The Trypanocidal Effect of Novel Quinolines: *In vitro* And *In vivo Studies*

Nefertiti, ASG[a], Batista, MM[a], Da Silva, PB[a], Batista, DGJ[a], Da Silva, CF[a], Peres, RB[a]; Torres-Santos, EC[b], Cunha-Junior, EF[b], Holt, E[c], Boykin, DW[c], Brun, R[de], Wenzler, T[de*] and Soeiro, MNC[a**]

[a] Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil.

[b] Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil.

[c] Department of Chemistry, Georgia State University, Atlanta, Georgia, USA.

[d] Swiss Tropical and Public Health Institute, Basel, Switzerland.

[e] University of Basel, Basel, Switzerland.

*Current address: Institute of Cell Biology, University of Bern, Bern, Switzerland

** Corresponding author at Av. Brasil, 4365 Manguinhos, Rio de Janeiro, Brazil. Phone: 55 21 2562-1368; fax: 055 21 2598-4577

Email address: soeiro@ioc.fiocruz.br (M.N.C. Soeiro)

Running title: Quinolines activity against trypanosomes

Abstract

The therapy for Human African Trypanosomiasis and Chagas Disease, caused by *Trypanosoma brucei* and *Trypanosoma cruzi* respectively, are limited providing minimal therapeutic options for the millions of individuals living in very poor communities. The effect of ten novel quinolines are evaluated herein through *in silico* and by phenotypic studies using *in vitro* and *in vivo* models. ADMET properties revealed that most molecules did not infringe Lipinski´s rules, which is a prediction of good oral absorption. They showed good probability of $CaCo_2$ permeability and for human intestinal absorption, low probability of mutagenicity and of hERG1 inhibition. *In vitro* screens against bloodstream forms of *T.cruzi* demonstrated that all quinolines were more active than the reference drug (benznidazole –Bz), except DB2171 and DB2192, with five (DB2187, DB2131, DB2186, DB2191 and DB2217), displaying EC_{50} <3 µM (<4-fold than Bz). Nine quinolines were more effective than Bz (2.7 µM) against amastigotes showing EC_{50} values ranging from 0.6 to 0.1 µM. All quinolines were also *in vitro* highly active on African trypanosomes showing EC_{50} values ≤ 0.25 µM. The most potent and highly selective candidates for each parasite species were tested *in vivo* models. Results for DB2186 were promising in mice with *T.cruzi and T.brucei* infection, reaching 70 % reduction of the parasitemia load and it cured 2 of 4 mice, respectively. DB2217 was *in vivo* also active and cured all 4 mice (100% cure rate) with *T.brucei* infection.

Key words: *Trypanosoma cruzi*, *Trypanosoma brucei*, experimental chemotherapy, quinolines, *in vitro, in vivo, in silico.*

Introduction

Currently more than one billion people live in poverty, without access to basic sanitation favoring the emergence and development of various diseases. The WHO grouped 18 pathologies caused by viruses, fungi, bacteria, protozoans and helminths, named neglected tropical diseases, that cause severe impact in on public health programs of developing countries but present low interest and investments for the development of early diagnosticis tools and safer/potent therapies by most pharmaceutical companies (1-3).

Human African trypanosomiasis (HAT) or sleeping sickness is a lethal disease in sub-Saharan Africa caused by two subspecies of *Trypanosome brucei* (*T.b.*). *T.b. gambiense (T.b.g.)* is endemic in western and central Africa and *T.b. rhodesiense (T.b.r)* is most prevalent in eastern and southern Africa (4). Both parasite subspecies are transmitted by the bite of an infected tsetse fly (genus Glossina). Clinical presentations vary according to the subspecies and the disease stage. The symptoms of the haemolymphatic stage are mostly nonspecific and include fever, headache and swelling of the lymph nodes. In the second meningoencephalitic stage, the trypomastigotes infect in addition to the blood and lymph system the central nervous system. Neurological symptoms such as as mental confusion and emotional lability as well as convulsions and alteration of the circadian rhythm, a characteristic giving the disease its name, accompany the second stage. Sleeping sickness is fatal, if left untreated (4).

Chagas disease (CD), also a neglected tropical disease, is endemic in 18 countries in Latin American, constituting a continuing serious public health problem, presenting a chronic progressive pathology that affects more than 6-8 million people worldwide (2). CD is caused by the protozoan *Trypanosoma cruzi,* and its transmission

occurs primarily via bug triatomine vectors and may also include other routes as blood transfusion, congenital (both in decline due to public health measures adopted by the endemic countries), due to laboratory accidents and by ingestion of food and drinks contaminated with the feces and/or the entire triatomines containing infective forms of the parasite (5). Current treatment of CD is based on two nitro-heterocyclic drugs, nifurtimox (Nif) and the 2-nitroimidazole benznidazole (Bz), introduced into clinical therapy over 5 decades ago (6).Recent clinical trials (7, 8) performed on chronic patients evaluating azoles inhibitors of CYP51 (prodrug of ravuconazole and posaconazole) and a nitroderivative (fexinidazole) showed high rates of therapeutic failure despite their excellent activity *in vitro* and *in vivo* using experimental models (mouse and canine models), arguing for generation of more predictive *in vitro* and *in vivo* data (6,9,10).

For HAT a total of five drugs are available. However, treatment recommodations fall back to one option for each subspecies and disease stage. Pentamidine (*T.b.g)* and suramin (*T.b.r*) are used as first line treatments for first-stage and melarsoprol (*T.b.r*) and a combination of eflornithine and nifurtimox (*T.b.g*) for second stage disease (1). The main general limitations of the current therapies for both HAT and CD include considerable adverse effects, high costs, require long periods of exposure, occurrence of natural and acquired resistant parasites and treatment failures especially in the later pathological stages (10).These findings underscore the urgent need to search for new trypanocidal agents with characteristics for each target product profile (for CD and HAT) (2, 5, 11). In this context, many compounds have been tested *in vitro* and *in vivo* but up to now only few candidates have been found (6, 12-14).

