

# Anti-parasitic effect of novel amidines against *Trypanosoma cruzi*: phenotypic and *in silico* absorption, distribution, metabolism, excretion and toxicity analysis

ALINE SILVA DA GAMA NEFERTITI<sup>1</sup>, MARCOS MEUSER BATISTA<sup>1</sup>,  
PATRÍCIA BERNARDINO DA SILVA<sup>1</sup>, EDUARDO CAIO TORRES-SANTOS<sup>2</sup>,  
EDEZIO F. CUNHA-JÚNIOR<sup>2</sup>, JULIUS GREEN<sup>3</sup>, ARVIND KUMAR<sup>3</sup>,  
ABDELBASSET A. FARAHAT<sup>3,4</sup>, DAVID WILSON BOYKIN<sup>3</sup>  
and MARIA DE NAZARE CORREIA SOEIRO<sup>1\*</sup>

<sup>1</sup> Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil

<sup>2</sup> Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil

<sup>3</sup> Department of Chemistry, Georgia State University, Atlanta, Georgia, USA

<sup>4</sup> Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

(Received 3 January 2017; revised 14 March 2017; accepted 16 March 2017)

## SUMMARY

New more selective and potent drugs are urgently need to treat Chagas disease (CD). Among the many synthetic compounds evaluated against *Trypanosoma cruzi*, aromatic amidines (AAs) and especially arylimidamides (AIAs) have potent activity against this parasite. Presently, the effect of four mono-amidines (DB2228, DB2229, DB2292 and DB2294), four diamidines (DB2232, DB2235, DB2251 and DB2253) and one AIA (DB2255) was screened *in vitro* against different forms (bloodstream trypomastigotes – BT and intracellular forms) and strains from discrete typing unit (DTU) I and VI of *T. cruzi* and their cytotoxic profile on mammalian host cells. Except for DB2253, all molecules were as active as benznidazole (Bz), resulting in 50% of reduction in the number of alive BT, with EC<sub>50</sub> ranging from 2.7 to 10.1 μM after 24 h of incubation. DB2255 was also the most potent against amastigotes (Tulahuen strain) showing similar activity to that of Bz (3 μM). *In silico* absorption, distribution, metabolism, excretion and toxicity analysis demonstrated probability of human intestinal adsorption, while mutagenicity and inhibition of hERG1 were not predicted, besides giving acceptable predicted volumes of distribution. Our findings contribute for better knowledge regarding the biological effect of this class of aromatic molecules against *T. cruzi* aiming to identify novel promising agent for CD therapy.

Key words: aromatic amidines, Chagas disease, experimental chemotherapy, toxicity, selectivity, *in silico* ADMET analysis.

## INTRODUCTION

Over 100 years ago, Carlos Chagas, a Brazilian researcher discovered a new disease, American trypanosomiasis or Chagas disease (CD) caused by a flagellated protozoan *Trypanosoma cruzi*. CD is endemic to 21 countries of Latin American, affecting more than 6 million individuals (WHO, 2016).

CD is currently emerging in non-endemic areas, such as North America, Europe and Oceania, mostly associated with the migration of infected carriers (Albajar-Viñas and Dias, 2014). CD presents in two stages: an acute and a later chronic phase, which after years or decades about 30–40% of patients progress to symptomatic forms, causing heart disease and/or digestive and neurological disorders (Teixeira *et al.* 2006; Marin-Neto *et al.* 2008; Coura and Dias, 2015). The two drugs currently

available for clinical treatment are the nitroderivates, nifurtimox (Nif) and benznidazole (Bz), were introduced about four decades ago into clinical use and up to now remain the only treatment options (Patterson and Wyllie, 2014). The major limitations of these compounds include the need for long-time administration and their considerable side-effects that in some cases leads to the discontinuation of treatment, therapeutic failure at the later chronic phase and exhibition of limited effectiveness against naturally resistant strains (Wilkinson *et al.* 2008). A novel candidate for CD therapy should present as drug characteristics: (i) efficacy upon the two phases of the disease, especially the later chronic stage; (ii) potency on different parasite discrete typing units (DTUs; I, II, V and VI) and forms relevant for human infection (trypomastigotes and amastigotes); (iii) low toxicity and absence of genotoxicity, mutagenicity and cardiotoxicity; (iv) be orally administered; (v) with good stability (3–5 years in climatic zone) with (vi) low costs (Chatelain and Konar, 2015; DNDi, 2016).

\* Corresponding author: Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil, 4365 Manguinhos, Rio de Janeiro, Brazil. E-mail: soeiro@ioc.fiocruz.br

