Trypanosoma cruzi Arequipa: a tool for Chagas disease drug discovery

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Abstract

Trypanosoma cruzi, the causative agent of Chagas disease, is a genuine parasite with a tremendous genetic diversity and a complex life cycle. Scientists have studied this disease for more than 100 years, and Chagas disease drug discovery has been a mainstay due to the absence of an effective treatment. Technical advances in several areas have contributed to a better understanding of the complex biology and life cycle of this parasite with the aim of designing the ideal profile of both drug and therapeutic options to treat CD. Here, we present *T. cruzi* Arequipa strain (MHOM/Pe/2011/Arequipa) as an interesting tool for CD drug discovery. We have characterized acute-phase parasitaemia and chronic-phase tropism in BALB/c mice, and we have determined the *in vitro* and *in vivo* benznidazole resistance profile of the different morphological forms of this strain. The tropism of this strain makes it an interesting tool for the screening of new compounds with a potential anti-Chagas profile for the treatment of this disease.

Trypanosoma cruzi **Arequipa: a tool for Chagas disease drug discovery**

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Summary

Trypanosoma cruzi , the causative agent of Chagas disease, is a genuine parasite with a tremendous genetic diversity and a complex life cycle. Scientists have studied this disease for more than 100 years, and Chagas disease drug discovery has been a mainstay due to the absence of an effective treatment. Technical advances in several areas have contributed to a better understanding of the complex biology and life cycle of this parasite with the aim of designing the ideal profile of both drug and therapeutic options to treat CD. Here, we present *T. cruzi* Arequipa strain (MHOM/Pe/2011/Arequipa) as an interesting tool for CD drug discovery. We have characterized acute-phase parasitaemia and chronic-phase tropism in BALB/c mice, and we have determined the*in vitro* and *in vivo* benznidazole resistance profile of the different morphological forms of this strain. The tropism of this strain makes it an interesting tool for the screening of new compounds with a potential anti-Chagas profile for the treatment of this disease.

Keywords: Arequipa strain, Benznidazole, Chagas disease, Chemotherapy, Drug discovery, *Trypanosoma cruzi* .

1. Introduction

Chagas disease (CD) or American Trypanosomiasis is a life-long and life-threatening disease caused by infection with the protozoan parasite *Trypanosoma cruzi* . According to the World Health Organization, CD is classified as a neglected tropical disease, the most important parasitic disease in America and, after AIDS and tuberculosis, the third most spread infectious disease in this region. In addition, CD is the most prevalent of poverty-caused and poverty-promoting disease in Latin America countries (Rassi Jr *et al.* , 2010; Urbina, 2010; Bern, 2015; DNDi. Drugs for Neglected Diseases Initiative, 2018; Lidani *et al.* , 2019).

Although CD was limited for many decades to Latin America as a silent and silenced disease, CD has become more widespread due to the increase mobility and migration, with large number of infected individuals, particularly in Europe, and recorded outbreaks in Australia, New Zealand and Japan (Pérez-Molina *et al.* , 2012; Jackson *et al.* , 2014; Strasen *et al.* , 2014; Lidani *et al.* , 2019). CD is currently a global health problema, with 6-8 million infected people, 14-50 thounsand deaths annually, and 70-100 million people ar risk of infection worldwide (Belaunzarán, 2015; PAHO. Pan American Health Organization, 2018; CDC. Centers for Disease Control and Prevention, 2019; Lidani *et al.* , 2019; Martín-Escolano *et al.* , 2020a; WHO. World Health Organization, 2021).

Despite many efforts, the long-term nature and the complex pathology of CD have resulted in a lack of effective treatments and vaccines. Approved treatments are limited to two nitroheterocyclic drugs, benznidazole (BNZ) and nifurtimox, developed more than 50 years ago, and lead to serious drawbacks. The aim is to find a specific treatment that allows the eradication of the parasite and, hence, the elimination of the signs and symptoms of CD. The development of new drugs, safer, more effective, that provide a shorter treatment course, and to devise paediatric formulations, preferably oral, is an important need (Hotez *et al.* , 2008; Bern, 2015; Morillo *et al.* , 2017; Aldasoro *et al.* , 2018). In this way, the urgency of further research to discover new therapeutic alternatives and tools for CD drug discovery is justified.

CD is far from innocuous and, in mammal hosts, it is an obligate intracellular parasite for replication (Tyler and Engman, 2001; Kessler *et al.* , 2017; Martín-Escolano *et al.* , 2020a). During the initial acute-phase, parasites can be detected in the bloodstream and they become widely disseminated in tissues and organs. Later, CD progresses to a long-lasting asymptomatic chronic-phase, which is characterized by an extremely low parasite burden. Around 30% of infected people will advance to a symptomatic chronic-phase, which is characterized by cardiomyopathy and organomegaly, outcomes for which there are few therapeutic options (Ribeiro *et al.* , 2012; Cunha-Neto and Chevillard, 2014; Morillo *et al.* , 2017). It is interesting to note that the outcome of the infection in a particular individual is the result of a set of complex interactions among the host genetic background and the genetic composition of the parasite, and modulated by environmental and social factors; all of which can be complicated by mixed infections and re-infections (Campbell *et al.* , 2004).