The presented work with quinolines is based on a high-throughput phenotypic screening of a library of 700,000 compounds by the Genomics Institute of the Novartis Research Foundation. It yielded over a hundred different scaffolds which were nontoxic

to human cells and were active (3.6 μM or less) against *T. b.* (15). One of the initial hits, 2-(2-benzamido) ethyl-4-phenylthiazole, has been extensively explored and a number of compounds which were highly active against *T. b. in vitro* were discovered (16). However, these compounds were only moderately effective in the STIB900 mouse model for *T. b. r.* infections, which was attributed, at least in part, to poor metabolic stability 16). We under took to explore *N*-(2-phenylquinolin-7-yl) benzamides and related compounds which retain a similar geometric relationship between the amide unit and the thiazole nitrogen atom hypothesized to be important for activity. In this quinoline system the "ethylamine link" of the original thiazole hit is incorporated into the quinoline ring and may improve metabolic stability. While our study was in progress excellent *in vivo* results against both early and late stage *T. b.* infections in mice for a benzothiazole analog of the inital hit were reported by the same group (16).

Thus, in this work we investigate the phenotypic activity of ten novel quinolines through whole-cell based assays *in vitro* by assaying different parasite forms (trypomastigotes and amastigotes) and strains (DTUs II and VI) of *T.cruzi (T.c.)* in addition to exploring their biological activity on bloodstream forms of *T.b.r in vitro*. Further, the toxicity profile of these quinolines was studied using different mammalian cells, and by their predictive pharmacological properties evaluated by pKCSM .Finally the most promising compounds were moved to animal models of *T.b.r*. and *T.c.* aiming to contribute to the identification of novel therapeutic options for these severe neglected pathologies.

Material and Methods

Compounds: The synthesis and characterization of the ten quinolines (**Table 1**) are found in the Supplemental Information. For *T.c* assays, benznidazole (Bz) (2-

nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as reference drug and stock solutions prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0.6% and 10% in assays *in vitro and in vivo,* respectively, which is not toxic to the parasite, mammalian cells and mice. For *T.b.r.* pentamidine and melarsoprol were used as reference drugs. Pentamidine (SIGMA) was dissolved in DMSO and melarsoprol (Arsobal, Aventis) in water.

Computational assessment of the drug-like properties: Absorption, distribution, metabolism, excretion and toxicity (ADMET and Lipinsky rule of five) properties of the studied quinolines were evaluated using the pkCSM approach, which uses graph-based signatures to develop predictive ADMET (17, 18).

Parasites: *T.cruzi*: Bloodstream trypomastigote (BT) forms of Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitemia. The purified parasites were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum as reported previously (19, 20). Trypomastigotes of Tulahuen strain expressing the *Escherichia coli* β-galactosidase gene were collected from the supernatant of *T.cruzi*-infected L929 cultures as reported (21, 22*).* ***T.brucei:*** The *T. b. rhodesiense* strain STIB900, a derivative of strain STIB704 was isolated from a patient in Ifakara, Tanzania, in 1982 (23). Blood stream forms were used for *in vitro* screening as well as for the acute mouse model, which mimics the first stage of HAT.

Mammalian cell cultures: For the toxicity assays on mammalian cells, primary cultures of cardiac cells (cc) obtained from mice embryos were plated in 96 well plates previously coated with 0.01% gelatin 19). L929 cell lineages were obtained as described in Romanha et al., (22). L6 cells (rat skeletal myoblast, ATCC CRL-1458) were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5.95 g/l HEPES, 2 g/L $NaHCO_3$ and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2 (24).

Cytotoxicity *in vitro* tests: The cc cells were incubated for 24 h at 37°C, with different concentrations of each compound (up to 400 µM) diluted in DMEM and then, the morphology, cell density and spontaneous contractibility evaluated by light microscopy and their cellular viability determined by the Presto Blue test as reported (21). L929 cells were incubated for 96 h at 37ºC, with different concentrations of each compound (up to 96 µM) diluted in RPMI and their cellular viability determined by the Alamar Blue test as reported (21). The results were expressed by following the manufacturer instructions and the value of LC_{50} that corresponds to the concentration that reduces the cellular viability by 50%, determined. The cytotoxicity assays performed using L6 cells were conducted with a 72 h compound exposure time as previously reported (24). The selectivity index (SI) was expressed by ratio between the values obtained for LC_{50} on the host or L6 cells and the EC_{50} obtained over the parasites.

Trypanocidal activity: For *T.c* assays**,** bloodstream trypomastigotes (BT) of the Y strain (DTU II) (25) ($5x10^6$ per mL) were incubated for 2 and 24 h at 37ºC in RPMI in the presence or not of serial dilution of the compounds (up to 32 µM). After compound incubation, the death rates of parasites were determined by light microscopy through the

