Cellular Effects of Reversed Amidines on *Trypanosoma cruzi*

Running title: *T. cruzi* treatment with reversed amidine

C.F. Silva¹, M.B. Meuser¹, E.M. De Souza¹, M.N.L. Meirelles², C.E. Stephens³, P. Som³,
D.W. Boykin³, and M.N.C. Soeiro¹*

Lab. Biologia Celular¹, Lab. Ultra-estrutura Celular² - Instituto Oswaldo Cruz,
FIOCRUZ, Rio de Janeiro, Brazil and Department of Chemistry³, Georgia State
University, Atlanta, GA,30303 USA.

*Corresponding author. Mailing address: Lab. de Biologia Celular, Dept. de Ultra-
estrutura e Biologia Celular, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365,
21040-900, Rio de Janeiro, RJ, Brazil. Phone: 0055 21 2598-4331. Fax: 00 55 21
2260-4434. email: soeiro@ioc.fiocruz.br
Abstract

Aromatic diamidines represent a class of DNA minor groove-binding ligands that exhibit high antiparasitic activity. Since the chemotherapy for Chagas disease is still an unsolved problem, and previous reports for diamidines and related analogues show high activity against both *in vitro* and *in vivo* *T. cruzi* infection, our present aim was to evaluate the cellular effect *in vitro* of three reversed amidines (DB889, DB 702 and DB 786) and one diguanidine (DB711), against both amastigotes and bloodstream trypomastigotes of *T. cruzi*, the aetiological agent of Chagas disease. Our data shows higher activity for the reversed amidines compared to the diguanidine with the following order of trypanocidal activity: DB889 > DB 702 > DB 786 > DB711. Transmission electron microscopy analysis showed that the reversed amidines induced many alterations in the nuclear morphology, swelling of the endoplasmatic reticulum and Golgi structures and consistent damages in the mitochondria and kinetoplasts of the parasites. Interestingly, in trypomastigotes treated by the reversed amidine DB889, multiple axoneme structures (flagellar microtubules) were curiously noted. Flow cytometry analysis confirmed that the treated-parasites presented an important loss of the mitochondrial membrane potential revealed by a decrease in the Rh123 fluorescence. Our results show promising activity for reversed amidines against the relevant mammalian forms of *T. cruzi*, displaying high trypanocidal effect at very low doses especially for DB889, which merits further *in vivo* evaluation.
INTRODUCTION

Aromatic diamidines are DNA minor groove-binding ligands (MGBLs), which present striking broad-spectrum antimicrobial effects (28). Although this class of compounds displays significant in vitro and in vivo activity against fungi, amoeba, bacteria and especially protozoan parasites, certain structures can show toxicity towards mammalian cells (23). In addition, aromatic diamidines in general lack oral bioavailability, which limits their use (28). To overcome these limitations, prodrugs such as the methamidoxime prodrug of furamidine (DB289), which is currently undergoing phase III clinical trials against human African trypanosomiasis, have been developed (29). *Trypanosoma cruzi* is the etiological agent of Chagas’ disease, a zoonosis considered a major public health problem in the developing countries of Central and South America (14). The disease is widespread in endemic areas of Latin America and it has been estimated that the overall prevalence of human infection is about 17 million cases and that approximately 120 million people are at risk of contracting the infection (30). However, up to now there is neither an effective vaccine nor a satisfactory treatment for the disease. Drug therapy depends mostly upon nitrofurans and nitroimidazoles such as nifurtimox and benznidazole (4, 26, 27).

Our previous studies revealed that furamidine and its N-phenyl substituted analogue (DB569) display activity against two kinetoplastid haemoflagellate members of the family *Trypanosomatidae*: *T. cruzi* and *L. (L) amazonensis*. Although both compounds have equivalent DNA binding properties, the phenyl-substituted analog exhibited higher activity against both parasites (8). DB569 was found to reduce the cardiac parasitism of *T. cruzi*-infected mice and also displayed
increased survival rates (9). In the present study we analyze the trypanocidal efficacy of three reversed amidines and one diguanidine against both intracellular amastigotes as well as bloodstream trypomastigotes of *T. cruzi* *in vitro*. Furthermore, by employing transmission electron microscopy and flow cytometry we identify possible targets of the drugs in the treated parasites.
MATERIALS AND METHODS

Drugs. The syntheses of DB702 and the diguanidine DB711 have been reported (24) and the synthesis of DB786 and DB889 was achieved by the same approach (Fig. 1). Stock solutions (5 mM) of the drugs were prepared in DMSO (dimethylsulphoxide) and fresh dilutions were prepared extemporaneously.

