# Inhibition of Host Cell Invasion and Intracellular Replication of *Trypanosoma cruzi* by $N,N'$-Bis(Benzyl)-Substituted Polyamine Analogs

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We studied the effects of two $N,N'$-bis(benzyl)-substituted polyamine analogs on the capacities of *Trypanosoma cruzi* to invade and multiply within a mammalian host cell. At concentrations as low as 1 µM, these compounds reduced significantly the infectivity of the parasite for rat heart myoblasts in a time-dependent manner. Pretreatment of virulent *T. cruzi* trypomastigotes, but not myoblast pretreatment, reduced the level of infectivity. The inhibitory effects started to subside 3 h after removal of the drugs and were no longer detectable after 4 h. A significant decrease in the rate of intracellular amastigote multiplication was also seen when the drugs were added to myoblast cultures which had been previously infected with untreated *T. cruzi*. These results show that $N,N'$-bis(benzyl)-substituted polyamine analogs meet the two most important criteria for potential chemotherapeutic agents against *T. cruzi* infection, namely, inhibition of both host cell invasion and intracellular replication by this parasite.

The unicellular parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, requires an intracellular localization to multiply in mammalian hosts. This stresses the importance of developing drugs that can inhibit host cell invasion and subsequent intracellular parasite replication. Such drugs are badly needed given the lack of a consistently effective, nontoxic drug to cure Chagas' disease (9). There is evidence that both host cell invasion by *T. cruzi* trypomastigotes and intracellular multiplication of the amastigote form are impaired when production of the polyamines agmatine and putrescine is decreased by the specific arginine decarboxylase inhibitor DL-α-difluoromethylarginine (7, 13). Polyamines play key roles in cell growth regulation, and polyamine synthesis inhibitors have been shown to affect some parasitic organisms (1, 8, 16). $N,N'$-Di-substituted tetraamines with the general formula RNH(CH$_2$)$_x$NH (CH$_2$)$_y$NH(CH$_2$)$_x$NHR have been reported to suppress the growth of several pathogenic protozoans (e.g., *Leishmania donovani*, *Plasmodium falciparum*, and *Plasmodium berghei*) in vitro and in infected animals (2–4) and could be suitable for exploring the role that polyamines and polyamine biosynthesis plays in *T. cruzi* infection. In this work, we used two $N,N'$-bis(benzyl)-substituted polyamine analogs and studied their effects on *T. cruzi* infectivity and replication using an in vitro model system of mammalian host cell infection.

Stock CD1(ICR) Swiss mice (Charles River Laboratory, Portage, Mich.) were infected subcutaneously with 10$^6$ *T. cruzi* (Tulahen isolate) organisms. Trypomastigotes were purified 10 to 12 days later from the blood by centrifugation through Isolymph (Gallard-Schlesinger, Carle Place, N.Y.) (5) followed by column chromatography through DEAE-cellulose (15). The eluted trypanosomes were washed twice with Dulbecco's modified minimal essential medium (GIBCO, Grand Island, N.Y.) containing 100 IU of penicillin and 100 µg of streptomycin per ml and supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis,

Mo.) (DMEM), counted microscopically with a hemacytometer, and adjusted to the desired concentration in the same medium.

To determine effects on *T. cruzi* infectivity, trypomastigotes were incubated with the desired drug at 37°C for 2 h, washed twice with DMEM by centrifugation, and added to untreated rat heart myoblast (RHM) (American Type Culture Collection CRL 1446) cultures. In experiments designed to define the kinetics of production of inhibitory effects, the organisms were incubated with the drug of interest for increasing lengths of time. After the flagellates were washed and resuspended in DMEM, they were added immediately to untreated RHM cultures. To establish whether the effect of a tested drug was reversible, we suspended the trypanosomes in solutions of the drug in DMEM at 37°C for 2 h. After a washing, the flagellates were resuspended in fresh DMEM, allowed to stand at 37°C for increasing lengths of time, and then added to RHM cultures. In all cases, aliquots of the *T. cruzi* suspensions were subjected to parallel mock treatments with DMEM alone. Each experimental and control condition was tested in triplicate. The method for determining *T. cruzi* infectivity has been described in detail (12, 17). Briefly, trypomastigotes pretreated with drug solutions at 37°C for 2 h were washed with DMEM and cocultured with confluent monolayers of RHMs for 3 h. The parasite/RHM ratio used in these experiments was 40:1. In some experiments, the RHMs were incubated with the appropriate drug at 37°C for 3 h and washed with DMEM before the addition of untreated parasites. All cultures were terminated by removing the free trypanosomes with DMEM. The slides were then fixed with methanol and stained with Giemsa. The cultures were examined microscopically, with no fewer than 200 cells screened at random. The results were expressed as the mean percents infected cells ± standard deviations and the average numbers of parasites per 100 screened RHMs ± standard deviations. The percent reduction (%R) in infectivity due to an experimental treatment was calculated by the following equation: %R = [(control value − experimental value)/(control value)] × 100.