direct quantification of the number of live parasites using a Neubauer chamber, and the EC_{50} concentration (the compound concentration that reduces the number of parasites by 50%) calculated (17). For assaying on intracellular forms of Y strain (DTU II), the most promising compound was further evaluated on its infection of primay cultures of cardiac cells (cc, using a ratio of 10 BT: 1 host cell). After 24 h of parasite interaction, the cultures were rinsed and incubated for 48 h with the compounds. After fixation with Bouin and staining with Giemsa solution, the percentage of cc infection and the mean number of parasites per infected cell were calculated through light microscopy for determination of EC_{50} values of the infection index (II) (II - % infected host cells X mean number of parasites per cell) (20). Culture-derived trypomastigotes of *T.cruzi* (Tulahuen strain expressing β-galactosidase; DTU VI) were used to infect L929 cultures using a ratio of 10:1 (parasite: host cell). After 2 h, the cultures were washed and cultivated for another 48 h for the establishment of infection. Then, the compounds were added using increasing non-toxic concentrations to the mammalian host cell followed by maintainance at 37ºC for 96 h for determination of EC_{50} values. After addition of 50 µL of the substrate (CPRG - chlorophenol red glycoside) 500 mM) in 0.5% Nonidet P40 and incubation at 37ºC for 18 h, the absorbance at 570 nm was measured, and results expressed as percent inhibition of infection rate (22). For *T.b.* assays, bloodstream forms of the *T.b.r* strain STIB900 were incubated in BMEM for 72 h in the presence of 3-fold serial dilutions at 37 °C in a humidified atmosphere containing 5% CO_2. Parasite viability was assessed with the viability marker Resazurin after a 3 days drug exposure time as previously reported (24).

Mouse acute toxicity: In order to determine the no-observed-adverse-effect level (NOAEL), increasing doses of the tested compounds (up to 200 mg/kg of body weight)

were injected by intraperitoneal (ip) route individually in Swiss female mice (21 to 23 g, n = 2 per assay of the tested compounds). Treated animals were inspected for toxic and sub-toxic symptoms according to the Organization for Economic Cooperation and Development (OECD) guidelines. Forty-eight h after compound injection, the NOAEL values were determined as reported (26). Biochemical analyses performed at 48 h post compound exposure was followed as reported at ICTB platform (Fiocruz/RJ) (26, 27).

Mouse infection and treatment: For *T.cruzi* acute models**,** Swiss Webster male and female mice (18-20 g) obtained from the animal facilities of ICTB were housed at a maximum of 7 per cage and kept in a specific pathogen free (SPF) room at 20–24°C under a 12/12 h light/dark cycle and provided with sterilized water and chow ad libitum. The animals were allowed to acclimate for 7 days before starting the experiments. Infection was performed by intraperitoneal (ip) injection of 10^4 bloodstream trypomastigotes (Y strain). Age-matched non-infected mice were maintained under identical conditions (20). Quinolines were first dissolved in DMSO and then freshly diluted with sterile distilled water. The stock solution of Bz was prepared in sterile distilled water with 3% Tween 80 (Sigma Aldrich). The animals were divided into the following groups (n >3 per group): uninfected (non-infected and non-treated); untreated (infected but treated only with vehicle); and treated (infected and treated with the compounds). The therapy was performed through the administration of 5-20 mg/kg at the parasitemia onset (5 dpi) and parasitemia peak (8 dpi) according to this animal model (28). Alternatively, the most promising quinoline derivatives were administrated for five consecutive days, starting at the 5 dpi, using up to 25 mg/kg/day (via ip) and 100 mg/kg/day Bz (po). In all assays, only mice with positive parasitemia were used in the infected groups. Parasitemia in *T.c.* assays were individually checked by direct

microscopic counting of parasites in 5 µL of blood, and mortality rates checked daily until 30 days post treatment and expressed as percentage of cumulative mortality (% CM) as described before (21).

For *T.brucei* models, efficacy experiments were performed as previously reported (29) with modifications to soften the stringency of the mouse model of infection and in line with the 3R principles for animal testing (reduce, refine, replace), the number of mice was reduced in the primary *in vivo* screen. Female NMRI mice were infected intraperitoneally (i.p.) with 10^4 *T.b.r.* STIB900 bloodstream trypanosomes. Experimental groups of two mice were treated with the new test compounds at 40 mg/kg i.p. on three consecutive days from day 1 to day 3 post infection (120 mg/kg i.p. total dose). A control group was infected but remained untreated. The tail blood of all mice was checked microscopically for parasitemia reduction 24 h and 96 h after the last dose. Parasite reduction of mice treated with the experimental compounds was compared with the untreated control mice. Mice were euthanized after 96 h post treatment, if parasites were still detected in the tail blood. Aparasitemic mice were further examined twice per week for 30 days or mice were euthanized after parasitemia relapses were detected. Mice that remained aparasitemic until day 30 were considered as cured. The follow-up efficacy study for compounds that achieved a parasite reduction of at least 98% in one of the two treated mice was comparable to groups of 4 infected mice and at a higher dosage of 50 mg/kg i.p. treated for 4 consecutive days at a higher dosage of 50 mg/kg i.p (200 mg/kg i.p. total dose). Pentamidine was used as positive drug control and it cured mice at 3x 4 mg/kg or 4x 1 mg/kg i.p.

Statistical Analyses: Statistical analyses performed by the ANOVA test with the level of significance set at $p \leq 0.05$.

Ethics: All animal procedures performed at FIOCRUZ were carried out in accordance with the guidelines established by the Committee of Ethics for the Use of Animals (CEUA LW16/14). All protocols and procedures using *T.brucei* animal models were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland.

Results

The phenotypic *in vitro* study using the ten quinolines derivates (**Table 1**) was performed upon *T.cruzi* and *T.brucei* parasites. Considering that all active drug candidates for *T.cruzi* must be assessed also against the relevant intracellular forms (Romanha et al., 2010), the initial step consisted of the analyses upon intracellular forms (Tulahuen strain transfected with β-galactosidase - DTU VI and Y strain - DTU II). Our findings upon Tulahuen strain showed that all quinolines were more potent than Bz when infected L929 cells were incubated for 96 h at 37 °C, with EC_{50} values ranging from 0.1 µM up to 2.05 µM, and selective indices from 48 up to 960 (**Table 1**). When screening against intracellular forms from Y strain lodge inside cc, the trypanocidal efficacy of quinolines was confirmed, as DB2187 exhibited a low EC_{50} value (1.03 ± 0.3 µM, data not shown).

