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In vitro and *In vivo* Biological Effect of Novel Arylimidamide Derivatives Against *Trypanosoma cruzi*

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Running Head: Trypanocidal activity of arylimidamides

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ABSTRACT

Chagas disease (CD), a neglected tropical disease caused by *Trypanosoma cruzi*, remains a serious public health problem in several Latin American countries. The available chemotherapies for CD have limited efficacy and exhibit undesirable side effects. Aromatic diamidines and arylimidamides (AIAs) have shown broad-spectrum activity against intracellular parasites including *T. cruzi*. Therefore our aim was to evaluate the biological activity of eight novel AIAs (16DAP002; 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070 and 27SMB009) against experimental models of *T. cruzi* infection *in vitro* and *in vivo*. Our data show that none of the compounds induced loss of cellular viability up to 32 µM. Two AIAs, 18SAB075 and 16DAP002, exhibited good *in vitro* activity against different parasite strains (Y and Tulahuen) and against the two relevant forms of the parasite for mammalian hosts. Due to the excellent selective indexes of 18SAB075, this AIA was moved to *in vivo* tests for acute toxicity and parasite efficacy, the later, employing non-toxic doses (NOAEL = 50mg/kg). In experimental acute models of *T. cruzi* infection, 18SAB075 reduced only up to 50% the parasitemia levels and led to 40% of protection against mortality (at 5 mg/kg), being less effective than the reference drug, benznidazole.

INTRODUCTION

Chagas disease is a neglected tropical disease caused by the intracellular flagellate protozoan *Trypanosoma cruzi*, which presents a complex life cycle with distinct morphological stages in its obligatory passage through vertebrate and invertebrate hosts [1]. Currently, there are approximately 10 million infected individuals in endemic areas of Latin America and many reports also point to the occurrence in non-endemic geographical areas such as the United States and Europe, mainly attributed to migration of infected people [2-6]. CD can be transmitted by Triatominae insect feces, blood transfusion, organ transplantation, laboratory accidents, and oral and congenital routes [7,8]. This pathology has the following two successive phases: a short acute phase characterized by a patent parasitemia, followed by a chronic phase in which most of the infected individuals remain asymptomatic (indeterminate form), but about one third may later on manifest cardiac and/or digestive complications, developed progressively for years or decades after infection [9,10]. Benznidazole (Bz) and Nifurtimox, introduced into clinical therapy about 40 years ago, are the only available drugs. Both have several shortcomings related to their required long periods of treatment, the high toxicity, variable results and low efficacy during the chronic phase, justifying the identification of novel therapies [11-13]. Aromatic diamidines and analogues exhibit broad spectrum activity against pathogenic microorganisms including *T. cruzi* [14]. Among the different tested derivatives of amidine compounds, the most effective against *T. cruzi* have been the called bis-arylimidamides (AIAs) like DB766 [15] and DB1831 [16]. Thus, presently, the biological activities of eight novel AIAs (16DAP002; 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070 and 27SMB009) were assayed against experimental models of *T. cruzi* infection *in vitro* and *in vivo*, using different parasite strains and also exploring toxicity aspects towards cardiac cell cultures and mouse models of acute toxicity.

MATERIALS AND METHODS

Synthesis of the arylimidamides. The eight arylimidamides, all isolated as their hydrochloride salts, were prepared by three general methods (see Supplementary data at AAC Online). Briefly, phenylimidamides 16DAP022 and 16SAB079 (Figure 1) were prepared by the reaction of with 2,7-diaminocarbazole (prepared by reduction of 2,7-dinitroocarbazole) [17] or *m*-xylylenediamine with (2-naphthyl)methyl benzothioimidate hydrobromide [18]. The 2-pyridylimidamide 18SAB075 (Fig. 2) was prepared by the reaction of 4,4□-diaminodiphenylacetylene [19,20] with 2-cyanopyridine by the method of Lange *et al.* [21] but using lithium (rather than sodium) bis(trimethylsilyl)amide. Reaction of the methyl imidate derivatives of 2- or 4-cyanopyridine with the appropriate α,ω-diamines or spermidine gave 2-pyridylimidamides 23SMB022, 23SMB026, and 23SMB054, and 4-pyridylimidamides 26SBM070 and 27SMB009 (Fig. 3).

Mammalian cell cultures. For the *in vitro* analysis of compound toxicity and effect against intracellular parasites (Y strain), primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously reported [22]. After purification, the CM were seeded at a density of 0.2 X 10^6 and 0.05 X 10^6 cell/well, respectively, into 24 and 96-well microplates containing gelatin-coated cover slips. The cultures were then maintained at 37° C in Dulbecco`s modified medium supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM $CaCl_2$, 1 mM *L*-glutamine and 2% chicken embryo extract (DMEM). For the further analysis of the effect on intracellular parasites for Tulahuen strain (parasites expressing the *Escherichia coli* β-galactosidase gene), monolayers of mouse L929 fibroblasts were cultivated (4 X 10^3 cell/well into 96-well microplates) at 37° C in RPMI-1640 medium (pH 7.2-7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (RPMIS), as reported [23].

