In vitro and *In vivo* Trypanosomicidal Action of Novel Arylimidamides Against *Trypanosoma cruzi*

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Summary

Arylimidamides (AIA) showed considerable biological activity against intracellular pathogens, including *Trypanosoma cruzi*. Presently, the analysis of twelve novel bis-AIAs and two mono-AIAs was performed against different strains of *T. cruzi in vitro* and *in vivo*. The most active was *m*-terphenyl bis-AIA 35DAP073, with EC_{50} value of 0.5 μM on trypomastigotes (Y strain), being 26-fold more effective than benznidazole (Bz, 13 μM). It was also active against Colombiana strain, (EC_{50} = 3.8 μM). The analysis against intracellular forms (Tulahuen strain) showed that this bis-AIA (EC_{50} = 0.04 μM) was about 100-fold more active than Bz (2 μM). Trypanocidal effect was dissociated to their ability to trigger intracellular lipid bodies within host cells detected by oil red labeling. Both active (35DAP073) and inactive (26SMB060) compounds displayed similar activation profiles. Due to high selectivity index, two AIAs (35DAP073 and 35DAP081) were moved to *in vivo*, but acute toxicity assays excluded the following tests using 35DAP081. Findings with 35DAP073 using Y strain revealed that two-days of therapy induced a dose-dependent action, leading to 96-46 % reduction of parasitemia. However, ten daily doses using Colombiana strain resulted in animal toxicity, impairing longer periods of treatment. Then the association of 0.5 mg/kg/day 35DAP073 with 100 mg/kg/day Bz was assayed for 10 consecutive days. The association resulted in a suppression of the parasitemia, elimination of neurological toxic effects and 100% survival of animal. Quantitative polymerase chain reaction showed considerable reduction of parasite load (60%) as compared to Bz or amidine alone. Our results support further investigations of this class aiming development of novel alternatives for Chagas disease therapy.

Keywords: Arylimidamides, experimental chemotherapy, Chagas disease, lipid bodies, combination therapy.

Introduction

Chagas disease (CD) is caused by the obligate intracellular protozoan *Trypanosoma cruzi.* More than a hundred years after its discovery (1), CD is still an important public health problem, affecting about 6 to 7 million people are estimated to be infected worldwide, mostly in Latin America (2). Of these infected individuals, 30-40% develops cardiomyopathy and / or digestive syndromes (3). For over four decades, Nifurtimox (3-methyl-4-(5'-nitrofurfurylideneamine tetrahydro-4H-1,4-tiazine-1 ,1-dioxide) and benznidazole (N-benzyl-2-nitroimidazole acetamide) introduced empirically in therapy to CD still remain the sole current options (4). Both have several limitations, including a variety of adverse effects and limited efficacy in later chronic phase (5,6), which strengthen the need for new trypanocidal compounds that overcome these limitations.

Classic aromatic diamidines (AD) are DNA minor groove binders with recognized antimicrobial and antitumor broad spectrum activity (7). ADs such as pentamidine, diminazene and propamidine have been used for decades in medicine and veterinary therapy, but despite their excellent anti-parasitic effects have relevant drawbacks that include low bioavailability and side effects. To overcome these limitations, new analogues have been synthesized and tested *in vivo* and *in vitro* (7). Several AD and related compounds have been screened against *T. cruzi* showing promising results at least in part due to their capacity

to alter the KDNA molecule (8, 9). Arylimidamides (AIAs, formerly called "reversed" amidines because of the reversed position of nitrogen and carbon atoms compared to classic aromatic amidines) are the most effective amidine analogues tested against *T. cruzi in vitro* and *in vivo* (9,10,11,12).

Lipid bodies (LB) consist of a nucleus of cholesteryl esters and triglycerides surrounded by a single monolayer of phospholipids. LB are considered not only lipid storage compartments but also intracellular sites for many biological events like cellular signaling and activation, regulation of lipid metabolism, membrane trafficking and regulation of inflammatory mediators (13,14). Recent studies proposed a dynamic role of LB in the control of pathogens infection due to their localization inside the parasitophorous vacuoles lodging intracellular parasites like *T.cruzi* (15).

Our present study focused on the analysis of twelve novel bis-AIAs and two mono-AIAs against bloodstream trypomastigotes and intracellular forms of *T. cruzi* (Tulahuen, Y and Colombiana strains) performed *in vitro*. The most potent and most selective compound, 35DAP073 underwent further screening in mouse experimental acute models of parasite infection (Y and Colombiana) alone or combined with the reference drug (Bz) aiming to contribute for the search of novel alternative therapeutic protocols for CD.

Materials and methods

Synthesis of the arylimidamides

The syntheses of the four *m*-terphenyl bis-arylimidamides 35DAP069, 35DAP073, 35DAP077, and 35DAP081, and the two mono-arylimidamides 35DAP092 and 38DAP096 (Figure 1) have been described previously (16),

wherein the final step was the reaction of the appropriate terphenyl amine or diamine with benzonitrile or 2-cyanopyridine using sodium bis(trimethylsilyl)amide in tetrayhydrofuran. Similar reactions involving 1,4-diphenylenediamine and 2,5- or 2,6-diaminopyridine were employed for the syntheses of 19SAB003, 19SAB005, 19SAB007, 28SMB008 (17) (Figure 2). Compounds 23SMB046, 23SMB050, 26SMB060, and 27SMB005 (Figure 3) were prepared by previously described methodology from the methyl imidate derivatives of 2- or 4-cyanopyridine and the appropriate α,ω-diaminoalkane (18). All fourteen compounds were isolated as their hydrochloride salts. Experimental details and physical data for the compounds shown in Figures 2 and 3 are given in the supplemental material (S1).

Stock Solutions of the tested compounds

The studied molecules were prepared in dimethyl sulfoxide (DMSO) - (Sigma Aldrich) - with the final concentration (dilution using RPMI) of the solvent never exceeding 0.6% and 10% for in vitro and in vivo analysis, respectively, which do not exert cellular toxicity upon on parasites and mammalian host cells (data not shown). Benznidazole (Bz) was purchased from Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil. Bz was dissolved in distilled and sterile water supplemented with 3% tween 80, which does not cause any detectable effect on mice (19).

