The toxic effects of piperine against Trypanosoma cruzi: Ultrastructural alterations and reversible blockage of cytokinesis in epimastigote forms

ARTICLE *in* PARASITOLOGY RESEARCH · MAY 2008

Impact Factor: 2.1 · DOI: 10.1007/s00436-008-0876-9 · Source: PubMed

10 AUTHORS, INCLUDING:

Gustavo Miranda Rocha
Federal University of Rio de Janeiro
12 PUBLICATIONS **111** CITATIONS
SEE PROFILE

Lucia Mendonça-Previato
Federal University of Rio de Janeiro
66 PUBLICATIONS **1,323** CITATIONS
SEE PROFILE

Tecia Carvalho
Federal University of Rio de Janeiro
66 PUBLICATIONS **1,153** CITATIONS
SEE PROFILE

Norton Heise
Federal University of Rio de Janeiro
38 PUBLICATIONS **882** CITATIONS
SEE PROFILE

Parasitol Res (2008) 102:1059–1067
DOI 10.1007/s00436-008-0876-9

ORIGINAL PAPER

The toxic effects of piperine against *Trypanosoma cruzi*: ultrastructural alterations and reversible blockage of cytokinesis in epimastigote forms

Leonardo Freire-de-Lima · Tatiana Santana Ribeiro · Gustavo Miranda Rocha · Bruno Alves Brandão · Alexandre Romeiro · Lucia Mendonça-Previato · José Osvaldo Previato · Marco Edilson Freire de Lima · Técia Maria Ulisses de Carvalho · Norton Heise

Received: 19 October 2007 / Accepted: 3 January 2008 / Published online: 29 January 2008

Abstract In a previous work, we have investigated the effects of piperine and several of its chemical derivatives on the proliferation of the protozoan parasite *Trypanosoma cruzi*. It was observed that natural piperine is more active against intracellular amastigotes than axenically grown epimastigotes with IC_{50} values of 4.91 and 7.36 μM, respectively. Despite its superior trypanocidal activity against the intracellular amastigotes, here, we show that piperine did not enhance microbiocidal characteristics of murine peritoneal macrophages (Mø) based on nitric oxide production. As shown by light and electron microscopy analysis, epimastigotes treated with sublethal concentrations of piperine presented a reversible cell cycle arrestment and become round shaped, with swelling of the mitochondrion matrix and intense intracellular vacuolization with structures displaying complex membrane invaginations. Similar to the effects of exposing epimastigotes to the antitumor and microtubule stabilizer taxol, multiplication of cell organelles such as the flagellum, kinetoplast, and nucleus occurred, but division into daughter cells was impaired. Unlike the effects caused by the anti-microtubular vinca alkaloids vincristine and vinblastine, which also induce cytokinesis arrestment in *T. cruzi* epimastigotes, piperine did not induce the formation of giant multinucleated cells. The data reinforce the selectivity of the mechanisms of action of piperine against *T. cruzi*.

L. Freire-de-Lima · A. Romeiro · L. Mendonça-Previato · J. O. Previato · N. Heise (✉)
Laboratório de Glicobiologia, Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Bloco G, Sala G1-019, Av. Carlos Chagas Filho 373, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ 21941-902, Brazil
e-mail: nheise@biof.ufrj.br

G. M. Rocha · B. A. Brandão · T. M. U. de Carvalho
Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil

T. S. Ribeiro · M. E. F. de Lima
Departamento de Química, Instituto de Ciências Exatas, Universidade Federal Rural do Rio de Janeiro, Km 7, BR 465, Seropédica, RJ 23890-000, Brazil

Introduction

The protozoan parasite *Trypanosoma cruzi* is the causative agent of American trypanosomiasis or Chagas disease, a human harmful disease affecting 16 to 18 million people in South and Central America (World Health Organization 2002). In June 2006, PAHO/WHO certified that most of the countries from the Southern Cone have eliminated Chagas disease transmission through both the principal insect vector and transfusion of blood and blood products (Pinto Dias 2006), but the emergence of several cases in non-endemic countries raised a new global campaign to eliminate the disease by 2010 (http://www.paho.org/). Chagas disease may lead to a severe chronic dysfunction of the cardiac muscle, the gastrointestinal tract, or the autonomous nervous system, and can be fatal in 5–10% of clinically affected patients (Prata 1994). Today, only benznidazole is in use for treating the acute form of Chagas

disease, as nifurtimox, used in the past in several Latin American countries, was discontinued because of its high degree of toxicity (Barret et al. 2003). Possible mechanisms of action of benznidazole against *T. cruzi* include the inhibition of DNA, RNA, and protein biosynthesis, and the enhanced generation of intermediary reactive oxygen species, like hydrogen peroxide and superoxide (Stoppani 1999). The deficiency of trypanosomatids in antioxidant enzymes like catalase (Boveris et al. 1980) and their lower activity for trypanothione peroxidase when compared to the glutathione peroxidase of mammalian cells (Henderson et al. 1987) makes the generation of such intermediate reactive species an attractive mechanism of toxicity against *T. cruzi*. There is currently no effective drug to cure chronically infected individuals (Urbina and Docampo 2003). In addition, the lack of interest of drug companies in clinically developing promising compounds for Chagas disease (Ceaser 2005), contributed to include this disease in the list of the world's most neglected tropical diseases. Therefore, new drugs and drug targets are needed to extend the range of chemicals available against the parasite.