A recent clinical trial, which included a 5-year follow-up, seeking the benefits of the trypanocidal therapy using Bz in patients with established Chagas' cardiomyopathy showed that although there was a significant reduction in total parasite load, this drug was not able to impair cardiac clinical deterioration (Morillo *et al.* 2015). These findings corroborate the need to find alternative therapies for CD. Aromatic amidines (AA) are dicationic molecules with many of them such as pentamidine (Pt) presenting DNA minor groove-binding characteristics (Soeiro *et al.* 2013). The anti-parasitic action of Pt has been known since 1937 (King *et al.* 1937) and in the ensuing years many analogues and derivatives have been synthesized and screened against parasitic organisms. Several of these molecules have demonstrated a wide spectrum of activity against human and veterinary pathogens such as leishmaniasis, human African trypanosomes and *T. cruzi* (De Souza *et al.* 2004; Soeiro *et al.* 2008, 2013; De Araújo *et al.* 2014). Among novel amidine molecules, the arylimidamides (AIAs) have shown very promising profiles and potent activity against intracellular parasites like *Neospora caninum*, *Leishmania* sp and *T. cruzi* (Soeiro *et al.* 2013). The present study investigates the anti-*T. cruzi* activity of additional novel amidines (four mono-amidines, four diamidines and one AIA) through phenotypic studies *in vitro* by assessing different forms and parasite strains besides determining their toxicity towards different host cell types (as L929 cell lines and primary cultures of cardiac cells) and their absorption, distribution, metabolism, excretion and toxicity (ADMET) properties from *in silico* predictions.

## MATERIAL AND METHODS

### Compounds

The synthesis of the four studied mono-amidines (2 – (5 – (4 – ((1 (quinolin – yl-1,2,3-triazol-4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole-6-carboximidamide hydrochloride (DB2228), 2-(5-(4-((1-(2-(naphthalen-1-yl) ethyl) – 1H-1,2,3-triazol-4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole-6-carboximidamide hydrochloride (DB2229), 2-(5-4-((1-(2-(2-(naphthalene-2-yloxy) ethoxy) ethyl)-1H-1,2,3-triazol-4-yl) methoxy) phenyl) thiophen- 2- yl-1H-benzo[d]imidazole – 6 - carboximidamide hydrochloride (DB2292) and 2-(5-(4-((1- (2-(2-(2-(naphthalene-2-yloxy) ethoxy) ethoxy) ethyl)-1H-1,2,3- triazol - 4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole-6-carboximidamide hydrochloride (DB2294)) has been previously described (Green, 2014). The synthetic route of the four diamidines (2,2'-((propane-1,3-diylbis (oxy)) bis (4,1-phenylene)) bis (1H-benzo [d]imidazole-6-carboximidamide) dihydrochloride

(DB2232), 4,4'-((1-phenyl-1H-pyrrole-2,5-diyl) dibenzimidamide dihydrochloride (DB2235), 2,2'-((1-phenyl-1 H-pyrrole-2,5-diyl) bis (4,1-phenylene)) bis (4,5-dihydro-1H-imidazole) dihydrochloride (DB2251), 2,2'-((1-phenyl-1H-pyrrole-2,5-diyl) bis (4,1-phenylene)) bis (1, 4, 5, 6-tetrahydropyrimidine) dihydrochloride (DB2253)) was also conducted using a methodology previously reported (Ismail *et al.* 2004; Farahat *et al.* 2011). The synthesis of the bis-AIA N, N''-((2-oxoimidazolidine-1,3-diyl) bis (3-iso-propoxy-4,1- phenylene)) dipicolinimidamide dihydrochloride (DB2255) was previously reported (Stephens *et al.* 2001) (Fig. 1). All compounds have been fully characterized by spectral methods (nuclear magnetic resonance [NMR] and mass spectrometry [MS]) and by satisfactory C, H, N analysis. Bz (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as reference drug. Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0.6% DMSO, which is not toxic to the parasite and mammalian cells.

### Parasites

Bloodstream trypomastigote (BT) forms of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitaemia. The purified parasites were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as reported previously (Batista *et al.* 2010). Trypomastigotes of Tulahuén strain expressing the *Escherichia coli*  $\beta$ -galactosidase gene (Buckner *et al.* 1996) were collected from the supernatant of infected cell cultures (L929 culture) as reported (Romanha *et al.* 2010).

### Cell cultures

For the toxicity assays on mammalian cells, primary cultures of cardiac cells were obtained from mice embryos plated onto coverslips in 96 well plates previously coated with 0.01% gelatin (Meirelles *et al.* 1986). L929 cell lineages were obtained as described and maintained in RPMI-1640 medium (pH 7.2–7.4) without phenol red (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine (RPMI), as reported previously (Romanha *et al.* 2010).

### Cytotoxicity *in vitro* tests

The cardiac cells were incubated for 24 h at 37 °C with different concentrations of each compound diluted in RPMI 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

