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Activity of "reversed" diamidines against *Trypanosoma cruzi* "in vitro"

C.F. Silva [a], Marcos Meuser Batista [a], Renata Alves Mota [a], Elen Mello de Souza [a], Chad E. Stephens [b], Phanneth Som [b], David Wilson Boykin [b], Maria de Nazaré C. Soeiro [a,*]

[a] Lab. Biologia Celular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil
[b] Department of Chemistry, Georgia State University, Atlanta, USA

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ABSTRACT

Chagas' disease is an important parasitic illness caused by the flagellated protozoan *Trypanosoma cruzi*. The disease affects nearly 17 million individuals in endemic areas of Latin America and the current chemotherapy is quite unsatisfactory based on nitroheterocyclic agents (nifurtimox and benznidazol). The need for new compounds with different modes of action is clear. Due to the broad-spectrum antimicrobial activity of the aromatic dicationic compounds, this study focused on the activity of four such diamidines (DB811, DB889, DB786, DB702) and a closely related diguanidine (DB711) against bloodstream trypomastigotes as well as intracellular amastigotes of *T. cruzi in vitro*. Additional studies were also conducted to access the toxicity of the compounds against mammalian cells *in vitro*. Our data show that the four diamidines compounds presented early and high anti-parasitic activity (IC50 in low-micromolecular range) exhibiting trypanocidal dose-dependent effects against both trypomastigote and amastigote forms of *T. cruzi* 2 h after drug treatment. Most of the diamidines compounds (except the DB702) exerted high anti-parasitic activity and low toxicity to the mammalian cells. Our results show the activity of reversed diamidines against *T. cruzi* and suggested that the compounds merit *in vivo* studies.

1. Introduction

Aromatic diamidines such as pentamidine isethionate, diminazene aceturate and furamidine are DNA minor groove-binding ligands, which present broad-spectrum antimicrobial activity [1]. Pentamidine (Pentacarinat®-Rhodia) first synthesized as a synthetic analog of insulin, represents the only compound from the aromatic diamidine class that is extensively used in the clinic. Pentamidine is used to treat early stage of African trypanosomosis, antimony-resistant leishmaniasis and *Pneumocystis jiroveci* infection, mostly in AIDS patients [2,3]. Although possessing high activity *in vitro* and *in vivo* against fungi, amoeba, bacteria and protozoan parasites, these compounds lack oral bioviability, which leads to important limitations to their use [1,4]. To overcome these limitations, prodrugs have been developed and one such compound, the methamidoxime prodrug of furamidine (DB289), is currently undergoing phase III clinical trials against human African trypanosomiasis [3]. Parasites from the Tripanosomatidae family such as *Trypanosoma brucei*, *Trypanosoma cruzi* and various species of *Leishmania* cause a variety of important diseases in humans and other mammals, being responsible for considerable human mortality and morbidity in developing countries [5].

* *Corresponding author*. Tel.: +55 21 25984331; fax: +55 21 25984577.
E-mail address: soeiro@ioc.fiocruz.br M. de N.C. Soeiro).
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T. cruzi is the etiological agent of Chagas' disease, a zoonosis, which is considered a major public health problem in the developing countries of Central and South America [6]. The most important transmission mechanism of this parasite among its hosts is by hematophagous reduviid vectors and the overall prevalence of human infection is about 17 million cases. Furthermore, approximately 120 million people are at risk of contracting the infection [7]. Myocardial damage is one of the most important pathological features responsible for the high morbidity and mortality rates. The current chemotherapy is mostly based on nitrofurans and nitroimidazoles, such as nifurtimox and benznidazole. These type compounds were empirically developed over three decades ago and are not very effective for the treatment of chronic disease [8,9].

Although much attention has being devoted to the trypanocidal effect of aromatic diamidines against African trypanosomosis, few studies have evaluated their effect against *T. cruzi*. Furamidine and its *N*-phenyl substituted analogue (DB569) display trypanocidal activity against *T. cruzi* and although both compounds have equivalent DNA binding properties; DB569 exhibits higher activity against a variety of strains and stages of *T. cruzi in vitro*, with inhibitory values in the low-micromolar range [10]. The *N*-phenyl substituted analog reduced the cardiac parasitism of *T. cruzi*-infected mice and also displayed increased mouse survival rates [11], encouraging additional studies with aromatic diamidines against this parasite. In this context, the present study aims to investigate the microbicidal efficacy of four different reversed diamidines and one diguanidine against the relevant parasite forms of *T. cruzi* found in the mammalian hosts the intracellular amastigotes and bloodstream trypomastigotes.