Piperine (1-piperoylpiperidine, Fig. 1a), the major plant alkaloid of fruit bodies of *Piper* species, inhibits enzymes of the pyruvate–malate cycle in rats (Malini et al. 1999), and presents anti-leishmanial (Raay et al. 1999), anti-inflammatory (Kumar et al. 2005; Woo et al. 2007), and bioavailability-enhancing properties for some nutritional substances and drugs (Atal et al. 1985; Szallasi 2005). In addition, piperine inhibits prostaglandin and leukotriene biosynthesis (Stöhr et al. 2001), and human P-glycoprotein (Bhardwaj et al. 2002; Khan et al. 2006). Previously (Ribeiro et al. 2004), we have investigated the effects of piperine and several of its chemical derivatives on the proliferation of epimastigote and amastigote forms of *T. cruzi*. The introduced chemical modifications did not enhance anti-*T. cruzi* activity, but chemical structure and biological activity correlations indicated the importance of the amide function and the central core of the natural piperine for maintaining its toxicity, and validated the use of piperine backbone as prototype for the chemical synthesis of new and more powerful compounds (Ribeiro et al. 2004).

Despite the described toxic activity against *Leishmania* (Kapil 1993; Raay et al. 1999) and *T. cruzi* (Ribeiro et al. 2004), there are no studies on the possible mechanisms of action of piperine on trypanosomatid protozoan. In the present work, we show the immunomodulatory effects of piperine over the capacity of noninfected or *T. cruzi*-infected murine macrophages (Mø) to produce nitric oxide (NO), and the cumulative morphological and ultrastructural changes and reversible cell cycle arrestment effects in epimastigotes of *T. cruzi* that are distinct from those caused by other known antimicrotubular agents.

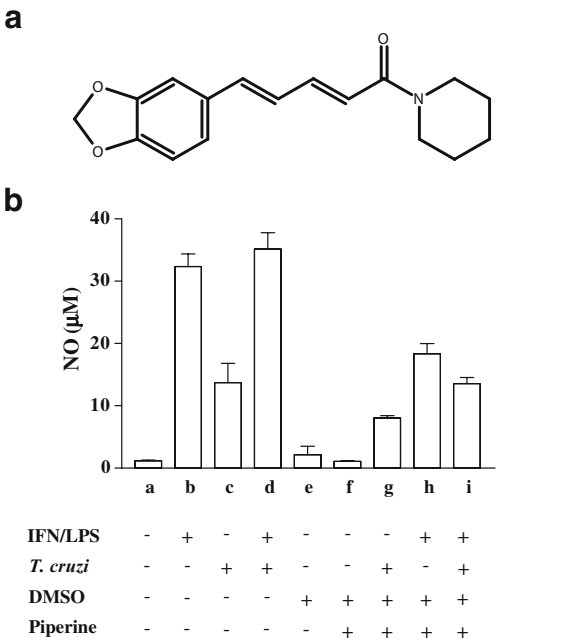

Fig. 1 The structure of piperine and its effects on the production of nitric oxide (NO) by murine macrophages. **a** Piperine (1-piperoylpiperidine), the major alkaloid found in fruit bodies of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). **b** Peritoneal murine macrophages infected (+) or not (−) with *T. cruzi* were treated in the absence (−) or presence (+) of 40 U mL^{-1} IFN-γ, 10 ng mL^{-1} LPS, 0.1% DMSO, or 5 μg mL^{-1} piperine, as indicated in the bottom. For details about all experimental procedures see Materials and methods section. After 48 h of incubation, the concentration of NO in the culture supernatants was determined by the method of Green et al. (1982). Each value corresponds to the mean±standard deviation (SD) for duplicates from two independent experiments

Materials and methods

Parasites

Epimastigote forms of Y-strain *T. cruzi* (from the culture collection of Fundação Oswaldo Cruz, Rio de Janeiro, R.J., Brazil) were axenically grown and maintained in complex medium containing 37 g L^{-1} brain heart infusion (BHI; Difco®, MA, USA), 10 μg L^{-1} hemin (Sigma®, MO, USA), 20 μg L^{-1} folic acid (Sigma), and 10% (*v*/*v*) fetal bovine serum (FBS; Gibco, BRL®) for 7 days at 28°C. Differentiation of epimastigotes into metacyclic trypomastigote (MCT) forms was achieved with a chemically defined triatomine artificial urine medium (Contreras et al. 1985). Tissue culture-derived trypomastigotes (TCT) were obtained after infection of confluent monolayers of Vero cells with MCTs to establish the intracellular cycle for 6 days and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% (*v*/*v*) FBS under an atmosphere of 5% CO_2 at 37°C (Andrews et al. 1985). The TCT forms were collected from the culture supernatants, washed with medium, and then used to infect murine peritoneal macrophages (see below).

Piperine treatment

For evaluation of the piperine effects on epimastigotes, parasites (between 2 and 11×10^5 mL^{-1}) were subcultured as described above in the absence or presence of different concentrations of piperine (from a 10-mg mL^{-1} stock solution in dimethyl sulfoxide (DMSO). Control conditions were established with organisms subcultured identically but containing equivalent amounts of DMSO to discard any effect of this solvent to the flagellates. The highest concentration of solvent (0.25% DMSO) did not have any significant effect on the growth of epimastigotes.

Reversibility of the piperine effects on the growth curve of epimastigotes in culture

Epimastigote forms of *T. cruzi* incubated with 10 μg mL^{-1} of piperine for up to 7 days in BHI medium were centrifuged at 3,000×*g* for 10 min at 4°C, washed twice in 137 mM NaCl, 2.7 mM KCl, 4.5 mM $Na_2HPO_4{\cdot}7H_2O$, 1.5 mM KH_2PO_4, pH 7.4 phosphate-buffered saline (PBS), and suspended in fresh media free of piperine or in media containing increasing amounts of the drug. Daily counts of cells were done with at least two independent experiments in triplicates.