The genetic diversity of *T. cruzi* is extensively known. Currently, *T. cruzi* is divided into seven DTUs, showing different genotypes and phenotypes, evolutionary relationships, ecological and epidemiological associations, pathogenesis, tropism and drug resistance (Zingales, 2017; Martín-Escolano *et al.* , 2020a). Besides the well-known limitations of current treatments, other drawbacks are the natural resistance of the *T. cruzi* genotype to the drugs used (Mejia *et al.* , 2012) and the cross-resistance (Wilkinson *et al.* , 2008; Mejia *et al.* , 2012). Here, we present *T. cruzi* Arequipa strain (MHOM/Pe/2011/Arequipa, isolated from a human from Arequipa, Peru) as an interesting tool for CD drug discovery. We have determined the *in vitro* BNZ resistance profile of the different morphological forms of *T. cruzi* Arequipa, we have characterized acute-phase parasitaemia and chronic-phase tropism in BALB/c mice, and we have determined the *in vitro* BNZ resistance profile of *T. cruzi* Arequipa in both acute and chronic phases of CD. The tropism of this strain makes it an interesting tool for CD drug discovery.

2. Material and Methods

2.1. Cultivation of *T. cruzi* Arequipa

2.1.1. Epimastigote forms

Epimastigote forms were cultured at 28 ºC in Gibco RPMI 1640 medium supplemented with 10% (*v* /*v*) heat-inactivated fetal bovine serum (hiFBS), 0.03 M hemin and 0.5% (*w* /*v*) BBL trypticase (Kendall *et al.* , 1990).

2.1.2. Transformation to metacyclic forms

Metacyclic trypomastigotes were induced by culturing a 7-day-old culture of epimastigote forms at 28 °C in Gibco Grace's Insect Medium supplemented with 10% (v/v) hiFBS (Isola *et al.* , 1986). Subsequently, parasites were incubated at a density of 5 × 10^8 mL^{-1} for 2 h at 28 degC in TAU medium (190 mM NaCl, 17 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 8 mM phosphate buffer, pH 6.0), and later at a density of 5 x 10^6 mL^{-1} for 4 days at 28 degC in TAU3AAG medium (TAU supplemented with 50 mM L-sodium glutamate, 2 mM L-sodium aspartate, 10 mM L-proline, 10 mM D-glucose) (Cardoso and Soares, 2010).

2.1.3. Amastigote and trypomastigote forms

Metacyclic trypomastigotes were used to infect Vero cells at 37o C in humidified 95 % air, 5 % CO_2 atmosphere in Gibco RPMI 1640 medium supplemented with 10% (*v* /*v*) hiFBS (Contreras*et al.* , 1985; Pless-Petig *et al.* , 2012). After 5-7 days of incubation, culture-derived trypomastigotes were collected every 24 h by centrifuging the supernatant at 3000g for 5 min, and used to infect new Vero cells cultures and BALB/c mice.

2.2. *In vitro* BNZ activity assays

2.2.1. BNZ activity against extracellular epimastigote forms

BNZ activity against epimastigote forms was determined using the method described by Rolon et al. (Rolon *et al.* , 2006) with some modifications. Briefly, 5 x 10^5 epimastigotes*mL^{-1} were treated by adding BNZ at a concentration range from 100 to 0.4 µM (200 µL·$well^{-1}$ volumes) in 96-well plates at 28 ºC for 72 h. Untreated growth controls were also included. 20 µL of resazurin sodium salt (0.125 mg·mL^{-1}) (Sigma-Aldrich) was added, and the plates were incubated for further 24 h. Finally, 5 µL of sodium dodecyl sulfate (10 % w/v) was added, and the absorbance was measured at 570 and 600 nm. The trypanocidal activity was expressed as the IC_{50} and IC_{90} (inhibitory concentrations 50 and 90) using GraphPad Prism 6 software. Each BNZ concentration was tested in triplicate in four separate determinations.

2.2.2. BNZ activity against intracellular amastigote forms

BNZ activity against amastigote forms was performed by seeding 1 × 10^4 Vero cells·$well^{-1}$ in 24-well plates with rounded coverslips at 37º C in humidified 95 % air, 5 % CO_2 atmosphere in Gibco RPMI 1640 medium supplemented with 10% (*v* /*v*) hiFBS. After 24h of incubation, Vero cells were infected with culture-derived trypomastigotes at a multiplicity of infection (MOI) ratio of 10:1. After 24 h of infection, nonphagocyted trypomastigotes were washed, and infected Vero cells were treated by adding BNZ at a concentration range from 100 to 0.4 µM (500 µL·$well^{-1}$ volumes) at 37º C in humidified 95 % air, 5 % CO_2 atmosphere for 72 h. Untreated growth controls were also included. Counting of amastigotes and infected cells was performed in methanol-fixed and Giemsa-stained preparations by random analysis of 500 host cells (Martín-Escolano *et al.* , 2020b). The infectivity index, defined as the average number of amastigote forms in infected cells multiplied by the percentage of infected cells, was also determined. The trypanocidal activity was expressed as the IC_{50} and IC_{90} (inhibitory concentrations 50 and 90) using GraphPad Prism 6 software. Each BNZ concentration was tested in triplicate in four separate determinations.

