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Cellular Effects of Reversed Amidines on *Trypanosoma cruzi*[▽]

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Aromatic diamidines represent a class of DNA minor groove-binding ligands that exhibit high levels of antiparasitic activity. Since the chemotherapy for Chagas' disease is still an unsolved problem and previous reports on diamidines and related analogues show that they have high levels of activity against *Trypanosoma cruzi* infection both in vitro and in vivo, our present aim was to evaluate the cellular effects in vitro of three reversed amidines (DB889, DB702, and DB786) and one diguanidine (DB711) against both amastigotes and bloodstream trypomastigotes of *T. cruzi*, the etiological agent of Chagas ' disease. Our data show that the reversed amidines have higher levels of activity than the diguanidine, with the order of trypanocidal activities being as follows: DB889 > DB702 > DB786 > DB711. Transmission electron microscopy analysis showed that the reversed amidines induced many alterations in the nuclear morphology, swelling of the endoplasmic reticulum and Golgi structures, and consistent damage in the mitochondria and kinetoplasts of the parasites. Interestingly, in trypomastigotes treated with the reversed amidine DB889, multiple axoneme structures (flagellar microtubules) were noted. Flow cytometry analysis confirmed that the treated parasites presented an important loss of the mitochondrial membrane potential, as revealed by a decrease in rhodamine 123 fluorescence. Our results show that the reversed amidines have promising activities against the relevant mammalian forms of *T. cruzi* and display high trypanocidal effects at very low doses. This is especially the case for DB889, which merits further in vivo evaluation.

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 activities against fungi, amoeba, bacteria, and especially protozoan parasites, certain structures can show toxicity toward 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 for the treatment of 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 areas of endemicity in 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 has been 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 hemoflagellate members of the family Trypanosomatidae: *T. cruzi* and *Leishmania amazonensis.* Although both compounds have equivalent DNA-binding properties, the phenyl-substituted analog exhibited higher levels of activity against both parasites (7). DB569 was found to reduce the cardiac parasitism of *T. cruzi*-infected mice and also resulted in increased survival rates (8). In the present study we analyzed the trypanocidal efficacies of three reversed amidines and one diguanidine against both intracellular amastigotes and bloodstream trypomastigotes of *T. cruzi* in vitro. Furthermore, by employing transmission electron microscopy and flow cytometry we identified possible targets of the drugs in the treated parasites.

MATERIALS AND METHODS

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

Parasites and cell cultures. The 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 were previously infected with trypomastigote forms (7). Bloodstream trypomastigotes were harvested from *T. cruzi*-infected Swiss mice by heart puncture on the day of peak parasitemia (18). For analysis of the effects of the drugs 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 (Sigma Aldrich) medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine. After 24 h of plating, the cultures were infected for 24 h at 37°C with trypomastigotes from tissue cultures by employing a parasite/host cell ratio of 10:1. 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 duplicate. 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 analysis of the effects of the drugs upon the bloodstream trypomastigote forms, the isolated parasites were incubated at 37°C for 2 and 24 h without drug or in the presence of increasing doses (0.0016 to 32 μM) of each compound diluted in Dulbecco's modified medium supplemented with 5%

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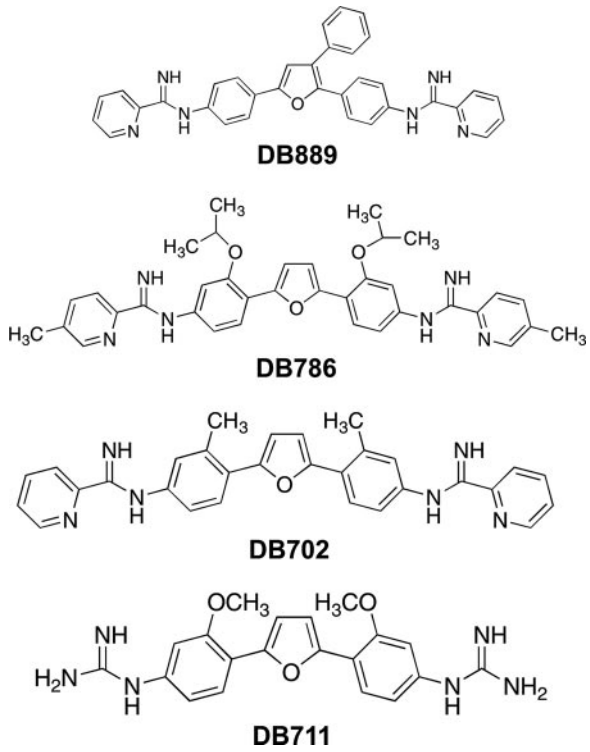