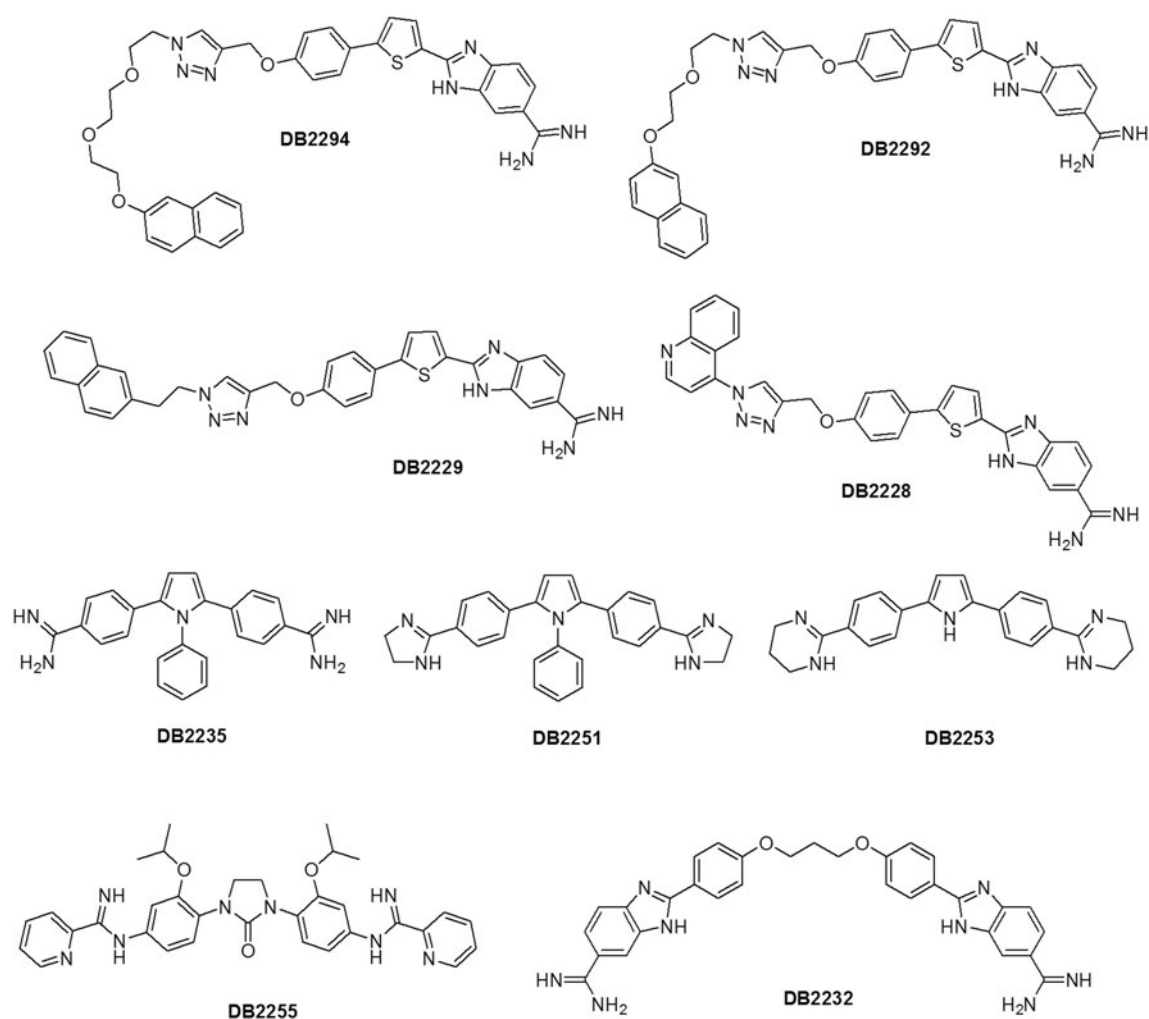


Fig. 1. Chemical structure of the nine selected amidines assayed in this work.

(Timm *et al.* 2014). L929 cell lineages incubated for 24 and 96 h at 37 °C, with different concentrations of each compound diluted in RPMI and their cellular viability determined by the AlamarBlue test as reported (Timm *et al.* 2014). The maximum concentration of each compound was 96  $\mu\text{M}$  due to molecule precipitation. The results expressed by following the manufacturer instructions and the value of  $\text{CC}_{50}$  that corresponds to the concentration that reduces in 50% the cellular viability, determined. Selective index (SI) expressed by ratio between the values obtained for  $\text{CC}_{50}$  over the host cells and the  $\text{EC}_{50}$  obtained over the parasites.

#### Trypanocidal activity

Bloodstream trypomastigotes (BT) of the Y strain (DTU II) (Zingales *et al.* 2009) ( $5 \times 10^6$  per mL) were incubated for 2 and 24 h at 37 °C in RPMI in the presence or not of serial dilution of the compounds (up to 32  $\mu\text{M}$ ). After compound incubation, the death parasite rates were determined by light microscopy through the direct quantification of

the number of live parasites using a Neubauer chamber, and the  $\text{EC}_{50}$  concentration (the compound concentration that reduces in 50% the number of parasites) was calculated (Timm *et al.* 2014). For the assay on intracellular forms, culture-derived trypomastigotes of *T. cruzi* (Tulahuen strain expressing  $\beta$ -galactosidase; DTU VI) (Zingales *et al.* 2009) used to infect L929 infected-cells cultures using a ratio of 10:1 (parasite: host cell). After 2 h, the cultures rinsed and further incubated for 48 h for establishment of the infection. Then, the compounds were added (initially using a fixed concentration of 10  $\mu\text{M}$  followed by other set of assays using increasing non-toxic concentrations to the mammalian host cell for determination of  $\text{EC}_{50}$  values) and the cultures maintained at 37 °C for 96 h. After addition of 50  $\mu\text{L}$  of the substrate [(CPRG – chlorophenol red glycoside) 500 mM] in 0.5% Nonidet P40 and incubation at 37 °C for 18 h, the absorbance at 570 nm was measured, and the results expressed as per cent of parasite growth inhibition (Romanha *et al.* 2010).