2.2.3. BNZ activity against extracellular trypomastigote forms

BNZ activity against culture-derived trypomastigote forms was performed by seeding 2 ×10^6 trypomastigotes·mL^{-1}, and after the addition of BNZ at a concentration range from 100 to 0.4 µM (200 µL·$well^{-1}$ volumes) in 96-well plates at 37º C in humidified 95 % air, 5 % CO_2 atmosphere for 24 h.

Untreated controls were also included. 20 µL of resazurin sodium salt (0.125 mg·mL^{-1}) (Sigma-Aldrich) was added, and the plates were incubated for further 4 h (Martín-Escolano *et al.* , 2019). Finally, trypanocidal activity was determined following the same procedure as described to assess the trypanocidal activity against epimastigote forms. Each compound concentration was tested in triplicate in four separate determinations.

Since the half-life of culture-derived trypomastigotes (without host cells) was determined to be 48 h, the assay was restricted to 24 h of treatment.

2.3. Characterisation of CD infection in a murine model

Female BALB/c mice – 10 weeks old and 20-25 g – were infected by intraperitoneal inoculation of 5.0×10^5 Bloodstream trypomastigotes (BTs) in 0.2 mL PBS. These parasites were obtained from previously infected mice.

2.3.1. Ethics statement

Mice were maintained under standard conditions – 12/12 h light-darkness cycle and 22 ± 3 °C temperature – with access to food and water ad libitum. Mice were handled by trained researchers in accordance with ethical considerations, and experiments were performed under the rules and principles of the international guide for biomedical research in experimental animals.

2.3.2. Characterisation of the acute CD

5 infected mice were used to characterise the acute CD, obtaining peripheral blood from the mandibular vein. 5 µL blood was obtained every 2 days from the 3rd day post-infection (dpi) until the day parasitaemia was not detected for 10 consecutive days. Blood was diluted 1:100 in PBS, and the parasitaemia was quantified using a Neubauer chamber (Martín-Escolano *et al.* , 2018) (**Scheme 1**).

2.3.3. Characterisation of the chronic CD

15 infected mice were used to characterise the chronic CD in a time-dependent manner, sacrificing 5 mice by cervical dislocation for each experimental time: 20th, 30th, and 60th dpi. 14 organs/tissues were harvested to evaluate the tropism: adipose tissue, bone marrow, brain, oesophagus, heart, kidney, large intestine, liver, lung, mesenteric tissue, muscle, small intestine, spleen and stomach (**Scheme 1**). Hearts and spleens were also weighed to evaluate splenomegaly and cardiomegaly. These organs/tissues were immediately flushed free of blood by infusion of pre-warmed PBS to avoid contamination with BTs, and they were then thawed and ground up using a Potter-Elvehjem. Organs/tissues DNA was extracted using Wizard Genomic DNA Purification Kit (Promega) (Martín-Escolano *et al.* , 2018). PCR was based on the Spliced Leader (SL) intergenic region sequence of *T. cruzi* (for detailed description, see Paucar et al. (Paucar *et al.* , 2019). Finally, the PCR products were visualised by UV illumination after a 2 % agarose gel electrophoresis for 90 min at 90 V, containing 1:10000 GelRed nucleic acid gel stain.

2.4. *In vivo* BNZ activity assays

2.4.1. Mouse infection and treatment

Infection was carried out by intraperitoneal inoculation as previously described. Treatment was performed by oral route once daily for 5 consecutive days once a) infection was confirmed (9thdpi) for mice treated in the acute-phase and b) it was established that the mice had entered the chronic-phase (75th dpi) for mice treated in the chronic-phase (**Scheme 2**).

Mice were divided into three groups (n = 5 per group): 0, negative control group (uninfected and untreated mice); I, positive control group (infected and untreated mice); II, BNZ group (infected and treated mice). BNZ was prepared at 2 mg·mL^{-1} in an aqueous suspension vehicle containing 5% (v/v) DMSO and 0.5% (w/v) hydroxypropyl methylcellulose (Francisco *et al.* , 2016). Doses of 20 mg·kg^{-1}day^{-1}(˜200 µL) were administered for 5 consecutive days in group II, and vehicle only was administered in groups 0 and I.

2.4.2. Parasitaemia counting

Parasitaemia counting from mice treated in the acute-phase was performed every 2 days from peripheral blood as stated above (Martín-Escolano*et al.* , 2018) (**Scheme 2**).