Parasites and cell cultures. Y stock of *Trypanosoma cruzi* was used throughout the experiments. Cell culture-derived trypomastigotes were isolated from the supernatant of Vero lineage cells (from green monkey kidney), which have been previously infected with trypomastigote forms (8). Bloodstream trypomastigotes were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the parasitaemia peak day (18). For the analysis of drug effects upon intracellular amastigotes, Vero cells were seeded at a density of $10^5$ cells/well into 24-well culture plates and sustained in RPMI 1640 (Roswell Park Memorial Institute- Sigma Aldrich) medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine. After 24 hours of plating, the cultures were infected for 24 hours at $37^\circ$C with tissue-cultured trypomastigotes, employing parasite: host cell ratio of 10:1. All the cell cultures were maintained at $37^\circ$C in an atmosphere of 5% CO$_2$ and air and the assays were run 3 times at least in duplicates. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA 0099/01), resolution 242/99.

Drug assays. For the analysis of the effect of the drugs upon the bloodstream trypomastigote forms, the isolated parasites were incubated at $37^\circ$C for 2 and 24
hours in the presence or not of increasing doses (0.0016-32 µM) of each compound diluted in Dulbecco’s modified medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine (DMES). After drug incubation, the trypomastigote death rates were determined by light microscopy through the direct quantification of the number of live and viable parasites (displaying typical motility and morphology as well) using a Neubauer chamber. For the analysis of the effect of the drugs upon intracellular amastigotes, after the initial host cell-parasite contact (24 hours), the cultures were washed to remove free parasites and treated or not for 24 hours at 37°C with graded concentrations (0.0014-10.6 µM) of the drugs. All the cell cultures were maintained at 37°C in an atmosphere of 5% CO₂ and air. Following the treatment, the infected cultures were fixed and processed as described above for ultrastructural analysis, or fixed in Bouin’s, stained with Giemsa solution as previously reported (1) and the mean number of infected host cells and of parasites per infected cells was then scored. Only characteristic parasite nuclei and kinetoplasts were counted as surviving parasites since irregular structures could mean parasites undergoing death. The drug activity was estimated by calculating the endocytic index (EI - percentage of infected cells versus mean number of parasite per infected cell).

**Flow cytometry analysis.** Bloodstream trypomastigotes (2×10⁶ cells/ml) were washed in phosphate buffered-saline and treated for 24 hours at 37°C with the respective IC 50 (previously determined) of each compound diluted in DMES. After treatment, the parasite suspension was incubated for 15 min at 37°C with 10 µg/ml rhodamine 123 (Rh123). Then the samples were immediately kept on ice until
analysis, as reported (21). Data acquisition and analysis were performed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, US) equipped with the Cell Quest software (Joseph Trotter, Scripps Research Institute, San Diego, CA, US). A total of 10,000 events were acquired in the region established as that corresponding to the bloodstream trypomastigotes and the alterations in the Rh123 fluorescence were quantified by calculating the mean percentage of treated and untreated parasite population that displayed depolarization of the mitochondrial membrane (marked as M2). All assays were run 3 times at least in duplicates and Student's t-test was applied to ascertain the statistical significance of the observed differences (p<0.05).

**Ultrastructural analysis.** $10^8$ bloodstream trypomastigotes were treated or not for 24 h at 37°C with the corresponding IC50 concentration of each drug. After incubation, the parasites were fixed for 60 min at 4 °C with 2.5% glutaraldehyde and 2.5 mM CaCl$_2$ in 0.1 M cacodylate buffer, pH 7.2 and post-fixed for 1 h at 4 °C with 1% OsO$_4$, 0.8% potassium ferricyanide and 2.5 mM CaCl$_2$ using this same buffer. Then, the samples were routinely processed for transmission electron microscopy (TEM) and examined in a Zeiss EM10C electron microscope (Oberkochen, Germany).