FIG. 1. Structures of the four drugs used in this study.

fetal bovine serum and 1 mM L-glutamine (DMES). After incubation with the drug, the trypomastigote death rates were determined by light microscopy through the direct quantification of the number of live and viable parasites (parasites displaying typical motility and morphology) by using a Neubauer chamber. For analysis of the effects of the drugs upon intracellular amastigotes, after the initial host cell-parasite contact (24 h), the cultures were washed to remove free parasites and were treated for 24 h at 37°C with graded concentrations (0.0014 to 10.6 μM) of the drugs or were left untreated. All the cell cultures were maintained at 37°C in an atmosphere of 5% CO_2 and air. Following the treatment, the infected cultures were fixed and processed as described above for ultrastructural analysis or were fixed in Bouin's fixative and stained with Giemsa solution, as reported previously (1). The mean number of infected host cells and the mean number of parasites per infected cell were then scored. Only parasites with characteristic nuclei and kinetoplasts were counted as survivors, since irregular structures could mean that parasites were undergoing death. The drug activity was estimated by calculating the endocytic index (the percentage of infected cells versus the mean number of parasites per infected cell).

Flow cytometry analysis. Bloodstream trypomastigotes (2×10^6 cells/ml) were washed in phosphate-buffered saline and were treated for 24 h at 37°C with each compound at its 50% inhibitory concentration (IC_{50}; which was determined previously) diluted in DMES. After treatment, the parasite suspension was incubated for 15 min at 37°C with 10 μg/ml rhodamine 123 (Rh123). The samples were then immediately placed on ice and kept on ice until analysis, as reported previously (21). Data acquisition and analysis were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest software (Joseph Trotter, Scripps Research Institute, San Diego, CA). 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 percentages of the treated and the untreated parasite populations that displayed depolarization of the mitochondrial membrane (marked M2). All assays were run three times at least in duplicate, and Student's *t* test was applied to ascertain the statistical significance of the observed differences ($P < 0.05$).

Ultrastructural analysis. A total of 10^8 bloodstream trypomastigotes were treated for 24 h at 37°C with the corresponding IC_{50} of each drug or were left untreated. 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 were postfixed for 1 h at 4°C with 1% OsO_4, 0.8% potassium ferricyanide, and 2.5 mM $CaCl_2$ with the same buffer. The samples were then routinely processed for transmission electron microscopy (TEM) and were examined in an EM10C electron microscope (Zeiss, Oberkochen, Germany).

RESULTS

In addition to the trypanocidal effects of the aromatic diamidines upon *T. cruzi* in vitro as well as in vivo (7, 8), our recent report showed that the reversed amidines have excellent activities against the bloodstream forms at 4°C in the presence of blood constituents (22). Our first approach in the present study was therefore to evaluate the activities of three different reversed amidines (DB889, DB702, and DB786) and one structurally related diguanidine (DB711) against bloodstream trypomastigotes of *T. cruzi* during treatment at 37°C. Our results showed that DB889 displayed dose- and time-dependent trypanocidal effects: as early as after 2 h of incubation with 1.18 μM, we found about 55% parasite lysis, with 100% parasite death achieved after 24 h of treatment with 3.5 μM DB889 (Fig. 2A). Similar data were noted when the trypomastigotes were incubated with the other two reversed amidines for 2 h: 55 and 50% parasite death for DB702 and DB786, respectively. However, when the parasites were incubated for 24 h with 3.5 μM, both drugs reduced the number of viable parasites by about 95% (Fig. 2B and C). In fact, DB786 was extremely effective even under nanomolar doses: the treatment of bloodstream forms with 0.014 μM DB786 for 24 h led to about 49% parasite death (Fig. 2C, inset). DB711 was less effective, with 32 μM DB711 causing 23 and 84% parasite death after 2 and 24 h of exposure, respectively (Fig. 2D).

