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In Vitro and *In Vivo* Studies of the Biological Activity of Novel Arylimidamides against *Trypanosoma cruzi*

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

Chagas disease (CD) is caused by *Trypanosoma cruzi* and affects more than 8 million people worldwide (1–5). Benznidazole (BZ) and nifurtimox (NF) are used for the treatment of CD, but because of their well-known toxicity and limited efficacy in the later chronic phase of the disease, new drugs are urgently needed (6–9). We have evaluated several classes of natural and synthetic compounds, including arylimidamides (AIAs), aromatic diamidine (AD) derivatives with extraordinary activity against *T. cruzi* and other 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). Here, we report the results of *in vitro* and *in vivo* activity studies and mutagenicity and selectivity assessments of new AIAs (6 bis-amidino analogues, DB1966, DB1967, DB1968, DB1979, DB1989, and DB1995, and 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.

We synthesized the mono- and bis-arylimidamides (see structures in Table 1) 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 trypomastigote forms were assayed as described previously (18, 23). Mammalian cell cytotoxicity of AIAs was evaluated on uninfected CC incubated up to 48 h at 37°C with each compound (0 to 32 μM); morphology, spontaneous contractibility, and cell death rates were measured for determination of the 50% effective compound concentrations (EC_{50}s) (24). For trypanocidal analysis, BT were incubated at 37°C for 24 h with nontoxic concentrations of the compounds to determine the EC_{50} (24). For analysis with intracellular amastigotes, after 24 h of parasite-host cell interaction, increasing nontoxic doses of the compounds were added for 48 h, and drug activity was estimated by calculating the infection index (II) as reported (12, 24). The data shown are the means ± standard deviations from 2 to 4 experiments run in duplicate. A bacterial reverse mutation (Ames) test and a cytotoxicity assay were performed as proposed by Maron and Ames (25) and Organization for Economic Cooperation and Development (OECD) test guideline 471 (26). Statistical analysis was performed by the analysis of variance test ($P \leq 0.05$) (22).

Male Swiss Webster mice (18 to 21 g) (Fundação Oswaldo Cruz Animal Facility [CECAL/FIOCRUZ], Brazil) were housed six per cage in a conventional room at 20 to 24°C under a 12/12-h light/dark cycle, with sterilized water and chow provided *ad libitum*. Infection was achieved by intraperitoneal (i.p.) injection of 10^4 BT (Y strain), and the mice (6 per group) were uninfected (noninfected and nontreated), untreated (infected and treated with vehicle), or treated with different doses of DB1989 and DB1967 (infected and treated with 0.2-ml i.p. daily doses up to 50 mg/kg of body weight). Infected mice were treated with 100 mg/kg/day BZ orally once a day. Treatment was given at the 5th (parasitemia onset) and 8th day postinfection (dpi) (parasitemia peak). Parasitemia levels, body weights, and percentage of cumulative mortality were checked until 30 days posttreatment, 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, and DB2002) had superior trypanocidal activities ($P \leq 0.05$) compared to that of BZ (EC_{50}, 13 μM). Five of the bis-AIAs (DB1966, DB1967, DB1968, DB1979, and DB1989) yielded EC_{50}s of ≤0.1 μM. The bis-AIA DB1989, the fastest-acting trypanocidal compound, provided an EC_{50} of 2.7 μM after 2 h (Table 1). Bis-AIAs also displayed the best

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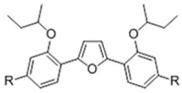
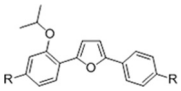
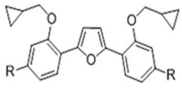
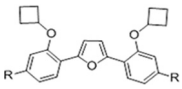
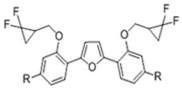
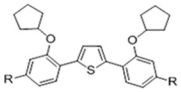
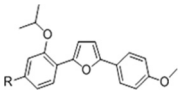
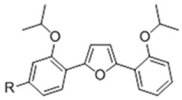
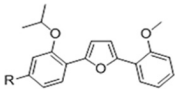
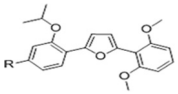
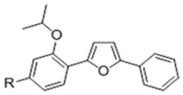
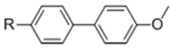
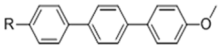
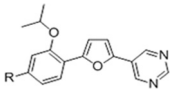
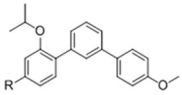
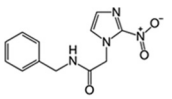
TABLE 1 AIA activity against bloodstream trypomastigotes and intracellular (amastigote) forms of *T. cruzi* (Y strain) and the corresponding selectivity indexes

