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EXPERIMENTAL THERAPEUTICS

Pharmacokinetics of Benznidazole in Experimental Chronic Chagas Disease Using the Swiss Mouse–Berenice-78 *Trypanosoma cruzi* Strain Model

Suzana Marques de Jesus,[a] Leonardo Pinto,[a] Fernanda de Lima Moreira,[b] Glauco Henrique Balthazar Nardotto,[b] Rodrigo Cristofoletti,[c] Luísa Perin,[a] Kátia da Silva Fonseca,[a] Pauliana Barbêdo,[a] Lorena Cera Bandeira,[a] Paula Melo de Abreu Vieira,[a,d] Claudia Martins Carneiro[a,e]

[a]Laboratory of Immunopathology, Nucleus of Biological Sciences Research, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, Brazil

[b]Laboratory of Clinical Pharmacokinetics, Department of Clinical, Toxicological, and Bromatological Analyses, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

[c]Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida, Orlando, Florida, USA

[d]Laboratory of Morphopathology, Department of Biological Sciences, Nucleus of Biological Sciences Research, Institute of Exact and Biological Sciences, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, Brazil

[e]Department of Clinical Analysis, School of Pharmacy, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, Brazil

Suzana Marques de Jesus and Leonardo Pinto contributed equally. Co-first-author order was determined by authors' ages.

ABSTRACT Chronic Chagas disease might have an impact on benznidazole pharmacokinetics with potential alterations in the therapeutic dosing regimen. This study aims to investigate the influence of chronic *Trypanosoma cruzi* infection on the pharmacokinetics and biodistribution of benznidazole in mice. Healthy ($n = 40$) and chronically *T. cruzi* (Berenice-78 strain)-infected ($n = 40$) Swiss female 10-month-old mice received a single oral dose of 100 mg/kg of body weight of benznidazole. Serial blood, heart, colon, and brain samples were collected up to 12 h after benznidazole administration. The serum and tissue samples were analyzed using a high-performance liquid chromatography instrument coupled to a diode array detector. Chronic infection by *T. cruzi* increased the values of the pharmacokinetic parameters absorption rate constant (K_a) (3.92 versus 1.82 h^{-1}), apparent volume of distribution (V/F) (0.089 versus 0.036 liters), and apparent clearance (CL/F) (0.030 versus 0.011 liters/h) and reduced the values of the time to the maximum concentration of drug in serum (T_{max}) (0.67 versus 1.17 h) and absorption half-life ($t_{1/2a}$) (0.18 versus 0.38 h). Tissue exposure (area under the concentration-versus-time curve from 0 h to time t for tissue [$AUC_{0-t,tissue}$]) was longer and higher in the colon (8.15 versus 21.21 $\mu g \cdot h/g$) and heart (5.72 versus 13.58 $\mu g \cdot h/g$) of chronically infected mice. Chronic infection also increased the benznidazole tissue penetration ratios ($AUC_{0-t,tissue}/AUC_{0-t,serum}$ ratios) of brain, colon, and heart by 1.6-, 3.25-, and 3-fold, respectively. The experimental chronic Chagas disease inflammation-mediated changes in the regulation of membrane transporters probably influence the benznidazole pharmacokinetics and the extent of benznidazole exposure in tissues. These results advise for potential alterations in benznidazole pharmacokinetics in chronic Chagas disease patients with possibilities of changes in the standard dosing regimen.

KEYWORDS Chagas disease, *Trypanosoma cruzi*, benznidazole, pharmacokinetics, preclinical drug studies

Chagas disease is a neglected tropical infectious disease caused by the intracellular hemoflagellate protozoan parasite *Trypanosoma cruzi*. Chagas disease remains endemic in Latin America, but migration has also led to its emergence in areas where

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Address correspondence to Leonardo Pinto, leonardopinto84@gmail.com.

