

Efficacy of voriconazole in a murine model of acute *Trypanosoma cruzi* infection

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Objectives: Antifungal triazole derivatives have been studied as possible alternatives for the treatment of Chagas' disease. Voriconazole has demonstrated *in vitro* activity against *Trypanosoma cruzi*, but its efficacy *in vivo* has not yet been tested. We aimed to determine the effect of voriconazole in a murine model of acute *T. cruzi* infection.

Methods: Treatment efficacy was evaluated by comparing parasitaemia, mortality and organ involvement (by histological examination) of infected mice.

Results: Treatment with voriconazole significantly lowered parasitaemia and mortality compared with controls, reduced the percentage of mice with amastigote nests in heart and skeletal muscle and moderately decreased myocardial inflammation.

Conclusions: Our findings support the potential of voriconazole for the treatment of acute Chagas' disease and motivate future animal studies using varying doses and treatment schemes. Further evaluation of voriconazole for clinical use in human Chagas' patients is warranted.

Keywords: Chagas' disease, benznidazole, mouse model

Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, constitutes an important public health problem in Latin America.^{1,2} In spite of the large number of infected patients, current treatment options are limited to two drugs: benznidazole and nifurtimox. However, treatment in many Latin American countries is largely dependent on either benznidazole or nifurtimox, but rarely both, due to the erratic availability of these compounds.³ Both drugs have significant side effects,⁴ especially in adults, and are not particularly efficacious in the chronic stage of the infection in adults. In this context, there is considerable need for new compounds to improve the chemotherapy of Chagas' disease.

Novel antifungal triazole derivatives, originally developed for the treatment of invasive fungal infections, have arisen as potential alternative treatments for Chagas' disease. Azoles inhibit *T. cruzi* ergosterol synthesis, which is fundamental for parasite growth and survival, and have pharmacokinetic properties

suitable for the treatment of this disseminated infection. In this report we evaluate the *in vivo* trypanocidal activity of voriconazole, a potent triazole antifungal agent with wide-spectrum activity already used in humans for the treatment of systemic mycoses, in a murine model of acute *T. cruzi* infection by determining its impact on parasitaemia, mortality and histological condition.

Methods

Experimental animals

Inbred 2-month-old female BALB/c mice were purchased from Central Bioterium (Veterinary Sciences Faculty, University of Buenos Aires, Argentina) and housed in the bioterium at Ricardo Gutiérrez Children's Hospital (Buenos Aires, Argentina). Procedures used for housing and handling animals were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.⁵ Protocols were approved by the Ricardo Gutiérrez Children's Hospital's Internal Ethics and Teaching

Committees. Animals were kept in plastic cages (four per cage) with commercial rodent diet (Ganave Rata Ratón®) and water available *ad libitum*, in controlled temperature and light conditions ($23 \pm 1^\circ\text{C}$ and cycles of 12 h in the dark and 12 h in the light, respectively).

Infection

Mice were infected by intraperitoneal injection of 500 Tulahuén strain trypomastigotes of *T. cruzi* (routinely maintained by serial passages in BALB/c mice). After infection was established, at 10 days post-infection (pi) mice with detectable parasitaemia were randomly divided into the following groups: voriconazole treatment ($n=8$), non-treated (NT) controls ($n=10$) and benznidazole treatment ($n=8$).

Drugs and treatment schedules

Voriconazole (pure product provided by Pfizer) was dissolved in a 4% polyethylene glycol 400 solution and administered at 40 mg per kg of body weight, once daily for 30 days. Benznidazole (Radanil®, Roche) was suspended in a 2% solution of carboxymethylcellulose and given at 100 mg per kg of body weight, once daily for 20 days. Treatment was administered by 150 μL oral gavage beginning immediately upon onset of parasitaemia (10 days pi) in all animals. The doses, length of treatment and route of administration were chosen based on published data.^{6–8}

Evaluation of treatment response

Parasitaemia was evaluated 3 days each week following a modified Pizzi-Brener method⁹ in which 5 μL of tail vein blood was compressed between a glass slide and an 18×18 mm coverslip and examined microscopically at $\times 400$ magnification. The number of parasites per mL of blood was determined by counting trypomastigotes in 50 fields and then multiplying that number by a conversion factor to express the final result in terms of 10^4 trypomastigotes/mL.¹⁰ Body weight, rectal temperature and clinical condition, including physical appearance and behaviour metrics based on previously established parameters,¹¹ were recorded weekly. Mortality was recorded daily.

