In vitro susceptibility of *Trypanosoma cruzi* strains from Santander, Colombia, to hexadecylphosphocholine (miltefosine), nifurtimox and benznidazole

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Introduction. The current chemotherapy for Chagas disease is unsatisfactory with only two drugs available for treatment. Research to discover new drugs for Chagas disease is urgent. Hexadecyl-phosphocholine (HPC, miltefosine) has been demonstrated to have in vitro activity against *Trypanosoma cruzi* parasites, but its activity on different Colombian *T. cruzi* strains is not known.

Objective. To evaluate the in vitro susceptibility of *T. cruzi* strains isolated from humans and vectors in Santander, Colombia, to miltefosine, nifurtimox and benznidazole.

Materials and methods. Eight *T. cruzi* Colombian strains and three reference strains (Esmeraldo, SilvioX10 and Y) were studied. Drug activities against extracellular epimastigotes and intracellular amastigotes were determined by microscopic counting. The results were expressed as the concentrations that inhibited 50% and 90% growth (IC<sub>50</sub> and IC<sub>90</sub>).

Results. For miltefosine a similar range of drug activity was observed against all the Colombian strains, all parasites being more susceptible to miltefosine than to the reference drugs. The intracellular amastigotes were more susceptible to miltefosine (IC<sub>50</sub> 0.08 to 0.63 µM and IC<sub>90</sub> 0.21 to 2.21 µM) than extracellular forms (IC<sub>50</sub> <0.92 to 2.29 µM and IC<sub>90</sub> 1.38 to 4.76 µM). For reference drugs, parasites were more susceptible to nifurtimox than to benznidazole and some differences in activity of benznidazole between *T. cruzi* strains was observed.

Conclusions. The results showed the significant in vitro activity of miltefosine against *T. cruzi* stages, and the expected results for the reference drugs. Further in vivo studies with miltefosine are planned.

Key words: *Trypanosoma cruzi*, Chagas disease, miltefosine, benznidazole, nifurtimox, drug therapy, Colombia.

Susceptibilidad *in vitro* a hexadecilfosfocolina (miltefosina), nifurtimox y benznidazole de cepas de *Trypanosoma cruzi* aisladas en Santander, Colombia

Introducción. Los tratamientos actuales para la enfermedad de Chagas son insatisfactorios y sólo existen dos medicamentos disponibles. La búsqueda de alternativas terapéuticas es prioritaria. La hexadecilfosfocolina (miltefosina) ha mostrado actividad *in vitro* contra *Trypanosoma cruzi*. Sin embargo, su actividad en aislamientos de *T. cruzi* obtenidos en Colombia aún no ha sido reportada.

Objetivo. Evaluar la susceptibilidad *in vitro* a miltefosina, nifurtimox y benznidazole de cepas de *T. cruzi* aisladas de humanos y vectores en Santander, Colombia.

Materiales y métodos. Se evaluó la susceptibilidad de los tres medicamentos en ocho cepas colombianas de *T. cruzi* y tres cepas de referencia: Esmeraldo, Silvio X10 y Y. La actividad de los compuestos fue determinada en epimastigotes extracelulares y amastigotes intracelulares, por conteo microscópico. Los resultados se expresaron en concentraciones inhibitorias 50 y 90 (IC<sub>50</sub> y IC<sub>90</sub>).
Resultados. Para la miltefosina, se observaron rangos similares en la actividad del medicamento entre las cepas colombianas; todos los parásitos fueron más susceptibles a la miltefosina que a los medicamentos de referencia. Los amastigotes intracelulares fueron más sensibles a la miltefosina (CI₅₀, 0,08 a 0,63 µM y CI₉₀, 0,21 a 2,21 µM) que las formas extracelulares (CI₅₀, <0,92 a 2,29 µM y CI₉₀, 1,38 a 4,76 µM). En los medicamentos de referencia, los parásitos fueron más susceptibles al nifurtimox que al benznidazole. Se observaron algunas diferencias en la actividad del benznidazole en las cepas estudiadas de T. cruzi.