Compound structure	Compound name	Bloodstream trypomastigotes					Amastigotes	
		EC_{50} (mean ± SD) (μM) at 37°C in RPMI at:		SI at 37°C at 24 h	EC_{50} (mean ± SD) (μM) at 4°C in blood at:		EC_{50} (mean ± SD) (μM) at 37°C in RPMI at 48 h	SI at 48 h
		2 h	24 h		2 h	24 h		
	DB1966[a]	>3.5	0.04 ± 0	30	>32	22 ± 3	0.09 ± 0.08	13
	DB1967[a]	>3.5	0.04 ± 0.02	88	>32	3.75 ± 0.3	0.03 ± 0.006	40
	DB1968[a]	>3.5	0.08 ± 0.02	15	>32	2.9 ± 0.4	0.1 ± 0.1	12
	DB1979[a]	3.5	0.1 ± 0.04	30	>32	10.8 ± 2.7	1 ± 1.4	3
	DB1989[a]	2.7 ± 1.6	0.05 ± 0.01	70	>32	3.9 ± 1.3	0.06 ± 0.03	20
	DB1995[a]	>32	1.0 ± 0.8	3	>32	25 ± 9	0.9 ± 0.35	1.2
	DB1980	>32	5.8 ± 4	0.2	>32	>32	>0.39	>0.39
	DB1996	>32	13 ± 12	0.09	>32	>32	>0.39	3
	DB1997	>32	15 ± 5	0.08	>32	>32	>0.39	3
	DB2001	>32	4.8 ± 1.5	0.2	>32	21 ± 6	>0.39	0.25
	DB2002	>32	20 ± 10	0.15	>32	>32	>0.39	3
	DB2003	13 ± 3	4.3 ± 1.9	0.9	>32	>32	>0.39	9
	DB2004	12 ± 3.9	2.9 ± 1.5	0.35	>32	>32	>0.39	2.5
	DB2006	12 ± 60	5.9 ± 10	0.5	>32	22 ± 0.9	>0.39	9
	DB2007	11 ± 60	1.2 ± 10	1	>32	>32	>0.39	3
	BZ	>50	13 ± 2	77	>250	>250	3.6 ± 1.7	>277

[a] Bis-AIA.

effect under blood bank conditions (in blood at 4°C); DB1967, DB1968, and DB1989 showed EC_{50}s ranging from 2.9 to 3.9 μM, while BZ was ineffective at up to 250 μM (Table 1). The most selective compounds against BT were DB1967 (selectivity index [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 at 37°C on *T. cruzi*-infected cultures (Table 1). Similar to the effect against BT, four bis-AIAs (DB1966, DB1967, DB1968, and DB1989) were the most effective against intracellular parasites (EC_{50}s of ≤0.1 μM) (Table 1). Mono-AIAs displayed very low selectivities, while the bis-AIAs DB1989 and DB1967 exhibited the highest SI levels (20 and 40, respectively) against the intracellular parasites (Table 1). A bacterial reverse mutation (Ames) test indicated no major mutagenic potential (mutagenic index, <2) with DB1989 (see Table S1 in the supplemental material) or BZ (data not shown). Due to their excellent *in vitro* activities against the two parasite forms and reasonable selectivities, DB1967 and DB1989 were evaluated *in vivo*. At 8 dpi (parasitemia peak), DB1989 reduced parasitemia (by 40, 76, and 75% with 12.5, 25, and 50-mg/kg/day doses, respectively), while BZ suppressed parasitemia (Fig. 1A). BZ resulted in 100% survival of the mice, but no dose of DB1989 prevented mortality triggered by the infection (Fig. 1B); the highest dose (50 mg/kg/day) produced higher mortality rates compared to that of the untreated group, possibly due to compound toxicity (a ponderal curve shows higher weight [Fig. 1C]). DB1967 produced dose-response suppression (67 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 such as DB766 are effective *in vitro* and *in vivo* against intracellular pathogens that cause human and animal pathologies (24, 27–29) and exhibit stronger activity than those of classical diamidines (possibly due to their lower pK_a values), better bioavailability, and improved cell membrane permeability (28). Similar to their effect against *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* strains, including naturally resistant strains, with a higher efficacy than those of the 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 the two parasite forms relevant to mammalian infection (the bloodstream and intracellular forms), demonstrating that two terminal amidino centers confer a higher parasiticidal effect than those bearing only one. The importance of the second amidino center is seen by comparing the results for DB1967 with those for DB2002 (500-fold activity difference; Table 1), which differ only in 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). The bis-AIAs DB1967, DB1968, and DB1989 maintained good trypanocidal activity at 4°C with 96% mouse blood, similar to the activities of other bis-AIAs, including DB766 (18), DB745 (30), and DB1831 (22).

