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# Phenyl substitution of furamidine markedly potentiates its anti-parasitic activity against *Trypanosoma cruzi* and *Leishmania amazonensis*

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**Abstract**

Furamidine (DB75) and related unfused aromatic diamidines have proven useful for the treatment of parasitic infections. These compounds were primarily developed to combat infections by *Pneumocystis carinii* and African trypanosomes but they are also active against other parasites. Here we have investigated the in vitro effects of DB75 and its phenyl-substituted analog DB569 on two kinetoplastid haemoflagellates Trypanosomatidae: *Trypanosoma cruzi* and *Leishmania (L) amazonensis*. The phenyl-amidine compound DB569 has equivalent DNA binding properties compared to DB75 but it was selected on the basis of its distinct tumor cell distribution properties. We found that DB569 is significantly more potent than DB75 at reducing the proliferation of the parasites, using either isolated parasites in cultures or with cardiomyocyte and macrophage host cells. DB569 is effective towards the intracellular forms of *T. cruzi* ($IC_{50}$ in the low-micromolar range) and it exhibits trypanocidal dose-dependent effects against trypomastigote forms of *T. cruzi* parasites obtained from the Y strain and Dm28c clone, which belong to two different biodemes. Fluorescence microscopy experiments indicated that both diamidines were mostly localized in the nucleus of the mammalian host cells and within the nuclei and kinetoplast of the parasites. Electron microscopy studies showed that the treatment of the parasites with DB75 and DB569 induces important alterations of the parasite nucleus and kinetoplast, at sites where their DNA target is localized. Altogether, the data suggest that the phenyl-substituted furamidine analogue DB569 is a potential new candidate for the treatment of the Chagas' disease and Leishmaniasis.


*Keywords:* Furamidine analogues; *Trypanosoma cruzi*; *Leishmania amazonensis*; Cardiomyocytes; Peritoneal macrophages; Electron microscopy

## 1. Introduction

Parasitic protozoa are unicellular pathogens with a complex life cycle displaying distinct morphological stages during their development in both vertebrate and invertebrate hosts. They can cause severe diseases, which represent major causes of mortality and morbidity in several endemic areas [1]. However, up to now no vaccines or safe chemotherapeutic agents are available for preventing or treating most of the protozoan infections in humans [2]. Among the protozoa, members of the Trypanosomatidae family include a large number of species that cause human diseases such as sleeping sickness, Chagas' disease and Leishmaniasis [3]. It is estimated that 16–18 and 12 million people suffer from Chagas' disease and Leishmaniasis, respectively [1].

Recent literature reviews clearly point to the need of finding more efficient and less toxic chemotherapeutic approaches for both Chagas' disease and Leishmaniasis [4–7]. In this respect, diphenylfuran diamidines represent an important, promising class of DNA-targeted anti-parasitic agents [8]. The best-known member of the series is compound DB75 commonly referred to as furamidine, a bis-amidine diphenylfuran derivative that displays potent anti-microbial activities against various organisms such as *Pneumocystis carinii*, *Giardia lamblia*, *Plasmodium falciparum* and *Trypanosoma rhodesiense* [8]. An amidoxime prodrug of furamidine is currently undergoing phase II clinical trials against human African trypanosomes [9].

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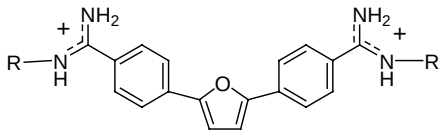


**DB75** R = H **(furamidine)**

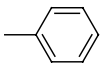
**DB569** R = 

Fig. 1. Structures of the two drugs used in this study.

In the present study we have explored the in vitro anti-parasitic activity of furamidine (DB75) and its phenyl-substituted analogue DB569 (Fig. 1) against two kinetoplastid haemoflagellates members of the family Trypanosomatidae: *Trypanosoma cruzi* and *Leishmania (L) amazonensis*, which have a comparable life cycle with alternating proliferative and non-proliferative stages. DB569 was selected on the basis of a recent structure–activity relationship study aimed at evaluating its subcellular distribution in tumor cells [10]. DB569 has quite similar DNA binding properties to those of DB75; it binds selectively to the minor groove of AT-rich DNA sequences but the two drugs were found to exhibit distinct cellular distribution profiles in tumor cells.