Mammalian cell cultures

Primary cultures of embryonic cardiac cells (CC) were obtained from Swiss mice as previously reported (20). After purification, the CC 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 as reported (18). The cardiac cultures were then sustained at 37° C in Dulbecco`s modified medium (DMEM - without phenol red - from Sigma Aldrich) supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM $CaCl_2$, 1 mM *L*-glutamine and 2% chicken embryo extract. Additionally, 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 and 2 mM glutamine (RPMI), as reported (21).

Cytotoxicity in vitro tests

CC and L929 cell cultures were incubated at 37°C for different periods of time (24-96 hours) with increasing concentrations of each compound (up to 96 µM) diluted in DMEM (without phenol red). Next, mammalian cells morphology and spontaneous contractibility (CC) were evaluated by light microscopy, and the cellular viability determined by a colorimetric assay using 10 µL AlamarBlue (Invitrogen) added to each well. After incubation for 24 h, the absorbance was determined (at 570 and 600 nm) and the results were determined following the manufacturer's instructions. Then, the values of LC_{50}, which corresponds to the concentration that reduces in 50 % the cellular viability calculated as reported (21).

Parasites

Bloodstream trypomastigote (BT) forms of Y and Colombiana strains of *T. cruzi* were obtained from the blood of infected male Swiss mice at the peak of parasitemia (12). Immediately after the purification step, the parasites were

ressuspended 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, as reported previously (18). The effect against intracellular forms was investigated through the use of L929 cell lineages infected with tissue culture-derived trypomastigotes (Tulahuen strain expressing the E. coli β-galactosidase gene), using a 10:1 parasite: host cell ratio. The incubation with the tested compounds was performed for 96 h, following previously established protocols (21). Alternatively, CC were infected (ratio 10:1) with bloodstream trypomastigotes (Y strain). After 24 h of interaction, the infected-cultures were rinsed and then exposed for 48 h with nontoxic concentrations of the amidines previously screened on mammalian host cells.

Trypanocidal analysis

BT forms of the Y and Colombiana strains (5 X 10^6 per mL) were incubated for up to 24 h at 37°C in RPMI in the presence of serial dilutions of the compounds (0 to 32 µM), the parasites incubated with culture medium alone were used as control. 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) calculated. Also, the EC_{90} (compound concentration that reduces 90 % of the number of parasites) was further calculated in the intracellular assays using the Y strain (18). For the assay on intracellular forms, cardiac cell cultures and L929 cell lines were used as hosts for the infection using the Y and Tulahuen strains, respectively. Briefly, Tulahuen-infected-L929 cultures were exposed to 10 µM

(corresponding the EC_{90} value of Bz, as reported by 18) of each compound diluted in RPMI and the compounds that presented ≥ 50% of reduction on the parasite infection index were further screened under increasing concentrations aiming to determine the EC_{50} values (18). After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500 μM) - (Sigma Aldrich) 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 *T. cruzi*-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 (21). Triplicate samples were run in the same plate and at least two assays performed in each analysis. For the analysis of the effect against intracellular amastigotes from Y strain, after 24 h of parasite-host cell interaction, the infected CC cultures were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CC were maintained at 37°C in an atmosphere of 5% CO_2 and air and the medium replaced every 24 h. Then, the samples were fixed and stained with Giemsa solution - (Sigma Aldrich) and the number of infected host cell and the number of parasite per infected host cell determined through light microscopy analysis (18). 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 (percentage of infected cells multiplied by the average number of intracellular amastigotes per infected host cell) (12). At least two assays were performed in duplicates in each analysis.

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Lipid Bodies labeling

To determine the biogenesis of lipid bodies (LB) in cardiac cell cultures exposed to amidines, untreated CC were incubated for 2-48 h with the corresponding EC_{50} values of the studied compounds (previously determined against intracellular forms from Y strain) and then fixed for 10 min with 3.7% formaldehyde in Ca^{2+}-Mg^{2+}-free HBSS (pH 7.4). Next, the samples were rinsed twice using distilled water, incubated for 5 min in absolute propylene glycol and stained for 10 min in 0.5% oil red O (Sigma-Aldrich). The cultures were incubated for 3 min with 85% propylene glycol, and finally rinsed twice using distilled water. The samples were incubated or not with 1 µg/mL 4,6-diamidino-2-phenylindole (DAPI) - (Sigma Aldrich) for DNA staining (host cells nuclei), rinsed with saline buffer, dried and mounted using aqueous mounting medium 2.5% 1.4-diazabicyclo-(2.2.2)octane (DABCO) and the fluorescence analyzed with a x63 oil objective in a Zeiss photomicroscope (Axiocam) equipped with epifluorescence (Zeiss Inc, Thornwood, New York), using a filter set for UV excited probes. Images were captured using the software AnalySIS OPTI. As positive control, 2 µM of Oleic acid (Sigma-Aldrich) was used to trigger lipid bodies' biogenesis (22).

***In vivo* acute toxicity**

In order to determine the no-observed-adverse-effect level (NOAEL), increasing doses of the tested compounds (up to 200 mg/kg of body weight) were injected by intraperitoneal (ip) route individually in Swiss Webster female mice (20 to 23 g, n = 2 per assay, two assays). Treated animals were inspected

for toxic and sub-toxic symptoms according to the Organization for Economic Cooperation and Development (OECD) guidelines. Forty-eight hours after compound injection, the NOAEL values were determined as reported previously (23).

Biochemical analysis

Forty-eight hours after compound administration, mouse blood was collected and immediately submitted to biochemical analysis for determination of plasma tissue markers including Urea (BUN), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Creatine Kinase (CK) that was performed at animal facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil, CECAL/Fiocruz platform) using Vitros 250 (Ortho Clinical-Johnson & Johnson), as reported previously (23).

