The efficacy of novel arylimidamides against *Trypanosoma cruzi in vitro*

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SUMMARY

The present study aimed to determine the *in vitro* biological efficacy and selectivity of 7 novel AIAs upon bloodstream trypomastigotes and intracellular amastigotes of *Trypanosoma cruzi*. The biological activity of these aromatic compounds was assayed for 48 and 24 h against intracellular parasites and bloodstream forms of *T. cruzi* (Y strain), respectively. Additional assays were also performed to determine their potential use in blood banks by treating the bloodstream parasites with the compounds diluted in mouse blood for 24 h at 4 °C. Toxicity against mammalian cells was evaluated using primary cultures of cardiac cells incubated for 24 and 48 h with the AIAs and then cellular death rates were determined by MTT colorimetric assays. Our data demonstrated the outstanding trypanocidal effect of AIAs against *T. cruzi*, especially DB1853, DB1862, DB1867 and DB1868, giving IC_{50} values ranging between 16 and 70 nanomolar against both parasite forms. All AIAs presented superior efficacy to benznidazole and some, such as DB1868, also demonstrated promising activity as a candidate agent for blood prophylaxis. The excellent anti-trypanosomal efficacy of these novel AIAs against *T. cruzi* stimulates further *in vivo* studies and justifies the screening of new analogues with the goal of establishing a useful alternative therapy for Chagas disease.

Key words: *Trypanosoma cruzi*, Chagas disease, chemotherapy, arylimidamides.

INTRODUCTION

Chagas disease, caused by the intracellular parasite *Trypanosoma cruzi*, is a neglected illness affecting 12–14 million people in South and Central American countries (Clayton, 2010*a*; Coura and Viñas, 2010). This disease is characterized by 2 sequential clinical phases: the acute phase which begins soon after parasite infection and is usually asymptomatic, and the chronic phase that may, after several years or even decades lead to cardiomyopathy and/or digestive megasyndromes in 30–40% of the infected individuals (Rassi *et al*. 2010; Laranja *et al*. 1951).

Nifurtimox (3-methyl-4-(5′-nitrofurfurylideneamine) tetrahydro-4H-1, 4-tiazine-1, 1-dioxide) and benznidazole (*N*-benzyl-2-nitroimidazole acetamide), were developed empirically more than 4 decades ago (Rodrigues Coura and de Castro, 2002), and remain the current treatments for Chagas disease. These compounds remain in use despite the fact that they are considered far from ideal because they cause multiple side effects, present limited efficacy, especially in patients with the late chronic stage of the disease (Rocha *et al*. 2007; Soeiro and de Castro, 2009; Bettiol *et al*. 2009; Machado *et al*. 2010), and have unfavourable pharmacokinetic properties (Caldas *et al*. 2008). These limitations emphasize the urgent need for development of new trypanocidal compounds to replace the current chemotherapies (Soeiro and de Castro, 2009).

In the last decade our group has focused on the study of a class of synthetic aromatic compounds that has shown promising activity and selectivity *in vitro* against a variety of pathogens, including *Giardia lamblia* (Bell *et al*. 1991), *Leishmania* sp. (Werbovetz, 2006; Wang *et al*. 2010), *Plasmodium* sp. (Bell *et al*. 1990; Hu *et al*. 2009; Purfield *et al*. 2009), *Pneumocystis carinii* (Francesconi *et al*. 1999), *Toxoplasma gondii* (Lindsay *et al*. 1991), and *Trypanosoma* sp. (Daliry *et al*. 2009; Batista *et al*. 2010, De Souza *et al*. 2011). Aromatic amidines (AA) and their analogues have also demonstrated effectiveness in *in vivo* models against a variety of pathogens including the causative agents of Leishmaniasis (Wang *et al*. 2010), Human African Trypanosomiasis (Mathis *et al*. 2007; Wenzler *et al*. 2009) and Chagas Disease

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(da Silva *et al*. 2008; Batista *et al*. 2010). In fact, pentamidine, a representative of the AA class has been widely used clinically against African trypanosomiasis (Apted, 1980), antimony-resistant leishmaniasis (Bryceson *et al*. 1985), and *P. carinii* pneumonia (Kim *et al*. 2008). Congener aromatic molecules, like arylimidamides (AIAs; previously described as 'reversed' amidines), exhibit submicromolar and nanomolar efficacy against *Leishmania donovani* promastigotes and in *L. donovani*-infected macrophage assays (Stephens *et al*. 2003; Rosypal *et al*. 2007, 2008). In addition, some AIAs demonstrated nanomolar IC_{50} values against *Trypanosoma cruzi* parasites *in vitro* (Stephens *et al*. 2003; Silva *et al*. 2007*a*; Pacheco *et al*. 2009; de Souza *et al*. 2010; Batista *et al*. 2010). In mouse models, employing Y and Colombian strains, the AIA DB766 effectively reduced the parasite load in the blood and cardiac tissue and presented efficacy similar to that of benznidazole, improving the electrocardiographic alterations, and providing 90 to 100% protection against animal mortality (Batista *et al*. 2010).

