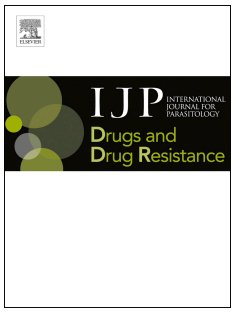
Accepted Manuscript



Assessing anti-*T. Cruzi* candidates *in vitro* for sterile cidality

Monica Cal, Jean-Robert Ioset, Matthias Fügi, Pascal Mäser, Marcel Kaiser

PII: S2211-3207(16)30042-2

DOI: 10.1016/j.ijpddr.2016.08.003

Reference: IJPDDR 151

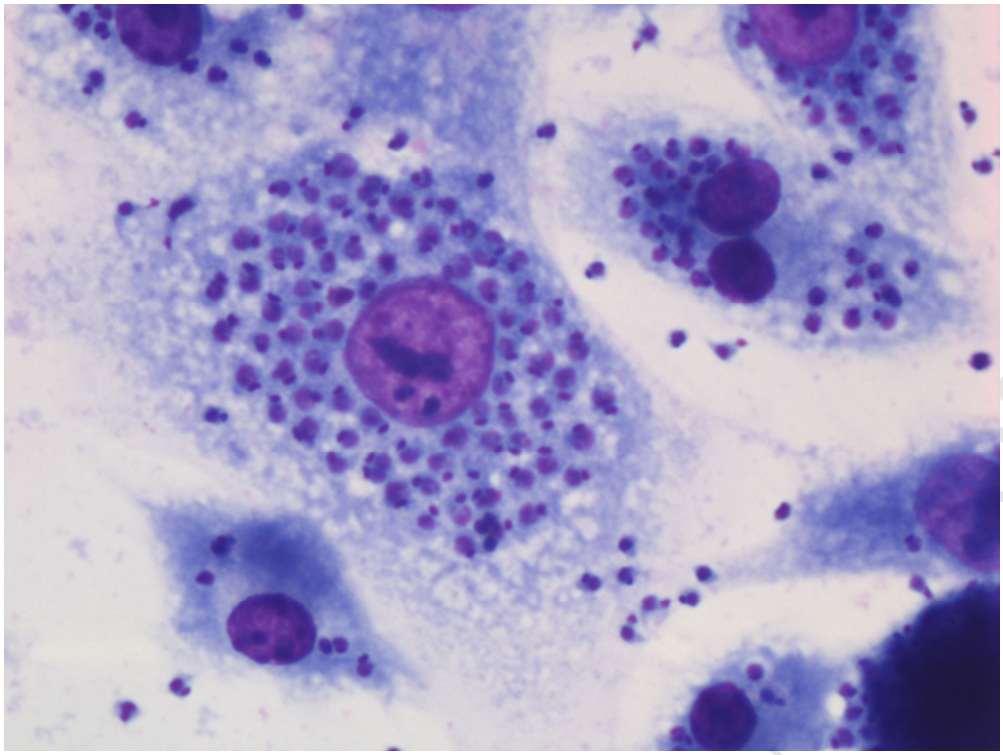
To appear in: *International Journal for Parasitology: Drugs and Drug Resistance*

Received Date: 18 May 2016

Revised Date: 24 August 2016

Accepted Date: 24 August 2016

Please cite this article as: Cal, M., Ioset, J.-R., Fügi, M., Mäser, P., Kaiser, M., Assessing anti-*T. Cruzi* candidates *in vitro* for sterile cidality, *International Journal for Parasitology: Drugs and Drug Resistance* (2016), doi: 10.1016/j.ijpddr.2016.08.003.



Assessing anti-*T.cruzi* candidates *in vitro* for sterile cidality

Monica Cal[1,2], Jean-Robert Ioset[3], Matthias Fügi[1,2], Pascal Mäser[1,2], Marcel Kaiser[1,2*]

[1] Swiss Tropical and Public Health Institute, CH-4051 Basel, Switzerland

[2] University of Basel, CH-4003 Basel, Switzerland

[3] Drugs for Neglected Diseases *initiative*, CH-1202 Geneva, Switzerland

[*]Corresponding author Swiss Tropical and Public Health Institute

Socinstrasse 57, 4051 Basel, Switzerland

E-mail marcel.kaiser@unibas.ch

Tel +41 61 284 8205

Fax +41 61 284 8101

Abstract

Total clearance of the *T. cruzi* infection – referred to herein as "sterile cure" – seems to be a critical prerequisite for new drug candidates for Chagas disease, ensuring long-term beneficial effects for patients in the chronic indeterminate stage. This requirement is notably supported by the recent findings of clinical studies involving posaconazole and fosravuconazole, where the majority of patients treated eventually relapsed after an apparent clearance of parasitaemia at the end of treatment. We have adapted an *in vitro* system to predict the ability of a compound to deliver sterile cure. It relies on mouse peritoneal macrophages as host cells for *Trypanosoma cruzi* amastigotes. The macrophages do not proliferate, allowing for long-term testing and wash-out experiments. Giemsa staining followed by microscopy provides a highly sensitive and specific tool to quantify the numbers of infected host cells. Combining macrophages as host cells and Giemsa staining as the read-out, we demonstrate that posaconazole and other CYP51 inhibitors are unable to achieve complete clearance of an established *T. cruzi* infection *in vitro* in spite of the fact that these compounds are active at significantly lower concentrations than the reference drugs benznidazole and nifurtimox. Indeed, a few macrophages remained infected after 96 h of drug incubation in the presence of CYP51 inhibitors– albeit at a very low parasite load. These residual *T. cruzi* amastigotes were shown to be viable and infective, as demonstrated by wash-out experiments. We advocate characterizing any new anti-*T.cruzi* early stage candidates for sterile cidality early in the discovery cascade, as a surrogate for delivery of sterile cure *in vivo*.

