Antiproliferative Effects and Mechanism of Action of SCH 56592 against Trypanosoma (Schizotrypanum) cruzi: In Vitro and In Vivo Studies

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Received 23 February 1998/Returned for modification 1 April 1998/Accepted 4 May 1998

We have investigated the antiproliferative effects of SCH 56592, a new experimental triazole, against Trypanosoma (Schizotrypanum) cruzi, the etiological agent of Chagas’ disease in Latin America. SCH 56592 blocked the proliferation of the epimastigote form of the parasite in vitro at 30 nM, a concentration 30- to 100-fold lower than that required with the reference compounds ketoconazole and itraconazole. At that concentration all the parasite’s endogenous sterols (ergosterol, 24-ethyl-cholesta-5,7,22-trien-3β-ol, and its 22-dihydro analogs), were replaced by methylated sterols (lanosterol and 24-methylene-dihydrolanosterol), as revealed by high-resolution gas chromatography coupled with mass spectrometry. This indicated that the primary mechanism of action of the drug was inhibition of the parasite’s sterol C-14 demethylase. Against the clinically relevant intracelluar amastigote form, grown in cultured Vero cells at 37°C, the MIC of SCH 56592 was 0.3 nM, again 33- to 100-fold lower than that of ketoconazole or itraconazole. In a murine model of acute Chagas’ disease, SCH 56592 given at ≥ 10 mg/kg of body weight/day for a total of 43 doses allowed 85 to 100% survival and 90 to 100% cure of the surviving animals, as verified by parasitological, serological, and PCR-based tests, while ketoconazole given at 30 mg/kg day allowed 60% survival but only 20% cure. In a murine model of chronic Chagas’ disease, SCH 56592 was again more effective than ketoconazole, providing 75 to 85% protection from death, with 60 to 75% parasitological cures of the surviving animals, while no parasitological cures were observed with ketoconazole. The results indicate that SCH 56592 is the most powerful sterol biosynthesis inhibitor ever tested against T. cruzi and may be useful in the treatment of human Chagas’ disease.

Chagas’ disease (American trypanosomiasis) is a parasitic disease caused by the kinetoplastid protozoon Trypanosoma (Schizotrypanum) cruzi, which afflicts 16 to 18 million people in Latin America and causes an estimated loss of 2.7 disability-adjusted years annually. It accounts for the largest parasitic disease burden in the region and the third largest worldwide, following malaria and schistosomiasis (42). Thirty to 40% of patients who survive the initial acute phase (ca. 90%) develop irreversible heart and gastrointestinal lesions over years or decades, and these frequently lead to death (30). Chemotherapy of this disease is still very unsatisfactory, since it is based on nitrofurans (nifurtimox; Bayer) and nitrimidazoles (benzimidazole; Roche), which act through the induction of oxidative or reductive damage of the parasite but which can also produce serious toxic effects in the host (6, 7, 29, 30). These compounds block the proliferation of the epimastigote form of the parasite in vitro at 30 nM, a concentration 30- to 100-fold lower than that required with the reference compounds ketoconazole and itraconazole. At that concentration all the parasite’s endogenous sterols (ergosterol, 24-ethyl-cholesta-5,7,22-trien-3β-ol, and its 22-dihydro analogs), were replaced by methylated sterols (lanosterol and 24-methylene-dihydrolanosterol), as revealed by high-resolution gas chromatography coupled with mass spectrometry. This indicated that the primary mechanism of action of the drug was inhibition of the parasite’s sterol C-14 demethylase. Against the clinically relevant intracelluar amastigote form, grown in cultured Vero cells at 37°C, the MIC of SCH 56592 was 0.3 nM, again 33- to 100-fold lower than that of ketoconazole or itraconazole. In a murine model of acute Chagas’ disease, SCH 56592 given at ≥ 10 mg/kg of body weight/day for a total of 43 doses allowed 85 to 100% survival and 90 to 100% cure of the surviving animals, as verified by parasitological, serological, and PCR-based tests, while ketoconazole given at 30 mg/kg day allowed 60% survival but only 20% cure. In a murine model of chronic Chagas’ disease, SCH 56592 was again more effective than ketoconazole, providing 75 to 85% protection from death, with 60 to 75% parasitological cures of the surviving animals, while no parasitological cures were observed with ketoconazole. The results indicate that SCH 56592 is the most powerful sterol biosynthesis inhibitor ever tested against T. cruzi and may be useful in the treatment of human Chagas’ disease.
has antiparasitic activity comparable or superior to that of D0870.