Conclusiones. Los resultados obtenidos de la actividad in vitro de miltefosina y de los medicamentos de referencia contra aislamientos de T. cruzi son satisfactorios y serán considerados en estudios posteriores in vivo.

Palabras clave: Trypanosoma cruzi, enfermedad de Chagas, miltefosina, benznidazol, nifurtimox, quimioterapia, Colombia.

Chagas disease, caused by the protozoan parasite Trypanosoma cruzi (Kinetoplastida: Trypanosomatidae), constitutes one of the largest parasitic disease burdens in Latin America where approximately 13-15 million of people are affected (1). Trypanosoma cruzi strains are divided into two main well-defined groups called T. cruzi I and T. cruzi II; five subgroups have been identified inside the T. cruzi II group (Ia, Ib, Ic, IId and Ile) (2). In Colombia, 5% of the population is infected in endemic areas and the predominance of T. cruzi I has been reported (3-7). The Santander Province is one of the territorial divisions where the prevalence of infection is highest (8,9). No vaccines are available, and the chemotherapy remains precarious with only two available drugs, the nitrofuran derivative, nifurtimox (Lampit, Bayer) and 2-nitroimidazole benznidazole (Radanil, Roche). Both are restricted mainly to the treatment of the acute phase or the congenital infection (10,11). Currently, these drugs have severe limitations, including high frequency of undesirable side effects, long protocols of treatment, and limited efficacy and availability (12,13). The research to discover new drugs for the treatment of Chagas disease is imperative.

Hexadecylphosphocholine (HPC; miltefosine), an alkylphosphocholine developed initially as an anticancer drug, constitutes the first oral treat-
Chagas. Biomédica. 2003;23 (Supl.1):119-20). Parasites originated from the domestic cycle in the biogeographical area of Santander, Colombia (Table 1). In addition, T. cruzi reference strains Silvio X10 (T. cruzi I) Esmeraldo and Y (T. cruzi II) were used. The parasites were maintained by serial passage in Liver Infusion Tryptose (LIT) medium supplemented with 10% heat inactivated fetal calf serum (hiFCS, Gibco, USA) and hemin (10 mg/L, Sigma) at 28 °C. They were used within four passages of isolation.

**Drugs**

Hexadecylphosphocholine (HPC, miltefosine) was obtained from A.G Scientific, INC, nifurtimox from Bayer (Germany) and benznidazole from Roche (Switzerland). Nifurtimox and benznidazole were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 1% (v/v). Miltefosine was dissolved directly in culture medium. Working solutions were prepared in LIT or RPMI 1640 culture media immediately before the assays.

**Epimastigotes assay**

Parasites were harvested during the exponential growth phase, diluted in LIT to 5x10^5 parasites/mL and incubated in a three-fold dilution series of miltefosine (0.92-25.0 µM), nifurtimox (1.1-261 µM) and benznidazole (1.2-288 µM) in 96 well multiwell plates (Becton Dickinson, New Jersey, USA), for 72 h at 28 °C. Each drug concentration was evaluated in triplicate and control cultures were maintained without drug. Parasite inhibition was determined microscopically by counting parasite numbers in a Neubauer haemocytometer.

**Intracellular amastigotes assay**

Vero cells, diluted to 3 x 10^5 cells/mL in RPMI 1640 medium plus 10% hiFCS, were plated in 16-well Lab-tek™ tissue culture chamber slides (Life Technologies, Paisley, UK) and allowed to adhere for 24 h at 37 °C in a 5% CO₂-95% air mixture. Adherent cells were infected with tissue derived trypomastigotes (TDT) at a ratio of parasites to cells of 10:1. The cultures were maintained in a 5% CO₂-95% air mixture at 37 °C. TDT were obtained by successive serial infections in Vero cells with released parasites from culture supernatants. Reference strains and the 215 strain were not tested in the amastigote assay due to a failure to generate sufficient TDT to infect cells. After 24 h, free parasites were removed by washing and infected cultures were incubated for 72 h with three fold dilution series of miltefosine (0.04 to 3.30 µM), nifurtimox (0.3-87.0 µM) and benznidazole (1.2 to 96.0 µM) for 72 h at 37 °C, in a 5% CO₂-95% air mixture. The cultures were then fixed with methanol and stained with Giemsa. Each drug concentration was evaluated in quadruplicate and control cultures were maintained without drug. Drug activity was determined by the percentage of infected cells in treated and untreated cultures.