Keywords

Trypanosoma cruzi; *in vitro* assay; posaconazole; macrophage; Giemsa; Chagas disease

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas disease, endemic in 21 countries of Latin America (WHO fact sheet 340). Migration and travel have additionally contributed to the spread of Chagas disease to other continents, including North America and Europe. The World Health Organization estimates that 6-7 million people are infected world-wide, leading to more than 10,000 deaths annually (WHO fact sheet 340). In the absence of a vaccine, the only treatment option is chemotherapy. However, the existing drugs benznidazole and nifurtimox have several limitations, notably in relation to their severe adverse effects and contraindications (Andrade et al., 1992; Bahia-Oliveira et al., 2000; Cancado, 2002; Urbina, 2010). Safer drugs are urgently needed. Over the last few years, the development of new anti-*T.cruzi* agents has focused on azoles as inhibitors of CYP450-dependent lanosterol demethylase (CYP51; 1.14.13.70) that act by blocking trypanosomatid ergosterol synthesis (Buckner and Urbina, 2012; Chatelain, 2015). Azoles display remarkable nanomolar range *in vitro* potency against *T. cruzi* as well as a good safety profile in humans (Buckner and Urbina, 2012; Soeiro Mde et al., 2013). Azoles have been in use as antifungal agents for decades, which has greatly facilitated their preclinical development for Chagas disease. Recently, the two triazoles posaconazole and fosravuconazole, a prodrug of ravuconazole, were tested in controlled clinical phase II studies (Urbina, 2015). However, both molecules failed to show sufficient levels of efficacy in chronic Chagas patients; after an initial phase of apparent clearance of parasitaemia following the end of treatment, 80% of the patients relapsed 10 months after the end of treatment in the posaconazole (CHAGAZASOL) study (Molina et al., 2014), while 71% relapsed 12 months after the end of treatment in the fosravuconazole study (Torrico, 2013), as determined by real time qPCR detection of *T. cruzi* DNA. These disappointing clinical results for azoles contrast with the relatively low treatment failures observed with benznidazole, which showed 80% and 94% sustained clearance of parasites at the same endpoint in the posaconazole and fosravuconazole trials respectively. The outcome of a clinical trial depends on several factors, pharmacokinetics and host immune system play an important role. The obtained posaconazole

levels in patients treated with 100-400 mg b.i.d. were clearly below the plasma levels in mice (20mg/kg/day) (Urbina, 2015). A prolonged treatment duration and a higher dose or an improved formulation to get higher plasma levels could lead to a better clinical outcome. While the full potential of azoles for combination chemotherapy has not yet been realized (Fügi et al., 2015), our working hypothesis, derived from the disappointing outcome of the clinical trials, is that any novel anti-*T.cruzi* hit or chemistry starting point emerging from the discovery pipeline should be assessed at an early stage for its potential to deliver sterile cidality against different *T. cruzi* genotypes (Moraes et al., 2014; Chatelain, 2015). Our aim is to develop an in vitro test for sterile cidality towards *T. cruzi* amastigotes. Posaconazole and benznidazole can serve as benchmarks for such a test (Fortes Francisco et al., 2015). Here we report the adaptation of *in vitro* assay protocols (McCabe et al., 1983) that can be performed on any *T. cruzi* strain without requiring sophisticated laboratory equipment, and the activity profiling of a small panel of CYP51 inhibitors in these assays to investigate whether the clinical relapses observed following posaconazole and fosravuconazole therapies could have been predicted, at least partly, from these simple *in vitro* surrogate assays.

2. Materials and methods

2.1 Cells and media

A *T. cruzi* Tulahuen C2C4 strain that expresses the β-galactosidase gene (LacZ) (Buckner et al., 1996) was cultured in RPMI-1640 supplemented with 10% inactivated FBS (iFBS) and 2 μM L-glutamine at 37°C and 5% CO_2. L6 rat skeletal myoblast cells (ATCC CRL-1458) were used as host cells for infection with transgenic *T. cruzi* trypomastigotes.

2.2 Drugs

Nifurtimox, posaconazole, fenarimol, clotrimazole, ecoconazole, itraconazole, ketoconazole and tioconazole were purchased from Sigma-Aldrich. Benznidazole was synthesized by Epichem Pty Ltd, Murdoch, Australia. Stock solutions of these drugs were prepared in DMSO 100% at 10 mg/ml and 1 mg/ml.

2.3 LacZ/CPRG assay

L-6 cells or murine peritoneal macrophages (MPM) were seeded, 1,000 cells per well (L6) or 4,000 cells per well (MPM), in 96-well microtiter plates (Costar) in 100 µl RPMI 1640 with 10% heat-inactivated FBS (Connectorate AG) and 2 µM L-glutamine at 37°C and 5% CO_2. At 24 h the medium was replaced by 100 µl fresh medium containing 5,000 (L6) or 4,000 (MPM) trypomastigote *T. cruzi*. At 72 h the medium was replaced by 100 µl (L6) or 200 µl (MPM) fresh medium with serial drug dilution from 30,000 to 0.5 ng/ml in 3-fold steps. After 96 h incubation, the plates were inspected microscopically, followed by the addition of CPRG/Nonidet solution (0.25 µM Chlorophenol red-ß-D-galactopyranoside and 0.25% Nonidet in PBS; 50 µl per well). After 5 h further incubation, the plates were read photometrically at 540 nm; IC_{50} and IC_{90} values as well as the Hill factor were calculated by the four parameter nonlinear regression model using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). All values are means from at least three independent assays.

