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Inhibition of *Trypanosoma cruzi* Growth in Mammalian Cells by Purine and Pyrimidine Analogs

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***Trypanosoma cruzi*, the causative agent of Chagas' disease, exhibits two different developmental stages in mammals, the amastigote, an intracellular form that proliferates in the cytoplasm of host cells, and the trypomastigote, an extracellular form that circulates in the bloodstream. We have already established an in vitro culture system using mammalian host cells (HeLa) infected with *T. cruzi* in which the time course of parasite growth is determined quantitatively. We adopted this system for the screening of anti-*T. cruzi* agents that would ideally prove to be effective against trypanosomes with no toxicity to the host cell. Of the purine analogs tested, allopurinol markedly inhibited the growth of amastigotes in a dose-dependent manner, with no lethal effect on trypomastigotes. 3′-Deoxyinosine and 3′-deoxyadenosine also suppressed *T. cruzi* growth inside the host cell, with the concentrations causing 50% growth inhibition being 10 and 5 μM, respectively, in contrast to a concentration causing 50% growth inhibition of 3 μM for allopurinol. Among the pyrimidine analogs examined, 3′-azido-3′-deoxythymidine (zidovudine) significantly reduced the growth of the parasite at concentrations as low as 1 μM. The anti-human immunodeficiency virus agents 2′,3′-dideoxyinosine and 2′,3′-dideoxyadenosine caused a decrease in amastigote growth, while 2′,3′-dideoxycytidine and 2′,3′-dideoxyuridine had no inhibitory effect. When Swiss 3T3 fibroblasts were used as host cells, allopurinol, 3′-deoxyinosine, 3′-deoxyadenosine, and 3′-azid-3′-deoxythymidine also markedly inhibited *T. cruzi* proliferation. These results indicate that our culture system is useful as a primary screening method for candidate compounds against *T. cruzi* on the basis of two criteria, namely, intracellular replication by the parasite and host-cell infection rate.**

Trypanosoma cruzi, the parasitic protozoan that causes Chagas' disease in Central and South America, exhibits three major developmental stages during its life cycle in the insect vector and mammalian hosts (2, 3). In insect vectors, the parasite occurs as the epimastigote form, while it shows trypomastigote and amastigote forms in mammals. The nondividing and infective trypomastigote form possesses a flagellum and circulates in the bloodstream. After penetration into mammalian host cells, preferably muscle cells, the trypomastigote form transforms into the amastigote form, which has no free flagellum. The amastigote multiplies by binary fission in the host-cell cytoplasm and eventually returns to the trypomastigote form, with the resulting destruction of infected cells before it reappears in the circulation.

There is no effective chemotherapy against this life-threatening flagellate parasite; an efficacious therapeutic agent is thus urgently needed. In an attempt to determine the growth and morphogenesis of *T. cruzi*, we established a culture system consisting of host HeLa cells infected with the protozoan parasite. Using this system, we were able to determine quantitatively the time courses of amastigote proliferation and trypomastigote propagation (8). This in vitro system also enabled us to test allopurinol and other purine analogs for their efficacies in inhibiting parasite growth inside host cells. Some of these results have been presented in a preliminary form (1). We report here that our culture system is useful as a primary screening method for candidate compounds with anti-*T. cruzi* activities and that purine and pyrimidine analogs including 3′-deoxyinosine and 3′-azido-3′-deoxythymidine (zidovudine; AZT) bring about a marked decrease in parasite growth. This is the first report of *T. cruzi* growth inhibition by anti-human immunodeficiency virus (anti-HIV) agents.

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MATERIALS AND METHODS

Chemicals. Eagle's minimum essential medium and Dulbecco's modified Eagle's medium were obtained from Nissui Pharmaceutical, Tokyo, Japan; fetal bovine serum was from Daiichi Pure Chemicals, Tokyo, Japan. Solutions of Diff-Quik and HSR (Kokusai Shiyaku, Kobe, Japan) were used to stain host mammalian cells infected with *T. cruzi* and to embed the stained specimens, respectively. 3′-Deoxyinosine was provided by Yamasa Corporation, Chiba, Japan. Allopurinol, 3′-deoxyadenosine, 3′-deoxyguanosine, AZT, 3′-deoxythymidine, 5′-deoxythymidine, 3′-azido-2′,3′-dideoxyuridine, 3′-deoxyuridine, 2′-azido-2′-deoxyuridine, 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyuridine (ddU) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were commercial products of the highest available grade. These compounds were dissolved in phosphate-buffered saline and were sterilized through a membrane filter (pore size, 0.22 μm).