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the disease is not endemic, including Europe, North America, Japan, and Australia. Currently, the World Health Organization estimates that approximately 7 million people are infected by *T. cruzi* worldwide and that 75 million are at risk of infection (1).

Chagas disease is characterized by acute and chronic phases of infection with different clinical forms. While infection can remain asymptomatic for many years, approximately 30 to 40% of individuals chronically infected by *T. cruzi* may develop the cardiac and/or digestive clinical forms (2, 3). During active *T. cruzi* infection, cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), transforming growth factor β (TGF-β), interleukin-12 (IL-12), IL-4, IL-10, IL-17, and IL-6 are released after macrophage and T lymphocyte activation (4–7).

Besides playing critical roles in pathogenesis and disease progression, *in vitro* and *in vivo* studies have shown that proinflammatory cytokines may alter the expression and activity of membrane transporters and cytochrome P450 (CYP) enzymes (8–12). Therefore, inflammatory disease-drug interactions may have an influence on the pharmacokinetics (PK) of drugs (8). Currently, mechanistic knowledge about the impact of parasitic infections on CYP-mediated drug metabolism and transporter-mediated kinetics remains limited for malaria (13, 14) and visceral leishmaniasis (15), being inexistent for Chagas disease.

During the last 50 years, benznidazole has been considered the trypanocidal drug of choice for treating Chagas disease. Benznidazole is not an ideal drug for curing Chagas disease because of its many limitations, including (i) variable efficacy, with therapeutic failure rates of around 20% for the acute phase and 80% for the chronic phase; (ii) varying natural susceptibility (or drug resistance) of *T. cruzi* strains; (iii) multiple adverse effects; and (iv) long-term treatment regimens (16, 17). These limitations could be related to unfavorable biopharmaceutical and pharmacokinetic properties such as low solubility and intestinal absorption, limited tissue and parasitic penetration, and high clearance rates (18–20). In fact, benznidazole is proposed to be a class 4 drug according to the biopharmaceutical classification system (low permeability and solubility) (21), showing low tissue distributions in healthy mice (22). Additionally, studies in HepG2 cells and rats have shown that benznidazole is a substrate and inducer of CYP3A4, glutathione *S*-transferase, P-glycoprotein (P-gp), and multiple-resistance protein 2 (23).

In this context, understanding the impact of *T. cruzi* infection on drug pharmacokinetics is essential to bridge phase I and II studies aiming to reduce attrition rates during clinical proof-of-concept trials designed for efficacy and safety assessments. The current benznidazole dosing regimen is based on pharmacokinetic studies in healthy subjects (24, 25). Nevertheless, the FDA highlights that benznidazole pharmacokinetics could be different in chronic Chagas disease patients (24). For example, due to the longer elimination half-life ($t_{1/2el}$) of benznidazole in patients with chronic Chagas disease, Soy et al. (26) recommended a reduction of the therapeutic dose.

Although the pharmacokinetics of benznidazole have been investigated in healthy mice, rats, rabbits, sheep, and dogs (27, 28), limited information on the preclinical pharmacokinetics and tissue distribution of benznidazole has been published (22, 29), leading to a limited understanding of the intrinsic and extrinsic mechanisms involved in its efficacy and toxicity. Furthermore, no standardized animal model has been reported in order to evaluate the drug pharmacokinetics in Chagas disease drug discovery and development. Therefore, the aim of this research was to investigate the impact of experimental chronic Berenice-78 (Be-78) *Trypanosoma cruzi* infection on systemic and tissue exposure of benznidazole in outbred Swiss mice.

RESULTS AND DISCUSSION

To the best of our knowledge, the Swiss mouse–Be-78 *T. cruzi* strain model is a novel experimental model for assessing translational benznidazole pharmacokinetics with available tissue distribution data in chronic Chagas disease.

