 10% from digestive or mixed alterations [1]. Infection triggers an immune response committed to parasite control that, during the lifelong infection, should keep parasites at bay with minimal tissue damage [2]. In this scenario, it is worthwhile to find drugs that target parasite viability and also alleviate symptoms of the chronic disease [3].

Despite efforts in drug discovery, only two drugs are currently used to treat Chagas disease, namely, benznidazole and nifurtimox. Both require long treatments, display severe side effects and have controversial efficacy in chronically *T. cruzi*-infected adults [3,4]. Therefore, the need for safe and efficacious treatment alternatives against Chagas disease is renewed. Drug repurposing has emerged as an attractive approach for the development of novel therapeutics for neglected diseases. This strategy implies expanding the utility of existing drugs to other therapeutic indications [5], facilitating rapid and cost-effective drug development. On the other hand, combination treatments are increasingly recommended in chemotherapy against other parasitic diseases [6].

Recently, Bellera et al published promising results of two repurposed candidates, benidipine and clofazimine, for the treatment of Chagas disease [7]. Benidipine is a calcium channel-blocking agent currently used for the treatment of hypertension and angina pectoris [8], and clofazimine is an antibiotic mainly indicated for the treatment of leprosy [9]. They were selected in silico for their predicted activity against cruzipain, the major cysteine protease of *T. cruzi*, and their inhibitory effects were confirmed *in vitro* and *in vivo* in a murine model of acute Chagas infection [7].

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In this work, the efficacy of benidipine and clofazimine was tested in a murine model of chronic Chagas disease infection. C3H/HeN mice infected with a myotropic *T. cruzi* strain were treated at the chronic phase. The parasite load in peripheral blood and heart and skeletal muscle as well as target tissue inflammation were assessed 60 days after the end of treatment.

2. Materials and methods

2.1. Animals and T. cruzi infection

C3H/HeN mice were bred and housed at the animal facilities of the Instituto de Microbiología y Parasitología Médica (IMPaM-UBA CONICET, Buenos Aires, Argentina). Mice were kept under standard conditions on a 12-h light : dark cycle in a temperature-controlled room (25 ± 2 °C) with food and water *ad libitum*. All animal procedures were approved by institutional regulations of the Committee for the Care and Use of Laboratory Animals of the Universidad de Buenos Aires (Buenos Aires, Argentina) in accordance with government regulations. All efforts were made to minimize the number of animals used and their suffering. Six-week-old male mice were infected by the intraperitoneal route with 1×10^5 bloodstream forms of the *T. cruzi* K98 strain [Discrete type Units (DTU) TcI clone of the CA-I isolate obtained from an Argentinean patient with chronic cardiomyopathy] [10].

Clinical evidence of disease was monitored and parasitaemia levels were measured by counting the number of circulating trypomastigotes from blood samples in a Neubauer chamber as described previously [11].

2.2. Pharmacologic treatment

Benidipine hydrochloride, clofazimine and benznidazole were acquired from Sigma-Aldrich (St. Louis, MO). Benidipine and benznidazole were suspended in 1% carboxymethyl cellulose (Sigma-Aldrich) and clofazimine was suspended in corn oil. Three groups of mice ($n = 4$ per group) were treated with either benznidazole 100 mg/kg/day, benidipine 15 mg/kg/day or clofazimine 30 mg/kg/day administered orally by gavage (0.1 mL) once a day during 30 consecutive days, starting 90 days post infection (dpi) with *T. cruzi*. The untreated infected control group ($n = 4$) was administered vehicle alone. Weight was recorded periodically and blood samples were obtained at different time points. Peripheral blood parasitaemia was measured by counting in a Neubauer chamber the number of parasites obtained from the tail vein blood diluted (1/10) in red blood cell lysis solution (0.83% Tris–NH_4Cl, pH 7.2). Sixty days after the end

