Amlodipine Increases the Therapeutic Potential of Ravuconazole upon *Trypanosoma cruzi* Infection

Yara Almeida Machado,^a Maria Terezinha Bahia,^b Ivo Santana Caldas,^a Ana Lia Mazzeti,^c Rômulo Dias Novaes,^a Breno Raimundo Vilas Boas,^a Lorena Júnia de Souza Santos,^d Olindo Assis Martins-Filho,^d Marcos José Marques,^a Livia de Figueiredo Diniz^a

^aLaboratório de Parasitologia Básica, Programa de Pós-Graduação em Ciências Biológicas, Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, Alfenas, Brazil

^bLaboratório de Doenças Parasitárias, Escola de Medicina & Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Brazil

^cLaboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

^dGrupo Integrado de Biomarcadores de Diagnóstico e Monitoração, Instituto René Rachou, FIOCRUZ-Minas, Belo Horizonte, Brazil

ABSTRACT Mining existing agents that enhance the therapeutic potential of ergosterol biosynthesis inhibitors (EBI) is a promising approach to improve Chagas disease chemotherapy. In this study, we evaluated the effect of ravuconazole, an EBI, combined with amlodipine, a calcium channel blocker, upon *Trypanosoma cruzi* experimental infection. *In vitro* assays confirmed the trypanocidal activity of both compounds in monotherapy and demonstrated an additive effect (sum of the fractional inhibitory concentration [Σ FIC] > 0.5) of the combined treatment without additional toxicity to host cells. *In vivo* experiments, using a murine model of the *T. cruzi* Y strain in a short-term protocol, demonstrated that amlodipine, although lacking trypanocidal activity, dramatically increased the antiparasitic activity of underdosing ravuconazole regimens. Additional analysis using long-term treatment (20 days) showed that parasitemia relapse until 60 days after treatment was significantly lower in mice treated with the combination (4 out of 14 mice) than ravuconazole monotherapy (10 out of 14 mice), even in the presence of immunosuppressant pressure. Furthermore, the combined therapy was well tolerated and protected the mice from mortality. The treatments also impacted on the cellular and humoral immune response of infected animals, inducing a reduction of serum cytokine levels in all ravuconazole-treated mice. Our findings demonstrate that amlodipine is efficacious in enhancing the antiparasitic activity of ravuconazole in an experimental model of *T. cruzi* infection and indicates a potential strategy to be explored in Chagas disease treatment.

KEYWORDS Chagas disease, calcium channel blockers, combination therapy, experimental chemotherapy, repurposing, synergism

Chagas disease, caused by the flagellate protozoan *Trypanosoma cruzi*, affects around 8 million people worldwide (1). In Latin America, where it is endemic, the infection remains one of the biggest public health problems, causing incapacity in infected individuals and more than 10,000 deaths per year (1). Due to the migration of *T. cruzi*-infected individuals, the parasitosis is an emerging infectious disease in regions of nonendemicity, such as the United States and Europe (2).

Two drugs are recognized by the World Health Organization to treat Chagas disease, benznidazole (Bz), and nifurtimox (Nfx). Although these drugs have considerable effectiveness for the treatment of acute infection, they have very limited activity in inducing parasitological cure at the chronic phase of the disease (3), when most patients are diagnosed. Furthermore, both medicines are administered as a long-term

Citation Machado YA, Bahia MT, Caldas IS, Mazzeti AL, Novaes RD, Vilas Boas BR, Santos LJD, Martins-Filho OA, Marques MJ, Diniz LDF. 2020. Amlodipine increases the therapeutic potential of ravuconazole upon *Trypanosoma cruzi* infection. Antimicrob Agents Chemother 64:e02497-19. <https://doi.org/10.1128/AAC.02497-19>.

Copyright © 2020 American Society for Microbiology. All Rights Reserved.

Address correspondence to Livia de Figueiredo Diniz, liviadinizcastro@gmail.com.

Received 13 December 2019

Returned for modification 10 February 2020

Accepted 5 May 2020

Accepted manuscript posted online 18 May 2020

Published 22 July 2020

TABLE 1 *In vitro* effect of ravuconazole and amlodipine against trypomastigotes and intracellular amastigotes of *Trypanosoma cruzi* (EC₅₀) and cytotoxicity in mammalian cells (CC₅₀)

Organism and parameter	Benznidazole (μ M)	Ravuconazole (nM)	Amlodipine (μ M)
Trypomastigote EC ₅₀	15.6 $\pm$ 5.52	79.5 $\pm$ 2.15	4.9 $\pm$ 0.14
Amastigote EC ₅₀	6.15 $\pm$ 1.90	1.84 $\pm$ 0.30	2.75 $\pm$ 0.06
Host cell CC ₅₀ (SI) ^a	>200 (>40)	>200 (>100)	33.2 $\pm$ 8.7 (12)

^aSI, selectivity index. This was calculated based on intracellular amastigotes.

treatment (60 days) and induce substantial adverse events that lead to treatment discontinuation, typically in 15% to 20% of patients (4).

During the search for new alternatives to treat *T. cruzi* infection, important knowledge of Chagas disease chemotherapy has been gathered in recent years. A vast number of anti-*T. cruzi* molecules have been evaluated, originating from either phenotypical or rational screening or from innovative strategies, including drug repurposing and combination therapy. Despite these efforts, a very limited number of molecules have demonstrated therapeutic efficacy in animal models of Chagas disease (5), an important step in the translational chain.

Antifungal lanosterol demethylase (CYP51) inhibitors, such as posaconazole and ravuconazole, are among the most promising candidates for Chagas disease treatment, as both compounds have been recently evaluated in clinical studies for Chagas disease (5). Of particular interest, ravuconazole is the active molecule of E1224 (or fosravuconazole), a water-soluble prodrug that was the first new chemical entity developed for Chagas disease in over 3 decades (6). Both ravuconazole and E1224 demonstrated potent and specific anti-*T. cruzi* activity in preclinical models (7–9). However, despite good tolerability and safety for clinical use, E1224 failed to induce a sustained antiparasitic effect in human chronic disease (6). Similar results were achieved with the azole derivative posaconazole (10, 11). However, considering the promising results obtained with ravuconazole and its prodrug in preclinical combination assays, the scarce panel of anti-*T. cruzi* active compounds *in vivo*, and the clinically favorable safety profile of azole molecules (6, 10), alternative therapeutic schemes based on ravuconazole/E1224, especially in combination with other drugs, should be explored.

In an ongoing effort to identify alternative rational and effective treatments, repositioning strategies based on antiarrhythmic and antihypertensive agents have indicated promising antiparasitic effects of calcium channel blockers (CCB) on *Plasmodium falciparum*, *Leishmania donovani*, and *T. cruzi* (12). Interestingly, there is evidence that CCB can interact positively with the reference drugs (Bz and Nfx) and azolic derivatives upon *T. cruzi* infection (13, 14). In particular, amlodipine, a calcium channel antagonist widely used for the treatment of hypertension, has demonstrated potent activity against trypanosomatids *in vitro* (12, 14). Although poorly explored, this drug also presented a promising effect in controlling *T. cruzi* infection when combined with an azolic derivative *in vivo* (14). Considering the antiparasitic potential of amlodipine and its favorable physicochemical properties to interact with azoles, we used *in vitro* and *in vivo* models to evaluate the relevance of a new chemotherapy combination based on ravuconazole and amlodipine in the treatment of *T. cruzi* infection.