Macrophages, infection with *T. cruzi*, and evaluation of NO production

Exudate cells removed from the peritoneal cavity of normal BALB/c mice (3×10^5 mL^{-1}) were suspended in complete RPMI 1640 medium (Sigma-Aldrich) containing 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10 μg mL^{-1} gentamicin (Sigma), MEM nonessential amino acids (Gibco), 10 mM HEPES, 50 μM 2-mercaptoethanol and 10% (*v*/*v*) FBS, plated inside 24-well plates (Corning, NY, USA), and maintained at 37°C in 5% CO_2 atmosphere to allow cells to adhere. After 90 min, non-adherent cells were removed with two washes in 1 mL RPMI each at 37°C, and (Mø) were incubated in 1 mL RPMI supplemented with 10% (*v*/*v*) FBS overnight as described above. Mø were then incubated in the presence of 1.5×10^6 Y-strain TCT forms (see 'Parasites' section above) per well in 1 mL, in a ratio of five parasites per macrophage in average. After 2 h, non-internalized parasites were removed, and infected Mø were cultured in complete RPMI medium (1 mL) alone, medium containing lipopolysaccharide (LPS; 10 ng mL^{-1}) plus interferon gamma (IFN-γ; 40 U mL^{-1}; both purchased from Pharmingen, CA, USA), or medium containing DMSO (0.1%) or piperine (5 μg mL^{-1}) at 37°C under 5% CO_2. After 2 days, NO levels produced by Mø cultures (assayed in duplicates from two independent experiments) were estimated using the method of Green et al. (1982) by reducing the accumulated nitrate to nitric acid (in a linear range between 1 and 80 μM) with nitrate reductase as described before (Saraiva et al. 2002). All procedures were conducted according to protocols approved by the Committee on Ethics and Regulations of Animal Use of the Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro.

Evaluation of piperine on epimastigote morphology and cell cycle with light microscopy

Parasites were subcultured in the absence or presence of 10 μg mL^{-1} piperine during 7 days as above. Cell growth was determined daily with a Neubauer hemocytometer, and 1- to 7-day-old cultures smears were Giemsa-stained after HCl treatment (Carvalho 1973). At least 500 randomly chosen microorganisms of each culture were evaluated and classified according to the number of kinetoplasts (K) and nuclei (N) per cell (Grellier et al. 1999; Robinson et al. 1995). Observations and photomicrographs were done with a Zeiss Axioplan-2 light microscope (Oberkochen, Germany).

Scanning electron microscopy and measurement of parasite dimensions

Epimastigotes cultured for 5 days in the absence or presence of 10 μg mL^{-1} piperine were washed three times in PBS and centrifuged for 5 min at 1,400×*g*, and fixed in 2.5% (*v*/*v*) glutaraldehyde, 4% (*w*/*v*) formaldehyde, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2 to 7.4, for 1 h at room temperature. Parasites were then collected by centrifugation, washed three times, suspended in 0.1 M sodium cacodylate buffer, pH 7.2 to 7.4, and allowed to adhere to glass coverslips treated with 0.1% (*w*/*v*) poly-L-lysine. Postfixation was carried out in 1% (*w*/*v*) osmium tetroxide, 0.8% (*w*/*v*) potassium ferrocyanide in 0.1 M sodium cacodylate buffer, pH 7.2 to 7.4, for 1 h. Samples were dehydrated in ascending ethanol series of 50 to 100% (*v*/*v*), critical-point dried with CO_2 in a Balzers CPD-20 apparatus (Balzers, Liechtenstein), and gold sputtered in a Balzers FL-9646 apparatus (Balzers, Liechtenstein). Fifty randomly chosen treated and control cells were measured at the Jeol JSM-5310 scanning electron microscope (Akishima Tokyo, Japan) considering the longitudinal and transversal axis of the cell body and the size of the flagellum. The χ^2 statistical test (α=0.05, $P<$ 0.05) was used to evaluate the significance of differences observed in measurements of treated and control parasites.

Transmission electron microscopy

Cultures of epimastigotes at days 1, 3, and 5 prepared in the absence or presence of 10 μg mL^{-1} of piperine were washed, fixed, postfixed, embedded, and ultrathin sectioned

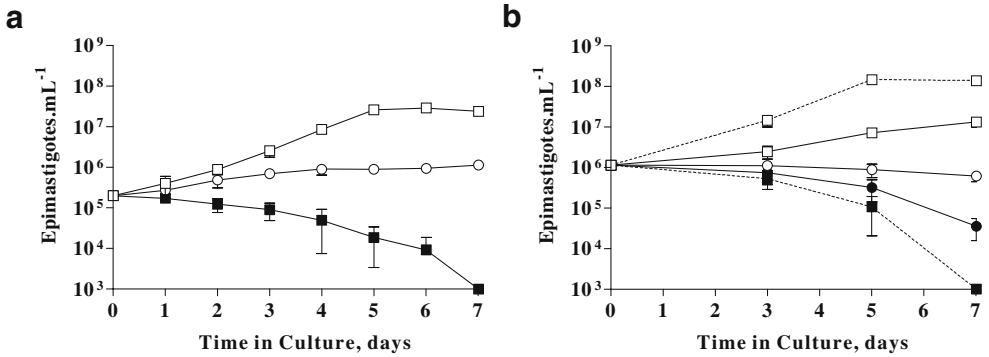

Fig. 2 Growth of *T. cruzi* in culture. **a** Epimastigotes (2×10^5 mL^{-1}) were cultured in absence (*empty squares*) and presence of 10 (*empty circles*) or 25 (*filled squares*) μg mL^{-1} piperine; the number of parasites per milliliter was determined daily. **b** Parasites maintained during 7 days in the presence of 10 μg mL^{-1} piperine in (**a**) were collected, washed twice in PBS and reinoculated (1.1×10^6 mL^{-1}) in media without (*empty squares*) or supplemented with 10 (*empty circles*) or 20 (*filled circles*) μg mL^{-1} piperine and the cell densities followed by direct counting for an additional 7 days. *Dashed lines* represent the growth curves of washed untreated parasites from (**a**) after reinoculation at the density of 1.1×10^6 mL^{-1} in the absence (*empty squares*) or presence of 25 μg mL^{-1} piperine (*filled squares*). The results are from two independent experiments done in triplicates ±SD

as described elsewhere (Braga et al. 2005; Romeiro et al. 2000). Observations and electron micrographs were done with a Zeiss EM-900 transmission electron microscope (Oberkochen, Germany).

