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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

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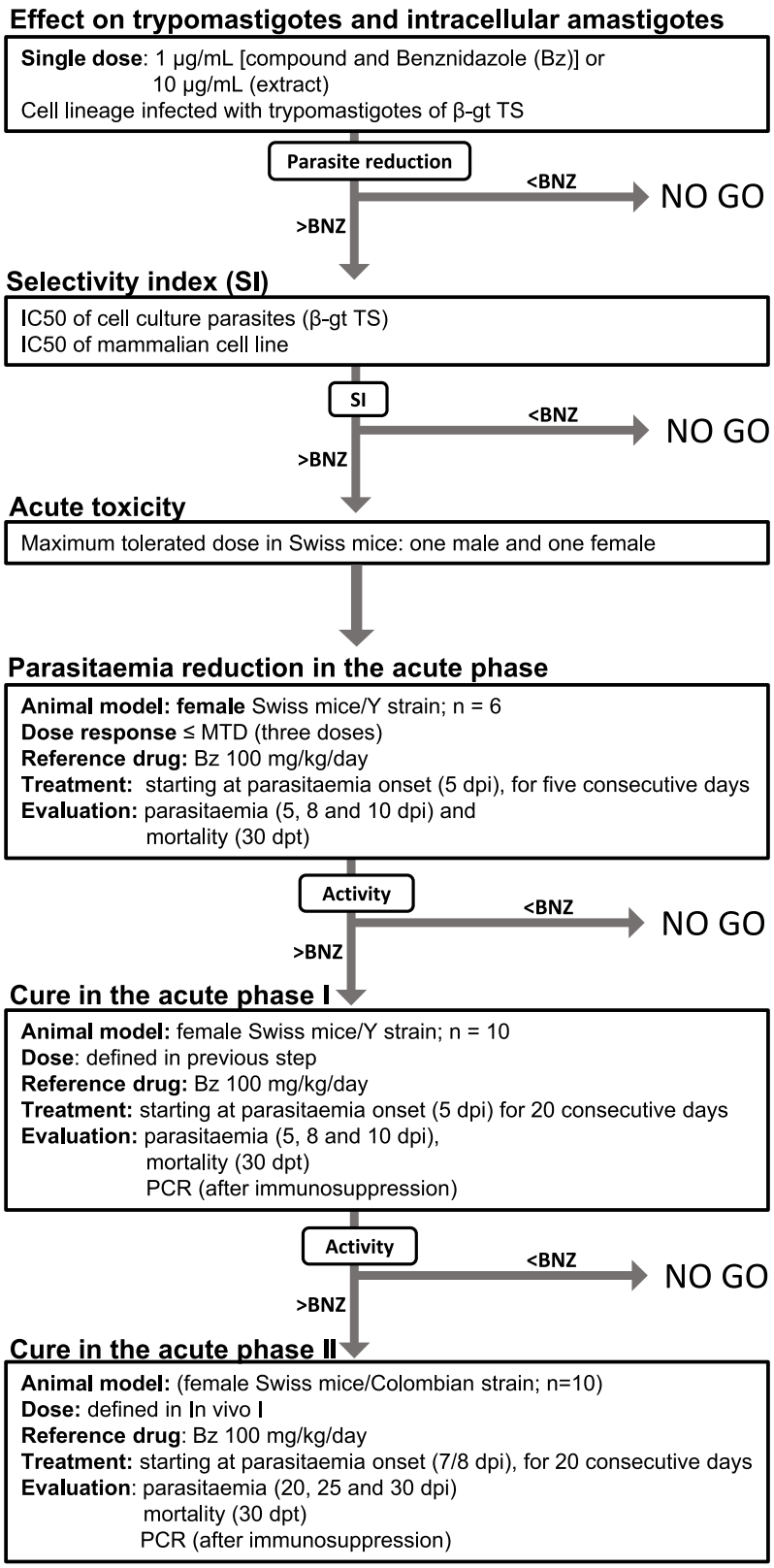

Fig. 1. Flow Chart designed as a general and standardized protocol for drug screening applied to chemotherapy for Chagas disease adapted from (Romanha et al., 2010).

β-gt TS: β-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 exists 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 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.

