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The biological activity of novel arylimidamides against *Trypanosoma cruzi: in vitro* and *in vivo* studies

Running title: AIAs effect against *T.cruzi*

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Abstract

Fifteen novel arylimidamides (AIAs) (6 bis-amidino and 9 mono-amidino analogues) were assayed against *T. cruzi in vitro* and *in vivo.* All bis-AIAs were more effective than mono-AIAs and two of them, DB1967 and DB1989, were further evaluated *in vivo*. Although both reduced parasitemia, protection against mortality was not achieved. Our results show that the number of amidino terminal units affects the efficacy against *T. cruzi*.

Keywords: *Trypanosoma cruzi,* experimental chemotherapy, arylimidamides

Chagas disease (CD) is caused by *Trypanosoma cruzi*, affecting more than 8 million people worldwide (1-5). Benznidazole (BZ) and Nifurtimox (NF) are used for treatment of CD but, due to their well-known toxicity and limited efficacy, especially in the later chronic phase, new drugs are urgently needed (6-9). Our group has evaluated several classes of natural and synthetic compounds, including arylimidamides (AIAs), aromatic diamidines (AD) derivatives with extraordinary activity against *T. cruzi* and others trypanosomatids, both *in vitro* (10-16) and *in vivo* (17, 18). In AIAs the imino group is linked via an "anilino" nitrogen while in classical amidines it is directly attached to an aryl ring, yielding reduced pK values (14). We report *in vitro* and *in vivo* activity studies, mutagenicity and selectivity assessment of new AIAs (6 bis-amidino analogues, DB1966, DB1967, DB1968, DB1979, DB1989 and DB1995; 9 mono-amidino analogues, DB1996, DB1997, DB1980, DB2001, DB2002, DB2003, DB2004, DB2006 and DB2007), which provide insight on the relevance of one or two terminal amidino units for biological activity.

Syntheses of the mono and bis-arylimidamides (Table 1) were performed as reported (19-21). Benznidazole (BZ, Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil) and gentian violet (Sigma-Aldrich) were used as reference drugs (22). Primary cultures of cardiac cells (CC) were obtained as reported (18, 23). The Y strain of *T. cruzi* was used and bloodstream trypomastigotes (BT) and intracellular forms were assayed as described (18, 23). Mammalian cell toxicity was evaluated on uninfected CC incubated up to 48 h/ 37°C with each compound (0 to 32 µM); morphology, spontaneous contractibility and cell death rates were measured for determination of EC_{50} values (compound concentration that reduces 50% of cellular viability) (24). For trypanocidal analysis, BT were incubated at 37°C/ 24 h with non-toxic concentrations of the compounds to determine the EC_{50} (drug concentration that reduces 50% of the number of the treated parasites) (24). For analysis with intracellular amastigotes, after 24 h of parasite - host cell interaction, increasing non-toxic doses of the compounds were added for 48 h and drug activity estimated by calculating the infection index (II) as reported (12,24). The data are means ± standard deviations from 2–4 experiments run in duplicate. Bacterial reverse mutation test and cytotoxicity assay (AMES) were performed as proposed by Maron and Ames (25) and OECD 471 (26). Statistical analysis was performed by ANOVA test ($p \leq 0.05$) (22).

Male Swiss Webster mice (18-21 g) (Fundação Oswaldo Cruz animal facilities CECAL/FIOCRUZ, Brazil), were housed six per cage, in a conventional room at 20-24°C under a 12/12h light/dark cycle, with sterilized water and chow *ad libitum*. Infection was achieved by intraperitoneal (ip) injection of 10^4 BT (Y strain) and mice grouped as (n=6): uninfected (non-infected and non-treated); untreated (infected and treated with vehicle) and treated with different doses of DB1989 and DB1967 (infected and treated

with 0.2 mL ip daily doses up to 50 mg/kg). Infected mice were orally treated with 100 mg/kg/day BZ, once a day. Treatment was given at 5th (parasitemia onset) and at 8th dpi (parasitemia peak). Parasitemia levels, body weight and percentage of cumulative mortality were checked until 30 days post treatment as reported (18). All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA 0028/09).