2.4 Giemsa assay

4,000 MPM were seeded into 16-chamber slides (Lab-tek) (McCabe et al., 1983). Incubation, infection with trypomastigote *T. cruzi* at MOI of 1, and drug exposure were performed as described above. After the 96 h drug exposure the medium was removed and the slides were fixed with methanol for 10 min, followed by staining with 10% Giemsa solution (Sigma). The infection status (infected/non-infected) of at least 400 cells was determined microscopically. If possible, the number of intracellular amastigotes was counted for 100 infected cells. At low infection rates, the number of intracellular amastigotes was determined for all infected cells. The infection rates of the untreated controls were between 85% and 95%. The results were expressed either as a percentage of infected host cells compared to untreated controls, or as mean numbers of amastigotes per infected macrophage. The *T. cruzi* population size was calculated as the number of intracellular amastigotes per 100 macrophages (% infection rate × number of amastigotes per infected cell). IC_{50} and IC_{90} values, as well as the Hill factor, were calculated from the sigmoidal dose-response curve using the

four parameter nonlinear regression model of the software Softmax Pro (Softmax Pro Molecular Devices Cooperation, Sunnyvale, CA, USA). All values are means from at least three independent assays. For assessment of cidality, the medium was removed after 96 h drug exposure and the adherent MPM were washed four times with 200 µl fresh medium. 200 µl fresh medium was added, followed by a further 168 h incubation. The medium was then removed, and the slides were fixed with methanol and stained with 10% Giemsa as described above.

3. Results and Discussion

3.1 Myoblasts vs. macrophages as host cells

Trypanosoma cruzi can infect practically every type of nucleated mammalian cell. Muscle cells, and cardiomyocytes in particular, are among the cells targeted by *T. cruzi* that contribute to the pathology of Chagas disease (Nagajyothi et al., 2012). Rat L6 are widely used as convenient and relevant host cells for testing compounds against the intracellular amastigote stage of *T. cruzi*. The standard assay requires 96 h exposure to a test compound followed by quantification of the parasites (Buckner et al., 1996). Longer incubation times with this specific cell line are not possible because L6 cells multiply with a population doubling time of about 15 h and the cultures would overgrow. Instead, we used mouse peritoneal macrophages as a non-dividing type of host cell. The infection rates of the macrophages with *T. cruzi* trypomastigotes were over 80%. The replication time of intracellular *T. cruzi* amastigotes is 18-20 hours and was determined in previous experiments. A selection of antifungal CYP51 inhibitors, 6 azoles plus the non-azole fungicide fenarimol, were tested against *T. cruzi*, along with the standard drugs benznidazole and nifurtimox, in parallel in L6 cells and macrophages. A *LacZ* transfected Tuluahen strain (C2C4) was used for ease of read-out with the chromogenic ß-galactosidase substrate CPRG (Chlorophenol red-ß-D-galactopyranoside). All CYP51 inhibitors had IC_{50} values in the low nanomolar range, whereas the reference drugs benznidazole and nifurtimox were clearly less potent, with IC_{50} values in the micromolar range (Table 1, left two columns). Posaconazole was over 1,000-fold more potent than benznidazole in both systems (Table 1). The IC_{50} values of all compounds tested were lower in macrophages than in

L6 cells (p=0.004; two-tailed Wilcoxon matched pairs test). IC_{90} values could not be determined accurately (Table 1) as the variance at the tail of the dose-response curve was too high. Thus the macrophages provided a highly sensitive test system, but the colorimetric signal from turn-over of CPRG was a suboptimal read-out for accurately measuring signals close to background from small numbers of residual *T. cruzi* amastigotes.

3.2 ß-galactosidase vs. Giemsa staining as read-out

As a more accurate alternative to CPRG-based colorimetric readout, we tested Giemsa staining followed by microscopic read-out to determine numbers of infected macrophages and, for selected compounds, the parasite load of infected cells. This more labor-intensive method of detection allowed the specific identification and accurate counting of *T. cruzi* amastigotes at the single cell level (see the Graphical Abstract for a sample image). The resulting IC_{50} values based on the infection rate were more than an order of magnitude higher than those determined by CPRG (Table 1). This can be explained by the fact that Giemsa staining determined the percentage of infected macrophages (regardless of the number of intracellular amastigotes), whereas the fluorescence signal from CPRG turn-over is proportional to the total number of amastigotes (regardless of the percentage of infected host cells). The IC values derived by Giemsa were thus not proportional to the total number of parasites, and the resulting IC_{50} values were comparably higher. The IC_{90} values obtained by microscopic determination of the infection rate following Giemsa staining are, however, in our opinion more meaningful than those obtained with CPRG, because single surviving amastigotes can be detected by microscopic counting. Figures 1 & 2A compare the two different kinds of dose-response curves. The reference drugs benznidazole and nifurtimox exhibited a smaller shift in IC_{50} from a decrease in overall parasitaemia (CPRG read-out) to a decrease in infected host cells (Giemsa read-out) than the seven antifungals. Furthermore, the Hill factors (HF) of the dose-response curves for the reference drugs were higher for the Giemsa read-out while the CYP51 inhibitors exhibited flatter curves for the Giemsa read-out than for CPRG (mean Hill factors of 0.90 *vs.* 1.8; p=0.047; two-tailed Wilcoxon matched pairs test). Importantly, the Giemsa dose-response