***In vivo* infection**

Swiss Webster male mice (18-20 g) obtained from the animal facilities of CECAL were housed at a maximum of 6 per cage and kept in a specific pathogen free (SPF) room at 20–24°C under a 12/12 h light/dark cycle and provided with sterilized water and chow ad libitum. The animals were allowed to acclimate for 7 days before starting the experiments. Infection was performed by intraperitoneal (ip) injection of 10^4 and 5 x 10^3 bloodstream trypomastigotes (Y and Colombiana strain, respectively). Age-matched non-infected mice were maintained under identical conditions (12)

Treatment schemes

AIAs were first dissolved in DMSO and then freshly diluted with sterile distilled water. The stock solution of benznidazole (N-benzyl-2-nitroimidazol acetamide) was prepared in sterile distilled water with 3% Tween 80 (Sigma Aldrich). The animals were divided into the following groups (5 animals per group): uninfected (non-infected and non-treated); untreated (infected but treated only with vehicle); and treated (infected and treated with the compounds). The therapy (once a day) was performed through different schemes (see Supplemental Material - S2): In the first set of assays, *T. cruzi*-infected mice (Y strain) were treated for only two days (at 5 dpi and at 8 dpi that corresponds to the parasitemia onset and peak in this experimental model), using 5-20 mg/kg/day AIA (ip) and with 100 mg/kg/day Bz (po). In the second set of experiments, mice were infected with Colombiana strain and at the 10 dpi (parasitemia onset in this animal model) were treated for ten consecutive days with 35DAP073 alone (5 mg/kg/day) or in association with Bz (0.5 mg/kg/day AIA plus 100 mg/kg/day of Bz). In all assays, only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates

Parasitemia was individually checked by direct microscopic counting of parasites in 5 µL of blood, and mortality rates checked daily until 30 days post treatment and expressed as percentage of cumulative mortality (% CM) as described before (18).

Blood parasite load by qPCR

For quantitative real time polymerase chain reaction (qPCR), 500 µL blood were diluted in 1:2 volume of guanidine solution (Sigma Aldrich) (Guanidine-HCl 6M/EDTA 0.2M), and heated for 90 sec. in boiling water. Guanidine-EDTA Blood Samples (GEB) were processed using the QIAamp DNA mini kit (QIAGEN) (24). Quantitative Real Time PCR Multiplex assays using TaqMan probes were performed targeting the *T. cruzi* satellite nuclear DNA and the internal amplification control - IAC (pZErO-2 plasmid containing an insert from the *A. thaliana* aquaporin gene), as described by (25). The standard curves for absolute quantification were constructed with serial dilutions of total DNA, obtained from a negative GEB sample spiked with 10^5 parasite equivalents per milliliter of blood (par. eq./mL), ranging from 10^5 to 0.5 par. eq./mL.

Ethics

All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

Statistical Analysis

The data represent means ± standard deviations from 2 experiments run in duplicate and statistical analysis performed by the ANOVA test with the level of significance set at $p \leq 0.05$ (23).

Results

The phenotypic analysis against BT (Y strain, DTU II) showed that all studied compounds were active against the parasite. 35DAP073, 35DAP081, 35DAP077 and 35DAP092 presented a time-dependent response, exhibiting EC_{50} values ranging from 0.7-28 µM and 0.5-10 µM after 2 and 24 h of incubation, respectively, while Bz resulted in EC_{50} value of 13 µM after 24 h (Table 1). Among these four compounds, the most active were 35DAP073 and 35DAP081 and both also displayed the highest selectivity indexes (64 and 53, respectively). The statistical analysis revealed that 35DAP073, 35DAP081 and 35DAP092 presented superior efficacy than Bz (p<0.05). When BT forms of Colombiana strain (DTU I) were assayed, both amidines showed trypanocidal effect showing EC_{50} values lower than 4µM after 24 h of exposure (Table 2). 35DAP073 and 35DAP081 were very active against intracellular forms from both Tulahuen (Table 3) and Y (Table 4) strains of *T. cruzi*, representatives of DTU VI and II, respectively, being more potent than Bz ($p \leq 0.05$). The m-terphenyl bis-AIA 35DAP073 showed the higher activity expressed by the lower EC_{90} values (87nM) after 48 h of incubation, being about 126-fold more effective than Bz, besides exhibiting the highest SI (2000) (Table 4).

As 35DAP073 was more effective against intracellular forms as compared to BT (Y strain, Tables 1 and 4), and biogenesis of lipid bodies (LB) may be induced by different stimuli (26), LB induction was evaluated during the incubation of uninfected CC with this amidine with a head-to-head comparison with another non active molecule against *T. cruzi in vitro* (26SMB060). After incubation for different time periods (2-48 h) our data revealed that both amidines were able to induce similar levels of LB biogenesis as a very fast phenomenon since only 2 h post-treatment resulted in strong LB formation as

compared to untreated group, and with similar profile as the positive control (oleic acid) (Figure 4). A great number of LB was also observed after 24 h of amidine exposure; which levels were maintained up to 48 h despite the oleic acid displayed a progressive and continuous formation of LB (increase in the number and volume) (Figure 4).

Next, due to the high activity and selectivity of 35DAP073 and 35DAP081, Swiss mice were used to determine their NOAEL values. 35DAP073 proved to be less toxic at all doses tested, presenting NOAEL 25 mg/kg while 35DAP081 showed a value < 12.5 mg/kg (Table 5). The plasma biochemical analysis ($p \leq 0.1$) after 48 h of compound administration did not show any statistical difference among all tested groups when ALT, AST, CK and BUN measurements were evaluated (Table 6). Due to these preliminary acute toxicity studies, only 35DAP073 was move to *in vivo* acute models of *T. cruzi* infection using no-toxic doses (up to 25 mg/kg/day). In parallel, a control group was orally treated with Bz (100 mg/kg/day) (Figure 5).

