In vitro analyses of the effect of aromatic diamidines upon Trypanosoma cruzi

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Objectives: Aromatic diamidines (ADs) have been recognized as promising antiparasitic agents. Therefore, in the present work, the in vitro trypanocidal effect of 11 ADs upon the relevant clinical forms of Trypanosoma cruzi was evaluated, as well as determining their toxicity to mammalian cells and their subcellular localization.

Methods: The trypanocidal effect upon trypomastigotes and amastigotes was evaluated by light microscopy through the determination of the IC50 values. The cytotoxicity was determined by the MTT colorimetric assay against mouse cardiomyocytes. For the subcellular localization, transmission electron microscopy and fluorescence approaches were used. The fluorescence intensity within the kinetoplast DNA (kDNA) and nuclear DNA (nDNA) of treated parasites was determined using the Image J program.

Results: Compounds 2, 5 and 7 showed the lowest IC50 values (micromolar range) against intracellular amastigotes and trypomastigotes. In the presence of blood, all the tested ADs exhibited a reduction of their activity. The compounds did not exhibit toxicity to cardiac cells and the highest selectivity index (SI) was achieved by compound 5 with an SI of >137 for trypomastigotes and compound 7 with an SI of >107 for intracellular parasites. The subcellular effects upon bloodstream forms treated with compounds 5 and 7 were mainly on kDNA, leading to its disorganization. The higher accumulation in the kDNA observed for all tested ADs was not directly related to their efficacy.

Conclusions: Our results show the high activity of this new series of ADs against both trypomastigote and amastigote forms, with excellent SIs, especially compound 7, which merits further in vivo evaluation.

Keywords: chemotherapy, Chagas disease, kDNA, intracellular localization

Introduction

Chagas disease, caused by Trypanosoma cruzi, is endemic in Latin America. The currently accepted drugs for its treatment are nifurtimox and benznidazole, and their efficacy for the chronic phase is still debatable; besides that they present side-effects frequently forcing the abandonment of the treatment. Since the use of pentamidine for human African trypanosomiasis, aromatic diamidines (ADs) have been recognized as promising antiparasitic agents, presenting striking broad-spectrum antimicrobial effects possibly related to their DNA binding properties. However, due to their poor oral bioavailability and unfavourable side-effects, new analogues have been synthesized and assayed in vitro and in vivo. Previous results from our laboratory showed high efficacy of ADs and analogues, such as reversed amidines or arylimidamides, against T. cruzi both in vitro and in vivo. In the present work we evaluate the in vitro activity of 11 ADs against the relevant clinical forms of T. cruzi and their toxicity to mammalian cells. Additionally, mechanistic fluorescence studies and transmission electron microscopy analysis were performed in order to investigate their intracellular distribution.

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Methods

Drugs
All dicarboxylic aromatic compounds tested in this study [Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)] were synthesized according to published procedures. Stock solutions of the drugs (5 mM) were prepared in DMSO.

Cell cultures and cytotoxicity assays
Primary cultures of embryonic cardiomyocytes (CMs) were purified following the method previously described. In order to rule out toxic effects of the compounds on the host cell, uninfected CM cultures were incubated at 37°C with compounds 1–11, for 24 h (10.7–96 μM) and 72 h (10.7–32 μM). The cell death rates were measured by the MTT colorimetric assay allowing the determination of LC50 values (compound concentration that reduces 50% of cellular viability). 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).

In vitro antitrypanosomal activity
Bloodstream trypomastigote forms of the Y strain of T. cruzi were obtained from infected albino Swiss mice at the peak of parasitaemia as previously reported. They were incubated for 24 h in the presence or not of serial dilutions of compounds 1–11 (0.04–32 μM). Alternatively, the treatment was performed with freshly isolated mouse blood at 4°C, with concentrations up to 96 μM. The parasite death rates were determined through direct analysis by light microscopy and calculating the IC50 values (compound concentration that reduces 50% of cellular viability). 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).

Table 1. IC50 and SI values of the tested ADs on T. cruzi

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM), 4°C, whole blood</th>
<th>IC50 (μM), 37°C, medium</th>
<th>SI</th>
<th>IC50 (μM), 72 h</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91.6 ± 20.2</td>
<td>&gt;32</td>
<td>&gt;3</td>
<td>&gt;32</td>
<td>&gt;1</td>
</tr>
<tr>
<td>2</td>
<td>&gt;400</td>
<td>2.7 ± 0.5</td>
<td>&gt;35.5</td>
<td>1.7 ± 0.8</td>
<td>&gt;19</td>
</tr>
<tr>
<td>3</td>
<td>229.3 ± 43.8</td>
<td>20.9 ± 3.8</td>
<td>&gt;46</td>
<td>5.1 ± 2.7</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>4</td>
<td>&gt;400</td>
<td>19.0 ± 2.8</td>
<td>&gt;5</td>
<td>11 ± 5.5</td>
<td>&gt;3</td>
</tr>
<tr>
<td>5</td>
<td>128.6 ± 18.3</td>
<td>0.7 ± 0.1</td>
<td>&gt;137</td>
<td>0.8 ± 0.2</td>
<td>&gt;40</td>
</tr>
<tr>
<td>6</td>
<td>&gt;400</td>
<td>&gt;32</td>
<td>&gt;3</td>
<td>&gt;32</td>
<td>&gt;1</td>
</tr>
<tr>
<td>7</td>
<td>128.6 ± 11.8</td>
<td>1.0 ± 0.1</td>
<td>&gt;96</td>
<td>0.3 ± 0.2</td>
<td>&gt;107</td>
</tr>
<tr>
<td>8</td>
<td>&gt;400</td>
<td>&gt;32</td>
<td>&gt;3</td>
<td>&gt;32</td>
<td>&gt;1</td>
</tr>
<tr>
<td>9</td>
<td>&gt;400</td>
<td>1.9 ± 0.9</td>
<td>&gt;50</td>
<td>16.4 ± 6.3</td>
<td>&gt;2</td>
</tr>
<tr>
<td>10</td>
<td>135.8 ± 33.7</td>
<td>16.3 ± 1.4</td>
<td>&gt;6</td>
<td>8.3 ± 1.4</td>
<td>&gt;4</td>
</tr>
<tr>
<td>11</td>
<td>&gt;400</td>
<td>&gt;32</td>
<td>&gt;3</td>
<td>16.0 ± 6.0</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