In all assays, at least three experiments ( $n \leq 3$ ) were done using  $\leq 2$  replicates.

### Computational assessment of the drug-like properties of the tested compounds

ADMET properties of the studied amidines evaluated using the pkCSM approach, which uses graph-based signatures to develop predictive ADMET (Pires *et al.* 2015).

### Statistical analysis

Statistical analysis was performed using Student's *t*-test with significance set at  $p \leq 0.05$ .

### Ethics

All procedures were conducted in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

## RESULTS

Initially, the biological assays were carried out to evaluate the activity of these molecules upon BT forms (Y strain – DTU II), and their respective toxicity towards cardiac cells. Our findings demonstrate that after a short period of incubation (2 h), six out of the nine drugs demonstrate trypanosomicidal activity against BT, exhibiting  $EC_{50}$  values lower than  $20 \mu\text{M}$ , while Bz was inactive (Table 1). DB2229 and DB2294 displayed an  $EC_{90}$  value  $<10 \mu\text{M}$  after only 2 h of compound treatment (Table 1) presenting a fast activity towards these forms. After 24 h, seven molecules (DB2228, DB2229, DB2232, DB2235, DB2255, DB2292 and DB2294) were more potent ( $EC_{50} \leq 8.3 \mu\text{M}$ ) than Bz ( $9.6 \mu\text{M}$ ), being DB2292 about 3-fold more active than the reference drug (Table 1). The toxicity profile assessed using cardiac cell cultures to exclude compounds and

concentrations that presented cardiotoxic characteristics evaluated by morphological, contractility and density analysis besides through cellular viability approach using a colorimetric methodology (PrestoBlue). Only DB2235 and DB2251 presented detectable toxicity up to the studied concentrations ( $CC_{50} = 49 \pm 21$  and  $62 \pm 23 \mu\text{M}$ , respectively) (Table 1).

Next, further assays analysed the activity on intracellular forms of *T. cruzi*, using the Tulahuen strain transfected with  $\beta$ -galactosidase, as previous reported (Romanha *et al.* 2010). The trypanocidal action after 96 h of incubation using a fixed concentration of  $10 \mu\text{M}$  showed that only the AIA DB2255 displayed a high inhibition of the parasite growth (88%), reaching similar activity to that of Bz (Table 2). Therefore, DB2255 was the only molecule selected for the next screening step, consisting of infection of L929 cells followed by incubation with non-toxic concentrations (up to  $32 \mu\text{M}$ ). DB2255 and Bz presented similar potency ( $EC_{50}$  values of  $3.6 \pm 0.39$  and  $3 \pm 1 \mu\text{M}$ , respectively), but the reference drug exhibited higher selectivity (data not shown).

Mathematic parameters of drug likeness including, absorption, distribution, metabolism, excretion and toxicity properties were calculated using the pkCSM approach that uses graph-based signatures to develop predictive of ADMET (Pires *et al.* 2015). *In silico* ADMET analysis demonstrated probability of human intestinal adsorption ( $>90\%$ ), while mutagenicity and inhibition of hERG1 were not predicted, besides giving acceptable predicted volumes of distribution (Tables 3 and 4).

## DISCUSSION

In the last 40 years the only available treatment for CD has been two nitrohetocyclic agents, Bz and

Table 1. *In vitro* activity of the amidines and benzimidazole on bloodstream trypomastigotes of the Y strain and on cardiac cells:  $EC_{50}$  and  $EC_{90}$  values after 2 and 24 h,  $CC_{50}$  values of CC after 24 h of incubation at  $37^\circ\text{C}$ , respectively, and the corresponding selectivity index (SI)

Compound	$EC_{50}$ (mean $\pm$ s.d.) $\mu\text{M}$		$EC_{90}$ (mean $\pm$ s.d.) $\mu\text{M}$		$CC_{50}$ (mean $\pm$ s.d.) $\mu\text{M}$	
	2 h	24 h	2 h	24 h	24 h	SI 24 h <sup>a</sup>
Bz	>32	$9.6 \pm 1.4$	>32	$30.6 \pm 0.64$	>1000	>104
DB2228	$9.3 \pm 0.75$	$8.3 \pm 3.2$	>32	$25.9 \pm 4.2$	>96	>12
DB2229	$6.3 \pm 3.7$	$2.7 \pm 0.3$	$9.8 \pm 1.3$	$8.1 \pm 1.37$	>96	>36
DB2292	$7.2 \pm 1$	$3.1 \pm 0.9^b$	$24.4 \pm 1.5$	$10.8 \pm 2.9$	>96	>31
DB2294	$5.2 \pm 2.9$	$3.9 \pm 1.3^b$	$9.6 \pm 1.7$	$9 \pm 0.5$	>96	>25
DB2232	$19.6 \pm 0.3$	$7.4 \pm 0.6$	>32	$30.6 \pm 1.1$	>96	>13
DB2235	>32	$5.27 \pm 3.7$	>32	$10.3 \pm 0.35$	$49 \pm 21$	9
DB2251	>32	$10.1 \pm 2.5$	>32	$28.9 \pm 1.8$	$62 \pm 23$	6
DB2253	>32	$19 \pm 8$	>32	>32	>96	>5
DB2255	$5.5 \pm 2.25$	$3.6 \pm 2.4^b$	>32	$6.46 \pm 3.2$	>96	>27

<sup>a</sup> Based on  $EC_{50}$  24 h.