2. Materials and methods

2.1. Drugs

The synthesis of DB702 and the diguanidine DB711 have seen reported [12] and the synthesis of DB786, DB811 and DB889 was achieved by the same approach (Fig. 1). Stock solutions (5 mM) of the drugs were prepared in DMSO (dimethyl sulfoxide) and fresh dilutions were prepared extemporaneously.

2.2. Cell cultures

For both infection and cytotoxic assays, Vero cell lineage cells (from green monkey kidney) were seeded at a density of 10^5 or 5×10^4 cells/well into 24- and 96-well culture plates, respectively, and sustained in RPMI 1640 medium (Roswell Park Memorial Institute, Sigma–Aldrich, USA) supplemented with 5% fetal bovine serum and 1 mM L-glutamine. Primary cultures of peritoneal mouse macrophages, also assayed for drug toxicity, were obtained as described elsewhere [13], seeded at a density of 5×10^4 cells/well into 96-well culture plates and sustained in Dulbecco's modified medium supplemented with 5% fetal bovine serum and 4 mM L-glutamine (DMES). All the cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and air and the assays were run three times at least in duplicates.

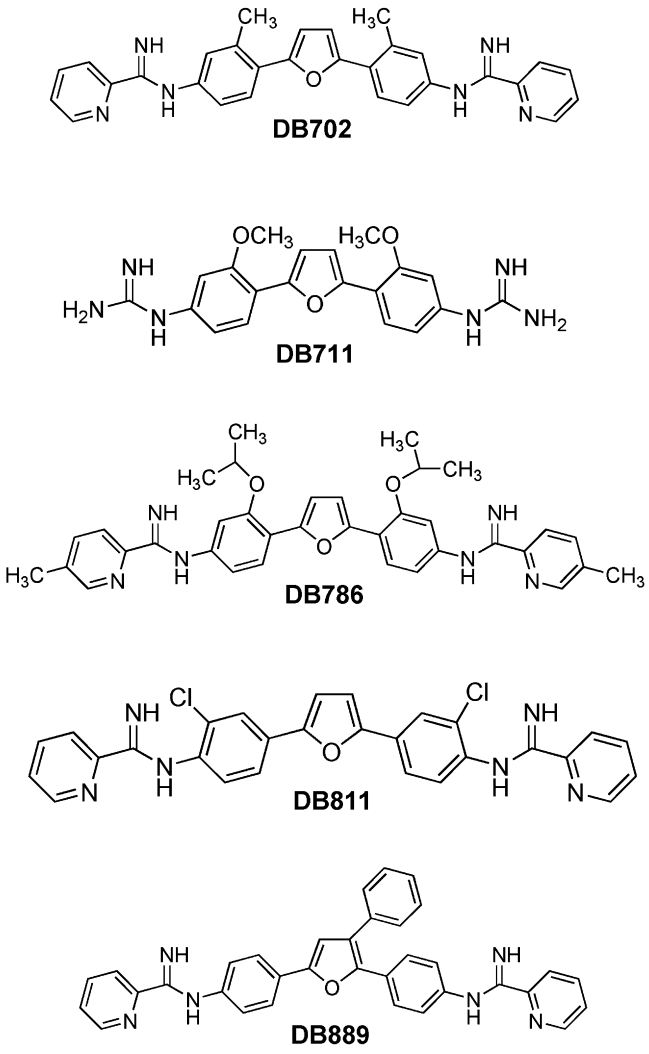

Fig. 1 – Structures of the five drugs used in this study.

2.3. Parasites

Y (moderately resistant to benznidazole and nifurtimox) [14] and Dm28c stocks of *T. cruzi*, representatives of biodemes II and I, respectively; were used throughout the experiments. Cell culture-derived trypomastigotes (Dm28c clone and Y strain) were isolated from the supernatant of Vero cells, which have been previously infected with trypomastigote forms [10]. Bloodstream trypomastigotes from Y strain were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the parasitaemia peak day [15]. 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.