Parasites. Bloodstream trypomastigote forms (BT) of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitemia. The purified parasites were

resuspended in Eagle medium modified by Dulbecco (DME) supplemented with 10% FBS (DMES) as reported [22]. The effect on intracellular forms was investigated by both the infection of L929 cell lineages with tissue culture derived trypomastigotes (Tulahuen strain expressing the *E. coli* β-galactosidase gene) and the infection of CM with bloodstream trypomastigotes (Y strain) using a 10:1 ratio, following previously established protocols [15,23]. Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0.6% and 10% for in vitro and in vivo analysis, which did not exert any toxicity (data not shown). Benznidazole (Bz) (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as reference drug.

Cytotoxicity *in vitro* tests. CM were incubated for 24 h at 37° C with different concentrations of each compound (up to 96 µM) diluted in DMEM (without phenol red), their morphology and spontaneous contractibility evaluated by light microscopy and then their cellular viability determined by the alamarBlue® assay. For this colorimetric bioassay, 10 µL alamarBlue® (Invitrogen) was added to each well and the plate further incubated for 24 h, after which the absorbances at 570 and 600 nm were measured. As negative controls, AlamarBlue® assay was also performed in the lack of cells, running only DMEM and DMEM containing each tested compound (at 96 µM). The results were expressed as percent difference in reduction between compound treated and vehicle treated cells by following the manufacturer's instructions and the value of EC_{50} corresponds to the concentration that reduces in 50 % the cellular viability [23].

Trypanocidal analysis. Bloodstream trypomastigotes of the Y strain (5 X 10^6 per mL) were incubated for 24 h at 37^{o}C in RPMI in the presence or absence of serial dilutions of the compounds (0 to 32 µM). After compound incubation, the parasite death rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the EC_{50} (compound concentration that reduces 50% of the number of parasites) was then calculated.

For the assay on intracellular forms, different parasite strain and protocols were performed using Y and Tulahuen strains. For Tulahuen-infected-L929 cells, the cultures were exposed to 1.0 µg/mL diluted in RPMIS and those compounds those that presented ≥ 50% of reduction on the parasite infection index were further screened under increasing concentrations aiming to determine the EC_{50} values [23]. After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500 µM) in 0.5% Nonidet P40 was added to each well and the plate incubated for 18 h at 37°C. Next the absorbance was measured at 570 nm. Uninfected and infected cultures submitted to vehicle and Bz exposure were run in parallel. The results are expressed as the percentage of *T. cruzi* growth inhibition in compound-tested cells as compared to the infected cells and untreated cells [23].

For the analysis of the effect against intracellular amastigotes from Y strain, after 24 h of parasite-host cell interaction, the infected cultures were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CM were maintained at 37°C in an atmosphere of 5% CO_2 and air and the medium replaced every 24 h. Then, untreated and treated infected CM were fixed and stained with Giemsa solution and the mean number of infected host cells and of parasites per infected cells scored as reported [16]. Only characteristic *T. cruzi* nuclei and kinetoplasts were counted as surviving parasites since irregular structures could mean parasites undergoing death. The compound activity was estimated by calculating the infection index (II - percentage of infected cells times the average number of intracellular amastigotes per infected host cell) [24]. Triplicate assays were run in the same plate and at least two assays performed in each analysis.

Mouse acute toxicity. In order to determine the NOAEL (No Observed Adverse Effect Level), each dose of the tested compounds (12.5 up to 200 mg/kg) was injected by intraperitoneal route (ip) individually in g Swiss Webster female mice (20-23 g). On days 2 and 3, mice were inspected for toxic and sub-toxic symptoms according to OECD guidelines (Organization for

Economic Co-operation and Development). Forty-eight hours after compound injection, the NOAEL values were determined and plasma biochemical analysis performed for alanine aminotransferase (ALT), urea and creatine kinase (CK) as reported [16].

Mouse infection and treatment schemes. Male Swiss mice were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (Rio de Janeiro, Brazil). Mice were housed at maximum 6 per cage and kept in a conventional room at 20-24ºC under a 12/12-h light/dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by ip injection of 10^4 bloodstream trypomastigotes (Y strain). The animals (18-21 g) were divided into the following groups: uninfected (non-infected and untreated); untreated (infected with *T. cruzi* and treated with vehicle); and treated – infected and treated (ip) with 0.5 up to 20 mg/kg/day test compound (up to 0.2 mL) or with 100 mg/kg/day Bz (po). The mouse treatment started at 5 days post infection (dpi) followed by (i) one second dose at 8 dpi or (ii) five consecutive daily doses. For Bz treatment, infected mice received 0.2 mL oral dose (gavage) following the same therapeutic schemes as above described [15].