curves of the reference drugs (Figure 2A) reached base-line, while those of the antifungals (Figure 2A) did not. Thus none of the CYP51 inhibitors tested led to a 100% clearance of parasitaemia from the infected cell population-. A small percentage of host cells remained infected, although with a very low parasite load of 1-4 parasites per infected cell, compared to >10 parasites in untreated infected control cells. The parasite load of untreated cells was very high 96 h post infection (>10 amastigotes/cell). For the purpose of Figure 2B, the average parasite load was counted up to 10 amastigotes/cell. The drop in mean parasite load is reflected by the drop in the CPRG signal. The CYP51 inhibitors showed a clear reduction of the parasite load per infected cell, which settled at a low load (Figure 2B). The standard drug benznidazole tends to show a continuous reduction of the parasite load. All compounds were tested to the maximum non-cytotoxic concentration. In Figure 2C, the calculated total number of intracellular *T. cruzi* amastigotes in 100 macrophages is plotted against drug concentration. This clearly shows the flat dose-response of CYP51 inhibitors and their inability to kill all parasites within 96 h of drug exposure. The drug exposure period of 96 h corresponds to 4-5 parasite generations. This should be long enough for a complete depletion of the preformed pool of sterols, which is a prerequisite for a cidal effect of the ergosterol biosynthesis inhibitors (Urbina et al 1998). However, we cannot exclude that an even longer exposure would kill all the intracellular *T. cruzi* amastigotes. We calculated that >10 parasites per 100 macrophages survive at the highest concentrations of CYP51 inhibitors. Benznidazole showed a good dose-response curve and at a concentration of 40 μM, only <1 parasite per 100 macrophages survived (Figure 2C). These results correlate with the previous observations made using high content imaging technology in a panel of *T. cruzi* I-VI genotype strains (Moraes et al., 2014), validating the inability of CYP51 inhibitors to lead to full clearance of *T. cruzi* amastigote populations following drug exposure, independant of the drug concentration tested.

3.3 A long-term in vitro assay for sterile cidality

To assess whether these few residual intracellular *T. cruzi* amastigotes were viable, we prolonged the *in vitro* test to a total duration of 11 days. The drug-containing medium was removed after 96 h

incubation, the adherent macrophages were washed twice, and fresh, drug free medium was added. After another 168 h incubation, the numbers of infected macrophages, and for selected compounds the parasite load of the infected cells, were determined by Giemsa staining. For benznidazole and nifurtimox, the resulting IC_{50} values only increased slightly (10-20%) compared to those after 96 h (Table 1, right). In contrast, the majority of the CYP51 inhibitors tested exhibited a strong shift (>200%) in IC_{50} from 96 h to 96+168 h tests. IC_{90} could not be determined, because the dose-response curves of the CYP51 inhibitors did not reach background levels. Figure 3A illustrates the shift in IC values based on the percentage of infected cells. Figures 3B and C show the corresponding mean number of intracellular amastigotes per infected cell and the estimated total number of parasites per 100 macrophages, respectively. The dose-response curves of the antifungals are flat in the 96 h drug exposure experiment (Figure 2A-C) whereas the curves are steeper in the wash-out experiment (Figure 3A-C). The wash-out experiments confirmed that the few residual amastigotes observed after 96 h incubation were viable and infective to other macrophages. Thus the tested antifungals posaconazole, clotrimazole, econazole, ticonazole and itraconazole were not able to deliver sterile cidality *in vitro*.

4. Conclusion

Mouse macrophages provide a highly sensitive system for testing molecules *in vitro* against *T. cruzi* intracellular amastigotes. Combined with Giemsa staining and microscopic read-out, this system allows drug sensitivity tests over long periods of time with high sensitivity and specificity, detecting single residual parasites. Wash-out experiments demonstrated that these residual parasites were alive and able to infect new host cells. When the percentage of infected host cells was quantified by Giemsa staining, all the CYP51 inhibitors tested displayed lower IC_{50} values than the reference drugs benznidazole and nifurtimox, but the dose-response curves were much flatter and did not reach baseline, showing that the CYP51 inhibitors were unable to clear all *T. cruzi* amastigotes. This indicates that measuring drug potency in terms of IC_{50} based on viability markers is an insufficient readout to predict for clearance of parasitaemia *in vitro* and – by extension – *in vivo*, in

particular if the parasites are persistent and the compound's effect is rather static than cidal. By using macrophages as host cells and Giemsa staining as the read-out, it was indeed possible to demonstrate that the azoles, as well as another non-azole based CYP51 inhibitors, do not deliver sterile cidality defined as full clearance of parasitaemia *in vitro*. To evaluate the anti-*T.cruzi* potential of a compound, in our opinion both the reduction in infection rate as well as the reduction in the total number of parasites is important. The approach presented may provide early stage evidence – and potentially offer a predictive preclinical tool – for the observed parasitological relapse of Chagas patients treated with azoles. The long-term incubation assay, relying on mouse peritoneal macrophages as host cells and Giemsa staining as the read-out, is not amenable to high throughput screening. It can be implemented as a secondary assay to profile and eventually prioritize anti-*T.cruzi* hits identified from screening campaigns, or it may serve as the starting point for developing a high-content assay amenable to higher throughput screening. Wash-out experiments demonstrated that the *T. cruzi* amastigotes that persist following *in vitro* drug exposure with CYP51 inhibitors are viable and replicating. While we cannot exclude that drugs which are unable to deliver sterile cidality in vitro might still work in vivo thanks to the contribution of the host's immune system or any other factor not captured under the experimental conditions of our assay, we hope that the presented assay will contribute to render the discovery cascade for Chagas disease more predictable.