**Statistical analyses**

The drug activities were expressed as the drug concentration needed to inhibit to 50% or 90% of parasite growth (IC₅₀ and IC₉₀). They were calculated by sigmoidal regression analysis (Msxlfit™; ID Business Solution, Guildford, UK). Results were expressed as mean ± standard deviation (SD) and statistical significance was determined by Student t test. Values of p <0.05 were considered significant. Each experiment was repeated twice.

**Results**

The activities of miltefosine, nifurtimox, and benznidazole against T. cruzi epimastigotes are
shown in table 2. All Colombian *T. cruzi* strains were more susceptible to miltefosine (IC$_{50}$ <0.92 to 2.29 ± 1.08 µM and IC$_{90}$ 1.38 ± 0.08 to 4.76 ± 1.54 µM) and nifurtimox (IC$_{50}$ 2.25 ± 0.34 to 5.05 ± 1.70 µM and IC$_{90}$ 12.80 ± 1.24 to 26.38 ± 2.09 µM) than to benznidazole (IC$_{50}$ 9.34 ± 0.78 to 27.30 ± 1.81 µM and IC$_{90}$ 60.00 ± 29.05 to >288 µM). Reference strains showed considerable variation in drug sensitivity: Esmeraldo was the least susceptible to miltefosine (IC$_{50}$ 3.92 ± 0.51 µM and IC$_{90}$ 7.13 ± 0.70 µM) and Y was the least susceptible to benznidazole (IC$_{50}$ 20.35 ± 3.04 µM and IC$_{90}$ >288 µM). The results were reproducible in the two independent experiments.

The activity range of miltefosine, nifurtimox and benznidazole against *T. cruzi* intracellular amastigotes is shown in table 3. All *T. cruzi* strains were highly susceptible to miltefosine (IC$_{50}$ 0.082 ± 0.01 to 0.63 ± 0.13 µM and IC$_{90}$ 0.21 ± 0.70 to 2.21 ± 0.07 µM). Nifurtimox and benznidazole were also active against intracellular amastigotes but with lower values than miltefosine.

Variation in the activity of miltefosine against each life cycle stage was observed. Intracellular amastigotes were more sensitive than epimastigotes (p=0.0000) (tables 2 and 3). Additionally, comparisons of values of IC$_{50}$ and IC$_{90}$ between genetic groups (*T. cruzi* I and *T. cruzi* II) showed no significant difference, with one exception for nifurtimox which showed significant differences in IC$_{90}$ values (p=0.0001) (figure 1).
Table 3. In vitro activities of miltefosine, nifurtimox and benznidazole against intracellular amastigotes of *Trypanosoma cruzi* parasites infecting Vero cells.

<table>
<thead>
<tr>
<th>Parasite code</th>
<th>No. Exp</th>
<th>% inf</th>
<th>Miltefosine</th>
<th>Nifurtimox</th>
<th>Benznidazole</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>IC₅₀ ±SD</td>
<td>IC₉₀ ±SD</td>
<td>IC₅₀ ±SD</td>
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<td>209</td>
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<td>0.44±0.06</td>
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<td>0.45±0.08</td>
<td>1.31±0.37</td>
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<tr>
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<td>Y*</td>
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<td>0.7 ± 0.2</td>
<td>ND</td>
<td>2.7</td>
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</tr>
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</table>

% inf: percent of infection before drug treatment; No. Exp: number of experiment; IC₅₀ and IC₉₀: drug concentration to inhibit 50 or 90 percent of parasite growth; ND: not determined. *Croft S, *et al*. 1996 (24).

Data are displayed as mean ± SD, as obtained from quadruplicate wells. Miltefosine was more effective against intracellular amastigotes of *T. cruzi* than nifurtimox (*p* = 0.0003) and benznidazole (*p*<0.0001).