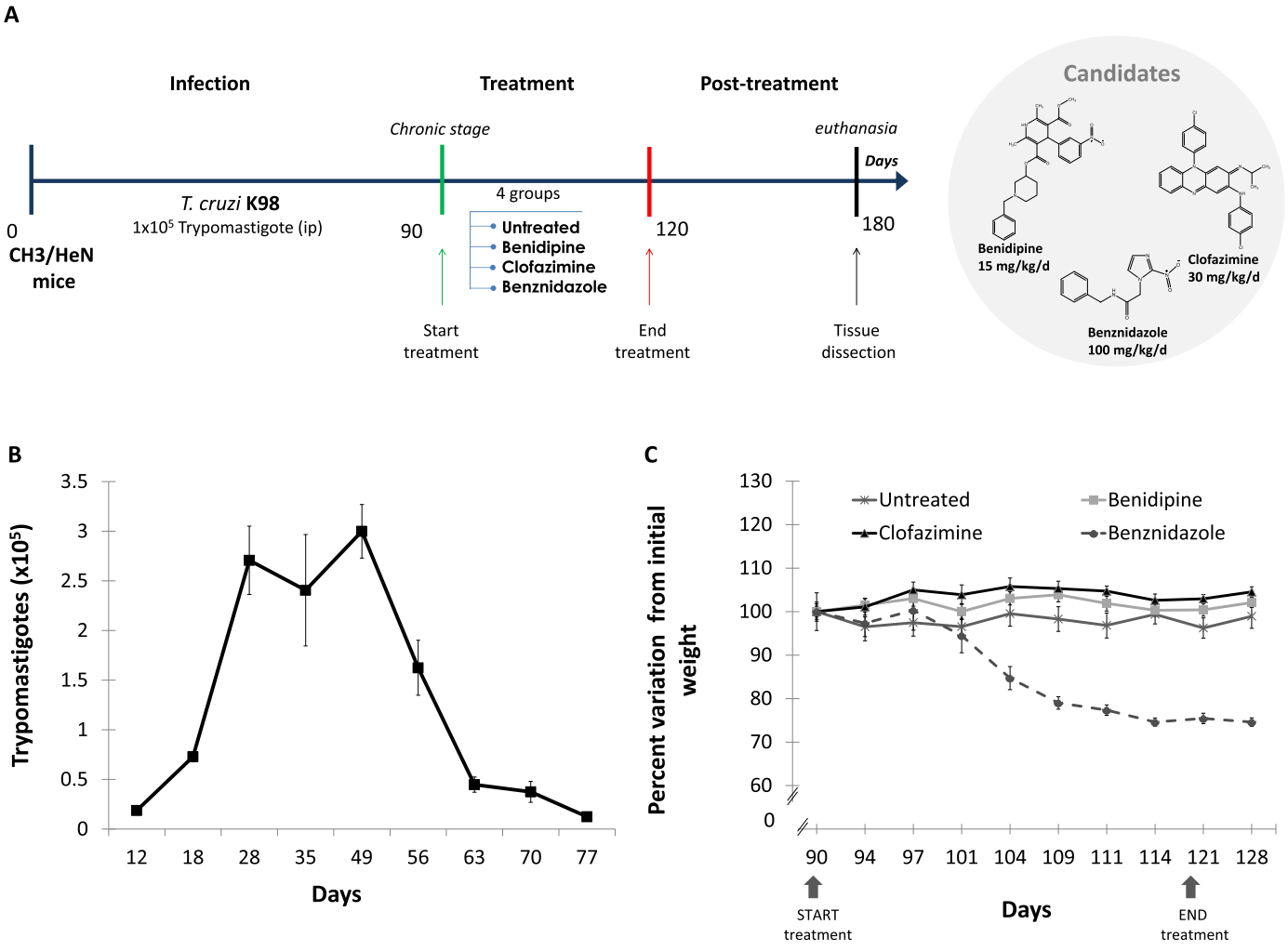

Fig. 1. Acute-phase parasitaemia levels and weight kinetics following treatment of mice chronically infected with *T. cruzi* K98 strain. (A) Experimental design. (B) Number of bloodstream parasites quantified by conventional microscopy as described in Section 2.2. (C) Percent variation from initial weight (90 days post-infection) starting from the first day of treatment until the end of treatment. Results are shown as the mean ± standard error of the mean.

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of treatment, the animals were euthanised and autopsied to collect tissues.

2.3. Immunoassays

Circulating levels of anti-*T. cruzi* immunoglobulin G (IgG) in serum were determined in blood samples collected at 0, 90 and 145 dpi and before euthanasia (180 dpi) by enzyme-linked immunosorbent assay (ELISA) using *T. cruzi* whole epimastigote lysate as described previously [11].