## 2. Material and methods

### 2.1. Drugs

The syntheses of D75 [11] and DB569 [10] have been reported. Stock solutions (5 mM) of the drugs were prepared in water and fresh dilutions were prepared extemporaneously.

### 2.2. Cell cultures

Primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously described [12]. After purification, the CM were seeded at a density of $0.15 \times 10^6$ cell/well into 24-well culture plates containing gelatin-coated cover slips and sustained in Dulbecco's modified medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FCS), 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract as described in the cited reference. Peritoneal mouse macrophages (PM) were obtained as described elsewhere [13], seeded at a density of $0.30 \times 10^6$ cell/well into 24-well culture plates containing cover slips and sustained in DMEM supplemented with 4 mM L-glutamine and 5% FCS (DMEMS). All cell cultures were maintained at 37 °C in an atmosphere of 5% $CO_2$ and air and the assays were run three to five times at least in duplicates.

### 2.3. Parasites

*L. (L) amazonensis* (MHOM/BR/77/LTB0016) parasites isolated from a human case of cutaneous leishmaniasis were used in all experiments [14]. Briefly, amastigotes were isolated from lesions of heavily infected animals and maintained at 25 °C in blood agar with brain heart infusion medium and an overlay of modified LIT (liver infusion-Trypticase) medium [15]. Afterwards, promastigote forms were obtained on the 7th day of cultivation, harvested by centrifugation at $1.500 \times g$ for 10 min at 4 °C and washed twice in 0.01 M phosphate-buffered saline (PBS) before the experiments. Y and Dm28c stocks of *T. cruzi* were used throughout the experiments. Cell culture-derived trypomastigotes (Dm28c clone) were isolated from the supernatant of CM, which have been previously infected with metacyclic forms [16]. Bloodstream trypomastigotes from Y strain were harvested by heart puncture from *T. cruzi* infected Swiss mice at the parasitaemia peak day [17].

### 2.4. In vitro infection assays

After 24 hours of platting, CM and PM were infected with trypomastigotes of *T. cruzi* for 2 and 24 hours at 37 °C employing parasite: host cell ratio of 10:1. After the initial host–parasite contact, the cultures were washed to remove free parasites and treated or not for different periods of time (2–24 hours) with graded concentrations of DB569 and DB75.

### 2.5. Drug assays

For the analysis of the effect of the diamidines on the extracellular flagellated forms, isolated parasites were incubated for 2 and 24 hours in the presence or not of 2–32 μM DB75 or DB569, followed by the determination of parasite viability and death rates through light microscopy using a Neubauer chamber. For the assessment of the anti-parasitic activity of the diamidines on intracellular parasites lodged into the host cells, infected cultures were incubated with increasing but non-toxic doses of the test drug. After 2 and 24 hours of treatment, the cultures were washed with PBS and fixed for 20 min with 4% paraformaldehyde solution (PFA) diluted in PBS.

### 2.6. Fluorescent analysis

After fixation, drug-treated cultures were incubated with 2 μg/mL propidium iodide (PI) for DNA staining to enable visualization of parasites and host cells nuclei and direct quantification of the parasite infection levels. Finally, the samples were mounted with 2.5% 1.4-diazabicyclo-(2.2.2)-octane (DABCO) and examined using a Zeiss photomicroscope equipped with epifluorescence (Zeiss INC, Thornwood, NY). The mean number of infected host cells was then counted in at least 200 host

cells of four independent experiments run in duplicates. Only characteristic parasite nuclei and kinetoplasts were counted as surviving parasites since irregular structures could mean parasites undergoing death. The drug activity was estimated by calculating the percentage of reduction in the number of infected host cell by counting the number of infected host cells in treated cultures/number of infected host cells in untreated cultures (run in parallel) × 100.

### 2.7. TUNEL reaction

The analysis of host apoptosis was carried out by the terminal deoxynucleotidyltransferase-mediated fluorescein dUTP nick end-labeling technique (TUNEL) using an apoptosis-detection kit (Boehringer Mannheim), as previously described [18].

