Biological, ultrastructural effect and subcellular localization of aromatic diamidines in *Trypanosoma cruzi*

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SUMMARY

No vaccines or safe chemotherapy are available for Chagas disease. Pentamidine and related di-cations are DNA minor groove-binders with broad-spectrum anti-protozoal activity. Therefore our aim was to evaluate the *in vitro* efficacy of di-cationic compounds – DB1645, DB1582, DB1651, DB1646, DB1670 and DB1627 – against bloodstream trypomastigotes (BT) and intracellular forms of *Trypanosoma cruzi*. Cellular targets of these compounds in treated parasites were also analysed by fluorescence and transmission electron microscopy (TEM). DB1645, DB1582 and DB1651 were the most active against BT showing IC_{50} values ranging between 0·15 and 6·9 μM. All compounds displayed low toxicity towards mammalian cells and DB1645, DB1582 and DB1651 were also the most effective against intracellular parasites, with IC_{50} values ranging between 7·3 and 13·3 μM. All compounds localized in parasite nuclei and kDNA (with greater intensity in the latter structure), and DB1582 and DB1651 also concentrated in non-DNA-containing cytoplasmic organelles possibly acidocalcisomes. TEM revealed alterations in mitochondria and kinetoplasts, as well as important disorganization of microtubules. Our data provide further information regarding the activity of this class of compounds upon *T. cruzi* which should aid future design and synthesis of agents that could be used for Chagas disease therapy.

Key words: *Trypanosoma cruzi*, Chagas disease, chemotherapy, aromatic diamidines.

INTRODUCTION

Chagas disease, a tropical parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*, which was discovered by Carlos Chagas exactly a century ago (Chagas, 1909). Currently, there are approximately 12–14 million infected individuals in endemic areas of Latin America and many reports also point to the occurrence in non-endemic geographical areas such as the United States and Europe, mainly attributed to population movement of infected people (Dias, 2007; Gascón *et al*. 2007; Rodriguez-Morales *et al*. 2008; Guerri-Guttenberg *et al*. 2008; Milei *et al*. 2009). Clinically, Chagas disease has 2 phases: the acute, which appears shortly after the infection, and the chronic phase, which can develop in about one-third of the infected individuals, after a silent period of years or decades called the indeterminate phase (Cunha-Neto *et al*. 2006). The main clinical manifestations of Chagas' disease include both cardiac and/or digestive alterations, the former being the most common (Marin-Neto *et al*. 1999; Teixeira *et al*. 2006; Rocha *et al*. 2007). Pathogenesis of chronic chagasic cardiopathy is still not clearly understood but growing evidence shows that the pathogenesis is a consequence of a sustained inflammatory process, with anti-parasitic and/or anti-self-immune response, associated with a low-grade persistent parasite presence (Higuchi *et al*. 2003; Rocha *et al*. 2007; Marin-Neto *et al*. 2008).

Up to now, current therapy of chagasic patients includes nifurtimox (5-nitrofuran-(3-methyl-4-(5'-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide – Bayer 2502), and benznidazole (2-nitroimidazole (N-benzyl-2-nitroimidazole acetamide – RO 7-1051). Both compounds were developed empirically over 3 decades ago and present variable results depending on the disease phase (effective in the acute and recent chronic phases of the infection), dose and duration of the treatment, natural susceptibility of *T. cruzi* isolates, in addition both show undesirable side effects (Filardi and Brener, 1987; Coura and De Castro, 2002; Soeiro and De Castro, 2009). Heterocyclic di-cations, such as furamidine (DB75) and analogues, are DNA minor groove-binding ligands that recognize sequences of at least 4 AT base pairs, and present striking

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broad-spectrum antimicrobial effects (Wilson *et al*. 2008; Checchi and Barrett, 2008; Soeiro *et al*. 2008). However, due to their poor oral bioavailability and unfavourable side-effects, numerous new di-cationic analogues have been synthesized with the goal to improve on these deficiencies (Werbovetz, 2006). Previous studies reported the efficacy of diamidines such as DB75, DB569(*N*-phenyl-substituted analogue of DB75) and DB1362 (a diarylthiophene diamidine), as well as arylimidamides, including DB889, DB786 and DB702 upon *T. cruzi in vitro* (De Souza *et al*. 2004; Silva *et al*. 2007*a*, *b*, 2008). Additional studies performed with murine experimental models demonstrated the effect of these cationic compounds *in vivo*, leading to considerable protection against mice mortality with an important decrease in cardiac parasitism and inflammation. Furthermore, they reversed the electrocardiography alterations due to parasite infection (De Souza *et al*. 2006, 2007; Silva *et al*. 2008), justifying further studies with such aromatic dications.