SI, selectivity index.

*Direct effect of the ADs on trypomastigotes performed after 24 h at 4°C in whole blood or at 37°C in RPMI.

**Effect on intracellular parasites measured by trypomastigote release into the supernatant of culture medium (96 h of infection) performed after 72 h of treatment at 37°C.

Statistical analysis was carried out using Student’s t-test, with the level of significance set at P<0.05. All the assays were run at least three times in duplicate.

Results
The direct effect of the ADs on bloodstream trypomastigotes was evaluated after 24 h of treatment at 37°C. The most active compounds were 2, 5, 7 and 9 with IC50s between 0.7 and 2.7 μM (Table 1). All compounds exhibited a strong reduction of their trypanocidal activity in the presence of blood (Table 1), which for the most active compounds varied from 128- to higher than 210-fold. Against interiorized parasites in CMs, compounds 2, 5 and 7 were the most active (Table 1), resulting in a 100% decrease in the number of bursting parasites at a dose of...
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10.7 μM after 72 h (data not shown). After 24 or 72 h of incubation, none of the ADs was toxic to CMs, exhibiting values of LC50 of >32 μM. Regarding the selectivity index (LC50/IC50 ratio), the compounds with the highest values were compounds 5 (>137) and 7 (>107), for trypomastigotes and intracellular parasites, respectively (Table 1).

Due to the high activity and selectivity of compounds 5 and 7, we next evaluated by transmission electron microscopy their main cellular targets in the treated bloodstream forms. The ultrastructural analysis showed that compounds 5 and 7 displayed similar effects, leading to the detachment of the nuclear envelope (Figure 1b, arrow) and plasmalemma (Figure 1c, arrows), besides inducing striking alterations in the mitochondria–kinetoplast complex (Figure 1b–d). Concerning the intracellular localization, we found that all drugs accumulated in the DNA-enriched structures: the nucleus and kinetoplast of trypomastigotes [Figure 1e and f and Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)] and intracellular amastigotes (Figure 1g), showing a kinetoplast DNA (kDNA)/nuclear DNA (nDNA) ratio of at least 1.5 for trypomastigotes (Table S1). However, the higher kDNA selectivity was not directly related to trypanocidal efficacy, since compound 5, although displaying the lower ratio (1.5), was the most active, and compound 6, which showed the highest accumulation in the kinetoplast (2.8), was the least active (Figure 1e and f, and Table S1).

Discussion

Pentamidine, berenil and other related aromatic compounds bind to AT minor groove sequences of trypanosomatid kinetoplasts resulting in excellent antiparasitic activity. Our present results confirm the high activity of ADs, which displayed biological activity at the submicromolar level, and which did not display cytotoxicity to the mammalian host cells, as also found previously. The potential applicability of these compounds in the prophylaxis of blood banks was evaluated by the direct effect on trypomastigotes at 4°C in the presence of whole blood. An important decrease in the trypanocidal effect was observed with all the ADs, which could be due to their association with serum components and/or to drug instability. Since the requirements for a new anti-T. cruzi drug include the identification of drugs with SIs ≥50, we found that compound 7 represents a very promising AD to be further assayed in vivo.

Our ultrastructural findings showed that the most active compounds, 5 and 7, induced detachments of the nuclear envelope and plasmalemma; however, the most frequent effect was upon the mitochondria–kinetoplast complex, corroborating previous reports. Although the ADs presented stronger staining in the kinetoplast of bloodstream forms as already reported using other analogues for African trypanosomes and T. cruzi, it was not correlated to their trypanocidal activity. Interestingly, previous studies performed with bloodstream parasites of Trypanosoma brucei also failed to correlate AD overall intracellular accumulation and localization with compound activity. However, this study represents the first report measuring the relative kDNA to nDNA accumulation of ADs in T. cruzi and the lack of correlation with their biological activity against this parasite. Although the exact mechanism of their action has not been clearly demonstrated, the data suggest that at least part of this activity may be related to the destruction of the kinetoplast leading to parasite death. Therefore, the association of ADs with the DNA seems to be an initial step followed by topological changes that can lead to molecule instability and destruction. Alternatively, these compounds could interfere with DNA-targeted enzymes and/or may act through direct inhibition of transcription, triggering cell death.

Funding

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


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