Parasite and host cells. The Tulahuene strain of *T. cruzi* (11) and the human cancer cell line HeLa, used as an in vitro host (4), were maintained and passaged in culture as described previously (8). Swiss 3T3 fibroblast cells were kindly provided by F. Hanaoka, Division of Cellular Biology, Institute for Molecular and Cellular Biology, Osaka University. The 3T3 cells were subcultured every 3 days at an initial cell density of 3×10^5 cells in 25-cm^2 plastic flasks (Corning-Iwaki Glass, Tokyo, Japan).

In vitro infection. Mammalian host cells were infected in vitro with *T. cruzi* trypomastigotes by a modification of the method described previously (8). A round coverslip (12 mm) was placed in each well of a 24-well plate (Corning-Iwaki Glass). Exponentially growing host cells (5×10^3 cells per ml per well), harvested from the preceding subcultures, were added to each well. After incubation at 37°C for 2 days in 5% CO_2 in air, the cells were infected with *T. cruzi* trypomastigotes (1×10^4 to 2×10^4 parasites) as described previously (8). The chemical compounds to be tested for their inhibitory effects on parasite growth were added immediately after infection.

Determination of the rate of infection and the number of parasites. The method for determining the rate of infection of host cells by *T. cruzi* has previously been described in detail (8). Briefly, host cells attached to the coverslip were fixed and stained with Diff-Quik in the wells of the 24-well plate. The coverslip was then transferred upside down to a glass slide, and the cells were finally embedded in HSR solution for observation under a light microscope. The percentage of infected host cells, i.e., those containing more than one amastigote,

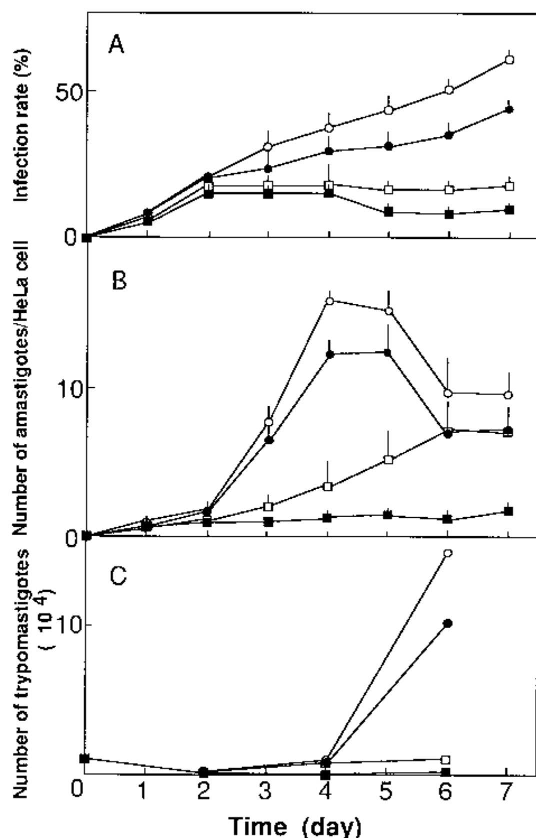

FIG. 1. Effects of allopurinol on the rate of infection and *T. cruzi* growth. HeLa cells were incubated for 2 days and were then infected with *T. cruzi* trypomastigotes. Other details are given in Materials and Methods. (A) Rate of infection; (B) mean number of amastigotes per infected HeLa cell; (C) number of trypomastigotes in the culture medium. ○, control; ●, 1 μM allopurinol; □, 10 μM allopurinol; ■, 100 μM allopurinol. The values are the means of five separate experiments for panels A and B, and the bars represent the standard deviations. For panel C, the average values of two separate determinations are presented.

and the mean number of amastigotes per infected cell were determined by analyzing more than 200 host cells distributed in randomly chosen microscopic fields. The number of trypomastigotes in the medium was determined as described previously (8).