Our data demonstrated a dose-dependent effect, leading to 96-46 % reduction of parasitemia with concentrations of up to 20 mg/kg/day (Figure 5). The animal survival rates achieved 100 % when mice were treated with 5 and 10 mg/kg/day of 35DAP073, similarly as 100 mg/kg/day Bz (Figure 5). However, as the tested compound did not result in complete suppression of the blood parasite load, next, a longer period of therapy (10 days using 5 mg/kg/day) was investigated on mice infected with $5x10^3$ bloodstream trypomastigotes of the Colombiana strain. Also, a combination with Bz (10 days using 0.5 mg/kg/day 35DAP073 and 100 mg/kg/day Bz) was assayed in parallel using the same experimental model, starting the therapy at the parasitemia onset. Our findings

showed that 5 mg/kg/day of 35DAP073 completely suppressed the parasitemia and gave 100% animal survival, as did Bz (Figure 6). However, at the end of the therapy period (last day of 35DAP073 administration), mice presented some undesirable neurological toxic disorders (tremors, shaking, ataxia) that were reverted after 48 h post-treatment (data not shown). The combination of 0.5 mg/kg/day of this amidine with Bz resulted in parasitemia suppression, 100% animal survival and no toxic events (Figure 6). The analysis by qPCR showed that although no statistical significance was found among the tested groups (p>0.05), the monotherapy with Bz and 35DAP073 resulted in mean values of 204 and 265 par.Eq/mL respectively, while the association of Bz with the AIA induced a higher reduction (60 %) of the blood parasite load (82 par.Eq/mL) (Figure 7).

Discussion

The current therapy for Chagas disease still remains unsatisfactory due to various side effects and limited efficacy (27). Amidines have been used for decades in human and veterinary medicine as anti-parasitic agents but show important limitations as parental administration and undesirable side effects (7). In order to overcome these limitations, the synthesis and phenotypic analysis of novel amidines have been conducted *in vivo* and *in vitro* (28, 12). Among them, arylimidamides (AIA) showed considerable biological activity against several intracellular pathogens, including *T.cruzi* (29), being the bis-AIAS the most effective among the group (19).

A previous study reported the synthesis of dicationic m-terphenyl derivatives and their biological effect against *T. brucei rhodesiense,*

374 *Plasmodium falciparum, Leishmania amazonensis* and *T. cruzi* (16). Among
375 them, bis-pyridylimidamides were the most potent against the intracellular
376 amastigote form of the *T. cruzi* (Tulahuen strain, DTU VI). Presently, we further
377 explored the phenotypic activity *in vitro* and *in vivo* of fourteen bis-and mono-
378 arylimidamides against bloodstream trypomastigotes (BT) and intracellular
379 forms of *T. cruzi,* extending the analysis to other parasite strain (Y and
380 Colombiana strains, belonging to DTUs II and I, respectively).

381 Structural variations of the test compounds in this study included those in
382 AIA moieties as well as the central cores of the molecules. Maximum potencies
383 were observed in 35DAP073 and 35DAP081, which each have two 2-
384 pyridylimidamide groups attached to a *m*-terphenyl nucleus, with at least one of
385 the two AIA groups *meta* to the central ring. These two compounds were more
386 potent than the corresponding bis-phenylimidamides 35DAP069 and
387 35DAP077. The difference in potencies between 35DAP073 and 35DAP069
388 was greater than 60-fold, but less pronounced between 35DAP081 and
389 35DAP077. The bis-pyridylimidamides 35DAP073 and 35DAP081 were also
390 more potent than the mono-pyridylimidamides 35DAP092 and 38DAP096, thus
391 demonstrating the importance of two AIA moieties. Compound 38DAP092, in
392 which the AIA group is *meta* to the central ring, was more potent than its
393 regioisomer 38DAP096 which bears a *para*-AIA moiety. None of the bis-AIAs
394 with lone aromatic rings or aliphatic chains between the two AIA functionalities
395 (Figures 2 and 3) showed detectable activity.

396 The most active molecule, the *m*-terphenyl bis-AIA 35DAP073, showed
397 after 24 h an EC_{50} value of 0.5 μM on trypomastigotes (Y strain), being more
398 effective than the reference drug, Bz (13 μM). This amidine was also active on

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highly naturally resistant strain (Colombiana), exhibiting EC_{50} = 3.8 µM. The analysis against intracellular forms (Tulahuen strain) showed that this bis-AIA (EC_{50} = 0.04 µM), was about 50-fold more active than Bz (2 µM). The higher effect against intracellular forms as compared to BT was dissociated to the ability of amidines to induce LB accumulation within mammalian host cells. Biogenesis of lipid bodies is a central event in several cellular homeostasis, as well as during intracellular pathogen infection (30, 31). LB are recognized as dynamic organelles composed of a triglyceride and cholesteryl ester core with a surrounding monolayer of phospholipid, cholesterol, and a variety of associated proteins with diverse functions in cell metabolism, signaling, and inflammation (14,32). Under inflammatory and infectious conditions, prostaglandins and others lipid mediators are mainly produced by LB (33). Also, recent findings showed that antimicrobial compounds may induce an accumulation of lipid droplets in the cytoplasm of yeasts (34) and protozoan (35). In *M. leprae* infected-cells, LBs show high ability to fuse forming giant LDs (36). Since amidines can modulate functional activities of mammalian host cells linked to the control of parasite proliferation and/or survival (37, 38) and LB act on cellular metabolism, inflammation and infectious conditions (14,32), presently we investigated whether the studied AIAs could interfere with LB biogenesis and parasitism control *in vitro*. Our data also demonstrate that 35DAP073 and 28SMB060 are able to induce LB biogenesis in cardiac cells in at all times tested (2-48 h), exhibiting a similar morphological profile. No correlation was found between trypanocidal effect of amidines and LB accumulation in CC but further studies are desirable to better understand the consequences of LB

levels induced by amidines in the parasite as well mammalian cells physiology since this modulation may influence the growth of intracellular parasites (36).