BT incubated for 24 h at 37°C showed that 12 of the 15 compounds (all but DB1996, DB1997, DB2002) gave superior trypanocidal activity ($p \leq 0.05$) compared to BZ (EC_{50} = 13 µM). Five of the bis-AIAs (DB1966, DB1967, DB1968, DB1979 and DB1989) yielded EC_{50} values ≤0.1 µM. The bis-AIA DB1989, the fastest acting trypanocidal compound, provided an EC_{50} value of 2.7 µM after 2 h (Table 1). Bis-AIAs also displayed the best effect under blood bank conditions (in blood, at 4 °C): DB1967, DB1968 and DB1989 showed EC_{50} values ranging from 2.9-3.9 µM, while BZ was ineffective up to 250 µM (Table 1). The most selective compounds against BT were: DB1967 (SI = 88) and DB1989 (SI = 70) (Table 1). The mono-AIAs DB1980, DB2001 and DB2004 were the most toxic against cardiac cell cultures at 48 h. Mono-AIAs were ineffective after 48 h/37°C on *T.cruzi*-infected cultures (Table 1). Similar to BT, four bis-AIAs (DB1966, DB1967, DB1968, and DB1989) were the most effective against intracellular parasites (EC_{50} values of $\leq$ 0.1µM - Table 1). Mono-AIAs displayed very low selectivity, while the bis-AIAs DB1989 and DB1967 exhibited the highest SI levels (20 and 40, respectively) against the intracellular parasites (Table 1). Bacterial reverse mutation test (Ames test) gave no major mutagenic potential (MI <2) using DB1989 (S1) or BZ (data not shown). Due to excellent *in vitro* activity against both parasite forms and reasonable selectivity both DB1967 and DB1989 were evaluated *in vivo*. At

8dpi (parasitemia peak), DB1989 reduced parasitemia (40, 76, 75% with 12.5, 25 and 50 mg/kg/day doses, respectively) while BZ suppressed parasitemia (Fig. 1A). BZ gave 100% survival of mice, but no dose of DB1989 prevented mortality triggered by the infection (Fig. 1B): the highest dose (50mg/kg/day) produced higher mortality rates compared to untreated group, possibly due to compound toxicity (ponderal curve shows higher weight-loss; Fig. 1C). DB1967 produced dose-response suppression (67 up to 87%) of parasitemia, but an earlier and higher mortality rate (100% for all DB1967 treated groups likely due to toxicity, data not shown).

AIAs like DB766 are effective *in vitro* and *in vivo* against intracellular pathogens that cause human and animal pathologies (24, 27-29), exhibiting stronger activity than classical diamidines possibly due to their lower pKa values, better bioavailability and improved cell membrane permeability (28). Similar to *Leishmania*, bis-AIAs are highly active against *T. cruzi*, (12,15,22). DB766 showed a selective effect against intracellular amastigotes and upon a large panel of *T.cruzi* including naturally resistant strains, with higher efficacy than reference drugs (18).

This work explores the correlation between the trypanocidal activity /selectivity of AIAs with one or two terminal amidino groups. Bis-AIAs were most potent against both parasite forms relevant to mammalian infection (bloodstream and intracellular forms), demonstrating that two terminal amidino centers confer higher parasiticidal effect than those bearing only one. The importance of the second amidino center is seen by comparing the results for DB1967 with that for DB2002 (500-fold activity difference; Table 1) which differ only by the absence of the second amidino group in DB1967. These results corroborate previous findings for classical diamidines, confirming the requirement of a diamidino unit for effectiveness against *T.cruzi* (15).

Bis-AIAs DB1967, DB1968 and DB1989 maintained good trypanocidal activity at 4°C with 96% mouse blood, similar to other bis-AIAs including DB766 (18), DB745 (31) and DB1831 (22).