Histology preparation and evaluation

Surviving mice were sacrificed at completion of the study (60 days pi). Segments of heart and skeletal muscle were collected from all animals in order to evaluate the inflammatory process and the presence of amastigote nests. Viable samples were fixed in buffered 10% formaldehyde, dehydrated and embedded in paraffin. Then, 5 μm thick sections were stained with haematoxylin and eosin. A single-blind evaluation of the specimens was performed by light microscopy and the parasite load was expressed as the presence or absence of amastigote nests. The degree of myocardial inflammation was scored as (0) absent, (1) focal, (2) multifocal, (3) diffuse with partial wall involvement or (4) diffuse with total wall involvement. Skeletal muscle inflammation was scored as (0) absent, (1) focal, (2) multifocal, (3) multifocal confluent or (4) diffuse.^{12,13} See Figure S1 (available as Supplementary data at JAC Online) for illustrative histological images of amastigote nests and the scoring regimen.

Statistical analysis

Survival curves of the three experimental groups were compared to determine whether treatment led to significantly longer life spans and decreased mortality using the Mantel–Cox log rank test and Fisher's exact test, respectively. Peak parasitaemia for each mouse was established in order to compare the response of voriconazole- and

benznidazole-treated groups with that of NT controls. Because parasitaemia data were not normally distributed (Shapiro–Wilk test, $P=0.031$ for benznidazole), we applied the non-parametric Kruskal–Wallis rank sum test to determine whether any significant differences occurred between the groups, followed by multiple two-sided pair-wise comparisons with the Wilcoxon rank sum test. Likewise, Kruskal–Wallis and Wilcoxon rank sum tests were used to compare the weight change among the groups at peak infection and at the end of the study. Later, these tests, along with Fisher's exact test, were used to see if voriconazole administration lowered the inflammation score or the proportion of mice with parasites in their myocardium and skeletal muscle.

Figure generation and the Mantel–Cox log rank test were performed using GraphPad Prism (GraphPad Prism, Version 5.0a for Mac OS X, GraphPad Software, San Diego, CA, USA; www.graphpad.com). All other analyses were conducted using R [R statistical language, V 2.12.0 for Mac OS X; R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. <http://www.R-project.org>]. The significance threshold ($P<0.05$) of all results was adjusted *post hoc* to account for multiple pair-wise comparisons using the Bonferroni correction (for pair-wise comparison testing, $P<0.017$ was significant and is indicated with an asterisk).

Results

Animal wellbeing

Throughout this study, mice treated with voriconazole displayed few adverse side effects. Weight loss and abnormal hunched posture were observed in two of eight animals during peak infection, followed by death. The remaining six mice lacked grooming and had slightly depressed behaviour at peak parasitaemia; however, the clinical condition of all these mice improved during the course of treatment. Similarly, benznidazole was well tolerated by mice. All eight subjects appeared physically healthy, with shiny, well-groomed fur and alert, active behaviour. In contrast to the treatment groups, NT mice presented with a soiled coat, a hunched guarding posture, reduced mobility and severely despondent behaviour and lethargic reflexes. This deteriorated state was accompanied with low body temperature and weight loss, concluding with death in 9 of 10 subjects.

All mice lost weight near maximum parasitaemia (Figure 1a), although, no statistical difference in weight change was observed between the groups at peak infection (Kruskal–Wallis, $P=0.208$). Animals treated with voriconazole gained weight prior to peak infection and afterwards, following a weight trajectory similar to uninfected healthy mice of the same age and sex.¹⁴ In contrast, NT and benznidazole-treated mice lost a considerable amount of weight—13% and 10% of their initial body weight at 24 days pi, respectively—and surviving mice did not begin to recover until 46 days pi. At the end of the study, benznidazole-treated subjects gained significantly less weight than voriconazole-treated mice ($P<0.001^*$).

At peak of infection, the body temperature (Figure 1b) of all three experimental groups was below the reference range for healthy mice. Mice receiving voriconazole continued to display low temperature until after treatment was discontinued. However, the temperature of benznidazole-treated subjects promptly improved to within the normal range. Markedly reduced temperature was observed in NT animals and only increased to normal values in the one surviving subject.