Acknowledgements

This piece of research was primarily funded by the Drugs for Neglected Diseases *initiative*. The Drugs for Neglected Diseases *initiative* received financial support for the work described in this article from the following donors: Department for International Development (UK), Reconstruction Credit Institution–Federal Ministry of Education and Research (KfW-BMBF; Germany), Bill & Melinda Gates Foundation (United States), and Médecins Sans Frontières. These donors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in, or financial conflict with, the subject matter or materials discussed in the article apart from those disclosed. We wish to thank Eric Chatelain for critical reading of the manuscript, Fred Buckner for kindly providing the *LacZ* transgenic *T. cruzi*, and Christiane Braghiroli and Guy Riccio for isolating the mouse macrophages.

References

Andrade S.G., Rassi A., Magalhaes J.B., Ferriolli Filho F., Luquetti A.O., 1992. Specific chemotherapy of Chagas disease: a comparison between the response in patients and experimental animals inoculated with the same strains. Trans R Soc Trop Med Hyg 86, 624-626.

Bahia-Oliveira L.M., Gomes J.A., Cancado J.R., Ferrari T.C., Lemos E.M., Luz Z.M., Moreira M.C., Gazzinelli G., Correa-Oliveira R., 2000. Immunological and clinical evaluation of chagasic patients subjected to chemotherapy during the acute phase of *Trypanosoma cruzi* infection 14-30 years ago. J Infect Dis 182, 634-638.

Buckner F.S., Urbina J.A., 2012. Recent Developments in Sterol 14-demethylase Inhibitors for Chagas Disease. Int J Parasitol Drugs Drug Resist 2, 236-242.

Buckner F.S., Verlinde C.L., La Flamme A.C., Van Voorhis W.C., 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrob Agents Chemother 40, 2592-2597.

Cancado J.R., 2002. Long term evaluation of etiological treatment of Chagas disease with benznidazole. Rev Inst Med Trop Sao Paulo 44, 29-37.

Chatelain E., 2015. Chagas Disease Drug Discovery: Toward a New Era. J Biomol Screen 20, 22-35.

Fortes Francisco A., Lewis M.D., Jayawardhana S., Taylor M.C., Chatelain E., Kelly J.M., 2015. The limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive in vivo imaging. Antimicrob Agents Chemother 59(8), 4653-61.

Fügi MA, Kaiser M, Tanner M, Schneiter R, Mäser P, Guan XL. Match-making for posaconazole through systems thinking. Trends Parasitol. 2015 Feb;31(2):46-51. doi: 10.1016/j.pt.2014.11.004.

McCabe RE, Araujo FG, Remington JS. Ketoconazole protects against infection with Trypanosoma cruzi in a murine model. Am J Trop Med Hyg. 1983 Sep;32(5):960-2.

Moraes C.B., Giardini M.A., Kim H., Franco C.H., Araujo-Junior A.M., Schenkman S., Chatelain E., Freitas-Junior L.H., 2014. Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. Sci Rep 4, 4703.

Nagajyothi F., Machado F.S., Burleigh B.A., Jelicks L.A., Scherer P.E., Mukherjee S., Lisanti M.P., Weiss L.M., Garg N.J., Tanowitz H.B., 2012. Mechanisms of *Trypanosoma cruzi* persistence in Chagas disease. Cell Microbiol 14, 634-643.

Soeiro Mde N., de Souza E.M., da Silva C.F., Batista Dda G., Batista M.M., Pavao B.P., Araujo J.S., Aiub C.A., da Silva P.B., Lionel J., Britto C., Kim K., Sulikowski G., Hargrove T.Y., Waterman M.R., Lepesheva G.I., 2013. In vitro and in vivo studies of the antiparasitic activity of sterol 14alpha-demethylase (CYP51) inhibitor VNI against drug-resistant strains of *Trypanosoma cruzi*. Antimicrob Agents Chemother 57, 4151-4163.

Torrico F., 2013. Results of proof-of-concept clinical trial in patients with chronic indeterminate Chagas disease. Annual Meeting of the American Society of Tropical Medicine and Hygiene E1224. Available from: http://www.dndi.org/about-us/our-people/team/354-media-centre/press-releases/1700-e1224.html accessed 28 April 2016.

Urbina J.A., 1998. Antiproliferative effects and mechanism of action of SCH 56592 against Trypanosoma (Schizotrypanum) cruzi: in vitro and in vivo studies. Antimicrob Agents Chemother 42(7):1771-7.

Urbina J.A., 2010. Specific chemotherapy of Chagas disease: relevance, current limitations and new approaches. Acta Trop 115, 55-68.

Urbina J.A., 2015. Recent clinical trials for the etiological treatment of chronic Chagas disease: advances, challenges and perspectives. J Eukaryot Microbiol 62, 149-156.

307 WHO, 2015. Chagas disease. Factsheet No. 340 (www.who.int/mediacentre/factsheets/fs340/en/)
308 accessed 28 April 2016.

