

ARTÍCULO ORIGINAL

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

Katherine Paola Luna¹, Indira Paola Hernández¹, César Mauricio Rueda¹,
María Magdalena Zorro¹, Simon L. Croft², Patricia Escobar¹

¹ Centro de Investigación en Enfermedades Tropicales (CINTROP), Facultad de Salud, Escuela de Medicina, Departamento de Ciencias Básicas, Universidad Industrial de Santander, Piedecuesta, Santander, Colombia.

² Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom

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 (IC₅₀ and IC₉₀).

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 (IC₅₀ 0.08 to 0.63 µM and IC₉₀ 0.21 to 2.21 µM) than extracellular forms (IC₅₀ <0.92 to 2.29 µM and IC₉₀ 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 (CI₅₀ y CI₉₀).

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 (CI_{50} , 0,08 a 0,63 μ M y CI_{90} , 0,21 a 2,21 μ M) que las formas extracelulares (CI_{50} , <0,92 a 2,29 μ M y CI_{90} , 1,38 a 4,76 μ M). 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 (IIa, IIb, IIc, IId and IIe) (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 nitrofurán 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-

ment for visceral and cutaneous leishmaniasis (14,15). It shows a broad spectrum of drug activity on several types of parasites such as *Leishmania* spp., *T. cruzi* and *Entamoeba histolytica* (16). In *T. cruzi* models, it is active against free and intracellular live forms of parasites as well as strains with different levels of susceptibility to benznidazole (17,18). Its mechanism of action is not clear, but parasite membranes are probably the primary target, possibly by interference with lipid metabolism (18,19). Additionally, synergism of miltefosine or edelfosine (another alkyl-phospholipid drug) in combination with ketoconazole has also been demonstrated (18-20).

The purpose of the current study was to evaluate the *in vitro* activities of miltefosine, nifurtimox and benznidazole against Colombian *T. cruzi* strains, as well as against the Silvio X10, Esmeraldo and Y reference strains.

Materials and methods

Parasites

Eight strains of *T. cruzi*, isolated from various hosts by hemoculture and xenoculture, were used (Luna KP, Jaramillo CL, Gutierrez R, Esteban L, Angulo VM. Aislamiento de *T. cruzi* en pacientes en fase crónica de la enfermedad de Chagas por medio de hemocultivo y xenocultivo. Biomédica. 2003;23 (Supl.1):119). They have been previously characterized by amplification of the mini-exon gene nontranscribed spacer (Gutierrez R, Luna KP, Angulo VM, Fernandes O. Eficiencia de los métodos parasitológicos de diagnóstico y caracterización de poblaciones de *Trypanosoma cruzi* en un área endémica de

Correspondence:

Patricia Escobar, CINTRIP, Facultad de Salud, Escuela de Medicina, Departamento de Ciencias Básicas, Universidad Industrial de Santander, Bucaramanga, Colombia.
Teléfono/fax: (0057) 656 3971
pescobar@uis.edu.co

Recibido: 28/11/08; aceptado:08/04/09

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 5×10^5 parasites/mL and incubated in a three-fold dilution series of miltefosine (0.92-25.0 μ M), nifurtimox (1.1-261 μ M) and benznidazole (1.2-288 μ M) 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 3×10^5 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 μ M), nifurtimox (0.3-87.0 μ M) and benznidazole (1.2 to 96.0 μ M) 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 (Mxflfit™; ID Business Solution, Guildford, UK). Results were expressed as mean \pm 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

Table 1. *Trypanosoma cruzi* parasites strains used in this study.