Following 24 h for incubation with trypomastigote forms of *T.cruzi* (Y strain - DTU II), except DB2171 and DB2192, all quinolines presented higher trypanocidal activity than Bz, exhibiting EC_{50} values ≤ 8 µM (**Table 1**). Among them, DB2187 and its analogue DB2186 were the most effective (EC_{50} ≤ 0.8 µM) being about 12-fold more potent than the reference drug. All molecules showed also a high trypanocidal activity on *T.brucei* bloodstream forms with EC_{50} ranging from 0.016 – 0.239 µM and with a strong selectivity for this parasite, with selectivity indices ranging from 88 to 5.455 (Table 1).

The cytotoxicity data of the studied quinolines using colorimetric assays as PrestoBlue (cardiac cells) and AlamarBlue (L929 cultures) showed that all molecules were tolerated with no detectable toxicity up to 96 μM after 24-96 h of incubation (data not shown). The lack of mammalian host toxicity was confirmed when DB2104, DB2131, DB2161, DB2171 and DB2191 were tested (up to 48 h) using higher concentrations on cardiac cells (up 400 μM) (data not shown). Cytotoxicity on L6 cells was also low and varied from 15 μM (DB2217) to >270 μM (DB2187). LC_{50} values on L6 of each compound can be deduced from the SI of *T.b.r.* (**Table 1**).

For both parasite species (*T.b.* and *T.c.*) DB2186 was the most potent molecule from this series without inhibiting mammalian cells. ADMET properties of quinolines predicted using the pkCSm tool revealed that DB2186, DB2187, DB2192 and DB2217 did not infringe on any of Lipinski´s rule of five which is a prediction of good oral absorption (**Table 2**). The quinolines showed good probability of permeability on Caco cells, with values above the adopted threshold of 0.9, probabilities higher than 89% of human intestinal absorption, with even a better oral absorption profile than Bz, and a positive prediction to be metabolized by CYP3A4 (**Table 3**). These quinolines have low probability of mutagenicity and no prediction to inhibit hERG1, although all show the possibilty of inhibiting hERG2 and a hepatotoxicity profile similar to Bz (**Table 4**). Evaluation of hepatic markers in biochemical analyses *in vivo* using a mouse models of acute toxicity demonstrated no alterations on the plasma levels of ALT (except for DB2192), AST, urea and CK in addition to no major clinical sign (except for losses in animal weight) when mice were given up to 200 mg/kg DB2187 and its derivatives DB2186, DB2191 and DB2192 and followed up to 48 h (data not shown). Next, based on the excellent phenotypic findings and lack of preliminary acute toxicity indications,

DB2187 and derivatives were moved to *in vivo* anti-parasitic analyses using mouse models.

Male mice inoculated with 10^4 bloodstream forms (*T.c.* Y strain) and treated (by ip) at 5 and 8 days after infection (dpi) using non-toxic concentrations of DB2187 gave a maximum reduction of 38% on the parasitemia at the peak (8 dpi) in comparison to the vehicle group but failed to protect against mortality induced by the parasite infection (**Table 5**). The administration of *T.cruzi*-infected male and female mice using non-toxic concentrations of DB2186, DB2191 and DB2192 at 25mg/kg/day for five consecutive days showed that DB2186 was the most active compound reaching 25 and 70 % of reduction on the blood parasitemia load when 25 mg/kg was given for five consecutive days while Bz completely suppressed parasitism (**Table 5**). Regarding the effects on mortality induced by *T.c.* experimental infection, female and male mice infected and only treated with vehicle displayed 50 and 17 % of survival whereas all the Bz-treated animals were alive. The tested quinolines were not able to provide significant protection against mortality (**Table 5**).

The quinolines were also evaluated for their antitrypanosomal efficacy in *T.b.r* infected mice. In the preliminary experiment, the compounds were tested in small groups of 2 mice and treated at 40 mg/kg/day i.p. for three consecutive days. Six compounds showed activity on the level of a strong parasitemia reduction (>98%) in at least one of the two mice and were well tolerated at the tested dosage (**Table 6**). These compounds have been further tested in 4 infected mice at a higher dosage of 50 mg/kg/day i.p. for four consecutive days. DB2186 and DB 2217 were the best molecules, and cured 2 of 4 infected mice and even all 4 infected mice, respectively (**Table 6**).

Discussion

Most drug development programs for neglected diseases are time-consuming (often more than 10 years), highly expensive (more than $1 Billion) and get only limited attention by the pharmaceutical industry. Up to now no vaccine is available for Chagas disease and for HAT and the current therapies available have strong liabilities, and thus novel therapeutic options are urgently needed. Drug discovery and development strategies include phenotypic screening of synthetic and natural molecules, the assessment of combination therapies, repurposing of medicines and drug development towards selective parasite targets (12, 27, 30). Our goal was to investigate the biological effect *in vitro* and *in vivo* of the ten novel quinoline derivatives (**Table 1**) against *T.cruzi and T.brucei* infection. We have conducted different analyses that include computational and cell-based screening as well as mouse models with trypanosome infections. As reported, *in silico* analyses has as advantages of low cost, fast processing, and the fact that compounds can be evaluated without synthesizing them, allowing large libraries to be explored. However, computational screens often fail to simulate the full complexity of biological systems and need to be complemented with experimental studies (31). Presently, the anti-parasitic activity of the novel quinolines was explored considering different aspects of the drug discovery cascade performed by *in silico, in vitro* (whole cell-based) and *in vivo* assays (the most biologically realistic), following current strategies for hit and lead identification for novel anti-*T.c*. and anti-*T.b.* drugs. Our findings demonstrated the promising *in vitro* activity of these compounds towards both bloodstream trypomastigotes (*T.c.* and *T.b.*) and intracellular forms (*T.c*.) of these parasites with most molecules exhibiting greater potency than the reference drug for Chagas disease (Bz). The quinolines have also potential to be developed for *T.b.* although they were less potent than the highly toxic compound melarsoprol or the *T.b.g.*