### 2.8. Statistical analysis

Statistical analysis was carried out using the Student's *t*-test, with the level of significance set at $P < 0.05$. The data are representative of three to five experiments run in duplicate.

### 2.9. Transmission electron microscopy (TEM) analysis

For TEM analysis, bloodstream trypomastigotes of *T. cruzi* were treated for 18 or 2 hours with 8 μM DB75 and DB569, respectively, washed with PBS and fixed for 1 hour with 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate buffer. After washing, the samples were post-fixed with 1% osmium tetroxide in the same buffer cacodylate. The parasites were then dehydrated in a graded series of acetone and finally embedded in epon. Sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 10 C transmission electron microscope.

## 3. Results

Furamidine (DB75) showed a modest dose and time-dependent trypanocidal effect on bloodstream trypomastigotes (Fig. 2A) whereas its phenyl-substituted analogue DB569 exhibited a much higher activity, reaching a low $IC_{50}$ value of 2 μM after 2 hours of drug incubation and presenting maximum death rates higher than 90% after 24 hours treatment with 8 μM (Fig. 2C).

We investigated the effect of DB569 upon trypomastigote forms obtained from a different biodeme of *T. cruzi*, the Dm28c clone, which belongs to the lineage 1. The lineage 2 (such as Y stock) predominates in the domestic cycle while lineage 1 is mainly represented in the sylvatic cycle [3]. As shown in Fig. 2C, DB569 also exhibits a trypanocidal dose-dependent effect against parasites from the Dm28c stock but these later parasites were less susceptible to the drug than Y parasites (Fig. 2C).

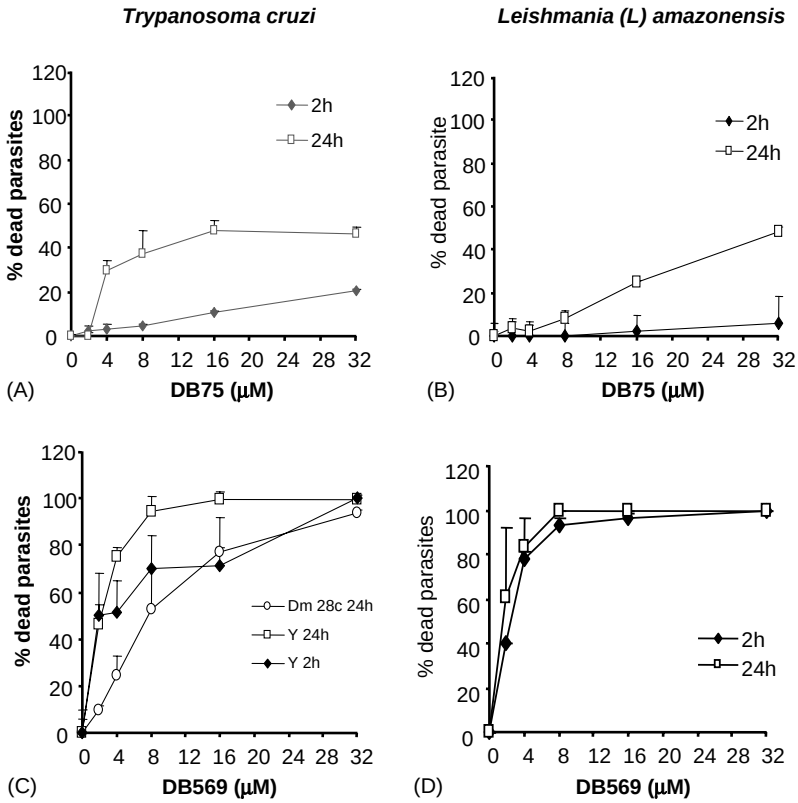



Fig. 2. Activity of (A and B) DB75 and (C and D) DB569 on (A and C) extracellular forms of *T. cruzi* and (B and D) promastigotes of *L. (L) amazonensis*. The percentage of dead parasites was measured after 2 and 24 hours of treatment.