To determine the rate of intracellular *T. cruzi* multiplica-

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TABLE 1. Effects of MDL 27695 or MDL 27699 on the infective capacity of *T. cruzi*[a]

| Expt | Reagent tested | Mean % infected cells ± SD (%R) | Mean no. of para-sites/100 cells ± SD (%R) |
|---|---|---|---|
| 1 | DMEM | 11.7 ± 1.1 | 14.4 ± 1.7 |
| | MDL 27695 | 4.6 ± 0.6[b] (60.6) | 4.4 ± 0.3[b] (69.4) |
| 2 | DMEM | 23.8 ± 2.1 | 27.6 ± 1.1 |
| | MDL 27699 | 12.7 ± 0.7[b] (46.4) | 14.3 ± 1.3[b] (48.1) |

[a] Cultures of RHMs were incubated with *T. cruzi* at 37°C for 3 h in the presence or absence of 1 µM polyamine analog. After being washed with DMEM, the RHM monolayers were fixed with methanol and stained with Giemsa.

[b] Significantly different (*P* ≤ 0.05; Mann-Whitney U test) from the corresponding control value obtained with DMEM alone.

tion, confluent RHM monolayers were cocultured with try-pomastigotes as described above, washed with DMEM to remove the free organisms, and incubated further in fresh medium for 15 h. During this time, >98% of the intracellular organisms transformed into the amastigote stage (18). Replicate sets of cultures then received fresh DMEM either alone or containing the drug to be tested and were further incubated for 24 h. All cultures were terminated and evaluated as described above. All conditions were tested in triplicate. It should be noted that upon reaching confluence, RHM growth comes to a virtual halt, reducing to an insignificant extent "parasite dilution" due to host cell growth.

*N,N'*-Bis{3-[(phenylmethyl)amino]propyl}-1,7-diaminoheptane (MDL 27695) and *N,N'*-bis{3-[(*p*-sulfomethyl-phenylmethyl)amino]propyl}-1,8-diaminooctane (MDL 27699) (6) were synthesized at the Marion Merrell Dow Research Institute (Cincinnati, Ohio). Solutions of these reagents were prepared in DMEM.

Each set of results is representative of at least two separate repeat experiments. All results are expressed as means ± standard deviations of triplicate determinations. Differences between means were considered significant if *P* was ≤0.05, determined by the Mann-Whitney U test.

Preliminary toxicity tests revealed that 1 µM MDL 27695 or MDL 27699 did not affect the motility or viability of *T. cruzi* and did not cause any discernible alteration in RHM morphology or viability (data not shown). Higher concentrations of these polyamine analogs up to 10 µM were not visibly toxic for the RHMs but reduced the motility of *T. cruzi* (data not shown). Because reduced parasite motility would decrease the frequency of its collisions with the host cells and produce misleading results, we selected 1 µM as the concentration of MDL 27695 or MDL 27699 to be used in experiments in which the parasite was directly exposed to these drugs.