Results

Effects of piperine on murine macrophages

We have described previously (Ribeiro et al. 2004) that 5–8 μM of piperine (Fig. 1a) was toxic against *T. cruzi*, but not to Mø maintained in vitro. In fact, addition of up to 35 μM (~10 μg mL^{-1}) of piperine did not interfere with the permeability of the Mø plasma membrane or with its phagocytic capacity (data not shown). Because the production of NO is one of the most potent microbiocidal mechanism used by Mø to destroy intracellular amastigotes during *T. cruzi* infection (Silva et al. 1995) and piperine was shown to interfere with the production of NO (Pradeep and Kuttan 2003) and pro-inflammatory cytokines (Pradeep and Kuttan 2004), we decided to evaluate the effects of piperine on the production of NO by *T. cruzi*-infected or noninfected Mø. As shown in Fig. 1b, piperine by itself was unable to induce NO production by noninfected Mø (Fig. 1b,f). As expected, both noninfected (Fig. 1b,b) or infected (Fig. 1b,d) Mø, but not treated with piperine, responded with a high production of NO after incubation with IFN/LPS. The capacity to elicit this response was reduced 45–60% when the Mø were treated in the presence of piperine (Fig. 1b,g–i). These results suggest that the major toxic effects of piperine was directed against the parasite itself and do not depend on the induction of microbiocidal mechanisms that involve NO production by the host Mø.

Effects of piperine on replication of epimastigotes

Previous studies with piperine demonstrated its toxicity against *Leishmania* parasites both in vitro (Kapil 1993) and in vivo (Raay et al. 1999), and against infective amastigote and noninfective epimastigote stage forms of *T. cruzi* in a dose-dependent fashion (Ribeiro et al. 2004). To investigate the possible mechanisms of action of piperine, intermediate sublethal concentrations of the drugs were tested against

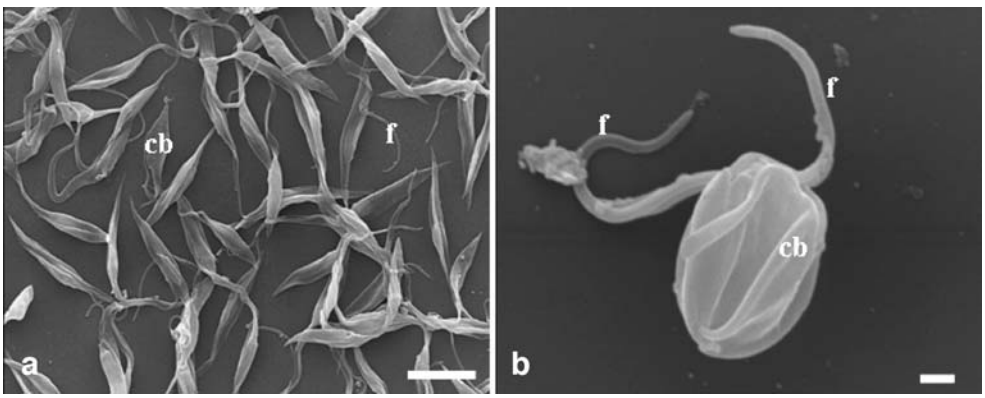

Fig. 3 Scanning electron microscopy of *T. cruzi* epimastigote forms. **a** Untreated control and **b** epimastigote cultured during 5 days with 10 μg mL^{-1} piperine showing the morphological changes in cell body (*cb*) and flagellum (*f*). The presence of rounded individuals with two flagella suggested an interference of piperine in the cell division cycle. *Bars*=10 μm (**a**) and 1 μm (**b**), respectively

epimastigotes in vitro (Fig. 2). As expected, when epimastigotes were cultured in the presence of 10–25 μg mL^{-1} of piperine during 7 days, a strong decrease in growth was observed when compared to untreated controls (Fig. 2a). However, unlike parasites from cultures treated with 25 μg mL^{-1}, those treated with 10 μg mL^{-1} of piperine remained motile and viable during the experiment as evaluated by the trypan blue dye exclusion (not shown). In addition, parasites treated in this intermediate condition were able to regain their ability to grow in culture if piperine was removed from the medium (Fig. 2b). However, the growth rate of the washed and pretreated parasite cultures