first stage drug pentamidine. The quinoline DB2186 was very active against both trypanosomes regardless of the parasite form displaying quite high selectivity indices even superior to those reported for novel hits for CD and HAT (11, 12, 32). Regarding *T.cruzi* screens, the quinolines were active against parasite strains from different DTUs (II and VI for Y strain and Tulahuen strains, respectively) furthermore showing low toxicity towards mammalian host cells, including primary cultures of cardiac cells that provides in a more sensitive manner, the potential for *in vivo* cardiotoxicity. These are very critical data since the heart represents an important target for *T.cruzi* infection and inflammation (33). In fact, the plasma biochemical analyses of quinoline-treated mice confirmed the low cardiotoxicity profile noticed by CK measurements. Thus, *in vitro* whole-cell based screening associated with theoretical analyses of the ADMET properties and mouse models of acute toxicity were used to select potential drug candidates to proceed to *in vivo* efficacy evaluations (17). The *in silico* properties of the novel quinolines were evaluated using the pkCSM tool and the overall findings predicted good oral absorption and probability of permeability on Caco cells and human intestinal absorption and low probability of mutagenicity and inhibition of hERG1. The preliminary acute murine toxicity assays fail to demonstrate increased levels of hepatic lesion markers such as ALT and AST except for DB2192 (statistically significant enhancement of ALT levels indicative of hepatic damage). To evaluate efficacy *in vivo for T.cruzi* infection, both female and male mice models were used and our data confirmed the more susceptible profile of male mice to *T.cruzi* experimental infection as compared to female (28). The findings also demonstrated that DB2186 was the most promising candidate for *T.cruzi* infection as well as for *T.brucei* murine models. It is important to consider that DB2186 exhibited consistently high selectivity indices for *T.c.* (123 and 640 for BT and intracellular forms) and *T.b.* (1.761 for BT). It is interesting to note that

DB2171 is much less active than two close analogues DB2186 and DB2192 pointing to the importance of the urea fragment for activity compared to the simple amide unit.

The limited water solubility of the quinolines may have impaired a more successful *in vivo* result which also was not improved by the use of other vehicles including cyclodextrin and carboxymethylcellulose (data not shown). It is possible that minor structural modifications can improve their solubility allowing further animal studies. The present set of results provide a basis for the development of novel quinoline derivatives following medicinal chemistry approaches presenting better solubility and improving their potency and thus contributing to the identification of more effective and safe medicines to treat neglected tropicl diseases such as Chagas disease and HAT.

Acknowledgments

The presente study was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Oswaldo Cruz, PDTIS, PAEF/CNPq/Fiocruz and CAPES. MNCS is research fellow of CNPq and CNE research. The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of its facilities. This work was also supported, in part, by the National Institutes of Health USA Grant No. AI06420 and by The Bill and Melinda Gates Foundation through a subcontract with the CPDD (to D.W.B. and R. B.).

References

1. World Health Organization. 2017. Working to overcome the global impact of neglected tropical diseases: first WHO report on neglected tropical diseases. World Health Organization, Geneva, Switzerland: http://www.who.int/neglected_diseases/2010report/en/

2. Drugs for Neglected Diseases Initiave. 2017. Drugs for Neglected Diseases Initiave, Geneva, Switzerland: https://www.dndi.org/

3. Mackey TK, Liang BA, Cuomo R, Hafen R, Brouwer KC, Lee DE. 2014. Emerging and reemerging neglected tropical diseases: a review of key characteristics, risk factors and the policy and innovation environment. Clin Microbiol Rev 4:949-79.

4. Büscher P, Cecchi G, Jamonneau V, Priotto G. Human African trypanosomiasis. 2017. Lancet pii: S0140-6736(17)31510-6.

5. Chatelain E. 2015. Chagas disease drug discovery: toward a new era. J Biomol Screen 1: 22-35.

6. Chatelain E, Konar N. 2015. Translational challenges of animal models in Chagas disease drug development: a review.Drug Design, Development and Therapy 9: 4807–4823.

7. Molina I, Salvador F, Sánchez-Montalvá A. 2015. The use of posaconazole against Chagas disease. Curr Opin Infect Dis 5:397-407.

8. Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A Jr, Rosas F, Villena E, Quiroz R, Bonilla R, Britto C, Guh l F, Velazquez E Bonilla L, Meeks B, Rao-Melacini P, Pogue J, Mattos A, Lazdins J, Rassi A, Connolly SJ, Yusuf S, BENEFIT, Investigators. 2015. Randomized Trial of Benznidazole for Chronic Chagas' Cardiomyopathy. N Engl J Med 14:1295-306.

9. Devine W, Thomas SM, Erath J, Bachovchin KA, Lee PJ, Leed SE, Rodriguez A, Sciotti RJ, Mensa-Wilmot K, Pollastri MP. Antiparasitic Lead Discovery: Toward Optimization of a Chemotype with Activity Against Multiple Protozoan Parasites. 2017. ACS Med Chem Lett 3:350-354.

10. Chatelain E. Chagas disease research and development: Is there light at the end of the tunnel? 2017. Comput Struct Biotechnol J 15:98-103.