All tested bis-AIAs have alkoxy groups of approximately the same size and similar *in vitro* activity (EC_{50} values ≤0.1 μM). DB1967 with only one 2-propoxy group has essentially the same antitrypanosomal activity as DB766 (18), which has two such groups; yet DB1967 is more toxic to animals than DB766 suggesting that two moderate sized alkoxy groups reduce animal toxicity. Generally, the activity of the mono-AIAs does not vary significantly with structure (Table 1). Most bis-AIAs were also less toxic towards cardiac cells than mono-AIAs. Presently, up to the maximum dose tested, genotoxicity was absent and only a mild mutagenicity profile was observed when DB1989 was assayed against *Salmonella enterica* Typhimurium TA98 strain (S1), which is suggestive of a frameshift mutation, probably during the DNA repair process or duplication process, adding GC pairs into the genome. Although OECD471 guidelines recommend going up to 5 mg of a tested compound, the high activity of DB1989 towards the bacteria strains impaired assaying higher AIA concentrations that may mask mutagenic aspects, demanding then, additional toxicological studies.

DB1989 and DB1967 were moved to *T.cruzi in vivo* models due to their high in vitro activity and reasonable selectivity. Although parasitemia reduced, neither DB1967 nor DB1989 protected against mortality. This is in contrast to results with DB766 (18) and DB1965, a mesylate salt form of DB 1831 (22) which showed in *vivo* efficacy comparable to that of BZ. The reduction of parasitemia observed with DB1967 correlates with the *in vitro* data obtained with both bloodstream and intracellular parasites (EC_{50} = 30-40 nM). As low toxicity was observed *in vitro*, the higher mortality

of DB1967-treated mice is likely due to an organ-specific toxicity (e.g. hepatotoxicity) or arises from metabolic products of the bis-AIA.

Our data confirm the importance of two amidino centers for the trypanocidal efficacy of arylimidamides against *T.cruzi* and demonstrated that mono-AIAs are both less effect and selective. Although very active *in vitro*, DB1989 and DB1967 failed to protect against *T.cruzi* infection *in vivo*, possibly due to toxicity. Since previous studies demonstrated in vivo efficacy comparable to that of BZ for other bis-AIAs, e.g. DB766 (18) and DB1965 (22), the synthesis of novel AIAs bearing bis terminal pyrimidines or pyridines merits further investigation as an approach for identification of new anti-*T. cruzi* agents.

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Figure Legends

Figure 1: *In vivo* effect of DB1989 (A-C) on acute mouse model of infection with Y strain of *T-cruzi*: Parasitaemia (A), mortality rates (B) and ponderal curve (C) are shown. The effects of DB1989 (ip) and BZ (p.o.) were followed using doses (up to 50 mg/kg/day for DB1989 and 100 mg/kg/day for BZ) administrated at the 5^{th} and 8^{th} dpi.

