The effect of benznidazole dose among the efficacy outcome in the murine animal model. A quantitative integration of the literature

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ABSTRACT

Despite more than 100 years since it was firstly described Chagas disease, only two drugs are available to treat Chagas disease: Nifurtimox launched by Bayer in 1965 and benznidazole launched by Roche in 1971.

Drug discovery initiatives have been looking for new compounds as an alternative to these old drugs. Although new platforms have been used with the latest technologies, a critical step on that process still relies on the in vivo model.

Unfortunately, to date, available animal models have limited predictive value and there is no standardization. With the aim to better understand the role of benznidazole, the current standard of care of Chagas disease, we performed this review. We intend to analyze the influence of the experimental design of the most used animal model, the murine model, in the assessment of the efficacy endpoint.

1. Introduction

Chagas disease remains one of the biggest public-health problems in Latin America and a challenge for clinical practitioners and basic researchers. An estimated 7 million people are infected with T. cruzi worldwide and it causes more than 7000 deaths per year as well as life-long morbidity and disability without early and successful antiparasitic treatment (Pérez-Molina and Molina, 2018).

At the present, much is known about Chagas disease, but much more remains to be known. One of the main obstacles the scientific community has to face is the complexity of the parasite (Panunzi and Agüero, 2014).

That fact not only elicits an insufficient understanding of the pathogenesis and immunology of T. cruzi infection, but also hampers the drug discovery process.

Despite more than 100 years since it was firstly described, only two drugs are available to treat Chagas disease: Nifurtimox launched by Bayer in 1965 and benznidazole launched by Roche in 1971 (Rodriques Coura and de Castro, 2002). New compounds have been evaluated to seek an alternative to these old nitroheterocyclic compounds. According to the results obtained in the in vitro or the in vivo models, drugs evaluated had to have shown a good therapeutic response in patients with Chagas disease. Unfortunately, none of the compounds tested in clinical trials so far has overcome in efficacy to benznidazole (Molina et al., 2014; Torrico, 2013). And according to the current pipeline, it seems that in the coming years the therapeutic arsenal that we can offer to our patients will be basically the same as 40 years ago.

Drug discovery process has been enriched with new technologies which brings an optimistic future into the quest of new compounds. However, between the in vitro process and the clinical proof of concept, a critical step relies on the in vivo model (Chatelain, 2015).

Unfortunately, to date, available animal models have limited predictive value and there is no standardization. In order to harmonize the assessment of any compound as a potential hit against Chagas disease, in 2010, a consensus document was created and coordinated by the Fiocruz Program for Research and Technological Development on
Effect on trypomastigotes and intracellular amastigotes

- **Parasite reduction**
  - \( \beta \)-gt TS: \( \beta \)-galactosidase-transfected Tulahuen strain
  - \( \beta \)-galactosidase-transfected Tulahuen strain
  - \( \beta \)-galactosidase-transfected Tulahuen strain

- **Selectivity index (SI)**
  - IC50 of cell culture parasites (\( \beta \)-gt TS)
  - IC50 of mammalian cell line

- **Acute toxicity**
  - Maximum tolerated dose in Swiss mice: one male and one female

- **Parasitaemia reduction in the acute phase**
  - **Animal model:** female Swiss mice/\( Y \) strain; \( n = 6 \)
  - **Dose response:** ≤ MTD (three doses)
  - **Reference drug:** Bz 100 mg/kg/day
  - **Treatment:** starting at parasitaemia onset (5 dpi), for five consecutive days
  - **Evaluation:** parasitaemia (5, 8 and 10 dpi) and mortality (30 dpi)

- **Activity**
  - >BNZ <BNZ

- **Cure in the acute phase I**
  - **Animal model:** female Swiss mice/\( Y \) strain; \( n = 10 \)
  - **Dose:** defined in previous step
  - **Reference drug:** Bz 100 mg/kg/day
  - **Treatment:** starting at parasitaemia onset (5 dpi) for 20 consecutive days
  - **Evaluation:** parasitaemia (5, 8 and 10 dpi), mortality (30 dpi), PCR (after immunosuppression)

- **Activity**
  - >BNZ <BNZ

- **Cure in the acute phase II**
  - **Animal model:** (female Swiss mice/Colombian strain; \( n = 10 \))
  - **Dose:** defined in \( \text{In vivo I} \)
  - **Reference drug:** Bz 100 mg/kg/day
  - **Treatment:** starting at parasitaemia onset (7/8 dpi), for 20 consecutive days
  - **Evaluation:** parasitaemia (20, 25 and 30 dpi), mortality (30 dpi), PCR (after immunosuppression)

- **Activity**
  - >BNZ <BNZ

**Fig. 1.** Flow Chart designed as a general and standardized protocol for drug screening applied to chemotherapy for Chagas disease adapted from (Romanha et al., 2010).