A typical time course for *T. cruzi* propagation in HeLa cells was as follows. When 5×10^3 HeLa cells were incubated for 2 days and then infected with 1×10^4 to 2×10^4 trypomastigotes, the parasites invaded the cells and underwent morphologic conversion to amastigotes within 1 day after infection. On days 2, 4, 6, and 8, the rate of host-cell infection increased to 21.9, 37.4, 48.3, and 64.0%, respectively. The average number of amastigotes per infected cell also increased to 2.2 on day 2 and 16.2 on day 4, but then decreased to 9.6 on day 6 and 8.3 on day 8. The number of trypomastigotes in the medium was 1.5×10^5 on day 6 and 1.8×10^5 on day 8. It is thus conceivable that on about day 5, the morphologic alteration from amastigotes to trypomastigotes takes place, with the latter disrupting host cells and then the trypomastigotes again appearing in the culture medium.

RESULTS

Effects of allopurinol on the host-cell infection rate and parasite growth. When allopurinol at concentrations of 1, 10, or 100 μM was added to logarithmically growing HeLa cells (5×10^3 cells per ml per well), neither growth inhibition nor morphological alteration of the cells occurred, and an apparently normal growth curve as a function of incubation time for up to 8 days was obtained (data not shown). Likewise, the concentrations of the various compounds tested in the study were chosen to be at levels that do not inhibit the growth of normal host cells.

TABLE 1. Effects of allopurinol and purine analogs on the rate of infection of HeLa cells and on the average number of amastigotes per infected HeLa cell on day 4 after infection by *T. cruzi* trypomastigotes

Compound	Concn (μM)	Infection rate (%)[a]	No. of amastigotes/ HeLa cell[a]
None (control)	0	36.9 ± 9.1	18.1 ± 4.2
3′-Deoxyinosine	0.5	32.9 ± 4.5	15.7 ± 4.2
	5	30.3 ± 6.2	12.5 ± 5.1
	50	18.2 ± 2.4[b]	4.2 ± 2.7[b]
3′-Deoxyadenosine	0.5	30.5 ± 4.9	16.4 ± 5.1
	5	22.5 ± 6.7[b]	8.0 ± 2.4[b]
	50	17.3 ± 5.1[b]	1.9 ± 1.4[b]
3′-Deoxyguanosine	0.5	34.0 ± 6.5	18.0 ± 3.8
	5	32.2 ± 3.5	16.3 ± 3.8
	50	27.0 ± 2.2[b]	7.1 ± 1.3[b]

[a] Values are means ± standard deviations of five separate determinations.
[b] $P < 0.05$.

Figure 1A illustrates the effect of allopurinol on the time course of *T. cruzi* infection. The infection rate in the control culture increased in a time-dependent manner to 37.5% on day 4 and 61.8% on day 7. The addition of 1, 10, or 100 μM allopurinol lowered the infection rate to 29.2, 18.1, or 15.3%, respectively, on day 4 and 44.4, 17.7, or 9.4%, respectively, on day 7. Allopurinol produced a clearly observed dose-dependent inhibition of the infection rate.

Allopurinol was also found to be highly inhibitory against *T. cruzi* amastigote replication in HeLa cells in vitro (Fig. 1B). This finding is in agreement with the data presented in a previous report (5). In the absence of allopurinol (control), the average number of amastigotes per infected HeLa cell increased to 16.0 on day 4 and decreased to 9.9 on day 7. The addition of 1, 10, or 100 μM allopurinol markedly lowered the amastigote number per infected cell to 12.8, 3.7, or 1.7, respectively, on day 4 and 8.1, 7.5, or 2.1, respectively, on day 7. On day 4, we could again observe a dose-dependent inhibition of parasite growth by allopurinol.

