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 trypanomastigotes 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 trypanomastigotes treated with the reversed amide 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.

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 trypanomastigotes 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 trypanomastigotes were isolated from the supernatant of Vero lineage cells (from green monkey kidney) which were previously infected with trypanomastigote forms (7). Bloodstream trypanomastigotes 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 10⁵ 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 trypanomastigotes 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% CO₂ 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 000901), resolution 242/99.

**Drug assays.** For analysis of the effects of the drugs upon the bloodstream trypanomastigote 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%
fetal bovine serum and 1 mM L-glutamine (DMEM). 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 (0.003 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% CO₂ 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 not observed differences in the IC₅₀ values established for each drug at 24 h (Table 1), the bloodstream trypanomastigotes 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 trypanomastigotes (Fig. 3B). The treatment of the bloodstream trypanomastigotes with the reversed amidines (DB889, DB702, and 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 IC₅₀ values established for each drug at 24 h (Table 1), the bloodstream trypanomastigotes 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 trypanomastigotes (Fig. 3B). The treatment of the bloodstream trypanomastigotes 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 trypanomastigotes 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...
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).

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amastigotes</th>
<th>Trypomastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB889</td>
<td>0.0086</td>
<td>0.09</td>
</tr>
<tr>
<td>DB702</td>
<td>0.24</td>
<td>0.45</td>
</tr>
<tr>
<td>DB786</td>
<td>1.14</td>
<td>0.015</td>
</tr>
<tr>
<td>DB711</td>
<td>&gt;10.6</td>
<td>19.4</td>
</tr>
</tbody>
</table>

* Values related to the EI, endocytic index (see Materials and Methods).
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...
much higher potencies (at very low micromolar doses) than the
diguanidinium against T. cruzi parasites. The present data show-
ing the excellent trypanocidal activities of these “reversed”
amidines confirmed the excellent results of previous studies performed
with bloodstream forms at 4°C in the presence of blood con-
stituents, indicating the potential use of this class of com-
pounds for the prophylactic treatment of banked blood (22).
In our present TEM analysis, all reversed amidines and, to
a lesser extent, the diguanidinium induced marked and frequent
alterations in the nuclei and mitochondria of both amastigotes
and trypomastigotes. These ultrastructural alterations corrobor-
ate the findings from previous reports of studies with pent-
amidinium 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 com-
partments 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 mitochon-
drion-kinetoplast complex. The alterations indicate interfer-
ence with the proton electrochemical potential gradient of the
mitochondrial membrane of the parasites, as reported previ-
ously during the treatment of trypomastigotes with other dia-
midine 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 interfere 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). Re-
cent 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 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 dia-
midine-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 mem-
brane potential, leading to the final destruction of the kDNA
(28). A recent report showed that an N-phenyl-substituted
analog of furamidines induces profound mitochondrial alter-
ations 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 pro-
found alteration in the organization of T. cruzi microtubules: the
drug provoked the partial loss of the subpellicular micro-
tubules in the intracellular amastigotes and induced an unusual
organization of multiple flagella in the nonproliferative, trypo-
mastigote stage. It is known that the microtubules in trypano-
somes 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 Trypanosoma-
tidae 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 organiza-
tion 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 pro-
vide a new chemotherapeutic approach, further investigations
are under way to seek to more fully understand this observa-
tion.

TEM also showed that DB889 induced shedding of the
membranes near the flagellar pocket of the intracellular amas-
tigotes, 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 trypanosoma-
tids. 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 inves-
tigation.

Due to the well-known side effects, the low bioavailability,
and the requirement for parenteral administration of the aro-
matic 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 re-
ported (22, 25), in the present study we found that the “re-
versed” 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 mi-
cromolar 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 penta-
midine 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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