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

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A B S T R A C T
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*.

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1. Introduction

*Trypanosoma cruzi* is the causative agent of Chagas disease, endemic in 21 countries of Latin America ([WHO, 2015]). 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, Bahia-Oliveira, Cancado, 1992; Bahia-Oliveira, 2000; Cancado, 2002; Urbina, 2006]). 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 trypanosomatida ergosterol synthesis ([Buckner, 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 tri-azoles 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

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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 (20 mg/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 curability against different T. cruzi genotypes (Moraes et al., 2014; Chatelain, 2015). Our aim is to develop an in vitro test for sterile curability 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 C24 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% CO₂. 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, econazole, itraconazole, ketoconazole and tioconazole were purchased from Sigma-Aldrich. Benznidazole was synthesized by Epicem 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, 1000 cells per well (L6) or 4000 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% CO₂. At 24 h the medium was replaced by 10 μl fresh medium containing 5000 (L6) or 4000 (MPM) trypomastigote T. cruzi. At T2 h the medium was replaced by 100 μl (L6) or 200 μl (MPM) fresh medium with serial drug dilution from 30,000–0.5 ng/ml in 3-fold steps. After 96 h incubation, the plate were inspected microscopically, followed by the addition of CPRG/Nonidet solution (0.25 μM Chlorophenol red-β-D-galactopyranoside and 0.25% Nonidet in PBS; 50 μl per well). After 5 further incubation, the plates were read photometrically at 540 nm. IC₅₀ and IC₉₀ 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

4000 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). IC₅₀ and IC₉₀ 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 curability, 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 (Nagajothy 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 h 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 Tulahuen strain (C24) was used for ease of read-out with the chromogenic β-galactosidase substrate CPRG (Chlorophenol red-β-D-galactopyranoside). All CYP51 inhibitors had IC₅₀ values in the low nanomolar range, whereas the reference drugs benznidazole and nifurtimox were clearly less potent, with IC₅₀ values in the micromolar range (Table 1, left two columns). Posaconazole was over 1000-fold more potent than benznidazole in both systems (Table 1). The IC₅₀ values of all compounds tested were lower in macrophages than in L6 cells (p = 0.004; two-tailed Wilcoxon matched pairs test). IC₉₀ 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. β-galactosidase vs. giemsa staining as read-out

As a more accurate alternative to CPRG-based colorimetric readout, we tested Giemsa staining followed by microscopic readout 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 IC50 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 IC50 values were comparably higher. The IC50 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. Figs. 1 and 2A compare the two different kinds of dose-response curves. The reference drugs benznidazole and nifurtimox exhibited a smaller shift in IC50 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 showed a clear reduction of the parasite load. All compounds were tested to the maximum non-cytotoxic concentration. In Fig. 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, 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 (Fig. 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, independent 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 IC50 values only increased slightly (10–20%) compared to those after 96 h (Table 4, right). In contrast, the majority of the CYP51 inhibitors tested exhibited a strong shift (>200%) in IC50 from 96 h to 96 + 168 h tests. IC50 could not be determined, because the dose-response curves of the CYP51 inhibitors did not reach background levels. Fig. 3A illustrates the shift in IC values based on the percentage of infected cells. Fig. 3B and C shows 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 (Fig. 2A–C) whereas the curves are steeper in the wash-out experiment (Fig. 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, econazol, tioconazole 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 IC50 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 IC50 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.

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