\( \beta \)-gt TS: \( \beta \)-galactosidase-transfected Tulahuen strain; dpi: days post infection; dpt: days post treatment; PCR: Protein Chain Reaction; MTD: maximum tolerated dose.
Chagas disease and the Drugs for Neglected Diseases Initiative (Romanha et al., 2010) (Fig. 1). Notwithstanding, according to the experiments published afterwards, we can affirm that it does not exist a homogenization in the design of the animal assays.

It is mandatory to elucidate data from animal model and mainly to be aware of its inherent limitations. The performance of experimental models is essential as the first step before reaching clinical trials in humans but, should be interpreted and evaluated with caution.

Therefore, with the aim to better understand the role of benznida-zole, the current standard of care of Chagas disease, we performed this review. We intend to analyze the influence of the experimental design of the most used animal model, the murine model, in the assessment of the efficacy endpoint.

2. Material and methods

A quantitative integration study of primary data from different indi-vidual studies was performed. A systematic review through MEDLINE (1985–2017), EMBASE (1985–2017), BIREME (1985–2017), LILACS (1985–2004), SCIELO (1985–2004) was conducted, using the following terms and keywords (with no language restriction): benznidazole, treatment, animal model, murine model, terms and keywords (with no language restriction): benznidazole, treatment, animal model, murine model, Trypanosoma cruzi. The last research was conducted on June 2017. For eligibility, studies were required to meet the following criteria: (a) Those which used murine model (mice), (b) at least one of the groups were treated with benznida-zole, treatment, animal model, murine model, Trypanosoma cruzi. The following data was retrieved for each study: type of infection (acute or chronic), type of mice, strain used, inoculums, dose of benznidazole and days of treatment, days post infection when treatment was started, cure criteria.

The primary outcome was the cure ratio. According to the heterogeneity of criteria used, we defined three levels of cure accuracy: minimum accuracy, where the cure was assessed by parasitemia detection through Fresh Blood Examination (FBE), (d) all data according methodology are reported. Assays using exclusively T. cruzi clones or strains isolated from patients were excluded for the analysis. Articles quest was performed by triplicate. After eliminating those duplicated, data were extracted.

The following data was retrieved for each study: type of infection (acute or chronic), type of mice, T. cruzi strain used, inoculums, dose of benznidazole and days of treatment, days post infection when treatment was started, cure criteria.

The primary outcome was the cure ratio. According to the heterogeneity of criteria used, we defined three levels of cure accuracy: minimum accuracy, where the cure was assessed by parasitemia detection through Fresh Blood Examination (FBE) plus serology or blood/tissue culture; medium accuracy, where besides the above, molecular biology techniques were used; maximum accuracy, where besides the above, an immunosuppression course with cyclophosphamide was administered previous the performance of molecular biology techniques.

3. Statistical analysis

Categorical data are presented as absolute numbers and propor-tions, and continuous variables are expressed as medians and inter-quartile range (IQR) or means and standard deviation (SD) if normal distribution was demonstrated (normal distribution of continuous variables was evaluated through the Kolmogorov-Smirnov test). Inter-group differences for continuous parameters were assessed by Student’s t-test if they presented a normal distribution or ANOVA with Bonferroni correction for multiple comparisons, and Mann-Whitney U test if they did not present a normal distribution. For categorical variables, general characteristics of the sample were assessed by percentages (chi-square test). Results were considered statistically significant if the 2-tailed P value was < 0.05.

We used a logistic regression model using robust estimate of var-iance, in order to relax the assumption of independence between the observations, defining each experiment as a cluster. The $F$ statistic was calculated. It describes the percentage of variation across studies that is due to heterogeneity rather than chance $F = 100\% \times (Q-df)/Q$, where $Q$ is the chi-squared statistic and df is its degrees of freedom (Higgins and Thompson, 2002). $F$ is an intuitive and simple expression of the in-consistency of studies’ results. Analyses were conducted with Stata software, version 13 (STATA Corp).