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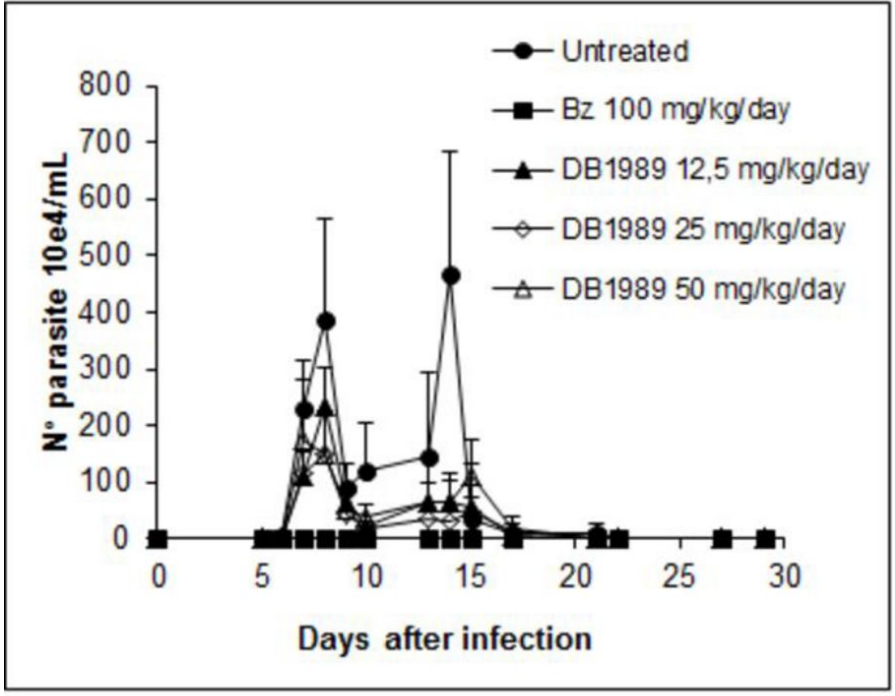
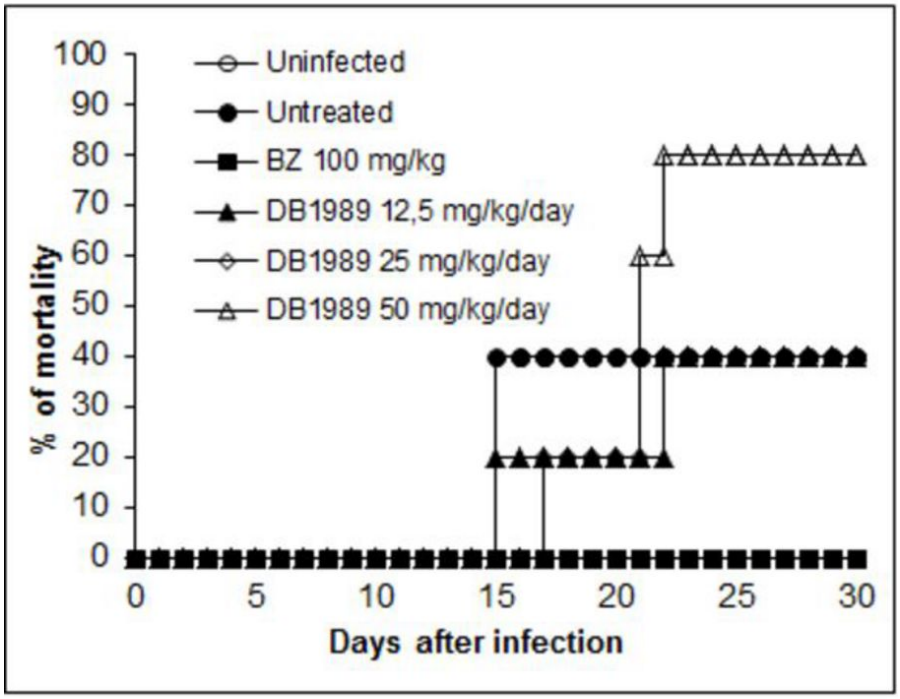