2.4.3. Immunosuppression and parasitaemia reactivation

After 100th dpi, mice with significantly decreased parasitaemia and established to be in the chronic-phase of the experiment, regardless of the treatment and undetectable by blood microscopy examination, were treated with cyclophosphamide (CP) (ISOPAC) every 4 days by i.p. injection with a dose of 200 mg·kg^{-1} for a maximum of three doses (Francisco*et al.* , 2016) (**Scheme 2**). Within 1 week after the last CP injection, parasitaemia was evaluated as stated above (**Scheme 2**).

2.4.4. Euthanasia and PCR analysis

Mice were bled out under gaseous anaesthesia (CO2) via heart puncture. Target organs/tissues were harvested, immediately flushed free of blood by infusion of pre-warmed PBS to avoid contamination with BTs, and they were then thawed and ground up using a Potter-Elvehjem. Organs/tissues DNA extraction and PCR was performed as stated above (Martin-Escolano*et al.* , 2018; Paucar *et al.* , 2019) (**Scheme 2**). Finally, the PCR products were visualised by UV illumination after a 2 % agarose gel electrophoresis for 90 min at 90 V, containing 1:10000 GelRed nucleic acid gel stain.

In addition, spleens and hearts were weighed to evaluate inflammation of this organ in the different groups of mice.

3. Results

3.1. Cell cultures and morphological forms

The different morphological forms of *T. cruzi* Arequipa were obtained from epimastigote forms cultures following the transformation and infection protocols of Vero cells *in vitro* and BALB/c mice*in vivo* . Representative images of each of the morphological forms are shown in **Figure 1** .

3.2. *In vitro* BNZ resistance profile

Inhibitory concentrations 50 and 90 (IC_{50} and IC_{90}, respectively) are shown in **Figure 2** . BNZ showed higher activity against the parasite forms relevant to human infection (intracellular amastigotes and trypomastigotes). The infection rate, the average number of amastigote forms per Vero cell and the infectivity index were also determined (**Figure 3A-3B**), giving and idea of the killing rate. It is widely known that the parasitic cure as soon as possible after infection can prevent parasite reproliferation and serious disease (Rao *et al.* , 2019). This topic has been notably supported, especially after the failure of the lastest candidates – posaconazole and ravuconazole – in clinical trials (Molina *et al.* , 2014; Martin-Escolano *et al.* , 2020a). BNZ is a fast-acting and cidal drug, and that is why here we observed a significant reduction in the infectivity index to practically zero at 50 µM.

3.3. Natural course of CD infection

First, the course of infection was monitored during the acute-phase (**Figure 4A**). Parasitaemia was detected from the 9th dpi, it showed the highest levels between the 22nd and 24th dpi, reaching more than 5 million BTs per mL of blood, and it was undetectable from the 45th dpi in all infected mice. Therefore, it can be established that the acute-phase ends on this day (Rassi Jr *et al.* , 2010; Pérez-molina and Molina, 2018).

However, parasites were detected at 20st dpi in 6 out of the 14 analysed organs/tissues, and 9 out of them were infected at the end of the acute-phase (40th dpi). The same organs/tissues were infected at 60th dpi.**Figure 4B** shows the PCR analysis of 14 organs/tissues for each experimental time. Therefore, this assay reveals the tropism of *T. cruzi* Arequipa for 9 target organs/tissues: adipose tissue, bone marrow, brain, oesophagus, heart, lung, muscle, spleen and stomach. Analysis of murine model has identified the gastrointestinal tract as a primary reservoir using different *T. cruzi* strains (Lewis*et al.* , 2014, 2016; Lewis and Kelly, 2016). Hence, the intestine may also be parasitized in some areas along its length using *T. cruzi* Arequipa, but we did not observe parasitization in the selected fragments for PCR. Other reason would be that the parasite load to

be below the PCR limit detection (˜1 parasite per 10 mg) (Francisco *et al.* , 2015), although three rounds of PCR were performed for all tissues/organs.

It should be noted that the chronic-phase begins when the amastigote forms are nested inside target organs/tissues (Guarner *et al.* , 2001; Rassi Jr *et al.* , 2010; Pérez-molina and Molina, 2018). Here, amastigote forms were already found nesting in 6 out of the 9 target organs/tissues at 20^{th} dpi; hence, the chronic-phase begins when parasitaemia has not yet peaked. It should be highlighted that the chronic-phase is characterized by a low and intermittent parasitaemia (Bilate and Cunha-Neto, 2008; Rassi Jr*et al.* , 2010; Pérez-molina and Molina, 2018), but not detectable by counting BTs. In addition, sophisticated parasite-detection bioluminiscence technologies are leading to a better appreciation of the spatiotemporal and quantitative dynamics of chronic infections (Lewis*et al.* , 2014, 2016, 2018), limited using the PCR method. In summary, acute-phase treatment should be performed once the parasitaemia is confirmed, that is, ˜9^{th} dpi; and parasitaemia should be monitored until ˜55^{th} dpi, when parasitaemia is not detected. For chronic-phase treatment, it should be performed from 70^{th} dpi, when this phase is established. Thereafter, assessment of cure should be confirmed by PCR of the 9 target organs/tissues in late chronic-phase.