Our present aim was to evaluate the *in vitro* activity of 6 aromatic diamidines upon bloodstream trypomastigotes and intracellular forms of *T. cruzi*. Moreover, fluorescence and transmission electron microscopy analyses were also performed in order to characterize the intracellular localization and cytoplasmatic distribution of the compounds as well as to evaluate their subcellular targets in the treated parasites.

MATERIALS AND METHODS

Drugs

The syntheses of the aromatic diamidines DB1582, DB1627, DB1645, DB1646, DB1651 and DB1670 (Fig. 1) were performed using standard procedures (Ismail *et al*. 2008). Stock solutions (5 mM) of the compounds were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the latter in the experiments never exceeding 0·6%, which did not exert any toxicity towards the parasite or mammalian host cells (data not shown).

Parasites

The Y strain of *T. cruzi* was used throughout the experiments. Bloodstream trypomastigote forms were obtained from infected albino Swiss mice at the peak of parasitaemia as previously reported (Meirelles *et al*. 1986).

Mammalian cell cultures

For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained following the previously described method (Meirelles *et al*. 1986). After purification, the CM were seeded at a density of $0{\cdot}1 \times 10^6$ cells/well into 24-well culture plates, or $0{\cdot}05 \times 10^6$ cell/well into 96-well microplates, containing gelatin-coated coverslips and sustained in Dulbecco's modified medium supplemented with 10% horse serum, 5% fetal bovine serum, 2·5 mM $CaCl_2$, 1 mM L-glutamine and 2% chicken embryo extract (DMEM). 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). All the cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and the assays were run at least 3 times in duplicate.

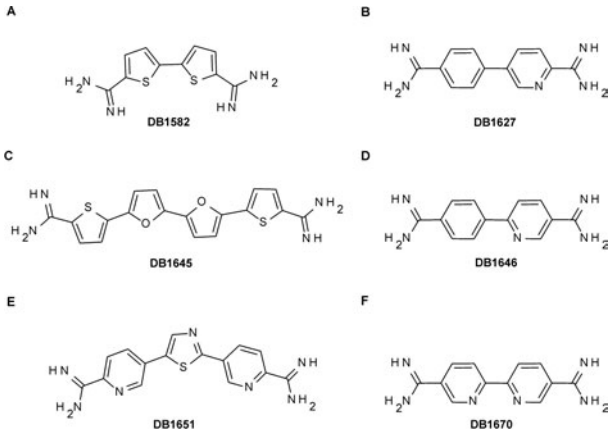

Fig. 1. Chemical structure of the six aromatic diamidines used in the present study.

Cytotoxicity tests

In order to rule out toxic effects of the compounds on host cells, uninfected cardiac cell cultures were incubated for 24 and 72 h at 37 °C in the presence or absence of the diamidines (10·6–96 μM) diluted in DMEM and then their morphology evaluated by light microscopy and the cell death rates measured by the MTT colorimetric assay (Mosmann, 1983). The absorbance was measured at 490 nm wavelength with a spectrophotometer (VERSAmax tunable, Molecular Devices, USA) allowing the determination of LC_{50} values (drug concentration that reduces 50% of cellular viability).

Trypanocidal analysis

Bloodstream trypomastigotes (5×10^6 per ml) were incubated for 24 h at 37 °C in RPMI 1640 medium (Roswell Park Memorial Institute – Sigma Aldrich, USA) supplemented with 10% of fetal bovine serum, in the presence or absence of serial dilutions of the compounds (0·04–32 μM). This short incubation time-period (24 h) is a standard protocol to avoid loss of cellular viability of these extracellular non-dividing parasite forms. In addition, the potential applicability of these compounds for blood bank

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prophylaxis was also evaluated by observation of the direct effect of the compounds on trypomastigotes at 4 °C maintained in freshly isolated mouse blood in the presence or absence of serial dilutions of the diamidines (up to 32 μM). After drug incubation, the parasite death rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the IC_{50} (drug concentration that reduces 50% of the number of the treated parasites) was then calculated. For analysis of the effect on intracellular parasites, after 24 h of parasite-host cell interaction (10 : 1 parasite : cardiac cell ratios), the parasitized cultures were washed to remove free parasites and then maintained at 37 °C in the presence or not of the compounds (0·04–32 μM), replacing the medium (with or without the respective drug) every 24 h. After 72 h of treatment that corresponds to 96 h of parasite infection, the supernatant of the infected cultures was recovered, the number of released parasites was quantified by light microscopy using a Neubauer chamber, and the corresponding IC_{50} values were averaged for at least 3 determinations done in duplicate.