Parasitemia, mortality rates and ponderal curve analysis. Parasitemia was individually checked by direct microscopic counting of parasites in 5 µL of blood, as described before [25]. Body weight was weekly evaluated, and mortality checked daily until 30 days post treatment and expressed as percentage of cumulative mortality (% CM) [15].

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW-16/2013).

RESULTS

The screening of the eight novel arylimidamides (Figs. 1-3) against bloodstream trypomastigotes (Y strain) revealed that after 2 h of incubation at 37°C, 18SAB075 and 16DAP002 showed considerable activity, displaying EC_{50} values of 11 and 14 µM (Table 1). After 24 h of incubation, both were more effective than Bz, displaying EC_{50} values of 0.3 and 3 µM, respectively, with the former molecule exhibiting about 43 fold higher trypanocidal effect than the reference drug Bz (Table 1). *In vitro* analysis of cytotoxicity on cardiac cell cultures performed by the alamarBlue® assay showed that up to 32 µM, none of the studied compounds caused loss of cellular viability (Table 1). AlamarBlue® performed in the lack of cells, with DMEM containing or not each tested compound (at 96 µM) showed none alteration in the absorbance measurements (data not shown). When tested on *T. cruzi*-infected host cells (Tulahuen strain transfected with β-galactosidase gene) under a standardized screening protocol using a fixed concentration of 1 µg/mL [23], again both 16DAP002 and 18SAB075 were effective (Table 2). Next, both AIAs were again screened against Tulahuen-infected host cells in order to determine the EC_{50} values. The findings showed that 18SAB075 was the most active with values of 1.5 µM after 96 h of incubation, comparable to Bz and 16DAP002 (Table 3). The further analysis of the effect of 18SAB075 against *T. cruzi*-infected cardiac cells using another strain (Y strain) confirmed the promising activity (EC_{50} = 0.9 µM) of this AIA, especially noticed at the mean number of infected cardiac cells data, without causing loss of host cell viability when the infected cultures were incubated with concentrations up to 32 µM (Table 4, A and B). Although no loss of viability was noticed up to ≤ 32 µM screened by alamarBlue® method, the light microscopy analysis demonstrated that 16SAB002 at concentrations > 3.5 µM induced cellular vacuolization and impaired the contractibility of the cardiac cells *in vitro*. Besides, no effect on the levels of *T. cruzi* infection was observed when non-toxic doses (≤ 3.5 µM) of this AIA were tested (data not shown).

Next, due to the higher selectivity indexes of 18SAB075 on bloodstream trypomastigotes (>106 – Table 1) and on intracellular forms (40 – Table 4), this compound was moved to *in vivo* analysis.

Our first *in vivo* step was to evaluate aspects of acute toxicity of 18SAB075 aiming to determine its NOAEL value. The administration of this AIA via the ip route to Swiss female mice (12.5 up to 200 mg/kg) followed for 48 h showed that none of the doses caused death. Up to 50 mg/kg no alteration on animal behavior (as compared to vehicle treated mice) was found. However, increased doses induced visible neurological disorders like tremors, ataxia and hyperactivity (data not shown). The gross pathology performed at 48 h after drug administration showed that 200 mg/kg induced hemorrhagic hepatic signs (data not shown). This hepatic injury was confirmed by the biochemical plasma analysis of the higher dose as increased levels of AST and ALT were found as compared to the animals from the other groups (data not shown). Then, the efficacy of 18SAB075 was tested in Swiss male mice inoculated with 10^4 bloodstream parasites using a therapeutic scheme employing doses administrated once a day, at 5 and 8 dpi (Fig. 4) that corresponds to the onset and parasitemia peak, respectively in this animal model (Batista e cols., 2010). Only parasitemia positive mice were used and Bz treatment was run in parallel using standard protocol (100mg/kg via p.o.), following the same therapeutic scheme as above described (Fig. 4). Although 20 and 10 mg/kg did not reduce or only slightly impaired (20%), respectively, the parasitemia levels, the lower dose (5 mg/kg) resulted in 50% of decrease in the parasitism peak decrease, also leading to 40% of animal survival while the other mice (except for Bz group), all died (Fig. 4).

Then, aiming to allow a longer plasma exposure of the compound but taking into consideration the use of maximum non-toxic doses (up to 50 mg/kg), additional studies were performed providing the AIA for 5 daily consecutive days, starting at 5 dpi (Fig. 5). As expected for this experimental mouse model of *T. cruzi* acute infection, infected and vehicle-treated animals

presented high parasitemia and no animal survival (Figs. 4 and 5). When 18SAB075 was administrated via ip route, none of the doses reduced parasitemia or protected against mortality. As expected, Bz completely suppressed the parasitemia and gave 100% of survival (Fig. 5). Also, none of the doses were able to protect the animals against the body weight loss (data not shown) induced by *T. cruzi* infection in this experimental acute model [26].