309

310

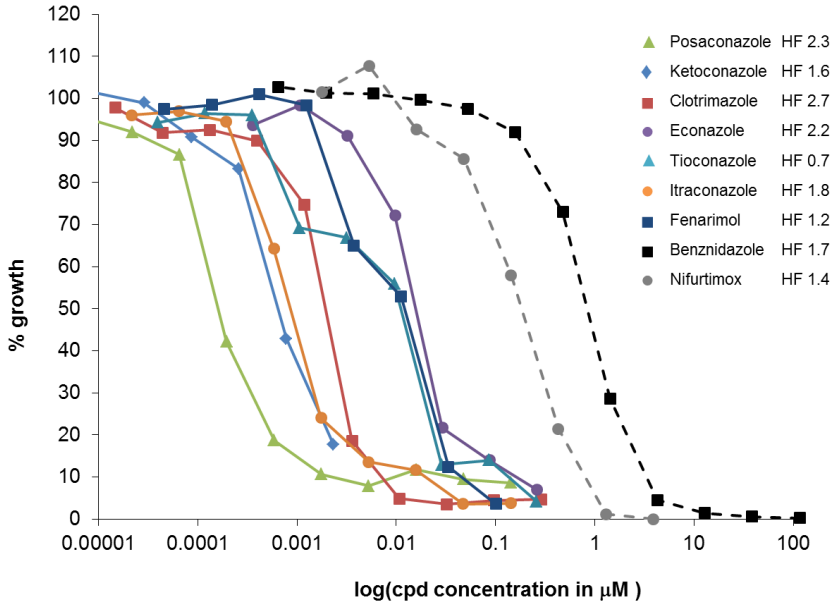
Figure 1. *In vitro* drug sensitivity of *T. cruzi* amastigotes in mouse macrophages after 96 h drug exposure. The solid lines represent the azoles and the dashed lines represent the reference drugs benznidazole and nifurtimox. The Hill factors (HF) of the dose response curves are indicated in the legend. Percentage growth of the parasites as quantified by colorimetric read-out of CPRG turn-over.

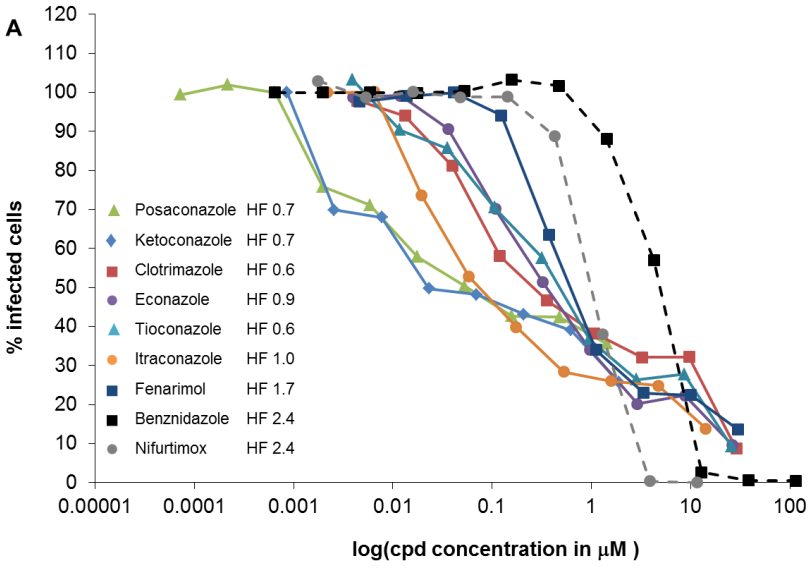
Figure 2. *In vitro* drug sensitivity of *T. cruzi* amastigotes in mouse macrophages after 96 h drug exposure. The solid lines represent the azoles and the dashed lines represent the reference drugs benznidazole and nifurtimox. Hill factors (HF) of the dose response curves are indicated in the legend. A) Percentage infected host cells as quantified by microscopic read-out of Giemsa stains. B) Mean numbers of amastigotes per infected macrophage as quantified by microscopic read-out of Giemsa stains. C) Estimated numbers of intracellular amastigotes in 100 macrophages. Calculation: % infection rate x mean number of amastigotes per infected cell.

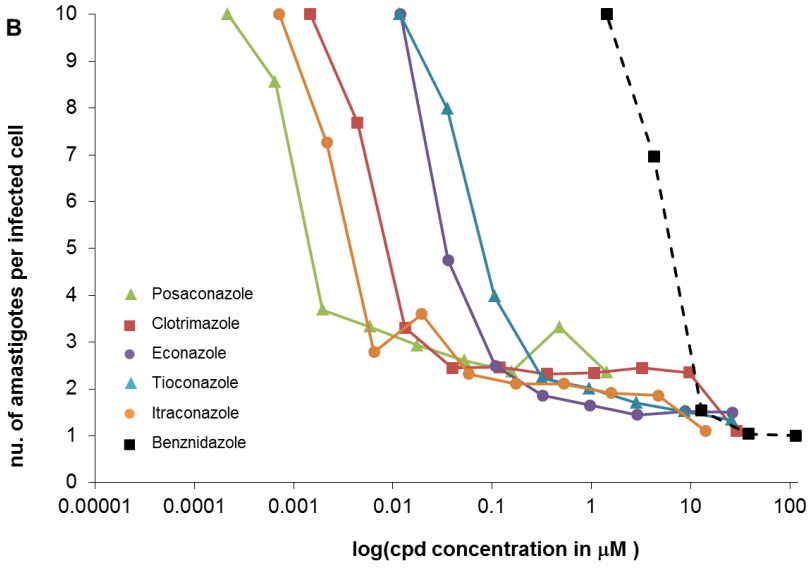
Figure 3. *In vitro* drug sensitivity of *T. cruzi* amastigotes in mouse macrophages after drug washout (96 h) and 168 h recovery period. The solid lines represent the azoles and the dashed lines represent the reference drugs benznidazole and nifurtimox. Hill factors (HF) of the dose response curves are indicated in the legend. A) Percentage infected host cells as quantified by microscopic read-out of Giemsa stains. B) Mean numbers of amastigotes per infected macrophage as quantified by microscopic read-out of Giemsa stains. C) Estimated numbers of intracellular amastigotes in 100 macrophages. Calculation: % infection rate x mean number of amastigotes per infected cell.