2. Material and methods

A quantitative integration study of primary data from different individual 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, *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 benznidazole (regardless the dose or duration), (c) efficacy was assed at least with parasitemia detection through Fresh Blood Examination (FBE), (d) all data regarding 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 (FBE) plus serology or blood/tissue culture; medium accuracy, where besides the above, molecular biology techniques were used; maximum accuracy, where besides the above, a immunosuppression course with cyclophosphamide was administered previous the performance of molecular biology techniques.

3. Statistical analysis

Categorical data are presented as absolute numbers and proportions, and continuous variables are expressed as medians and interquartile 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). Intergroup 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 variance, in order to relax the assumption of independence between the observations, defining each experiment as a cluster. The I^2 statistic was calculated. It describes the percentage of variation across studies that is due to heterogeneity rather than chance $I^2 = 100\%$ x (Q-df)/Q. where Q is the chi-squared statistic and df is its degrees of freedom (Higgins and Thompson, 2002). I^2 is an intuitive and simple expression of the inconsistency 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 designed taking into account both, acute and chronic model (see Tables 3 and 4).

The most commonly mouse used 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 observational 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 formulations 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 most 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).

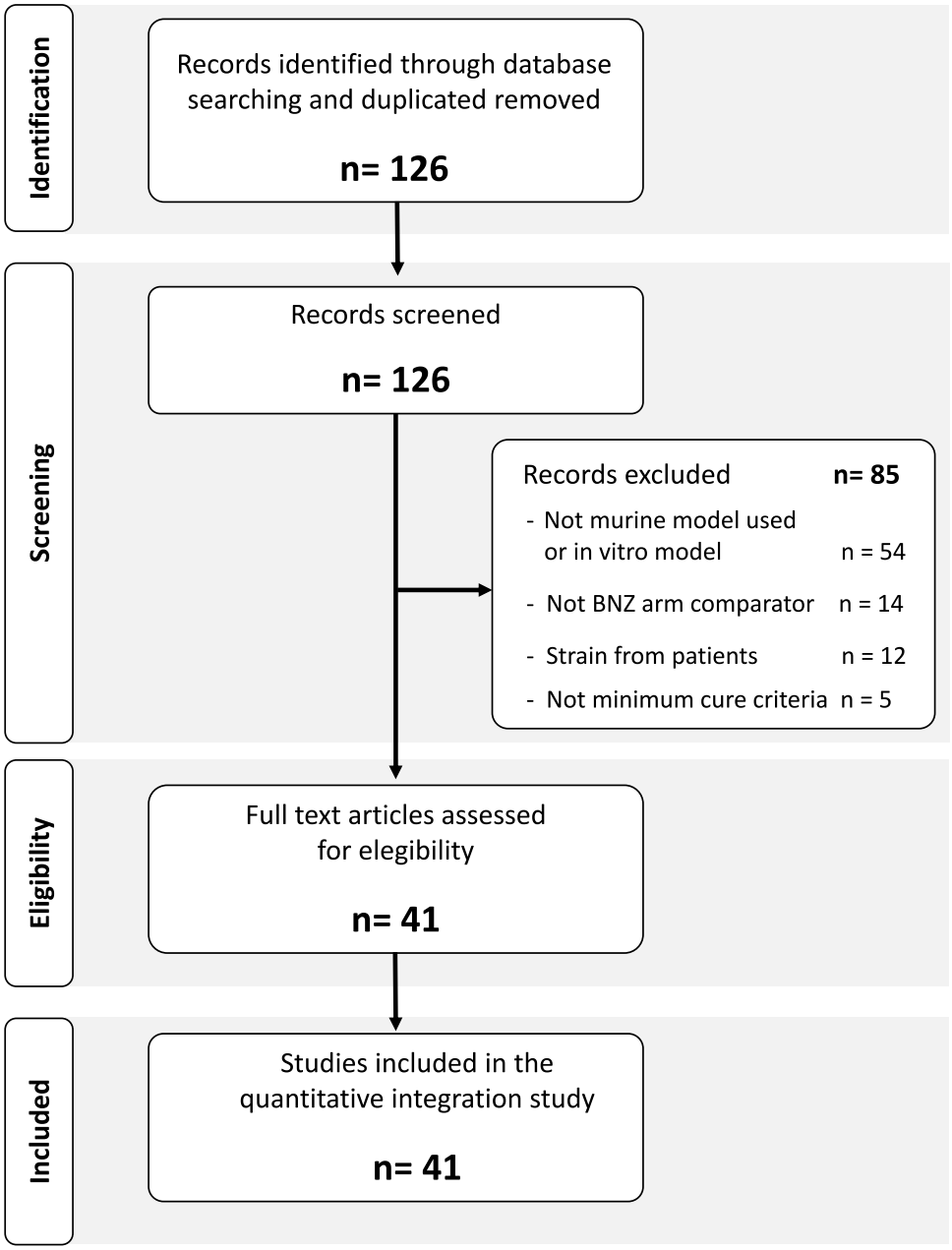

Fig. 2. PRISMA study-selection flowchart for systematic search.