DISCUSSION

Presently, structural variations include seven different linkers between the two AIA moieties and three different outer aromatic rings. Those molecules containing multiple or fused aromatic rings in their linkers were active against the bloodstream and intracellular parasites. The most active compound, 18SAB075, contains a diphenylacetylene linker, which offers extended conjugation. The second most active, 16DAP002, contains a carbazole system as its linker, which also offers extended conjugation but a more rigid conformation due to the fused rings. Both compounds displayed AIA nitrogen atoms attached directly to aromatic rings. Xylene derivative 26SMB070, which was less active, bears an aromatic ring in its linker, but the nitrogen atoms are attached to aliphatic carbons. Analogues of the two most active compounds containing the same linkers but different outer rings were not included in this study.

Chemotherapeutic options for treating chagasic patients is currently dependent on only two nitro derivative drugs, namely benznidazole (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) and nifurtimox (5-nitrofuran, Bayer 2502; Bayer, Germany) [27]. Both exhibit severe side effects resulting in discontinuation of the treatment, low effectiveness, especially in the later chronic phase, demanding the identification of novel drugs to treat this devastating chronic pathology. However, there is a lack of interest from most pharmaceutical companies for drug discovery of new anti-*T. cruzi* agents mainly due to the long time and high costs associated to a drug pipeline for this as well as other neglected parasitic

illnesses that afflict millions of the poorest people worldwide [28]. In this context, the present aim was to explore the possibility of finding new amidine derivatives with considerable activity against *T. cruzi* in *vitro* and *in vivo*, which possess the potential to act as clinical candidates against this parasitic neglected disease. In this vein, eight novel AIAs were screened against bloodstream and intracellular forms of the parasite and two of them, 18SAB075 and 16 DAP002, demonstrated higher activity, with EC_{50} values below 4 µM. Both compounds were effective against different strains (Y and Tulahuen) and due to the high selectivity, 18SAB075 was further examined within *in vivo* to ascertain preliminary acute toxicity and for efficacy determination using acute mouse model of *T. cruzi* infection (male Swiss mice infected with Y strain). The follow up of acute toxicity signs for 48 h after 18SAB075 administration via ip showed that although up to 50mg/kg none alterations could be noticed, considerable toxic side effects like ataxia, hyperactivity and tremors were found with doses 100 and 200 mg/Kg. The gross pathology confirmed that the higher dose also induced hemorrhagic hepatic signs that were corroborated by increased plasma levels of hepatic markers like AST. Thus, efficacy analysis was further explored only using doses ≤ 20mg/kg/day, never exceeding cumulative doses of 50 mg/kg. Bz was included as a reference drug given at its effective dose of 100 mg/kg/day [23]. The ip administration of 18SAB075, in all assayed therapeutic schemes (two and five days of treatment) using non-toxic doses failed to demonstrate the activity of this diphenylacetylene AIA *in vivo* while Bz not only suppressed the parasitemia but also induced 100% protection against mortality due to the experimental infection as described [26]. Previous data using bis-AIAs like the 2,5-bis[2-(2-propoxy)-4-(2-pyridylimino)aminophenyl]furan - DB 766 [15] and a related analog, DB 1831 [16] showed their high activity *in vivo* in the acute experimental mouse models of *T. cruzi*, exhibiting similar trypanocidal effect as Bz. In fact, under the use of a same highly stringent experimental *in vivo* model as presently performed for 18SAB075, both previous studies reported that the presence of pyrimidine and pyridine units of bis-AIAs seemed to

be advantageous for *T. cruzi* activity [14], supporting the continuity of pre-clinical studies of novel related molecules aiming to find new alternatives for treating Chagas disease.

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REFERENCES

[1] **De Souza W.** 2009. Structural organization of *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz **104**:89-100.

[2] **Dias JC.** 2007. Southern Cone Initiative for the elimination of domestic populations of Triatoma Infestans and the interruption of transfusion Chagas disease: historical aspects, present situation, and perspectives. Mem. Inst. Oswaldo Cruz **102**:11-18.

[3] **Gascón J, Albajar P, Cañas E, Flores M, Gómez i Prat J, Herrera RN, Lafuente CA, Luciardi HL, Moncayo A, Molina L, Muñoz J, Puente S, Sanz G, Treviño B, Sergio-Salles X.** 2007. Diagnosis, management and treatment of chronic Chagas' heart disease in areas where *Trypanosoma cruzi* infection is not endemic. Rev. Esp. Cardiol. **60**:285-293.

[4] **Rodriguez-Morales AJ, Benitez JA, Tellez I, Franco-Paredes C.** 2008. Chagas disease screening among Latin American immigrants in non-endemic settings. Travel Med. Infect. Dis. **6**:162–163.

[5] **Guerri-Guttenberg RA, Grana DR, Ambrosio G, Milei J.** 2008. Chagas cardiomyopathy: Europe is not spared! Eur. Heart J. **29**:2587-2591.