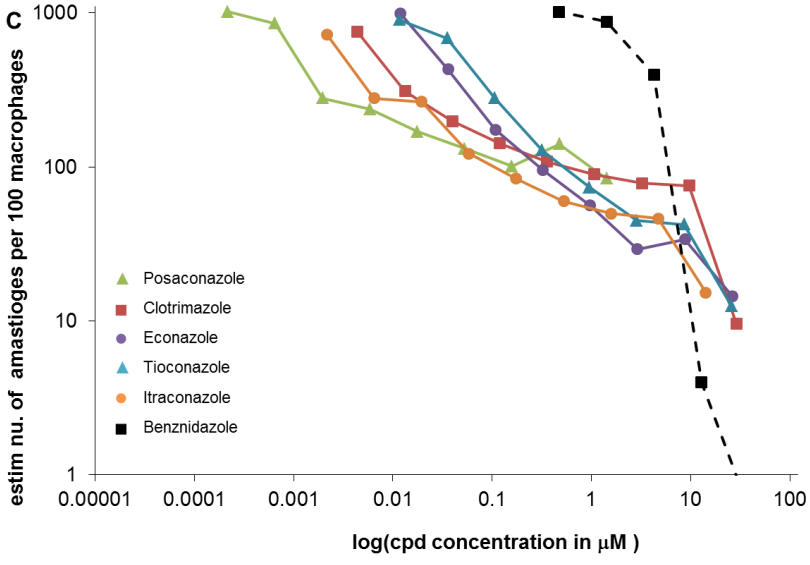
Table 1. *In vitro* drug sensitivity tests for *T. cruzi*. Values are the means of at least 3 replicates ± S.D. in nM (L6, rat L6 myoblasts; MPM, murine peritoneal macrophages; CPRG, Chlorophenol red-ß-D-galactopyranoside).

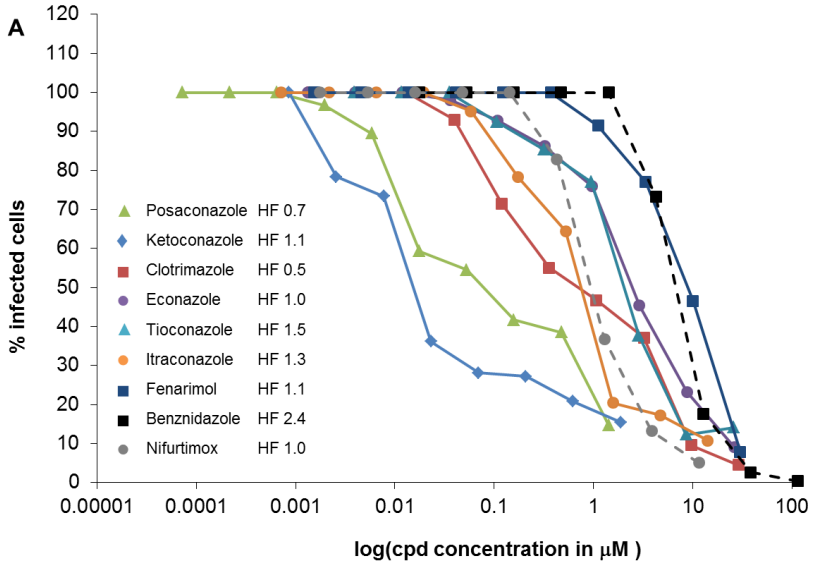
		L6 96h, CPRG	**MPM** 96h, CPRG	**MPM** 96h, Giemsa	**MPM** 96+168h, Giemsa
Benznidazole	IC50	1,800 ± 540	850 ± 190	4,800 ± 1,100	5,800 ± 2,900
	IC90	6,600 ± 2,800	3,100 ± 790	11,000 ± 650	18,000 ± 9,700
Nifurtimox	IC50	470 ± 160	180 ± 45	990 ± 200	1,100 ± 720
	IC90	2,200 ± 760	770 ± 7	2,700 ± 290	4,300 ± 3,900
Posaconazole	IC50	0.7 ± 0.3	0.3 ± 0.07	46 ± 2.8	280 ± 460
	IC90	57 ± 49	1.4 ± 1.0	>1,400	>4,300
Ketoconazole	IC50	1.7 ± 1.5	0.4 ± 0.4	34 ± 35	n/a
	IC90	67 ± 64	1.7 ± 0.4	>1,900	n/a
Clotrimazole	IC50	20 ± 15	1.7 ± 0.6	470 ± 440	4,200 ± 520
	IC90	310 ± 190	7.3 ± 2.9	>29,000	>29,000
Econazole	IC50	60 ± 11	16 ± 5.2	320 ± 210	1,100 ± 130
	IC90	1,300 ± 530	170 ± 24	18,000 ± 9500	>26,000
Tioconazole	IC50	72 ± 10	10 ± 7.8	500 ± 410	1,400 ± 110
	IC90	970 ± 360	21 ± 26	>26,000	>26,000
Itraconazole	IC50	5.7 ± 4.3	0.9 ± 0.4	99 ± 58	1,200 ± 150
	IC90	69 ± 51	5.7 ± 4.3	>1,400	>14,000
Fenarimol	IC50	160 ± 6.0	12 ± 9.1	540 ± 57	5,700 ± 5,000
	IC90	>300	33 ± 39	>30,000	>30,000