2.4. Trypanocidal assays

For the analysis of the effect of the drugs upon the bloodstream trypomastigote forms, isolated parasites were incubated at 4 °C for 2 and 24 h in the presence of increasing doses (0.043–32 μM) of each compound diluted in DMES or in whole blood collected from *T. cruzi*-infected mice. After drug

incubation, the parasite death rates were determined through direct analysis by light microscopy, using a Neubauer chamber. Untreated parasites were used as control. The IC50 (drug concentration that reduces 50% of the number of the treated parasites) was then calculated.

2.5. Cytotoxicity assays

In order to rule out toxic effects of the drugs on the host cell, uninfected Vero cell line and peritoneal macrophages were incubated for 24 h/37 °C in presence or not of crescent doses (10.6–96 μM) of the drugs, and then their morphology and viability were evaluated by light microscopy using the trypan blue exclusion assay [16].

2.6. Infection assays and effect of diamidines upon intracellular parasites

After 24 h of platting, Vero cell cultures were infected for 2 and 24 h at 37 °C with cell culture-derived trypomastigotes of *T. cruzi* (for Dm28c and Y parasites, respectively) employing parasite: host cell ratio of 10:1. After the initial host–parasite contact, the cultures were washed to remove free parasites and treated for different periods of time (2–72 h) at 37 °C with graded concentrations (0.33–10.6 μM) of the drugs diluted in culture medium. Infected cultures not submitted to the drug treatment were used as control. The culture medium was replaced every 24 h. After the drug exposure, the infected cultures were fixed and stained with Giemsa solution as reported [13]. The mean number of infected host cells and of parasites per infected cells was then scored in 400 host cells in three independent experiments each was run in duplicate. Only characteristic parasite nuclei and kinetoplasts were counted as surviving parasites since irregular structures could mean parasites undergoing death [10]. The drug activity was estimated by calculating the inhibition levels of the endocytic index (EI—percentage of infected cells versus mean number of parasite per infected cell).

3. Results

Initially, we investigated the effect of the diguanidine DB711 and the ''reversed'' diamidines DB702, DB889 and DB786 upon trypomastigotes, which represent the main infective stage of *T. cruzi*. Our data showed that DB889, DB702 and DB786 gave a time-dependent trypanocidal effect when the bloodstream parasites were incubated with the drugs diluted in the culture medium (Fig. 2A–C). DB889 exerted a high dose-dependent activity after 2 h of treatment, reaching about 53% of parasite death with 10.6 μM of the reversed diamidine (Fig. 2A). After 24 h of incubation with 0.13 μM DB889, we found about 62% of dead parasites, reaching 89% of death with the dose of 10.6 μM (Fig. 2A). When the trypomastigotes were assayed for 2 h with the dose of 10.6 μM, DB702 and DB786 presented 45.8 and 57% of parasite death, respectively. However, compared to DB889, when the parasites were incubated for 24 h with 0.13 μM, DB702 and DB786 reduced the number of viable parasites by only about 23 and 21%, respectively (Fig. 2B and C). The diguanidine DB711 was much less active as it only slightly reduced the number of viable parasites, reaching maximum parasite death rates of 49% after 24 h of parasite drug exposure with 32 μM (Fig. 2D).