The presence of either MDL 27695 or MDL 27699 in the culture medium during *T. cruzi*-RHM interaction caused significant reductions in both the proportion of infected RHMs and the number of parasites per 100 RHMs (Table 1). To establish whether the cells affected by the polyamine analogs were *T. cruzi*, the RHMs, or both, we separately pretreated each of these cell types, washed them to remove nonincorporated reagent, and then cocultured them with the untreated counterpart. As can be seen in Table 2, pretreatment of *T. cruzi* with either MDL 27695 or MDL 27699 significantly inhibited infectivity whereas pretreatment of the RHMs did not alter their susceptibility to *T. cruzi* infection. It should be pointed out that while the level of

TABLE 2. Effects of pretreatment of *T. cruzi* or RHMs with MDL 27695 or MDL 27699 on capacity of parasite to infect untreated RHMs or susceptibility of RHMs to infection by untreated *T. cruzi*

| Cell type[a] | Treatment | Mean % infected cells ± SD (%R) | Mean no. of para-sites/100 cells ± SD (%R) |
|---|---|---|---|
| *T. cruzi* | DMEM | 20.6 ± 2.7 | 30.0 ± 6.0 |
| | MDL 27695 | 6.3 ± 1.9[b] (69.4) | 7.2 ± 2.7[b] (76.0) |
| | MDL 27699 | 10.7 ± 1.6[b] (48.0) | 12.9 ± 2.7[b] (57.0) |
| RHM | DMEM | 19.6 ± 1.2 | 20.4 ± 1.7 |
| | MDL 27695 | 18.3 ± 1.0 (6.6) | 19.1 ± 1.7 (6.3) |
| | MDL 27699 | 18.1 ± 0.6 (4.0) | 19.0 ± 0.9 (6.8) |

[a] For *T. cruzi*, the parasites were incubated with DMEM either alone or containing the indicated drug (1 µM) at 37°C for 2 h. After being washed with fresh medium, the organisms were added to untreated RHM cultures. For RHMs, monolayers were incubated with DMEM either alone or containing the indicated drug (1 µM) at 37°C for 3 h. After cells were washed with fresh medium, the cultures received untreated *T. cruzi* and were terminated by washing and fixation 3 h later.

[b] Significantly different (*P* ≤ 0.05; Mann-Whitney U test) from the corresponding control value obtained with DMEM alone.

inhibition of *T. cruzi* infectivity varied among repeat experiments (data not shown), the inhibitory effects of MDL 27695 and MDL 27699 were systematically demonstrable and statistically significant (*P* ≤ 0.05).

Because the parasite load of the RHM cultures was evaluated by visible microscopy and this method does not distinguish membrane-attached from cytoplasmic organisms, it was not clear whether we were measuring parasite penetration, membrane attachment, or a combination of both. To settle this issue we determined the number of trypomastigotes per 100 RHMs immediately after removal of the free trypomastigotes (i.e., after a 2-h coculture period) and the number of trypomastigotes plus amastigotes 12 h later. In a typical experiment, the number of trypomastigotes per 100 RHMs immediately after removal of the free organisms was 15.8 ± 1.0, whereas the value recorded 12 h later was 14.5 ± 0.7, of which 90% were amastigotes.

A 30-min incubation with either MDL 27695 or MDL 27699 was necessary to produce significant inhibition of *T. cruzi* infectivity (Fig. 1A). The extent of these effects increased as treatment time was extended, reaching a maximal level after 1 h. To establish whether MDL 27695- or MDL 27699-induced inhibition of *T. cruzi* infectivity was permanent or reversible, trypomastigotes were treated with these polyamine analogs for 2 h, washed with medium to remove free reagent, and resuspended in fresh medium. Preliminary studies failed to show any significant recovery within the first 2 h (data not shown), but subsequent experiments using longer periods of incubation in medium revealed a partial recovery after 3 h and complete restoration of infectivity after 4 h (Fig. 1B).

To test whether MDL 27695 or MDL 27699 affected the rate of intracellular *T. cruzi* growth, the drugs were added to infected RHM cultures after virtually all of the trypomastigotes had transformed into amastigotes. After a 24-h incubation period, the average numbers of parasites per 100 host RHMs in cultures containing the polyamine analogs were found to be significantly lower than those occurring in the control cultures (Fig. 2).