In parallel, we tested the compounds against *L. (L) amazonensis*. DB75 showed a dose dependent activity against the promastigote forms (Fig. 2B) and a considerably superior activity was measured with DB569 (Fig. 2D). Therefore, both diphenylfuran diamidine compounds exert anti-parasitic activity against *T. cruzi* and *L. (L) amazonensis* extracellular parasites. The phenyl-substituted compound DB569 exhibits lower $IC_{50}$ values than the parent compound.

The effect of the two compounds on intracellular parasites was studied using mammalian cells. In order to rule out toxic effects of the drugs on the host cell, different doses of DB75 and DB569 were incubated with uninfected host cells and the cell viability was determined. High concentrations of both drugs (>32 μM) after 24 hours of treatment induced alterations in the cell physiology due to impaired cardiomyocytes contractions and intense vacuolization in both heart cells and peritoneal macrophages leading after longer periods of time (24–48 hours) to cell detachment. A marked increased nuclear fragmentation (i.e. a higher number of TUNEL positive cells) was noted after incubation of the host cells with a high dose of DB569 for 48 hours (data not shown). For the assessment of the anti-parasitic activity of the two compounds against the intracellular forms of the parasites, the infected cultures were incubated with selected non-toxic doses (≤16 μM) of each drug.

We assayed the cardiomyocyte primary cultures as host cells since the cardiac cells represent one of the most affected tissues during infections. The trypanocidal activity of the two diamidines was studied using the two aforementioned *T. cruzi* stocks: the Y strain (type II biodeme) and the Dm28c clone (type I biodeme). When cardiomyocytes were infected with Y parasites and further incubated for 2 and 24 hours with different doses of DB75, the number of infected cells only slightly decreased in the treated cultures as compared to untreated infected cardiac cells (Table 1). In contrast, a considerable reduction in the percentage of infected cardiomyocytes was observed when infected cultures were treated with DB569, reaching 73% reduction with a 2 μM exposure to the drug for 24 hours (Table 1). When cardiac cells were infected with Dm28c parasites and then treated for 2 and 24 hours it was noted that DB569 had trypanocidal activity towards the intracellular parasites, however, not to the extent of that with the Y stock, causing less than 30% reduction in the parasite infection on 24 hours exposure to 2 μM dose (Table 1).

To further explore the effectiveness of DB569 towards the intracellular forms of *T. cruzi*, the effect of this drug during the interaction of Dm28c stock with another host cell type, peritoneal macrophages was assayed. After treatment for 24 hours with 2 μM a reduction of about 78% in the number of infected host cells was noted (Table 1), confirming the high trypanocidal activity of this

Table 1
Reduction (%) in the number of *T. cruzi*-infected host cells after treatment for 24 hours with DB75 and DB569

| Drug (μM) | Cardiomyocytes | | | Macrophages |
|---|---|---|---|---|
| | Y stock | | Dm28c stock | |
| | DB75 | DB569 | | |
| 0 | 0 | 0 | 0 | 0 |
| 2 | 16±2 | 73±8 * | 26±22 | 78.5±3.5 * |
| 4 | 5±7 | 74.5±1.2 * | 63±2 * | 89±11 * |
| 8 | 16±1.8 | 95±3 * | 77±11 * | 89±8 * |
| 16 | 16±6 | 99±1 * | 91±4 * | 88.5±10.6 * |

The activity of the drugs was estimated by calculating the percentage of reduction in the number of infected host cells by counting the number of infected host cells in treated cultures/number of infected host cells in untreated cultures (run in parallel) × 100; (*means statistically significance $P \leq 0.05$).