On the basis of the IC_{50} values established for each drug at 24 h (Table 1), the bloodstream trypomastigotes were treated and processed for transmission electron microscopy to investigate the morphological damage induced by the compounds at the ultrastructural level. Untreated parasites presenting typical organelles, such as the endoplasmic reticulum, nucleus, mitochondrion, and flagellum, can easily be identified (Fig. 3A). Note the single giant mitochondrion that branches throughout the parasite and that 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 with the reversed amidines (DB889, DB702, and DB786) for 24 h caused several alterations mostly related to swelling of the endoplasmic reticulum (Fig. 3C) and Golgi structures (Fig. 3D, inset); mitochondrial swelling, with the presence of membranous structures and disorganization of the kinetoplast (Fig. 3C to F); profound alterations in the nuclear morphology, including membrane swelling (Fig. 3C, D, and F); 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 microtubule organization (Fig. 3H, inset), including multiple axoneme structures (flagellar microtubules) (Fig. 3C and F). The analysis of DB711-treated trypomastigotes also showed alterations related to the mitochondrion-kinetoplast complex (Fig. 3F), in addition to intracellular dilated membrane profiles (Fig. 3G).

As the ultrastructural findings revealed frequent and extensive mitochondrial damage, we next assayed by flow cytometry

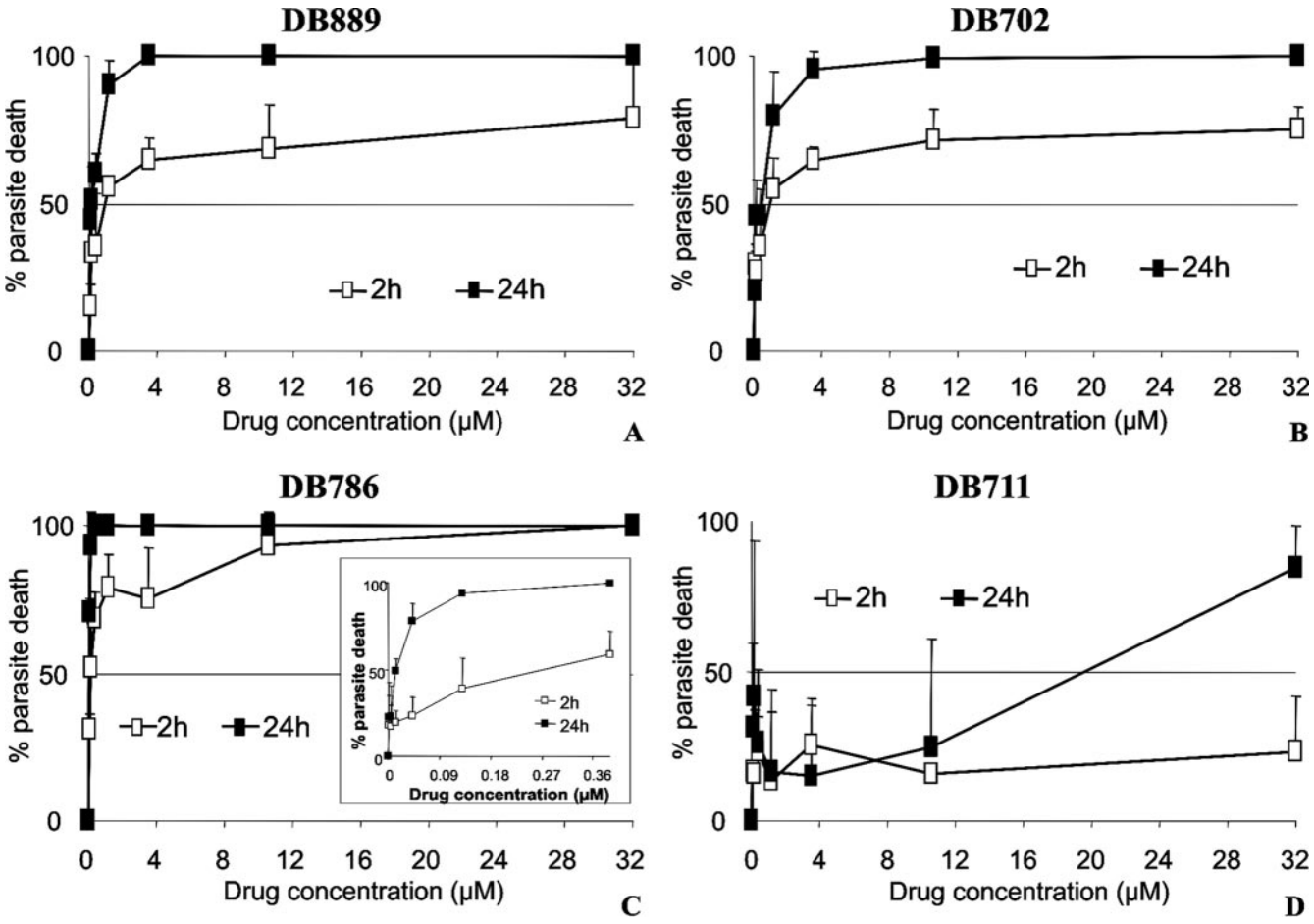


FIG. 2. Activities of DB889 (A), DB702 (B), DB786 (C), and DB711 (D) against trypomastigote forms of *T. cruzi*. The percentage of dead parasites was measured by light microscopy after 2 and 24 h of treatment at 37°C.

analysis whether these drugs could interfere with the mitochondrial membrane potential of the parasites, as has been reported to occur after treatment of *T. cruzi* with other aromatic diamines (9). The incubation of bloodstream trypomastigotes with the reversed amidines resulted in an important decrease in the mitochondrial membrane potential (MMP), as noticed by the low fluorescence intensity peaks marked M2 (Fig. 3I to L). Analysis of the means and standard deviations of three independent assays confirmed that treatment with DB889, DB702, and DB786 statistically reduced the MMPs in about 59% ± 11% ($P < 0.014$), 54% ± 5% ($P < 0.011$), and 55% ± 4.5% ($P < 0.009$) of the bloodstream forms, respectively; in contrast, in the untreated group only about 17% ± 13% of the parasites displayed decreased MMPs (Fig. 3I to L).

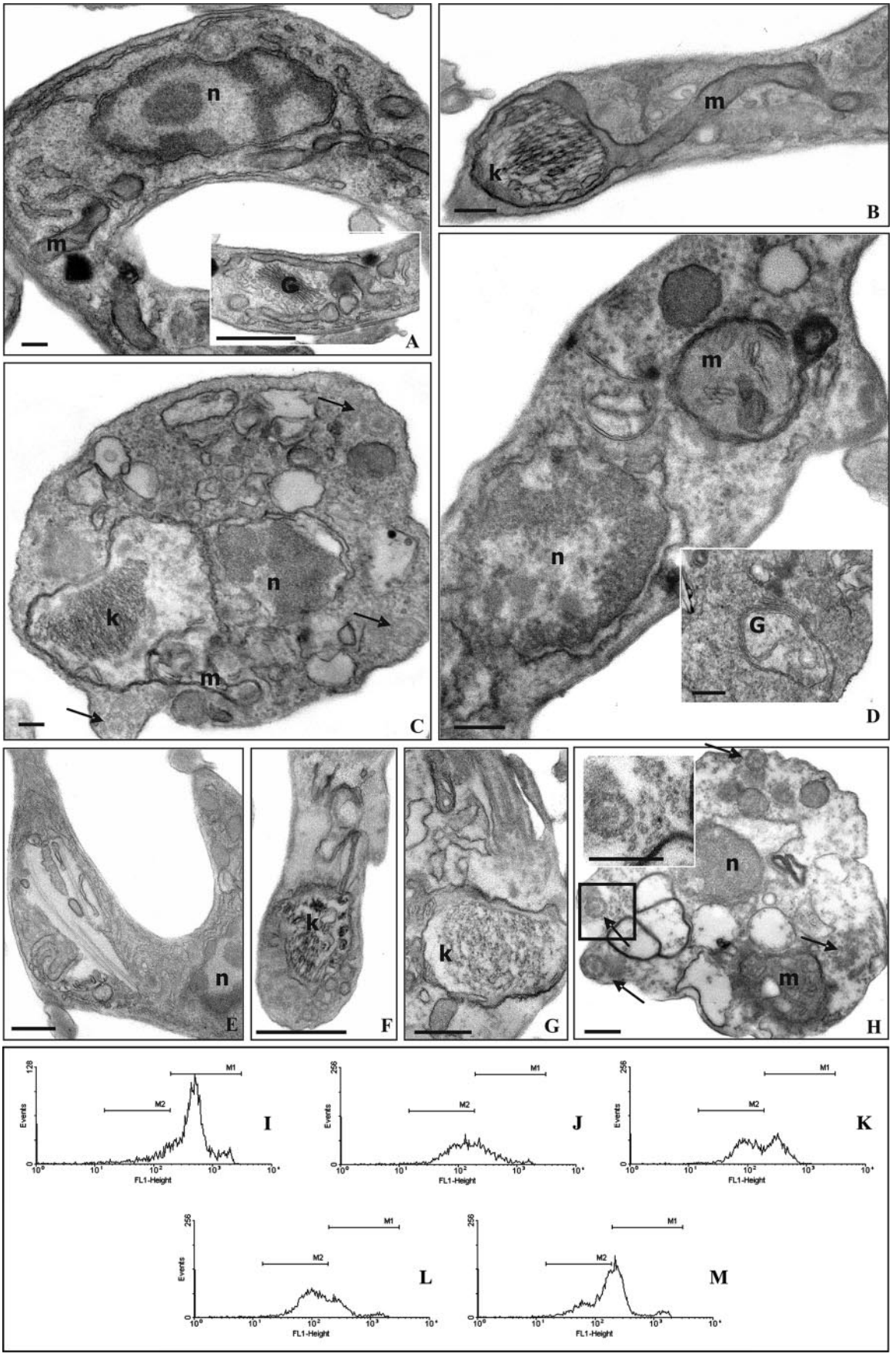
TABLE 1. IC_{50} values for amastigotes and trypomastigotes treated with drugs at 37°C for 24 h