The decrease in the average amastigote numbers on day 6 in control and 1 μM allopurinol-treated cultures coincided with the increase in trypomastigote numbers (Fig. 1B and C). This coincidence implies that 1 μM allopurinol fails to achieve complete killing of the amastigotes and that the parasites multiply, transform into trypomastigotes, and reappear in the medium. In contrast, at higher concentrations of 10 and 100 μM allopurinol, no trypomastigotes appear (Fig. 1C). Therefore, 10 μM or more allopurinol completely inhibits the host cell infection rate, amastigote replication, and trypomastigote propagation. These results are consistent with the fact that trypanosomatids are incapable of synthesizing purines de novo and rely solely on salvaging preformed purines; allopurinol is converted to aminopurinol triphosphate via several metabolic steps and is eventually incorporated into the parasite RNA (6). Allopurinol shows little killing effect on *T. cruzi* trypomastigotes in vitro, since preincubation of trypomastigotes with 100 μM allopurinol did not alter the time course of infection or amastigote growth (data not shown). Allopurinol riboside produces similar inhibitory effects on parasite infectivity and replication. In the experiments described below, the effects of various chemicals were measured essentially as described here for allopurinol. For clarity, however, only the data for infection

TABLE 2. Effects of AZT and pyrimidine analogs on the rate of infection of HeLa cells and on the average number of amastigotes per infected HeLa cell on day 4 after infection by *T. cruzi* trypomastigotes

Compound	Concn (μM)	Infection rate (%)[a]	No. of amastigotes/ HeLa cell[a]
None (control)	0	32.3 ± 1.4	17.2 ± 4.3
AZT	0.001	31.0 ± 1.9	17.4 ± 1.1
	0.01	31.5 ± 1.8	14.3 ± 1.9
	0.1	32.9 ± 3.1	13.3 ± 2.8
	1	26.7 ± 3.8[b]	8.9 ± 2.7[b]
	10	23.5 ± 6.9[b]	8.6 ± 3.8[b]
	50	19.8 ± 8.1[b]	6.8 ± 2.1[b]
3′-Deoxythymidine	1	29.3	15.7
	10	29.7	15.9
	50	29.6	15.2
5′-Deoxythymidine	1	30.3	22.1
	10	32.4	19.8
	50	32.1	18.8
3′-Azido-dideoxyuridine	1	32.4	16.2
	10	30.6	17.3
	50	30.2	16.4
3′-Deoxyuridine	1	32.8	17.7
	10	30.8	17.9
	50	31.9	16.5
2′-Azido-deoxyuridine	1	33.4	16.5
	10	33.1	15.1
	50	33.4	17.7
ddU	1	33.4	18.9
	10	33.1	17.7
	50	32.0	20.1

[a] For the control and AZT-treated cells, the values are means ± standard deviations of five separate determinations. For the cells treated with the remaining compounds, the values are averages of two separate determinations.
[b] $P < 0.05$.

rate and average amastigote number obtained on day 4 are presented.

Effects of purine analogs on the host-cell infection rate and parasite growth. Using allopurinol as a positive control, we extended our examination to 3′-deoxypurine nucleosides, and the results are summarized in Table 1. Whereas the control culture had an infection rate of 36.9%, high concentrations (50 μM) of 3′-deoxyinosine, 3′-deoxyadenosine, and 3′-deoxyguanosine reduced the rate to 18.2, 17.3, and 27.0%, respectively. Parasite growth, measured by the average number of amastigotes per infected cell, was inhibited by these three compounds, with concentrations causing 50% growth inhibition (IC_{50}s) of 10, 5, and 50 μM, respectively; this is in contrast to an IC_{50} of 3 μM for allopurinol.

Effects of pyrimidine analogs on the host-cell infection rate and parasite growth. It was of particular interest that among the pyrimidine derivatives tested (Table 2) AZT inhibited both the infection rate and amastigote growth in HeLa cells most strongly, with an IC_{50} of 0.5 to 1.0 μM. Even at a low concentration of 0.1 μM, AZT exhibited a certain degree of inhibition against *T. cruzi* growth. Other 3′-deoxypyrimidine nucleosides, 3′-deoxythymidine and 3′-deoxyuridine, had no inhibitory effect. Compounds containing an azido group, 3′-azido-2′,3′-dideoxyuridine and 2′-azido-2′-deoxyuridine, were not effective. Neither 5′-deoxythymidine nor ddU exhibited growth inhibition under the conditions used. AZT also inhibited the growth of epimastigotes by 20% at a higher concentration of 10 μM (data not shown).