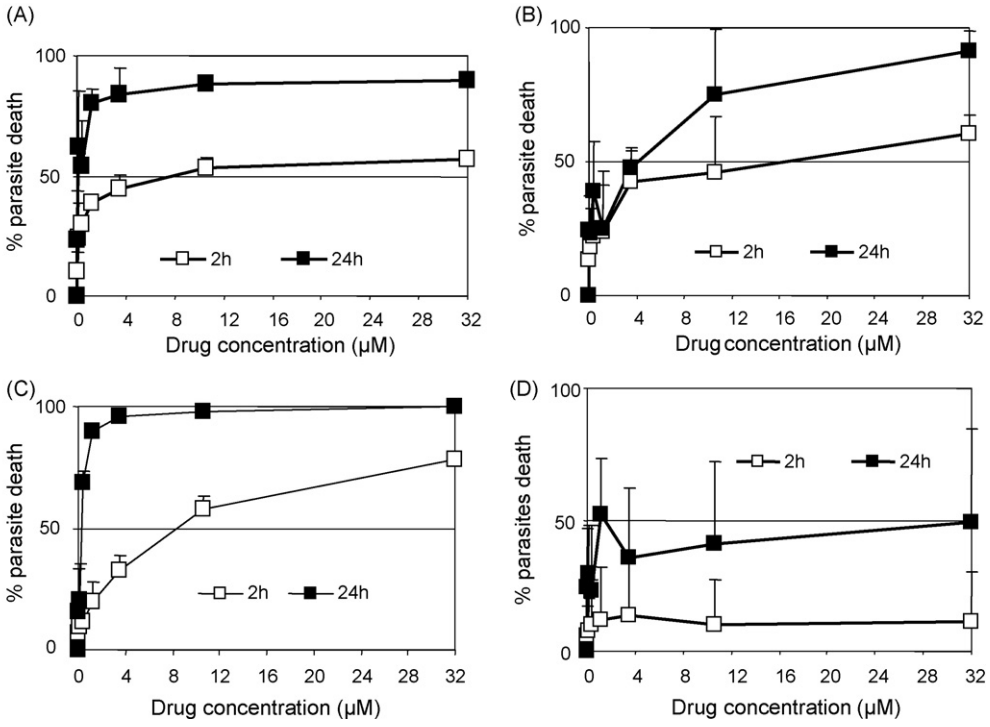

Fig. 2 – Activity of DB889 (A), DB702 (B), DB786 (C) and DB711 (D) upon trypomastigotes of *T. cruzi* (Y strain) *in vitro*. The effect upon the parasites was evaluated during the treatment at 4 °C with the drugs diluted in the culture medium. The percentage of dead parasites was measured after 2 and 24 h of treatment.