[6] **Milei J, Guerri-Guttenberg RA, Grana DR, Storino R.** 2009. Prognostic impact of Chagas disease in the United States. Am. Heart J. **157**:22-29.

[7] **Nóbrega AA, Garcia MH, Tatto E, Obara MT, Costa E, Sobel J, Araujo WN.** 2009. Oral transmission of Chagas disease by consumption of açaí palm fruit, Brazil. Emerg Infect. Dis. **15**:653-655.

[8] **Teixeira AR, Nitz N, Guimaro MC, Gomes C, Santos-Buch CA.** 2006. Chagas disease. Postgrad. Med J. **82**:788-798.

[9] **Rocha MO, Teixeira MM, Ribeiro AL.** 2007. An update on the management of Chagas cardiomyopathy. Expert Rev. Anti Infect. Ther. **5**:727-743.

[10] **Soeiro MNC, de Castro SL, de Souza EM, Batista DGJ, da Silva CF, Boykin DW**. 2008. Diamidines activity upon trypanosomes: The state of the art. Curr. Mol. Pharmacol. **1**:151-161.

[11] **Filardi LS, Brener Z.** 1987. Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. Trans. R. Soc. Trop. Med. Hyg. **81**:755-759.

[12] **Coura JR, de Castro SL.** 2002. A critical review on Chagas disease chemotherapy. Mem Inst Oswaldo Cruz 97: 3-24.

[13] **Soeiro MNC, de Castro SL.** 2009. *Trypanosoma cruzi* targets for new chemotherapeutic approaches. Expert Opin. Ther. Target **13**:105-121.

[14] **Soeiro MNC, Werbovetz K, Boykin OW, Wifson WO, Wang MZ, Hemphifl A.** 2013. Novel amidines ond analogues as promising agents against intracellular parosites: a systematic review. Parasitology **140**:929-951.

[15] **Batista DGJ, Batista MM, Oliveira GM, Borges P, Lannes-Vieira J, Britto CC, Junqueira A, Lima MM, Romanha AJ, Sales Junior PA, Stephens CE, Boykin DB, Soeiro MNC.** 2010. Arylimidamide DB766: A potential chemotherapeutic candidate for Chagas disease treatment. Antimicr. Agents Chemother. **54**:2940-2952.

[16] **da Silva CF, Batista DGJ, Oliveira GM, de Souza EM, Hammer ER, da Silva PB, Daliry A, Araujo JS, Britto C, Rodrigues AC, Liu Z, Farahat AA, Kumar A, Boykin DW, Soeiro MNC.** 2012. *In vitro* and *in vivo* investigation of the efficacy of arylimidamide DB1831 and its mesylated salt form-DB1965-against *Trypanosoma cruzi* infection. PLoS One **7**:e30356.

[17] **Leclerc M, Morin J-F**. Conjugated polycarbazole derivatives and process for the preparation thereof. US20020103332A1, 2002.

[18] **Stephens CE, Tanious F, Kim S, Wilson WD, Schell WA, Perfect JR, Franzblau SG, Boykin DW**. 2001. Diguanidino and "reversed" diamidino 2,5-diarylfurans as antimicrobial agents. ***J. Med. Chem.*** **44:**1741-1748.

[19] **Chandra R, Oya S, Kung M-P, Hou C, Jin L-W, Kung HF**. 2007. New diphenylacetylenes as probes for positron emission tomographic imaging of amyloid plaques. *J Med Chem* **50:**2415-23.

[20] **Nishimura D, Oshikiri T, Takashima Y, Hashidzume A, Yamaguchi H, Harada A**. 2008. Relative rotational motion between α-cyclodextrin derivatives and a stiff axle molecule. J. Org. Chem. **73:**2496-2502.

[21] **Lange JHM, van Stuivenberg HH, Coolen HKAC, Adolfs TJP, McCreary AC, Keizer HG, Wals HC, Veerman W, Borst AJM, de Looff W, Verveer PC, Kruse CG**. 2005. Bioisosteric replacements of the pyrazole moiety of rimonabant: Synthesis, biological properties, and molecular modeling investigations of thiazoles, triazoles, and imidazoles as potent and selective cb1 cannabinoid receptor antagonists. J. Med. Chem. **48:**1823-1838.