Benznidazole systemic and tissue exposure profiles after the administration of a

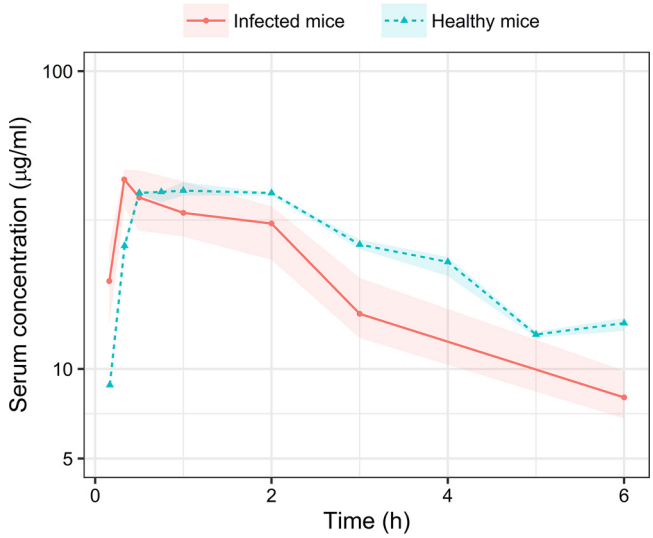

FIG 1 Serum concentration-versus-time curves of benznidazole after a single oral dose of 100 mg/kg in healthy and chronically *T. cruzi* (Berenice-78 strain)-infected Swiss mice. Data are expressed as medians (solid and dotted lines) and interquartile ranges (IQ25–75) (shaded area).

single oral dose of 100 mg/kg of body weight in healthy and chronically *T. cruzi*-infected mice are shown in Fig. 1 and 2. Chronic infection by *T. cruzi* increased the values of the pharmacokinetic parameters absorption rate constant (K_a) (3.92 versus 1.82 h^{-1}), apparent volume of distribution (*V*/*F*) (0.089 versus 0.036 L), and apparent clearance (CL/*F*) (0.030 versus 0.011 liters/h) and reduced the values of the time to reach the maximum concentration of drug in serum (T_{max}) (0.67 versus 1.17 h) and absorption half-life ($t_{1/2a}$) (0.18 versus 0.38 h) compared with healthy mice (Table 1). As benznidazole absorption seems to be accelerated (higher K_a and lower T_{max} and $t_{1/2a}$ values) in infected mice, it could explain the faster elimination (higher CL/*F* value). Furthermore, the unchanged elimination rate constant (K_{el}) (~0.33 h^{-1}) is the rational explanation for the increased *V*/*F*. The proportional changes of 2.7-fold in *V*/*F* and CL/*F* values regarding infected versus healthy mice resulted in unchanged elimination half-life ($t_{1/2el}$) values. These results suggest that chronic infection by *T. cruzi* alters benznidazole pharmacokinetics, which may be due to inflammation-mediated changes in the expression and activity of membrane transporters (8, 10, 30).

Benznidazole is a poorly permeable compound and a substrate of P-gp-mediated efflux (21–23, 31, 32). Therefore, it is plausible to hypothesize that the higher benznidazole absorption rate observed in infected mice was due to the potential downregulation of P-gp expression, which has already been observed for several inflammatory/infectious diseases (33, 34). Further mechanistic studies coadministering benznidazole with P-gp inhibitors are needed to fully characterize the disease-mediated alteration in benznidazole absorption across the enterocyte membrane.

Figure 2 shows the concentrations of benznidazole in the brain, colon, and heart over time curves of healthy and infected mice after a single oral dose of benznidazole. Chronic infection increased the peak concentration as well as the extent of benznidazole exposure in all three studied tissues compared with healthy mice (Table 2). The magnitude of the change in benznidazole penetration under disease conditions was higher in the colon and heart (Table 2). This might be due to the preferential tropism of the Berenice-78 strain of *T. cruzi* for heart muscle and intestine, as demonstrated in chagasic patients (35) and animal models such as the outbred Swiss mouse model (36, 37). These results suggest that a permeability-limited but not a perfusion-rate-limited model is controlling the benznidazole tissue distribution. Disease-mediated changes in the permeability of the barriers and/or the expression and function of transporters