MATERIALS AND METHODS

Parasite. For the in vitro studies, the EP (8) and Y strains of T. cruzi were used with similar results; for the in vivo studies, the Y and Bertoldo (34, 38) strains were used. Live T. cruzi was handled according to established guidelines (12).

In vitro studies. The epimastigote form of the parasite was cultivated in a modification of liver infusion-tryptose (LIT) medium (8) supplemented with 10% newborn calf serum (Gibco) at 28°C with strong agitation (120 rpm); the cultures were initiated with a cell density of $2 \times 10^6$ epimastigotes per ml, and the drugs were added when cell density reached $1 \times 10^7$ epimastigotes per ml. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics, Inc., Hialeah, Fla.) and by direct counting with a hemocytometer. Cell viability was followed by trypan blue exclusion using light microscopy. Amastigotes were cultured in Vero cells maintained in minimal essential medium supplemented with 2% fetal calf serum in a humidified 95% air–5% CO₂ atmosphere at 37°C as previously described (16, 35–37, 39). Cells were infected with 10 tissue culture-derived trypomastigotes per cell for 2 h and then were washed three times with phosphate-buffered saline to remove nonadherent parasites. Fresh medium with or without drugs was added, and the cells were incubated for 96 h, with a change of medium at 48 h. Quantification of the number of infected cells and of the number of parasites per cell by use of light microscopy and statistical analysis of the results were carried out as described elsewhere (16, 35–37, 39).

Studies of lipid composition. For the analysis of the effects of drugs on the sterol composition of the epimastigotes, total lipids from control and drug-treated cells were extracted and fractionated by silicic acid column chromatography and gas-liquid chromatography (16, 38–40). The neutral lipid fractions were preliminarily analyzed by thin-layer chromatography (on Merck 5721 silica gel plates with heptane-isopropyl ether-glacial acetic acid [60:40:4] as the developing solvent) and conventional gas-liquid chromatography (isotactic separation in a 4-m glass column packed with 5% OV-1 on Chromosorb 100/200 mesh, with nitrogen as the carrier gas at 24 ml/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, the neutral lipids were separated in a capillary high-resolution column (25 m by 0.20 mm [inner diameter]) Ultra-2 column, 5% phenyl-methyl-siloxane, 0.33-μm film thickness) in a Hewlett-Packard 5890 series II gas chromatograph equipped with an HP5971A mass-sensitive detector. Lipids were injected in ethyl acetate, and the column was kept at 50°C for 1 min; the temperature was then increased to 270°C at a rate of 25°C min⁻¹ and finally to 300°C at a rate of 1°C min⁻¹. The carrier gas (He) flow was kept constant at 1.0 ml min⁻¹. The injector temperature was 250°C, and the detector temperature was kept at 280°C.

In vivo studies. Studies were carried out as described previously (17, 37, 39) by following the initial studies of McCabe et al. (19–21) for the acute model and the procedures described by Urbina and coworkers (34, 38) for the chronic model. For the acute model, groups of 8 to 11 outbred NMRI albino female mice weighing 25 to 30 g were inoculated intraperitoneally with $10^5$ blood trypomastigotes of the Y strain, and treatment was initiated 24 h later. For the chronic model, animals were inoculated with $10^4$ blood trypomastigotes of the Bertoldo strain, and treatment was initiated in surviving animals when no circulating parasites could be observed, usually 45 to 60 days postinfection (p.i.). The drugs were suspended in aqueous 2% methylcellulose plus 0.5% Tween 80 and given by gavage; controls (untreated animals) received the vehicle as a placebo, which had no detectable toxic effects. Treatment was given once daily for 28 consecutive days, followed by a 7-day rest and another 15 days of treatment. Surviving animals were monitored for up to 100 to 120 days p.i. Parasitemia was measured.
in a hemocytometer by using tail blood. The Kaplan-Meier nonparametric method was used to estimate the survival functions of the different experimental groups, and rank tests were used to compare them; these analyses were done by using the Survival Tools for StatView 4.5 run in a Power Macintosh 7100/66 computer. Hemocultures were carried out by inoculating 2 ml of liver infusion medium with 0.4 ml of blood obtained from experimental animals by cardiac puncture; the cultures were microscopically examined for the presence of proliferative epimastigote forms weekly for 4 weeks. Surviving animals were sacrificed, and organs (spleen, heart, and liver) were minced individually in 1 ml of sterile phosphate-buffered saline with 10 mM D-glucose. Portions (0.4 ml) of