329 [22] **Meirelles MNL, Araujo-Jorge TC, Miranda CF, De Souza W, Barbosa HS.** 1986.
330 Interaction of *Trypanosoma cruzi* with heart muscle cells: ultrastructural and cytochemical
331 analysis of endocytic vacuole formation and effect upon myogenesis *in vit*ro. Eur. J. Cell
332 Biol. **41**:198-206.
333 [23] **Romanha AJ, Castro SL, Soeiro Mde N, Lannes-Vieira J, Ribeiro I, Talvani A, Bourdin**
334 **B, Blum B, Olivieri B, Zani C, Spadafora C, Chiari E, Chatelain E, Chaves G, Calzada**
335 **JE, Bustamante JM, Freitas-Junior LH, Romero LI, Bahia MT, Lotrowska M, Soares**
336 **M, Andrade SG, Armstrong T, Degrave W, Andrade ZA.** 2010. *In vitro* and *in vivo*
337 experimental models for drug screening and development for Chagas disease. Mem. Inst.
338 Oswaldo Cruz **105**:233-238.
339 [24] **da Silva CF, Batista MM, Mota RA, de Souza EM, Stephens CE, Som P, Boykin DW,**
340 **Soeiro MNC.** 2007. Activity of "reversed" diamidines against *Trypanosoma cruzi in vitro*.
341 Biochem. Pharmacol. **73**:1939-1946.
342 [25] **de Souza EM, Menna-Barreto R, Araújo-Jorge TC, Kumar A, Hu Q, Boykin DW, Soeiro**
343 **MNC**. 2006. Antiparasitic activity of aromatic diamidines is related to apoptosis-like death
344 in *Trypanosoma cruzi*. Parasitology **133**:75-79.
345 [26] **da Silva CF, Batista DJ, Siciliano JA, Batista MM, Lionel J, de Souza EM, Hammer ER,**
346 **da Silva PB, De Mieri M, Adams M, Zimmermann S, Hamburger M, Brun R, Schühly**
347 **W, Soeiro MNC.** 2013. Effects of psilostachyin A and cynaropicrin against Trypanosama
348 cruzi in vitro and in vivo. Antimicrob. Agents Chemother. **57**:5307-5314.
349 [27] **Soeiro MNC, de Castro SL.** 2011. Screening of potential anti-*Trypanosoma cruzi* candidates:
350 *In vitro* and *in vivo* studies. Open Med. Chem. J **5**:21-30.
351 [28] **Soeiro MN, de Souza EM, da Silva CF, Batista DG, Batista MM, Pavão BP, Araújo JS,**
352 **Aiub CA, da Silva PB, Lionel J, Britto C, Kim K, Sulikowski G, Hargrove TY,**
353 **Waterman MR, Lepesheva GI.** 2013. *In vitro* and *in vivo* studies of the antiparasitic

activity of sterol 14 ∝-demethylase (CYP51) inhibitor VNI against drug-resistant strains of *Trypanosoma cruzi*. Antimicrob. Agents Chemother. **57**:4151-4163.

LEGENDS

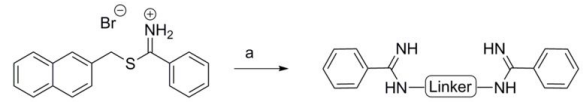
Figure 1: Synthesis of 16DAP002 and 16SAB079. Reagents and conditions: (a) appropriate diamine, ethanol (plus acetonitrile for 16DAP022), 0-25 °C, overnight (66-71%).

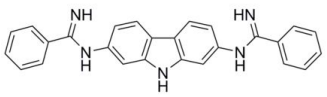
Figure 2: Synthesis of 18SAB075. Reagents and conditions: (a) 2-cyanopyridine, lithium bis(trimethylsily)amide, tetrahydrofuran, 25 °C, 2 days, (16%).

Figure 3: Synthesis of 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009. Reagents and conditions: (a) sodium methoxide, methanol, 25 °C, 1 week; (b) appropriate α,ω-diamine (or spermidine for 23SMB054), 4 M HCl in dioxane, methanol, 25 °C (5-92%).

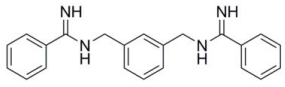
Figure 4: *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with 10^4 bloodstream trypomastigotes (Y strain). The activity of 5, 10 and 20 mg/kg/day 18SAB075 (ip) and of benznidazole (100 mg/kg/day by gavage) given at 5 and 8 dpi was evaluated through the (A) parasitemia levels and (B) cumulative mortality.

Figure 5: *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with 10^4 bloodstream trypomastigotes (Y strain). The activity of 0.5, 1 and 5 mg/kg/day 18SAB075 (ip) and of benznidazole (100 mg/kg/day by gavage) given for 5 consecutive days (5-9 dpi) was evaluated through the (A) parasitemia levels and (B) cumulative mortality.





16DAP002



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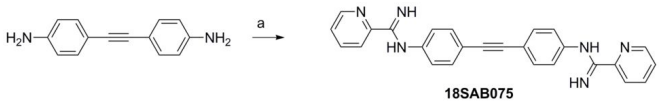