The mechanisms of action of AAs and congeners are still poorly understood; however, biophysical studies (Bailly *et al*. 1999; Wang *et al*. 2000; Mazur *et al*. 2000; Farahat *et al*. 2011) demonstrated their ability to bind to the minor-groove of DNA at AT-rich sites, which could explain at least in part, their microbicidal activity. This association could impair important biological events associated with the cellular cycle and also prevent the association of protein/factors to the parasite DNA, arresting steps such as DNA replication and protein expression, ultimately leading to parasite death (Werbovetz, 2006; Soeiro *et al*. 2010; Wang *et al*. 2010).

In a screening effort to further explore the antiparasitic activity of these promising cationic compounds aiming to identify potential novel candidates for Chagas disease therapy, the biological efficacy of 7 AIAs was evaluated *in vitro* against bloodstream and intracellular forms of *T. cruzi*. Additionally, their toxicity was determined against primary cultures of cardiac host cells. Our data demonstrate the high trypanocidal effect of most of these aromatic compounds, such as DB1867, that displayed higher efficacy than the reference drug, being about 50 to 600 times more active against both bloodstream and intracellular forms, respectively. The high selectivity of these novel AIAs confirmed that they may represent novel therapeutic options for Chagas' disease treatment.

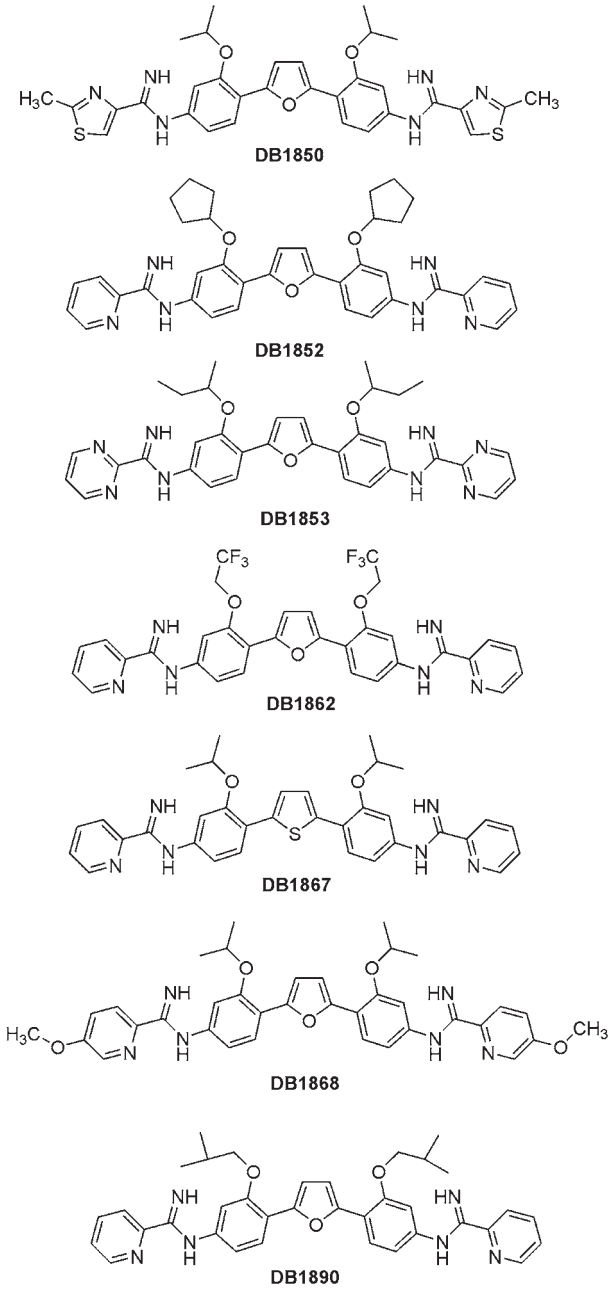

Fig. 1. Chemical structure of the compounds used in the study.

MATERIALS AND METHODS

Compounds

The synthesis of the 7 compounds (DB1850, DB1852, DB1853, DB1862, DB1867, DB1868, DB1890 – Fig. 1) was performed according to methods we have previously described (Stephens *et al*. 2003; Wang *et al*. 2010) and will be published elsewhere. Benznidazole (Bz, Rochagan; Roche) was used as reference drug as reported (Batista *et al*. 2010). Stock solutions were prepared in dimethyl sulfoxide (DMSO), with the final concentration in the *in vitro* experiments never exceeding 0·6%, which did not exert any toxicity toward the parasite or mammalian host cells (data not shown).

Parasites

The Y strain of *T. cruzi* was used in the present study. Bloodstream trypomastigote forms (BT) were

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Table 1. IC_{50} and LC_{50} (μM) values of AIAs and benznidazole against BT forms of *Trypanosoma cruzi* and cardiac cells, respectively, with their respective SI values

	LC_{50} values	IC_{50} values		
	RPMI			Blood
Compounds	37 °C	37 °C (SI)	4 °C	
DB1850	32	0·19 ± 0·01 (168)	Nd	2·35 ± 0·75
DB1852	32	0·06 ± 0·01 (>533)	>32	>32
DB1853	7·3	0·016 ± 0·01 (457)	Nd	0·14 ± 0·07
DB1862	32	0·06 ± 0·01 (533)	Nd	0·79 ± 0·82
DB1867	32	0·02 ± 0·0 (>2461)	nd	0·70 ± 0·19
DB1868	32	0·06 ± 0·02 (533)	0·157 ± 0·064	0·28 ± 0·08
DB1890	25	0·01 ± 0·0 (> 2461)	>32	>32
Bz	1000	12·94 ± 1·9 (>77)	>250	>250

BT = Bloodstream trypomastigotes.
SI* = selectivity index corresponds to the ratio LC_{50}/IC_{50}: SI was calculated on LC_{50} values of 24 h.
Bz = Benznidazole.
Nd = Not done.

obtained from *T. cruzi*-infected Swiss mice at the peak of parasitaemia (Meirelles *et al*. 1986). For the analyses on intracellular forms, parasites lodged within cardiac cell cultures were employed, as previously reported (Silva *et al*. 2007*a*).