Table 1
Inoculums used according to the experiment design and *T. cruzi* strain.

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

Numbers are expressed in parasite number and range

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

Table 2
Effect of benznidazole dose in to the cure ratio taking into account the experiment design. Combined univariated and multivariated analyses.

		DAILY DOSE vs CURE				ACCUMULATE DOSE vs CURE			
		N = 1178 Overall OR [CI]*	N = 479 Sensible Strain OR [CI]	N = 435 Y strain OR [CI]	N = 264 Resistant Strain OR [CI]	Overall OR [CI]	Sensible Strain OR [CI]	Y strain OR [CI]	Resistant Strain OR [CI]
ACUTE MODEL	**TOTAL**	**1.28 [1.06–1.54]**	1.81 [1.26–2.60]	—		1.00 [1.00–1.01]	1.00 [1.00–1.01]	1.01 [1.00–1.03]	1.01 [1.00–1.01]
	CURE	**MIN**	1.47 [1.05–2.05]	1.75 [1.20–2.55]	—		1.00 [0.99–1.01]	1.03 [1.01–1.04]	1.01 [0.98-1.04]	1.01 [1.00–1.02]
	LEVEL	**MEDIUM**	1.08 [0.93–1.24]	1.02 [0.87–1.19]	1.44 [0.92–2.25]	—	1.00 [0.99–1.00]	1.00 [0.99–1.00]	1.02 [0.99–1.04]	—
		MAXIMUM	1.66 [1.12–2.45]	1.64 [1.27–2.13]	1.95 [1.61–2.36]	—	1.00 [1.00–1.01]	1.01 [0.99–1.02]	1.03 [1.02–1.05]	1.01 [1.00–1.01]
CHRONIC MODEL	**TOTAL**	**1.02 [0.90–1.16]**	1.25 [1.02-1.51]	—	—		0.99 [0.99–1.00]	1.00 [0.99–1.01]	1.00 [0.99–1.01]	1.00 [0.99–1.00]
	CURE	**MIN**	0.86 [0.78–0.95]	0.90 [0.76–1.06]	—	—	0.99 [0.99–1.00]	0.99 [0.98–1.00]	—	—
	LEVEL	**MEDIUM**	—	—	—	—	—	—	—	—
		MAXIMUM	—	—	—	—	0.99 [0.97–1.00]	—	—	—
Predictors.				**Odds Ratio [CI]**	**P-value**					
Dose (mg/Kg)				1.34 [1.12–1.60]	0.001					
Sensible strain				14.86 [4.29–59.41]	0.001					
Y strain				4.99 [1.47-16.89]	0.010					

CI = 95% confidence interval, OR = Odds ratio, — = not possible to calculate.
MIN (minimum cure level accuracy): the cure was assessed by parasitemia detection through (FBE) plus serology or blood/tissue culture
MEDIUM (minimum cure level accuracy): the cure was assessed by parasitemia detection through (FBE) plus serology or blood/tissue culture plus molecular biology techniques
MAXIMUM (maximum cure level accuracy): the cure was assessed by parasitemia detection through (FBE) plus serology or blood/tissue culture plus molecular biology techniques previous an immunosuppressant course with cyclophosphamide.
* :Odds ratio have been calculated based on 10 mg increase of both daily and accumulate dose

genetic background which determine the occurrence of hypersensitivity phenomena (Salvador et al., 2015). The only side effect classically related with drug exposure is polyneuritis; it is possible to observe its appearance when the accumulative dose exceeds 18g, therefore it could be easily prevented (Cancado, 2002). Furthermore, previous experiences with higher daily doses have been recorded (400 mg/day) without an increased ratio of adverse events (Morillo, 2019).

These results might seem controversial, since future clinical trials are based on lower dose regimens of benznidazole. The idea to reduce the daily dose came from two population pharmacokinetics studies (Soy et al., 2015; Altcheh et al., 2014). Both studies reach to the conclusion that benznidazole treatment regimen against Chagas disease in adults might be overdosed.