11. Don R, Ioset JR. 2013. Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. Parasitology 141:140-6.

12. Field MC, Horn D, Fairlamb AH, Ferguson MA, Gray DW, Read KD, De Rycker M, Torrie LS, Wyatt PG, Wyllie S, Gilbert IH. 2017. Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. Nat Rev Microbiol 4:217-231.

13. Keenan M, Chaplin JH. 2015. A new era for chagas disease drug discovery? Prog Med Chem 54:185-230.

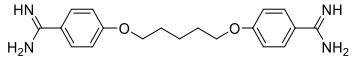
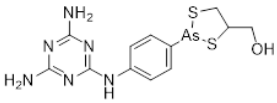
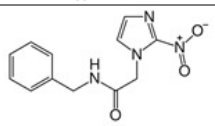
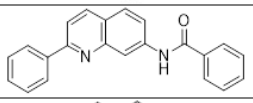
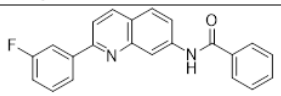
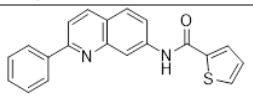
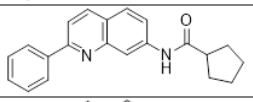
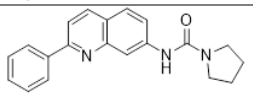
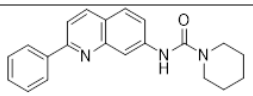
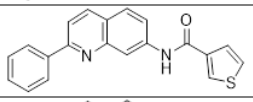
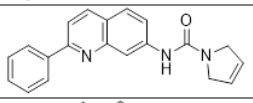
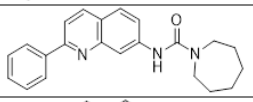
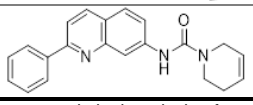
448 14. Bahia MT, Nascimento AF, Mazzeti AL, Marques LF, Gonçalves KR, Mota LW,
449 Diniz Lde F, Caldas IS, Talvani A, Shackleford DM, Koltun M, Saunders J, White KL,
450 Scandale I, Charman SA, Chatelain E. 2014. Antitrypanosomal activity of fexinidazole
451 metabolites, potential new drug candidates for Chagas disease. Antimicrob Agents
452 Chemother 58:4362-70.
453
454 15. Tatipaka HB, Gillespie JR, Chatterjee AK, Norcross NR, Hulverson M A, Ranade
455 RM, Nagendar P, Creason SA, McQueen J, Duster NA, Nagle A, Supek F, Molteni V,
456 Wenzler T, Brun R, Glynne R, Buckner FS, Gelb MH, Substituted 2-
457 phenylimidazopyridines: A new class of drug leads for human african trypanosomiasis.
458 2014. J Med Chem 57: 828−835.
459
460 16. Patrick DA, Wenzler T, Yang S, Weiser PT, Wang MZ, Brun R, Tidwell RR.,
461 2016. Synthesis of novel amide and urea derivatives of thiazol-2-ethylamines and their
462 activity against trypanosoma brucei rhodesiense. Bioorg Med Chem 24: 2451−2465.
463
464 17. Nefertiti ASG, Batista MM, Da Silva PB, Torres-Santos EC, Cunha-Júnior EF,
465 Green J, Kumar A, Farahat AA, Boykin DW, Soeiro MNC. 2017. Anti-parasitic effect of
466 novel amidines against *Trypanosoma cruzi*: phenotypic and *in silico* absorption,
467 distribution, metabolism, excretion and toxicity analysis. Parasitology Open 5: 1 - 8.
468
469 18. Pires DE., Blundell TL, Ascher DB. 2015. pkCSM: Predicting Small-Molecule
470 Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures. J Med Chem
471 9:4066-72.

472 19. Meirelles MN, De Araujo-Jorge T, C Miranda, CF, De Souza W, Barbosa HS.
473 1986. Interaction of *Trypanosoma cruzi* with heart muscle cells: ultrastructural and
474 cytochemical analysis of endocytic vacuole formation and effect upon myogenesis *in*
475 *vitro.* European Journal Cell Biology 41:198-206.
476
477 20. Batista DG, Pacheco MG, Kumar A, Branowska D, Ismail MA, Hu L, Boykin DW,
478 Soeiro MN. 2010. Biological, ultrastructural effect and subcellular localization of aromatic
479 diamidines in *Trypanosoma cruzi*. Parasitology 2: 251-9.
480
481 21. Timm BL, da Silva PB, Batista MM, da Silva FHG, da Silva CF, Tidwell RR,
482 Patrick DA, Jones SK, Bakunov, SA, Bakunova, SM, Soeiro, MNC. 2014. *In vitro* and *In*
483 *vivo* Biological Effect of Novel Arylimidamide Derivatives Against *Trypanosoma cruzi.*
484 Antimicrob Agents Chemother 7: 3720-6.
485
486 22. Romanha AJ, de Castro SL, Soeiro MNC, Lannes-Vieira J, Ribeiro I, Talvani A,
487 Bourdin B, Blum B, Olivieri B, Zani C, Spadafora C, Chiari E, Chatelain E, Chaves G,
488 Calzada JE, Bustamante JM, Freitas-Junior LH, Romero LI, Bahia MT, Lotrowska M,
489 Soares M, Andrade SG, Armstrong T, Degrave W, Andrade ZA. 2010. *In vitro* and *in*
490 *vivo* experimental models for drug screening and development for Chagas disease.
491 Mem Inst Oswaldo Cruz 105:233-238.
492
493 23. Brun R, Schumacher R, Schmid C, Kunz C, Burri C. 2001. The phenomenon of
494 treatment failures in human African trypanosomiasis. Trop Med Int Health 6:906–914.
495