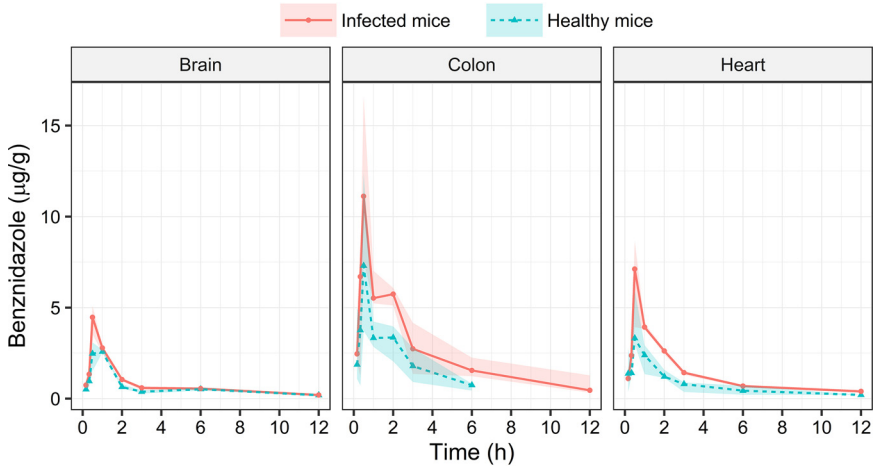

FIG 2 Tissue concentration-versus-time curves of benznidazole after a single oral dose of 100 mg/kg in healthy and chronically *T. cruzi* (Berenice-78 strain)-infected Swiss mice. Data are expressed as medians (solid and dotted lines) and interquartile ranges (IQ25–75) (shaded area).

seem to lead to an altered target site distribution of total benznidazole concentrations. Whether the *T. cruzi* disease model is downregulating efflux and/or upregulating uptake transporters responsible for the benznidazole tissue distribution is still unknown and should be the subject of further studies. Cytokines and other mediators of the cellular inflammatory response could be involved in the regulation of membrane transporters in chronic infection of Chagas disease. Future studies should evaluate the role of inflammation biomarkers in drug transporter activity in experimental and clinical infection by *T. cruzi*.

Contrary to our results, the noninfluence of experimental chronic Chagas disease on the pharmacokinetics of oral benznidazole at 100 mg/kg was previously reported for the BALB/c mouse-CL Brener *T. cruzi* strain model (38). A plausible explanation is differences in the *T. cruzi* strains (CL Brener versus Be-78), mouse breeds (BALB/c versus Swiss), and time of chronic infection. According to Soy et al. (26) and the FDA (24), the benznidazole pharmacokinetics could be different between chronic Chagas disease patients and healthy subjects; thus, a suitable animal model of choice should demonstrate this difference in order to generate adequate data to translate to humans (39). Furthermore, in Chagas disease drug discovery and development, benznidazole is used as a drug reference to compare with a new drug candidate (40, 41). Our research group has demonstrated an intensive inflammation process in several organs, includ-

TABLE 1 Serum pharmacokinetic parameters of benznidazole after a single oral dose of 100 mg/kg in healthy and chronically *T. cruzi* (Berenice-78 strain)-infected Swiss mice[a]

	Median value (IQ25–75) for group	
Parameter	**Infected mice**	**Healthy mice**
K_a (h^{-1})	3.92* (3.22–4.66)	1.82 (1.73–1.88)
C_{max} (μg/ml)	44.24 (39.78–52.22)	41.74 (40.86–42.87)
T_{max} (h)	0.67* (0.60–0.76)	1.17 (1.16–1.18)
$t_{1/2a}$ (h)	0.18* (0.15–0.23)	0.38 (0.37–0.40)
$AUC_{0-\infty}$ (μg · h/ml)	158.09 (141.34–181.98)	199.67 (191.53–200.57)
$t_{1/2el}$ (h)	1.92 (1.79–1.99)	2.33 (2.10–2.43)
V/*F* (liters)	0.089* (0.07–0.10)	0.036 (0.03–0.04)
CL/*F* (liters/h)	0.030* (0.02–0.04)	0.011 (0.010–0.012)
K_{el} (h^{-1})	0.36 (0.35–0.39)	0.30 (0.29–0.33)