<sup>b</sup> Student's *t*-test statistical analysis of studied compound and Bz: ( $P < 0.05$ ).

Table 2. Activity of the amidines and benzimidazole on L929 cell lines infected with *Trypanosoma cruzi* (Tulahuen strain transfected with  $\beta$ -galactosidase) after 96 h of incubation with 10  $\mu$ M of each compound

Compounds	% of parasite growth inhibition	CC <sub>50</sub>
Bz	83 $\pm$ 5	>100
DB2228	46 $\pm$ 18	>96
DB2229	50 $\pm$ 10	>96
DB2292	39 $\pm$ 22	>96
DB2294	7 $\pm$ 5	>96
DB2232	22 $\pm$ 10	>96
DB2235	58 $\pm$ 14	>96
DB2251	70 $\pm$ 17	>96
DB2253	70 $\pm$ 17	>96
DB2255	88 $\pm$ 11	>96

Nif, despite their severe side effects and low efficiency during the later chronic phase (Wilkinson and Kelly, 2009; Don and Ioset, 2013). The limitations of these therapies highlight the urgent need to find more effective and safer new compounds. Many compounds have been developed and screened with different experimental models of neglected diseases including CD (Bilbe, 2015). The azole anti-fungal inhibitors posaconazole (Pos) and ravuconazole (Rav) that act on the sterol 14 $\alpha$ -demethylase (CYP51) enzyme although were very potent *in vitro* and *in vivo* (using dog and mouse models) (Urbina *et al.* 1998; Diniz *et al.* 2010; Keenan and Chaplin, 2015) unfortunately failed during clinical trials performed by the Drugs for Neglected Diseases initiative (Molina *et al.* 2014). In addition, another recent clinical trial called 'Benzimidazole Evaluation for Interrupting Trypanosomiasis' (BENEFIT) designed to evaluate the efficacy and safety of Bz compared with placebo, did not demonstrate protection by this drug against clinical outcomes among patients with chronic CD. Often, in drug development for CD, as well as for other pathologies, there is a lack of direct translation between pre-clinical *in vitro* and *in vivo* results and clinical outcomes. Experimental chemotherapy for CD presents serious challenges in part due to experimental difficulties related to reliable demonstration of a sterile cure, particularly during the chronic stage of infection when parasite burden is low and tissue distribution is not fully understood (Chatelain and Konar, 2015; Francisco *et al.* 2015).

Our group has studied the *in vitro* and *in vivo* activity of AA and analogues and the bulk of the data revealed very promising action of these cations against intracellular pathogens, including *T. cruzi* (Soeiro *et al.* 2013). Presently, nine aromatic compounds were evaluated by phenotypic and *in silico* studies. The mono-amidines (DB2228, DB2229, DB2292 and DB2294) with tethered aryl rings

chosen due to previous observation that this class display potent effect against this parasite (Simões-Silva *et al.* 2016). Three of the four diamidines (DB2235, DB2251 and DB2253) are analogues of furamidine and one (DB2232) is an extended bis-amidino benzimidazole, which represents another class of highly active diamidines. Lastly, one novel bis-AIA (DB2255) results from a simple modification of the structure of the highly active anti-*T. cruzi* compound DB766 (Batista *et al.* 2010). In DB2255, the central furan ring of DB766 replaced with a non-aromatic 5-membered imidazolidin-2-one ring.

Results of calculations using the pkCSM approach for estimation of ADMET and other drug-like properties are important to consider at an early stage in the drug discovery process (Pires *et al.* 2015). The *in silico* estimation of ADMET properties showed that only DB2229, DB2235, DB2251 and DB2253 are likely to permeate Caco2 cells, with values near of the adopted threshold of 0.9. In addition, DB2228, DB2229, DB2251, DB2253, DB2255, DB2292 and DB2294 are predicted to show good adsorption (above 90%) by human intestines and reasonable predicted volume of distribution. Regarding the toxicity predictors, none expected to be mutagenic nor inhibitors of hERGI, whereas all compounds are expected to inhibit hERGII and have hepatotoxic profile as has also the reference drug, Bz.

Regarding the biological phenotypic assays, seven of out nine amidines presently screened against bloodstream forms resulted in parasite death rates similar to Bz including mono-amidines DB2228, DB2229, DB2292, DB2294, diamidines DB2232, DB2235 and AIA DB2255. Another important characteristic of some (DB2228, DB2229, DB2235, DB2292 and DB2294) was the ability to fast kill the parasite exhibiting anti-trypomastigote activity after 2 h of exposure while Bz was completely inactive at this time of drug exposure. When these aromatic compounds were tested against the intracellular amastigotes the bis-AIA DB2255 that was one of the best molecules against BT forms, also presented anti-parasitic effect in the same range as Bz, even using a different parasite strain and DTUs (Y and Tulahuen for BT and intracellular forms, corresponding to DTU II and VI, respectively). These data corroborate our previous findings that demonstrated the promising trypanocidal phenotypic effect of bis-AIAs (De Araujo *et al.* 2014; Timm *et al.* 2014). Data using trypomastigotes collected from infected cell lines reported EC<sub>50</sub> values of 2.8 and 15.2  $\mu$ M for pentamidine exposure using Y and Dm28c strains, respectively (Díaz *et al.* 2014). However, DB2255 was less potent than other studied AIAs such as DB766 which gives EC<sub>50</sub> values at <0.1  $\mu$ M (Batista *et al.* 2010). This result demonstrates that to achieve high anti-*T. cruzi* activity using the DB766 scaffold a central five membered