3.4. *In vivo* BNZ resistance profile

First, parasitaemia profiles were monitored during the acute-phase (**Figure 5A**). Significant differences were observed between untreated and BNZ-treated mice, showing a remarkable parasitaemia reduction even at subcurative BNZ doses. Second, the experimental cure was determined using a double checking widely used in *in vivo*trypanocidal assays: immunosuppression and PCR (**Figure 5A-B**) (Santos *et al.* , 2010; Francisco *et al.* , 2016; Martín-Escolano *et al.* , 2020c). Mice whose parasitaemia and PCR remain negative after immunosuppression (enhancing the reactivation of any residual infection) are considered cured.

Finally, organomegaly of spleens and hearts was measured (**Figure 6**). They are manifested in both the acute- and chronic-phases of CD, allow us to characterise the pathogenesis of the *T. cruzi*Arequipa strain, and also allow us an indirect evaluation of the BNZ efficacy since they are directly associated with the parasitic load (Martín-Escolano *et al.* , 2018, 2019, 2020d). Splenomegaly and cardiomegaly occurred in experimentally infected mice in both phases of CD, during which the spleens of infected mice were approximately twice the mass of those from uninfected mice. The hearts also suffered a significant enlargement. Importantly, treatment with BNZ reduced infection-induced splenomegaly and cardiomegaly, even at subcurative doses and in the absence of a parasitological cure, since it is linked to a reduction of the parasite load (Francisco *et al.* , 2015).

4. Discussion

It is widely known that, besides the well-known limitations of current treatments, other drawbacks are the natural resistance of the *T. cruzi* genotype to the drugs used and the cross-resistance of current drugs (Wilkinson *et al.* , 2008; Mejia *et al.* , 2012). Here, the *in vitro* resistance of the three morphological forms of*T. cruzi* Arequipa to the reference drug BNZ was characterized. *T. cruzi* Arequipa shows moderate resistance to BNZ compared to other strains used for CD drug discovery. It is well known that resistance to BNZ is strain-dependent, with IC_{50} values ranging from 1 µM to over 200 µM (Vela *et al.* , 2021). Therefore, ignoring the parasite's genetic variability during CD drug discovery is not advisable.

Regarding *T. cruzi* infection, it should be noted that it is dependent on the genetic composition of the infecting *T. cruzi*strain (Toledo *et al.* , 2004; Santos *et al.* , 2010; Rodriguez *et al.* , 2014) and the genetic background of the animal model used as a host (Caldas *et al.* , 2008), that is, the host-parasite interactions (Campbell *et al.* , 2004). Hence, it is really important to know the infective capacity of the strains used for CD drug discovery, that is, virulence (quantitative) and tropism (qualitative) in BALB/c mice, before *in vivo* assays (Rodriguez*et al.* , 2014). *T. cruzi* is able to parasitize a large variety of cells (de Souza *et al.* , 2010; Rodriguez *et al.* , 2014; Lewis and Kelly, 2016; Lewis *et al.* , 2018) and its tissue homing ability has been reported to be strain-specific (Tibayrenc and Telleria, 2010). Here, the acute-phase parasitaemia and the chronic-phase tropism of *T. cruzi* Arequipa were evaluated in BALB/c mice in order to use this strain as a suitable tool for CD drug discovery.

It should be noted that most *in vivo* chemotherapy has focused on acute-phase infections, partially because it is simpler to monitor the course of parasitaemia (Canavaci *et al.* , 2010; Romanha *et al.* , 2010; Buckner, 2011; Chatelain and Konar, 2015). However, the ability to cure chronic-phase infections is the main need from a clinical viewpoint. Effectiveness of current drugs is especially limited during the chronic-phase of CD (Wilkinson *et al.* , 2008; Chatelain, 2014; Scarim *et al.* , 2018), so chronic-phase infections should be the main research focus in animal models (DNDi. Drugs for Neglected Diseases Initiative, 2018). Notably, this strain does not cause mortality, which is ultimately useful for comparing the*in vivo* trypanocidal efficacy of potential compounds and the reference drug BNZ in late-chronic phase.

T. cruzi Arequipa shows an attractive tropism for the evaluation of potential new compounds for the treatment of CD. *T. cruzi*Arequipa shows tropism towards: 1) brain, which allows us to evaluate the ability of potential compounds to cross the blood-brain barrier; 2) adipose tissue, which allows us to evaluate the solubility of compounds in lipid rich environments; and 3) heart, which allows us to use this strain as a model for cardiac CD. It has to be highlighted that these organs/tissues are the ones that have maintained the infection most after treatment using current potential candidates for the treatment of CD (including BNZ), showing nested parasites in them (Ferreira *et al.* , 2011; Tanowitz *et al.* , 2016); lesser drug accessibility or parasite susceptibility in these environments could be the reasons for the lower efficacy (Nagajyothi *et al.* , 2013; Tanowitz *et al.* , 2016). It has been postulated that inappropriate pharmacokinetics/pharmacodynamics between current drugs and tissue location of parasites is linked to the inability to reliably cure chronic infections (Urbina, 2002; Perin *et al.* , 2017).