Cardiac cell cultures and cytotoxicity assays

For the evaluation of toxicity and compound activity against intracellular parasites, primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice and purified following the method previously described (Meirelles *et al*. 1986). In order to rule out toxic effects of the compounds on the host cells, uninfected CM cultures were incubated at 37 °C with compounds for 24 h and 48 h (0·1–96 μM). Untreated cultures were used as control samples. The cell death rates were measured by the MTT colorimetric assay allowing the determination of LC50 values (compound concentration that reduces 50% of cellular viability) (da Silva *et al*. 2010). All cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and air, and the assays were run at least 3 times in duplicate. All procedures were carried out in accordance with the guidelines established and approved by the FIOCRUZ Committee of Ethics for the Use of Animals (0099/01).

Trypanocidal assays

The effect of the compounds against BT was evaluated through assaying 5×10^6 parasites/ml for 24 h at 37 °C in RPMI 1640 medium supplemented with 10% of fetal bovine serum, in the presence of serial dilutions of the AIAs (0·001–32 μM). Untreated parasites were used as control samples. Alternatively, experiments were performed at 4 °C for 24 h, with BT maintained in the presence of increasing concentrations of each compound (0·001–32 μM) with or without mouse blood contents (96%). In these later assays, 4 μl of each compound (at 50% times the final dose) were added in 196 μl of blood from *T. cruzi*-infected mice. Parasite death rates were determined by light microscopy using a Neubauer chamber that allows the direct visualization and quantification of the number of motile and live parasites, and then IC_{50} (drug concentration that reduce 50% of the number of the treated parasites) calculated (Silva *et al*. 2007*a*, *b*).

For the analysis of the effect against intracellular parasites, after 24 h of parasite-host cell interaction (ratio 10:1), the infected cardiac cultures were washed to remove the free parasites and then maintained, at 37 °C in an atmosphere of 5% CO_2 and air, in the presence of non-toxic concentrations of each AIA (from 0·004 up to 1·18 μM), replacing the medium (with the respective compound) every 24 h. Infected cultures not treated were used as control. After 48 h, all cultures were fixed and stained with Giemsa solution. The endocytic index (EI) was used to compare the compound activity and was calculated by multiplying the percentage of infected cells by the mean number of parasites per infected cell (da Silva *et al*. 2008). Next, the IC_{50} was calculated based on the drug concentration that reduced 50% of the EI.

In all assays, the Selectivity Index (SI) was calculated as follows: SI = ratio LC_{50}/IC_{50}.

RESULTS

The biological activity of 7 novel arylimidamides (AIAs) was initially assayed against bloodstream trypomastigotes (BT) (Table 1, Fig. 2). When the compounds were diluted in the culture medium (RPMI) at 37 °C, all showed a dose-dependent

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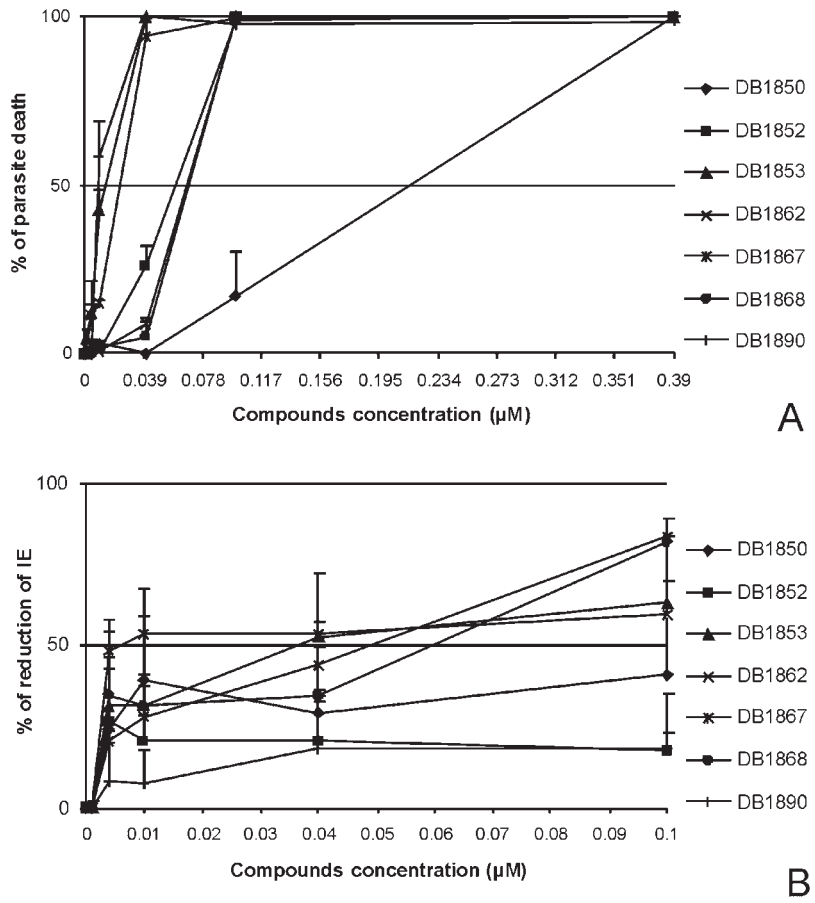