Transmission electron microscopy (TEM) analysis

For TEM analysis, bloodstream trypomastigotes were treated for 2–24 h at 37 °C with the compounds at the concentration of their IC_{50} values, rinsed with PBS and fixed for 60 min at 4 °C with 2·5% glutaraldehyde and 2·5 mM $CaCl_2$ diluted 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 the same buffer. The parasites were dehydrated in a graded series of acetone and finally embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM-1011 electron microscope.

Fluorescence microscopy analysis

For fluorescence analysis, bloodstream trypomastigotes (3×10^6 cells/ml) and infected cardiac cell cultures (24 h of infection) were treated for 1 h with 10 μg/ml of each compound. Subsequently, the parasites and infected cultures were washed with PBS, fixed with 4% paraformaldehyde, mounted with 2·5% 1.4-diazabicyclo-(2.2.2)octane (DABCO) and fluorescence analysed with a x63 oil objective in a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc, Thornwood, New York), using a filter set for UV excited probes. Images were captured using the software AnalySIS OPTI.

RESULTS

The studies conducted to evaluate the efficacy of 6 cationic compounds upon bloodstream trypomastigotes of *T. cruzi* showed that DB1645, DB1582 and

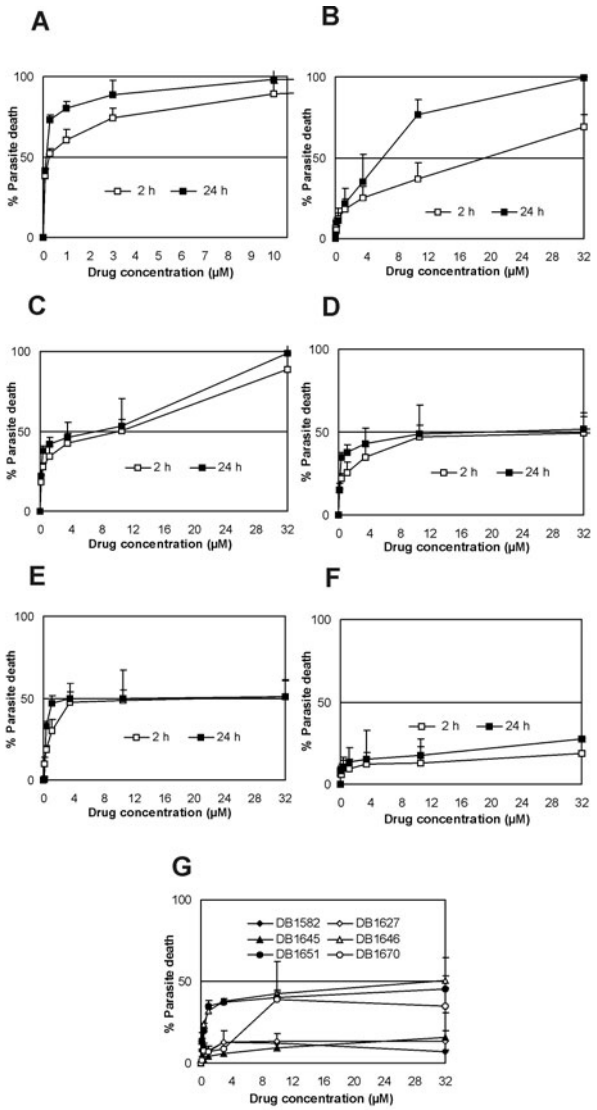

Fig. 2. Activity of the aromatic diamidines upon bloodstream trypomastigotes of *Trypanosoma cruzi in vitro*. (A) DB1645, (B) DB1582, (C) DB1651, (D) DB1646, (E) DB1670 and (F) DB1627. Effect upon the parasites evaluated during the treatment at 37 °C with the drugs diluted in the culture medium. (G) Effect upon the parasites evaluated during the treatment at 4 °C with the drugs diluted in mouse blood. The percentage of dead parasites was measured after 2 and 24 h of treatment.