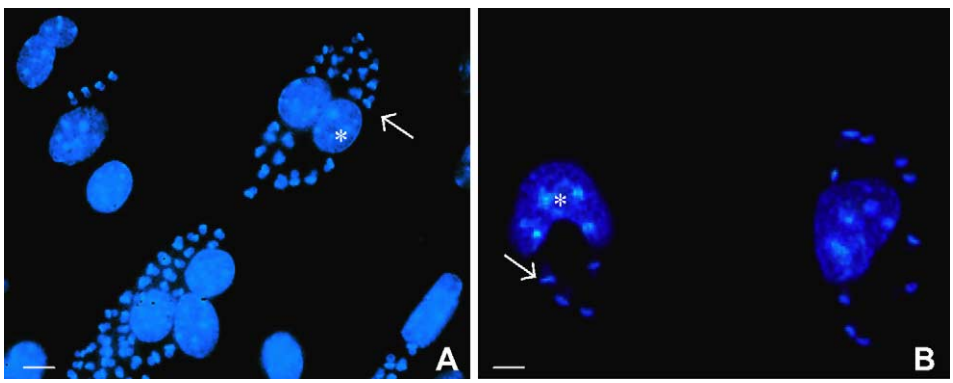



Fig. 3. Fluorescence analysis of the DB75 and DB569 localization within host cells and intracellular forms of *T. cruzi*. (A) Drug localization within cardiomyocytes infected for 24 hours with *T. cruzi* (Dm28c stock) and treated for 2 hours with 2 μM DB569. (B) Furamidine localization in peritoneal macrophages infected for 2 hours with Dm28c trypomastigotes and then treated for another 2 hours with 8 μM DB75. Note the furamidine analogues labeled in the host cell nuclei (*) and within the kinetoplast and nuclei of the intracellular parasites (arrow). Bar = 20 μM.