Table 3. *In silico* ADME

	DB2228	DB2229	DB2232	DB2235	DB2251	DB2253	DB2255	DB2292	DB2294	Bz
Absorption										
CaCo <sub>2</sub> permeability (log cm s <sup>-1</sup> )	0.316	0.858	0.086	0.824	1.124	0.998	0.026	0.163	0.199	0.479
Intestinal absorption (human, %)	97.185	100	86.259	82.685	94.223	93.881	90.08	96.671	93.916	68.885
Skin permeability (logKp)	-2.741	-3.011	-2.947	-3.713	-3.51	-3.629	-2.838	-2.818	-2.759	-2.893
Distribution										
VD <sub>ss</sub> (human) (log VD <sub>ss</sub> (L kg <sup>-1</sup> ))	0.645	0.709	0.318	0.482	0.93	0.705	0.449	0.573	0.521	-0.104
Fraction unbound (human)	0.307	0.2	0.278	0.35	0.278	0.316	0.187	0.213	0.221	0.503
BBB permeability	-1.185	-0.85	-1.086	-0.157	0.13	0.007	-1.265	-1.249	-1.465	-0.619
CNS permeability	-1.913	-2.298	-2.944	-2.439	-2.095	-2.151	-2.526	-2.766	-3.072	-2.995
Metabolism										
CYP2D6 substrate	No	No	No	No	No	No	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP1A2 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitor	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No
Excretion										
Total clearance log(ml min <sup>-1</sup> kg <sup>-1</sup> )	1.117	1.322	1.74	1.177	0.598	0.628	0.605	1.459	1.649	0.625

Table 4. *In silico* toxicity

	DB2228	DB2229	DB2232	DB2235	DB2251	DB2253	DB2255	DB2292	DB2294	Bz
AMES toxicity	No	No	No	No	No	No	No	No	No	Yes
Max. tolerated dose (human) (log mg kg <sup>-1</sup> day <sup>-1</sup> )	0.346	-0.896	-0.719	-0.109	-0.494	-0.233	-0.624	-0.838	-0.741	0.984
hERG I inhibitor	No	No	No	No	No	No	No	No	No	No
hERG II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Oral rat acute toxicity (ID50) (mol kg <sup>-1</sup> )	2.776	3.007	2.577	2.451	2.887	2.785	2.841	2.758	2.53	2.454
Oral rat chronic toxicity (LOAEL) log(mg kg <sup>-1</sup> _bw day <sup>-1</sup> )	2.577	0.511	0.981	1.483	1.085	1.213	0.407	0.471	0.436	1.649
Hepatotoxicity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Skin sensitisation	No	No	No	No	No	No	No	No	No	No
<i>T. Pyriformis</i> toxicity pIGC50 (log ug L <sup>-1</sup> )	0.285	0.3	0.308	0.946	0.475	0.807	0.329	0.29	0.287	1.227
Minnnow toxicity log LC50 (mM)	0.276	0.125	0.822	1.156	0.726	0.925	-0.069	-0.025	-0.19	1.649

hetero aromatic ring is required. In addition, it is important to take into consideration that a hit compound for CD must be active against both parasite stages and upon the different DTUs in order to be given in the distinct endemic areas of this neglected disease (Chatelain, 2015).

#### ACKNOWLEDGEMENTS

The authors acknowledge the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of its facilities.

#### FINANCIAL SUPPORT

The present 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) and Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, PROEP/CNPq/Fiocruz. M.N.C.S. is a research fellow of CNPq and CNE research. This work was also supported, in part, by the National Institutes of Health USA Grant No. RO1AI64200 and by The Bill and Melinda Gates Foundation through a subcontract with the CPDD (to D.W.B.).

#### CONFLICT OF INTEREST

None.