**RESULTS**

In addition to the trypanocidal effect of aromatic diamidines upon *T. cruzi* in *vitro* as well as *in vivo* (8,9), our recent report showed an excellent activity of reversed amidines during the treatment of bloodstream forms at 4°C in the
presence of blood constituents (22). Then, our first approach in the present study was to evaluate the activity of three different reversed amidines (DB889, DB702 and DB786), and one structurally related diguanidine (DB711) against bloodstream trypomastigotes of *T. cruzi*, during their treatment at 37°C. Our results showed that DB889 displayed a dose and time-dependent trypanocidal effect: as early as after 2 hours of incubation with 1.18 µM we found about 55% of parasite lysis, reaching a 100% of death after 24 hours of treatment with 3.5 µM (Fig. 2A). Similar data were noted when the trypomastigotes were incubated for 2 hours with the other two reversed amidines: 55 and 50% parasite death for DB702 and DB786, respectively. However, when the parasites were incubated for 24 hours with 3.5µM, both drugs reduced the number of viable parasites by about 95% (Fig. 2B and 2C). In fact, DB786 was extremely effective even under nanomolar doses: the treatment of bloodstream forms for 24 hours with 0.014µM DB786 lead to about 49% of parasite death (Fig.2C Inset). DB711 was the less effective, presenting 23 and 84% of death with 32 µM after 2 and 24 hours of parasite drug exposure, respectively (Fig. 2D).

Based on the IC$_{50}$/24h values established for each drug (Table), the bloodstream trypomastigotes were treated and processed for transmission electron microscopy to investigate morphological damage induced by the compounds studied at the ultrastructural level. Untreated parasites presenting typical organelles such as the endoplasmatic reticulum, nucleus, mitochondria and flagellum (arrow) can be easily identified (Fig. 3A). Note the single giant mitochondrion that branches throughout the parasite and contains a large
condensation of mitochondrial DNA, called the kinetoplast, which has a basket-like shape characteristic of the trypomastigotes (Fig. 3B). The treatment of the bloodstream trypomastigotes for 24 h with the reversed amidines (DB889, DB702 and DB786) caused several alterations mostly related to: swelling of the endoplasmatic reticulum (Fig. 3C) and Golgi (Fig. 3D, inset); mitochondrial swelling with presence of membranous structures and disorganization of the kinetoplast (Fig. 3C, 3D, 3E and 3F), profound alterations in the nuclear morphology including membrane swelling (Fig. 3C, 3D and 3F), and in addition intense vacuolization of the cytoplasm (Fig. 3F). An interesting finding was that parasites treated with DB889, the most effective compound, presented profound alterations related to the microtubule organization (Fig. 3F, inset) including showing multiple axoneme structures (flagellar microtubules) (Fig. 3C and 3F, arrow). The analysis of DB711-treated trypomastigotes also showed alterations related to the mitochondria-kinetoplast complex (Fig. 3H), besides intracellular dilated membrane profiles (Fig. 3G).

As the ultrastructural findings revealed frequent and extensive mitochondrial damage, we next assayed by flow cytometry analysis if these drugs could interfere with the mitochondrial membrane potential of the parasites as reported after treatment of T. cruzi by other aromatic diamines (10). The incubation of bloodstream trypomastigotes with the reversed amidines resulted in an important decrease of the mitochondrial membrane potential (MMP), as noticed by the low fluorescence intensity peaks marked as M2 (Fig. 3I-L). The mean and standard deviation analysis of three independent assays confirmed that the treatment with
DB889, DB702 and DB786 statistically reduced the MMP in about 59±11
\(p<0.014\), 54±5 \(p<0.011\), and 55±4.5\% \(p<0.009\) of the bloodstream forms,
contrasting to the untreated group, where only about 17±13\% of the parasites
already displayed decreased MMP (Fig. 3I-L). On the other hand, the treatment of
the parasites with the diguanidine DB711 did not result in statistically significant
\(p<0.31\) reduction of MMP (Fig. 3M).