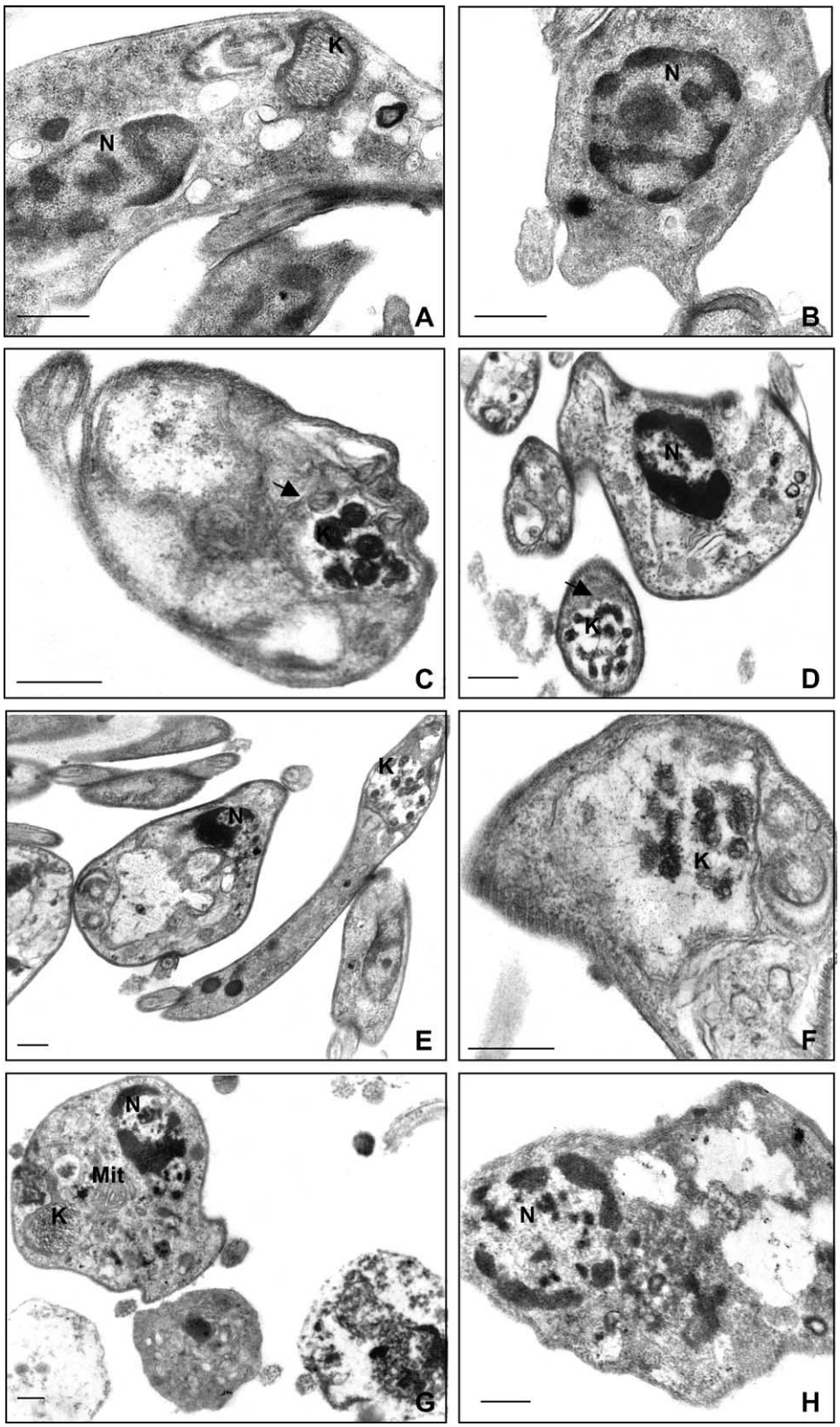



Fig. 4. Ultrastructural analysis of the effects of DB75 and DB569 in bloodstream trypomastigote forms of *T. cruzi*. (A and B) Untreated parasites show typical organelles such as nucleus with a centrally located nucleolus and chromatin mostly localized in the periphery (N) and mitochondrion containing a large condensation of DNA, the kinetoplast (K), with the characteristic basket-like shape. (C–F) The parasites treated with 8 μM DB569 for 2 hours showed damaged kinetoplast with signs of fragmentation (arrow) and severe alterations on the nucleus (N). (G–H) The incubation of bloodstream trypomastigotes with 2 μM DB75 for 18 hours also showed alterations in the nucleus (N), swollen mitochondria (N) and altered kinetoplast (K). Bar = 0.5 μM.

compound. Both evolutive forms found in the mammalian host are susceptible to furamidine type compounds, with DB569 being more effective than DB75.

Taking advantage of the intrinsic blue fluorescence of the diphenylfuran unfused aromatic system we monitored the distribution of the compounds in the host cells and *T. cruzi* by fluorescence microscopy. Both analogues were mostly localized within the host cell nuclei such as cardiomyocytes (Fig. 3A) and peritoneal macrophages (Fig. 3B). In the parasite the drugs localize within both nuclei and the kinetoplast (Fig. 3), which could be identified by co-localization with propidium iodide labeling (data not shown).

Electron microscopy measurements were performed to investigate the morphological damage induced by the diamidine derivatives at the ultrastructural level. In the untreated parasites, two organelles, the nucleus (Fig. 4A and B) and the kinetoplast, which is the hallmark of the Kinetoplastida order, were easily identified. The kinetoplast has a characteristic basket-like shape in the trypomastigotes (Fig. 4A). When incubated with either DB569 or DB75, the parasites displayed altered morphology in the kinetoplast–nucleus complex. Swollen mitochondria, fragmentation of the kinetoplast and profound alterations in the nuclear morphology were observed (Fig. 4C–H).