DB1651 present a time-dependent trypanocidal effect, reaching sub- and micromolar IC_{50} values of 0·15, 6 and 6·9 μM, respectively, after 24 h of incubation at 37 °C (Fig. 2A–C, Table 1). On the other hand, DB1646 and DB1670 showed only modest activity against bloodstream parasites with IC_{50} values of 31 and 32 μM, respectively. DB1627 did not exert any trypanocidal effect, displaying an IC_{50} value higher than 32 μM (Fig. 2D–F, Table 1). However, further analysis of the trypanocidal effect upon bloodstream forms incubated in the presence of mouse blood, at 4 °C, resulted in a significant reduction of the activity of all compounds, giving IC_{50} values $\geqslant 32$ μM (Fig. 2G).

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Table 1. IC_{50} and SI values for the effect of the aromatic diamidines on *T. cruzi*

	Bloodstream trypomastigotes[1]		Intracellular parasites[2]	
Compounds	IC_{50} (μM)	SI[3]	IC_{50} (μM)	SI[3]
DB1582	6·0	15·9	11·8	8·1
DB1627	>32	3	>32	3
DB1645	0·15	640	7·3	13
DB1646	31	3	23·3	4·1
DB1651	6·9	13·9	13·3	7·2
DB1670	32	3	>32	3

SI = Selectivity index corresponds to the ratio LC_{50}/IC_{50}.
[1] Direct effect of the aromatic diamidines on bloodstream trypomastigotes performed after incubation for 24 h at 37 °C in RPMI.
[2] Effect on intracellular parasites measured by the determination of trypomastigotes released into the supernatant culture medium (96 h of infection) performed after 72 h of treatment at 37 °C.
[3] $LC_{50} > 96\ \mu$M.

Next, to assess possible toxic effects towards mammalian host cells, uninfected cardiac cultures were treated for 24–72 h/37 °C with increasing doses (up to 96 μM) of each di-cation and their morphology and viability were evaluated. The data demonstrated that after 24 h of incubation with 96 μM of all compounds induced less than a 20% loss in cellular viability (data not shown). Following treatment for 72 h, DB1627, DB1645, DB1651 and DB1670 resulted in 36, 21, 45 and 22% loss of cellular viability at 96 μM drug concentration, while both DB1582 and DB1646 gave a <20% reduction (data not shown).

The assessment of the anti-parasitic activity of the 6 compounds against the intracellular forms was performed by incubating the *T. cruzi*-infected cultures (24 h of parasite contact) with selected non-toxic doses (up to 32 μM) and the number of parasites released into the supernatant was quantified after 96 h of infection (corresponding to 72 h of drug treatment). A dose-dependent effect was observed, resulting in a decline in the number of parasites released, with IC_{50} values of 7·3, 11·8 and 13·3 μM for DB1645, DB1582, and DB1651, respectively (Fig. 3A–C, Table 1). While DB1646 showed a moderate effect (IC_{50} 23·3 μM) (Fig. 3D), both DB1670 and DB1627 did not show trypanocidal activity against intracellular parasites similar to the results found with bloodstream extracellular parasites (Fig. 3E–F).

The IC_{50} and LC_{50} findings allowed determination of the selectivity index (SI) for each compound tested. The results demonstrated that the most active compounds also displayed the highest SI values for both extracellular bloodstream trypomastigotes as well as for intracellular forms, as can be seen for DB1645, DB1582 and DB1651 which were 640, 15·9 and 13·9-fold, respectively (Table 1). Similarly, high SI values (13, 8·1 and 7·2), for DB1645, DB1582 and DB1651, respectively, in intracellular parasites (Table 1) were found.