Parasite strains	Classification ^a	Host
215	Z3	Human
209	<i>T. cruzi</i> I	Human
204	<i>T. cruzi</i> I	Human
132	<i>T. cruzi</i> I	Human
070106	<i>T. cruzi</i> I	<i>Rhodnius prolixus</i>
320101	<i>T. cruzi</i> I	<i>R. prolixus</i>
421102	<i>T. cruzi</i> I	<i>Triatoma dimidiata</i>
187401	<i>T. cruzi</i> I	<i>T. dimidiata</i>
Silvio X10	<i>T. cruzi</i> I	Human
Esmeraldo	<i>T. cruzi</i> II	Human
Y	<i>T. cruzi</i> II	Human

^a Classification from amplification of mini-exon gene non-transcribed spacer by Fernández, et al., 2001 (Gutiérrez, et al., 2003)

shown in table 2. All Colombian *T. cruzi* strains were more susceptible to miltefosine ($IC_{50} < 0.92$ to $2.29 \pm 1.08 \mu M$ and $IC_{90} 1.38 \pm 0.08$ to $4.76 \pm 1.54 \mu M$) and nifurtimox ($IC_{50} 2.25 \pm 0.34$ to $5.05 \pm 1.70 \mu M$ and $IC_{90} 12.80 \pm 1.24$ to $26.38 \pm 2.09 \mu M$) than to benznidazole ($IC_{50} 9.34 \pm 0.78$ to $27.30 \pm 1.81 \mu M$ and $IC_{90} 60.00 \pm 29.05$ to $>288 \mu M$). Reference strains showed considerable variation in drug sensitivity: Esmeraldo was the least susceptible to miltefosine ($IC_{50} 3.92 \pm 0.51 \mu M$ and $IC_{90} 7.13 \pm 0.70 \mu M$) and Y was the least susceptible to benznidazole ($IC_{50} 20.35 \pm 3.04 \mu M$ and $IC_{90} >288 \mu M$). 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 ($IC_{50} 0.082 \pm 0.01$ to $0.63 \pm 0.13 \mu M$ and $IC_{90} 0.21 \pm 0.70$ to $2.21 \pm 0.07 \mu M$). 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).

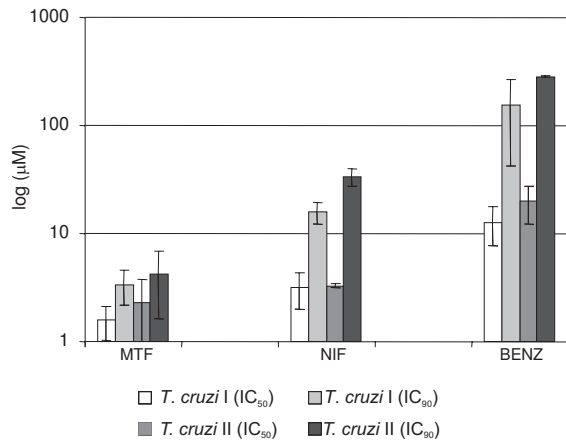


Figure 1. Susceptibility of *T. cruzi* I and *T. cruzi* II epimastigotes to miltefosine, nifurtimox and benznidazole.

MTF: miltefosine; NIF: nifurtimox, BENZ: benznidazole, IC_{50} and IC_{90} : drug concentration to inhibit 50 or 90 percent of epimastigotes. The each bar represents the mean \pm SD obtained from triplicate wells of a representative experiment. Values of p for each case were: IC_{50} : MTF ($p=0.68$) NIF ($p= 1.00$) BENZ ($p= 0.09$) and IC_{90} MTF ($p=0.46$) BENZ (0.1989) NIF ($p=0.0001$). $p < 0.05$ were considered statistically significant.

Table 2. In vitro activities of miltefosine, nifurtimox and benznidazole against extracellular epimastigotes of *Trypanosoma cruzi* parasites.