Comparable numbers of parasites were associated with 100 RHM immediately after removal of the free trypomastigotes and 12 h later, indicating that no significant parasite
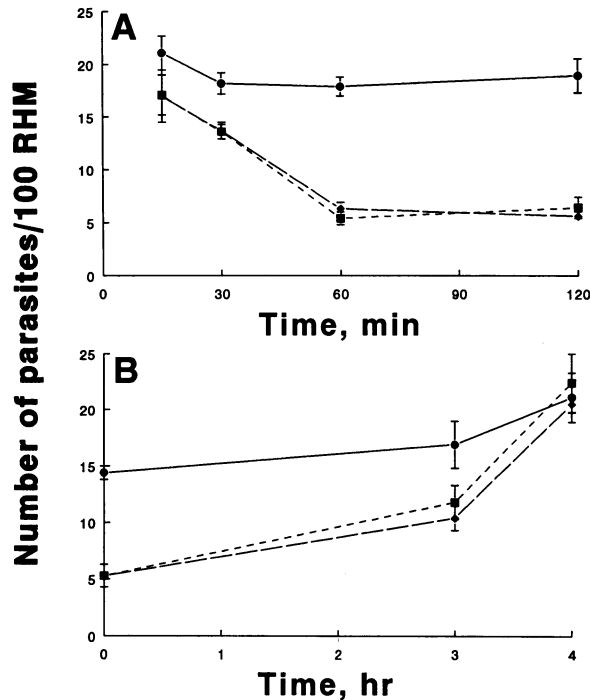
FIG. 1. Effects of MDL 27695 or MDL 27699 on *T. cruzi* infectivity. (A) Kinetics of RHM infection by trypomastigotes treated with medium alone or containing either 1 μM MDL 27695 or 1 μM MDL 27699. The organisms were incubated at 37°C for the indicated periods, washed with medium, and cocultured with untreated RHM monolayers for 3 h. The differences between the values obtained with MDL 27695 and MDL 27699 at 30, 60, and 120 min and the corresponding control values were statistically significant ($P \leq 0.05$; Mann-Whitney U test). (B) Reversibility of the effects of MDL 27695 or MDL 27699. The parasites were incubated with medium either alone or containing 1 μM MDL 27695 or 1 μM MDL 27699 at 37°C for 2 h. After being washed with and resuspended in medium, the organisms were incubated further for the indicated periods and then cocultured with untreated RHM for 3 h. Only the differences between the experimental values obtained at 0 and 3 h and the corresponding control values were statistically significant ($P \leq 0.05$; Mann-Whitney U test). ●, DMEM; ■, MDL 27695; ◆, MDL 27699.
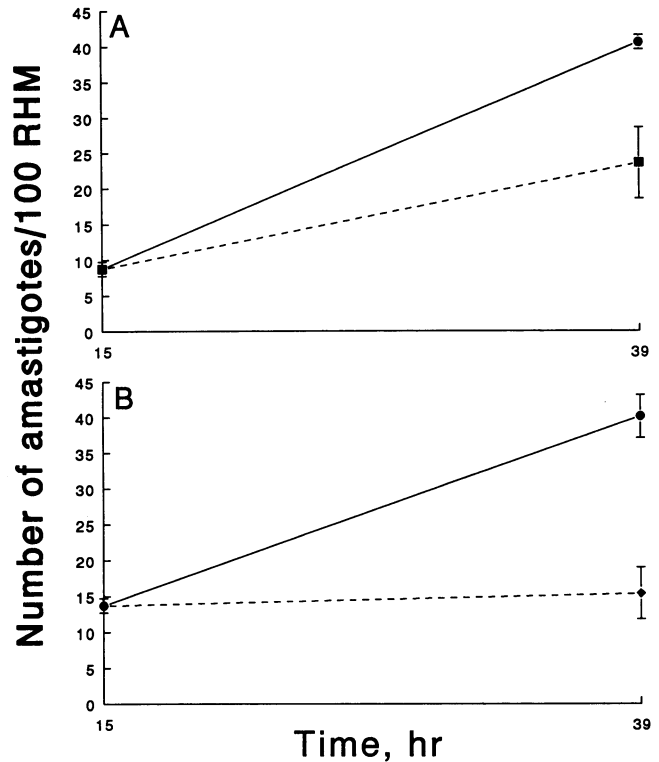