Fig. 2. Activity *in vitro* of arylimidamides at 37 °C against bloodstream trypomastigotes (A) and intracellular forms (B) of *Trypanosoma cruzi* (Y strain).

trypanocidal response (Fig. 2). In these experiments the compounds exhibited excellent *in vitro* activity achieving IC_{50} values in the low micromolar range (between 0·01 and 0·19 μM) (Table 1, Fig. 2). The two most active compounds against BT at 37 °C were DB1890 and DB1867, which presented IC_{50} values of 0·01 μM and 0·02 μM and SI values of 2461 and 1600, respectively, while DB1850 was the least active, with values of 0·19 μM and 168 for IC_{50} and SI, respectively (Table 1). All the AIAs showed superior activity to BZ, presenting from 68 to 1200-fold higher efficacy than the reference drug (Table 1).

The drug activity in the presence of blood at 4 °C was evaluated with the goal to determine the applicability of these compounds for blood-bank prophylaxis. As it can be seen in Table 1, DB1850, DB1853, DB1862, DB1867 and DB1868 maintained considerable trypanocidal effect (at submicromolar range) at 4 °C, presenting superior efficacy to that of Bz (Table 1). However, both DB1852 and DB1890 showed a pronounced decrease in activity (>32 μM). Under these conditions, Bz did not show any activity at 4 °C against trypomastigote forms (Table 1).

It is possible that the decreased activity may be related to the low temperature of the assay instead of only the presence of blood, therefore the effect of some compounds was investigated at 4 °C but in the absence of blood, using only culture medium (RPMI). Compounds were selected based on their previous data: DB1868 that maintained its high

Table 2. IC_{50} and LC_{50} values of AIAs and benznidazole against intracellular forms of *Trypanosoma cruzi* and cardiac cells, respectively, with their respective SI values

Compounds	IC_{50} values of EI reduction on intracellular parasites multiplication after 48 h of treatment (μM)	Selective Index	LC_{50} values after 48 h of treatment upon cardiac cells (μM)
DB1850	0·184 ± 0·077	82	15
DB1852	0·538 ± 0·218	>59	32
DB1853	0·044 ± 0·034	294	13
DB1862	0·016 ± 0·02	>2000	32
DB1867	0·058 ± 0·047	>551	32
DB1868	0·062 ± 0·004	48	3
DB1890	0·9 ± 0·0007	>35	32
Bz	2·77 ± 1·96	>360	1000

SI*, selectivity index corresponds to the ratio LC_{50}/IC_{50} : SI was calculated on IC_{50} and LC_{50} values of 48 h.
Bz, benznidazole.

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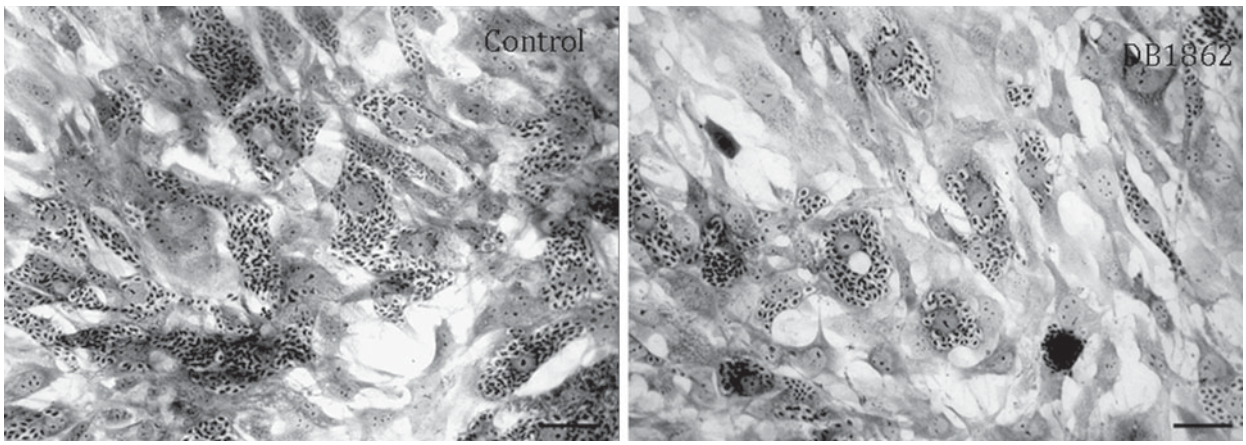

Fig. 3. Light microscopy analysis of *Trypanosoma cruzi*-infected cardiac cells submitted (B) or not (A) to 0·13 μM DB 1862 treatment.

trypanocidal activity compared to two other AIAs that displayed loss of activity (DB1852 and DB1890). For comparative analyses the reference drug Bz, was also included in the assay. Interestingly, lowering the temperature of incubation was sufficient to drop the activity of DB1852, DB1890 and Bz while DB1868, alone, was unaffected by the temperature (see IC_{50} values in Table 1).