496 24. Bakunov SA, Bakunova SM, Wenzler T, Ghebru M, Werbovetz KA, Brun R,
497 Tidwell RR. 2010. Synthesis and antiprotozoal activity of cationic 1,4-Diphenyl-1- H-
498 1,2,3-triazoles. J Med Chem 53: 254.
499
500 25. Zingales B, Andrade SG, Briones MR, Campbell DA, Chiari E, Fernandes O, Guhl
501 F, Lages-Silva E, Macedo AM, Machado CR, Miles MA, Romanha AJ, Sturm NR,
502 Tibayrenc M, Schijman AG. 2009. A new consensus for *Trypanosoma cruzi* intraspecific
503 nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo
504 Cruz 7:1051-4.
505
506 26. Da Silva CF, BatistaDda G, Oliveira GM, de Souza EM, Hammer ER, da Silva
507 PB, Daliry A, Araujo JS, Britto C, Rodrigues AC, Liu Z, Farahat AA, Kumar A, Boykin
508 DW, Soeiro Mde N C. 2012. *In vitro* and *in vivo* investigation of the efficacy of
509 arylimidamide DB1831 and its mesylated salt form--DB1965-against *Trypanosoma cruzi*
510 infection. PLoS One 1: e30356.
511
512 27. Soeiro MNC, Werbovetz K, Boykin DW, Wilson WD, Wang MZ, Hemphill A. 2013.
513 Novel amidines and analogues as promising agents against intracellular parasites: a
514 systematic review. Parasitology (8):929-51.
515
516 28. Guedes-da-Silva FH, Batista DG, da Silva CF, Meuser MB, Simões-Silva MR, de
517 Araújo JS, Ferreira CG, Moreira OC, Britto C, Lepesheva GI, Soeiro Mde N. 2015.
518 Different Therapeutic Outcomes of Benznidazole and VNI Treatments in Different
519 Genders in Mouse Experimental Models of *Trypanosoma cruzi* Infection. Antimicrob
520 Agents Chemother 12:7564-70.

521 29. Wenzler T, Yang S, Patrick DA, Braissant O, Ismail MA, Tidwell RR, Boykin DW,
522 Wang MZ, Brun R. *In vitro* and *in vivo* evaluation of 28DAP010, a novel diamidine for
523 treatment of second-stage African sleeping sickness. 2014. Antimicrob Agents
524 Chemother 8:4452-63.
525
526 30. Peña I, Pilar Manzano M, Cantizani J, Kessler A, Alonso-Padilla J, Bardera AI,
527 Alvarez E, Colmenarejo G, Cotillo I, Roquero I, de Dios-Anton F, Barroso V, Rodriguez
528 A, Gray DW, Navarro M, Kumar V, Sherstnev A, Drewry DH, Brown JR, Fiandor JM,
529 Julio Martin J. 2015. New compound sets identified from high throughput phenotypic
530 screening against three kinetoplastid parasites: an open resource. Sci Rep 5:8771.
531
532 31. Williams K, Bilsland E, Sparkes A, Aubrey W, Young M, Soldatova LN, De Grave
533 K, Ramon J, de Clare M, Sirawaraporn W, Oliver SG, King RD. 2015. Cheaper faster
534 drug development validated by the repositioning of drugs against neglected tropical
535 diseases. J R Soc Interface 104:20141289.
536
537 32. Katsuno K, Burrows JN, Duncan K, Hooft van Huijsduijnen R, Kaneko T, Kita K,
538 Mowbray CE, Schmatz D, Warner P, Slingsby BT. 2015. Hit and lead criteria in drug
539 discovery for infectious diseases of the developing world. Nat Rev Drug Discov 11:751-
540 8.
541
542 33. Rassi A Jr, Marin JA Neto, Rassi A. 2017. Chronic Chagas cardiomyopathy: a
543 review of the main pathogenic mechanisms and the efficacy of aetiological treatment
544 following the BENznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial.
545 Mem Inst Oswaldo Cruz 3:224-235.

Table 1: Antitrypanosomal activities (µM) of novel quinolines against bloodstream trypomastigotes of *T. brucei* and intracellular and bloodstream forms of *T.cruzi* and corresponding selective indices (SI).

EC_{50} (Mean ± SD) and SI (*) values				
Chemical Structure	**Compound**	***T. b.r.*** **BT forms**	***T. cruzi*** **BT forms**	***T. cruzi*** **Intracellular forms**
	pentamidine	0.003 (11436*)	NT	NT
	melarsoprol	0.004 (1275*)	NT	NT
	benznidazole	NT	9.6 ± 1.4 (>104*)	2.7 ± 1 (370*)
	DB2104	0.213 (971*)	6.1 ± 2.8 ** (>66*)	0.54 ± 0.2 (>178*)
	DB2131	0.204 (229*)	2.5 ± 1.1 ** (>160*)	0.6 ± 0.3 (>160*)
	DB2161	0.076 (1164*)	8 ± 2.9 (>50*)	0.5 ± 0.038 (>192*)
	DB2171	0.239 (983*)	15 ± 8 (>27*)	2.05 ± 0.6 (>47*)
	DB2186	0.016 (1761*)	0.78 ± 0.46 ** (>123*)	0.15 ± 0.01 (>640*)
	DB2187	0.050 (>5455*)	0.8 ± 0.2 ** (>137*)	0.36 ± 0.12 (>233*)
	DB2191	0.057 (647*)	2.6 ± 0.64 ** (>154*)	0.1 ± 0 (>960*)
	DB2192	0.045 (3828*)	24 ± 5.8 (>4*)	0.1 ± 0.001 (>960*)
	DB2212	0.123 (>2118*)	7.2 ± 3.2 (>12*)	0.3 ± 0.039 (>320*)
	DB2217	0.170 (88*)	2.7 ± 1.2 ** (>36*)	0.41 ± 0.27 (>234*)

**Anova statistical analysis of studied compound and Bz: ($p < 0.05$).

Table 2: Physicochemical parameters and Lipinski's rule of five.