RESULTS

First, we checked the *in vitro* effect of ravuconazole and amlodipine in monotherapy and in combination against trypomastigotes and intracellular amastigotes of *T. cruzi*. As shown in Table 1, amlodipine presented anti-*T. cruzi* activity in the micromolar range, with similar 50% effective concentrations (EC₅₀s) against amastigotes (2.75 $\pm$ 0.06 μ M) and trypomastigotes (4.9 $\pm$ 0.14 μ M). As expected, ravuconazole was highly effective against amastigotes at the nanomolar level (EC₅₀, 1.84 $\pm$ 0.30 nM), but its capacity to induce the death of trypomastigotes was limited (Table 1). In addition, Bz was active in the low micromolar range against intracellular amastigotes and trypomastigotes (Table 1). Regarding *in vitro* toxicity to mammalian cells, ravuconazole presented no

FIG 1 *In vitro* toxicity of ravuconazole and amlodipine combinations on mammalian cells. Reduction of viability of H9c2 cells incubated for 72 h at different concentrations of ravuconazole (Rav) and amlodipine (Amlo) as monotherapy or combination. Different letters above the bars indicate statistical differences among the groups ($P < 0.05$), and groups that have the same letters do not differ statistically ($P > 0.05$). Data are expressed as the mean $\pm$ the standard deviation (SD) of two independent experiments performed in triplicate. Groups were compared using Tukey's multiple-comparison test.

evidence of toxicity at concentrations up to 100 nM (Fig. 1A), while amlodipine demonstrated a moderate concentration-dependent toxicity profile (50% lethal concentration [LC_{50}], $33.2 \pm 8.7 \mu\text{M}$), resulting in a selective index of 11.8 in our model (Table 1). The addition of ravuconazole did not increase the host cell toxicity of the partner drug (Fig. 1).

In the next step, we checked if amlodipine could improve the trypanocidal effect of ravuconazole. The nature of the interaction between the drugs against trypomastigotes was screened using a fixed-ratio protocol, and the data were analyzed at the EC_{50} level. As shown in Fig. 2A and B, a leftward shift of the combined-therapy curve was identified

FIG 2 *In vitro* effects of ravuconazole and amlodipine combinations on *T. cruzi* trypomastigotes and amastigotes. (A and B) Dose-response curves for ravuconazole (Rav) (A) and amlodipine (Amlo) (B) alone (black lines) and in fixed-ratio combinations (colored lines) against trypomastigote forms of the *T. cruzi* Y strain. (C and D) Graphical representations of the interaction between ravuconazole and amlodipine against trypomastigotes (C) and amastigotes (D).

FIG 3 Effect of ravuconazole (Rav) and amlodipine (Amlo) in monotherapy or combined on parasitemia in mice infected by the *T. cruzi* Y strain. Oral treatment started 5 days after infection. The drugs were administered daily for 5 consecutive days ($n = 6$ mice/group). INT, infected untreated; Bz, benznidazole; mpk, $\text{mg} \cdot \text{kg}^{-1}$. The inset shows the area under the parasitemia curve. Different letters above the bars indicate statistical differences among the groups ($P < 0.05$), and groups that have the same letters do not differ statistically ($P > 0.05$).

for ravuconazole, but not for amlodipine, suggesting an additive effect resulting from different mixtures. The value of the mean sum of the fractional inhibitory concentration (ΣFIC) was 1.53, confirming the additive nature of ravuconazole and amlodipine association, represented in Fig. 2C. Interestingly, when intracellular amastigotes were assayed using a 1:1 concentration of each drug in a 2-fold serial dilution, an additive effect near synergism was detected (Fig. 2D), with $\Sigma\text{FIC} = 0.56$.

To verify the effects of ravuconazole and amlodipine combined therapy upon the *T. cruzi* infection *in vivo*, we used a short treatment protocol (5 consecutive days of drug administration starting on day 5 postinfection) employing ravuconazole underdosing schemes in monotherapy (0.5, 1.0, and 5.0 $\text{mg} \cdot \text{kg}^{-1}$) and in combination with amlodipine at 10 $\text{mg} \cdot \text{kg}^{-1}$. Figure 3 shows the parasitemia curves of Swiss mice infected with the *T. cruzi* Y strain, treated or not treated with the different schemes. The parasitemia peak was detected 8 days postinfection with an average of $1.09 \pm 0.13 \times 10^6$ parasites/0.1 ml of blood from untreated mice. Ravuconazole at doses as low as 1 $\text{mg} \cdot \text{kg}^{-1}$ was barely effective in reducing parasitemia, while at 5 $\text{mg} \cdot \text{kg}^{-1}$ it was similar to the reference dose of Bz at 100 $\text{mg} \cdot \text{kg}^{-1}$ (Fig. 3). In contrast, the mice treated with 0.5 $\text{mg} \cdot \text{kg}^{-1}$ ravuconazole and those left untreated showed a similar peak of parasitemia. In addition, amlodipine was unable to prevent parasite proliferation (Fig. 3), and mice experienced parasitemia levels similar to the control and ravuconazole 0.5 $\text{mg} \cdot \text{kg}^{-1}$ groups ($P > 0.05$) (Fig. 3, inset). Interestingly, the association with 10 $\text{mg} \cdot \text{kg}^{-1}$ amlodipine dramatically increased the anti-*T. cruzi* activity of ravuconazole at 0.5 $\text{mg} \cdot \text{kg}^{-1}$, inducing parasitemia suppression in all combination-treated mice during the evaluated period (Fig. 3). The treatments were also well tolerated, and no mortality was detected among animals receiving ravuconazole, in monotherapy or combined therapy, or in Bz-treated mice. In contrast, 60% of mice receiving amlodipine as monotherapy or those left untreated died by 14 days postinfection, when the experiment terminated (data not shown).

Aiming to check if amlodipine could improve the antiparasitic effect of ravuconazole in a sustainable manner, a second set of assays was conducted employing a higher concentration of this azole (5.0 $\text{mg} \cdot \text{kg}^{-1}$) in monotherapy and in combination with 10 $\text{mg} \cdot \text{kg}^{-1}$ of amlodipine. Here, we performed long-term treatment (20 days), and the therapeutic efficacy was investigated using two different and independent protocols, in the presence or absence of cyclophosphamide-induced immunosuppression. In mice

TABLE 2 *In vivo* effects of ravuconazole and amlodipine combination upon experimental *Trypanosoma cruzi* murine infection^a

Experiment group ^b	AUC (mean $\pm$ SD) ^{c,e}		Positive tests (%) ^d	
	Exp 1	Exp 2	Exp 1	Exp 2
Infected and untreated	2,786 $\pm$ 977.8 ^{&}	11,14.5 $\pm$ 487 [#]	6/6 (100%)	8/8 (100%)
Benznidazole 100 mg $\cdot$ kg ⁻¹	14.1 $\pm$ 5.8	7.5 $\pm$ 5.3	3/6 (50%)	1/8 (87.5%)
Amlodipine 10 mg $\cdot$ kg ⁻¹	3,089 $\pm$ 1,777.3 ^{&}	868.4 $\pm$ 335.4 [#]	6/6 (100%)	8/8 (100%)
Ravuconazole 5 mg $\cdot$ kg ⁻¹	14.2 $\pm$ 5.8	8.75 $\pm$ 6.4	5/6 (83.3%)	5/8 (62.5%)
Ravuconazole 5 mg $\cdot$ kg ⁻¹ plus amlodipine 10 mg $\cdot$ kg ⁻¹	19.2 $\pm$ 22.2	5.1 $\pm$ 3.8	2/6 (33.3%)	2/8 (25%)
Ravuconazole 2.5 mg $\cdot$ kg ⁻¹	ND	8.25 $\pm$ 8.2	ND	8/8 (100%)
Ravuconazole 2.5 mg $\cdot$ kg ⁻¹ plus amlodipine 10 mg $\cdot$ kg ⁻¹	ND	7.5 $\pm$ 4.6	ND	4/8 (50%)