4. Results

A total of 126 articles were identified. Forty-one fulfilled the inclusion criteria (Fig. 2). In 29 of them, the design of the experimental assay was based in the acute model exclusively and in 5 out of them was based in the chronic model. In seven articles, the experiment was de-signed taking into account both, acute and chronic model (see Tables 3 and 4).

The most commonly used models was the Swiss Webster Outbred mice, followed by the BALB/c and the C57BL/6. By far, the most utilized T. cruzi strain was Y strain, followed by CL Brener (which is actually a clone derived from the original CL strain), Colombiana and marginally VL10, Brazil and Tulahuen. The trypomastigote inoculums varied significantly according to type of model; in the acute model the inoculum was 5000 parasite forms (IQR 10000-1000) and 1000 parasite forms (IQR 1000-30) in the chronic model ($p < 0.001$). Note that the inoculums also depend on the strain (see Table 1).

From the 90s, molecular biology techniques were incorporated as a method to assess cure in 24 out 35 studies with an acute model experiment published. For chronic model experiments, in 5 out of 12.

Regarding to the recommendations about the methodology to be used in the drug discovery process published in 2010, were followed in 13 out 36 articles with an acute model design and in 4 out 12 articles with a chronic model design, published beyond 2011.

Analyzing the overall effect of the dose on the efficacy outcome (defined according the criteria of each experiment), it exists a direct effect between either the daily dose (mg/kg) of benznidazole or the accumulated dose (calculated through the daily dose for the days of treatment) with the probability of cure regardless the T. cruzi strain used. In the acute murine model, an increase of ten mg in the daily dose or in the accumulated dose of benznidazole increases in 1.28 points (CI 1.06–1.54) or 1 point (CI 1.00–1.01) respectively, the probability of cure (Table 2 and Fig. 3). That positive effect is higher when the Y strain is utilized or the level of cure requirement is high. In the chronic model although the effect of the daily dose is still positive (OR: 1.02, CI 0.90–1.16), is less evident than in the acute one.

In the multivariate analyses, apart from the susceptible T. cruzi strains (sensible strain: OR 14.86 and partially resistant OR: 4.99), only the daily dose of benznidazole showed a significant relationship with cure (OR: 1.34, CI 1.12–1.6). More data on Table 2.

5. Discussion

The current therapeutic regimen of benznidazole was empirically introduced at the end of the decade of seventies based on clinical ob-servational studies (Cerisola, 1977; Coura et al., 1978). Because of fewer adverse events and equal parasite suppression, the lower dose tested (5–7.5 mg/kg/day) has been chosen and used till nowadays.

Despite the relevant advances and drug discovery efforts, new for-mulations have failed to demonstrate superiority compared to benznidazole (Molina et al., 2014; Torrico, 2013; Morillo, 2019). Moreover, and according to the pipeline and preliminary results of experimental drugs, there is not going to be any new drug at a commercial level in the forthcoming years. Therefore, all the current efforts are focused on evaluating dose-optimization regimens (Molina).

In this new scenario, where benznidazole dosage is being rethought, the focus should be shifted towards basic research and especially animal models. For several (mainly practical) reasons, the most widely used animal model is the murine. Albeit that the murine model concentrates more results related to the treatment with benznidazole, it has some controversial aspects. One of the main constraints we had to face in our revision, were the important heterogeneity between the experiment design, where less than 50% followed the recommendations published in 2010 (Romanha et al., 2010).
It is important to note that among all variables analyzed, the dose, either diary dose or accumulative dose, was the one who had the major effect over the efficacy outcome. That is to say: the greater the dose or the drug exposure, the greater the probability of cure.

That hypothesis could be plausible taking into account its mechanism of action. Although it remains unclear, it seems that the nitrogenized free radicals produced by the action of trypanosomatid nitroreductases and not detoxified by the parasite's redox enzymatic system may cause direct damage over key structures of host and the parasite (Hall and Wilkinson, 2012).

In parallel, novel time-to-kill assays have highlighted the effect of drug concentration among efficacy. These assays are designed in order to determine the pharmacodynamics compound concentration versus the total time of exposure needed to achieve efficacy. These assays have revealed that nitroheterocyclic compounds are concentration-dependent trypanocidal drugs and therefore more efficacious at higher doses (Moraes et al., 2014).