Concerning the drug exposure, there is also contrary evidence. Recent studies that explore new regimes in mouse model showed that fewer days of treatment (5 days, 25% of the established standard) in the chronic model, achieved the same cure rates than standard treatment period (Francisco et al., 2016).

In clinical practice, shorter regimens are being currently evaluated ClinicalTrials.gov Identifier: NCT03191162 and NCT03378661 in the framework of clinical trials, being at that time results pending to be published. Previously two experiences have been reported with schemes using a lower overall dose of benznidazole. Viotti et al. analyzed the efficacy (seroconversion) of patients which had to withdraw treatment because of side effects (Alvarez et al., 2012). Eighty-one adult patients with Chagas disease were followed after receiving treatment with benznidazole incompletely for a median of 10 days. Twenty percent of these patients (16/81) were considered to be healed. The same group conducted a study assessing a new scheme of benznidazole with intermittent doses of benznidazole at 5 mg/kg/day in two daily doses every 5 days for a total of 60 days among 20 patients in the chronic phase of the disease. Although the efficacy endpoint should be analyzed carefully because of the low follow up period, the adverse effects ratio was similar than previously reported in literature (50%) but with a lower discontinuation rate (in only one case was treatment suspended) (Álvarez et al., 2016).

It would therefore appear that exist contradictory data to argue both opposite hypotheses, although the level of evidence seems not enough to rule out none of them. Thus, new clinical trial will be needed in order to confirm the dose and drug exposure effect of benznidazole over the efficacy.

Interestingly, the type of animal model has not shown any significant effect in the efficacy outcome. It is widely accepted that when testing antitrypanocidal drugs, mainly nitroheterocyclic derivates, the acute murine model is superior to the chronic one (Rodriques Coura and de Castro, 2002; Bern, 2011). That fact added to the easy-performing, the rapidity in obtaining results and lower cost, has lead a greater prominence of the utilization of acute model during the drug discovery process, to the detriment of the chronic one. Apart from the obvious biases our study has (inherent to the heterogeneity of the experiment designs, as the strain used, inoculums, etc…) probably the scarce number of chronic model assays included in the analyses, could have limited the weight of the comparison. Nevertheless could be reasonable to understand that in the chronic model has lower parasite load and more localized infection compared with the acute stages of the diseases, leading to a theoretically better response, as has been demonstrated by Francisco et al. (Francisco et al., 2016) (although some authors have pointed out the possibility of a higher susceptibility to benznidazole in those genetically engineered luminescent CL Brener strains) (Urbina and McKerrow, 2015). From a clinical point of view, to cure the chronic stage of the disease is one of the priorities or at least where the current standard of cure fails. Therefore, taking into account the current poor therapeutic scenario and the urgent need in having more efficient drugs for chronic patients, to rule out any candidate based only on acute model assays not appears to be the most optimal drug discovery strategy.