FIG. 2. Inhibition of intracellular *T. cruzi* replication by MDL 27695 or MDL 27699. Trypomastigotes were cocultured with RHM at 37°C for 3 h. After the free organisms were removed, the cultures were incubated for 15 h to allow transformation into the amastigote form. At this time, replicate sets of cultures received medium either alone (●) (A and B) or containing 10 μM MDL 27695 (■) (A) or 10 μM MDL 27699 (◆) (B). Sets of triplicate cultures were terminated 24 h later. The differences between the values obtained at this time with MDL 27695 or MDL 27699 and the corresponding control value were statistically significant ($P \leq 0.05$; Mann-Whitney U test).

multiplication had occurred during the intervening time. Since 90 and >98% of the organisms associated with the RHM after 12 and 15 h, respectively, were in the cytoplasmic, amastigote form, it can be inferred that virtually all of the trypomastigotes initially associated with the RHM had an intracellular localization. Therefore, the finding that the number of *T. cruzi* associated with RHMs was reduced in the presence of either MDL 27695 or MDL 27699 largely represents inhibition of the invasive capacity of the parasite. These effects resulted from a selective inhibitory action on the parasite, since they were readily reproduced when trypomastigotes, but not RHMs, were pretreated with the polyamine analogs.

The production of significant inhibition of *T. cruzi* infectivity by MDL 27695 or MDL 27699 required a 30-min treatment, and greater levels of impairment were noted after additional incubation. This time dependence might reflect parasite incorporation or biochemical conversion of sufficient amounts of inhibitor to effectively alter infectivity. Alternatively, the *N,N'*-substituted polyamine analogs might have led to biochemical alterations inconsistent with normal

infectivity. In this context, it is noteworthy that MDL 27695 has been shown to inhibit RNA and DNA synthesis in malaria parasites (4). The notion that molecules important in the invasion process are depleted would be in keeping with the time-dependent recovery of infectivity observed after removal of nonincorporated MDL 27695 or MDL 27699. This recovery may be due to degradation or excretion of the polyamine analogs or, conceivably, turnover of parasite molecules that bind these analogs.

The *N,N'*-bis(benzyl)-substituted polyamine analogs used in this work were effective in curtailing intracellular *T. cruzi* amastigote multiplication. However, the need to treat the cells that harbor the parasite made it difficult to ascertain whether inhibition of parasite growth resulted from biochemical effects of the amastigotes or the host cells.

Difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, inhibits infection by African trypanosomes (reviewed in reference 14), whereas *T. cruzi* infectivity in our assay system is altered by difluoromethylarginine (DFMA), a specific arginine decarboxylase inhibitor (10), and not by DFMO (11). This contrast points to major differences in polyamine biosynthesis by African and American trypanosomes, possibly related to the extracellular nature of the former and the requirement of a cytoplasmic localization for *T. cruzi* to multiply in mammalian hosts.

Inhibition of host cell invasion and intracellular replication by *T. cruzi* are the two most important criteria which must be met by drugs considered for potential chemotherapeutic use. Although the inhibitory effects of MDL 27695 and MDL 27699 were similar to that previously reported for DFMA (10), they were produced at a molar concentration 12,000 times lower than that of DFMA. Whether administration of *N,N'*-bis(benzyl)-substituted polyamine analogs would have a beneficial effect in *T. cruzi*-infected mammalian hosts is an important question deserving further study.