On the other hand, it could be said that increasing the dose could be risky since the toxicity of the drug could also be increased. To date, has not been able to be demonstrated the relationship between adverse reactions and drug blood concentration (Pinazo et al., 2013; Salvador et al., 2015). By the contrary, it seems that could exist a

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**Table 1**

Inoculums used according to the experiment design and *T. cruzi* strain.

| Strain   | Acute model | Chronic Model | p       |
|----------|-------------|---------------|---------|
| Y        | 5000 (5000–10000) | 30 (30–1000) | < 0.001 |
| CL       | 5000 (1000–10000) | 1000 (30–10000) | < 0.001 |
| Colombiana | 10000 (5000–10000) | 1000 (30–10000) | 0.82    |
| VL 10    | 5000 (5000–100000) | 5000 (5000–5000) | P=0.01  |
| Tulahuen | 1000 (50–1000)  | 1000 (1000–10000) |        |
| Brazil   | 1000 (1000–10000) | 1000 (1000–1000) | 0.08    |
|          | P < 0.001    | P < 0.001     |         |

Numbers are expressed in parasite number and range.
Table 2

| EFFECT OF BENZNIDAZOLE DOSE IN THE CURE RATIO TAKING INTO ACCOUNT THE EXPERIMENT DESIGN. COMBINED UNIVARIATE AND MULTIVARIATE ANALYSES. |
|---------------------------------------------------------------|
| **Total** vs CHRONIC MODEL **Total** | **N = 264 Resistant Strain | **OR** | **CI** |
| **N = 479 Sensible Strain** | | **OR** | **CI** |
| DAILY DOSE vs CURE | accumulate dose vs CURE | 1.28 [0.96-1.66] | 1.26 [0.94-1.69] |
| **ACUTE MODEL** | **MIN** [0.39-4.99] | 1.00 [0.89-1.00] | 1.00 [0.89-1.00] |
| **MEDIUM** [0.39-4.99] | 1.00 [0.89-1.00] | 1.00 [0.89-1.00] |
| **MAXIMUM** [0.39-4.99] | 1.00 [0.89-1.00] | 1.00 [0.89-1.00] |
| **CHRONIC MODEL** | **MIN** [0.39-0.96] | 0.99 [0.98-1.00] | 0.99 [0.98-1.00] |
| **MEDIUM** [0.39-0.96] | 0.99 [0.98-1.00] | 0.99 [0.98-1.00] |
| **MAXIMUM** [0.39-0.96] | 0.99 [0.98-1.00] | 0.99 [0.98-1.00] |

*CI = 95% confidence interval, OR = Odds ratio*
Table 3
Experimental design and assessment of the efficacy endpoint of the murine model treated with Benznidazole in the acute phase of Chagas disease available in the literature.

| Paper | Laboratory mice | Infection | Strain | Inoculums Route | Dose mg/kg/d Days DPI | Efficacy assessment | Results (%) | Survival | Cure |
|-------|----------------|-----------|--------|----------------|-----------------------|---------------------|-------------|----------|------|
| Filardi 1984 (Filardi and Brener, 1984) | Swiss (M) 18-20 g | CL | 10^5 Tryp | 100 | 20 | 1 | Parasitaemia | 100 | 66.6 | 6.6 | 13.3 |
| Araújo 2000 (Araújo et al., 2000) | Swiss (F) 20-24 g | CL | 10^4 Tryp | 25 | 20 | 8-10 | Parasitaemia | 0 | 0 | 9.1 |
| Fournet 2000 (Fournet et al., 2000) | BALB/c (F/M) 6-8 weeks | CL Brener | 5 x 10^3 Tryp | 25 | 30 | 4 | Parasitaemia | 31 | 85.7 | 44.9 | 60 | 0 | 20 |
| Molina 2000 (Molina et al., 2000) | Swiss (F) 18-20 g | CL | 10^4 Tryp | 100 | 28d + 15d | 4 | Parasitaemia | 100 | 80 | 50 |
| Olivieri 2002 (Olivieri et al., 2002) | Swiss (M) 16-20 g | Y | 10^4 Tryp | 62.5 | 14 | 7 | Parasitaemia | 78.5 | — |
| Romanha 2002 (Romanha et al., 2002) | BALB/c C57BL/6 (F) | Y | 5 x 10^5 Tryp | 100 | 20 | 4 | FBE | 100 | 100 |
| Saraiva 2002 (Saraiva et al., 2002) | BALB/c (M) 6 weeks | Y | 10^5 Tryp | 100 | 20 | 1 | Parasitaemia | 100 | 100 |
| Corrales 2005 (Corrales et al., 2005) | Swiss (M) 8 weeks | Tulahuen | 10^3 Tryp | 200 | 30 | 13 | Spleen culture | 95 | 68.4 |
| Ferraz 2007 (Ferraz et al., 2007) | C57BL/6 (M) 8-10 weeks | Y | 5 x 10^5 Tryp | 100 | 20 | 4 | Hemoculture | WT 86 | KOIFN: 0 | KOIL12: 39 |
| Ferraz 2009 (Ferraz et al., 2009) | C57BL/6 (M) 8-10 weeks | Y | 5 x 10^5 Tryp | 100 | 20 | 4 | Hemoculture | WT: 86.2 | KOCD4: 65.5 | KOCD8: 22.2 |
| Batista 2010 (Batista et al., 2010) | Swiss (F/M) 6 | Y | 10^5 Tryp | 100 | 10 | 5 | Parasitaemia | 100 | 0 |