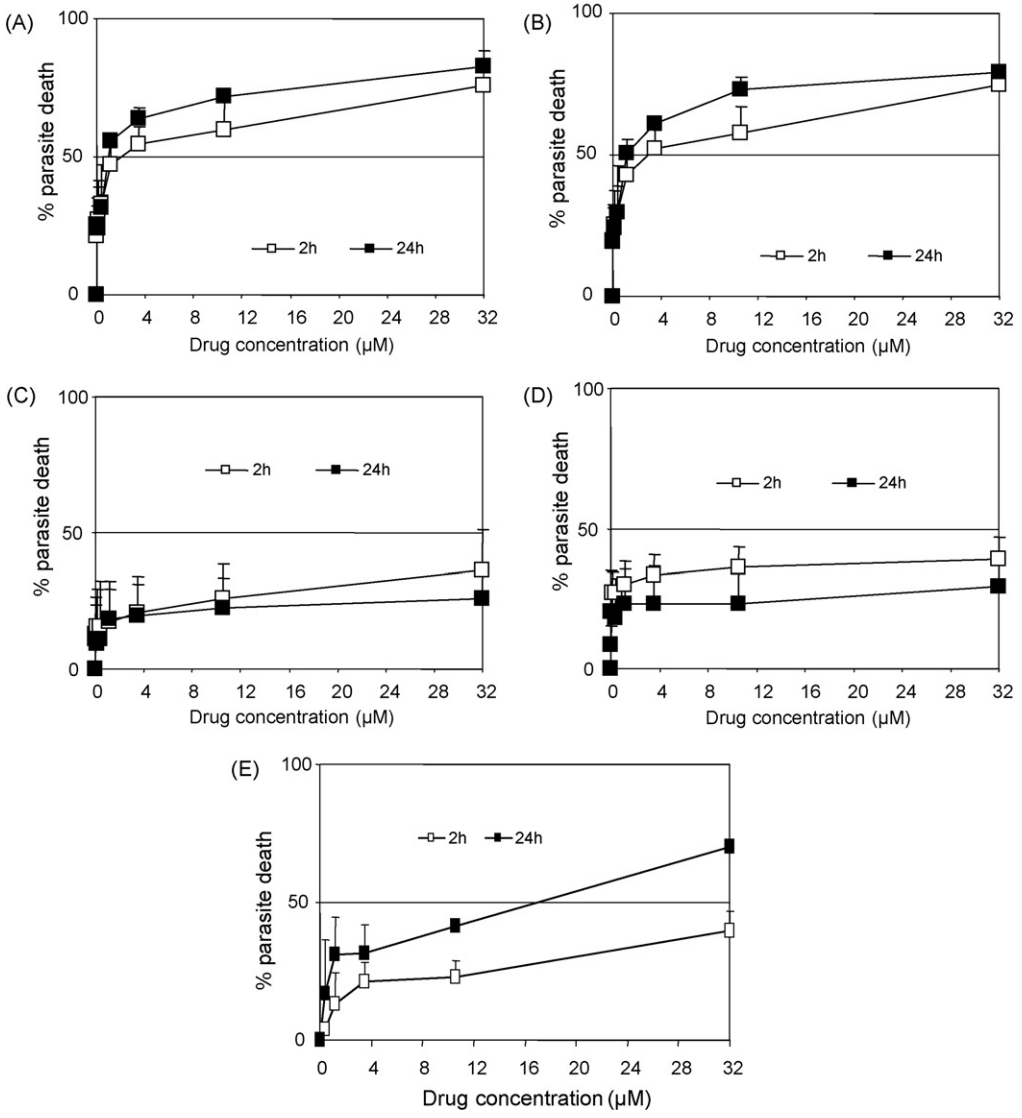

Fig. 3 – Activity of DB889 (A), DB702 (B), DB786 (C), DB711 (D) and DB811 (E) against bloodstream trypomastigotes of *T. cruzi* (Y strain) *in vitro*. The effect upon the parasites was evaluated during the treatment at 4 °C with the drugs diluted in whole blood. The percentage of dead parasites was measured after 2 and 24 h of treatment.

In the next step, we analyzed the effect of the compounds upon bloodstream forms in the presence of whole blood. Again, as above noted, the trypanocidal activities of the reversed diamidines DB889 and DB702 were the highest as compared to the others drugs (Fig. 3A–E). When the bloodstream forms were exposed to DB889 and DB702 in the presence of blood, we observed a substantial decrease in the parasite viability: after 2 and 24 h of incubation we found IC50 values of about 3.5 and 1 μM, respectively for both drugs (Fig. 3B). On the other hand, our data showed that after 2 and 24 h of treatment with DB711 and DB786, both IC50 values were higher than 32 μM (Fig. 3C and D).

The effect of the diamidine DB811 upon bloodstream parasites exhibited intermediate activity compared to the previous compounds: after 2 and 24 h of treatment with 32 μM DB811 in the presence of blood we found 39 and 70% of parasite death, respectively (Fig. 3E).

Clearly, the presence of blood greatly reduced the effect of these compounds as compared to the incubation of the parasites only in culture medium (Figs. 2 and 3). For example, after 24 h of incubation with DB889 in the presence of blood we found IC50 value of about 1 μM (Fig. 3A), while the treatment with the drug diluted in culture medium lead to IC50 value of 0.089 μM (Fig. 2A). The trypanocidal activity of DB786 was considerably affected by the presence of blood: the treatment for 24 h with 3 μM diluted in DMES resulted in 96% of parasite death while the same protocol performed in blood lead to only 19% parasite death (Figs. 2C and 3C).

The analysis of drug toxicity was performed by incubating uninfected host cells with different doses of each compound and then evaluating the cell viability by the trypan blue exclusion analysis. The toxicity analysis showed that the incubation of Vero cell with DB889, DB711 and DB786 *in vitro* only resulted in loss of cellular viability (10–20%) when higher