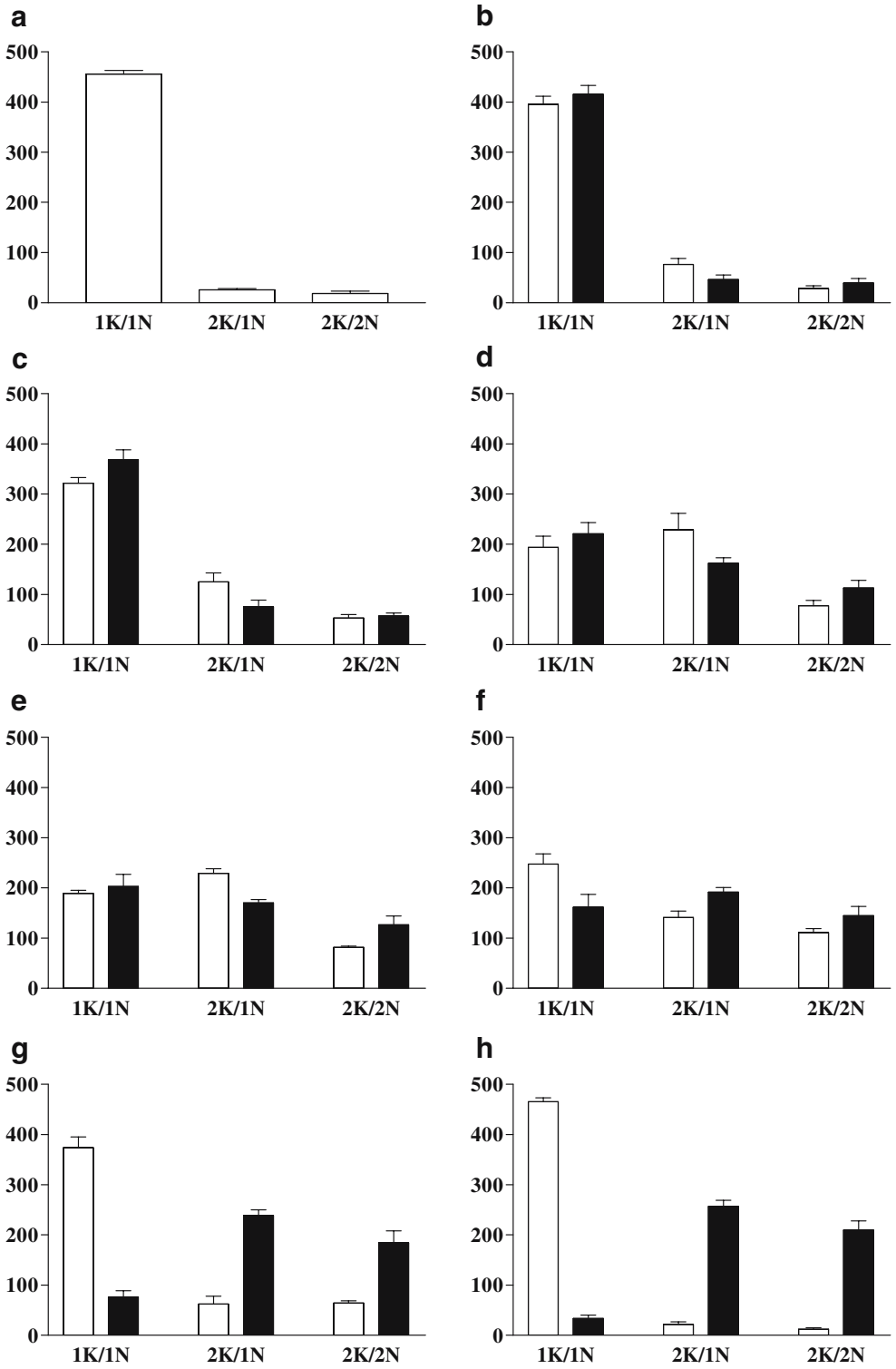

Fig. 4 Effects of Piperine in the cell cycle of axenically grown epimastigotes. Giemsa-stained epimastigotes (500 per each day) obtained from cultures maintained in the absence (*white bars*) or presence of 10 μg mL^{-1} piperine (*black bars*) were evaluated at days 0 (**a**), 1 (**b**), 2 (**c**), 3 (**d**), 4 (**e**), 5 (**f**), 6 (**g**), and 7 (**h**) for the presence of one kinetoplast and one nucleus (1K/1N), two kinetoplasts and one nucleus (2K/1N), and two kinetoplasts and two nuclei (2K/2N). The values represent the mean of quadruplicates±SD of two independent experiments

transferred to drug-free medium was lower when compared to naive untreated cultures (Fig. 2b, open squares and dashed lines). On the other hand, if the pretreated and washed parasites were inoculated in cultures containing increasing drug amounts, they do not survive (Fig. 2b, closed squares and dashed lines).

Ultrastructural changes in epimastigotes treated with piperine

The inhibition of *T. cruzi* replication by piperine was followed by the formation of bizarre morphologic forms as observed by scanning electron microscopy, clearly demonstrating the dose-related emergence of altered forms with time after treatment (Fig. 3). Piperine-treated epimastigotes presented trigonal or round morphologies with an accumulation of biflagellated forms (Fig. 3b), unlike the characteristic fusiform morphology presented by normal untreated cells (Fig. 3a). Careful measurements of 50 individuals in each condition indicated changes of 40 and 42%, respectively, in the longitudinal length of the cell body and flagella of treated cells (α=0.05, P<0.05). There was no significant alteration regarding the transversal axis of the cell body when comparing treated and untreated parasites.

To quantify a possible effect of piperine arresting the cell cycle of epimastigotes, a set of kinetic experiments was