To evaluate the toxicity of each AIA, uninfected cardiac cultures were incubated for 24 and 48 h with different doses of the compounds and the cellular viability evaluated by light microscopy and MTT colorimetric assays. Cellular viability was not significantly reduced by most of the AIAs after incubation for 24–48 h with doses up to 32 μM (Tables 1 and 2).

We further investigated the effect of the compounds on intracellular parasites by incubating *T. cruzi*-infected cardiac cells with selected non-toxic doses of each AIA. The direct quantification of the number of parasites in *T. cruzi*-infected cultures after 48 h of treatment showed a dose-dependent effect using all compounds (Figs 2 and 3). As found for BT forms, most AIAs exhibited excellent biological activity with IC_{50} values in the low micromolar range: between 0·016 and 0·9 μM (Table 2). All AIAs were again more potent than Bz, with some of them showing considerably superior efficacy, as can be seen for DB1853 and DB1862, which were about 60 and 170-fold more potent than Bz, respectively (Table 2, Figs 2 and 3).

The promising performance of this set of compounds was also observed by the evaluation of the selectivity index (SI). For the BT forms the SI values were between 168 and 2461 (Table 1), and for intracellular forms were >35 and >2000 (Table 2). Taken together the compound DB1862 displayed the best overall performance, with IC_{50} of 0·06 and 0·016 μM and with SI values of ~533 and >2000, for BT and amastigotes, respectively (Tables 1 and 2).

DISCUSSION

Aromatic amidines (AA), such as pentamidine, propamidine, and diminazene aceturate, are DNA minor groove binders that have long been used in infectious disease chemotherapy because they exhibit broad-spectrum anti-microbial effects (Soeiro *et al*. 2009, Soeiro and de Castro, 2011). However, they display toxicity, require parenteral administration and present low bioavailability (Werbovetz, 2006), justifying the search for more effective analogues. Many AIAs have shown superior trypanocidal and leishmanicidal activity to that of the related aromatic amidines (De Souza *et al*. 2004, 2006, 2010; Soeiro *et al*. 2005; Silva *et al*. 2007*a*, *b*; Pacheco *et al*. 2009; Batista *et al*. 2010; Wang *et al*. 2010). In contrast to the original diamidines, in which the imino group is directly attached to an aryl ring, AIAs, previously named reversed amidines, present the imino group attached to an 'anilino' nitrogen (Soeiro and de Castro, 2011). Therefore our aim was to evaluate the activity and selectivity of additional novel AIAs against clinically relevant forms of Chagas disease.

When assayed against bloodstream trypomastigotes, these AIAs were able to induce a high level of parasite lyses in a dose-dependent manner, showing IC_{50} values in the submicromolar range. With the exception of DB1850, all the compounds had IC_{50} values lower than 0·07 μM, confirming the excellent activity of the AIA class of compounds against *T. cruzi* as previously reported (Stephens *et al*. 2003; Silva *et al*. *2007a*, *b*; Pacheco *et al*. 2009; de Souza *et al*. 2010; Batista *et al*. 2010). The difference found in the activity of the most active compounds (DB1852, DB1853, DB1862, DB1867, DB1868, DB1890) compared to the less active compound (DB1850), could be due to binding to different targets, or even different modes of uptake and/or extrusion of the drugs. Pentamidine and other amidines such as DB75 are actively transported into

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African trypanosomes via the P2, HAPT1, and LAPT1 system of transporters (Lanteri *et al*. 2006) and accumulate at very high concentrations in the parasite mitochondria (Mathis *et al*. 2006). However, until now, no report has appeared regarding the internalization of amidines by *T. cruzi*. The superior activity of AIA compared to AA compounds could be due to their lower pK values and greater lipophilic character which may contribute to their efficient entrance into cells (Mathis *et al*. 2007). The IC_{50} values against BT forms are reasonably consistent except for that of DB1850. The lower activity of 1850 may be due the markedly different terminal groups (5-membered ring thiazoles for DB1850 and 6-membered ring pyridine or pyrimidine for the others). The 6 compounds with the higher activity against BT forms show that a range of variations in the *O*-alkyl groups (OCH_2CF_3 to *O*-*c*-pentyl) are tolerated. However, additional experimental data are needed to evaluate all of these hypotheses. Interestingly, all the compounds were significantly more active against BT forms than the current drug used for Chagas disease treatment, Bz.

The effectiveness of AIAs at 4 °C in the presence of blood constituents was assessed in order to evaluate their potential use in prophylaxis for blood banking. This analysis may also identify the potential loss of compound activity due to plasma protein-compound interactions and may help to understand, in part, the pharmacokinetics and mechanism of action of these compounds. The incubation of BT under these experimental conditions showed that most AIAs maintained their activity (at submicromolar level) in the presence of blood (DB1850, DB1853, DB1862, DB1867 and DB1868), which confirms their potential as candidates for blood prophylaxis (Silva *et al*. 2007*a*; Batista *et al*. 2010; da Silva *et al*. 2011).

