

In vitro* trypanocidal activity of DB745B and other novel arylimidamides against *Trypanosoma cruzi

Cristiane França Da Silva¹, Angela Junqueira², Marli Maria Lima³, Alvaro José Romanha⁴, Policarpo Ademar Sales Junior⁴, Chad E. Stephens⁵, Phanneth Som⁶, David W. Boykin⁶ and Maria de Nazaré Correia Soeiro^{1*}

¹Laboratório de Biologia Celular do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; ²Laboratório de Doenças Parasitárias do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; ³Laboratório de Eco-epidemiologia da doença de Chagas do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; ⁴Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou, Fiocruz-MG, MG, Brazil; ⁵Department of Chemistry and Physics, Augusta State University, Augusta, GA, USA; ⁶Department of Chemistry, Georgia State University, Atlanta, GA, USA

*Corresponding author. Tel: +55-21-25621368; Fax: +55-21-25621432; E-mail: soeiro@ioc.fiocruz.br

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Objectives: As part of a search for new therapeutic opportunities to treat chagasic patients, *in vitro* efficacy studies were performed to characterize the activity of five novel arylimidamides (AIAs) against *Trypanosoma cruzi*.

Methods: The trypanocidal effect against *T. cruzi* was evaluated by light microscopy through the determination of IC₅₀ values. Cytotoxicity was determined by MTT assays against mouse cardiomyocytes.

Results: Our data demonstrated the trypanocidal efficacy of these new compounds against bloodstream trypomastigotes and intracellular amastigotes, exhibiting IC₅₀ values ranging from 0.015 to 2.5 and 0.02 to 0.2 μM, respectively. One of the compounds, DB745B, was also highly active against a broad panel of isolates, including those naturally resistant to benznidazole. DB745B showed higher *in vitro* efficacy than the reference drugs used to treat patients (benznidazole IC₅₀=12.94 μM) and to prevent blood bank infection (gentian violet IC₅₀=30.6 μM).

Conclusions: AIAs represent promising new chemical entities against *T. cruzi* and are also potential trypanocidal agents to prevent transfusion-associated Chagas' disease.

Keywords: Chagas' disease, chemotherapy, *T. cruzi*

Introduction

Chagas' disease (CD) is a neglected disease of poor, rural and forgotten populations, representing one of the main public health problems in 22 developing countries of Latin America.¹ Nifurtimox and benznidazole are recommended for all acute, early chronic and reactivated cases, but produce variable results mostly related to the endemic area. Both exhibit considerable undesirable side effects, are administered over 30 or more days and are not very effective against the late chronic phase.^{2,3} Another challenge is blood prophylaxis in endemic areas, since the only trypanosomicidal agent (gentian violet) has toxicity problems, gives the blood a purple colour and may stain the skin and mucosa of recipients.⁴

In vitro and *in vivo* studies have shown the promising efficacy of diamidines and congeners, mainly arylimidamides (AIAs), against *Trypanosoma cruzi*.^{2,5,6} Because recent findings also reported the pharmacological properties and biological efficacy

of AIAs, such as DB745, against *Leishmania* in models of *in vitro* and *in vivo* infection,⁷ in this study the trypanocidal activity of five novel AIAs was evaluated *in vitro* against different strains of *T. cruzi*.

Methods

Drugs

All amidines (see Figure S1; available as Supplementary data at JAC Online) were synthesized according to published procedures.⁸ Benznidazole (LAFEPE, Brazil) and gentian violet (Sigma-Aldrich) were used as previously reported.⁵

Cardiac cell cultures and cytotoxicity assays

To rule out toxic effects against mammalian cells, uninfected primary cultures of embryonic cardiomyocytes (CMs) were incubated at 37°C for 24 and 72 h and LC₅₀ values were determined by MTT colorimetric assays.⁵

Table 1. Trypanocidal effect of arylimidamides and benznidazole against *T. cruzi* (Y strain)^a