All tested bis-AIAs have alkoxy groups of approximately the same size and with similar *in vitro* activities (EC_{50}s of ≤0.1 μM). DB1967, with only one 2-propoxy group, has essentially the same antitrypanosomal activity as that of DB766 (18), which has two such groups; yet, DB1967 is more toxic to animals than DB766,

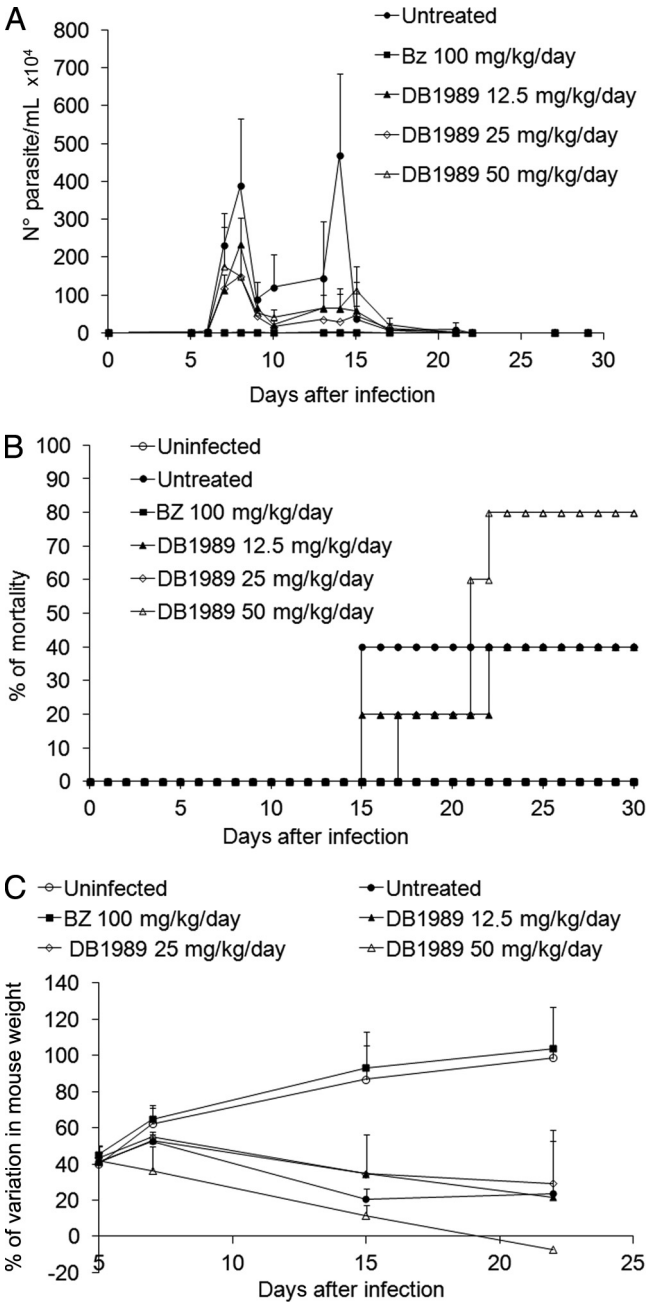


FIG 1 *In vivo* effect of DB1989 on acute mouse model of infection with the Y strain of *T. cruzi*. Parasitemia (A), mortality rates (B), and ponderal curve (C) are shown. The effects of DB1989 (i.p.) and BZ (oral) were followed using doses (up to 50 mg/kg/day for DB1989 and 100 mg/kg/day for BZ) administered at the 5th and 8th dpi.

suggesting that two moderately sized alkoxy groups reduce animal toxicity. Generally, the activities of the mono-AIAs do not vary significantly with structure (Table 1). Most bis-AIAs were also less toxic toward cardiac cells than were the 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 the *Salmonella enterica* Typhimurium TA98 strain (see Table S1 in the supplemental material), which is suggestive of a frameshift mutation, probably during the

DNA repair or duplication process, adding GC pairs into the genome. Although OECD test guideline 471 recommends using up to 5 mg of a tested compound, the high activity of DB1989 toward the bacterial strains impaired assaying higher AIA concentrations that may mask mutagenic aspects, demanding additional toxicological studies.

DB1989 and DB1967 were moved to *T. cruzi in vivo* models due to their high *in vitro* activities and reasonable selectivities. Although parasitemia was reduced, neither DB1967 nor DB1989 protected against mortality. This is in contrast to results with DB766 (18) and DB1965, a mesylate salt form of DB1831 (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 bloodstream and intracellular parasites (EC_{50}s of 30 to 40 nM). As low toxicity was observed *in vitro*, the higher mortality rate of the DB1967-treated mice is likely due to an organ-specific toxicity (e.g., hepatotoxicity) or arose 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 less effective and selective than bis-AIAs. 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* efficacies 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 identifying new anti-*T. cruzi* agents.

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