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Mass % with the following concn of SCH 56592:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 (control) 0.01 μM 0.03 μM 0.3 μM 3.0 μM</td>
</tr>
<tr>
<td>EXOGENOUS:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHOLESTEROL</td>
<td></td>
<td>9.6 8.1 9.8 11.9 12.3</td>
</tr>
<tr>
<td>ENDogenous, 14-DESMETHYL:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-METHYL-5,7,17,22-CHOLESTA-10-EN-30-OL (ERGOSTEROL)</td>
<td></td>
<td>15.3 2.0 &lt;1 &lt;1 &lt;1</td>
</tr>
<tr>
<td>24-ETHYL-5,7,17-CHOLESTA-10-EN-30-OL</td>
<td></td>
<td>17.0 4.5 &lt;1 &lt;1 &lt;1</td>
</tr>
<tr>
<td>ERGOSTA-5,7-DIEN-30-OL</td>
<td></td>
<td>35.1 2.0 &lt;1 &lt;1 &lt;1</td>
</tr>
<tr>
<td>24-ETHYL-5,7-CHOLESTA-6,10-DIEN-30-OL</td>
<td></td>
<td>17.1 9.8 &lt;1 &lt;1 &lt;1</td>
</tr>
<tr>
<td>ENDogenous, 14-METHYL:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SQUALENE</td>
<td></td>
<td>5.9 7.9 23.7 22.9 39.1</td>
</tr>
<tr>
<td>24-METHYLENE-CHYDOROLANOSTEROL</td>
<td></td>
<td>&lt;1 56.8 55.5 48.4 39.5</td>
</tr>
<tr>
<td>4,14-DIMETHYL-ERGOSTA-5,8(14),10-TRIEN-30-OL (ORTURIFOLIOLE)</td>
<td></td>
<td>&lt;1 4.8 4.7 2.5 &lt;1</td>
</tr>
<tr>
<td>LANOSTEROL</td>
<td></td>
<td>&lt;1 4.1 6.3 14.3 9.1</td>
</tr>
</tbody>
</table>

Sterols were extracted from *T. cruzi* epimastigotes cultured in LIT medium in the absence or in the presence of the indicated concentrations of SCH 56592 for 120 h; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass spectrometry as described in Materials and Methods.
these suspensions were inoculated in juvenile (15 to 20-g) animals. Hemoinoculation (50 μl of blood diluted to 100 μl with sterile phosphate-buffered saline) was done subcutaneously in 10- to 12-day-old mice. Xenodiagnosis was done with 10 2nd-stage Rodnius prolixus nymphs per mouse. After 2 weeks the feces were analyzed for T. cruzi metacyclic forms, and the examination was repeated weekly thereafter for 1 month. The presence of circulating anti-T. cruzi antibodies was detected by immunoprecipitation of 125I-labeled total-surface epimastigote antigens with experimental sera in the presence of protein A, followed by analysis of the precipitate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. DNA extraction and PCR of blood samples were carried out as described previously (2, 3, 41), by using the T. cruzi-specific primers 5'-AAATAATGTACGG(T/G)GAGATGCATGA-3' and 5'-GGTTCGATTGGGGTTGGTGTAAT-3'.

**Drugs.** SCH 56592 was provided by Schering-Plough Research Institute through David Loebenberg, while ketoconazole and itraconazole were provided by Janssen Pharmaceutica, Caracas, Venezuela. The drugs were added to cultures as dimethyl sulfoxide solutions; the final dimethyl sulfoxide concentration in the culture medium never exceeded 1% (vol/vol), and dimethyl sulfoxide had no effect by itself on the proliferation of the parasites or Vero cells.