^aND, not done.^bThe treatments were initiated on day 5 postinfection and administered for 20 days orally (p.o.).^cArea under the parasitemia curve until 12 days postinfection.^dNumber of positive tests until 60 days after treatment. Exp 1, parasitemia reactivation after immunosuppression with cyclophosphamide. Exp 2, natural reactivation of parasitemia and blood PCR.^eSimilar superscript symbols (& or #) indicate statistical similarity in the same experiment.

not subjected to cyclophosphamide treatment, therapeutic failure was detected by natural parasitemia reactivation and blood PCR. For both approaches, the results were compared with the monotherapies and Bz-treated mice at the standard dose (Table 2). Our results show that all infected and untreated mice presented high parasitemia levels and 50% to 100% mortality, occurring on average at 12 to 20 days postinfection. As observed in the short-term experiment, amlodipine failed to demonstrate significant anti-*T. cruzi* activity, with area under the parasitemia curve (AUC) values very close to those of untreated animals ($P > 0.05$) in both protocols and with mortality rates of 85% to 100%. In contrast, ravuconazole at 5 mg $\cdot$ kg⁻¹, as monotherapy and in combination with amlodipine, similar to Bz, completely suppressed parasitemia, with no difference in the AUC of these groups (Table 2). One hundred percent survival was observed in these groups until 60 days after infection.

Figure 4A shows that monotherapy-based treatments were well tolerated by healthy mice, with no impact on body weight. Conversely, combined therapy induced a slight decrease in weight gain compared to the untreated group ($P = 0.004$). However, this effect was suppressed after the cessation of treatment (data not shown). Regarding infected mice, all the drug schemes, except for amlodipine, had positive effects on the outcomes of acute infection by the Y strain, preventing the marked loss of weight observed in untreated mice (Fig. 4B). In addition, plasma levels of the liver transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) detected on the last day of treatment were not increased in uninfected mice in response to the different treatments (Fig. 4C, left). On the other hand, *T. cruzi* infection increased AST levels ($P < 0.05$), while ravuconazole and amlodipine monotherapies (two surviving mice) did not amplify this effect. Furthermore, the combined treatment, as well as benznidazole, reduced AST levels (Fig. 4C, right). In general, intragroup ALT levels were heterogeneous and similar among the experimental groups (Fig. 4D).

Despite the potent suppression of parasitism, a parasitemia relapse was detected in all experimental groups after the completion of treatment, especially among those treated with monotherapies. Relapse rates of 83.3% (5/6) and 62.5% (5/8) were observed in the ravuconazole monotherapy-treated groups in the presence or absence of the immunosuppressant, respectively. On the other hand, the combined therapy decreased infection recrudescence by about 2.5-fold compared to ravuconazole monotherapy, even after the immunosuppression protocol (Table 2). Considering treatment with a low dose of ravuconazole (2.5 mg/kg), the combined therapy reduced the *T. cruzi* positivity of parasitological and molecular tests by 2-fold (Table 2). These results emphasize that the effect of ravuconazole was sustainably potentiated by amlodipine, and this combination was more effective in reducing parasite levels than ravuconazole monotherapy.

Considering the anti-*T. cruzi* humoral response up to 60 days after the end of the treatment, our findings corroborate the reduction in parasite load achieved by ravu-

FIG 4 Influence of 20 days of treatment with ravuconazole and amlodipine on weight gain and hepatic function of health and infected mice. (A and B) Weight gain in uninfected (A) and infected (B) mice treated with ravuconazole at $5 \text{ mg} \cdot \text{kg}^{-1}$ (Rav), amlodipine at $10 \text{ mg} \cdot \text{kg}^{-1}$ (Amlo), or a combination (Rav at $5 \text{ mg} \cdot \text{kg}^{-1}$ plus Amlo at $10 \text{ mg} \cdot \text{kg}^{-1}$). (C) AST and (D) ALT values detected on plasma samples at the last day of the treatment. NINT, uninfected untreated mice; INT, infected untreated group. Different letters above the bars indicate statistical differences among the groups ($P < 0.05$), and groups that have the same letters do not differ statistically ($P > 0.05$). *, statistical difference compared to infected untreated animals.

conazole treatment and demonstrated by parasitological tests. Figure 5 presents the impact of treatment with the drugs alone or in combination on anti-*T. cruzi* IgG serum levels 30 days (Fig. 5A) and 60 days (Fig. 5B) after treatment. Increased levels of antibodies were detected in sera of infected untreated and amlodipine-treated mice (Fig. 5A). In contrast, in the groups receiving ravuconazole, as monotherapy and combined with amlodipine, antibody levels were significantly lower ($P < 0.0001$) and similar to those detected in Bz-treated animals (Fig. 5A). Even at 60 days after infection, the same pattern was maintained (Fig. 5B).

The cytokine profile of animals from the different experimental groups was assessed in serum samples 30 days after the end of the treatment. The results show that all cytokines evaluated were significantly increased by *T. cruzi* infection (Fig. 6). Amlodipine as a monotherapy failed to interfere with this interleukin expression pattern and was similar to the infected and untreated control. Conversely, the production of pro- and anti-inflammatory cytokines was markedly reduced in mice treated with ravuconazole alone, especially when combined with amlodipine. In this case, the levels were similar to those observed in Bz-treated animals (Fig. 6). In particular, levels of tumor necrosis factor- α (TNF- α), an important molecule involved in Chagas disease pathology, were significantly reduced ($P < 0.017$) by the combined therapy compared to ravuconazole monotherapy (Fig. 6).

DISCUSSION

Recent drug development efforts for Chagas disease have focused on repurposing drugs, particularly antifungal azoles that inhibit the biosynthesis of ergosterol targeting

FIG 5 Anti-*T. cruzi* IgG levels 30 and 60 days after treatment with ravuconazole plus amlodipine. (A and B) Specific IgG antibodies in serum samples collected from *T. cruzi*-infected mice 30 (A) and 60 (B) days after treatment with ravuconazole at 5 mg · kg⁻¹ (Rav) and amlodipine at 10 mg · kg⁻¹ (Amlo) in monotherapies or combination (Rav+Amlo) for 20 days. NINT, noninfected and untreated mice; INT, infected and untreated mice; Bz, benznidazole. Different letters above the symbols indicate statistical differences among the groups ($P < 0.05$), and groups that have the same letters do not differ statistically ($P > 0.05$).