2.4. Quantitation of parasite burden in tissues by quantitative PCR (qPCR)

DNA was extracted from tissue specimens (heart and skeletal muscle) by phenol extraction followed by ethanol precipitation as described previously [12]. DNA was extracted from blood (200 μL) using a QIAamp® DNA Mini Blood Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For the PCR reaction, *T. cruzi* satellite nuclear repeat and single-copy murine-specific tumor necrosis factor-alpha (TNFα) gene were amplified as described previously [12]. The ΔΔCt method was used for relative quantification in tissues of the number of parasite genome-equivalents of treated vs. untreated mice. Data were expressed as the fold decrease ($2^{-\Delta\Delta Ct}$) in parasite genome-equivalents relative to untreated infected controls. For blood samples, a standard *T. cruzi* curve was generated from blood contaminated with 10-fold dilutions of *T. cruzi* K98 strain trypomastigotes. Ct values were extrapolated to obtain the number of parasites per millilitre of blood.

2.5. Histopathological analysis

Heart tissue and skeletal muscle (quadriceps) were collected and were fixed in 10% formalin (Laboratorios Oliveri S.R.L., Quilmes Oeste, Argentina) for histopathological analysis. Paraffin-embedded tissue sections stained with haematoxylin and eosin (Biopur S.R.L., Rosario, Argentina) were evaluated for the presence of tissue damage and parasite nests. Images were obtained with a Nikon ECLIPSE E600 microscope (Nikon Corp., Tokyo, Japan). For the analysis, four images were randomly selected from fields of tissue sections on three slides per animal using Image J software (National Institutes of Health, Bethesda, MD). Diffuse inflammatory infiltrates were quantified as the number of nuclei per area in four randomly selected fields per image, and focal inflammatory infiltrates were quantified as the maximum area occupied per image by the infiltrates. Adipose replacement was quantified by counting the number of adipocytes per image. Fibrosis replacement analysis was performed semiquantitatively on tissue sections stained with Masson's trichrome stain (Biopack S.R.L., Buenos Aires, Argentina).

3. Results

3.1. Treatment of chronically T. cruzi-infected mice with the cruzipain inhibitors benidipine and clofazimine

The myotropic *T. cruzi* strain K98 is moderately virulent during acute infection and causes severe myositis mainly in skeletal tissues but also in cardiac tissues at the chronic stage [13]. Circulating parasites registered by conventional microscopy at the acute phase of infection fell below the detection level from 77 dpi. Infected mice showed posture and gait abnormalities from 60 dpi. During pharmacologic treatment, no significant variations from their initial weight were observed among benidipine-treated, clofazimine-treated and untreated mice. In contrast, animals treated with benznidazole suffered progressive weight reduction that reached 25% without weight recovery (Fig. 1). This observation is compatible with anorexia and weight loss, reported as common side effects mainly in pediatric patients treated with this drug [14]. Serum *T. cruzi*-specific IgG levels displayed no significant differences among groups at 180 dpi (data not shown). This was expected as in humans conventional serological markers respond very slowly after treatment with benznidazole or nifurtimox [15].

3.2. Cruzipain inhibitors are able to reduce parasite burden in target tissues

By conventional methods, bloodstream forms are undetectable and amastigote nests are seldom observed in cardiac and skeletal tissues from chronically K98-infected mice [10]. Therefore, we estimated parasite levels in tissue and blood by qPCR at 180 dpi, 2 months after the end of treatment. Both cruzipain inhibitors were superior to benznidazole at reducing parasite levels in cardiac muscle (Fig. 2A). Benidipine also displayed a superior capacity to reduce