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				Treatment		Results (%)	
Author Year	Animal	n°	Strain	Inoculums Route	Dose mg/kg/d	Days	DPI	Efficacy assessment	Survival	Cure
Filardi 1984 (Filardi and Brener, 1984)	Swiss (M) 18-20 g	15	CL	10^5 Tryp IP	100	20	1	Parasitaemia	—	100
			MR							100
			Y							66.6
			Colombian							6.6
			VL-10							13.3
Araújo 2000 (Araújo et al., 2000)	Swiss (F) 20–24 g	10–14	CL	10^4 Tryp IP	25	20	8–10	Parasitaemia	—	0
			Y				12–15	Hemoculture		0
			Colombian				12–15	Xenodiagnosis		0
			CL		50		8–10	Circulating anti *T. cruzi*		9.1
			Y				12–15			0
			Colombian				12–15			0
			CL		100		8–10			100
			Y				12–15			30.8
			Colombian				12–15			0
Fournet 2000 (Fournet et al., 2000)	BALB/c (F/M) 6-8 weeks	10-15	CL Brener	5×10^3 Tryp IP	25	30	4	Parasitaemia	—	31
								ELISA Immunoblotting		
Molina 2000 (Molina et al., 2000)	Swiss (F) 18-20 g	—	CL	10^4 Tryp IP	100	28d + 15d	4	Parasitaemia	90 80	100
			Y					Hemoculture		50
			Colombian					Xenodiagnosis	60 70 70	50
			SC-28					Circulating anti *T. cruzi*		28.6
			VL-10							14.3
			CL			20		IS-CFM	IC: 90 IS: 70 IC: 90 IS: 50 IC: 60 IS: 50	100
										85.7
			Y							44.9
										60
			Colombian							0
										20
Olivieri 2002 (Olivieri et al., 2002)	Swiss (M) 16-20 g 6-8 weeks	—	Y	10^4 Tryp IP	62,5	14	7	Parasitaemia	78.5	—
Romanha 2002 (Romanha et al., 2002)	BALB/c	16	Y	5×10^3 Tryp IP	100	20	4	FBE	100	100
	C57BL/6 (F)							Hemoculture		
Saraiva 2002 (Saraiva et al., 2002)	BALB/c (M) 6 weeks	5	Y	10^5 Tryp IP	100	20	1	Parasitaemia Spleen culture	100	100
Corrales 2005 (Corrales et al., 2005)	Swiss (M) 8 weeks	—	Tulahuen	10^3 Tryp IP	200	30	13	Parasitemia	95	68.4
								Hemoculture, ELISA PCR (Blood), Histology		
Ferraz 2007 (Ferraz et al., 2007)	C57BL/6 (M) 8-10 weeks	—	Y	5×10^3 Tryp —	100	20	4	Parasitemia Hemoculture	WT 100 KOIFN: 0 KOIL12: 83	86 0 39
Ferraz 2009 (Ferraz et al., 2009)	C57BL/6 (M) 8-10 weeks	—	Y	5×10^3 Tryp —	100	20	4	Parasitemia Hemoculture	WT: 100 KOCd4: 6.3 KOCd8: 86.2 KOB: 66.7	86.2 0 65.5 22.2
Batista 2010 (Batista et al., 2010)	Swiss (F/M)	6	Y	10^4 Tryp IP	100	10	5	Parasitemia	100	0

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Table 3 (*continued*)

Paper	Laboratory mice		Infection				Treatment		Results (%)	
Author Year	Animal	n°	Strain	Inoculums Route	Dose mg/kg/d	Days	DPI	Efficacy assessment	Survival	Cure
	8 weeks							Hemoculture		
		6	Colombian	10^2 / 5 × 10^3 Tryp IP			Parasitemia onset	Histology PCR (Blood), ECG	100	0
Davies 2010 (Davies et al., 2010)	Swiss (F) 20 g	12	Tulahuen	10^3 Tryp IP	60	—	5	PCR	66	100
Olivieri 2010 (Olivieri et al., 2010)	Swiss (F)	—	Y	10^4 Tryp IP	100	20	4	Parasitemia	100	50
	18-20 g							Hemoculture Histopathology Serology anti *T. cruzi*		
Batista 2011 (Batista et al., 2011)	Swiss (M)	15	Y	10^4 Tryp IP	50	20	5	PCR (Blood)	93,3	0
	20-24 g							Hemoculture		
Maximiano 2011 (Maximiano et al., 2011)	Swiss (F) 18-23 g	6	Y	5 × 10^3 Tryp IP	100	7	4	Parasitemia	100	100
					50				83.3	0
					25				66.7	0
Bahia 2012 (Bahia et al., 2012)	Swiss (F)	10	Y	5 × 10^3 Tryp IP	100	20	4	FBE	100	50
			CL				7-8	Blood culture	100	70
			VL-10					PCR	90	0
			Colombian					IS-CFM	100	0
Buckner 2012 (Buckner et al., 2012)	Swiss (F)	7	Y	5 × 10^3 Tryp IP	100	20	4	Parasitemia and PCR		66.6
	18-24 g							IS-CFM		
Cencig 2012 (Cencig et al., 2012)	BALB/cJ (F)	—	Tulahuen	10^3 Tryp IP	100	5	10	Parasitemia and PCR	100	0
	7 weeks					10	10	IS-CFM	100	100
			Y			5	5		100	0
						10	5		100	12.5
Da Silva 2012 (da Silva et al., 1831 a)	Swiss (M)	5	Y	10^4 Tryp IP	100	20	5	Parasitaemia	100	0
								PCR, Hemoculture		
Diniz 2013 (Diniz et al., 2013)	Swiss (F)	—	Y	5 × 10^3 Tryp IP	100	7	4	Parasitaemia	100	0
	18-24 g				50	7	4	PCR	66.7	0
					25	7	4	IS-CFM	33.3	0
					100	20	4		100	70
					100	10	4		100	0
			VL-10		100	20	7		100	0
			Y		75	20	7			20
Soeiro 2013 (Soeiro et al., 2013)	Swiss (M)	6	Y	10^4 Tryp IP	100	5, 20 and 26	5	Parasitaemia PCR	100	0
			Colombian	5 × 10^3 Tryp IP			11-12	Hemoculture IS-CFM	100	0
Strauss 2013 (Strauss et al., 2013)	Swiss	20	Tulahuen	50 Tryp IP	100	20	7	Parasitaemia, ELISA	100	0
					50			PCR, Histology	100	0
Bahia 2014 (Bahia et al., 2014)	Swiss (F)	—	Y	5 × 10^3 Tryp IP	10	20	4	Parasitaemia	43	0
	20-24 g				25			ELISA	86	0
					50			PCR	100	0
					100			Histology	100	80
Branquinho 2014 (Branquinho et al., 2014)	Swiss (F)	8	CL	10^4 Tryp IP	50	10	1	Parasitaemia	100	100
	20-25 g					20	4	Hemoculture	100	100
			Y			10	1	PCR	100	0
						20	4	Serology	100	75
Bustamante 2014 (Bustamante et al., 2014)	C57BL/6	—	CL	10^3 Tryp IP	100	40	15	Parasitaemia	—	100
						10		Hemoculture		12.5
						20		PCR		62.5
			Colombian			40		IS-CFM		72
						60				54.5
			Brazil			40				81.8
						10 doses				
						40				81.8
						12 doses				
						60				89.5
						13 doses				
						40				100