Compound	Amastigotes[a]	Trypomastigotes
DB889	0.0086	0.09
DB702	0.24	0.45
DB786	1.14	0.015
DB711	>10.6	19.4

[a] Values related to the EI, endocytic index (see Materials and Methods).

On the other hand, treatment of the parasites with the diguanidine DB711 did not result in a statistically significant ($P < 0.31$) reduction of the MMP (Fig. 3M).

Since amastigotes represent the multiplicative intracellular forms of *T. cruzi* found in the mammalian hosts and recent findings indicate that these reversed amidines have excellent activities against amastigotes from different stocks of the parasite (22), we next evaluated by transmission electron microscopy the alterations induced by the compounds at doses corresponding to the IC_{50}s upon intracellular amastigotes localized within the host cells (Table 1). Ultrastructural images of an untreated intracellular amastigote show typical structures, such as the nucleus, the mitochondrion (within the characteristic kinetoplast in the shape of a bar), flagellar pocket, and flagellum (Fig. 4A and B). As already noted during the treatment of the bloodstream trypomastigotes (Fig. 3), the alterations related to amastigote nuclei and mitochondria were always the most common and frequent effects induced by the reversed amidine (Fig. 4). In addition to these alterations in the mitochondria (swelling, disorganization of the kinetoplast, and the presence of low electrodense structures) (Fig. 4D and E) and in the nuclear morphology (Fig. 4C), other effects included the vacuolization and loss of the cytoplasm components (Fig. 4C, asterisks); the disorganization of the subpel-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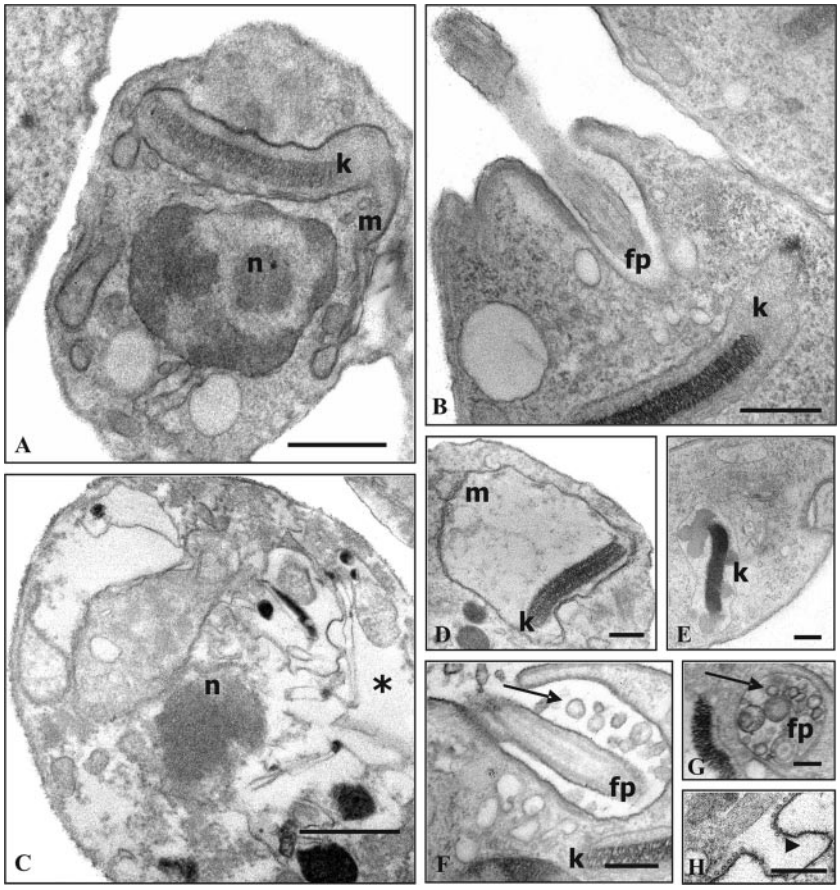