CYP51 (5, 15–17). Ergosterol is essential for providing structure and function of *T. cruzi* membranes (7, 15, 17), and a number of azoles originally developed to treat fungal diseases have shown strong anti-*T. cruzi* activity *in vitro* and *in vivo* (15, 16). However, in contrast with remarkable activity in preclinical models, the azole derivatives have not translated into sustainable clearance of parasitemia in patients with chronic Chagas disease (6, 10). In agreement with these results, Moraes et al. (18) reported *T. cruzi* genotypes resistant to ergosterol biosynthesis inhibitors *in vitro*, including posaconazole and ravuconazole (18). In another set of *in vivo* experiments, using a more sensible readout in nonclinical assays, posaconazole was found to be significantly inferior to benznidazole as a treatment for both acute and chronic *T. cruzi* murine infections (19). In this context, the use of azole derivatives in monotherapy to treat *T. cruzi* infection has been discouraged (6). On the other hand, some authors have discussed whether the therapeutic failure observed with posaconazole and ravuconazole in clinical trials may be related to suboptimal drug exposure and inadequate drug levels in tissues (5, 6, 10, 11, 15, 17). In particular, ravuconazole has a poor pharmacokinetic (PK) profile, and this issue has prompted the development of prodrugs; E1224 is the first new entity specifically developed for Chagas disease (6). This prodrug improved PK properties and had higher cure rates in acute infection caused by a Bz-partially resistant strain (Y), but it was unable to cure established infections in experimental models infected by a resistant strain (Colombiana) (9). Similarly, E1224 had a suppressive but not sustained effect on parasite clearance in human chronic Chagas disease (6).

Although the reasons for drug resistance in *T. cruzi* are not understood (20, 21), the aforementioned data highlight the importance of exploring strategies to increase the therapeutic potential of azole derivatives in Chagas disease (10, 17).

Accumulating evidence has indicated that combination therapy is a promising approach to achieve a better response in the treatment of *T. cruzi* experimental infection (5, 9, 16, 22–24). In this context, a proof-of-concept evaluation of new benznidazole-sparing regimens in combination with E1224 and in monotherapy is ongoing. The results of the BENDITA study (Benznidazole New Doses Improved Treatment and Associations; ClinicalTrials.gov identifier [NCT03378661](https://clinicaltrials.gov/ct2/show/study/NCT03378661)) demonstrated that shorter protocols with benznidazole were efficient in controlling the parasitism, and E1224 did not increase the effect of benznidazole, but the analysis of the combination arms to assess its potential indication for certain groups of patients with specific needs is in progress (25).

FIG 6 Serum levels of cytokines in Swiss mice 30 days after treatment with ravuconazole and amlodipine in monotherapy and combination. Plasma levels of cytokines detected in noninfected (NI) and infected mice untreated (INT) or treated with ravuconazole at 5 mg $\cdot$ kg $^{-1}$ (Rav), amlodipine at 10 mg $\cdot$ kg $^{-1}$ (Aml), and a combination at the same doses (Rav+Aml). MFI, median fluorescence intensity. Different letters above the bars indicate statistical differences among the groups ($P < 0.05$), and groups that have the same letters do not differ statistically ($P > 0.05$).

Previous reports have indicated that ergosterol inhibitors can interact positively with antagonists of calcium channels against fungal organisms (26, 27) and trypanosomatids (12–14). Here, we investigate if amlodipine, a CCB used clinically as an antihypertensive agent, could increase the anti-*T. cruzi* activity of ravuconazole *in vitro* and *in vivo*.

Using an acute murine model of infection by the *T. cruzi* Y strain in a short-term strategy, we first demonstrated that amlodipine, despite its *in vitro* activity, did not exert any promising trypanocidal effect *in vivo*. However, this drug dramatically increased the trypanocidal effect of ravuconazole subdoses, reducing the AUC values by more than 36-fold compared to azole monotherapy at the same dose (Fig. 2). In this short-term protocol, we treated the animals for only 5 days with ravuconazole at very low doses in order to facilitate observation of effects of drug combinations on bloodstream parasitemia. Thus, in an attempt to investigate the long-lasting outcomes of the ravuconazole plus amlodipine combination, we further evaluated it in a long-term protocol using ravuconazole at a higher dose, 5 mg $\cdot$ kg $^{-1}$. Given that parasitemia control and protection of mortality are parameters achieved with ravuconazole in monotherapy at doses as low as 1.0 mg $\cdot$ kg $^{-1}$ (Fig. 2), the outcomes of ravuconazole (5 mg $\cdot$ kg $^{-1}$) plus amlodipine (10 mg $\cdot$ kg $^{-1}$) combined treatment on parasite load were measured overall using parasitemia relapse after treatment. We used a stringent strategy based on distinct protocols in the presence (experiment 1) or absence (experiment 2) of cyclophosphamide immunosuppression (Cyl). In addition, in experiment 2, an alternative protocol using ravuconazole at 2.5 mg $\cdot$ kg $^{-1}$ was included. The findings show that ravuconazole effected lasting parasitism inhibition in a dose-dependent manner, with parasitemia detection after the end of treatment (natural reactivation or PCR) in 100% and 62.5% of mice treated with 2.5 and 5.0 mg $\cdot$ kg $^{-1}$, respectively. Using the immunosuppression protocol, the failure rate in the ravuconazole-treated group

(5 mg · kg⁻¹) reached 83.3% (Table 2). Previous reports, using similar experimental models, demonstrated that ravuconazole (7, 9) and its prodrug E1224 (9) have a limited dose-response relationship; however, in such studies, minimum doses of 5 or 10 mg · kg⁻¹ were administered. Interestingly, mice treated with ravuconazole and amlodipine in combination experienced lower rates of parasitemia rebound, regardless of the protocol employed to investigate therapeutic failure. Considering the results of both experiments together, 10 out of 14 (71.4%) mice treated with ravuconazole at 5 mg · kg⁻¹ as monotherapy experienced therapeutic failure, while this index dropped to 28.6% (4 out of 14) when the same dose was associated with amlodipine. The effect of the drug combination was evident with an even lower dose of ravuconazole, 2.5 mg · kg⁻¹; in this case, the association with the partner drug reduced the detection of parasites after treatment by 50% (Table 2). These results were corroborated by the serological evaluation, showing a notable attenuation in the humoral response until 60 days after the end of treatment related to parasite control, as observed in another studies (8, 9, 22, 24).

Amlodipine is among the best-studied agents that reverse resistance of *Plasmodium* to antimalarial agents (28), and accumulating evidence suggests that some CCBs can enhance the antifungal activity of azole derivatives by interfering with efflux pumps and/or influencing calcium signaling pathways (26, 27). The drug has also exhibited broad-spectrum antibacterial activities and *in vitro* synergistic antimicrobial effects on multidrug-resistant microorganisms (29). Notably, positive interactions between CCBs and nitroheterocyclic or azolic compounds have been documented against *T. cruzi* infections in experimental models (13, 14).

Here, we demonstrated that amlodipine increases the anti-*T. cruzi* effect of ravuconazole in a sustainable manner, considering the experimental follow-up up to 60 days after the end of treatment. Given the complete absence of trypanocidal activity observed for the CCB in monotherapy *in vivo* and the additive profile resulting from the association with ravuconazole observed *in vitro*, it is possible to hypothesize that the positive effect observed in the animal model may result from a pharmacological interaction between the drugs *in vivo*. Amlodipine is metabolized by CYP3A4 into inactive metabolites (30). Ravuconazole is a moderate inhibitor of CYP3A4 (31), and inhibition of this enzyme could lead to increased exposure levels of the partner drug and thus contribute to increasing its trypanocidal activity in host tissues *in vivo*. Alternatively, one may hypothesize that amlodipine could interfere with efflux pump activity (29, 32), thereby increasing the bioavailability of ravuconazole. Thus, combination therapy using ravuconazole and amlodipine can be considered promising and deserves further detailed investigation related to the pharmacokinetic/pharmacodynamic (PK/PD) properties of the drug combination.