(continued on next page)
| Paper                        | Animal     | Strain | Inoculum Route | Dose mg/kg/d | Days DPI | Efficacy assessment | Survival | Cure  |
|-----------------------------|------------|--------|----------------|--------------|----------|---------------------|----------|-------|
| 8 weeks                     |            |        |                |              |          |                     |          |       |
| Davies 2010 (Davies et al., 2010) | 20 g    | Colombian | 10^2 / 5 × 10^3 Tryp IP | 50 | 60 | Parasitemia onset | 100 | 0    |
| Olivieri 2010 (Olivieri et al., 2010) | —     | Y | 10^4 Tryp IP | 100 | 20 | 4 | Parasitemia | 100 | 50   |
| Batista 2011 (Batista et al., 2011) | 20 g    | Y | 10^4 Tryp IP | 50 | 20 | 5 | Hemoculture | 83.3 | 0    |
| Maximiano 2011 (Maximiano et al., 2011) | 18-23 g | Y | 5 × 10^3 Tryp IP | 100 | 7  | 4 | Hemoculture | 100 | 100  |
| Bahia 2012 (Bahia et al., 2012) | 10 g     | Y | 5 × 10^3 Tryp IP | 100 | 20 | 4 | FBE | 100 | 50   |
| Buckner 2012 (Buckner et al., 2012) | 7 g      | Y | 5 × 10^3 Tryp IP | 100 | 20 | 4 | IS-CFM and PCR | 100 | 0    |
| Cencig 2012 (Cencig et al., 2012) | 18-24 g | Y | 10^3 Tryp IP | 100 | 5  | 10 | IS-CFM | 100 | 100  |
| Da Silva 2012 (da Silva et al., 2013) | 5 g     | Y | 10^4 Tryp IP | 100 | 20 | 5 | Parasitaemia | 100 | 0    |
| Diniz 2013 (Diniz et al., 2013) | 18-24 g | Y | 5 × 10^3 Tryp IP | 100 | 7  | 4 | PCR, Hemoculture | 100 | 0    |
| Soeiro 2013 (Soeiro et al., 2013) | 6 g      | Y | 10^4 Tryp IP | 100 | 20 | 5 | Parasitaemia, IS-CFM | 100 | 0    |
| Strauss 2013 (Strauss et al., 2013) | 20 g    | Y | 5 × 10^3 Tryp IP | 50 | 10 | 20 | PCR, Hemoculture | 100 | 0    |
| Bahia 2014 (Bahia et al., 2014) | 20-24 g | Y | 5 × 10^3 Tryp IP | 25 | 10 | 20 | PCR | 100 | 0    |
| Branquinho 2014 (Branquinho et al., 2014) | 8 g     | CL | 10^4 Tryp IP | 50 | 10 | 1 | Hemoculture | 100 | 100  |
| Bustamante 2014 (Bustamante et al., 2014) | 57BL/6 | Y | 10^3 Tryp IP | 100 | 40 | 15 | Parasitaemia | 100 | 100  |

(continued on next page)
One of the inherent limitations of quantitative integration studies is the heterogeneity of data. Trying to minimize this heterogeneity, a model has been constructed composed of homogenous groups in terms of dose administered, strain, level of cure and model used as shown in Table 2. Parameter $I^2$ indicates the proportion of the variation among studies regarding of the total variation, that is to say the proportion of the total variation that is attributable to the heterogeneity, in our study we obtained moderate to high results that is why we decided to use the robust logistic model.