**RESULTS AND DISCUSSION**

**In vitro studies.** Figure 2 presents the effects of SCH 56592 on the epimastigote form of T. cruzi, a form equivalent to that present in its reduvid vectors, grown in modified LIT medium at 28°C (8). It can be seen that with all the concentrations tested there were no immediate effects on epimastigote proliferation, but at ≥30 nM, growth arrest and subsequent rounding up and lysis of the cells (verified by light microscopy and trypan blue exclusion) ensued. This delayed lytic effect is a characteristic effect of sterol biosynthesis inhibitors, associated with the time required for the complete depletion of the parasite’s endogenous sterols (16, 36–40). This was directly confirmed by analysis of the parasite’s sterols with high-resolution gas-liquid chromatography coupled to mass spectrometry. Table 1 shows that in epimastigotes incubated for 120 h with concentrations of the SCH 56592 equal to or greater than the MIC (30 nM), all the endogenous 4,14-desmethyl sterols (ergosterol, 24-ethyl-cholesta-5,7,22-trien-3β-ol, and precursors) were replaced by 4,14-dimethyl and -trimethyl sterols such as lanosterol, 24-methylene-dihydrolanosterol, and 4,14-dimethyl-ergosta-8,24(24')-3β-ol (obtusifoliol). This confirmed that the primary target of the drug was the parasite’s sterol C-14-demethylase, as was seen in fungi (24, 32, 43). Together with the accumulation of the C-14-methyl sterols, significant amounts of the apolar precursor squalene were detected.
reaching ca. 40% of the total weight of sterols and precursors in the cells at 3 μM SCH 56592 (100 times the MIC). This accumulation is most probably due to mass-action effects and indicated a very effective blockade of the metabolic flow at the level of sterol C-14-demethylase, which in turn suggested tight binding of this drug to its target enzyme. In fact, the concentration of SCH 56592 required for complete endogenous sterol depletion and growth arrest, associated with subsequent cell lysis of the epimastigotes (Fig. 2), was 33- to 100-fold lower than that required for ketoconazole (5, 14, 35, 37), D0870 (16, 38), or itraconazole (data not shown). These results are in line with those of Sanglard et al. (32), who have recently shown that azole-resistant isolates of Candida albicans which had mutations in their sterol 14α-demethylase, which in turn suggestedtight binding of this drug to its target enzyme. In fact, the concentration of SCH 56592 required for complete endogenous sterol depletion and growth arrest, associated with subsequent cell lysis of the epimastigotes (Fig. 2) was 33- to 100-fold lower than that required for ketoconazole (5, 14, 35, 37), D0870 (16, 38), or itraconazole (data not shown). These results are in line with those of Sanglard et al. (32), who have recently shown that azole-resistant isolates of Candida albicans which had mutations in their sterol 14α-demethylase which sharply reduced their affinity for fluconazole or itraconazole nevertheless had normal affinity for SCH 56592.

Against the clinically relevant intracellular amastigote form, proliferating in cultured Vero cells at 37°C, SCH 56592 was again remarkably active. Figure 3A shows that the minimal concentration of SCH 56592 required to eradicate the parasite from the host cells was just 0.3 nM, again 33 to 100 times lower than that required with ketoconazole (Fig. 3B), D0870 (16, 38), or itraconazole (data not shown). It can also be seen from Fig. 3 that SCH 56592 had no effects on the proliferation of the host cells at 100 nM (>300 times the MIC); the same was also observed even at 1 μM (data not shown), indicating a very specific antiparasitic activity. Taken together, these results indicated that SCH 56592 is, in vitro, the most potent sterol biosynthesis inhibitor and antiproliferative agent ever tested against both proliferative stages of T. cruzi.