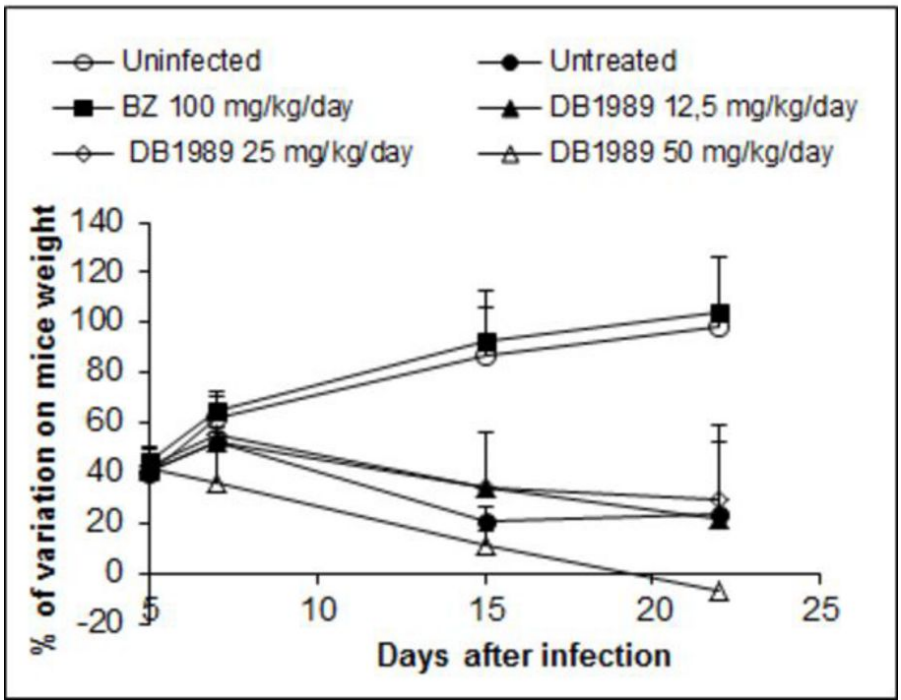
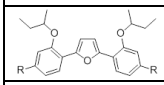
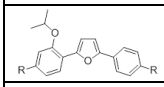
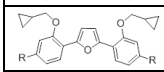
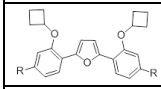
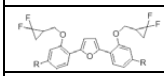
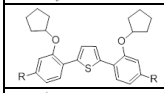
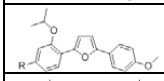
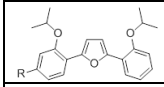
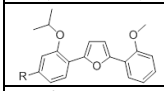
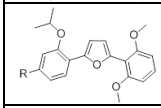
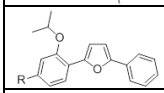
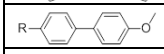
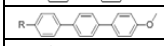
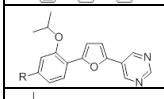
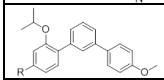
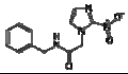


Table I: AIAs activity (μM) against bloodstream and intracellular (amastigotes) forms of *T. cruzi* (Y strain) (EC_{50} - μM) and the corresponding Selectivity Indexes (SI)

Compounds		Bloodstream Trypomastigotes					Amastigotes	
		EC_{50}: 37ºC / RPMI		SI 37ºC	EC_{50} 4ºC / Blood		EC_{50} 37ºC /RPMI	SI
Structure	Name	2 h	24 h	24 h	2 h	24 h	48 h	48 h
	DB1966*	>3.5	0.04±0	30	>32	1.2	0.09±0.08	13
	DB1967*	>3.5	0.04±0.02	88	>32	1.2	0.03±0.006	40
	DB1968*	>3.5	0.08±0.02	15	>32	3.5	0.1±0.1	12
	DB1979*	3.5	0.1±0.04	30	>32	1.2	1±1.4	3
	DB1989*	2.7±1.6	0.05±0.01	70	>32	1.1	0.06±0.03	20
	DB1995*	>32	1.0±0.8	3	>32	0.15	0.9±0.35	1.2
	DB1980	>32	5.8±4	0.2	>32	1.17	>0.39	>0.39
	DB1996	>32	13±12	0.09	>32	1.17	>0.39	3
	DB1967	>32	15±5	0.08	>32	0.09	>0.39	3
	DB2001	>32	4.8±1.5	0.2	>32	1.17	>0.39	0.25
	DB2002	>32	20±10	0.15	>32	3.5	>0.39	3
	DB2003	13±3	4.3±1.9	0.9	>32	0.97	>0.39	9
	DB2004	12±3.9	2.9±1.5	0.35	>32	3.5	>0.39	2.5
	DB2006	12±60	5.9±10	0.5	>32	1.17	>0.39	9
	DB2007	11±60	1.2±10	1	>32	1000	>0.39	3
	BZ	>50	13 ± 2	77	>250	>250	3.6±1.7	> 277

* Bis-AIAs