Next, due to the excellent selective indexes of 35DAP073 and 35DAP081, both were tested for acute toxicity analysis. The results showed values of 25 and 12.5 mg/kg NOAEL for 35DAP073 and 35DAP081. Then, *in vivo* efficacy studies were conducted with 35DAP073, using concentrations < 25 mg/kg. Our data demonstrated that the infection of mouse models using Y and Colombiana strains resulted in a dose-dependent action of 35DAP073, leading to 96-46 % reduction of parasitemia and 100 % of animal survival, with similar effect of Bz. Unfortunately, as the ten-daily dose (using 5mg/kg via ip) resulted in animal toxic aspects, the combined therapy of 35DAP073 with Bz was performed aiming to reduce the toxic aspects of the studied amidine. The monotherapy using only 0.5mg/kg/day was not approached as previous data with related amidines demonstrated a mild effect of parasitemia control using doses ≤ 5mg/kg/day (23). As also found by our group (28) using the combination of Bz with the amidine DB289, presently the association of 0.5 mg/kg 35DAP073 with Bz improved the control of the parasite proliferation providing a 9-fold enhancement of activity as compared to that of Bz alone. qPCR analysis showed that the combination therapy was the best protocol regimen exhibiting parasitemia suppression with no detectable animal toxicity. In this sense, our results support further experimental investigations of this compound class in association with licensed anti-parasitic drugs aiming to contribute for the identification of novel alternatives to treat parasitic neglected pathologies as Chagas disease.

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Legend of the Figures

Figure 1: Structures of mono- and bis-arylimidamide derivatives of *m*-terphenyl.

Figure 2: Synthesis of bis-arylimidamide derivatives of benzene and pyridine. Reagents and conditions: (a) sodium bis(trimethylsilyl)amide, terahdyrofuran, 25°C.

Figure 3: Synthesis of bis-arylamidamide deratives of *n*-alkanes. Reagents and conditions: (a) 4 M HCl in dioxane, methanol, 25 °C.

Figure 4: Differential interference contrast (DIC) microscopy analysis of oil red labeling of cardiac cells untreated (A-C) and submitted to oleic acid (D-F), 35DAP073 (G-I) and 26SMB060 (J-L) for 2 (A,D,G,J), 24 (B, E,H, K) and 48

(C,F, I, L) hours of exposure at 37°C. Detailed oil red labeling of cardiac cells by DIC (M, O, Q and S) and fluorescence microscopy using DAPI staining of mammalian cell nuclei (N, P, R and T) after 24 h of incubation with 35DAP073 (Q and R) and 35DAP060 (S and T) showing the accumulation of lipid bodies randomly distributed throughout the cytoplasm.

Figure 5: Effect of 35DAP073 (20-5 mg/kg/day) and of Benznidazole (100 mg/kg/day) administration at 5 and 8 day after infection (dpi) on mouse experimental model of *T. cruzi* infection (Y strain). (A) Parasitemia levels and (B) Percentage of cumulative mortality.

Figure 6: Effect of 10 daily administration of 35DAP073 (5 mg/kg/day), Benznidazole (100 mg/kg/day) and 35DAP073 + Bz (0.5 mg/kg/day and 100 mg/kg/day, respectively) on mouse experimental models of *T. cruzi* infection (Colombiana strain), starting the compound administration at the 10 dpi. (A) Parasitemia levels and (B) Percentage of cumulative mortality.

Figure 7: Blood qPCR analysis of the parasite equivalent per milliliter (Par. Eq. / mL) from uninfected and infected mice (using Colombiana strain) submitted to each different treatment regimen: Monotherapies (benznidazole 100 mg/kg/day and 35DAP073 5 mg/kg/day) and combined therapy (benznidazole (100 mg/kg/day) + 35DAP073 (0.5 mg/kg/day)). Each symbol represents an individual value and the bars represent respective mean values.