In vivo studies. In a murine model of acute Chagas’ disease previously described (17, 37, 39), a fulminant infection is produced by inoculating 10⁶ bloodstream trypomastigotes of the virulent Y stock per mouse, leading to the death of all untreated animals in 5 weeks (Fig. 4). Oral treatment was started 24 h p.i. and given daily for 28 consecutive days, followed by a 7-day rest and another 15 days of treatment. As reported previously (17, 34, 37–39), ketoconazole given at 30 mg/kg/day was very effective in suppressing the proliferation of the parasite while the drug pressure was present, but the parasite was not eradicated, as seen by the subsequent increase in parasitemia (Fig. 4B) and the concomitant death of the animals receiving this treatment 70 to 80 days p.i. (Fig. 4A). At low (5- to 10 mg/kg/day) dosages of SCH 56592, some animals displayed delayed parasitemia and subsequently died, as did those receiving ketoconazole, but at higher dosages, no evidence of circulating parasites was found during the observation period (100 to 120 days p.i.), suggesting sterilization of the treated animals. At those higher dosages (15 to 25 mg/kg/day), survival levels varied from 75 to 100%. Parasitological cures (Table 2) were assessed by a combination of xenodiagnosis, hemoculture, hemoinoculation in baby mice, subinoculation of organs in naive animals, xenodiagnosis, and the presence of circulating anti-T. cruzi antibodies as described in references 38 and 39.

### Table 2. Effects of SCH 56592 and ketoconazole on survival and parasitological cure in a murine model of acute Chagas’ disease

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total no. of doses</th>
<th>Results at 114 days p.i.</th>
<th>No. of animals surviving/total no. of animals</th>
<th>No. of negative parasitological and serological tests/no. tested</th>
<th>No. of negative blood PCR tests/no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
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<tr>
<td>Ketoconazole, 30 mg/kg/day</td>
<td>43</td>
<td>6/10</td>
<td>2/10</td>
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<tr>
<td>SCH 56592</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg/day</td>
<td>43</td>
<td>9/9</td>
<td>7/9</td>
<td>7/9</td>
<td>7/9</td>
</tr>
<tr>
<td>10 mg/kg/day</td>
<td>43</td>
<td>5/6</td>
<td>5/6</td>
<td>5/6</td>
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</tr>
<tr>
<td>15 mg/kg/day</td>
<td>43</td>
<td>9/9</td>
<td>8/9</td>
<td>7/9</td>
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</tr>
<tr>
<td>20 mg/kg/day</td>
<td>43</td>
<td>6/8</td>
<td>6/8</td>
<td>6/8</td>
<td>6/8</td>
</tr>
<tr>
<td>25 mg/kg/day</td>
<td>43</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

* Female NMRI albino female mice weighing 25 to 30 g were inoculated intraperitoneally with 10⁶ blood trypomastigotes (Y stock). Treatment was initiated 24 h later and given daily for 28 days, followed by a 7-day rest and another 15 days of treatment. Drugs were suspended in 2% methylcellulose plus 0.5% Tween 80 and were given by gavage. Control (untreated) animals received the vehicle as a placebo. Parasitological cures were assessed by hemoculture, hemoinoculation in baby mice, subinoculation of organs in naive animals, xenodiagnosis, and the presence of circulating anti-T. cruzi antibodies as described in references 38 and 39.

### Table 3. Effects of SCH 56592 and ketoconazole on survival and parasitological cure in a murine model of chronic Chagas’ disease

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total no. of doses</th>
<th>Results at 177 days p.i.</th>
<th>No. of animals surviving/total no. of animals</th>
<th>No. of negative parasitological and serological tests/no. tested</th>
<th>No. of negative blood PCR tests/no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>7/11</td>
<td>2/11</td>
<td>1/11</td>
<td>1/11</td>
</tr>
<tr>
<td>Ketoconazole, 30 mg/kg/day</td>
<td>43</td>
<td>9/11</td>
<td>1/11</td>
<td>1/11</td>
<td>1/11</td>
</tr>
<tr>
<td>SCH 56592</td>
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<td></td>
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</tr>
<tr>
<td>10 mg/kg/day</td>
<td>43</td>
<td>8/11</td>
<td>6/11</td>
<td>6/11</td>
<td>6/11</td>
</tr>
<tr>
<td>15 mg/kg/day</td>
<td>43</td>
<td>10/12</td>
<td>6/12</td>
<td>6/12</td>
<td>6/12</td>
</tr>
</tbody>
</table>