## 4. Discussion

At present, specific chemotherapy for the Chagas' disease remains unsatisfactory mainly because the *T. cruzi* parasite displays a wide range of host tissue tropism where it can replicate within infected mammalian hosts [6,7]. Considering the promising anti-parasitic activity of diamidines, and furamidine in particular, against African trypanosomes we reasoned that this compound might also be a good candidate to treat Chagas' disease. Since furamidine and analogues have previously revealed potent activities against various microorganisms including *P. carinii*, *P. falciparum* and *T. rhodesiense* [8], we decided to evaluate its effect on the two evolutive stages of *T. cruzi* found in the mammalian hosts and towards promastigote forms of *L. (L) amazonensis*. Furthermore, the phenyl-substituted diamidine DB569 which has quite similar DNA-binding capacity to that of DB75 but was shown to exhibit a distinct uptake profile in cancer cells [10] was studied. The substitution of the amidine ends of furamidine by phenyl groups does not alter the capacity of the drug to bind selectively within the minor groove of DNA at AT-rich sites. Surface plasmon resonance study indicated that the affinity of DB569 for AT sites is only reduced by a factor of about 2 compared to DB75 but remains high, in the $10^7\ M^{-1}$ range [10]. The dicationic nature of these two diamidines is essential to their DNA binding capacity. The related monocations show a much lower affinity for DNA and little or no activity [19]. The present study shows that the two drugs exert anti-parasitic activities against the two parasites tested and in both cases, DB569 was found to be significantly more potent than DB75. The substitution of the amidine function with a phenyl group enhances the trypanocidal activity of the drug, both with trypomastigotes and intracellular amastigote forms of *T. cruzi* and the promastigote forms of *L. (L) amazonensis*. It is also interesting to note that DB569 presented a higher in vitro activity than DB75 with the two different parasite stocks corresponding to biodemes I and II. However, Dm28c parasites, which lead to an earlier invasion and higher proliferation rates as compared to Y stock [16,20], are less sensitive than Y parasites suggesting that different mechanisms for drug action may be involved. This aspect will obviously deserve to be explored further.

In agreement with previous studies employing tumor cell lines [10], we found that furamidine is weakly toxic to mammalian cells. Only high drug concentrations (>32 μM) induced alterations in the cellular physiology of the host cells leading to an intense DNA fragmentation of both the cardiac and phagocyte cells, which can be suggestive of apoptosis.

The natural fluorescence of the diphenylfuran unfused aromatic system is useful to localize the compounds into cells by means of fluorescence microscopy. This method has proved informative and, earlier we found a different distribution for the two drugs in tumor cells. DB75 was exclusively nuclear whereas DB569 was largely located in cytoplasmic granules, which corresponded to mitochondria [10]. Here the situation is different as we observed identical distribution profiles for the two compounds with both accumulated in the nuclear compartment and the kinetoplast of *T. cruzi*. The nuclear membrane of the parasite is perhaps more permeable to the phenyl-substituted analogue than the nuclear membrane of mammalian cells. The mechanism by which these cationic molecules reach the intracellular milieu remains largely unknown. Specific transporters have been characterized for pentamidine in *T. brucei* [21] but it is not known at present if furamidines use the same transport machinery to accumulate in trypanosomes or other parasites. Given that their DNA binding properties are similar, it is conceivable that the superior activity of DB569 compared to DB75 on *T. cruzi* arises either from interaction with another non-DNA target or from a different uptake capacity. The substitution of the amidine functions with a phenyl group enhances the lipophilicity of the molecule and lowers the $pK_a$ of the amidine group. The modulation of these physicochemical parameters could facilitate the delivery of the molecule to these intracellular parasites, explaining, at least in part, the improved anti-parasitic activity.

Transmission electron microscopy was applied to explore the damages induced by the drugs in bloodstream *T. cruzi* parasites. The ultrastructural analysis clearly showed that the morphological changes were more severe

in the nucleus and kinetoplast, which resulted in the mitochondrial enlargement and final fragmentation of the kinetoplast into condensed bodies. These ultrastructural alterations are directly reminiscent to those previously reported with pentamidine and analogues for in vitro treatments of *L. (L) amazonensis* [22], *L. tropica* [23,24] and *L. major* [25] and in vivo treatment of *L. donovani* and *L. major* in mouse models [26]. This suggests a common mechanism of action. Pentamidine and furamidine represent comparable classes of DNA minor groove binders and it is particularly interesting to note that they cause damage principally in DNA-containing organelles. The ultrastructural perturbations of the kinetoplast may results from drug binding to catenated kDNA, which has a high AT content [27]. Several structure–activity relationship studies have reported a satisfactory correlation between the ability of furamidine analogues to bind to AT sites in DNA and their anti-parasitic activity [28–33].

In summary, the present data indicate that furamidine displays anti-parasitic activities against two kinetoplastid haemoflagellates members of Trypanosomatidae family, *T. cruzi* and *L. (L) amazonensis*, and the phenyl substitution of its amidine termini significantly enhances the anti-parasitic activity. The furamidine analogue DB569 appears as a promising candidate for further evaluations in vivo.

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