#### REFERENCES

- Albajar-Viñas, P. and Dias, J. C. (2014). Advancing the treatment for Chagas' disease. *New England Journal of Medicine* **15**, 1942–1943.
- Batista, D. G., Batista, M. M., de Oliveira, G. M., do Amaral, P. B., Lannes-Vieira, J., Britto, C. C., Junqueira, A., Lima, M. M., Romanha, A. J., Sales Junior, P. A., Stephens, C. E., Boykin, D. W. and Soeiro, M. N. (2010). Arylimidamide DB766, a potential chemotherapeutic candidate for Chagas' disease treatment. 2010. *Antimicrobial Agents and Chemotherapy* **54**, 2940–2952.
- Bilbe, G. (2015). Infectious diseases. Overcoming neglect of kinetoplastid diseases. *Science* **29**, 974–976.
- Buckner, F. S., Verlinde, C. L. M. J., La Flamme, A. C. and van Voorhis, W. C. (1996). Efficient technique for screening drugs activity against *Trypanosoma cruzi* using parasites expressing  $\beta$ -galactosidase. *Antimicrobial Agents and Chemotherapy* **40**, 2592–2597.
- Chatelain, E. (2015). Chagas disease drug discovery: toward a new era. *Journal of Biomolecular Screening* **20**, 22–35.
- Chatelain, E. and Konar, N. (2015). Translational challenges of animal models in Chagas disease drug development: a review. *Journal of Drug Design Development and Therapy* **19**, 4807–4823.
- Coura, J. R. and Dias, J. C. (2015). Epidemiology, control and surveillance of Chagas disease: 100 years after its discovery. *Memórias do Instituto Oswaldo Cruz* **104**(Suppl.), 31–40.
- De Araújo, J. S., Da Silva, C. F., Batista, D. G., Da Silva, P. B., Meuser, M. B., Aiub, C. A., da Silva, M. F., Araújo-Lima, C. F., Banerjee, M., Farahat, A. A., Stephens, C. E., Kumar, A., Boykin, D. W. and Soeiro, M. N. (2014). *In vitro* and *in vivo* studies of the biological activity of novel arylimidamides against *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **58**, 4191–4195.
- De Souza, E. M., Lansiaux, A., Bailly, C., Wilson, W. D., Hu, Q., Boykin, D. W., Batista, M. M., Araújo-Jorge, T. C. and Soeiro, M. N. (2004). Phenyl substitution of furamide markedly potentiates its anti-parasitic activity against *Trypanosoma cruzi* and *Leishmania amazonensis*. *Biochemical Pharmacology Journal* **15**, 593–600.
- Díaz, M. V., Miranda, M. R., Campos-Estrada, C., Reigada, C., Maya, J. D., Pereira, C. A. and López-Muñoz, R. (2014). Pentamidine exerts *in vitro* and *in vivo* anti *Trypanosoma cruzi* activity and inhibits the polyamine transport in *Trypanosoma cruzi*. *Acta Tropica Journal* **134**, 1–9.

- Diniz, L. de F., Caldas, I. S., Guedes, P. M., Crepalde, G., de Lana, M., Carneiro, C. M., Talvani, A., Urbina, J. A. and Bahia, M. T. (2010). Effects of ravuconazole treatment on parasite in dogs experimentally Infected with *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **54**, 2979–2986.
- Don, R. and Ioset, J. R. (2013). Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. *Parasitology* **141**, 140–146.
- Drugs for Neglected Diseases Initiative (2016). *Chagas Disease Target Product Profile*. <http://www.dndi.org/diseases-projects/portofolio.html> (accessed June 4, 2016).
- Farahat, A. A., Paliakov, E., Kumar, A., Barghash, A. E., Goda, F. E., Eisa, H. M., Wenzler, T., Brun, R., Liu, Y., Wilson, W. D. and Boykin, D. W. (2011). Exploration of larger central ring linkers in furamide analogues: synthesis and evaluation of their DNA binding, antiparasitic and fluorescence properties. *Bioorganic and Medicinal Chemistry* **1**, 2156–2167.
- Francisco, A. F., Lewis, M. D., Jayawardhana, S., Taylor, M. C., Chatelain, E. and Kelly, J. M. (2015). Limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive *in vivo* imaging. *Antimicrobial Agents and Chemotherapy* **59**, 4653–4661.
- Green, J. (2014). Synthesis of aza-heterocyclic monoamidines as potential DNA minor groove binders, anti-trypanosomals, and boron neutron capture therapy agents. Dissertation, Georgia State University. [http://scholarworks.gsu.edu/chemistry\\_diss/101](http://scholarworks.gsu.edu/chemistry_diss/101).
- Ismail, M. A., Brun, R., Wenzler, T., Tanious, F. A., Wilson, W. D. and Boykin, D. W. (2004). Dicationic biphenyl benzimidazole derivatives as antiprotozoal agents. *Bioorganic and Medicinal Chemistry* **15**, 5405–5413.
- Keenan, M. and Chaplin, J. H. (2015). A new era for chagas disease drug discovery? *Progress in Medicinal Chemistry* **54**, 185–230.
- King, H., Lourie, E. M. and Yorke, W. (1937). New trypanocidal substances. *Lancet* **230**, 1360–1336.
- Marin-Neto, J. A., Rassi, A., Jr., Morillo, C. A., Avezum, A., Connolly, S. J., Sosa-Estani, S., Rosas, F., Yusuf, S. and Benefit, I. (2008). Rationale and design of a randomized placebo-controlled trial assessing the effects of etiologic treatment in Chagas' cardiomyopathy: the benzimidazole evaluation for interrupting trypanosomiasis (BENEFIT). *American Heart Journal* **156**, 37–43.
- Meirelles, M. N., de Araujo-Jorge, T. C., Miranda, C. F., de Souza, W. and Barbosa, H. S. (1986). Interaction of *Trypanosoma cruzi* with heart muscle cells: ultrastructural and cytochemical analysis of endocytic vacuole formation and effect upon myogenesis *in vitro*. *European Journal of Cell Biology* **41**, 198–206.
- Molina, I., Gómez, i Prat, J., Salvador, F., Treviño, B., Sulleiro, E., Serre, N., Pou, D., Roure, S., Cabezos, J., Valerio, L., Blanco-Grau, A., Sánchez-Montalvá, A., Vidal, X. and Pahissa, A. (2014). Randomized trial of posaconazole and benzimidazole for chronic Chagas' disease. *New England Journal of Medicine* **15**, 1899–1908.
- Morillo, C. A., Marin-Neto, J. A., Avezum, A., Sosa-Estani, S., Rassi, A., Jr., Rosas, F., Villena, E., Quiroz, R., Bonilla, R., Britto, C., Guhl, F., Velazquez, E., Bonilla, L., Meeks, B., Rao-Melacini, P., Pogue, J., Mattos, A., Lazdins, J., Rassi, A., Connolly, S. J., Yusuf, S. and BENEFIT, I. (2015). Randomized trial of benzimidazole for chronic Chagas' cardiomyopathy. *New England Journal of Medicine* **373**, 1295–1306.
- Patterson, S. and Wyllie, S. (2014). Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. *Trends in Parasitology* **30**, 289–298.
- Pires, D. E., Blundell, T. L. and Ascher, D. B. (2015). pkCSM: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. *Journal of Medicines Chemistry* **14**, 4066–4072.
- Romanha, A. J., Castro, S. L., Soeiro, M. N., Lannes-Vieira, J., Ribeiro, I., Talvani, A., Bourdin, B., Blum, B., Olivieri, B., Zani, C., Spadafora, C., Chiari, E., Chatelain, E., Chaves, G., Calzada, J. E., Bustamante, J. M., Freitas-Junior, L. H., Romero, L. I., Bahia, M. T., Lotrowska, M., Soares, M., Andrade, S. G., Armstrong, T., Degraeve, W. and Andrade, Z. A. (2010). *In vitro* and *in vivo* experimental models for drug screening and development for Chagas disease. *Memórias do Instituto Oswaldo Cruz* **105**, 233–238.
- Simões-Silva, M. R., Nefertiti, A. S. G., De Araújo, J. S., Batista, M. M., Da Silva, P. B., Bahia, M. T., Menna-Barreto, R. S., Pavão, B. P., Green, J., Farahat, A. A., Kumar, A., Boykin, D. W. and Soeiro, M. N. C. (2016). Phenotypic screening *in vitro* of novel aromatic amidines against *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **60**, 4701–4707.
- Soeiro, M. N., de Castro, S. L., de Souza, E. M., Batista, D. G., Silva, C. F. and Boykin, D. W. (2008). Diamidine activity against