In order to determine whether the reduction in activity observed for some AIAs (DB1852 and DB1890) was due to the low incubation temperature and not to compound instability and/or binding to blood elements, some of the AIAs were evaluated, along with BT, by substituting mouse blood for RPMI culture medium. The data demonstrated that these AIAs show decreased activity when incubated at 4 °C with RPMI, suggesting that the drop in activity could be due to impaired compound uptake due to a transport-mediated uptake (de Koning, 2001) and/or due to decreased cellular target metabolism. Further studies are underway to clarify the effect observed in the present study.

These novel AIAs also exhibited considerable activity against intracellular parasites at doses that did not cause host cell damage, presenting superior activity when compared to Bz. In this case, a slightly different SAR (Structure-Activity Relationship) is seen in that DB1852 and DB1890 are less active against intracellular parasites as compared to BT. The cause of this difference is unclear but may be due to subtle differences in uptake, mechanisms of action and/or different targets. A viable drug candidate must be active against both clinical relevant forms, killing BT that are released from the cells as well as targeting the intracellular amastigotes. The present study shows that certain AIAs present this desirable characteristic. DB 1862 showed very high SI against both forms, and thus deserves to be considered for *in vivo* studies.

Taken together the set of compounds evaluated here showed excellent biological activity against clinically relevant forms of *T. cruzi*, showing high SI values and limited toxicity towards mammalian cells. The efficacy of these compounds against *T. cruzi* encourages *in vivo* evaluation as well as screening of new analogues in search of a useful alternative therapy for Chagas disease.

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REFERENCES

Apted, F. I. (1980). Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Eastern Hemisphere. *Pharmacology & Therapeutics* **11**, 391–413.

Bailly, C., Dassonneville, L., Carrasco, C., Lucas, D., Kumar, A., Boykin, D. W. and Wilson, W. D. (1999). Relationships between topoisomerase II inhibition, sequence-specificity and DNA binding mode of dicationic diphenylfuran derivatives. *Anti-Cancer Drug Design* **14**, 47–60.

Batista, D. da 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.de N. (2010). Arylimidamide DB766, a potential chemotherapeutic candidate for Chagas' disease treatment. *Antimicrobial Agents and Chemotherapy* **54**, 2940–2952.

Bell, C. A., Cory, M., Fairley, T. A., Hall, J. E. and Tidwell, R. R. (1991). Structure-activity relationships of pentamidine analogs against *Giardia lamblia* and correlation of antigiardial activity with DNA-binding affinity. *Antimicrobial Agents and Chemotherapy* **35**,1099–107.

Bettiol, E., Samanovic, M., Murkin, A. S., Raper, J., Buckner, F. and Rodriguez, A. (2009). Identification of three classes of heteroaromatic compounds with activity against intracellular *Trypanosoma cruzi* by chemical library screening. *PLoS Neglected Tropical Diseases* **3**, e384.

Bryceson, A. D., Chulay, J. D., Mugambi, M., Were, J. B., Gachihi, G., Chunge, C. N., Muigai, R., Bhatt, S. M., Ho, M., Spencer, H. C., Meme, J. and Anabwani, G. (1985). Visceral leishmaniasis unresponsive to antimonial drugs. II. Response to high dosage sodium stibogluconate or prolonged treatment with pentamidine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 705–714.

Caldas, I. S., Talvani, A., Caldas, S., Carneiro, C. M., de Lana, M., da Matta Guedes, P. M. and Bahia, M. T. (2008). Benznidazole therapy during acute phase of Chagas disease reduces parasite load but does not prevent chronic cardiac lesions. *Parasitology Research* **103**, 413–21.

Clayton, J. (2010). Chagas disease: pushing through the pipeline. *Nature, London* S12–S15.

Coura, J. R. and Viñas, P. A. (2010). Chagas disease: a new worldwide challenge. *Nature Outlooks* **465** (7301 suppl), S6–S7.

da Silva, C. F., Batista, M. M., Batista, D.da G., de Souza, E. M., da Silva, P. B., de Oliveira, G. M., Meuser, A. S., Shareef, A. R., Boykin, D. W. and Soeiro, M. de N. (2008). *In vitro* and *in vivo* studies of

https://doi.org/10.1017/S0031182011001429 Published online by Cambridge University Press

the trypanocidal activity of a diarylthiophene diamidine against *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **52**, 3307–3314.

da Silva, C. F., da Silva, P. B., Batista, M. M., Daliry, A., Tidwell, R. R. and Soeiro, M. de N. (2010). The biological *in vitro* effect and selectivity of aromatic dicationic compounds on *Trypanosoma cruzi*. *Memorias do Instituto Oswaldo Cruz* **105**, 239–245.

Daliry, A., Da Silva, P. B., Da Silva, C. F., Batista, M. M., De Castro, S. L., Tidwell, R. R. and Soeiro, M. de N. (2009). *In vitro* analyses of the effect of aromatic diamidines upon *Trypanosoma cruzi*. *Jounal of Antimicrobial Chemotherapy* **64**, 747–750.

de Koning, H. P. (2001). Transporters in African trypanosomes: role in drug action and resistance. *International Journal for Parasitology* **31**, 512–522.