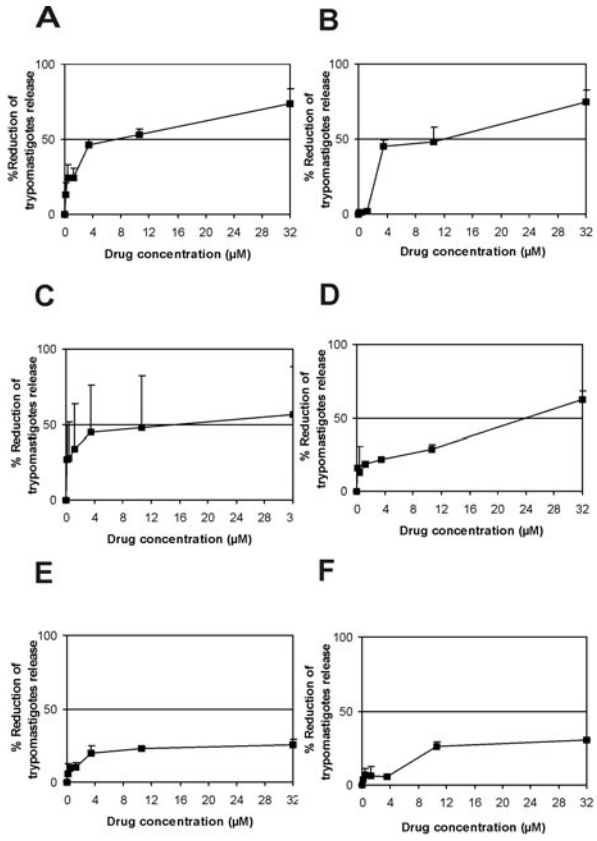

Fig. 3. Effect of the compounds upon intracellular parasites localized in *Trypanosoma cruzi*-infected cardiac cell cultures through the quantification of the number of parasites released into the supernatant of untreated and drug-treated infected cultures after 72 h of treatment at 37 °C. (A) DB1645, (B) DB1582, (C) DB1651, (D) DB1646, (E) DB1670 and (F) DB1627.

Due to the characteristics of the tested compounds, blue fluorescence is emitted when excited by UV light, the intracellular localization and distribution of these heterocyclic compounds in both

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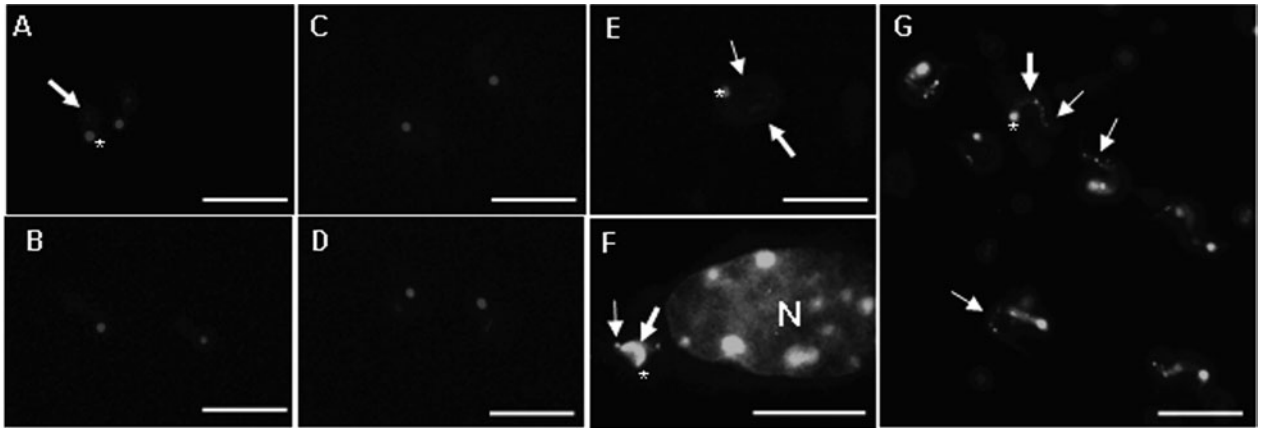

Fig. 4. Intracellular localization of the compounds in bloodstream (A–E, G) and amastigote (F) forms of *Trypanosoma cruzi* after 1 h of incubation with 10 μg/ml of each diamidine. (A) DB1627, (B) DB1646, (C) DB1645, (D) DB1670, (E, F) DB1651 and (G) DB1582. The accumulation of aromatic diamidines was higher in the kinetoplast (white asterisk) than in the nucleus (thick white arrow). Several non-DNA-containing organelles (thin white arrows) distributed within the cytoplasm of intracellular amastigotes (F) and bloodstream forms (E and G) can be noticed. Labelling in the nucleus of cardiac cells was also observed (F). N = Nucleus of host cell. Scale bar = 5 μm.

bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi* were detected by fluorescence microscopy. The data showed that in both parasite forms, the diamidine compounds presented a striking localization within the kDNA (asterisk) and at much lower levels in the parasite nuclei (thick white arrow) (Fig. 4A–G). Interestingly, DB1651 and DB1582 were also localized in several punctated non-DNA-containing organelles (thin white arrows) distributed within the cytoplasm of intracellular amastigotes (Fig. 4F) and bloodstream forms (Fig. 4E and G).

