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

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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*.

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 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 trypanosomatida 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

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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 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, econazole, 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, 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% CO_2. At 24 h the medium was replaced by 100 μl fresh medium containing 5000 (L6) or 4000 (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 30,000–0.5 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-β-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

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). 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 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 (C2C4) was used for ease of read-out with the chromogenic β-galactosidase substrate CPRG (Chlorophenol red-β-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 1000-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