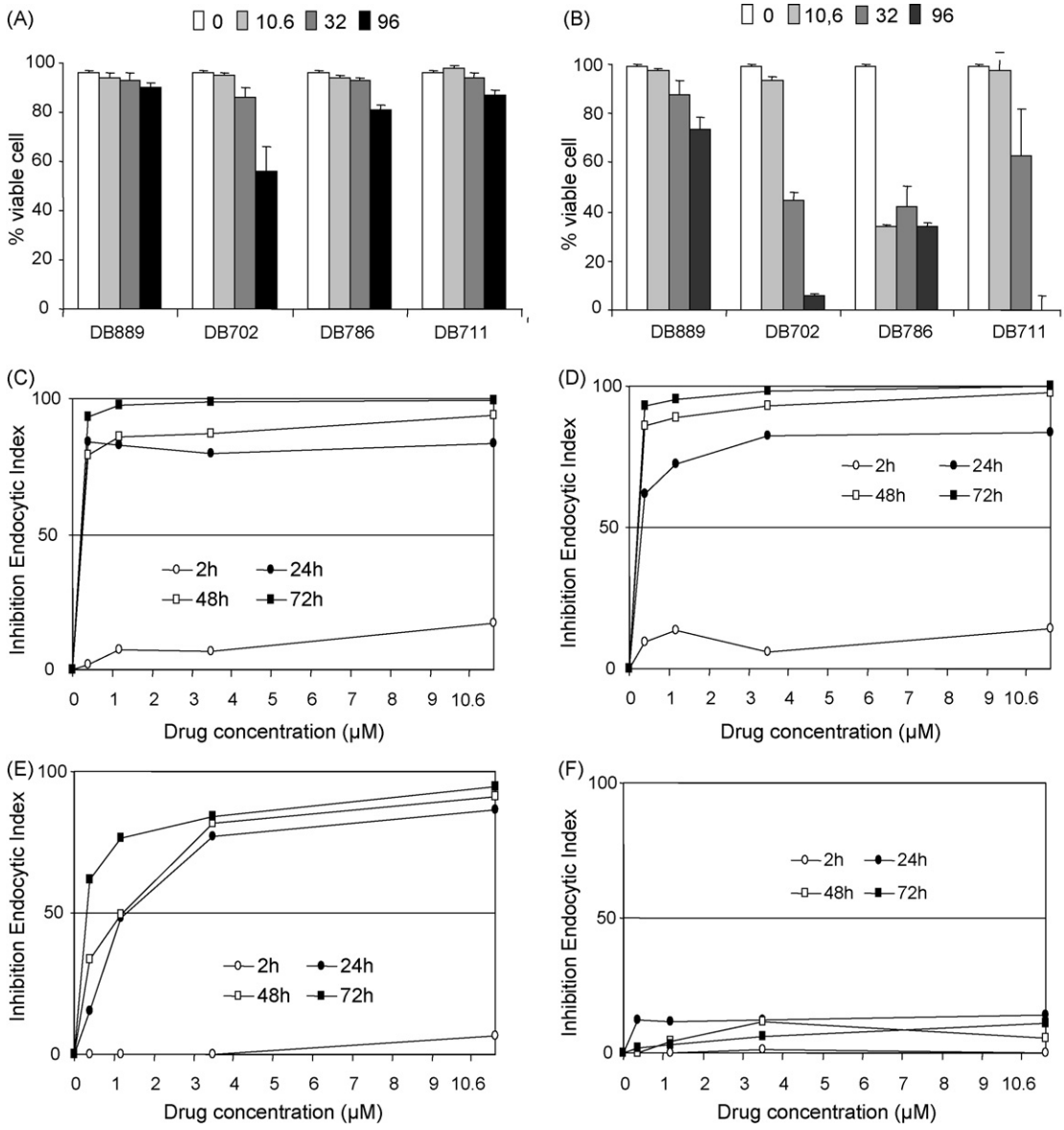

Fig. 4 – Effect of the reversed diamidines and the diguanidine upon mammalian host cells *in vitro*. (A) Vero cell line and (B) primary culture of peritoneal macrophages. (C–F) Effect of the reversed diamidines and the diguanidine upon intracellular amastigotes lodge in *T. cruzi*-infected cultures (Y strain). The activity of drugs noted by the inhibition of the endocytic index (EI) is presented for (C) DB889, (D) DB702, (E) DB786 and (F) DB711.

doses (>32 μM) were used, while the incubation with DB702 decreased the viability by about 44% of treated-cell lineage cultures (Fig. 4A). Nevertheless, when primary cultures of peritoneal macrophage were incubated with the same doses as previously studied, we found a higher toxicity especially for DB702, DB786 and DB711: the dose of 32 μM lead to 55, 58 and 38% of loss in cell viability, respectively (Fig. 4B). The assessment of the anti-parasitic activity of the compounds against the intracellular forms of *T. cruzi* employed infected cultures incubated with selected non-toxic doses (10.6 μM) of each drug.

We next investigated the effect of these compounds upon intracellular amastigotes from two stocks of *T. cruzi*: Dm28c clone and Y strain. Since our data did not reveal significant differences (data not shown) concerning the drugs sensitivity to both stocks, we will only present the data regarding Y strain.