Finally, in order to investigate the cellular targets of these di-cationic compounds, transmission electron microscopy analysis was performed with drug-treated bloodstream parasites. Ultrastructural analysis of untreated bloodstream forms showed typical morphology for the nucleus, mitochondrion, kinetoplast, subpelicular microtubules (double black arrows) and flagellum (Fig. 5A–B). The treatment of parasites for 2–24 h with the di-cationic compounds (DB1582, DB1627, DB1645, DB1646, DB1651 and DB1670) induced several ultrastructural alterations mostly related to (i) severe mitochondrial damage, with organelle swelling (Fig. 5C, I and K), (ii) intense kDNA network disruption leading to its total fragmentation and disappearance (Fig. 5C, F, I and K), (iii) important disorganization in the plasma membrane (thick arrow) with bleb formation (Fig. 5J, arrowhead), appearance of concentric membranes between cytoplasm and plasmalemma unit, with its complete dissociation from the parasite body (Fig. 5F) and flagellum (Fig. 5E and G, thick arrow), (iv) nuclear alterations (Fig. 5J) and loss of cytoplasmatic constituents (Fig. 5J). Striking alterations could also be noticed in subpellicular (Fig. 5D, double arrow) and flagellar microtubules (Fig. 5E,H and J, inset, arrow) of the parasite under the action of the diamidine compounds. Commonly, multiple axoneme profiles were found in non-dividing bloodstream parasites incubated with the compounds without any evidence of other duplicated organelles (Fig. 5H and J, asterisks).

DISCUSSION

At the Centennial of the discovery of Chagas disease, no alternative therapy has been found to replace the two old and non-specific nitroheterocyclic drugs. The discovery of new effective and less-toxic compounds that could be applied to this neglected pathology is urgently needed (Soeiro and De Castro, 2009; Soeiro *et al*. 2008).

Studies were presently conducted *in vitro* to determine the efficacy of 6 synthetic heterocyclic di-cationic compounds (DB1645, DB1582, DB1651, DB1646, DB1670 and DB1627) against bloodstream trypomastigotes and intracellular forms of *T. cruzi*. The compounds studied can be divided into 3 groups, molecules with the size of DB75 or larger (DB1645, DB1651), a curved molecule smaller than DB75 (DB1582) and linear molecules smaller than DB75 (DB1627, DB1646, DB1670). The first 2 groups (DB1645, DB1651 and 1582) showed good activity whereas the smaller linear molecules were not effective. In addition, the cytoplasmatic localization and cellular targets of all these compounds in parasites were investigated. As noted above, the results showed that both parasite stages, which are relevant to mammalian infection, are sensitive to 3 of these compounds – DB1645, DB1582 and DB1651, presenting time- and dose-dependent antiparasitic activity, at sub- and micromolar IC_{50} levels. However, none were as potent as the arylimidamides, which were previously assayed against *T. cruzi* and demonstrated an excellent trypanocidal effect at the nanomolar level (Silva *et al*. 2007*a*, *b*; Pacheco *et al*.

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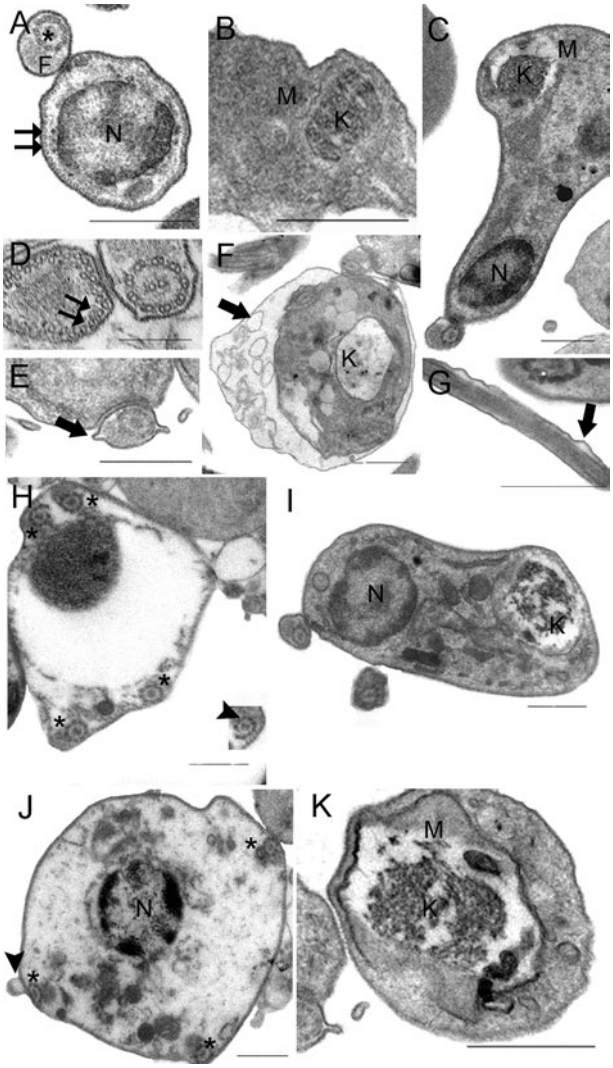