### REFERENCES

1. **Bacchi, C. J., and P. P. McCann.** 1987. Inhibition of polyamine metabolism. Biological significance and basis for new therapies, p. 317–354. *In* P. P. McCann, A. E. Pegg, and A. Sjoerdsma (ed.), Parasitic protozoa and polyamines. Academic Press, Inc., Orlando, Fla.
2. **Baumann, R. J., W. L. Hanson, P. P. McCann, A. Sjoerdsma, and A. J. Bitonti.** 1990. Suppression of both antimony-susceptible and antimony-resistant *Leishmania donovani* by a bis(benzyl)polyamine analog. Antimicrob. Agents Chemother. **34:**722–727.
3. **Baumann, R. J., P. P. McCann, and A. J. Bitonti.** 1991. Suppression of *Leishmania donovani* by oral administration of a bis(benzyl)polyamine analog. Antimicrob. Agents Chemother. **35:**1403–1407.
4. **Bitonti, A. J., J. A. Dumont, T. L. Bush, M. L. Edwards, D. M. Stemerick, P. P. McCann, and A. Sjoerdsma.** 1989. Bis(benzyl)polyamine analogs inhibit the growth of chloroquine-resistant human malaria parasites (*Plasmodium falciparum*) in vitro and in combination with α-difluoromethylornithine cure murine malaria. Proc. Natl. Acad. Sci. USA **86:**651–655.
5. **Budzko, D. B., and F. Kierszenbaum.** 1974. Isolation of *Trypanosoma cruzi* from blood. J. Parasitol. **60:**1037–1038.
6. **Edwards, M. L., D. M. Stemerick, A. J. Bitonti, J. A. Dumont, P. P. McCann, P. Bey, and A. Sjoerdsma.** 1991. Antimalarial polyamine analogues. J. Med. Chem. **34:**569–574.
7. **Kallio, A., P. P. McCann, and P. Bey.** 1981. DL-α-(Difluoromethyl)arginine: a potent enzyme-activated irreversible inhibitor of bacterial arginine decarboxylases. Biochemistry **22:**3163–3166.
8. **Kelly, M., P. P. McCann, and J. Schindler.** 1985. Alterations in polyamine metabolism during embryonal carcinoma cell differentiation in vitro. Dev. Biol. **111:**510–514.
9. **Kierszenbaum, F.** 1984. The chemotherapy of *Trypanosoma cruzi* infection (Chagas' disease), p. 133–163. *In* J. M. Mansfield (ed.), Parasitic diseases. Marcel Dekker, Inc., New York.
10. **Kierszenbaum, F., J. J. Wirth, P. P. McCann, and A. Sjoerdsma.** 1987. Inhibition of the capacity of *Trypanosoma cruzi* to infect and multiply within mammalian host cells by treatment with inhibitors of arginine decarboxylase. Proc. Natl. Acad. Sci. USA **84:**4278–4282.
11. **Kierszenbaum, F., J. J. Wirth, P. P. McCann, and A. Sjoerdsma.** 1987. Impairment of macrophage function by inhibitors of ornithine decarboxylase activity. Infect. Immun. **55:**2461–2464.
12. **Lima, M. F., and F. Kierszenbaum.** 1984. Effects of treatment of trypomastigote forms of *Trypanosoma cruzi* or host cells with ethidium bromide on cell infection and intracellular fate of the parasite. J. Parasitol. **70:**911–917.
13. **Majumder, S., J. J. Wirth, A. J. Bitonti, P. P. McCann, and F. Kierszenbaum.** 1992. Biochemical evidence for the presence of arginine decarboxylase activity in *Trypanosoma cruzi*. J. Parasitol. **78:**371–374.
14. **McCann, P. P., C. J. Bacchi, A. J. Bitonti, F. Kierszenbaum, and A. Sjoerdsma.** 1989. Inhibition of ornithine or arginine decarboxylase as an experimental approach to African or American trypanosomiasis. Adv. Exp. Med. Biol. **150:**727–735.
15. **Mercado, T. I., and K. Katusha.** 1979. Isolation of *Trypanosoma cruzi* from the blood of infected mice by column chromatography. Prep. Biochem. **9:**97–106.
16. **Pegg, A. E., and P. P. McCann.** 1982. Polyamine metabolism and function. Am. J. Physiol. **243:**C212–C221.
17. **Wirth, J. J., and F. Kierszenbaum.** 1984. Fibronectin enhances macrophage association with invasive forms of *Trypanosoma cruzi*. J. Immunol. **133:**460–464.
18. **Yakubu, M. A., B. Basso, and F. Kierszenbaum.** 1992. DL-α-Difluoromethylarginine inhibits intracellular *Trypanosoma cruzi* multiplication by affecting cell division but not trypomastigote-amastigote transformation. J. Parasitol. **78:**414–419.