After treatment of *T. cruzi*-infected cultures with DB889, DB702 and DB786 we found a time-dependent effect (Fig. 4C–E), leading to a considerable reduction in both percentage of infected cells and mean number of parasite per infected cells, evaluated by the endocytic index. As previously noted during the treatment of trypomastigotes, the two reversed diamidines DB889 and DB702 displayed the highest activity against the intracellular parasites (Fig. 4C and D): when the *T. cruzi*-infected cultures were incubated for 24 h with 0.33 μM DB889 (Fig. 5) and DB702, we found 83 and 61% of inhibition in the EI, respectively. When 10.6 μM of these diamidines was added for 24 h into the infected cultures, we observed about 83% of inhibition in the EI (Figs. 4C and D and 5). Further treatment for 72 h resulted in IC50 values of 0.17 μM for both compounds, leading to 93% inhibition of infection with the dose of 0.33 μM

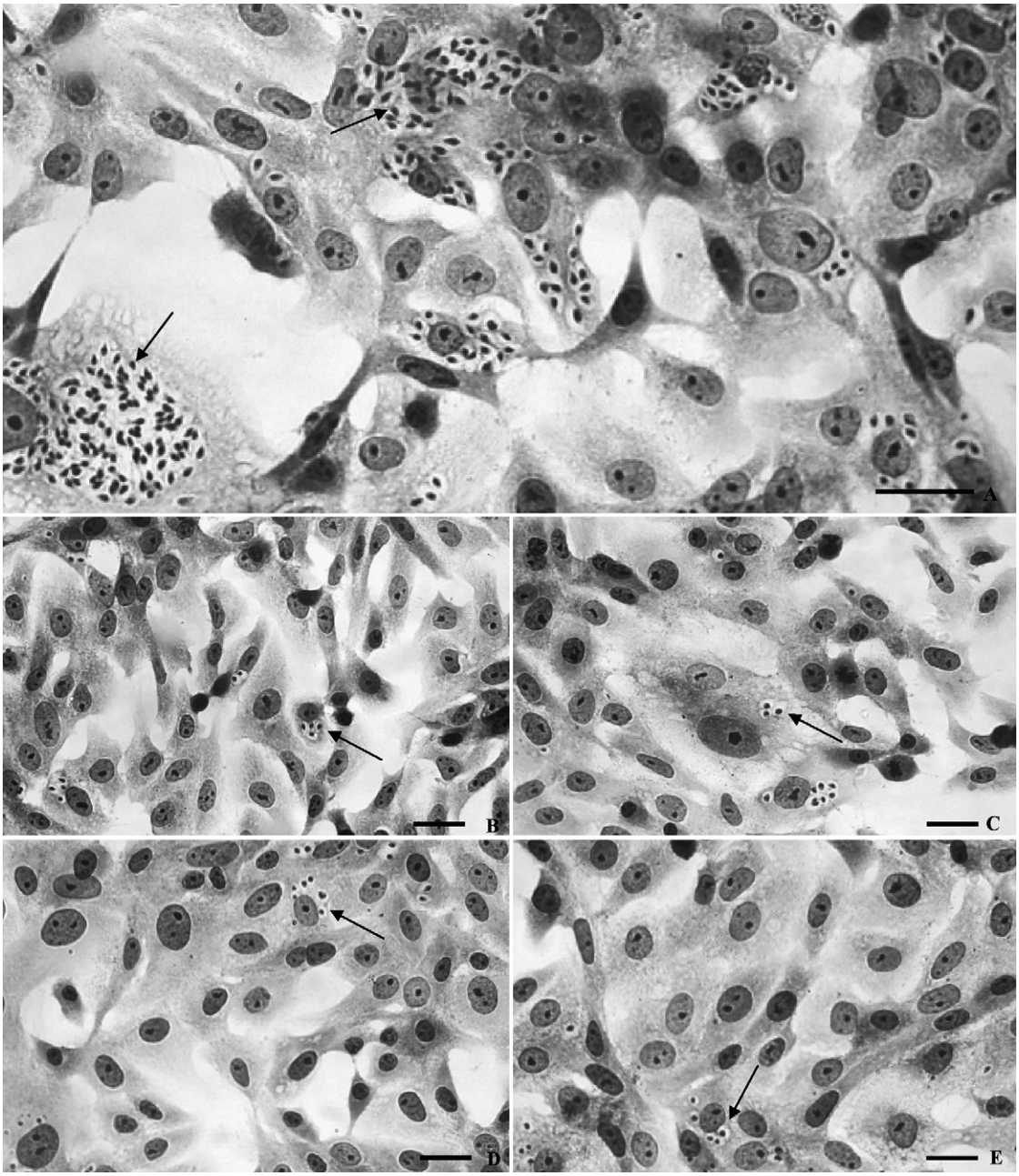

Fig. 5 – Light microscopy of *T. cruzi*-infected cells (Y strain), treated or not for 24 h with different doses of DB889. Untreated (A), and treated with 0.33 μM (B), 1.18 μM (C), 3.5 μM (D), and 10.6 μM (E). Arrow: intracellular parasites. Bar = 20 μm.