Fig. 5. Transmission electron micrographs of untreated bloodstream trypomastigote forms of *Trypanosoma cruzi* (A–B) and treated with the heterocyclic compounds for 2 (E–F, I and K) and 24 h (C–D, G–H and J). (C) DB1646, (D–F and K) DB1645, (G) DB1670, (H, J) DB1582 and (I) DB1651. (A–B) Untreated parasites showed characteristic nucleus (N), mitochondria (M), kinetoplast (K), subpellicular (double arrow) and axonemal (asterisks) microtubules. The compounds induced alterations in subpellicular (D, double arrow) and axonemal microtubules (H, inset, thin black arrow), detachment of plasma membrane (thick black arrow) of parasite body (F) and flagellum (E and G) and bleb formation (J, arrowhead), dramatic damage in the complex mitochondria-kinetoplast with kDNA disorganization and fragmentation (C, F, I and K) and multiple axoneme profiles (H–J, asterisks). Flagellum = F. A, C, E, F and H–J Scale bar = 0,5 μm. B, D and G Scale bar = 1 μm.

2009). The difference in efficacies of the diamidines and the arylimidamides is likely to be related to the significant differences in their physical properties. The amidines are highly basic molecules with pK_a values near 11, whereas the arylimidamides pK_a values are near 7. Consequently, at physiological pH amidines are protonated and thus cationic molecules and the arylimidamides are essentially neutral. This large difference in properties will significantly affect absorption and distribution and likely plays an important role in the differences in activity of the 2 classes of compounds.

Our present study also showed differences regarding drug susceptibility among intracellular parasites and bloodstream trypomastigotes, the latter being more sensitive. This disparity could possibly reflect differences in compound uptake and/or active extrusion, and/or different mechanisms of action upon non-dividing trypomastigotes and the highly multiplicative intracellular stages of the parasite, which are localized in the cytoplasm of infected host cells. In fact, although the precise mechanism of action of these cationic heterocyclic compounds has not been fully elucidated, it is likely that multiple modes of action are operative and then their transport represents a fundamental step in their action and contributing, in part, to their selectivity (Wilson *et al*. 2008; de Koning, 2001). Therefore it is possible that non-viable transport of the drugs to the cytoplasmic milieu (of both host cells and *T. cruzi*) could account for the lower susceptibility found in the intracellular parasites. However, the fact that these di-cationic compounds are localized in the nuclei of cardiac cells as well as within the kinetoplast and nuclei of amastigotes confirms their ability to cross the host cell plasmatic membrane reaching the intracellular parasites. Similar observations have been reported for other diamidines such as DB569 (De Souza *et al*. 2004). While several potential transporters that effectively carry diamidines have been studied in other parasites, including African trypanosomes, *Leishmania* species and *Plasmodium falciparum* (Carter *et al*. 1995; Bray *et al*. 2003; Barrett and Gilbert, 2006), the mechanisms of uptake of diamidines by *T. cruzi* is still unknown and deserves further investigation in order to understand the different profiles of susceptibility among the different evolutive forms of this protozoan.

The efficacy of these heterocyclic diamidines upon bloodstream forms was also evaluated in the presence of mouse blood, at 4 °C, considering the possible application of these compounds for prophylaxis of banked blood. Our present results show that all compounds display reduced activity in the presence of blood possibly due to their propensity to bind serum proteins as previously reported for other compounds or possibly due to drug instability or metabolism in the presence of blood constituents (Santa-Rita *et al*. 2006; Silva *et al*. 2008).