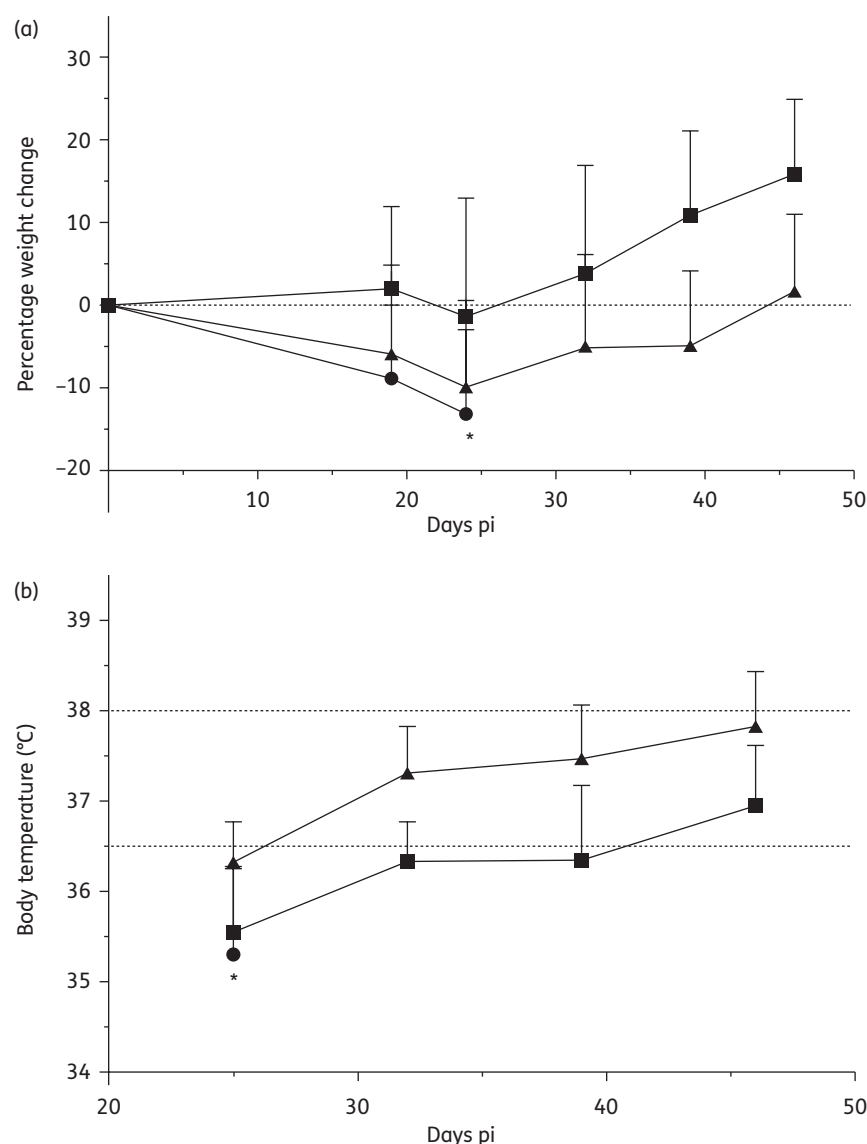


Figure 1. (a) Percentage change in body weight of infected mice measured during the acute phase of Chagas' infection. Initial baseline weight (0% change) is indicated by the broken line. (b) Body temperature of mice obtained through rectal measurements. Range for normal corporal temperatures in mice (36.5–38°C) is indicated with broken lines. Error bars show standard deviations. NT mice, circles; benznidazole-treated mice, triangles; voriconazole-treated mice, squares. The asterisk indicates the last timepoint when the number of surviving NT subjects was greater than or equal to three ($n < 3$ not shown).

Parasitaemia treatment response

In our model, mice infected with the Tulahuén strain of *T. cruzi* exhibited the classical pattern of parasitaemia during the acute phase of infection (Figure 2). Trypomastigotes were detected in all animals by 10 days pi, peaking at 26 days pi and declining gradually to low values by 42 days pi in the surviving animals. At 26 days pi, mean peak parasitaemia (expressed as 10^4 trypomastigotes/mL \pm standard deviation) was 1275.75 ± 781.19 for NT mice, 238.00 ± 195.27 for voriconazole-treated mice and 0.00 ± 0.00 for benznidazole-treated mice. Non-parametric analysis of variance (ANOVA; Kruskal–Wallis) showed a statistically significant difference among groups ($P < 0.001^*$). NT animals

had significantly higher parasitaemia levels than animals treated with voriconazole ($P = 0.0014^*$) or benznidazole ($P < 0.001^*$). Treatment with voriconazole lowered peak parasitaemia at 26 days pi by 81%. Treatment with benznidazole proved significantly more effective than voriconazole ($P < 0.001^*$), rendering a 100% reduction (i.e. negative parasitaemia in all treated mice).