18SAB075

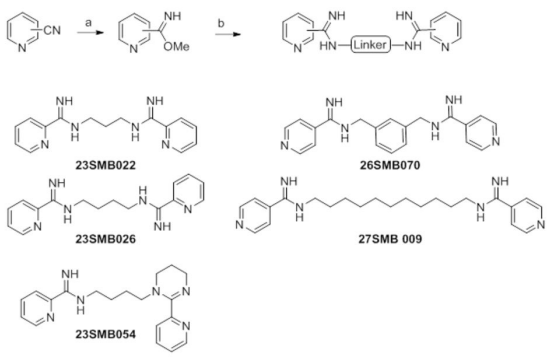
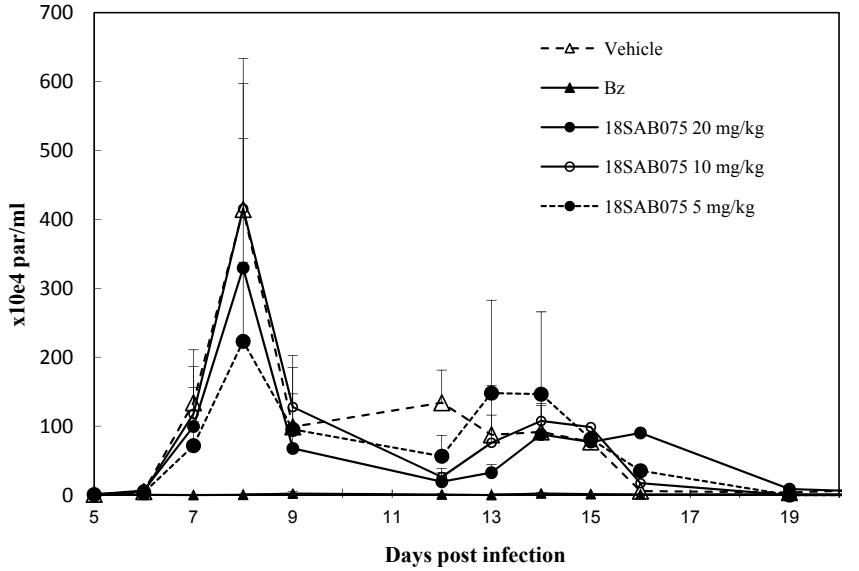
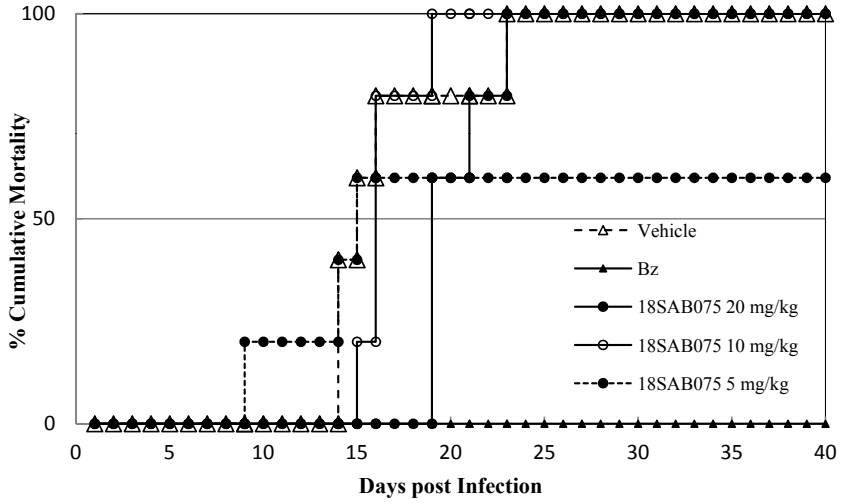
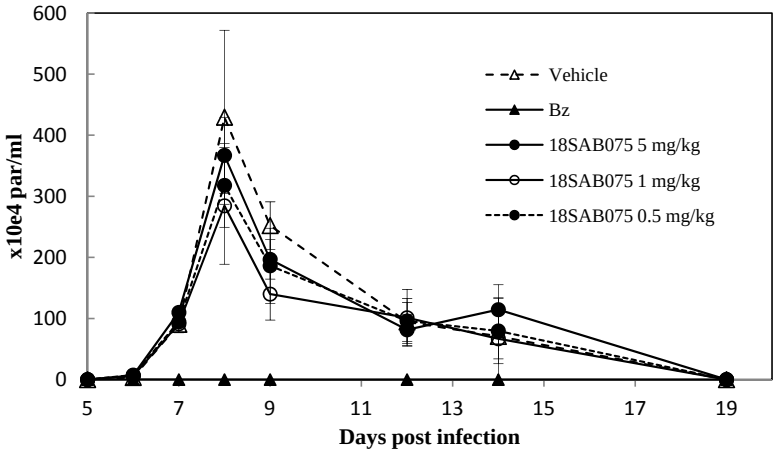