The fluorescence of many of these heterocyclic compounds makes it possible to follow their distribution in *T. cruzi* as was previously performed with African trypanosomes (Mathis *et al*. 2006, 2007; Wilson *et al*. 2008). As found with African trypanosomes incubated with the heterocyclic diamidines

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DB75 and DB820 (Mathis *et al*. 2006, 2007), we also observed that all compounds display very strong fluorescence in the kinetoplast and, with less intensity, in the parasite nucleus. DB1651 and DB1582 also accumulated in non-DNA-containing punctuated organelles preferentially localized in the anterior portion of bloodstream trypomastigotes and near the nuclei and kinetoplast regions of amastigotes of *T. cruzi*. According to this intracellular distribution and morphology, and as suggested by previous studies with African trypanosomes (Mathis *et al*. 2007), these organelles are possibly acidocalcisomes, although other non-DNA-containing organelles can not be excluded. Acidocalcisomes are acidic calcium-storage organelles found in a diverse range of organisms, being first described in trypanosomes (Vercesi *et al*. 1994; Docampo *et al*. 1995). It is possible, as suggested for African trypanosomes, that the localization of these compounds within these acidic organelles of *T. cruzi* could also play a role in their mechanism of action and/or act as storage sites (Mathis *et al*. 2006, 2007). Previous studies performed with *T. brucei* reported that the localization of DB75 and DB820 (and their analogues) within non-DNA compartments was a time-dependent event (Mathis *et al*. 2006). However, in the studies performed here only 1 h of drug incubation was involved. Therefore, future studies are planned to further investigate whether longer periods of treatment (with DB1627, DB1645, DB1646 and DB1670) would lead to their localization in these cytoplasmatic organelles.

Since alterations in the fine structure of parasites evaluated by transmission electron microscopy provide insights into the nature of drug-induced lesions, allowing deduction of possible modes of action (Rodrigues and de Souza, 2008; de Souza, 2008), ultrastructural aspects of *T. cruzi* treated with the 6 compounds was investigated. The most prominent and usual alterations noticed with all compounds were related to changes in mitochondrial structure and the disorganization of the kDNA, as has been previously detected in *T. cruzi* treated with other diamidines (De Souza *et al*. 2004; Silva *et al*. 2008) as well as arylimidamides (Silva *et al*. 2007*b*). Other interesting findings were the dramatic alterations of microtubule organization induced by these heterocyclic compounds. In bloodstream trypomastigotes, the non-proliferative stage of *T. cruzi*, we observed that DB1582 induced disruption of microtubules axonemal organization and an unusual organization of multiple flagella without any evidence of flagellum duplication, as also previously reported for arylimidamides (Silva *et al*. 2007*b*). Some of the parasites treated with DB1645 also presented alterations in the structural organization of sub-pellicular microtubules in addition to modifications in the basic structure of (9 + 2) axonemal microtubules, showing more than 2 central microtubules. Microtubules are dynamic and very stable structures that play a role in several biological processes of protozoal parasites including cellular structural maintenance (subpellicular microtubules); motility (flagellar microtubules); and proliferation (basal body and mitotic spindles) (Menna-Barreto *et al*. 2009). Although different studies have been conducted using drugs that target microtubules such as taxol, colchicine and vinblastine, no major alterations were noticed in both subpellicular and flagellar microtubules of *T. cruzi* possibly due to the high content of acetylated tubulin and/or poly-glutamylation of tubulin (Souto-Padron *et al*. 1993; Dantas *et al*. 2003). Since these structures in trypanosomatids are very resistant to microtubule disrupters compared to those in mammalian cells they may represent interesting targets for drug development. Further investigations are needed to better understand the effect, if any, of these heterocyclic dicationic compounds upon *T. cruzi* microtubules.

Despite their high activity against a broad spectrum of microorganisms, a major concern for diamidines and related compounds is their selectivity (Soeiro *et al*. 2005). Therefore it is quite promising that these compounds show low toxicity towards mammalian cells. The most effective compound, DB1645, which gave excellent IC_{50} values against bloodstream trypomastigotes and intracellular parasites, exerted very low toxicity even after 72 h of treatment of cardiac cell cultures, leading to high selectivity indices (640 and 13, respectively). The identification of effective and selective compounds is a crucial element in drug development and the results reported herein justify further study of this class of compounds in experimental models of *T. cruzi* infection.

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