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Table 3 (*continued*)

Paper	Laboratory mice		Infection				Treatment		Results (%)	
Author Year	Animal	n°	Strain	Inoculums Route	Dose mg/kg/d	Days	DPI	Efficacy assessment	Survival	Cure
Caldas 2014 (Caldas et al., 2014)	Swiss (F)	6	VL-10	5 × 10^3 Tryp IP	100	20	10	Parasitemia	—	0
								PCR (Blood/Heart)		
Carneiro 2014 (Carneiro et al., 2014)	Swiss (F)	7	Y	2 × 10^3 Tryp IP	1.0 mg kg-1 day-1/free	10	5	Parasitemia	30	—
	6-8 weeks							PCR (Heart)		
Ndao 2014 (Ndao et al., 2014)	CD-1 (M) 30 g	10	Brazil	10^4 Tryp —	50	28	2	Parasitaemia	90	77.8
	6-8 weeks									
								PCR (Blood/Tissue)		
		10						Parasitaemia and PCR IS-CFM	70	50
Olmo 2014 (Olmo et al., 2014)	BALB/c (F)	6	CL Brener	5 × 10^5 Tryp IP	25	20	14	Parasitemia	100	0
	6-8 weeks							Serology PCR (Heart) IS-CFM		
Khare 2015 (Khare et al., 2015)	C57BL/6	8	CL	10^3 Tryp IP	100	10	45	Parasitemia	100	0
		8			100	15	40	PCR	100	87.5
		8			100	20	35	IS-CFM	100	100
		5			10	20	35		100	0
		5			30	20	35		100	0
		21			100	20	35		100	100
		8	Y		100	20	35		100	87.5
Fortes 2015 (Francisco et al., 2015)	BALB/c	6	CL Brener	10^3 Tryp	100	20	14	PCR	100	100
	8-12 weeks			IP				Bioluminiscence IS-CFM		
Fontes 2015 (Assíria Fontes Martins et al., 2015)	Swiss (F) 18-22 g	—	Y	5 × 10^3 Tryp IP	50	20	4	Parasitemia	100	0
					75			PCR (Blood)	100	20
					100			Serology IS-CFM	100	70
Guedes-Da-Silva 2015 (Guedes-da-Silva et al., 2015)	Swiss (F/M)	10	Y	10^4 Tryp	100	30	z6	Parasitemia	100	30
	18-20 g	10	Colombiana	5 × 10^3 Tryp	100	60	10	PCR IS-CFM	90	11
Santos 2015 (Santos et al., 2015)	C57BL/6 10 weeks 23.5-3.2 g	10	Y	5 × 10^3 Tryp IP	100	15	4	Parasitemia PCR IS-CFM	100	NS
De Mello 2016 (de Mello et al., 2016)	Swiss (F) 20-25 g	8	Y	10^5 Tryp IP	100	20	4	Parasitemia Serology Hemoculture PCR	100	62.5
Francisco 2016 (Francisco et al., 2016)	BALB/c (F)	-	CL Brener	10^3 Tryp	10	5	14	Bioluminiscence	-	-
	6-8 weeks	6		IP	30	10		(Ex vivo and In vivo,	100	0
		6			100	20		after IS-CFM)	100	0
		-			100*	5			-	-
		6				10			100	0
		6				20			100	33
		30				5			100	0
		6				10			100	0
		15				20			100	93
		-				5			100	0
		6				10			-	-
		-				20			-	-
		-								