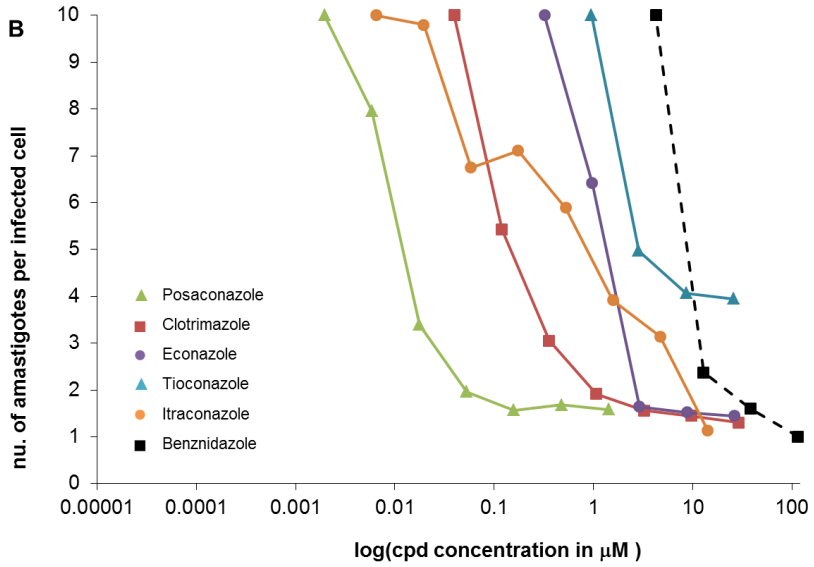

120
110
100
90
80
70
60
50
40
30
20
10
0
% growth
0.00001
0.0001
0.001
0.01
0.1
1
10
100
log(cpd concentration in µM)
Posaconazole HF 2.3
Ketoconazole HF 1.6
Clotrimazole HF 2.7
Econazole HF 2.2
Tioconazole HF 0.7
Itraconazole HF 1.8
Fenarimol HF 1.2
Benznidazole HF 1.7
Nifurtimox HF 1.4

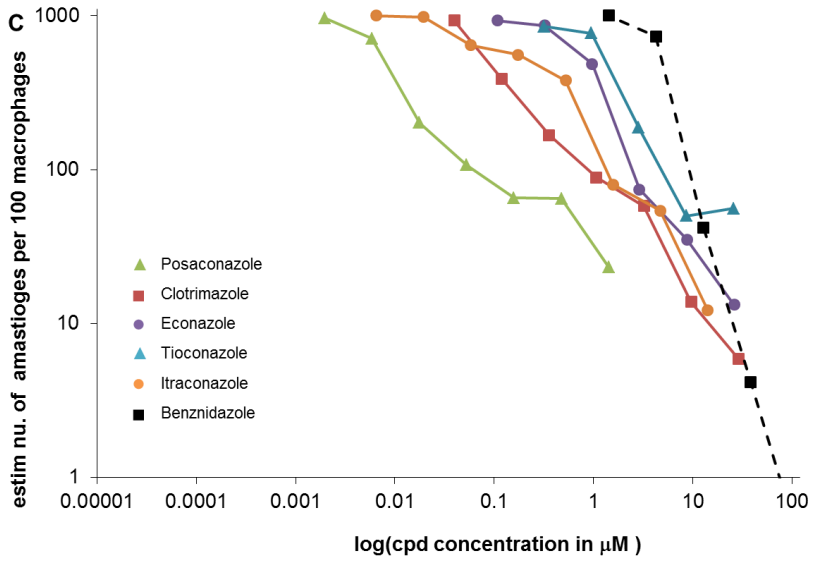

A
% infected cells
log(cpd concentration in µM)
Posaconazole HF 0.7
Ketoconazole HF 0.7
Clotrimazole HF 0.6
Econazole HF 0.9
Tioconazole HF 0.6
Itraconazole HF 1.0
Fenarimol HF 1.7
Benznidazole HF 2.4
Nifurtimox HF 2.4


B
nu. of amastigotes per infected cell
log(cpd concentration in µM)
Posaconazole
Clotrimazole
Econazole
Tioconazole
Itraconazole
Benznidazole


C
estim nu. of amastioges per 100 macrophages
log(cpd concentration in µM)
Posaconazole
Clotrimazole
Econazole
Tioconazole
Itraconazole
Benznidazole


A
% infected cells
log(cpd concentration in µM)
Posaconazole HF 0.7
Ketoconazole HF 1.1
Clotrimazole HF 0.5
Econazole HF 1.0
Tioconazole HF 1.5
Itraconazole HF 1.3
Fenarimol HF 1.1
Benznidazole HF 2.4
Nifurtimox HF 1.0


B
nu. of amastigotes per infected cell
log(cpd concentration in µM)
Posaconazole
Clotrimazole
Econazole
Tioconazole
Itraconazole
Benznidazole


C
estim nu. of amastioges per 100 macrophages
log(cpd concentration in µM)
Posaconazole
Clotrimazole
Econazole
Tioconazole
Itraconazole
Benznidazole

Highlights (max 85 char.)

- A highly sensitive *Trypanosoma cruzi* in vitro drug test for sterile cidality
- This test demonstrates failure of CYP51 inhibitors to deliver sterile cure