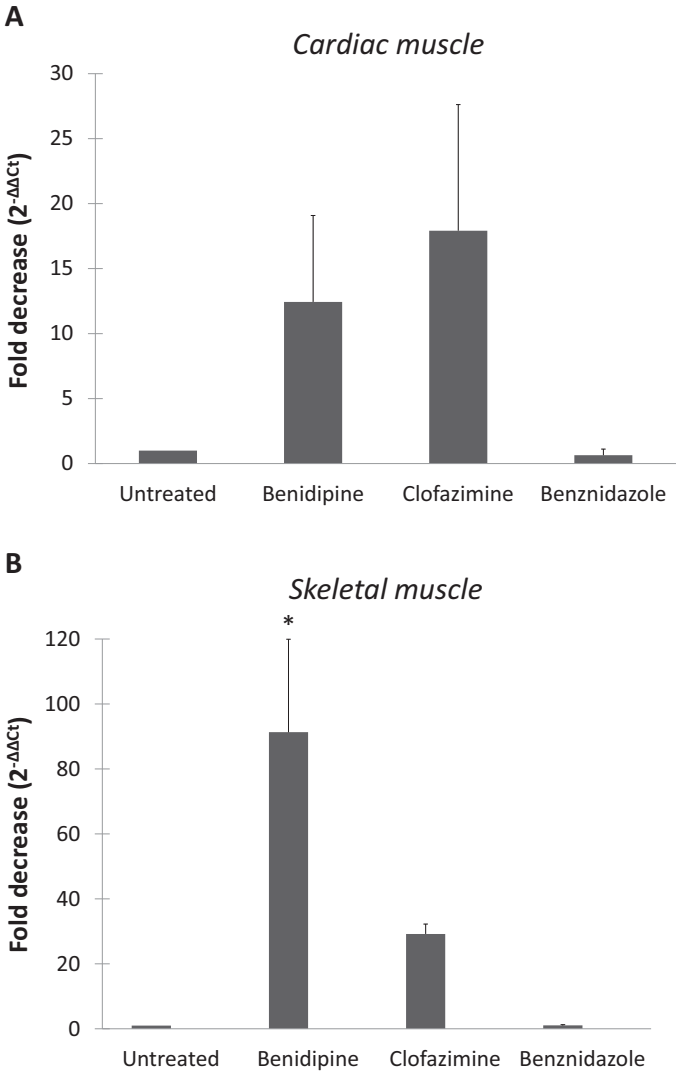
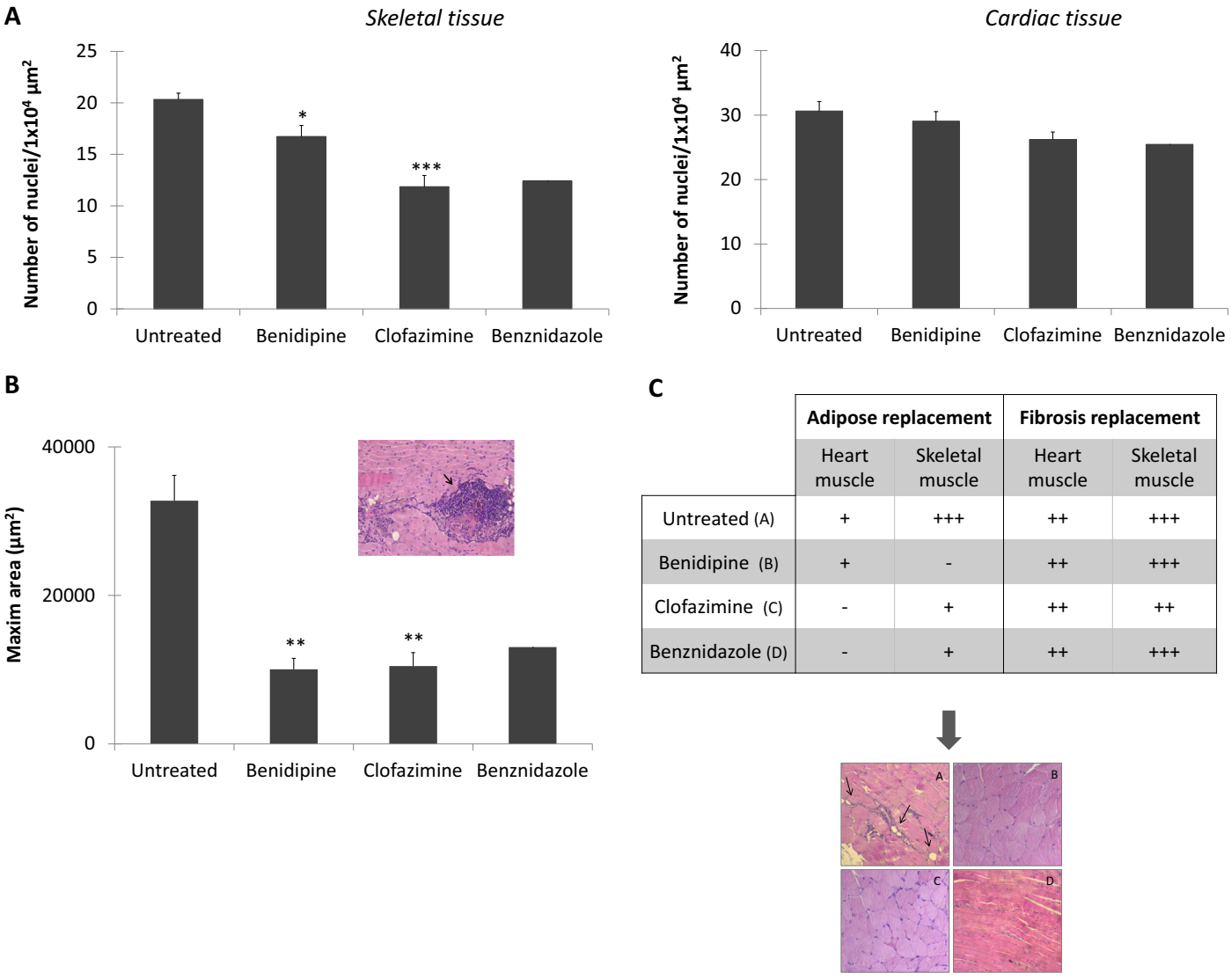