Mortality

Survival rates at the conclusion of the trial were 10% (1/10) for NT mice, 75% (6/8) for mice treated with voriconazole and 100% (8/8) for mice treated with benznidazole. Voriconazole-treated

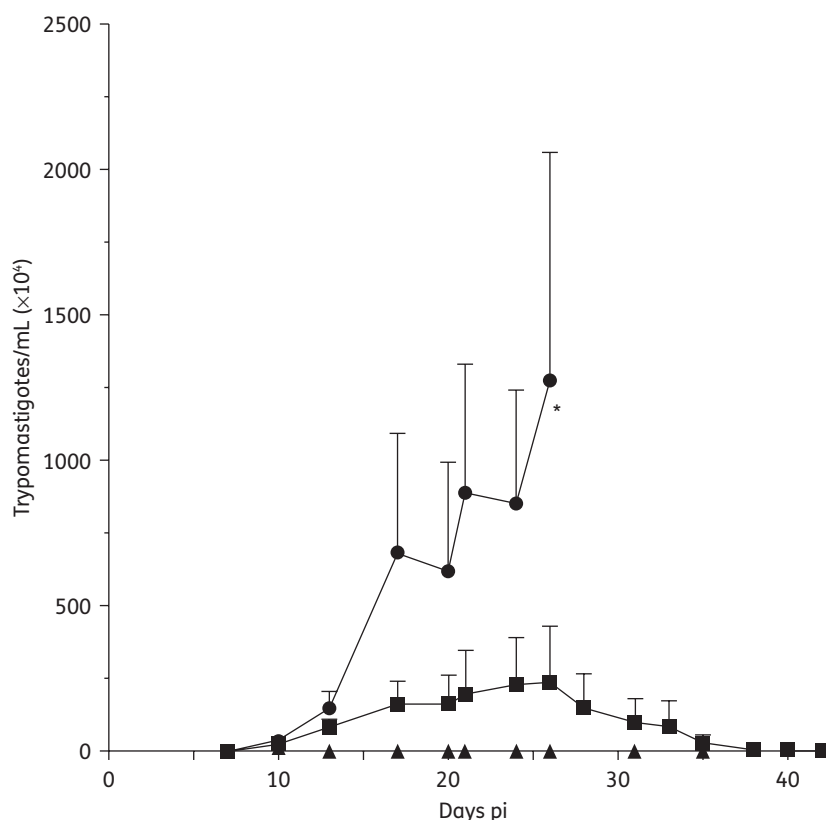


Figure 2. Parasitaemia curve of infected mice that were either NT controls (circles) or treated with voriconazole (squares) or benznidazole (triangles) followed through 42 days pi. Error bars show standard deviations. The asterisk indicates the last timepoint when the number of surviving NT subjects was greater than or equal to three ($n < 3$ not shown).

mice were statistically more likely to survive ($P=0.013^*$) and lived significantly longer ($P=0.007^*$) than NT mice. Similarly, as expected, comparison of the survival curves (Figure 3) indicates that benznidazole treatment prolonged survival compared with NT mice ($P<0.001^*$) and lowered the risk of mortality ($P<0.001^*$). However, no statistically significant differences could be demonstrated between the benznidazole and voriconazole treatment groups regarding lifespan ($P=0.144$) and survival odds ($P=0.467$).