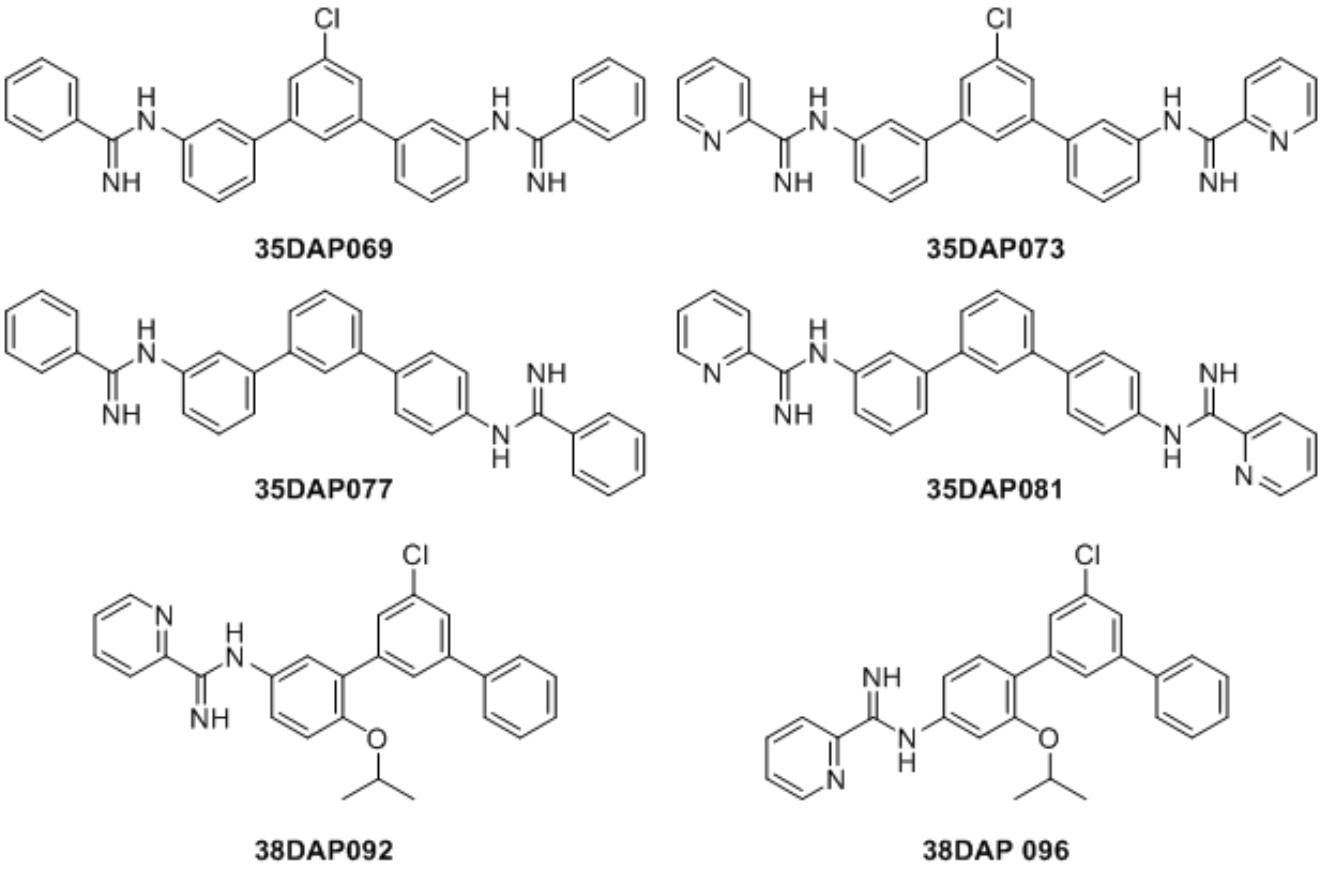
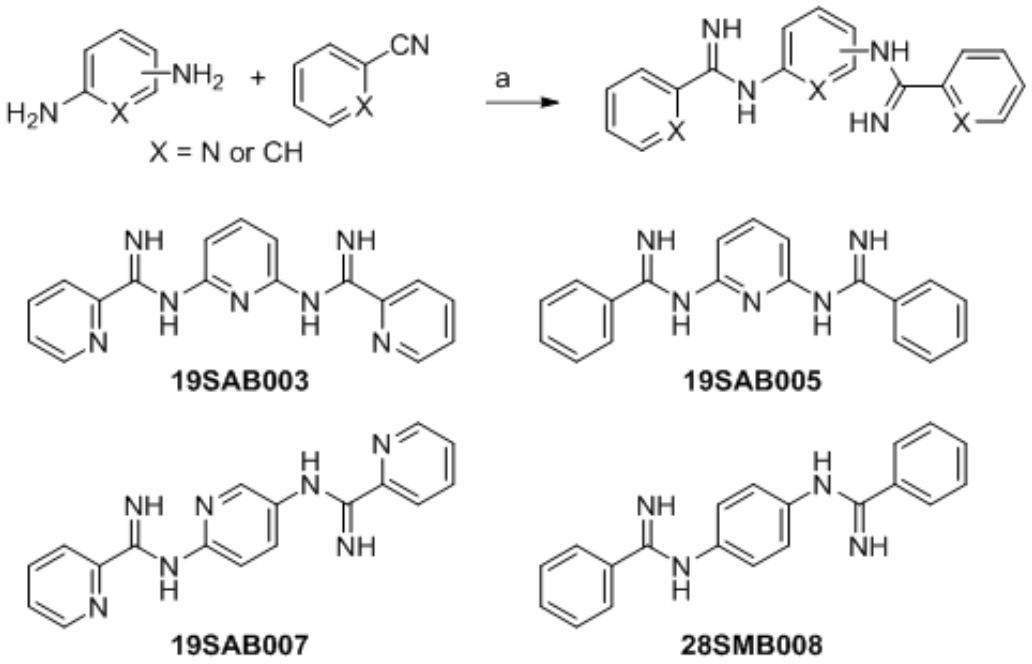


Figure 1: Structures of mono- and bis-arylimidamide derivatives of *m*-terphenyl.



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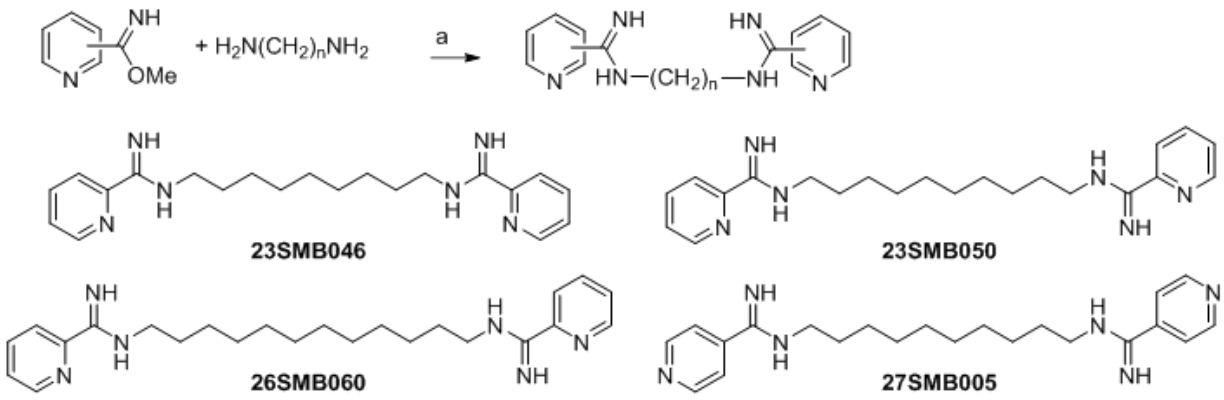

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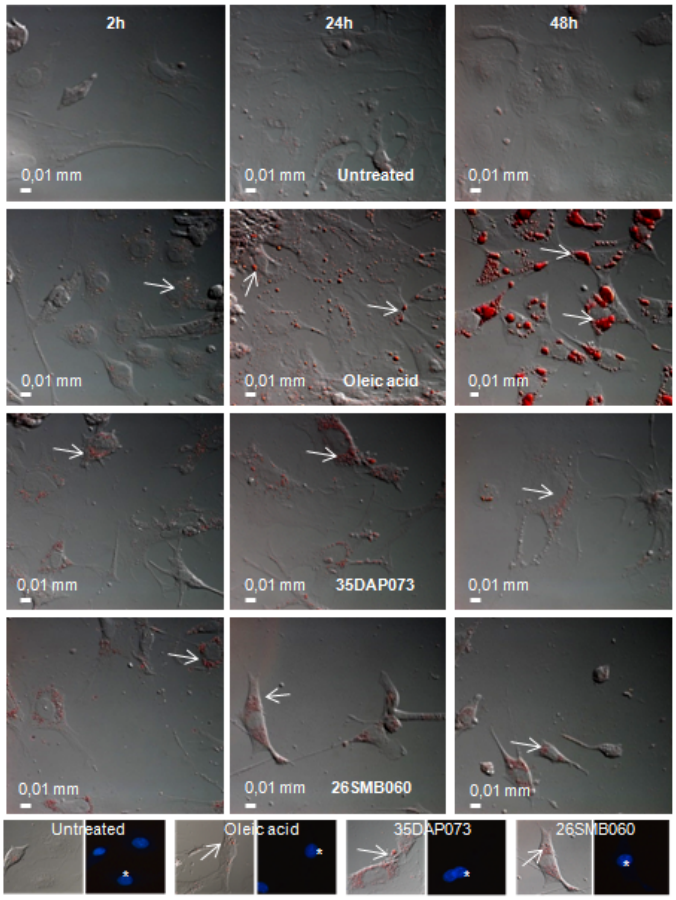