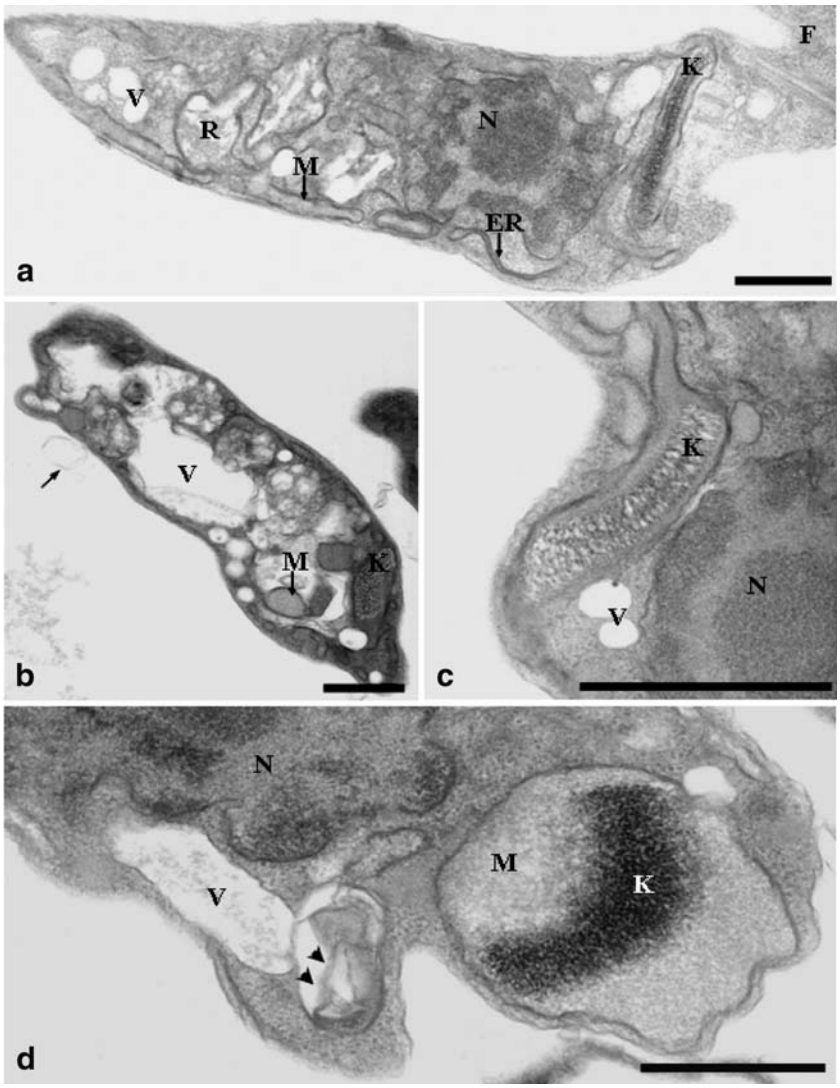

Fig. 5 Transmission electron microscopy of *T. cruzi* epimastigotes after 1 and 3 days of treatment with 10 μg mL^{-1} of piperine. **a** Control untreated parasite cultured in BHI medium with 0.1% DMSO for 1 day showing the nucleus (*N*), kinetoplast (*K*), reservosome (*R*), mitochondrion (*M*), flagellum (*F*), vacuole (*V*), and endoplasmic reticulum (*ER*). (**b**, **c**) Epimastigotes after treatment for 1 day with 10 μg mL^{-1} piperine, showing intense vacuolization (V) of the cytoplasm and membrane blebs detaching from the parasite surface (**b**, arrow), but no intense mitochondrion swelling (C). **d** Epimastigote after 3 days of treatment with piperine evidencing the swollen mitochondrion matrix (*M*) around the kinetoplast (*K*) and the presence of vacuolar structures displaying several complex membrane invaginations (*arrowheads*) suggesting autophagocytosis (*Bars*=1 μm)

done where Giemsa-stained parasites were analyzed for the presence and number of nuclei (N) and kinetoplasts (K). A total of 500 parasites from the beginning until day 7 of culture in the absence or presence of piperine were evaluated by light microscopy and the results presented in Fig. 4. Because *T. cruzi* cultures cannot be synchronized, an arbitrary time zero was set to show that the majority of the cells contained 1K and 1N (Fig. 4a). After a short lag period (Fig. 4b), cultures entered into a cycle of divisions where no significant difference between treated and untreated parasites was observed (Fig. 4b–f). However, after 6–7 days of culture, while the controls contained 1K/1N, the majority of the treated parasites presented 2K/1N or 2K/2N (Fig. 4g–h).

To evaluate the ultrastructural alterations caused by piperine, a kinetic study was done comparing the effects of the drug after 1, 3, and 5 days of treatment by transmission electron microscopy (Figs. 5 and 6). At day 1, plasma membrane blebs (Fig. 5b, arrow), a large number of intracellular vacuoles (V), and only a small compression of the kinetoplastid DNA (K) were observed in treated parasites (Fig. 5b and c) when compared to controls (Fig. 5a). No further alterations on nucleus (N) or mitochondria (M) were detected. After 3 days however, treated parasites showed an increase of membranous structures (not shown), and the swelling of the mitochondrion matrix with separation of the mitochondrial membrane from the kinetoplastid DNA (Fig. 5d), a scenario that was not observed in untreated parasites (data not shown). Finally, after 5 days of treatment, whereas control cells presented normal morphology (data not shown), treated parasites showed the presence of several vacuoles with membranous structures (Fig. 6a, arrows and arrowhead), mitochondrial swelling (M), and more than one kinetoplastid DNA structure that duplicated without completion of cytokinesis (Fig. 6b and c). The results obtained with the ultrastructural analysis were in agreement with the observations made under light microscopy (data not shown).

Discussion

The toxic effects of piperine have been described before against *Leishmania donovani* (Kapil 1993; Raay et al. 1999) and *T. cruzi* (Ribeiro et al. 2004), but its mode of action is not known. Although it has been initially suggested that the drug inhibits leishmanial type I DNA topoisomerase (Kapil 1993), there is no experimental data supporting this or other possible biochemical targets in the parasites' metabolism. In the present study, we have shown, for the first time, the kinetics of the morphological alterations induced by piperine on *T. cruzi* epimastigote forms and identified its capability to reversibly arrest cytokinesis when parasites were treated with sublethal concentrations of the drug.