Since amastigotes represent the multiplicative intracellular forms of \textit{T. cruzi}
found in the mammalian hosts and recent findings showed an excellent activity of
these reversed amidines upon amastigotes from different stocks of the parasite
(22), we next evaluated by transmission electron microscopy the alterations
induced by the compounds, with doses corresponding to the IC50 values, upon
intracellular amastigotes localized within the host cells (Table). Ultrastructural
image of untreated intracellular amastigote shows typical structures such as
nucleus, mitochondria (within the characteristic kinetoplast in the shape of bar),
flagellar pocket and flagellum (Fig. 4A and 4B). As already noted during the
treatment of the bloodstream trypomastigotes (Fig. 3), alterations related to the
amastigotes nuclei and mitochondria were always the most common and frequent
effects induced by the reversed amidine (Fig. 4). Besides these alterations in the
mitochondria (swelling, disorganization of the kinetoplast and presence of low
eletrodense structures) (Fig. 4D and 4E), and in the nuclear morphology (Fig. 4C),
other effects also included the vacuolization and loss of the cytoplasm components
(Fig. 4C, asteriks), disorganization of the subpellicular microtubules, which are
absent in distended membrane areas (Fig. 4H), and an intense vesicular profiles in the flagellar pocket (Fig. 4F and 4G).

DISCUSSION

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas disease, an illness that kills at least 50000 individuals every year in endemic areas of Latin America (26). The conventional treatment of Chagas’ disease depends upon nitrofurans and nitroimidazoles (Nifurtimox and benznidazole) that are not satisfactory due to their inefficiency in the chronic phase, their serious side effects and the need of administration under medical supervision (4, 26). These arguments justify an urgent search for new compounds for the treatment of the Chagasic patients and diamidines are considered potential drugs since they present broad-spectrum activity against several parasitic agents both *in vivo* and *in vitro* (23). In the present study we explored the effects on the parasite targets for three “reversed” amidines and one diguanidine, which display related structures. Our data suggest that small variation in chemical structure can result in significant differences in the antiparasitic potency: even after short periods of incubation (2 hours) the three amidines displayed much higher potency (with very low micromolar doses) against *T. cruzi* as compared to the diguanidine-treated parasites. The present data showing the excellent trypanocidal activity of these “reversed” amidines confirmed previous results performed with bloodstream forms at 4°C in the presence of blood constituents, aiming the potential use of this class of compounds for the prophylaxis of banked blood (22).
In our present transmission electron microscopy (TEM) analysis, all reversed amidines, and to a lesser extent the diguanidine, induced marked and frequent alteration in the nuclei and mitochondria of both amastigotes and trypomastigotes. These ultrastructural alterations corroborate previous reports with pentamidine and analogues for in vitro treatments of *L. (L) amazonensis* (5), *L. tropica* (13), *L. major* (11), *T. cruzi* (8) as well as in vivo treatment of *L. donovani* and *L. major* in mouse models (16), suggesting a common mechanism of action, at least with part, for these dicationic compounds. However, since aromatic diamidines have also been localized within non-DNA-containing cytoplasmic organelles such as acidocalcisomes, in African trypanosomes (17), the possible involvement of these later compartments in the mechanism of action of diamidines upon *T. cruzi* must be considered and represents an interesting matter, which is currently under investigation.

The flow cytometry analysis corroborated the TEM confirming that reversed amidines act on the mitochondria-kinetoplast complex. The alterations indicate an interference with the proton electrochemical potential gradient of the mitochondrial membrane of the parasites as previously reported during the treatment of trypomastigotes with other diamidine compounds (10). The perturbations of the kinetoplast may result from dication binding to catenated kDNA, which has a high AT content (2) reinforcing the concept that this class of MGBLs interferes with the kDNA of the trypanosomatids. In fact, the exact mode(s) of action of the diamidines towards trypanosomatids is still unclear but strong evidence indicates that these MGBLs interfere in the kinetoplast function through selective association
to the unique AT rich regions of the kinetoplastids minicircle kDNA, perhaps involving DNA-processing enzymes (27). Recent reports show that at least part of the antileishmanial activity of pentamidine can also be related to the selective action of drug against *Leishmania* kinetoplast and/or nuclear topoisomerase I, which may represent some of the drug's targets (15). It has been proposed that although the lethal event has not been fully determined, the death of diamidine-treated kinetoplastids is probably a result of a series of occurrences, which involves the mitochondrial swelling, as we presently noted, caused by the dissipation of the membrane potential leading to the final destruction of the kDNA (28). A recent report showed that a \(N\)-phenyl-substituted analog of furamidine induces profound mitochondrial alteration in the drug-treated trypomastigotes of *T. cruzi* leading to the parasite apoptosis-like death (10).