6. Conclusions

An extra effort in order to standardize a predictive Chagas disease in vivo model need to be done and validated in order to improve its predictability and to ease its comparison and reproducibility.

Dose of benznidazole (diary dose and accumulative dose) is strongly...
Table 4
Experimental design and assessment of the efficacy endpoint of the murine model treated with Benznidazole in the chronic phase of Chagas disease available in the literature.

| Paper | Laboratory mice | Infection | Treatment | Efficacy assessment | Results (%) |
|-------|-----------------|-----------|-----------|--------------------|-------------|
| Author Year | Animal n° Strain Inoculums Route | Dose mg/kg/d Days DPI | | |
| Andrade 1991 (Andrade et al., 1991) | Swiss 18-20 g Colombian | 5 × 10⁴ | 100 60 | 90-157 Subinoculation | 0 |
| Fournet 2000 (Fournet et al., 2000) | BALB/c (F/M) 33 | CL Brener | 10⁴ Tryp IP | 25 30 | 60 Hemoculture Xenodiagnosis Parasitaemia 24 33.3 |
| Molina 2000 (Molina et al., 2000) | Swiss (F) 18-20 g | CL 30 Tryp IP | 100 20 | 120 — ELISA Immunoblotting Parasitaemia 40 0 |
| Molina 2000 bis (Molina et al., 2000) | Swiss (F) 18-20 g 12 | Colombo 30 Tryp IP | 100 20 | 120 Hemoculture Xenodiagnosis Circulating anti T. cruzi IS-CFM 30 0 Parasitaemia — 27.3 |
| Nakayama 2001 (Nakayama et al., 2001) | Balb/c (F/M) 6-8 weeks 10 | CL Brener | 10⁴ Tryp IP | 25 30 | 60 Elisa Parasitaemia 100 30 |
| Siqueira Portella 2009 (Portella and Andrade, 2009) | Swiss (F) 15-20 g 25 | Colombiano | 10⁴ IP | 100 90 | 120 Hemoculture Parasitaemia 35.5 |
| Canavaci 2010 (Canavaci et al., 2010) | Balb/c 34 | 21SF-C3 10³ Tryp IP | 100 40 | 55 IS-CFM Parasitaemia 100 |
| Bahia 2012 (Bahia et al., 2012) | Swiss (F) 10 | VL-10 5 × 10⁴ Tryp IP | 100 20 | 120 IS-CFM Parasitaemia 100 |
| Cencig 2012 (Cencig et al., 2012) | BALB/cJ 7 weeks — Tulahuen | 10³ Tryp IP | 100 10 | 60 Blood culture, PCR IS-CFM — 100 |
| Bustamante 2014 (Bustamante et al., 2014) | C57BL/6 — Y | Colombiano | 10³ Tryp IP | 5 10 5 5 10 5 5 10 5 | Hemoculture, PCR IS-CFM 100 |
| Fortes 2015 (Francisco et al., 2015) | BALB/c 6-12 weeks 5 | CL Brener | 10³ Tryp IP | 100 | 74 Bioluminescence (Ex vivo and In vivo, after IS-CFM) 100 |
| De Mello 2016 (de Mello et al., 2016) | Swiss (F) 20-25 g 10 | Y | 500 Tryp IP | 100 20 | 90 Serology | 80 |
| Francisco 2016 (Francisco et al., 2016) | BALB/c 6-8 weeks 6 | CL Brener | 10³ Tryp IP | 100 20 | 90 Bioluminescence (Ex vivo and In vivo, after IS-CFM) 100 |

(—) Not shown or not reported; (M) Male; (F) Female; (Tryp) Tripomastigote; (DPI) Days post infection; (IP) Intraperitoneal; (FBE) Fresh blood examination; (IS-CFM) Variable ciclophosphamide immunosuppression scheme; (PCR) Protein Chain Reaction.

* 50 mg/kg/day administered bid.
Regardless the \textit{T. cruzi} strain used, exist a positive effect between both the benznidazole dose expressed by daily mg/kg or total accumulated dose (daily mg/kg for the days of treatment). The higher the dose used, the higher the probability of cure.

associated with the efficacy outcome.

In future clinical trials, new regimens with higher dose schemes (daily dose or accumulate dose) could be considered.

Chronic murine model for assessing the efficacy of new anti trypanocidal drugs should be reconsidered.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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