In an attempt to perform a comprehensive analysis of the outcomes of combined therapy in the infected host, rather than the antiparasitic effect, the cytokine profile was evaluated 30 days after the end of the treatment (Fig. 6). Interestingly, the parasitism suppression observed in the early phase of infection led to a reduction in proinflammatory cytokine levels, even 30 days after treatment cessation. In contrast to amlodipine, reduced levels of all evaluated cytokines were observed in ravuconazole monotherapy-treated mice. In addition, the levels of TNF- α , an important molecule involved in the pathogenesis of Chagas-related heart disease, were significantly lower in combination-treated mice than with azole monotherapy. Considering that the exacerbated proinflammatory microenvironment is associated with tissue damage and the progression of cardiac lesions in Chagas disease (33), a marked reduction in cytokine levels could positively impact the host. Similar results have been reported in a canine model of Chagas disease treated with ravuconazole, where long-lasting parasitemia suppression yielded beneficial effects on the immune response and thus prevented the progression of cardiac lesions, even in the absence of a parasitological cure (8).

Regarding the toxicity of the treatments, our data corroborate the safety profile previously described for ravuconazole (6–9, 24) and amlodipine (14, 34) in murine

models, with no weight loss or elevation of liver injury markers in treated mice (Fig. 4). The combined therapy, despite slightly decreasing the mass gain in uninfected mice, prevented the loss of body weight and the mortality observed with infected and untreated mice, without evidence of liver cytotoxicity; ravuconazole as a monotherapy had a similar effect (Fig. 4). These results indicate that the combination had no additional toxicity and that the control of parasitism protected the mice against the deleterious effects of infection.

Our data also prompt a discussion about the extrapolation of *in vitro/in vivo* assays. The data obtained from the murine model of infection did not translate our *in vitro* findings, including the nature of the interaction between the drugs. While ravuconazole had very limited activity on extracellular forms (Table 1) with time-dependent activity (data not shown), the *in vitro* effect of amlodipine was similar for both the amastigote and trypomastigote stages, but with no activity *in vivo*. Moreover, the nature of the interaction was classified as indifferent *in vitro*, but the *in vivo* effect was like a synergistic interaction. Considering that the protozoan is able to infect several cell types, the most accurate *in vitro* model with the best predictive value remains to be identified (35). Our results agree with another report (14) that showed promising *in vivo* results for a number of drug combinations that were merely additive *in vitro*. Although there are scarce preclinical data about *in vitro/in vivo* correlation in the field of anti-*T. cruzi* chemotherapy, these data encourage the *in vivo* evaluation of *in vitro* nonantagonist and nontoxic drug combinations.

Taken together, our findings demonstrate that amlodipine has no antiparasitic activity *in vivo*, but it enhances the therapeutic effect of ravuconazole in an experimental model of *T. cruzi* infection. Considering that this work was performed in an acute model treated with ravuconazole at suboptimal doses, further studies are required to determine the PK/PD properties of E1224-amlodipine combinations as well as their potential to treat chronic infection caused by Bz- and azole-resistant *T. cruzi* lineages. Bearing in mind the advanced stage of ravuconazole/E1224 evaluation upon Chagas disease and the clinical availability of amlodipine, this drug combination could be applicable in the short term. Finally, this work reinforces the importance of exploring *in vitro* and *in vivo* strategies for evaluating drug combinations in order to find experimental models with higher translational value. Even though the combined therapy strategy has been validated in recent clinical studies of Chagas disease treatment (3, 6, 11, 25), the definition of adequate preclinical models to more confidently predict the efficacy of drug combinations in human disease remains to be defined. The generation of data that would help to fill this gap in order to find criteria to move preclinical drug combinations to clinical trials is paramount.

MATERIALS AND METHODS

Parasites. *Trypanosoma (Schizotrypanum) cruzi* strain Y (DTU II) (36) was used in this study. The Y strain was selected for its high degree of virulence and partial resistance to benznidazole (37).

Mammalian cell cultures. The embryonic rat ventricular cell line H9c2 was used for both drug toxicity and infection assays. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 100 µg/ml penicillin-streptomycin (38). Cell cultures were maintained at 37°C in an atmosphere with 5% CO₂/air mixture. All assays were independently conducted at least two times.

Compounds. (i) Test compounds. Ravuconazole, or 4-[2-[(1R,2R)-2-(2,4-difluorophenyl)-2-hydroxy-1-methyl-3-(1H-1,2,4-triazol-1-yl)propyl]-4-thiazolyl]benzonitrile, was purchased from Sigma-Aldrich (Chemical Abstracts Service [CAS] number 182760-06-1; St. Louis, MO, USA); amlodipine-3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate was purchased from Teuto Bras (Brazil).

(ii) Reference compound. Benznidazole-2-nitroimidazole-(N-benzil-2-nitro-1-imidazoleacetamide), purchased from LAFEPE (Pernambuco State Pharmaceutical Laboratory, Brazil), was used as the reference treatment.

For *in vitro* studies, stock solutions of ravuconazole (10 mM), amlodipine (100 mM), and benznidazole (100 mM) were prepared in dimethyl sulfoxide (DMSO). The dilutions of each drug, in monotherapy or combinations, were prepared in fresh culture medium (DMEM) on the day of the assay. The final DMSO concentration never exceeded 0.5%. For *in vivo* studies, ravuconazole, amlodipine, and benznidazole were administered in aqueous suspensions containing 0.5% wt/vol methyl cellulose.

Cell toxicity assessment. Noninfected H9c2 cells were incubated at 37°C for 48 h with increasing concentrations of ravuconazole (up to 100 nM), amlodipine (up to 100 μ M), and benznidazole (up to 100 μ M). The cell viability was determined in a spectrophotometer by resazurin assay (22). The results were expressed as the difference in resazurin reduction between cells incubated or not incubated with the drugs. The toxicity of the combinations was assessed by combining the higher concentrations of ravuconazole and amlodipine followed by five points of 1:2 serial dilutions.

Anti-*T. cruzi* assays. (i) Monotherapies. The effect of ravuconazole and amlodipine in monotherapy *in vitro* was investigated on different *T. cruzi* evolutive forms. For the antitrypomastigote assay, tissue culture-derived trypomastigotes (TCT) were obtained from the first burst of H9c2 cells previously infected with blood trypomastigotes of the *T. cruzi* Y strain. The parasites were collected from the monolayer supernatant, purified as previously reported (22), and further resuspended in fresh culture medium. Parasites (1×10^6) were incubated for 24 h in 96-well plates, with different concentrations of the drugs in 1:2 serial dilutions (0 to 120 nM for ravuconazole and 0 to 25 μ M for amlodipine). The rates of parasite death were quantified by light microscopy to determine the EC₅₀ (39).

The anti-mastigote assay was carried out using H9c2 cells infected with TCT of the Y strain, which have been shown to infect at least 40% of exposed cells. Host cells (1×10^4 cell/well) were dispensed into 24-well tissue culture plates containing coverslips. After 24 h, the cells were infected with TCT at a multiplicity of infection (MOI) of 10 and incubated for 24 h. Following this period, the cultures were washed to remove noninternalized parasites and were further incubated with the drugs. The top concentrations used were 10 μ M for amlodipine and 16 nM for ravuconazole followed by five 1:2 dilutions. All tissue culture slides were maintained at 37°C in a 5% CO₂/air mixture. After 72 h, the cultures were fixed with methanol, stained with Giemsa, and microscopically examined to determine the percentage of cells infected in the absence and presence of the drugs. These data were used to calculate EC₅₀ values.