A curious and unique finding was that DB889 lead to profound alteration in *T. cruzi* microtubules' organization: the drug provoked partial loss of the subpellicular microtubules in the intracellular amastigotes and induced an unusual organization of multiple flagella in the non proliferative stage, trypomastigote. It is known that the microtubules in trypanosomes are the main component of the flagellar axoneme and of the subpellicular microtubule corset, whose relative positions determine the morphology of each cell stage of the life cycle of these parasites (12). All members of the Trypanosomatidae family display a flagellum that emerges from the flagellar pocket, showing a basic structure of 9 + 2 axonemal microtubules (7). Alterations in the structure and organization of microtubules are not a common event noted in these parasites even after treatment with different types of drugs.
Since, the development of strategies to interfere with these important structures represent a unique chance of providing a new chemotherapeutic approach, further investigations are under way to seek to more fully understand this observation.

TEM also showed that DB889 induced a shedding of membranes near the flagellar pocket of the intracellular amastigotes suggestive of higher exocytic activity of the parasites since the flagellar pocket is one of the main sites where endocytosis and exocytosis take place in trypanosomatids. Similar alterations have been reported in *T. cruzi* (3) and other kinetoplastids (20) when subjected to treatment with other drugs. This issue deserves further investigation.

Due to well-known side effects, low bioavailability and the requirement of parenteral administration of the aromatic diamidines, the search for novel aromatic dications has been intensively investigated (28). However, these previous studies have largely focused on African trypanosome infections and few studies have been designed to investigate the potential effect of aromatic diamidines against *T. cruzi* (23, 28). As already reported (25, 22), we presently found a high activity of the “reversed” amidines against *T. cruzi* upon the parasite forms present in the vertebrate hosts (intracellular amastigotes and bloodstream trypomastigotes), at very low micromolar doses that do not affect the viability of mammalian cells. The mechanism by which these dicationic molecules reach the intracellular milieu remains largely unknown and it is possible that the nuclear membrane of the parasite (presently found with striking alterations), as well as the mitochondrial membrane, may be more permeable to dications than the nuclear membrane of
mammalian cells. In fact, specific transporters have been characterized for pentamidine in trypanosomatids such as T. brucei (6).

The high activity of DB889, effective at low micromolar doses, warrants further in vivo studies in experimental models with the goal of establishing an effective scheme of therapy of T. cruzi infection.

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REFERENCES


LEGEND TO FIGURES

Fig. 1: Structures of the four drugs used in this study.

Fig. 2: Activity of DB889 (A), DB702 (B), DB786 (C) and DB711 (D) on trypomastigote forms of *T. cruzi*. The percentage of dead parasites was measured, after 2 and 24 hours of treatment at 37ºC, by light microscopy.

Fig. 3: Transmission electron micrographs (A-H) and flow cytometry (I-M) analysis of trypomastigote forms of *T. cruzi* treated with compounds of interest. A-B: Untreated parasites; C, D and F: DB889-treated parasites, E: DB702-treated parasites; G-H: DB711-treated parasites. Note the swelling mitochondria and kinetoplast (C-F and H), the alterations of the nuclear morphology (C, D and H) and in the microtubule organization (F, inset). Multiple flagellar structures (C and F, arrow); dilated membrane profiles (G, asteriks) can be found. I-M: Histograms show a representative assay displaying the fluorescence intensity of untreated (I) and diamidine treated-parasites after their incubation with Rh123: (J) DB889, (K) D702; (L) DB786; and (M) DB711. The high fluorescence intensity peaks are marked as M1, whereas the low fluorescence intensity peaks, which represent decreased mitochondrial membrane potential, are marked as M2. kinetoplast (k); mitochondria (m); nucleus (n); endoplasmatic reticulum (er) and Golgi (g). Bar = A-H: 1 µm, H inset: 0.5 µm.

Fig. 4: Transmission electron micrographs of intracellular amastigotes of *T. cruzi*. Untreated parasites (A and B) or treated with (C) DB889, (E) DB702 and (G and H)
DB786. Note alterations in the nuclear morphology (C), vacuolization and loss of the cytoplasm components (C, asterisk); disorganization of the kinetoplast (D), swelling and presence of low electron dense structures in the mitochondria (E), intense vesicular profiles in the flagellar pocket (F-G - arrow) and disorganization of the subpellicular microtubules (H - arrowhead). Nucleus (n), mitochondria (m); kinetoplast (k) and flagellar pocket (fp). Bar = A: 0.5 µm; B-H: 1 µm.