[a]Data are expressed as medians and interquartile ranges (IQ25–75). C_{max}, maximum plasma concentration; $AUC_{0-\infty}$, area under the plasma concentration-versus-time curve from time zero to infinity; *V*, volume of distribution; CL, total clearance; $t_{1/2el}$, elimination half-life; K_{el}, elimination rate constant; K_a, absorption rate constant; $t_{1/2a}$, absorption half-life; T_{max}, time to reach C_{max}. *, $P < 0.05$ by a Mann-Whitney test.

TABLE 2 Tissue pharmacokinetic parameters of benznidazole after a single oral dose of 100 mg/kg in healthy and chronically *T. cruzi* (Berenice-78 strain)-infected Swiss mice[a]

Parameter and tissue	Value for group	
	Infected mice	Healthy mice
Median C_{max} (μg/g) (IQ25–75)		
Brain	3.53* (2.92–4.47)	2.53 (1.87–2.58)
Colon	7.56* (6.34–11.12)	3.73 (3.05–7.30)
Heart	3.93* (3.77–7.12)	3.00 (1.92–3.32)
Median T_{max} (h) (IQ25–75)		
Brain	0.5	1.0
Colon	0.5	0.5
Heart	0.5	0.5
Median AUC_{0-t} (μg · h/g) (IQ25–75)		
Brain	7.97 (6.97–9.17)	6.23 (5.08–7.27)
Colon	21.21* (18.59–28.74)	8.15 (6.71–13.76)
Heart	13.58* (12.35–15.60)	5.72 (4.90–8.63)
$AUC_{0-t,tissue}/AUC_{0-t,serum}$ ratio (%)		
Brain	5	3
Colon	13	4
Heart	9	3

[a]Data are expressed as medians and interquartile ranges (IQ25–75). C_{max}, maximum plasma concentration; AUC_{0-t}, area under the plasma concentration-versus-time curve from 0 h to time *t*; T_{max}, time to reach C_{max}; $AUC_{0-t,tissue}/AUC_{0-t,serum}$ ratio, tissue penetration ratio. *, $P < 0.05$ by a Mann-Whitney test.

ing heart and intestine, mediated by inflammatory biomarkers (e.g., IFN-γ, TNF-α, and IL-10) in the chronic Swiss mouse–Be-78 *T. cruzi* strain model (36, 37) that can influence drug metabolism enzyme and drug transporter activities. Based on our results, the Swiss mouse–Be-78 *T. cruzi* strain model may be an appropriate experimental model to evaluate the impact of inflammation-mediated chronic infection on translational drug pharmacokinetics for Chagas disease.

Therefore, the results obtained in the present study indicate the impact of experimental chronic Chagas disease on benznidazole pharmacokinetics in mice, advising for a potential change in the dosing regimen in clinical pharmacotherapy. These results support previous clinical studies that suggest that the standard dosing regimen might be significantly different in patients (26, 42, 43). Future clinical and preclinical studies should evaluate the role of chronic and acute Chagas disease in benznidazole pharmacokinetics and a possible change in the standard dosing regimen.

Conclusions. In summary, experimental chronic Chagas disease using the Swiss mouse–Be-78 *T. cruzi* strain model altered the benznidazole pharmacokinetics, probably mediated by inflammatory biomarkers produced during chronic infection. Chronic infection by the Be-78 *T. cruzi* strain increased benznidazole exposure in the heart and colon. Thus, our study supports alterations in benznidazole membrane permeability during chronic infection, which might be by downregulating efflux but also upregulating the uptake of drug transporters. These results advise for a potential change in benznidazole pharmacokinetics in chronic Chagas disease patients.