Figure 3







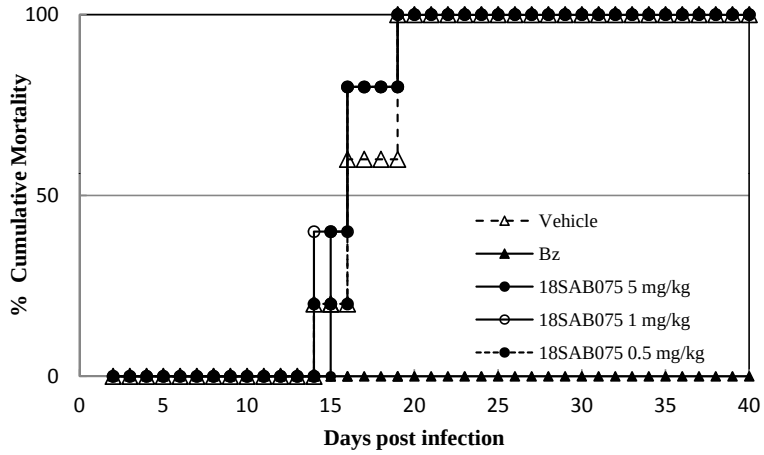


TABLE I Trypanocidal activity (EC_{50}) of the studied arylimidamides against bloodstream trypomastigotes of *T. cruzi* (Y strain) and their selectivity index (SI) related to cardiomyocytes (EC_{50})

Compounds	**EC_{50} (µM)**			**SI[a]**
	Trypomastigotes		Cardiomyocytes	
	2 h	24 h	24 h	24 h
18SAB075	11 ± 1	0.3 ± 0	>32	>106
16DAP002	14 ± 3	3 ± 1	>32	>11
27SMB009	>100	85 ± 21	>32	>0.4
23SMB054	>100	>100	>32	-
23SMB022	>100	>100	>32	-
23SMB026	>100	>100	>32	-
26SMB070	>100	>100	>32	-
16SAB079	>100	>100	>32	-
Benzinidazole	>100	13 ± 2[b]	1000	77

[a]Treatment for 24 h at 37°C; Selectivity index (SI) = EC_{50} cardiomyocytes/EC_{50} parasite
[b] Silva et al 2011

TABLE II Activity of arylimidamides (at 1 µg/mL) against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) after treatment for 96 h at 37°C

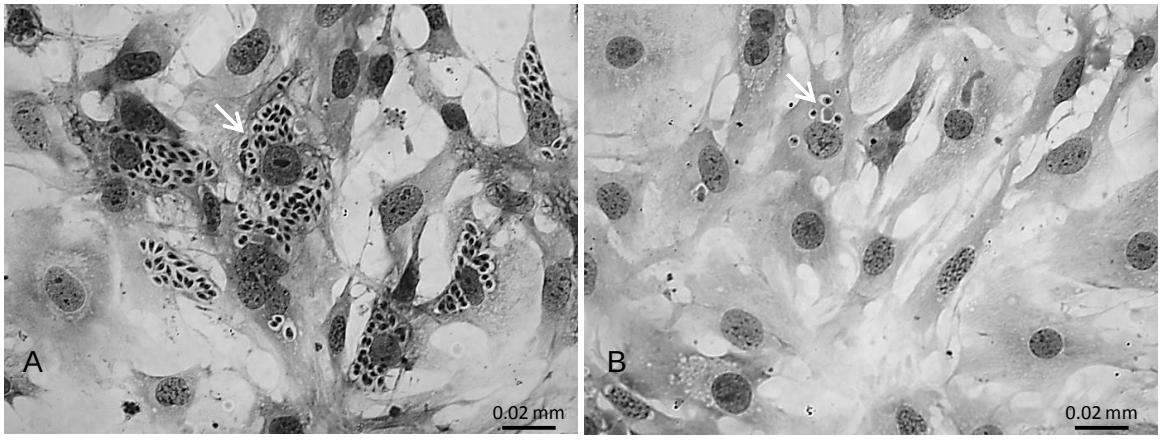
Compounds	% Inhibition of the host cell infection
16DAP002	58 ± 31
18SAB075	50± 7
26SMB070	15 ± 8
16SAB079	4 ± 5
23SMB054	3 ± 4
23SMB022	1 ± 1
27SMB009	0 ± 0
23SMB026	0 ± 0
Benznidazole	82 ± 3

TABLE III *In vitro* effect (EC_{50} values) of arylimidamides against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) after treatment for 96 h at 37°C

Compounds	**EC_{50} (µM)**
16DAP002	4.1 ± 1.8
18SAB075	1.5 ± 0.2
Benznidazole	2.6 ± 0.8

TABLE IV Activity (EC_{50} values) and selective index (SI) of 18SAB075 against intracellular forms of *T. cruzi* (Y strain) after treatment for 48 h at 37°C

18SAB075	**EC_{50} (µM)**	**SI**
% Host cell Infection	0.8 ± 0.1	40
Mean number of parasite/per infected host cell	6.0 ± 2.0	5
Infection Index*	0.9 ± 0.1	36



- *Host cell infection x number of parasite/infected host cell as reported in Batista et al.. 2010
- A: *T.cruzi*-infected cardiac cells incubated with vehycle
- B: *T.cruzi*-infected cardiac cells incubated with 32 µM.
- Arrow: Intracellular parasites