There are several mammalian antimicrotubular agents capable of arresting cell division in kinetoplastids, including taxol, ansamitocin P3, vinblastine, vincristine, and hemiasterlin, each of them proposed to target different stages and particular structures/phases during the division process (Baum et al. 1981; Grellier et al. 1999; Havens et al. 2000). Trypanosomatids have a subpellicular microtubular skeletal network disposed just below the surface membrane of the entire parasite cell body, except for the area comprising the flagellar pocket. The first indication of division in *T. cruzi* is the replication of the basal body and flagellum. Mitosis starts with the nuclear membrane intact and after duplication of intracellular organelles; these organisms divide by longitudinal binary fission beginning at the anterior part of the cell and progressing to the posterior end (de Souza and Meyer 1974). As division proceeds, a new set of subpellicular microtubules must be synthesized (de Souza 2002), and this apparently happens when parasites were incubated with piperine because new cell bodies containing duplicated nuclei, kinetoplasts, and flagella can be observed after cell division started. Together, these observations indirectly exclude DNA topoisomerases as potential targets for the drug. Inhibition of cell division

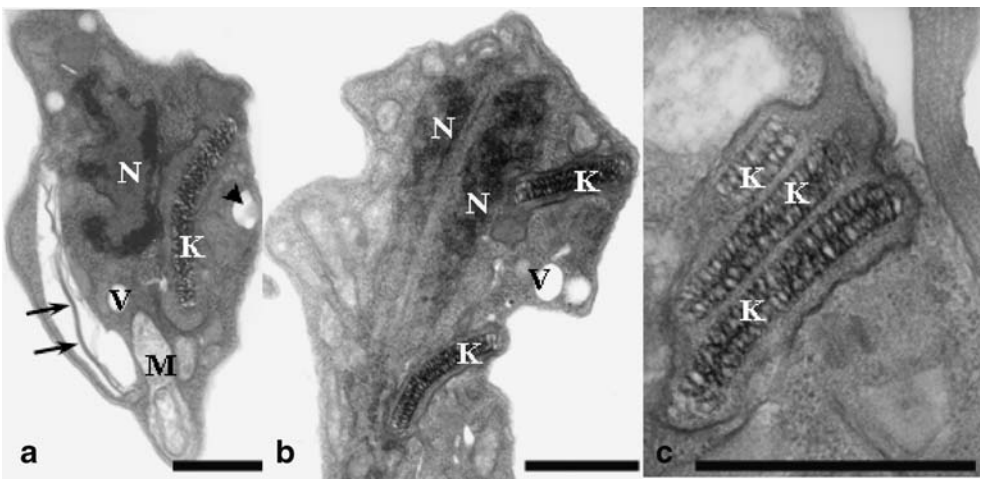

Fig. 6 Transmission electron microscopy of *T. cruzi* epimastigotes induced after 5 days of treatment with 10 μg mL^{-1} of piperine. **a** Mitochondrion (*M*) cristae were strongly affected after treatment with piperine. The cytoplasm was more vacuolated (*V*, *arrowhead*), and sometimes filled with membranous structures (*arrows*). **b**, **c** The k-DNA (*K*) was densely compacted, and some parasites contained a triple k-DNA without a complete division of the parasite (Bars=1 μm)

by piperine appears to be reversible because when the drug is removed, division proceeds again. It is not clear if this apparent reversibility represents the recovery and division of affected individuals or whether the increase in the number of parasites on removal of piperine is solely due to replication of unaffected epimastigotes (Baum et al. 1981). Like the effects observed with the microtubule stabilizer taxol, cessation of replication was followed by the formation of morphologically altered parasites in which multiplication of all identifiable organelles took place, but division into daughter cells was impaired (Baum et al. 1981). However, unlike the effects caused by the anti-microtubular vinca alkaloids vincristine and vinblastine that also induce cytokinesis inhibition (Grellier et al. 1999), piperine did not induce the production of giant multinucleate cells. Despite the inhibition of replication, the organisms remained viable after treatment with lower amounts of piperine and continued to exhibit motility (data not shown).

Piperine killed *T. cruzi* parasites when used in higher concentrations (Ribeiro et al. 2004). The presence of several vacuolated membranous structures in treated parasites with sublethal concentrations of the drug suggests that its effects are not restricted to a single specific point of the metabolism. Previously, piperine was shown to inhibit enzymes of the pyruvate–malate cycle in the testis of rats, with special emphasis on malate dehydrogenase, malic enzyme, and isocitrate dehydrogenase (Malini et al. 1999). Indeed, several dehydrogenases involved in the carbohydrate intermediary metabolism of *T. cruzi* have been shown to be inhibited in a dose-dependent manner by piperine like glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and malate dehydrogenase, regardless of the intracellular compartment where each isoform is found at a time and which include the cytosolic, the mitochondrial, or the glycosomal compartments (unpublished results). Supporting these enzymatic activities as potential targets for the inhibition by piperine in *T. cruzi* is the fact that chemically modified species that have lost their toxicity against the parasite (Ribeiro et al. 2004) became also poor inhibitors of the parasite dehydrogenases (unpublished results).

Several immunomodulatory effects of piperine have been described, including the inhibition of NO and tumor necrosis factor alpha (TNF-α) production by activated murine macrophages (Pradeep and Kuttan 2003), nuclear factor-B, c-Fos, CREB, ATF-2 and proinflammatory cytokine gene expression in melanoma cells (Pradeep and Kuttan 2004), and cell adhesion molecules on endothelial cells (Kumar et al. 2005). As recently observed (Pradeep and Kuttan 2003) and confirmed here, piperine caused an inhibition of NO production by activated murine macrophages, one of the most potent microbiocidal mechanism used by macrophages to destroy intracellular amastigotes during *T. cruzi* infection (Silva et al. 1995). Despite these observations, piperine was extremely toxic to intracellular amastigotes (Ribeiro et al. 2004) rendering its mechanism of toxic action against the parasites independent of additional NO killing mechanisms mediated by the host macrophages. However, the participation of reactive oxygen intermediates in the mechanism of piperine action that are also known as toxic molecules of the immune system to help in controlling microbial pathogens (Bogdan et al. 2000) cannot be ruled out and should be investigated in the future.