Fig. 2. Quantification of parasite burden in tissues by quantitative PCR (qPCR). Parasite burden in (A) cardiac muscle and (B) skeletal muscle of *Trypanosoma cruzi*-infected mice treated with benidipine, clofazimine or benznidazole by oral gavage for 30 days starting at 90 days' post-infection (dpi) and untreated controls (1% carboxymethyl cellulose). Tissue parasite burden was determined by qPCR at 180 dpi as described in Section 2.4. *T. cruzi* satellite sequences were not detected in heart and muscle tissue DNAs from uninfected controls. The mean Ct value for the tumor necrosis factor-alpha (TNFα) gene was 22.27 ± 0.19. Results are expressed as fold decreases ($2^{-\Delta\Delta Ct}$). Data show the mean ± standard error of the mean ($n = 3$). * $P < 0.05$ vs. untreated (Student's *t*-test).



	Adipose replacement		Fibrosis replacement	
	Heart muscle	Skeletal muscle	Heart muscle	Skeletal muscle
Untreated (A)	+	+++	++	+++
Benidipine (B)	+	-	++	+++
Clofazimine (C)	-	+	++	++
Benznidazole (D)	-	+	++	+++

Fig. 3. Inflammatory changes in skeletal muscle and cardiac tissue of chronically infected treated and untreated mice. (A) Diffuse inflammation index of skeletal and cardiac muscles calculated as the number of nuclei per 1×10^4 µm^2. (B) Focal inflammation calculated in skeletal muscle as the maximum area occupied by mononuclear cells around vessels in each image. The black arrow shows a perivascular inflammatory focus. (C) Adipose replacement was assessed by counting the number of adipocytes per image using the following score: +, <4 adipocytes per image; ++, 4–9 adipocytes per image; and +++, >9 adipocytes per image. Fibrosis in muscle was assessed by microscope observation: +, low; ++, medium; or +++, high fibrosis replacement. Kruskal–Wallis test and Dunn's post-test were used. A P-value of <0.05 was considered significant. Results are expressed as the mean ± standard error of the mean. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ vs. untreated.

parasite levels in skeletal muscle ($P < 0.05$) when administered to chronically infected mice (Fig. 2B). Regarding peripheral blood parasite levels, no significant differences were observed between groups. Parasite genome was consistently detected at 180 dpi by specific qPCR in all groups (mean 16.3 ± 3.7 trypomastigotes/mL of blood).