(ii) Combined therapy. For combination experiments, the top concentrations of each drug were defined from the predetermined EC₅₀ values to ensure that the EC₅₀ fell near the midpoint of a five-point 2-fold dilution series. The top concentrations were used to prepare solutions at ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of ravuconazole to amlodipine (for trypomastigote experiments) or 1:1 of ravuconazole to amlodipine for anti-mastigote assays (9, 22). All experiments were run in duplicate, and the results are given as means $\pm$ standard deviations from at least two independent experiments. *In vitro* data were analyzed using the software Compusyn (ComboSyn, Inc., Paramus, NJ, USA).

***In vivo* efficacy studies. (i) Mouse infection and ethics.** Female Swiss mice (18 to 23 g) obtained from the animal facility at the Universidade Federal de Alfenas (Alfenas, Minas Gerais, Brazil) were housed in a conventional room at 20 to 24°C under a 12 h/12 h light/dark cycle. The animals were supplied with commercial feed and drinking water *ad libitum*. The animals ($n = 6$ to 8/group) were inoculated intraperitoneally with 5.0×10^3 bloodstream trypomastigotes of the *T. cruzi* Y strain. The parasites were obtained from the blood of previously infected animals. All procedures were approved by the Institutional Ethics Committee in Animal Research (protocol number 14/2016).

***In vivo* assay for ravuconazole and amlodipine combinations. (i) Short-term protocol.** The first set of experiments was designed to determine if amlodipine would be able to increase the anti-*T. cruzi* activity of suboptimal doses of ravuconazole. Infected mice were treated with ravuconazole at 5.0, 1.0, and 0.5 mg $\cdot$ kg⁻¹ of body weight (7, 9) and with amlodipine at a fixed dose of 10 mg $\cdot$ kg⁻¹ (14) in monotherapy and combined. After parasitemia detection (5 days postinoculation), the drugs were administered by gavage for 5 consecutive days. Animals treated with both drugs received ravuconazole and then amlodipine after 30 min. Mice were euthanized 14 days postinoculation, and parasitemia and body weight and mortality were registered. Results were compared to those reached with ravuconazole and amlodipine in monotherapies and with infected and untreated animals.

(ii) Efficacy protocol. The second set of experiments was designed to determine the impact of amlodipine on the therapeutic potential of ravuconazole in a long-term treatment protocol (20 days). Infected Swiss mice were randomly divided ($n = 7$ to 8/group) and treated at the onset of parasitemia (5 days after infection) for 20 consecutive days with ravuconazole at 5.0 or 2.5 mg $\cdot$ kg⁻¹ and amlodipine at 10 mg $\cdot$ kg⁻¹, in monotherapy or combination. Therapeutic failure was determined from (i) parasitemia recrudescence in the presence of immunosuppression with cyclophosphamide (experiment 1) and (ii) natural parasitemia relapse detected by fresh blood examination until 30 days after treatment, followed by PCR in blood samples of negative cases (experiment 2). We also evaluated the outcomes of long-term treatment using ravuconazole (5.0 mg $\cdot$ kg⁻¹) alone and combined with amlodipine (10 mg $\cdot$ kg⁻¹) on the host toxicity profile (body weight and serum transaminases) and on the immune response (anti-*T. cruzi* IgG antibodies and Th1/Th2/Th17 cytokine levels). Mortality was checked daily until 60 days postinfection, when the animals were euthanized. In all protocols, the results were compared to those achieved with monotherapies at the same dose and with the reference treatment (100 mg $\cdot$ kg⁻¹ Bz). A group of infected animals receiving no treatment was used as a control of infection. Finally, two groups of uninfected animals were included; one remained untreated, and the other was treated with ravuconazole (5.0 mg $\cdot$ kg⁻¹) and amlodipine (10 mg $\cdot$ kg⁻¹) alone and in combination.

Fresh blood examination. To determine the natural reactivation of infection after treatment, the parasitemia was evaluated for up to 30 days posttreatment by fresh blood examination. To do so, 5 ml of blood collected from the tail vein was examined, and the parasite number was estimated as described previously (40). To detect parasitemia recrudescence after the immunosuppressant protocol, 30 days after the treatment, the mice were subjected to immunosuppression (Cyl), which consisted of three cycles of

50 mg cyclophosphamide/kg of body weight intraperitoneally (i.p.) for 4 consecutive days, with 3-day intervals between cycles (41). The parasitemia was evaluated during the cycles and up to 10 days thereafter.

Real-time PCR-based parasitic detection assay. Genomic DNA was purified from blood samples collected 30 and 55 days posttreatment. DNA extraction was performed using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) with some modifications (42). PCRs were performed to amplify *T. cruzi* DNA using the TaqMan system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and using primers for *T. cruzi* (CzFw 5'-CCACCATTCA TAAATGGAAACAAA-3' and CzRv 5'-CTCGGCTGATCGTTTCGA-3') and murine TNF- α (TNF-F 5'-GCC CAGACCTCACACTCA-3' and TNF-R 5'-AACTGCCCTT CCTCCATCTTAA-3'). It was also used oligonucleotide probes for *T. cruzi* (5'-FAM-ACCACAACGTGTGATGC-3'-MGB-NFQ) and for TNF- α (5'-VIC-TAAGTGTCCACACCTC-3'-MGB-NFQ) (23). Amplification cycles were carried out in an ABI 7500 real-time PCR system (Applied Biosystems). The cycles consisted of an initial denaturation hold of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C with fluorescence acquisition. All samples were analyzed in duplicate, and negative samples as well as reagent controls were processed in parallel for each assay.

Anti-*T. cruzi* IgG antibody assay. *T. cruzi*-specific antibodies were measured in plasma samples collected 30 and 55 days after infection, as reported by Caldas et al. (41). Briefly, enzyme-linked immunosorbent assay (ELISA) plates were coated with alkaline-extracted *T. cruzi* antigens obtained from the Y strain during the exponential growth phase in LIT medium. Anti-mouse IgG-peroxidase-conjugated antibody (Sigma Chemical Co., St. Louis, MO, USA) was used as the secondary antibody. The mean absorbance for 10 negative-control samples plus two standard deviations was used as the cutoff to discriminate positive and negative results.

Cytokine analyses. Cytokine levels were evaluated in plasma samples collected 30 days after the end of the treatment. Tumor necrosis factor- α (TNF- α), interferon gamma (IFN- γ), interleukin-6 (IL-6), IL-2, IL-4, IL-10, and IL-17A were measured with flow cytometry using a BD cytometric bead array (CBA) mouse Th1/Th2/Th17 cytokine kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Samples were acquired in a BD FACSVerse flow cytometer, and data analyses were performed using CBA analysis FCAP Array software (BD Biosciences).

In vivo toxicity assay. Quantification of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was performed with the plasma samples obtained on the last day of the treatment. Enzymatic assays were performed by spectrophotometry using commercial enzymatic colorimetric kits and the instructions provided by the manufacturer (Labtest, Lagoa Santa, Minas Gerais, Brazil).

Statistical analysis. All data are presented as means $\pm$ standard deviations. To classify the nature of the *in vitro* interaction, the fractional inhibitory concentration at the EC₅₀ level (FIC₅₀) and the sum of FICs (Σ FICs) were calculated as described previously (9, 22). An overall mean Σ FIC was calculated for each combination and used to classify the nature of the interaction. Isobolograms were constructed plotting the FIC₅₀ of ravuconazole against amlodipine. Normality in the data distribution was checked with the Shapiro-Wilk test. Statistical analysis was performed individually for each assay, and the data were compared with Student's *t* test or with one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test using GraphPad Prism 7 (California, USA). The differences were considered significant if the *P* value was less than or equal to 0.05.