Discussion

Differences in the drug susceptibility of *T. cruzi* strains according to genetic group, host and geographical origin have been described (21-23). However, investigations performed to date have mainly evaluated susceptibilities to benznidazole and nifurtimox. Both of these standard drugs have limited efficacy in the chronic form of Chagas disease and they produce toxic side effects (12). Therefore, there is an urgent need for studies that evaluate the activity of new anti-trypanosomal drugs.

This is the first study that shows the in vitro susceptibility to miltefosine of *T. cruzi* strains isolated from humans and vectors in a Colombian endemic area. All the parasites strains and stages tested were highly susceptible to miltefosine. Similar results were obtained by other authors using *T. cruzi* reference strains confirming the broad activity of this drug (17-19,24). Miltefosine was significantly more active against intracellular amastigotes than epimastigotes (*p*<0.001) (Table 3). This has been demonstrated in earlier studies as well (17,19). Initially, phospholipid-derived drugs were discovered as a new class of biological response modifiers, with therapeutic activity in cancer mediated by an increase in the cytotoxic properties of macrophages (25).

Saraiva *et al*. (2002) observed that miltefosine may activate macrophages *in vitro* and the antiparasitic activity of this compound on intracellular amastigotes was independent of nitric oxide production (17). The participation of some cytokines was suggested. However, the host cell mechanisms involved in the inhibition or parasite killing after miltefosine treatment is still unclear.

Against *Leishmania* parasites, a significant variation of the *in vitro* susceptibility to miltefosine has been described for several species of *Leishmania* (26). In patients with cutaneous leishmaniasis, it is more effective in lesions caused by *L. panamensis* as compared to those by *L. braziliensis* (27). In contrast with the results obtained in the present study, miltefosine was found to be more active against promastigotes of *Leishmania* than against intracellular
amastigotes infecting bone marrow derived macrophages (26). This difference with *T. cruzi* may be due to intrinsic differences of both parasites. However, Croft (2003) proposed that in comparative studies between extracellular and intracellular stages one must consider that: (i) the effective drug concentration in host cells may be higher or lower than that outside the cells, (ii) the division rates of the stages differ, or (iii) the assay temperatures may differ (16).

In experimental *in vivo* models, miltefosine has also proved to be active against Leishmania and *T. cruzi* when it was administered orally (17,21,28,29). Unfortunately, only two studies with *T. cruzi* have been reported; both showed a suppressive effect against *T. cruzi* on BALB/c-infected mice, although they used different protocols of treatment and infection (17,21). Further research in a murine or another more appropriate *in vivo* model is necessary to determine aspects such as pharmacokinetic properties, dose-response relationship and protocols of treatment.

We also investigated the susceptibility of *T. cruzi* strains to two nitroheterocyclic compounds (nifurtimox and benznidazole). Although all strains were susceptible to both drugs, we noted that nifurtimox was more active than benznidazole both in epimastigotes (p<0.001) and intracellular amastigotes (P=0.0004). Benznidazole showed greater variation in its activity against different Colombian *T. cruzi* strains. This could be related to the different mechanisms of action of both drugs (30,31). Additionally, in contrast to the results obtained with miltefosine, the epimastigotes of strains isolated from vectors were more susceptible to nifurtimox (p=0.03) and benznidazole (p= 0.01) than those isolated from humans (table 2). Several studies have reported a wide variation in the susceptibility of a large number of *T. cruzi* strains isolated from different hosts and geographic areas to these nitroheterocyclic compounds (22,23,32-34). The existence of predominant strains with particular levels of susceptibility to these drugs in specific geographical areas has been also reported (22,23). In these cases, the parasite drug susceptibility before clinical treatment must be considered as a factor. Moreover, for a better understanding of the response to drugs, the local strains should be characterized genetically and biologically, as completed in this study (Luna KP, Jaramillo CL, Rueda CM, Hernández IP, Zorro MM, Angulo VM, *et al*. Biological and genetic characteristics of *Trypanosoma cruzi* isolates from an endemic area of Santander, Colombia. Bol Malariol Sal Amb. 2007;47 (Supl 1):139-40).