(—) Not shown or not reported; (M) Male; (F) Female; (Tryp) Tripomastigote; (DPI) Days post infection; (IP) Intraperitoneal; (ECG) Electrocardiogram; (FBE) Fresh blood examination; (IC) immunocompetent; (IS) immunosuppressed; (WT) Wild-type; (KO) knockout; (IS-CFM) Variable ciclophosphamide immunosuppression scheme; (PCR): Protein Chain Reaction; (NS) Not shown

* 50mg/kg/day administered bid

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		Results (%)	
Author Year	Animal	n°	Strain	Inoculums Route	Dose mg/kg/d	Days	DPI	Efficacy assessment	Survival	Cure
Andrade 1991 (Andrade et al., 1991)	Swiss 18–20 g	Colombian	5×10^4	100	60	90-157	Subinoculation	—	0	
				—				Hemoculture Xenodiagnosis		
Fournet 2000 (Fournet et al., 2000)	BALB/c (F/M)	33	CL Brener	10^3 Tryp IP	25	30	60	Parasitaemia	24	33.3
								ELISA Immunoblotting		
Molina 2000 (Molina et al., 2000)	Swiss (F) 18-20 g	12	CL	30 Tryp IP	100	20	120	Parasitaemia	40	0
		12	Y					Hemoculture Xenodiagnosis Ciculating anti *T. cruzi*	40	0
		12	Colombian					IS-CFM	30	0
Molina 2000 bis (Molina et al., 2000)	Swiss (F) 18–20 g	12	CL	30 Tryp IP	100	20	120	Parasitaemia	—	27.3
		12	Y					Hemoculture		18.2
		12	Colombian					Xenodiagnosis Circulating anti *T. cruzi*		0
Nakayama 2001 (Nakayama et al., 2001)	Balb/c (F/M) 6–8 weeks	10	CL Brener	10^3 Tryp IP	25	30	60	Parasitemia ELISA	100	30
Siqueira Portella 2009 (Portella and Andrade, 2009)	Swiss Webser 15–20 g	25	Colombian	10^4 IP	100	90	120	Parasitemia	—	20.8
		34	21SF-C3				90	Hemoculture Subinoculation		35.5
Canavaci 2010 (Canavaci et al., 2010)	Balb/c	10	CL	10^3 Tryp	100	40	55	Parasitemia	—	100
				IP				IS-CFM Serology		
Bahia 2012 (Bahia et al., 2012)	Swiss (F)	10	VL-10	5×10^3 Tryp IP	100	20	120	FBE	100	10
								Blood culture, PCR IS-CFM		
Cencig 2012 (Cencig et al., 2012)	BALB/cJ (F) 7 weeks	—	Tulahuen	10^3 Tryp IP	100	10	60	Parasitaemia	—	100
						5		Quantitative PCR		0
			Y			10		IS-CFM		12.5
						5				0
Bustamante 2014 (Bustamante et al., 2014)	C57BL/6	—	Colombian	10^3 Tryp IP	100	40d	120	Parasitaemia	—	0
			Brazil			60 days 13 doses	130	Hemoculture, PCR IS-CFM		100
Fortes 2015 (Francisco et al., 2015)	BALB/c 8-12 weeks	5	CL Brener	10^3 Tryp IP	100	20	74	PCR	100	100
		6				10		Bioluminiscence	100	100
		6				5		(Ex vivo and In vivo, after IS-CFM)	100	100
De Mello 2016 (de Mello et al., 2016)	Swiss (F) 20–25 g	10	Y	500 Tryp IP	100	20	90	Parasitemia Serology Hemoculture PCR	80	0
Francisco 2016 (Francisco et al., 2016)	BALB/c (F) 6–8 weeks		CL Brener	10^3 Tryp IP	10	5	-	Bioluminiscence	-	-
					30	10		(Ex vivo and In vivo, after IS-CFM)	100	0
					100	20			100	17
					100*	5			100	0
						10			100	67
						20			100	100
						5			100	100
						10			100	100
						20			100	100
						5			-	-
						10			100	100
						20			-	-

(—) 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.