ACKNOWLEDGMENTS

We thank Programa de Desenvolvimento Tecnológico em Insumos para a Saúde (PDTIS-FIOCRUZ) for providing the flow cytometry facilities and to Caroline Vicente Oliveira and Leonardo Cabral for helping with the experimental work.

This study was funded by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Brazil, and financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES) (finance code 001) and the Universidade Federal de Alfenas, Brazil.

We have no conflicts of interest to declare.

REFERENCES

1. WHO. 2019. Chagas disease. <https://www.who.int/chagas/epidemiology/en/>. Accessed 01/11/2019.
2. Lidani KCF, Andrade FA, Bavia L, Damasceno FS, Beltrame MH, Messias-Reason IJ, Sandri TL. 2019. Chagas disease: from discovery to a worldwide health problem. *Front Public Health* 7:166. <https://doi.org/10.3389/fpubh.2019.00166>.
3. Kratz JG, Garcia Bournissen F, Forsyth CJ, Sosa-Estani S. 2018. Clinical and pharmacological profile of benznidazole for treatment of Chagas disease. *Exp Rev Clin Pharmacol* 11:943–957. <https://doi.org/10.1080/17512433.2018.1509704>.
4. Pérez-Molina JA, Molina I. 2018. Chagas disease. *Lancet* 391:82–94. [https://doi.org/10.1016/S0140-6736\(17\)31612-4](https://doi.org/10.1016/S0140-6736(17)31612-4).
5. Urbina JA. 2015. Recent clinical trials for the etiological treatment of chronic Chagas disease: advances, challenges and perspectives. *J Eukaryot Microbiol* 62:149–156. <https://doi.org/10.1111/jeu.12184>.
6. Torrico F, Gascon J, Ortiz L, Alonso-Vega C, Pinazo M-J, Schijman A, Almeida IC, Alves F, Strub-Wourgaft N, Ribeiro I, Santana G, Blum B, Correia E, Garcia-Bournissen F, Vaillant M, Morales JR, Pinto Rocha JJ, Rojas Delgadillo G, Magne Anzoleaga HR, Mendoza N, Quechover RC, Caballero MYE, Lozano Beltran DF, Zalabar AM, Rojas Panozo L, Palacios Lopez A, Torrico Terceros D, Fernandez Galvez VA, Cardozo L, Cuellar G, Vasco Arenas RN, Gonzales I, Hoyos Delfin CF, Garcia L, Parrado R, de la Barra A, Montano N, Villarroel S, Duffy T, Bisio M, Ramirez JC, Duncanson F, Everson M, Daniels A, Asada M, Cox E, Wesche D, Diderichsen PM, Marques AF, Izquierdo L, et al. 2018.

- Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomised, placebo-controlled trial. *Lancet Infect Dis* 18:419–430. [https://doi.org/10.1016/S1473-3099\(17\)30538-8](https://doi.org/10.1016/S1473-3099(17)30538-8).
7. Urbina JA, Payares G, Sanoja C, Lira R, Romanha AJ. 2003. *In vitro* and *in vivo* activities of ravuconazole on *Trypanosoma cruzi*, the causative agent of Chagas disease. *Int J Antimicrob Agents* 21:27–38. [https://doi.org/10.1016/S0924-8579\(02\)00273-X](https://doi.org/10.1016/S0924-8579(02)00273-X).
 8. Diniz LF, Caldas IS, Guedes PMDM, Crepalde G, de Lana M, Carneiro CM, Talvani A, Urbina JA, Bahia MT. 2010. Effects of ravuconazole treatment on parasite load and immune response in dogs experimentally infected with *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 54:2979–2986. <https://doi.org/10.1128/AAC.01742-09>.
 9. Diniz LF, Mazzeti AL, Caldas IS, Ribeiro I, Bahia MT. 2018. Outcome of E1224-benznidazole combination treatment for infection with a multidrug-resistant *Trypanosoma cruzi* strain in mice. *Antimicrob Agents Chemother* 62:e00401-18. <https://doi.org/10.1128/AAC.00401-18>.
 10. Molina I, Gómez I Prat J, Salvador F, Treviño B, Sulleiro E, Serre N, Pou D, Roure S, Cabezas J, Valerio L, Blanco-Grau A, Sánchez-Montalvá A, Vidal X, Pahissa A. 2014. Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. *N Engl J Med* 370:1899–1908. <https://doi.org/10.1056/NEJMoa1313122>.
 11. Urbina JA. 2017. Pharmacodynamics and follow-up period in the treatment of human *Trypanosoma cruzi* infections with posaconazole. *J Am Coll Cardiol* 70:299–300. <https://doi.org/10.1016/j.jacc.2017.03.611>.
 12. Reimão JQ, Mesquita JT, Ferreira DD, Tempone AG. 2016. Investigation of calcium channel blockers as antiprotozoal agents and their interference in the metabolism of *Leishmania (L.) infantum*. *Evid Based Complement Alternat Med* 2016:1523691. <https://doi.org/10.1155/2016/1523691>.
 13. Neal RA, van Bueren J, McCoy NG, Iwobi M. 1989. Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovani* by verapamil. *Trans R Soc Trop Med Hyg* 83:197–198. [https://doi.org/10.1016/0035-9203\(89\)90642-1](https://doi.org/10.1016/0035-9203(89)90642-1).
 14. Planer JD, Hulverson MA, Arif JA, Ranade RM, Don R, Buckner FS. 2014. Synergy testing of FDA-approved drugs identifies potent drug combinations against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 8:e2977. <https://doi.org/10.1371/journal.pntd.0002977>.
 15. Villalta F, Rachakonda G. 2019. Advances in preclinical approaches to Chagas disease drug discovery. *Expert Opin Drug Discov* 14:1161–1174. <https://doi.org/10.1080/17460441.2019.1652593>.
 16. Bahia MT, Diniz LF, Mosqueira VC. 2014. Therapeutic approaches under investigation for treatment of Chagas disease. *Exp Opin Invest Drugs* 23:1225–1237. <https://doi.org/10.1517/13543784.2014.922952>.
 17. Lepesheva GI, Friggeri L, Waterman MR. 2018. CYP51 as drug targets for fungi and protozoan parasites: past, present and future. *Parasitology* 145:1820–1836. <https://doi.org/10.1017/S0031182018000562>.
 18. Moraes CB, Giardini MA, Kim H, Franco CH, Araujo-Junior AM, Schenkman S, Chatelain E, Freitas-Junior LH. 2014. Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. *Sci Rep* 4:4703. <https://doi.org/10.1038/srep04703>.
 19. Francisco AF, Lewis MD, Jayawardhana S, Taylor MC, Chatelain E, Kelly JM. 2015. Limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive *in vivo* imaging. *Antimicrob Agents Chemother* 59:4653–4661. <https://doi.org/10.1128/AAC.00520-15>.
 20. Sánchez-Valdéz FJ, Padilla A, Wang W, Orr D, Tarleton R. 2018. Spontaneous dormancy protects *Trypanosoma cruzi* during extended drug exposure. *Elife* 26:e34039. <https://doi.org/10.7554/eLife.34039>.
 21. Petravičius PO, Costa-Martins AG, Silva MN, Reis-Cunha JL, Bartholomeu DC, Teixeira MMG, Zingales B. 2019. Mapping benznidazole resistance in trypanosomatids and exploring evolutionary histories of nitroreductases and ABCG transporter protein sequences. *Acta Trop* 200:105161. <https://doi.org/10.1016/j.actatropica.2019.105161>.
 22. Mazzeti AL, Diniz LDF, Gonçalves KR, WonDollinger RS, Assíria T, Ribeiro I, Bahia MT. 2019. Synergic effect of allopurinol in combination with nitroheterocyclic compounds against *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 63:e02264-18. <https://doi.org/10.1128/AAC.02264-18>.
 23. Gonçalves-Santos E, Vilas-Boas DF, Diniz LF, Veloso MP, Mazzeti AL, Rodrigues MR, Oliveira CM, Fernandes VHC, Novaes RD, Chagas-Paula DA, Caldas IS. 2019. Sesquiterpene lactone potentiates the immunomodulatory, antiparasitic and cardioprotective effects on anti-*Trypanosoma cruzi* specific chemotherapy. *Int Immuno Pharmacol* 77: 105961. <https://doi.org/10.1016/j.intimp.2019.105961>.
 24. Novaes RD, Sartini MVP, Rodrigues JPF, Gonçalves RV, Santos EC, Souza RLM, Caldas IS. 2016. Curcumin enhances the anti-*Trypanosoma cruzi* activity of benznidazole-based chemotherapy in acute experimental Chagas disease. *Antimicrob Agents Chemother* 60:3355–3364. <https://doi.org/10.1128/AAC.00343-16>.
 25. Drugs for Neglected Diseases Initiative (DNDi). The BENDITA study: a phase II study to assess safety, tolerability, and efficacy of different benznidazole regimens, alone and in combination with fosravuconazole. Chagas Bendita study briefing document (2019). https://www.ndi.org/wp-content/uploads/2019/03/2page_BenditaStudyOverview_ENG.pdf.
 26. Zeng Q, Zhang Z, Chen P, Long N, Lu L, Sang H. 2019. *In vitro* and *in vivo* efficacy of a synergistic combination of itraconazole and verapamil against *Aspergillus fumigatus*. *Front Microbiol* 10:1266. <https://doi.org/10.3389/fmicb.2019.01266>.
 27. Liu S, Yue L, Gu W, Li X, Zhang L, Sun S. 2016. Synergistic effect of fluconazole and calcium channel blockers against resistant *Candida albicans*. *PLoS One* 11:e0150859. <https://doi.org/10.1371/journal.pone.0150859>.
 28. Pereira MR, Henrich PP, Sidhu ABS, Johnson D, Hardink J, Van Deusen J, Lin J, Gore K, O'Brien C, Wele M, Djimde A, Chandra R, Fidock DA. 2011. *In vivo* and *in vitro* antimalarial properties of azithromycin-chloroquine combinations that include the resistance reversal agent amlodipine. *Antimicrob Agents Chemother* 55:3115–3124. <https://doi.org/10.1128/AAC.01566-10>.
 29. Hu C, Li Y, Zhao Z, Wei S, Zhao Z, Chen H, Wu P. 2018. *In vitro* synergistic effect of amlodipine and imipenem on the expression of the AdeABC efflux pump in multidrug-resistant *Acinetobacter baumannii*. *PLoS One* 13:e0198061. <https://doi.org/10.1371/journal.pone.0198061>.
 30. Nishio S, Watanabe H, Kosuge K, Uchida S, Hayashi H, Ohashi K. 2005. ANO interaction between amlodipine and simvastatin in patients with hypercholesterolemia and hypertension. *Hypertens Res* 28:223–227. <https://doi.org/10.1291/hyres.28.223>.
 31. Watanabe S, Tsubouchi I, Okubo A. 2018. Efficacy and safety of fosravuconazole L-lysine ethanolate, a novel oral triazole antifungal agent, for the treatment of onychomycosis: a multicenter, double-blind, randomized phase III study. *J Dermatol* 45:1151–1159. <https://doi.org/10.1111/1346-8138.14607>.
 32. Campos MC, Castro-Pinto DB, Ribeiro GA, Berredo-Pinho MM, Gomes LH, da Silva Bellieny MS, Goulart CM, Echevarria A, Leon LL. 2013. P-glycoprotein efflux pump plays an important role in *Trypanosoma cruzi* drug resistance. *Parasitol Res* 112:2341–2351. <https://doi.org/10.1007/s00436-013-3398-z>.
 33. Acevedo GR, Girard MC, Gómez KA. 2018. The unsolved jigsaw puzzle of the immune response in Chagas disease. *Front Immunol* 9:1929. <https://doi.org/10.3389/fimmu.2018.01929>.
 34. Palit P, Ali N. 2008. Oral therapy with amlodipine and lacidipine, 1,4-dihydropyridine derivatives showing activity against experimental visceral leishmaniasis. *Antimicrob Agents Chemother* 52:374–377. <https://doi.org/10.1128/AAC.00522-07>.
 35. Franco CH, Alcântara LM, Chatelain E, Freitas-Junior L, Moraes CB. 2019. Drug discovery for Chagas disease: impact of different host cell lines on assay performance and hit compound selection. *Trop Med Infect Dis* 4:82. <https://doi.org/10.3390/tropicalmed4020082>.
 36. Zingales B, Andrade SG, Briones MRS, Campbell DA, Chiari E, Fernandes O, Guhl F, Lages-Silva E, Macedo AM, Machado CR, Miles MA, Romanha AJ, Sturm NR, Tibayrenc M, Schijman AG. 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Mem Inst Oswaldo Cruz* 104:1051–1054. <https://doi.org/10.1590/s0074-02762009000700021>.
 37. Filardi LS, Brener Z. 1987. Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Trans R Soc Trop Med Hyg* 81:755–759. [https://doi.org/10.1016/0035-9203\(87\)90020-4](https://doi.org/10.1016/0035-9203(87)90020-4).
 38. Moraes KC, Diniz LF, Bahia MT. 2015. Role of cyclooxygenase-2 in *Trypanosoma cruzi* survival in the early stages of parasite host-cell interaction. *Mem Inst Oswaldo Cruz* 110:181–191. <https://doi.org/10.1590/0074-02760140311>.
 39. Simões-Silva MR, De Araújo JS, Peres RB, Da Silva PB, Batista MM, De Azevedo LD, Bastos MM, Bahia MT, Boechat N, Soeiro MNC. 2019. Repurposing strategies for Chagas disease therapy: the effect of imatinib

- and derivatives against *Trypanosoma cruzi*. *Parasitology* 146:1006–1012. <https://doi.org/10.1017/S0031182019000234>.
40. Brener Z. 1962. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. *Rev Inst Med Trop Sao Paulo* 4:389–396.
41. Caldas S, Santos FM, de Lana M, Diniz LF, Machado-Coelho GL, Veloso VM, Bahia MT. 2008. *Trypanosoma cruzi*: acute and long-term infection in the vertebrate host can modify the response to benznidazole. *Exp Parasitol* 118:315–323. <https://doi.org/10.1016/j.exppara.2007.08.016>.
42. Caldas S, Caldas IS, de Figueiredo Diniz L, de Lima WG, Oliveira RDP, Cecílio AB, Ribeiro I, Talvani A, Bahia MT. 2012. Real-time PCR strategy for parasite quantification in blood and tissue samples of experimental *Trypanosoma cruzi* infection. *Acta Trop* 123:170–177. <https://doi.org/10.1016/j.actatropica.2012.05.002>.