Arrow: corpuscles lipid

Asterisk: cardiac cell nucleus

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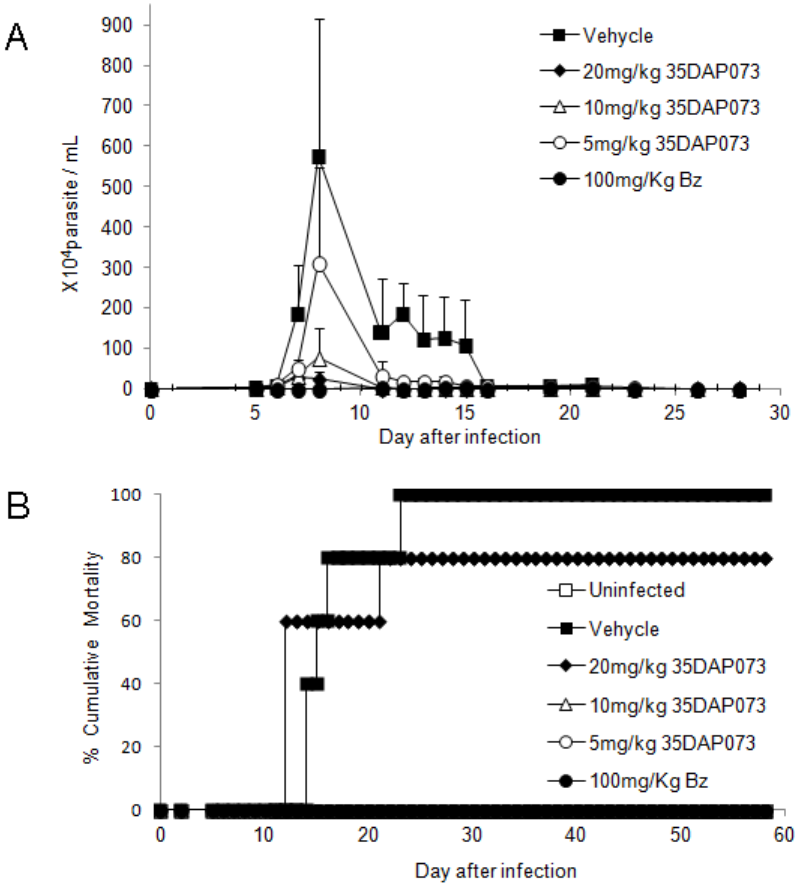

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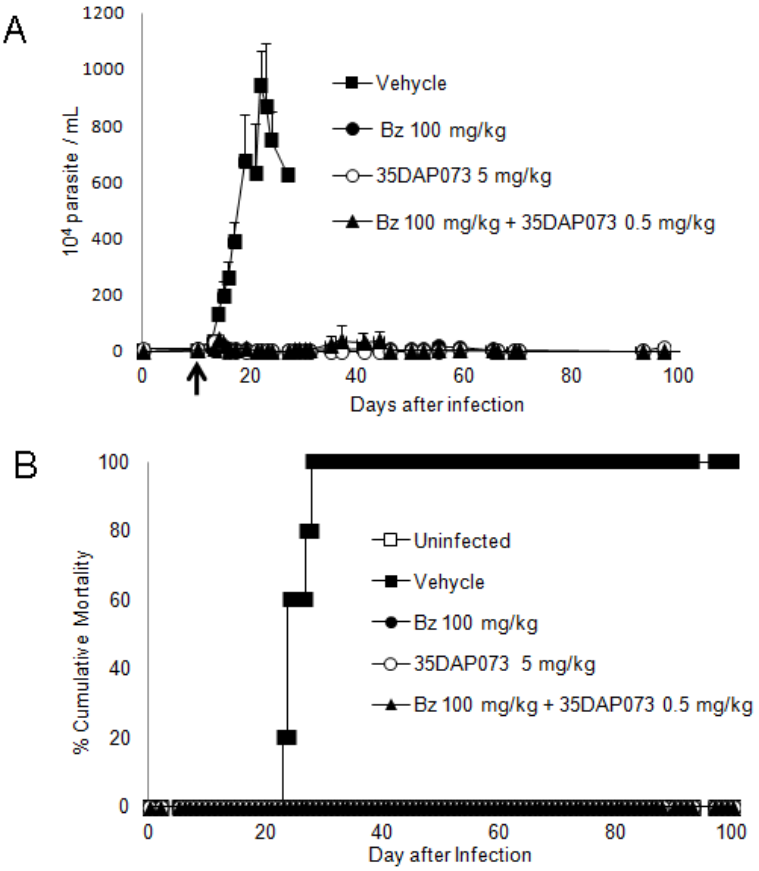