3.3. Effect of cruzipain inhibitors on Chagas disease immunopathology in target tissues

Chronic human infection with *T. cruzi* triggers sustained inflammatory events that lead to functional alterations in cardiac muscles and conductive fibres and, finally, heart remodelling [16]. We evaluated the inflammation processes in target tissues, observing major differences in skeletal muscles that are more severely targeted by the K98 strain (Fig. 3) [10]. Skeletal tissue from infected untreated animals showed diffuse and focal mononuclear infiltrates, fibrosis and adipose replacement. Animals treated with benidipine, clofazimine and benznidazole showed a significant reduction in the relative number of diffuse infiltrates in this muscle (18%, 42% and 38%, respectively) compared with untreated mice (Fig. 3A). No differences were found in cardiac tissue. Focal inflammation appears predominantly around blood vessels in skeletal muscle but is seldom found in cardiac tissue from chronically infected mice. Benidipine, clofazimine and benznidazole reduced the extension of inflammatory foci in skeletal tissues by 67%, 68% and 60%, respectively (Fig. 3B). Moreover, extensive destruction of muscle fibres is followed by fibrosis and adipose replacement. Treated animals reduced their adipose replacement scores in contrast to untreated animals (Fig. 3C). The effect of treatment on the process of fibrosis in target tissues was less clear. The qualitative fibrosis analysis revealed no significant differences among groups. At 180 dpi, no parasite nests were detected in the microscopic examination of tissue sections from treated and untreated mice, a feature already described for this parasite strain by Solana et al [10].

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4. Discussion

Treatment of chronic infection with *T. cruzi* is challenging as parasites are virtually restricted to selected tissues where the intracellular forms are found at very low levels. Here we demonstrate that repositioned drugs achieved a reduction in parasite burden in cardiac and skeletal tissue of chronic chagasic mice treated with benidipine and clofazimine compared with untreated mice. The ability of the cruzipain inhibitors to control parasite load in tissues was superior to that of the reference drug benznidazole. This feature probably reflects a superior accessibility of benidipine and clofazimine to tissues where parasites are restricted (e.g. the lipophilic clofazimine reaches and accumulates in fat tissue, a reservoir of parasites at the chronic stage).

The mechanisms responsible for cardiomyopathy are not completely understood, but myocardial tissues from chronic chagasic cardiomyopathy patients show inflammation and fibre injury with a scarce number of amastigote nests [17]. The disappointing results of the BENEFIT trial taught us that the parasiticidal activity of a drug is not sufficient to reverse or even arrest the clinical symptoms and signs of chronic Chagas cardiomyopathy [4]. An ideal drug against Chagas disease should protect tissues from injury caused by chronic infection. Chronically infected mice treated with the novel cruzipain inhibitors reduced quantitative and qualitative parameters of inflammation compared with untreated mice. This was significant in skeletal muscle, which is the most compromised in our model. Reduction of tissue damage can be attributed to the parasiticidal properties of the cruzipain inhibitors. Nonetheless, the anti-inflammatory/immunosuppressive properties of clofazimine have already been reported [9,18] and recent data have suggested the secondary cardioprotective properties of benidipine related to its antioxidant and antiapoptotic nature [19]. It is important to note that both repurposed drugs ameliorate the clinical effects of Chagas disease; however they do not necessarily result in sterile cure.

In summary, we have expanded the study of two repurposed drug candidates in a chronic model of Chagas disease. The results presented here reveal their capacity to reduce the parasite load in target tissues and the inflammatory effects of chronic infection in tissues. These aspects make them promising drugs against the disease. As expected for these repurposed drugs largely used in clinical settings, the general condition of mice during and after treatment show that the drugs are well tolerated, a convenient feature considering the rate of adverse effects with benznidazole and nifurtimox. Taking into account that the latter are prodrugs activated within *T. cruzi* by the same mitochondrial nitroreductase [20], the different mechanisms of action of cruzipain inhibitors make them suitable candidates to use in combination with currently used drugs for Chagas disease. Further studies should be performed to test the synergism with benznidazole and nifurtimox in view of combined therapies.

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Competing interests: None declared.

Ethical approval: All animal procedures were approved by institutional regulations of the Committee for the Care and Use of Laboratory Animals of the Universidad de Buenos Aires (Buenos Aires, Argentina) [approval No. 1874/2014] in accordance with government regulations [SENASA, resolution No. RS617/2002, Argentina].

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