Histopathology

In voriconazole-treated mice, parasites were detected in the myocardium in two of eight animals (25%) and in the skeletal muscle in three of eight animals (37.5%). The two animals that had nests in both skeletal and myocardial tissue were the same two mice that did not survive until study completion. Amastigote nests were not observed in the skeletal muscle from mice treated with benznidazole; however, one of the eight mice (12.5%) displayed parasites in the myocardium. In NT animals, five out of six (83.3%) and four out of six (66.7%) were positive in the myocardium and the skeletal muscle, respectively. The only NT subject that survived was also the only animal to not have nests present in either tissue type. There was no significant difference between the benznidazole- and voriconazole-treated groups' proportion of mice containing parasites in either muscle type. Likewise,

voriconazole-treated and NT mice were not statistically different regarding the percentage of mice with nests in heart ($P=0.103$) or skeletal ($P=0.592$) muscle. After adjustment of the significance threshold to account for multiple pair-wise comparisons, benznidazole-treated and NT myocardial proportions were also not significantly different ($P=0.026$); however, benznidazole treatment did lead to significantly fewer mice with amastigote nests in their skeletal muscle compared with NT controls ($P=0.015^*$). These non-significant P values are probably due to the small number of viable histological samples studied, as the results illustrate that voriconazole reduces the number of mice with parasites compared with NT controls.

Voriconazole- and benznidazole-treated animals displayed myocardial inflammatory scores that ranged from 0 to 2, whereas the NT control group had scores ranging from 0 to 3 (Figure 4). Non-parametric ANOVA (Kruskal-Wallis) showed no statistically significant difference among the groups' myocardium inflammation scores ($P=0.236$). In the skeletal muscle, scores continued to range between 0 and 2 for benznidazole-treated mice. However, significantly higher inflammation of the skeletal muscle was observed in voriconazole-treated and NT control mice compared with subjects receiving benznidazole, with scores from 1 to 4 ($P=0.002^*$) and 1 to 3 ($P=0.005^*$), respectively. Voriconazole treatment was not statistically more effective than no treatment in reducing skeletal muscle inflammation ($P=0.815$).

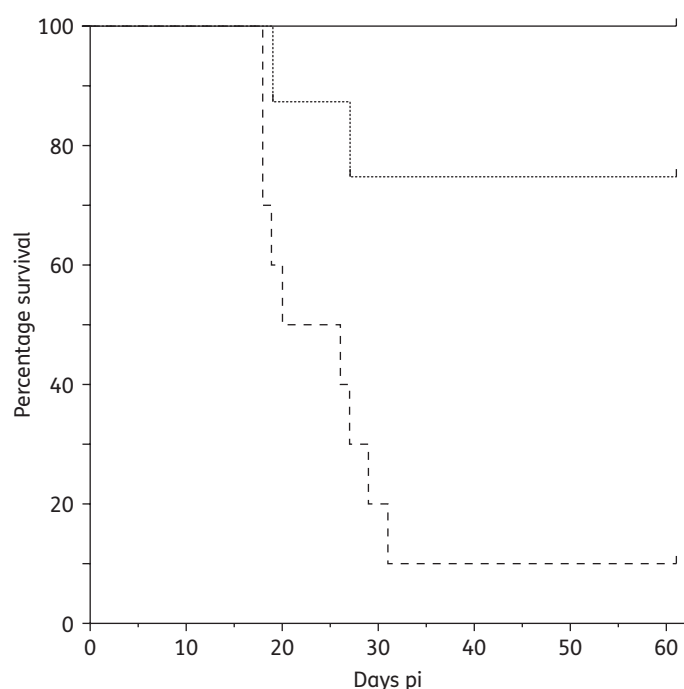


Figure 3. Cumulative survival curves of mice infected with *T. cruzi* throughout the acute stage of parasitosis. NT mice, dashed broken line; benznidazole-treated mice, continuous line; voriconazole-treated mice, dotted broken line.

Discussion

Voriconazole was selected as a promising chemotherapeutic agent because previous studies have demonstrated that voriconazole possesses *in vitro* activity against *T. cruzi*,¹⁵ and the clinical pharmacology and toxicity of this drug are well studied in adults and children, with relatively few adverse events reported.^{16–18} Experimentally, several other azole derivatives have also been tested, including posaconazole,¹⁹ ravuconazole²⁰ and albaconazole.²¹ However, unlike voriconazole, clinical experience with these drugs in humans is limited and the safety and pharmacokinetic profiles are still not fully understood.

Even though *in vitro* voriconazole activity against *T. cruzi* is relatively weak compared with other azole derivatives, such as ketoconazole and posaconazole, its benign toxicity profile allows potentially higher doses to be used to overcome this relatively low potency.²² Benznidazole likewise displays weak *in vitro* anti-*T. cruzi* activity, but is remarkably effective *in vivo* and the reason for this discordance is not understood.¹⁵ Thus, we speculated that voriconazole might also display high activity in an animal model.