TABLE 3. Effects of anti-HIV agents on the rate of infection of HeLa cells and on the average number of amastigotes per infected HeLa cell on day 4 after infection by *T. cruzi* trypomastigotes

Compound	Concn (μM)	Infection rate (%)[a]	No. of amastigotes/ HeLa cell[a]
None (control)	0	37.0 ± 5.0	15.9 ± 3.1
ddI	0.1	41.1 ± 6.4	15.2 ± 1.1
	1	34.0 ± 4.5	14.2 ± 3.8
	10	33.7 ± 2.5	11.0 ± 1.5[b]
	50	23.4 ± 0.7[b]	10.9 ± 1.8[b]
ddA	0.1	31.3 ± 4.2	20.3 ± 0.9
	1	27.4 ± 1.9[b]	13.4 ± 6.0
	10	25.0 ± 3.2[b]	13.1 ± 3.7
	50	25.2 ± 4.9[b]	9.3 ± 0.3[b]
ddC	1	37.3 ± 2.8	15.3 ± 0.6
	10	32.2 ± 4.4	19.7 ± 3.5
	50	27.9 ± 7.0	21.8 ± 3.5

[a] Values are means ± standard deviations of four separate determinations.
[b] $P < 0.05$.

Effects of anti-HIV agents on the host-cell infection rate and parasite growth. Since AZT is a very potent inhibitor of *T. cruzi* growth, we also examined whether other anti-HIV compounds that target the viral reverse transcriptase also suppress parasite growth in our in vitro screening system (Table 3). ddI at concentrations from 0.1 to 50 μM caused a decrease in amastigote growth to a lesser extent than AZT did at equivalent concentrations; ddA showed inhibitory effects similar to those of ddI, while ddC and ddU (Table 2) failed to inhibit *T. cruzi* growth.

Effects of nucleoside analogs on the rate of infection and parasite growth in 3T3 fibroblast cells. We used Swiss 3T3 fibroblasts as host cells to study those nucleoside analogs that possessed inhibitory activity in HeLa cells. Allopurinol and 3′-deoxypurines inhibited the infection rate and amastigote replication in 3T3 fibroblasts (Table 4). The IC_{50}s for parasite growth were 15, 5, 0.6, and 12 μM for allopurinol, 3′-deoxyinosine, 3′-deoxyadenosine, and 3′-deoxyguanosine, respectively. AZT was the most effective inhibitor, at a low concentration (1 μM), of the growth of amastigotes in 3T3 cells, with an IC_{50} of 0.2 to 0.3 μM.

DISCUSSION

From the results obtained in the present study, particularly with allopurinol, we consider it useful to use our in vitro host-parasite culture as a primary screening system and allopurinol as a positive control for the screening of anti-*T. cruzi* agents. This system uses the exponentially growing mammalian host cells, the growth of which may be more markedly inhibited by various chemical compounds than that of host cells at the stationary phase. The concentrations of compounds tested may thus have been chosen on the basis of a strict criterion. Using this in vitro screening system, we have found very potent inhibitors of parasite growth: 3′-deoxyinosine, 3′-deoxyadenosine, AZT, ddA, and ddI. 3′-Deoxyinosine has been reported to be a potent inhibitor of the growth of *Leishmania* promastigotes (13) and *T. cruzi* epimastigotes (7), both of

TABLE 4. Effects of nucleoside analogs on the rate of infection and on the average number of amastigotes per infected Swiss 3T3 cell on day 4 after infection by *T. cruzi* trypomastigotes

Compound	Concn (μM)	Infection rate (%)[a]	No. of amastigotes/ 3T3 cell[a]
None (control)	0	21.7 ± 5.4	20.9 ± 7.9
Allopurinol	1	13.9 ± 2.5[b]	18.9 ± 4.6
	10	12.4 ± 5.6[b]	12.8 ± 3.5[b]
	100	5.6 ± 2.5[b]	3.2 ± 2.5[b]
3′-Deoxyinosine	1	16.2 ± 5.1	13.0 ± 7.8
	10	12.0 ± 3.8[b]	9.5 ± 5.4
	50	9.6 ± 5.0[b]	2.8 ± 1.9[b]
3′-Deoxyadenosine	1	10.0 ± 4.2[b]	8.9 ± 8.3[b]
	10	8.4 ± 3.9[b]	1.2 ± 0.1[b]
	50	5.6 ± 3.2[b]	1.1 ± 0.1[b]
3′-Deoxyguanosine	1	17.8 ± 8.4	16.6 ± 7.1
	10	14.2 ± 8.9	12.2 ± 4.8[b]
	50	9.9 ± 6.2[b]	4.4 ± 2.7[b]
AZT	0.01	20.5 ± 3.1	17.9 ± 3.5
	0.1	15.9 ± 3.9[b]	12.6 ± 3.7
	1	14.8 ± 3.8[b]	7.4 ± 3.2[b]
	10	13.7 ± 5.7[b]	6.8 ± 5.0[b]