Compounds	BTs				Intracellular parasites		
	IC ₅₀ (μM) 4°C	IC ₅₀ (μM)	SI	LC ₅₀ (μM)	IC ₅₀ (μM)	LC ₅₀ (μM)	SI
DB667	>32	0.078 ± 0.008	410	32	0.20 ± 0.01	10.6	53
DB709	>32	0.09 ± 0.03	352	32	0.02 ± 0.01	10.6	530
DB745B	0.66 ± 0.253	0.015 ± 0.002	2133	32	0.03 ± 0.004	10.6	353
DB749	>32	2.5 ± 0.73	13	32	0.02 ± 0.004	8.58	441
DB946	32	0.05 ± 0.002	640	32	0.03 ± 0.006	8.58	286
Benznidazole	>250	12.94 ± 1.93	77	1000	2.77 ± 1.96	1000	360

IC₅₀, drug concentration that reduces the number of parasites by 50%; LC₅₀, drug concentration that reduces cell viability by 50%; SI, corresponds to the ratio LC₅₀/IC₅₀ (for BTs and intracellular parasites calculated on LC₅₀ values at 24 and 72 h of incubation at 37°C, respectively). The IC₅₀ and LC₅₀ values were averaged for at least three determinations done in duplicate.

^aThe activity of the compounds against bloodstream trypomastigotes (BTs) and intracellular parasites was evaluated during their incubation at 4°C (as indicated) or otherwise at 37°C for 24 h and 72 h.

Parasites

Different strains of *T. cruzi* were used (see Table S1; available as Supplementary data at JAC Online). Bloodstream trypomastigotes (BTs) were obtained from Swiss mice infected with *T. cruzi*.⁵ Intracellular amastigotes lodged within CMs were employed as reported previously.⁶

Antitrypanosomal activity

BTs were incubated with compounds (0–32 μM) for 24 h at 37°C or were treated for 5–60 min with 0.1–10 μg/mL DB766, DB745B, DB709 and the diamidine DB569.⁶ To identify a possible candidate for blood bank prophylaxis, BTs were maintained at 4°C for 24 h in freshly isolated mouse blood (96% and 50%) in the presence or absence of serial dilutions of the compound (up to 32 μM).⁵ The parasite death rates were determined through direct analysis by light microscopy, allowing the calculation of IC₅₀ values.⁵ For the analysis on intracellular parasites, after 24 h of infection using BTs (ratio 10:1), infected CMs were incubated for 72 h with the compounds (0–10.6 μM) and the number of released parasites quantified for determination of IC₅₀ values.²

All procedures were carried out in accordance with the guidelines approved by Fiocruz CEUA 0099/01. Statistical analysis was carried out using analysis of variance (ANOVA), with the level of significance set at $P \leq 0.05$.

Results

DB667, DB709, DB745B, DB749 and DB946 gave a dose-dependent trypanocidal effect against BTs (Y strain) (see Figure S2; available as Supplementary data at JAC Online). DB709, DB749 and DB946 presented IC₅₀ values of 0.09 ± 0.03, 2.5 ± 0.73 and 0.05 ± 0.002 μM, respectively. DB745B was the most effective, showing an IC₅₀ value of 0.015 ± 0.002 μM (Table 1).

To compare the efficacy of DB745B with other AIAs previously studied (DB766)⁵ and with a well-known trypanocidal diamidine (DB569),⁶ BTs were incubated for 15 min with different concentrations of each compound (0.1–10 μg/mL). The lower concentrations revealed large differences between the activities of DB745B and DB766; 78% and 27% parasite death, respectively, with 1 μg/mL (data not shown). To further explore the efficacies of these compounds, a time–kill study was conducted using the

higher concentration (10 μg/mL). After 5 min a statistically significant difference was found between DB745B and DB766 ($P = 0.009$); DB745B induced 51% parasite lysis, whereas DB569 and DB766 induced only 16% and 27% parasite lysis, respectively (Figure S2a). After 60 min both AIAs induced more than 96% parasite lysis, whereas DB569 produced about 50% (Figure S2b).

When assayed at 4°C using 96% mouse blood, DB745B presented the highest activity, exhibiting IC₅₀ = 0.66 ± 0.25 μM. DB667, DB709 and DB749 gave IC₅₀ values >32 μM, while DB946 showed modest activity (IC₅₀ = 22 μM) (Figure S2c).