Although many animal models exist for Chagas' disease, mice were chosen as the most suitable *in vivo* model in this study for several reasons. Practically, mice are economical and their immune system has been extensively studied. In addition, an inbred line of infected mice already exists that reproduces histopathological lesions and clinical manifestations similar to the human condition. While acute Chagas' disease is often asymptomatic and difficult to detect in other animal models, the infection with pathogenic strains in mice is evident—with high

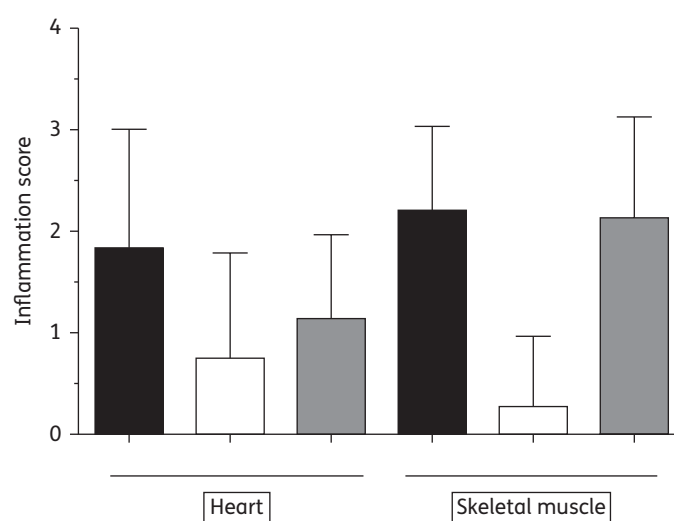


Figure 4. Inflammation score of heart and skeletal muscle samples from mice infected with *T. cruzi*. Increasing score indicates more severe histopathological lesions. Error bars show standard deviations. NT mice, black bars; benznidazole-treated mice, white bars; voriconazole-treated mice, grey bars.

parasitaemia and mortality—and therefore has been proposed as a useful method to evaluate and compare the therapeutic efficacy of new compounds.^{23,24} The Tulahuén strain of *T. cruzi* was chosen for infection because it displays a reproducible parasitaemia curve and is not resistant to benznidazole treatment.²⁵

In our murine model of acute *T. cruzi* infection, voriconazole treatment was well tolerated in mice, with few side effects observed and healthy weight maintained throughout the study. The benznidazole-treated mice gained significantly less weight in spite of appropriate parasitological response, suggesting the observed toxicity is drug related. Even though benznidazole is not commonly associated with anorexia or weight loss in humans, many patients report gastrointestinal discomfort at the beginning of treatment.²⁶ Therefore, voriconazole might be a suitable alternative in patients whose digestive intolerance causes benznidazole treatment to be suspended.

Treatment with voriconazole significantly reduced peak parasitaemia compared with NT mice. Additionally, significantly increased lifespan and decreased mortality were observed in voriconazole-treated mice compared with NT control mice. Histopathological analyses show that voriconazole decreases the proportion of mice with parasite nests present in their heart and skeletal muscles. Results also suggest that voriconazole is moderately effective in reducing inflammation of the myocardium, but does not lessen inflammation of the skeletal muscle. Furthermore, this experiment presented a reliable murine model to use in the search for new trypanocidal agents, as the results obtained with benznidazole were consistent with the treatment response observed in humans.

Additional studies using voriconazole need to be performed with a greater number of subjects, varying doses and different treatment methods. One particular treatment scheme of interest is combination benznidazole/voriconazole therapy, which has synergic potential as the compounds act on different cellular

targets, and could thus reduce the toxicity of benznidazole and possibly improve chemotherapeutic success in benznidazole-resistant strains of *T. cruzi*.²⁷ In addition to examining the trypanocidal activity of voriconazole at higher doses, our future research may attempt to improve the absorption of voriconazole, and therefore increase effective blood concentration, by adding grapefruit juice to the diet, as reported in other work.²⁸ We also plan to investigate voriconazole's impact on chronic Chagas' infection and check for any toxicity caused by long-term treatment. In conclusion, our findings illustrate the possibility of identifying, among commercially available compounds, drugs such as voriconazole that could improve the treatment of Chagas' disease.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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