[a] Values are means ± standard deviations of three separate determinations.
[b] $P < 0.05$.

which occur in insect vectors of different species. 3′-Deoxypurine nucleosides contain 3′-deoxyribose and an intact purine base, while allopurinol, a hypoxanthine analog, contains a modified purine ring with an intact ribose moiety. The hydroxy group at the 3′ position of ribonucleotides is essential for the elongation of the RNA strand. Therefore, the absence of this group from 3′-deoxypurine nucleosides, which would be converted to the corresponding 3′-deoxypurine nucleotides in *T. cruzi* amastigotes, may result in the production of truncated RNA molecules. In accordance with this view, the 3′-deoxypurine nucleosides tested in the present study were all potent inhibitors of *T. cruzi* growth, with 3′-deoxyinosine appearing to be the most promising; 3′-deoxyadenosine, although very effective in the present study, has been demonstrated to have extensive cytotoxicity for mammalian cells (10).

Of the pyrimidine analogs tested, only a thymidine derivative with 3′-azido and 2′,3′-dideoxy groups, AZT, was a potent inhibitor, while a uridine derivative with the same modified groups was not. This suggests that AZT interferes with DNA synthesis rather than RNA synthesis in *T. cruzi* amastigotes. Other anti-HIV agents, ddI and ddA, acted as potent inhibitors of *T. cruzi* growth in HeLa cells, whereas ddC was ineffective, possibly indicating unique modes of action of these 2′,3′-dideoxynucleosides on parasite growth that are different from those on viral replication. *T. cruzi* amastigotes appear to preferentially incorporate purine analogs rather than pyrimidine analogs, since ddU was also ineffective.

The inhibition of infectivity and parasite growth by AZT is not apparently dose dependent, particularly at the high concentrations (Table 2). It has been reported that the intracellular half-life of an AZT metabolite, AZT-triphosphate, is approximately 1.3 h, whereas that of a ddI metabolite is 12 to 24 h (14). The concentration of AZT would be very low at the time of evaluation on day 4 after its addition, because AZT was added only once to the culture medium in our system.

In HIV, the reverse transcriptase is a target of AZT. It has been reported that the reverse transcriptase genes in the *T. cruzi* genome exist as a family of spliced leader-associated retrotransposons (12). Our preliminary study suggests that a reverse transcriptase gene is transcribed throughout the three developmental life stages, the epimastigote, trypomastigote, and amastigote forms. Although reverse transcriptase activity was weakly detected in the cytosolic fraction of a homogenate of epimastigotes, it was not inhibited by the addition of AZT under the conditions used. This suggests that AZT might target some other enzyme(s), e.g., trypanosomal DNA polymerase, a unique enzyme with susceptibility to aphidicholine different from those of other species (9).

Allopurinol, 3′-deoxypurines, and AZT, which all showed inhibitory effects on the infection rate and amastigote replication of *T. cruzi* in HeLa cells, exhibited quite similar effects in Swiss 3T3 fibroblasts (Table 4). In detail, however, the IC_{50} of allopurinol on parasite growth was 3 μM in HeLa cells, while it was 15 μM in 3T3 cells. On the other hand, the IC_{50} of AZT was three to five times higher in HeLa cells than in 3T3 cells. On day 8 after infection, the rate of infection was 64.0% in HeLa cells, while it reached 92.0% in 3T3 cells (data not shown). Although some differences in host cell potency existed between HeLa and 3T3 cells, both were useful in the search for trypanocidal drugs. The results indicate that our in vitro system is applicable as a primary screening method for searching for potential drugs against Chagas' disease.

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