FIG. 4. Transmission electron micrographs of intracellular amastigotes of *T. cruzi*. The parasites were untreated (A and B) or were treated with DB889 (C), DB702 (E), and DB786 (G and H). Note the alterations in the nuclear morphology (C), the vacuolization and loss of the cytoplasm components (C, asterisk), disorganization of the kinetoplast (D), the swelling and the presence of low electrondense structures in the mitochondrion (E), the intense vesicular profiles in the flagellar pocket (F and G, arrows), and the disorganization of the subpellicular microtubules (H, arrowhead). n, nucleus; m, mitochondrion; k, kinetoplast; fp, flagellar pocket (fp). Bars, 0.5 μm (A) and 1 μm (B to H).

licular microtubules, which are absent in distended membrane areas (Fig. 4H); and intense vesicular profiles in the flagellar pocket (Fig. 4F and G).

DISCUSSION

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas' disease, an illness that kills at least 50,000 individuals every year in areas of Latin America where the disease is endemic (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 for administration under medical supervision (4, 26). These arguments justify an urgent search for new compounds for the treatment of patients with Chagas' disease, and diamidines are considered potential drugs since they present broad-spectrum activities against several parasitic agents both in vivo and in vitro (23). In the present study we explored the effects of three "reversed" amidines and one diguanidine which have related structures on the parasite targets. Our data suggest that small variations in chemical structure can result in significant differences in the antiparasitic potencies: even after short periods of incubation (2 h), the three amidines displayed

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

much higher potencies (at very low micromolar doses) than the diguanidine against *T. cruzi* parasites. The present data showing the excellent trypanocidal activities of these "reversed" amidines confirmed the results of previous studies performed with bloodstream forms at 4°C in the presence of blood constituents, indicating the potential use of this class of compounds for the prophylactic treatment of banked blood (22).

In our present TEM analysis, all reversed amidines and, to a lesser extent, the diguanidine induced marked and frequent alterations in the nuclei and mitochondria of both amastigotes and trypomastigotes. These ultrastructural alterations corroborate the findings from previous reports of studies with pentamidine and its analogues and their effectiveness for the in vitro treatment of *L. amazonensis* (5), *Leishmania tropica* (13), *Leishmania major* (11), and *T. cruzi* (7), as well as the in vivo treatment of *L. donovani* and *L. major* in mouse models (16), suggesting that these dicationic compounds have a common mechanism of action, at least in part. 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 the latter 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 results of flow cytometry analysis corroborated those of TEM, confirming that reversed amidines act on the mitochondrion-kinetoplast complex. The alterations indicate interference with the proton electrochemical potential gradient of the mitochondrial membrane of the parasites, as reported previously during the treatment of trypomastigotes with other diamidine compounds (10). The perturbations of the kinetoplast may result from dication binding to catenated kinetoplast DNA (kDNA), which has a high A+T 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 toward trypanosomatids is still unclear, but strong evidence indicates that these MGBLs interfere in the kinetoplast function through a selective association with the unique A+T-rich regions of the kinetoplastid 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 the drug against the *Leishmania* kinetoplast and/or nuclear toposoimerase 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 involve mitochondrial swelling, as we noted in the present study, caused by the dissipation of the membrane potential, leading to the final destruction of the kDNA (28). A recent report showed that an *N*-phenyl-substituted analog of furamidine induces profound mitochondrial alterations in the drug-treated trypomastigotes of *T. cruzi*, leading to the apoptosis-like death of the parasite (9).