De Souza, E. M., da Silva, P. B., Nefertiti, A. S., Ismail, M. A., Arafa, R. K., Tao, B., Nixon-Smith, C. K., Boykin, D. W. and Soeiro, M. N. (2011). Trypanocidal activity and selectivity *in vitro* of aromatic amidine compounds upon bloodstream and intracellular forms of *Trypanosoma cruzi*. *Experimental Parasitology* **127**, 429–435.

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 furamidine markedly potentiates its antiparasitic activity against *Trypanosoma cruzi* and *Leishmania amazonensis*. *Biochemical Pharmacology* **68**, 593–600.

De Souza, E. M., Nefertiti, A. S., Bailly, C., Lansiaux, A. and Soeiro, M. N. (2010). Differential apoptosis-like cell death in amastigote and trypomastigote forms from *Trypanosoma cruzi*-infected heart cells *in vitro*. *Cell and Tissue Research* **341**, 173–180.

De Souza, E. M., Oliveira, G. M., Boykin, D. W., Kumar, A., Hu, Q. and Soeiro, M. N. C. (2006). Trypanocidal activity of the phenyl-substituted analogue of furamidine DB569 against *Trypanosoma cruzi* infection *in vivo*. *Journal of Antimicrobial Chemotherapy* **58**, 610–614.

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 furamidine analogues: Synthesis and evaluation of their DNA binding, antiparasitic and fluorescence properties. *Bioorganic & Medicinal Chemistry* **19**, 2156–2167.

Francesconi, I., Wilson, W. D., Tanious, F. A., Hall, J. E., Bender, B. C., Tidwell, R. R., McCurdy, D. and Boykin, D. W. (1999). 2,4-Diphenyl furan diamidines as novel anti-*Pneumocystis carinii* pneumonia agents. *Journal of Medicinal Chemistry* **42**, 2260–2265.

Hu, L., Arafa, R. K., Ismail, M. A., Patel, A., Munde, M., Wilson, W. D., Wenzler, T., Brun, R. and Boykin, D. W. (2009). Synthesis and activity of azaterphenyl diamidines against *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*. *Bioorganic & Medicinal Chemistry* **17**, 6651–6658.

Kim, S. Y., Dabb, A. A., Glenn, D. J., Snyder, K. M., Chuk, M. K. and Loeb, D. M. (2008). Intravenous pentamidine is effective as second line *Pneumocystis pneumonia* prophylaxis in pediatric oncology patients. *Pediatric Blood & Cancer* **50**, 779–783.

Lanteri, C. A., Stewart, M. L., Brock, J. M., Alibu, V. P., Meshnick, S. R., Tidwell, R. R. and Barrett, M. P. (2006). Roles for the *Trypanosoma brucei* P2 transporter in DB75 uptake and resistance. *Molecular Pharmacology* **70**,1585–1592.

Laranja, F. S., Dias, E. and Nobrega, G. (1951). Clinical aspect and treatment of Chagas' disease. *Prensa Medicine Argentina* **38**, 465–484.

Lindsay, D. S., Blagburn, B. L., Hall, J. E. and Tidwell, R. R. (1991). Activity of pentamidine and pentamidine analogs against *Toxoplasma gondii* in cell cultures. *Antimicrobial Agents and Chemotherapy* **35**,1914–1916.

Machado, F. S., Tanowitz, H. B. and Teixeira, M. M. (2010). New drugs for neglected infectious diseases: Chagas' disease. *British Journal of Pharmacology* **160**, 258–259.

Mathis, A. M., Holman, J. L., Sturk, L. M., Ismail, M. A., Boykin, D. W., Tidwell, R. R. and Hall, J. E. (2006). Accumulation and intracellular distribution of antitrypanosomal diamidine compounds DB75 and DB820 in African trypanosomes. *Antimicrobial Agents and Chemotherapy* **50**, 2185–2191.

Mathis, A. M., Bridges, A. S., Ismail, M. A., Kumar, A., Francesconi, I., Anbazhagan, M., Hu, Q., Tanious, F. A., Wenzler, T., Saulter, J., Wilson, W. D., Brun, R., Boykin, D. W., Tidwell, R. R. and Hall, J. E. (2007). Diphenyl furans and aza analogs: effects of structural modification on *in vitro* activity, DNA binding, and accumulation and distribution in trypanosomes. *Antimicrobial Agents and Chemotherapy* **51**, 2801–2810.

Mazur, S., Tanious, F. A., Ding, D., Kumar, A., Boykin, D. W., Simpson, I. J., Neidle, S. and Wilson, W. D. (2000). A thermodynamic and structural analysis of DNA minor-groove complex formation. *Journal of Molecular Biology* **300**, 321–337.

Meirelles, M. N., 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.

Pacheco, M. G., da Silva, C. F., de Souza, E. M., Batista, M. M., da Silva, P. B., Kumar, A., Stephens, C. E., Boykin, D. W. and Soeiro, M. de N. (2009). *Trypanosoma cruzi*: activity of heterocyclic cationic molecules *in vitro*. *Experimental Parasitology* 123, 73–80.