Currently, drugs against CD present variable activity in the acute- and chronic-phases of the disease, and the effectiveness of treatments, especially during the chronic-phase, is not as effective as they should be (Martín-Escolano *et al.* , 2020a). For these reason, the*in vivo* resistance of *T. cruzi* Arequipa in both acute- and chronic-phase was evaluated. The treatment strategy using BALB/c mice was as follows: 1) Drugs were administered orally because it is the preferred route for the treatment of parasitic diseases in developing countries, it leads to better patient compliance, and it has a low cost (Espuelas *et al.* , 2012; DNDi. Drugs for Neglected Diseases Initiative, 2018); 2) Given that a compound showing a reasonable parasitaemia reduction following 5 days of treatment can be defined as a lead compound (Chatelain, 2014), the treatment guideline was for 5 consecutive days in order to evaluate the resistance of *T. cruzi*Arequipa to oral BNZ treatment. Moreover, to evaluate if potential compounds (for further research) show higher trypanocidal activity than BNZ in the first *in vivo* screening phase (Romanha *et al.* , 2010), the treatment was at subcurative doses of BNZ (20 $mg{\cdot}kg^{-1}$ per day). As expected, mice treated in the chronic-phase showed lower infection levels than those treated in the acute-phase. It is widely known that BNZ is more effective in chronic phase, probably because the parasite burden is significantly low and limited to far few locations (Francisco *et al.* , 2016).

5. Conclusions

CD is still considered a global health problem with significant epidemiological and socioeconomic implications. In recent years, technical advances in several areas have contributed to a better understanding of the biology and life cycle of this parasite, which will make it possible to design the ideal profile of both drugs and therapeutic options for treating CD. Here, we present *T. cruzi*Arequipa strain as an interesting tool for drug discovery against CD. For *in vitro* assays, several different strains should be used for the evaluation of the spectrum of action of potential compounds. However, for *in vivo* assays, and in order to fulfil the principles of the 3Rs (Replacement, Reduction and Refinement) in animal research, *T. cruzi* Arequipa can be the strain of choice. The tropism of this strain makes it ideal for the evaluation of potential compounds with the aim of selecting those with the best anti-Chagas profile.

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Conflicts of Interest

The authors declare no conflict of interest.

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Scheme 1. Timeline of *in vivo* characterisation of Chagas disease on BALB/c mice infected with 5.0x10^{5}bloodstream trypomastigotes of *T. cruzi* Arequipa strain. dpi, days post-infection.

Scheme 2. Timeline of BNZ activity assays on BALB/c mice infected with 5.0x10^5 bloodstream trypomastigotes of*T. cruzi* Arequipa strain. *Oral treatment at 20 mg*kg-1 per day for 5 consecutive days; dpi, days post-infection.

Figure 1. Representative images of the different morphologic forms of *Trypanosoma cruzi* Arequipa stained with Giemsa. A, Epimastigote forms; B, Metacyclic trypomastigote forms; C, Vero cells infected by intracellular amastigote forms; D, Culture-derived trypomastigote forms infecting Vero cells; E, Mouse peripheral blood infected by bloodstream trypomastigotes (1:100 dilution in PBS); F, Mouse peripheral blood infected by bloodstream trypomastigotes (blood smear); G, Mouse peripheral blood infected by bloodstream trypomastigotes (after enrichment buffer for trypomastigote forms).

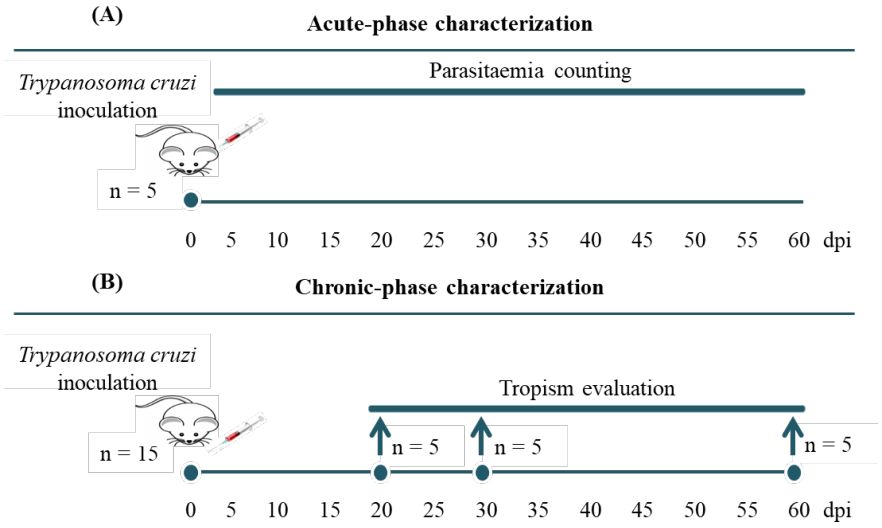
Figure 2. Dose-response curves and inhibitory concentrations (IC) 50 and 90 of benznidazole against the three morphological forms of*T. cruzi* Arequipa using GraphPad Prism 6 software.