MATERIALS AND METHODS

Animals and ethics. Swiss 10-month-old female mice, weighing 45 to 50 g, were housed under proper handling conditions with access to food and water *ad libitum*. The Ethics Committee on Animal Experimentation of the Federal University of Ouro Preto, Minas Gerais, Brazil, approved the protocol (2016/58).

Treatment schedule, sample collection, and extraction. Mice were divided into two groups of 40 animals each: uninfected (healthy) and infected with an intraperitoneal inoculum of 5×10^3 trypomastigote forms of the Berenice-78 *T. cruzi* strain. Mice were infected at 30 days of age, and infection was confirmed by parasitemia detection in fresh blood. After 9 months of *T. cruzi* inoculation in the infected mouse group, both groups received a single oral dose of 100 mg/kg benznidazole in an aqueous solution of 0.5% methylcellulose administered by gavage. Serial blood samples were collected 0.16, 0.33, 0.5,

1, 2, 3, 6, and 12 h after benznidazole administration ($n = 5$ mice/time point), and serum samples were obtained by centrifugation. Samples were processed according to a method previously described by Perin et al. (29). *In totum* heart, colon, and brain were collected from five animals at each sampling time, weighed, processed, and stored as homogenized tissue in phosphate buffer (pH 7.4) (29).

Benznidazole analysis in serum and tissues. The samples were extracted and analyzed using a bioanalytical method developed and validated by our research group (22, 29). A high-performance liquid chromatography (HPLC) system (Prominence LC20AT; Shimadzu, Kyoto, Japan) was coupled to a diode array detector (DAD) SPD-M20A model operating at 324 nm with an analytical C_{18} column (Gemini-NXVR; Phenomenex, Torrance, CA, USA) (150 mm by 4.6 mm; 5 μm) and a C_{18} column guard (model AJ0-7597VR; Phenomenex, Torrance, CA, USA) (4 mm by 3 mm) and maintained at 40°C. The mobile phase was composed of a mixture of water and acetonitrile (70:30, vol/vol) with a 1.0-ml/min isocratic flow rate. The injection volume was 20 μl, and the run time was 7 min.

The method (29) was validated according to EMA guidelines (44), and partial validation was executed in order to confirm reproducibility by the following assays: selectivity, linearity, precision, and accuracy. The calibration curves were linear ($r^2 > 0.99$) in the range of 0.1 to 100 μg/ml. Precision varied from 2.29 to 12.33%, and accuracy varied from −12.91 to 14.33%, confirming the reproducibility of the method.

Systemic and tissue drug distribution analyses. Benznidazole pharmacokinetic parameters were estimated from the serum concentration-versus-time profiles using the one-compartment model of Phoenix 64/WinNonLin version 7.0 (Pharsight, Certara Company).

For tissue distribution studies, the amount of benznidazole was expressed in terms of micrograms per gram of tissue and calculated using the equation $C_t = (C_s \times V_s)/P$, where C_t represents the total tissue concentration (micrograms per gram), C_s is the supernatant concentration, V_s is the supernatant volume, and P is the weight of the tissue sample. The pharmacokinetic parameters from tissue concentrations versus time were determined using a one-compartment PK model. Benznidazole tissue penetration was determined as the ratio of the area under the concentration-versus-time curve from 0 h to time t for tissue ($AUC_{0-t,tissue}$) to the $AUC_{0-t,serum}$.

Statistical analysis. The statistical analysis was performed using R software version 3.6.1 (R Foundation for Statistical Computing). The data are expressed as medians and 25% to 75% interquartile ranges (IQ25–75). A Mann-Whitney nonpaired test was employed to compare the data of healthy versus chronically infected groups. Differences in median values were considered significant at a P value of <0.05.

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