Purfield, A. E., Tidwell, R. R. and Meshnick, S. R. (2009). The diamidine DB75 targets the nucleus of *Plasmodium falciparum*. *Malaria Journal* **8**, 1–9.

Rassi, A. Jr., Rassi, A. and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* **375**, 1388–1402.

Rocha, M. O., Teixeira, M. M. and Ribeiro, A. L. (2007). An update on the management of Chagas cardiomyopathy. *Expert Review of Anti-Infective Therapy* **5**, 727–743.

Rodriques Coura, J. and de Castro, S. L. (2002). A critical review on Chagas disease chemotherapy. *Memórias do Instituto Oswaldo Cruz* **97**, 3–24.

Rosypal, A. C., Tidwell, R. R. and Lindsay, D. S. (2007). Prevalence of antibodies to *Leishmania infantum* and *Trypanosoma cruzi* in wild canids from South Carolina. *Journal of Parasitology* **93**, 955–957.

Rosypal, A. C., Werbovetz, K. A., Salem, M., Stephens, C. E., Kumar, A., Hall, J. E. and Tidwell, R. R. (2008). Inhibition by Dications of *in vitro* growth of *Leishmania major* and *Leishmania tropica*: causative agents of old world cutaneous leishmaniasis. *Journal of Parasitology* **94**, 743–749.

Silva, C. F., Batista, M. M., Mota, R. A., de Souza, E. M., Stephens, C. E., Som, P., Boykin, D. W. and Soeiro, M. de N. (2007*a*). Activity of "reversed" diamidines against *Trypanosoma cruzi "in vitro"*. *Biochemical Pharmacology* **73**, 1939–1946.

Silva, C. F., Meuser, M. B., De Souza, E. M., Meirelles, M. N., Stephens, C. E., Som, P., Boykin, D. W. and Soeiro, M. N. (2007*b*). Cellular effects of reversed amidines on *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **51**, 3803–3809.

Soeiro, M. N. and de Castro, S. L. (2009). *Trypanosoma cruzi* targets for new chemotherapeutic approaches. *Expert Opinion Therapy Targets* **13**, 105–121.

Soeiro, M. De N. and de Castro, S. L. (2011). Screening of potential anti-*Trypanosoma cruzi* candidates: in vitro and in vivo studies. *Open Medicinal Chemistry Journal* **5**, 21–30.

Soeiro, M. N. C., Daliry, A., Silva, C. F., Batista, D. G. J., de Souza, E. M., Oliveira, G. M., Salomão, K., Menna-Barreto, R. F. S. and de Castro, S. L. (2010). Electron microscopy approaches for the investigation of the cellular targets of trypanocidal agents in Trypanosoma cruzi In *Microscopy: Science, Technology, Applications and Education* 1, 191–203. Formatex Research Center, Badajoz, Spain.

Soeiro, M. de N., Dantas, A. P., Daliry, A., Silva, C. F., Batista, D. G., de Souza, E. M., Oliveira, G. M., Salomão, K., Batista, M. M., Pacheco, M. G., Silva, P. B., Santa-Rita, R. M., Barreto, R. F., Boykin, D. W. and Castro, S. L. (2009). Experimental chemotherapy for Chagas disease: 15 years of research contributions from *in vivo* and *in vitro* studies. *Memórias do Instituto Oswaldo Cruz* **104**, 301–310.

Soeiro, M. N., De Souza, E. M., Stephens, C. E. and Boykin, D. W. (2005). Aromatic diamidines as antiparasitic agents. *Expert Opinion on Investigational Drugs* **14**, 957–972.

Stephens, C. E., Brun, R., Salem, M. M., Werbovetz, K. A., Tanious, F., Wilson, W. D. and Boykin, D. W. (2003). The activity of diguanidino and "reversed" diamidino 2,5-diarylfurans versus *Trypanosoma cruzi* and *Leishmania donovani*. *Bioorganic & Medicinal Chemistry Letters* **13**, 2065–2069.

Wang, L., Bailly, C., Kumar, A., Ding, D., Bajic, M., Boykin, D. W. and Wilson, W. D. (2000). Specific molecular recognition of mixed nucleic acid sequences: an aromatic dication that binds in the DNA minor groove as a dimer. *Proceedings of the National Academy of Sciences, USA* **97**, 12–16.

Wang, M. Z., Zhu, X., Srivastava, A., Liu, Q., Sweat, J. M., Pandharkar, T., Stephens, C. E., Riccio, E., Parman, T., Munde, M., Mandal, S., Madhubala, R., Tidwell, R. R., Wilson, W. D., Boykin, D. W., Hall, J. E., Kyle, D. E. and Werbovetz, K. A. (2010). Novel arylimidamides for treatment of visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy* **54**, 2507–2516.

Wenzler, T., Boykin, D. W., Ismail, M. A., Hall, J. E., Tidwell, R. R. and Brun, R. (2009). New treatment option for second-stage African sleeping sickness: *in vitro* and *in vivo* efficacy of aza analogs of DB289. *Antimicrobial Agents and Chemotherapy* **53**, 4185–4192.

Werbovetz, K. (2006). Diamidines as antitrypanosomal, antileishmanial and antimalarial agents. *Current Opinion in Investigational Drugs* **7**, 147–157.

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