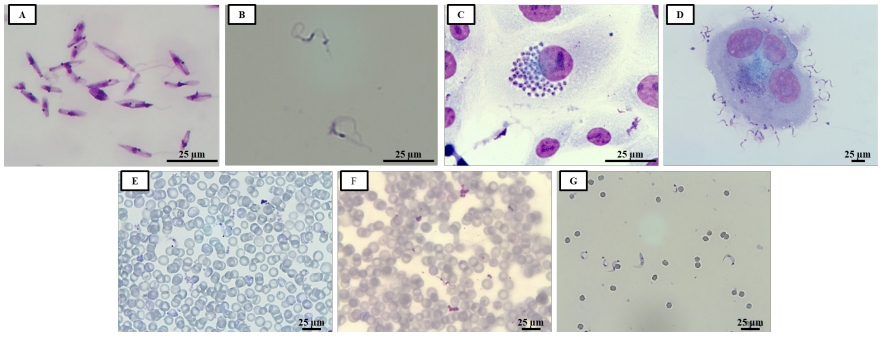
Figure 3. (A) Percentage of infected Vero cells and average number of intracellular amastigote forms of *Trypanosoma cruzi*Arequipa per Vero cell of benznidazole-treated infected cultures. (B) Infectivity index (average number of amastigote forms in infected cells multiplied by the percentage of infected cells) of benznidazole-treated infected cultures. (C) Representative images of Vero cells infected, benznidazole-treated and Giemsa stained.

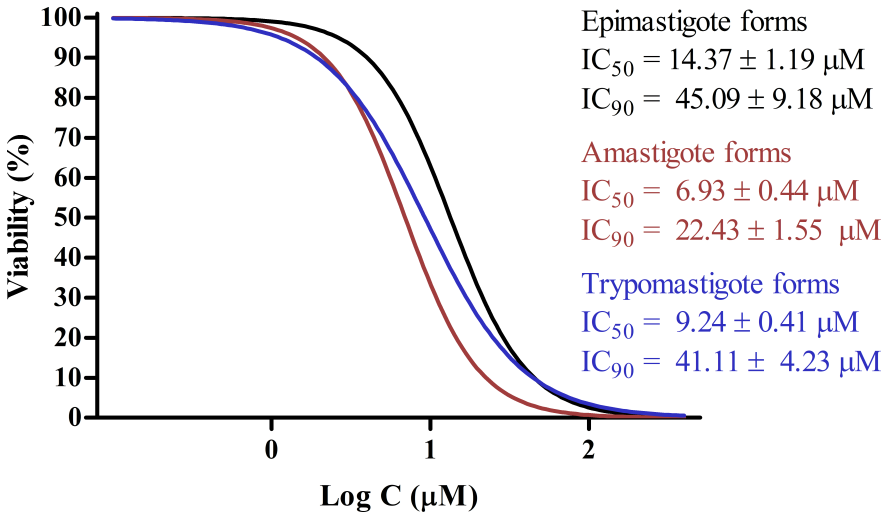
Figure 4. (A) Acute-phase parasitaemia (blue) and tropism (red) in BALB/c mice after intraperitoneal inoculation of $5x10^5$ *T. cruzi* Arequipa bloodstream trypomastigotes. Values constitute means of 5 mice +- standard deviation. Tropism bars represent the percentage of organs/tissues with nested amastigote forms with respect to the 9 target organs/tissues. (B) Tropism evaluation by PCR analyses of 14 potential target organs/tissues with the Spliced Leader (SL) intergenic region sequence of *T. cruzi* . Table show the positive mice for each organ/tissue; n = 5 for each experimental time.