Parasite code	Miltefosine		Nifurtimox		Benznidazole	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
215	1.17±0.04	2.06±0.14	<3.2	26.38±2.09	27.30±1.81	>288
209	1.75±0.19	3.60±0.27	5.05±1.71	16.06±2.94	21.03±3.55	>288
204	0.93±0.01	2.36±0.05	4.24±0.32	17.56±1.85	20.39±1.22	>288
132	0.96±0.04	2.36±0.16	3.81±1.91	15.11±2.47	11.65±4.15	114.46±29.76
070I06	<0.92	1.38±0.08	2.25±0.34	21.60±2.04	11.40±0.43	66.70±11.51
320I01	2.29±1.08	4.22±1.29	2.47±0.50	18.76±7.67	9.48±2.31	60.00±29.05
421I02	1.96±0.67	4.76±1.54	3.80±1.22	12.80±1.24	11.34±3.31	>288
1874-01	1.97±0.09	4.61±0.38	2.31±0.27	14.39±1.08	9.34±0.78	61.48±5.92
SilvioX10	1.85±0.28	3.82±0.48	1.56±0.40	10.58±0.71	7.33±1.53	27.39±3.72
Esmeraldo	3.92±0.51	7.13±0.70	<3.2	38.84±7.50	12.12±0.49	277.91±43.12
Y	1.73±0.16	3.50±0.26	3.50±0.58	35.28±9.47	20.35±3.04	>288

IC_{50} and IC_{90} : drug concentration to inhibit 50 or 90 percent of epimastigotes growth. Data are displayed as mean \pm SD of two independent experiments performed in triplicate. Miltefosine was more effective against epimastigotes of *T. cruzi* than nifurtimox ($p=0.004$) and benznidazole ($p<0.001$).

Table 3. In vitro activities of miltefosine, nifurtimox and benznidazole against intracellular amastigotes of *Trypanosoma cruzi* parasites infecting Vero cells.

Parasite code	No. Exp	% inf	$\mu\text{M} \pm \text{SD}$					
			Miltefosine		Nifurtimox		Benznidazole	
			IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
209	1	43.6	0.16±0.005	0.44±0.06	>1.1	7.35±4.52	3.66±0.10	23.09±5.94
	2	43.9	0.22±0.07	0.45±0.08	1.31±0.37	6.82±4.04	5.90±0.81	23.72±16.73
204	1	66.8	0.213±0.04	0.98±0.22	1.80±0.48	9.63±7.15	8.16±1.77	46.46±23.64
	2	66.5	0.22±0.003	0.72±0.09	1.43±0.26	5.03±2.95	6.32±1.38	74.14±15.14
132	1	47.3	0.10±0.006	0.34±0.13	1.82±0.35	6.63±1.91	5.98±0.76	25.37±8.58
	2	66.5	0.34±0.08	2.12±0.11	1.52±0.43	8.94±5.02	11.49±3.26	66.92±21.68
070I06	1	97.9	0.63±0.13	0.91±0.07	2.62±0.48	9.98±3.67	7.72±1.47	58.60±13.60
	2	89.7	0.082±0.01	0.21±0.07	1.45±0.28	5.17±3.12	5.73±1.10	38.61±17.40
320I01	1	45	0.09±0.02	1.31±0.16	ND	ND	5.93±1.43	>96
	2	70	0.21±0.003	0.43±0.05	3.44±0.43	11.37±1.50	6.16±1.14	29.18±14.55
421I02	1	38.8	0.33±0.03	0.51±0.19	1.63±0.51	44.83±12.30	3.33±0.424	30.69±1.98
	2	69.6	0.13±0.03	1.99±0.87	3.23±1.72	>87	13.38±5.21	>96
187401 I	1	72.7	0.44±0.08	1.56±0.80	3.55±2.05	22.90±8.71	22.03±8.07	>96
	2	78.4	0.13±0.03	1.00±0.43	0.47±0.24	28.34±4.23	6.81±3.70	14.00±8.80
Y*		ND	0.7 ± 0.2	ND	2.7	ND	ND	ND

% 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 \pm 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.

Financing

This work was supported by the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología "Francisco Jose de Caldas" Colciencias (Grant 1102-04-12928) and by the Universidad Industrial de Santander, Bucaramanga, Colombia.

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