* Female NMRI albino mice (25 to 30 g) were inoculated intraperitoneally with 10⁶ blood trypomastigotes (Bertoldo stock). Treatment was initiated 52 days later, when no circulating parasites were observed, and was given daily for 28 days, followed by a 7-day rest and another 15 days of treatment. Drugs were suspended in 2% methylcellulose plus 0.5% Tween 80 and were given by gavage. Control (untreated) animals received the vehicle as a placebo. Parasitological cures were assessed by hemoculture, hemoinoculation in baby mice, subinoculation of organs in naive animals, xenodiagnosis, and the presence of circulating anti-T. cruzi antibodies as described in references 38 and 39.
such as Nifurtimox and ketoconazole, did not produce significant levels of parasitological cures.

A model of the chronic form of the disease previously described (34, 38) was also investigated. Animals were infected with $10^4$ trypomastigotes of the cardiotropic Bertoldo strain, which produced a slowly developing parasitemia that peaked ca. 25 days p.i. but was effectively controlled by most infected animals. Animals that survived this initial phase (ca. 70%) developed a condition in which their general physical condition deteriorated slowly but they survived for several months, although sudden death was also observed. Treatment was started 45 to 60 days p.i., when no circulating parasites were found. SCH 56592 at 10 to 15 mg/kg/day, given for a total of 43 doses as described above, provided 75 to 85% protection from death and 60 to 75% parasitological cures of the surviving animals, while ketoconazole at 30 mg/kg/day had no significant effect on the level of parasitological cures compared with those for untreated controls (Table 3). Autopsy of control (untreated) animals revealed a significant increase (ca. 50%) in the size of the heart and spleen, with focal inflammatory lesions, characteristic of human and murine chagasic cardiomyopathy (29, 30), while the organs from animals receiving SCH 56592 at curative doses had normal morphology. These results are again comparable only to those previously reported for D0870 (34, 38), which was the first report of parasitological cure of chronic experimental Chagas’ disease. More recently (22a), we have found that SCH 56592 is capable of inducing parasitological cures of experimental T. cruzi infections caused by strains which are moderately to strongly resistant to benznidazole and nifurtimox (10); this result also was previously obtained only with SCH 56592 (22). As we have discussed in previous publications (17, 37), the effective doses of antifungal azoles are, on a weight basis, approximately 10 times smaller in humans than in mice. If this is also valid for SCH 56592, it will suggest that the doses of this compound required for anti-T. cruzi activity in humans will be well within the range which is already known to be well tolerated (13).

In the case of D0870, we have previously argued (16, 38) that its exceptional in vivo anti-T. cruzi activity could be explained only by its special pharmacokinetic properties (4, 9), since its in vitro anti-T. cruzi activity was comparable to that of ketoconazole or nifurtimox, and these drugs have no curative activity in our in vivo models. For SCH 56592, both its high intrinsic anti-T. cruzi activity, reflected in the in vitro results, and the long terminal half-lives and large volumes of distribution in animals (11, 25) and humans (13) should contribute to its in vivo antiparasitic activity. In conclusion, we have shown that SCH 56592 has outstanding and specific in vitro activity against both proliferative stages of T. cruzi, particularly against the clinically relevant intracelular amastigote form. We have also demonstrated that this compound exhibits curative rather than suppressive activity in murine models of acute and chronic Chagas’ disease, and in this respect it is similar or superior to D0870 (34, 38). SCH 56592 is currently in Phase I/II clinical trials as a systemic antifungal agent and should be a logical candidate for clinical trials for the treatment of human Chagas’ disease.

ACKNOWLEDGMENTS

This work received financial support from the UNDP/World Bank/World Health Organization Programme for Research and Training in Tropical Diseases (grant 930161), the National Research Council of Venezuela (CONICIT; grant RP-IV-110034), and the Instituto Venezolano de Investigaciones Científicas. J.A.U. is a John Simon Guggenheim Foundation Fellow.

We gratefully acknowledge the technical support of Gonzalo Visbal and Renée Lira.

REFERENCES


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