(Fig. 4C and D). The other reversed diamidine DB786 also presented a high activity, displaying a reduction in the EI of about 86 and 94% with 10.6 μM after 24 and 72 h of drug incubation (Fig. 4E). The diguanidine DB711 was the less effective giving IC50 values greater than 10.6 μM even after 72 h of incubation (Fig. 4F).

4. Discussion

The elimination of Chagas disease depends on several strategies including (i) the interruption of the vectorial transmission, (ii) the systematic screening of blood donors in the endemic countries, and (iii) the treatment of all acute cases. However, the current therapy for Chagas disease remains unsatisfactory mostly due to limited efficacy and high incidences of side effects [8], justifying the search for alternative trypanocidal drugs. In this context, our present aim was to investigate the activity of reversed diamidines and diguanidine against bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi*.

Aromatic diamidines have been successfully used to combat a wide variety of parasites that cause important human and veterinary infections [4,17]. While the effects on African trypanosomes have been extensively studied relatively little data exist for aromatic diamidines against *T. cruzi*

[1]. In our previous study we showed that furamidine displays anti-parasitic activities against extracellular and intracellular forms of *T. cruzi* and that the *N*-phenyl substitution of its amidine termini significantly enhanced the anti-parasitic activity [10]. We also noted that both compounds induced apoptosis-like death characteristics in trypomastigote forms of *T. cruzi* and that DB569, the analog that displayed superior trypanocidal activity also presented higher ability to induce apoptosis-like death in the treated parasites [18]. In addition, in mouse experimental model, DB569 significantly reduced the cardiac parasitism, partially increased the animal survival rates and lowered the levels of alanine aminotransferase and creatinine indicating a protective role of diamidines against the pathological events found in *T. cruzi* infection [11]. Structural variations on the cationic centers of the diamidines have been performed and the data showed the potential activity of ''reversed'' amidines against both *T. cruzi*, *L. donovani* and *Leishmania infantum* [19–21].

Our assays evaluating the effect of the drugs against trypomastigotes were performed at 4 °C in the presence or absence of blood due to the potential use of these compounds for the prophylaxis of banked blood. The incubation of the bloodstream trypomastigotes under these different experimental conditions showed that the presence of blood decreased the tripanocidal activity of the compounds possibly due to the drug association with serum components as already reported previously during the treatment of *T. cruzi* with lysophospholipids [22,23]. Although we noted a reduction of the diamidines, except for DB786, DB889 and DB702 activity towards the parasite still presented IC values with low-micromolecular doses (about 1 μM) in the presence of blood. In fact, we found that DB889 and DB702 were the most effective diamidines as compared to the other diamidines and to the diguanidine compound. The higher activity of DB889 and DB702 may be due to differences in lipophilicity.

In agreement with our previous studies showing that furamidine is weakly toxic to mammalian cells [10], our present data showed that only high drug concentrations (>32 μM) of the reversed diamidines induced alterations in the cellular physiology of the host cells. Recent data also reported the low mammalian toxicity of some reversed amidines, which presented excellent potency *in vitro* against *L. infantum* [21]. However, differences related to the drug toxicity were noticed when we employed different cell sources, indicating different susceptibility to the compounds due to the mammalian cells state. We also found that the three reversed diamidines exert anti-parasitic activities against the intracellular forms of *T. cruzi*, however we found that against the bloodstream forms, DB889 and DB702 were significantly more potent than DB786. As was observed for the trypomastigotes, the diguanidine compound DB711 was not effective against intracellular amastigotas *in vitro*. Our present data confirms previous results that showed the superior tripanocidal activity of the dicationic molecules containing reversed amidine cationic groups as compared to diguanidino cationic groups [21], as well as compared to other dicationic compounds [21]. It is important to note that although we previously reported a different activity of aromatic diamidines against the parasite stocks biodemes I and II [10], in the present study we did not find significant differences regarding the effect of reversed amidines upon intracellular amastigotes from Y and Dm28c. These differences deserve to be explored further.

Our present data describes the potential effect of the reversed diamidines against *T. cruzi*, which supports further screening of new analogs, which could be used alone or in combinations with other drugs for the treatment of Chagas' disease.

Acknowledgments

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