A curious and unique finding was that DB889 led to a profound alteration in the organization of *T. cruzi* microtubules: the drug provoked the partial loss of the subpellicular microtubules in the intracellular amastigotes and induced an unusual organization of multiple flagella in the nonproliferative, trypomastigote stage. 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 family Trypanosomatidae display a flagellum that emerges from the flagellar pocket and that shows a basic structure of nine and two axonemal microtubules (10). Alterations in the structure and organization of the 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 represents a unique chance to provide a new chemotherapeutic approach, further investigations are under way to seek to more fully understand this observation.

TEM also showed that DB889 induced shedding of the membranes near the flagellar pocket of the intracellular amastigotes, which is suggestive of the 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 they have been subjected to treatment with other drugs. This issue deserves further investigation.

Due to the well-known side effects, the low bioavailability, and the requirement for parenteral administration of the aromatic diamidines, the search for novel aromatic dications has been intensivel (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 (22, 25), in the present study we found that the "reversed" amidines had high levels of activity against the parasite forms of *T. cruzi* 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 largely remains unknown, and it is possible that the nuclear membrane of the parasite (which was found to have striking alterations in the present study), 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 level of activity of DB889, which was effective at low micromolar doses, warrants further in vivo studies with experimental models with the goal of establishing an effective scheme of therapy for *T. cruzi* infections.

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REFERENCES

1. **Araujo-Jorge, T. C., E. P. Sampaio, W. De Souza, and M. N. Meirelles.** 1989. *Trypanosoma cruzi*: the effect of variations in experimental conditions on the levels of macrophage infection *in vitro*. Parasitol. Res. **75:**257–263.
2. **Baselin, M., M. A. Badet-Denisot, and M. Robert-Gero.** 1998. Modification of kinetoplast DNA minicircle composition in pentamidine-resistant *Leishmania*. Acta Trop. **70:**43–61.