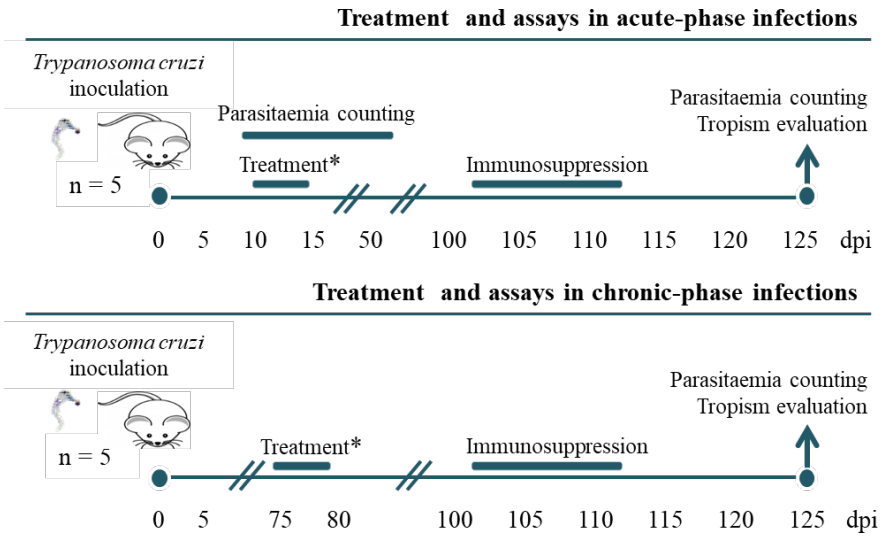
Figure 5. (A) Parasitaemia profiles of untreated and benznidazole(BNZ)-treated mice during the acute-phase of Chagas disease over 60 days, and parasitaemia reactivation after immunosuppression of untreated and BNZ-treated mice during the acute- and chronic-phase of Chagas diseae. Treatment days are represented in grey. Values are the means of five mice +- standard deviation. (B) Tropism evaluation by PCR analyses of the target organs/tissues with the Spliced Leader (SL) intergenic region sequence of *T. cruzi* of untreated mice and benznidazole(BNZ)-treated mice during both the acute- and chronic-phase of Chagas disease, Lanes: (M), base pair marker; (-), PCR negative control; (+), PCR positive control; (1-9), target organs/tissues: (1), adipose; (2), bone marrow; (3), brain; (4), esophagus; (5), heart; (6), lung; (7), muscle; (8), spleen; (9), stomach. * 4/5 of the corresponding organs/tissue PCR products showed 300 bp band on electrophotesis; 3/5 of the corresponding organs/tissue PCR products showed 300 bp band on electrophotesis.

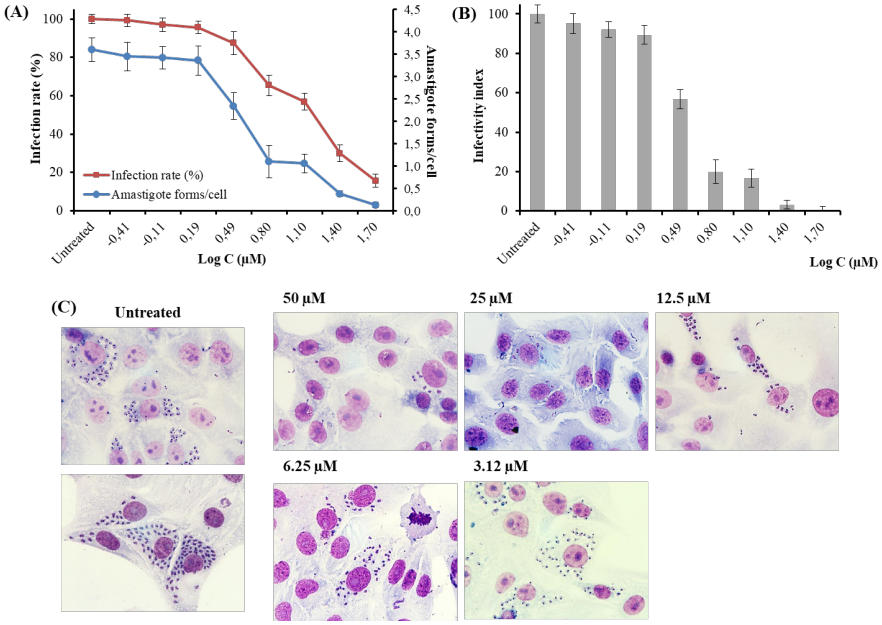
Figure 6. Organomegaly of spleens and hearts of uninfected, untreated and BNZ-treated mice at the euthanasia day. Values are the means of five mice +- standard deviation.

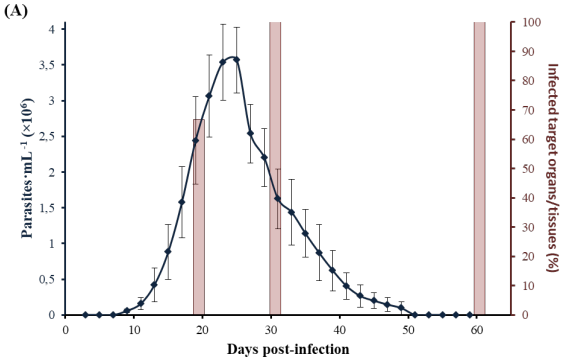












(B)

Organs/tissues	Days post-infection		
	20	30	60
Adipose tissue	2/5	3/5	4/5
Bone marrow	3/5	4/5	5/5
Brain	2/5	4/5	4/5
Oesophagus	0/5	3/5	4/5
Heart	4/5	5/5	5/5
Kidney	0/5	0/5	0/5
Large intestine	0/5	0/5	0/5
Liver	0/5	0/5	0/5
Lung	3/5	4/5	4/5
Mesenteric tissue	0/5	0/5	0/5
Muscle	3/5	5/5	5/5
Small intestine	0/5	0/5	0/5
Spleen	0/5	4/5	5/5
Stomach	0/5	3/5	4/5