Parameters	Water solubility (mg/L)	Donors	Acceptors	LogP	MW
DB2104	2.394	2	4	5.154	324.383
DB2131	1.041	2	4	5.293	342.373
DB2161	1.416	3	4	5.215	330.412
DB2171	2.775	2	4	5.030	316.404
DB2186	3.887	2	4	4.529	317.392
DB2187	-5.9	1	2	4.9	333.419
DB2191	1.416	3	4	5.215	330.412
DB2192	5.356	2	4	4.305	315.376
DB2212	0.934	2	4	5.309	345.446
DB2217	2.587	2	4	4.695	329.403
Bz	376.248	1	5	0.11	260.253

Table 3: *In silico* ADME.

	DB2104	DB2131	DB2161	DB2171	DB2186	DB2187	DB2191	DB2192	DB2212	DB2217	Bz
ABSORPTION											
Caco2 permeability (log cm/s)	1.491	1.163	1.773	1.765	1.373	1.21	1.773	1.361	1.158	1.384	0.479
Intestinal absorption (human,%)	94.747	89.969	89.46	90.659	90.877	91.515	89.46	91.353	90.1	90.964	68.885
Skin Permeability (logKp)	-2.763	-3.167	-3.118	-3.092	-3.214	-3.012	-3.118	-3.238	-3.198	-3.23	-2.893
DISTRIBUTION											
VDss (human) (L/kg)	0.345	5.521	5.140	6.124	4.721	-0.115	5.140	4.624	5.508	4.989	0.787
Fraction unbound (human)	0	0.278	0.292	0.3	0.321	0.064	0.292	0.331	0.275	0.307	0.503
BBB permeability	0.264	0.265	0.241	0.254	0.201	0.269	0.241	0.2	0.227	0.213	-0.619
CNS permeability	-1.013	-2.778	-2.687	-2.687	-2.691	-1.639	-2.687	-2.691	-2.782	-2.737	-2.995
METABOLISM											
CYP2D6 substrate	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP1A2 inhibitior	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP2C19 inhibitior	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP2C9 inhibitior	Yes	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitior	No	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitior	No	No	No	No	No	No	No	No	No	No	No
EXCRETION											
Total Clearance (ml/min/kg)	3.793	6.998	12.023	10.162	8.433	0.545	12.023	6.714	6.823	6.368	4.217

Table 4: *In silico* toxicity.

Parameters	DB2104	DB2131	DB2161	DB2171	DB2186	DB2187	DB2191	DB2192	DB2212	DB2217	Bz
AMES toxicity	No	No	No	No	No	No	No	No	No	No	Yes
Max. tolerated dose (human)	17.418	1.393	1.758	1.807	1.758	0.784	1.758	1.766	1.330	1.535	9.638
hERG I inhibitor I	No	No	No	No	No	No	No	No	No	No	No
hERG II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Oral Rat Acute Toxicity (LD_{50})	2.535	2.906	2.924	2.719	2.817	2.856	2.924	2.783	2.885	2.819	2.454
Oral Rat Chronic Toxicity (LOAEL) (mg/kg_bw/day)	289.068	46.345	61.094	80.168	47.973	1.878	61.094	43.451	46.345	42.756	44.566
Hepatotoxicity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Skin Sensitisation	No	No	No	No	No	No	No	No	No	No	No
T. Pyriformis toxicity $pIGC_{50}$ (ug/L)	5.929	29.923	33.884	31.405	31.842	1.256	33.884	31.333	28.642	30.479	16.866
Minnow toxicity LC_{50} (mM)	0.586	4.732	3.365	4.325	6.039	0.739	3.365	7.244	3.524	5.534	44.566

Table 5: Antitrypanosomal activity of quinolines in mouse models of *T.cruzi* infection (Y strain) using 25 mg/kg (i.p.) for five consecutive days starting at the parasitemia onset (5dpi).

Compound	Gender	% Parasite variation at the parasitemia peak (8 dpi)	% Cumulative mortality at 30 days post therapy
Benznidazole*	Female Male	100 100	0 0
Vehicle	Female Male	- -	50 83
DB2187**	Male	-38	100
DB2186	Female Male	-25 -70	75 50
DB2191	Female Male	-9 -27	50 100
DB2192	Female Male	+74 +28	33 100

*Benznidazole was tested at 100 mg/kg p.o.
**DB2187 was tested at 20 mg/kg ip at 5 and 8 dpi.

Table 6: Antitrypanosomal activity of quinolines in a mouse model of STIB900 *T. b. r.* infection.

Compound	% Parasite reduction x days after treatment at 3x 40 mg/kg i.p.		Cures at 4 x 50 mg/kg i.p.
	1 day	3 days	
pentamidine*	100 100	100 100	4/4*
DB2104	<98 <98	<98 <98	NT
DB2131	<98 98	100 <98	0/4
DB2161	<98 <98	100 <98	0/4
DB2171	<98 <98	99 <98	0/4
DB2186	100 100	<98 <98	2/4
DB2187	<98 98	100 <98	0/4
DB2191	<98 <98	<98 <98	NT
DB2192	<98 <98	<98 <98	NT
DB2212	<98 <98	<98 <98	NT
DB2217	<98 <98	<98 100	4/4

NT= not tested. * Pentamidine was tested at 3x 4 mg/kg i.p. and 4x 1 mg/kg i.p. and cured all infected mice.