DB745B was also assayed against other strains with different patterns of natural resistance to benznidazole and nifurtimox (Table S1).⁵ For comparative purposes we also included the diamidine DB75, which displays only modest activity against *T. cruzi*.⁶ Although no effect was found for both DB75 and DB749 (IC₅₀ ≥ 31 μM), DB745B showed significant activity, regardless of the drug resistance parasite phenotype, giving IC₅₀ values ranging from 0.3 to 0.7 μM, and greater efficacy than gentian violet (Table 2). DB745B and DB667 tested against a broader panel of *T. cruzi* strains (855, 875, MS1523 and RBVIII) showed that although both were more active than benznidazole, DB667 was less active than DB745B (data not shown).

The five novel AIAs did not cause significant loss of cardiac cell viability after treatment for 24 and 72 h, displaying LC₅₀ values ≥ 32 and 10.6 μM, respectively (data not shown). The incubation of *T. cruzi*-infected CMs with non-toxic concentrations of AIAs (≤ 3.5 μM) resulted in strong inhibition of parasite burden, presenting a dose-dependent and greater activity than benznidazole (Figure S2d and Table 1).

Regarding selectivity indexes (SIs), except for DB749, all the other AIAs displayed high SIs against BTs and intracellular parasites, ranging from 352 to 2133 and 53 to 530, respectively (Table 1).

Discussion

A systematic lead discovery programme performed by the Consortium for Parasitic Drug Development (<http://www.thecpdd.org/>) demonstrated that novel AIAs such as DB745B and

Table 2. *In vitro* activity of DB75, DB745B, DB749 and gentian violet against BTs from different *T. cruzi* strains^a

Compound	IC ₅₀ values (μM)			
	YuYu	Colombiana	Y	CL
DB75	>32	>32	>32	>32
DB745B	0.701	0.468	0.308	0.387
DB749	>32	>32	>32	31
Gentian violet	30.6	30.6	30.6	30.6

^aThe activity of the compounds against BTs was evaluated for 24 h during their incubation at 4°C with 50% mouse blood.⁵

DB766 are effective against *Leishmania* infection *in vitro* and *in vivo*, do not exhibit mutagenicity, display low acute toxicity, have moderate oral bioavailability, are distributed to different tissues such as the liver and spleen, present large volumes of distribution and have an elimination half-life ranging from 1 to 2 days in mice.⁷ As DB766 also presented potent anti-*T. cruzi* activity,⁵ we screened for the trypanocidal effect of five novel AIAs, including DB745B. All AIAs exhibited considerable activity against *T. cruzi*, but DB745B was the most active, even in the presence of blood constituents. The loss of activity exhibited by DB667, DB709, DB749 and DB946 may be related to their association with and/or inactivation by serum components, as reported previously.²

The efficacy of DB745B in the presence of blood is a desirable characteristic shared by a few other AIAs, such as DB766,⁵ that was noted during the evaluation of new trypanocidal agents for use in blood banks. Although the transfusion procedures that have been implemented have reduced the number of blood-related new infections, these procedures are not universally followed. Also, the only trypanocidal agent available for chemical prophylaxis of blood in areas of high endemicity is gentian violet, which is a toxic cationic dye that has several limitations.¹

As recommended for drug screening against CD,⁹ a promising agent should: (i) be active against bloodstream and intracellular forms; (ii) be active against a large panel of parasite isolates, including those that express natural resistance to benznidazole and nifurtimox; (iii) present efficacy equal to or better than the reference drugs; and (iv) display a high SI (≥ 50). In this study, all these requirements were fulfilled, especially by DB745B. All AIAs were more active than benznidazole (e.g. DB745B is about 860 and 90 times more effective against BTs and intracellular parasites, respectively). DB745B is effective against a large panel of strains (855, 875, MS1523 and RBVIII strains are present in peridomestic and sylvatic ecotopes), including those that express natural resistance to benznidazole,¹⁰ and is more active than the diamidines DB569 and DB75, confirming previous studies that revealed the superior activity of AIAs compared with diamidines against *T. cruzi*.^{2,6} Dose-dependent and timepoint studies demonstrated that DB745B is faster acting than DB766, requiring further studies to explore the possibility that different transport mechanisms and/or cellular targets

may be operating. Our findings warrant additional *in vivo* studies with DB745B using acute and chronic experimental models of *T. cruzi* infection with the goal of identifying novel lead AIA candidates against this parasite.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Table S1 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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