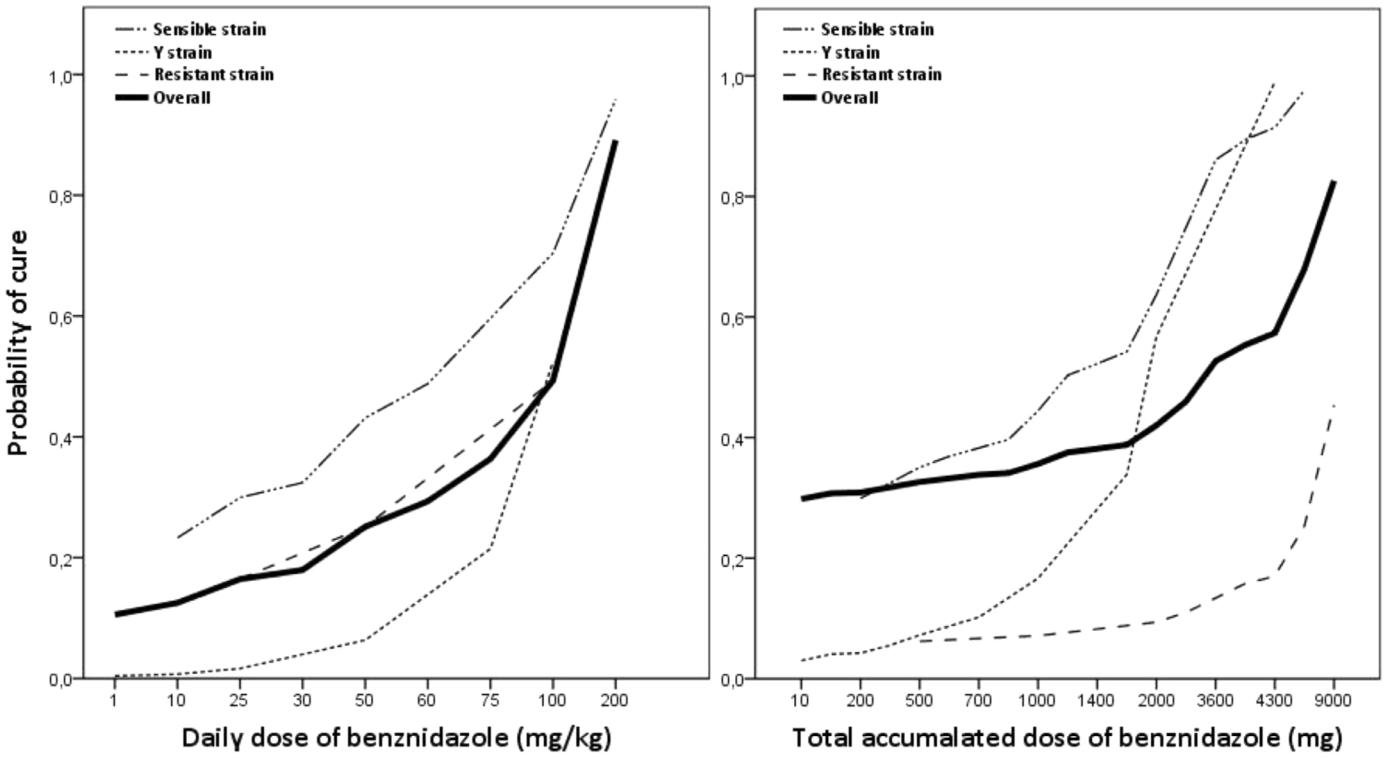

Fig. 3. Relationship between the benznidazole dose over the efficacy (cure criteria according to each experiment design) in the acute murine model. Regardless the *T. cruzi* strain used, exist a positive effect between both the benznidazole dose expressed by daily mg/kg or total accumulated dose (daily 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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