Many authors have explored possible links between the phylogenetic diversity of *T. cruzi* and biological properties (24,35-38). Correlations between the susceptibility to benznidazole and genetic groups of *T. cruzi* have been described (24,38). However, in the current study, no significant differences were observed in the drug susceptibility *in vitro* between *T. cruzi* I and *T. cruzi* II strains (figure 1). This is in agreement with Villareal *et al.* (2004) (39), although due to the low number of strains analyzed in the current work, the results obtained must be considered as preliminary data.

In conclusion, the current *in vitro* study described the susceptibility to miltefosine, nifurtimox and benznidazole of *T. cruzi* strains isolated from humans and vectors in the same transmission area of Chagas disease in Colombia. Miltefosine showed greater activity than the used reference drugs and no differences in the susceptibility according to host origin were observed. These were considered promising results. Further investigation with these strains in murine models of experimental Chagas disease will be necessary to establish the potential of miltefosine for the treatment of human Chagas disease before undertaking clinical studies.

**Conflicts of interest**

The authors declare that there are no conflicts of interest on the results published in this paper.

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References


(miltefosine), ET-18-OCH(3) (edelfosine) and amphoter- 

27. Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, 
et al. Miltefosine for new world cutaneous leishmaniasis. 

28. Escobar P, Yardley V, Croft SL. Activities of 
hexadecylphosphocholine (miltefosine), AmBisome, and 
sodium stibogluconate (Pentostam) against Leishmania 
donovani in immunodeficient scid mice. Antimicrob 

29. Murray HW, Delph-Etienne S. Visceral leishmanicidal 
activity of hexadecylphosphocholine (miltefosine) in 
mice deficient in T cells and activated macrophage 

30. Maya JD, Repetto Y, Agosin M, Ojeda JM, Téllez R, 
Gaule C, et al. Effects of nifurtimox and benznidazole upon 
glutathione and trypanothione content in epimastigote, 
trypanmastigote and amastigote forms of Trypanosoma 

31. de Castro SL. The challenge of Chagas’ disease 
chemotherapy: an update of drugs assayed against 

32. Andrade SG, Rassi A, Magalhaes JB, Ferrioli Filho F, 
Luquetti AO. Specific chemotherapy of Chagas disease: 
a comparison between the response in patients and 
experimental animals inoculated with the same strains. 

33. Toledo MJ, Guilherme AL, da Silva JC, de Gasperi 
MV, Mendes AP, Gomes ML, et al. Trypanosoma cruzi: 
chemotherapy with benznidazole in mice inoculated 
with strains from Parana state and from different 
endemic areas of Brazil. Rev Inst Med Trop Sao Paulo. 

34. Murta SM, Gazzinelli RT, Brener Z, Romanha AJ. 
Molecular characterization of susceptible and naturally 
resistant strains of Trypanosoma cruzi to benznidazole 

35. Laurent JP, Barnabe C, Quesney V, Noel S, Tibayrenc 
M. Impact of clonal evolution on the biological diversity of 

36. de Lana M, da Silveira A, Barnabe C, Quesney V, 
Noel S, Tibayrenc M. Trypanosoma cruzi: compared 
vectorial transmissibility of three major clonal genotypes 

37. Toledo MJ, de Lana M, Carneiro CM, Bahia MT, 
Machado-Coelho GL, Veloso VM, et al. Impact of 
Trypanosoma cruzi clonal evolution on its biological 

38. Toledo MJ, Bahia MT, Carneiro CM, Martins-Filho 
OA, Tibayrenc M, Barnabe C, et al. Chemotherapy 
with benznidazole and itraconazole for mice infected 
with different Trypanosoma cruzi clonal genotypes. 

39. Villarreal D, Barnabe C, Sereno D, Tibayrenc M. Lack of 
correlation between in vitro susceptibility to benznidazole 
and phylogenetic diversity of Trypanosoma cruzi, the 