- trypanosomes: the state of the art. *Current Molecular Pharmacology* **1**, 151–161.
- Soeiro, M. N., Werbovetz, K., Boykin, D. W., Wilson, W. D., Wang, M. Z. and Hemphill, A.** (2013). Novel amidines and analogues as promising agents against intracellular parasites: a systematic review. *Parasitology* **140**, 929–951.
- Stephens, C. E., Tanious, F., Kim, S., Wilson, W. D., Schell, W. A., Perfect, J. R., Franzblau, S. G. and Boykin, D. W.** (2001). Diguandino and “reversed” diamidino 2,5-diarylfurans as antimicrobial agents. *Journal of Medicinal Chemistry* **24**, 1741–1748.
- Teixeira, A. R., Nascimento, R. J. and Sturm, N. R.** (2006). Evolution and pathology in chagas disease—a review. *Memórias do Instituto Oswaldo Cruz* **101**, 463–491.
- Timm, B. L., da Silva, P. B., Batista, M. M., da Silva, F. H., da Silva, C. F., Tidwell, R. R., Patrick, D. A., Jones, S. K., Bakunov, S. A., Bakunova, S. M. and Soeiro, M. N.** (2014). *In vitro* and *in vivo* biological effects of novel arylimidamide derivatives against *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **58**, 3720–3726.
- Urbina, J. A., Payares, G., Contreras, L. M., Liendo, A., Sanoja, C., Molina, J., Piras, M., Piras, R., Perez, N., Wincker, P. and Loebenberg, D.** (1998). Antiproliferative effects and mechanism of action of SCH 56592 against *Trypanosoma (Schizotrypanum) cruzi*: *in vitro* and *in vivo* studies. *Antimicrobial Agents and Chemotherapy* **42**, 1771–1777.
- Wilkinson, S. R. and Kelly, J. M.** (2009). Trypanocidal drugs: mechanisms, resistance and new targets. *Expert Reviews in Molecular Medicine Journal* **11**, 1–25.
- Wilkinson, S. R., Taylor, M. C., Horn, D., Kelly, J. M. and Cheeseman, I. A.** (2008). A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5022–5027.
- World Health Organization** (2016). *What is Chagas Disease? (WHO) Third WHO Report on Neglected Tropical Diseases*. Department of Control of Neglected Tropical Diseases, World Health Organization, Geneva.
- Zingales, B., Andrade, S. G., Briones, M. R., Campbell, D. A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A. M., Machado, C. R., Miles, M. A., Romanha, A. J., Sturm, N. R., Tibayrenc, M., Schijman, A. G.** and Second Satellite Meeting (2009). A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Memórias do Instituto Oswaldo Cruz* **104**, 1051–1054.