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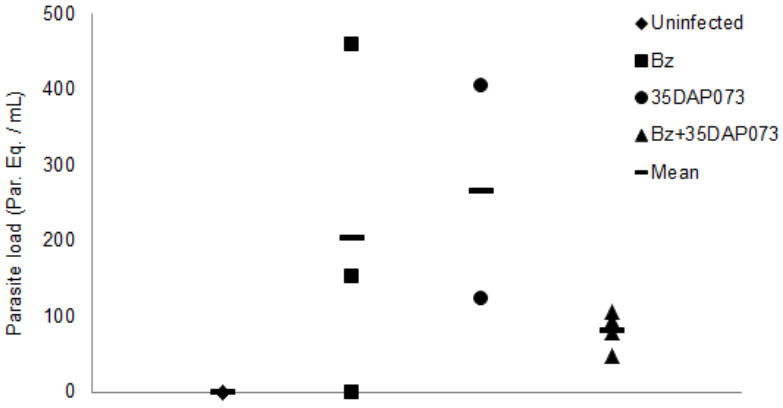

Mean and standard deviation (±) values of the parasite equivalents per milliliter of blood (Par.Eq/mL) by qPCR analysis

Mice Groups	Par.Eq/mL
Uninfected	0 ± 0
35DAP073	265 ± 199
Benznidazole	204 ± 233
Benznidazole + 35DAP073	82 ± 24

Figure 7: Blood qPCR analysis of the parasite equivalent per milliliter (Par. Eq. / mL) from uninfected and infected mice (using Colombiana strain) submitted to each different treatment regimen: Monotherapies (benznidazole 100 mg/kg/day and 35DAP073 5 mg/kg/day) and combined therapy (benznidazole (100 mg/kg/day) + 35DAP073 (0.5 mg/kg/day)). Each symbol represents an individual value and the bars represent respective mean values.

Table 1 *: In vitro* activity (EC_{50} values at µM) and selectivity index (SI) of the tested compounds on bloodstream trypomastigotes from Y strain of *Trypanosoma cruzi*

Compounds	EC_{50} (µM)		SI
	2 h	24 h	24 h
35DAP096	>32	>32	>1
35DAP073	0.7 ± 0.14	0.5 ± 0.2**	64
35DAP077	28 ± 0.3	10 ± 5.5	2
35DAP081	2 ± 0.8	0.6 ± 0.2**	53
38DAP092	16 ± 11	7 ± 1.2**	5
38DAP096	>32	24 ± 1	1.3
19SAB003	>32	>32	>1
19SAB005	>32	>32	>1
19SAB007	>32	>32	>1
28SMB008	>32	>32	>1
23SMB046	>32	>32	>1
23SMB050	>32	>32	>1
26SMB060	>32	>32	>1
27SMB005	>32	>32	>1
Benznidazole	>100	13 ± 2	77*

* Timm et al. 2014

Anova statistical analysis: **($p < 0.05$)

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Table 2 : *In vitro* activity (EC_{50} values at µM) of the tested compounds on bloodstream trypomastigotes of Colombiana strain of *Trypanosoma cruzi* after at 24 h of treatment at 37C

Compounds	EC_{50} (µM)
35DAP073	3.8 ± 2.8
35DAP081	1.9 ± 0.4

Table 3 : *In vitro* activity (EC_{50} values at µM) and selectivity index (SI) of the tested compounds on intracellular forms of *Trypanosoma cruzi* (Tulahuen-β galactosidase strain), using infected-L929 cell lines treated for 96h at 37C

	EC_{50} (µM)	SI
35DAP073	0.04 ± 0.005**	480
35DAP081	0.79 ± 0.06**	18
Benznidazole	2 ± 0.8	51

Anova statistical analysis: **($p \leq 0.05$)

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Table 4: *In vitro* activity (EC_{50} values at µM) and selectivity index (SI) of the tested compounds on intracellular forms of *Trypanosoma cruzi* (Y strain) present in cardiac cell cultures, 48 h after incubation at 37C

	EC_{50}	EC_{90}	SI (EC_{50})
35DAP073	0.016 ± 0.007**	0.087 ± 0.009**	2000
35DAP081	0.23 ± 0.06**	0.9 ± 0.05**	139
Benznidazole	3.6 ± 1.7	11 ± 2.7	277

Anova statistical analysis: **($p \leq 0.05$)

Table 5: Acute toxicity analysis – Escalating doses using a single mice per dose (starting at 12.5 mg/kg up to 200 mg/kg – via ip – using 0.1mL final volume per Swiss female mice).

Compound	0 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	NOAEL
35DAP073	NDE	NDE	NDE	Loss of mice body weight	Tremors, ataxia	death	25 mg/kg
35DAP081	NDE	Ataxia*, tremors, excitation and vocalization	Ataxia, tremors, excitation and vocalization	Ataxia, tremors, excitation and vocalization	Ataxia, tremors, excitation and vocalization	Ataxia, tremors, excitation and vocalization	<12.5 mg/kg

NDE: No detectable effect

NOAEL (No observed adverse effect level): For non-invasive parameters

* Effects observed after 2 hours of compound administration

Table 6: Plasma Biochemical[1] analysis of female mice after 48 h of compound administration

	Compound	0 mg/kg*	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
ALT	35DAP073	57 ± 8	58 ± 0	51 ± 0	88 ± 51	77 ± 41	ND
	35DAP081		56 ± 0.7	67 ± 13	82 ±8	102 ± 23	116 ± 11
CK	35DAP073	935 ± 226	635 ± 80	368 ± 0	701 ± 260	870 ± 646	ND
	35DAP081		503 ± 145	509 ± 145	737 ± 0	1681 ± 310	1162 ± 452
AST	35DAP073	183 ± 16	109 ± 0	113 ± 0	152 ± 37	242 ± 105	ND
	35DAP081		105 ± 16	143 ± 21	285 ± 55	422 ± 132	376 ± 229
BUN	35DAP073	46 ± 11	51 ± 16	34 ± 0	43 ± 2	55 ± 12	ND
	35DAP081		37 ± 7	29 ± 7	31 ± 10	29 ± 0	60 ± 29

[1] Mean ± SD values of two independent assays

* One representative assay

ND (not determined) due to animal death at the higher dose.

Reference values (CECAL/Fiocruz): Blood urea nitrogen (BUN) (up to 29 mg/dL); Alanine aminotransferase (ALT) (up to 132 U/L); Aspartate transaminase (AST) (up to 247 U/L) and Creatine kinase (CK) (up to 1070 U/L)

Anova statistical analysis of all samples ($p \geq 0.05$)