3. **Braga, M. V., and W. De Souza.** 2006. Effects of protein kinase and phosphatidylinositol-3 kinase inhibitors on growth and ultrastructure of *Trypanosoma cruzi*. FEMS Microbiol. Lett. **256:**209–216.
4. **Coura, R. J., and S. L. De Castro.** 2002. A critical review on Chagas' disease chemotherapy. Mem. Inst. Oswaldo Cruz **97:**3–24.
5. **Croft, S. L., and R. P. Brazil.** 1982. Effect of pentamidine isethionate on the ultrastructure and morphology of *Leishmania mexicana amazonensis* in vitro. Ann. Trop. Med. Parasitol. **76:**37–43.
6. **De Koning, H. P.** 2001. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol. Pharmacol. **59:**586–592.
7. **De Souza, E. M., A. Lansiaux, C. Bailly, W. D. Wilson, Q. Hu, D. W. Boykin, M. M. Batista, T. C. Araújo-Jorge, and M. N. C. Soeiro.** 2004. Phenyl substitution of furamidine markedly potentiates its antiparasitic activity against *Trypanosoma cruzi* and *Leishmania amazonensis*. Biochem. Pharmacol. **68:**593–600.
8. **De Souza, E. M., G. M. Oliveira, D. W. Boykin, A. Kumar, Q. Hu, and M. N. Soeiro.** 2006. Trypanocidal activity of the phenyl-substituted analogue of furamidine DB569 against *Trypanosoma cruzi* infection in vivo. J. Antimicrob. Chemother. **58:**610–614.
9. **De Souza, E. M., R. Menna-Barreto, T. C. Araujo-Jorge, A. Kumar, Q. Hu, D. W. Boykin, and M. N. Soeiro.** 2006. Antiparasitic activity of aromatic diamidines is related to apoptosis-like death in *Trypanosoma cruzi*. Parasitology **133:**75–79.
10. **De Souza, W.** 2002. From the cell biology to the development of new chemotherapeutic approaches against trypanosomatids: dreams and reality. Kinetoplastid Biol. Dis. **1:**3.
11. **Fusai, T., Y. Boulard, R. Durand, M. Paul, C. Bories, D. Rivollet, A. Astier, R. Houin, and M. Deniau.** 1997. Ultrastructural changes in parasites induced by nanoparticle-bound pentamidine in a *Leishmania major*/mouse model. Parasite **4:**133–139.
12. **Gallo, J. M., and E. Precigout.** 1988. Tubulin expression in trypanosomes. Biol. Cell **64:**137–143.
13. **Hentzer, B., and T. Kobayasi.** 1977. The ultrastructural changes of Leishmania tropica after treatment with pentamidine. Ann. Trop. Med. Parasitol. **71:**157–166.
14. **Hirst, S. I., and L. A. Stapley.** 2000. Parasitology: the dawn of a new millennium. Parasitol. Today **16:**1–3.
15. **Jean-Moreno, V., R. Rojas, D. Goyeneche, G. H. Coombs, and J. Walker.** 2006. *Leishmania donovani*: differential activities of classical topoisomerase inhibitors and antileishmanials against parasite and host cells at the level of DNA topoisomerase I and in cytotoxicity assays. Exp. Parasitol. **112:**21–30.
16. **Loiseau, P. M., N. Mbongo, C. Bories, Y. Boulard, and D. G. Craciunescu.** 2000. In vivo antileishmanial action of Ir-(COD)-pentamidine tetraphenylborate on *Leishmania donovani* and *Leishmania major* mouse models. Parasite **7:**103–108.
17. **Mathis, A. M., J. L. Holman, L. M. Sturk, M. A. Ismail, D. W. Boykin, R. R. Tidwell, and J. E. Hall.** 2006. Accumulation and intracellular distribution of antitrypanosomal diamidine compounds DB75 and DB820 in African trypanosomes. Antimicrob. Agents Chemother. **50:**2185–2191.
18. **Meirelles, M. N. L., T. C. Araújo-Jorge, and W. De Souza.** 1982. Interaction of *Trypanosoma cruzi* with macrophages *in vitro:* dissociation of the attachment and internalization phases by low temperature and cytochalasin B. Z. Parasitenkd. **68:**7–14.
19. Reference deleted.
20. **Santa-Rita, R. M., A. Henriques-Pons, H. S. Barbosa, and S. L. De Castro.** 2004. Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against *Leishmania amazonensis*. J. Antimicrob. Chemother. **54:** 704–710.
21. **Santa-Rita, R. M. H. S. Barbosa, and S. L. De Castro.** 2006. Ultrastructural analysis of edelfosine-treated trypomastigotes and amastigotes of *Trypanosoma cruzi*. Parasitol. Res. **100:**187–190.
22. **Silva, C. F., M. M. Batista, R. A. Mota, E. M. de Souza, C. E. Stephens, P. Som, D. W. Boykin, and N. Soeiro Mde.** 2007. Activity of "reversed" diamidines against *Trypanosoma cruzi "in vitro."* Biochem. Pharmacol. **73:**1939–1946.
23. **Soeiro, M. N. C., E. M. De Souza, C. E. Stephens, and D. W. Boykin.** 2005. Aromatic diamidines as antiparasitic agents. Expert Opin. Investig. Drugs **14:**957–972.
24. **Stephens, C. E., F. Tanious, S. Kim, W. D. Wilson, W. A. Schell, J. R. Perfect, S. G. Franzblau, and D. W. Boykin.** 2001. Diguanidino and "reversed" diamidino 2,5-diarylfurans as antimicrobial agents. J. Med. Chem. **44:**1741–1748.
25. **Stephens, C. E., R. Brun, M. M. Salem, K. A. Werbovetz, F. Tanious, W. D. Wilson, and D. W. Boykin.** 2003. The activity of diguanidino and 'reversed' diamidino 2,5-diarylfurans versus Trypanosoma cruzi and Leishmania donovani. Bioorg. Med. Chem. Lett. **13:**2065–2069.
26. **Stewart, M., S. Krishna, R. S. Burchmore, R. Brun, H. P. de Koning, D. W. Boykin, R. R. Tidwell, J. E. Hall, and M. P. Barrett.** 2005. Detection of arsenical drug resistance in *Trypanosoma brucei* with a simple fluorescence test. Lancet **366:**486–487.
27. **Urbina, J. A., and R. Do Campo.** 2003. Specific chemotherapy of Chagas disease: controversies and advances. Trends Parasitol. **19:**495–501.
28. **Werbovetz, K.** 2006. Diamidines as antitrypanosomal, antileishmanial and antimalarial agents. Curr. Opin. Investig. Drugs **7:**147–157.
29. **Wilson, W. D., B. Nguyen, F. A. Tanious, A. Mathis, J. E. Hall, C. E. Stephens, and D. W. Boykin.** 2005. Dications that target the DNA minor groove: compound design and preparation, DNA interactions, cellular distribution and biological activity. Curr. Med. Chem. **5:**389–408.
30. **World Health Organization.** 2002. The world health report. World Health Organization, Geneva, Switzerland.