The data presented herein suggest that the toxic effects of piperine against *T. cruzi* can affect several biochemical targets in the parasites. Non-trypanocidal concentrations of the drug can be used in the future, together with taxol and vinca alkaloids, as a tool to explore new aspects involving different steps of the cell division of *T. cruzi* and other members of the kinetoplastida.

Acknowledgements This investigation received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), PADCT-FAPERJ and International Foundation for Science (IFS). Fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) to T. S. Ribeiro and from CNPq to L. Freire-de-Lima are gratefully acknowledged. The authors declare that the experiments presented herein comply with current laws of Brazil, country in which they were performed.

References

Andrews NW, Alves MJM, Schumacher RI, Colli W (1985) *Trypanosoma cruzi*: protection in mice immunized with 8-methoxypsolareninactivated trypomastigotes. Exp Parasitol 60:255–262

Atal CK, Dubey RK, Singh JJ (1985) Biochemical basis of enhanced drug availability by piperine, evidence that piperine is a potent inhibitor of drug metabolism. J Exp Ther 2329:258–262

Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S (2003) The trypanosomiases. Lancet 362:1469–1480

Baum SG, Wittner M, Nadler JP, Horwitz SB, Dennis JE, Schiff PB, Tanowitz HB (1981) Taxol, a microtubule stabilizing agent, blocks the replication of *Trypanosoma cruzi*. Proc Natl Acad Sci USA 78:4571–4575

Bhardwaj RK, Glaeser H, Becquemont L, Klotz U, Gupta SK, Fromm MF (2002) Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4. J Pharmacol Exp Ther 302:645–650

Bogdan C, Röllinghoff M, Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 12:64–76

Boveris A, Sies H, Martino EE, Docampo R, Turrens JF, Stoppani AO (1980) Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi*. Biochem J 188:643–638

Braga MV, Magaraci F, Lorente SO, Gilbert I, de Souza W (2005) Effects of inhibitors of $\Delta^{24(25)}$-sterol methyl transferase on the ultrastructure of epimastigotes of *Trypanosoma cruzi*. Microsc Microanal 11:1–10

Carvalho ALM (1973) Estudos sobre a posição sistemática, a biologia e transmissão de Tripanosomatídeos encontrados em *Zelus leucogrammus* (Perty, 1834) (Hemiptera: Reduvidae). Rev Pat Trop 2:223–274

Ceaser M (2005) Battling with Chagas disease in South America. Lancet Infect Dis 5:470–471

Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S (1985) In vitro differentiation of *Trypanosoma cruzi* under chemically defined conditions. Mol Biochem Parasitol 16:315–327

de Souza W (2002) Basic cell biology of *Trypanosoma cruzi*. Curr Pharma Des 8:269–285

de Souza W, Meyer H (1974) On the fine structure of the nucleus in *Trypanosoma cruzi* in tissue culture forms. Spindle fibers in the dividing nucleus. J Protozool 21:48–52

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate and [^{15}N]nitrate in biological fluids. Anal Biochem 126:131–138

Grellier P, Sinou V, Garreau-de Loubresse N, Bylen E, Boulard Y, Schrevel J (1999) Selective and reversible effects of Vinca alkaloids on *Trypanosoma cruzi* epimastigote forms: blockage of cytokinesis without inhibition of the organelle duplication. Cell Motil Cytoskeleton 42:36–47

Havens CG, Bryant N, Asher L, Lamoreaux L, Perfetto S, Brendle JJ, Werbovetz KA (2000) Cellular effects of leishmanial tubulin inhibitors on *L. donovani*. Mol Biochem Parasitol 110:223–236

Henderson GB, Fairlamb AH, Cerami A (1987) Trypanothione-dependent peroxide metabolism in *Crithidia fasciculata* and *Trypanosoma brucei*. Mol Biochem Parasitol 24:39–45

Kapil A (1993) Piperine: a potent inhibitor of *Leishmania donovani* promastigotes in vitro. Planta Med 59:474

Khan IA, Mirza ZM, Kumar A, Verma V, Qazi GN (2006) Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*. Antimicrob Agents Chemother 50:810–812

Kumar S, Arya P, Mukherjee C, Singh BK, Singh N, Parmar VS, Prasad AK, Ghosh B (2005) Novel aromatic ester from *Piper longum* and its analogues inhibit expression of cell adhesion molecules on endothelial cells. Biochemistry 44:15944–15952

Malini T, Arunakaran J, Aruldhas MM, Govindarajulu P (1999) Effects of piperine on the lipid composition and enzymes of the pyruvate–malate cycle in the testis of the rat in vivo. Biochem Mol Biol Int 47:537–545

Pinto Dias JC (2006) Chagas disease: successes and challenges. Cad Saúde Pública, Rio de Janeiro 22:2020–2021

Pradeep CR, Kuttan G (2003) Effect of piperine on the inhibition of nitric oxide (NO) and TNF-alpha production. Immunopharmacol Immunotoxicol 25:337–346

Pradeep CR, Kuttan G (2004) Piperine is a potent inhibitor of nuclear factor-kappa B (NF-kB), c-Fos, CREB, ATF-2 and proinflammatory cytokine gene expression in B16F-10 melanoma cells. Int Immunopharmacol 4:1795–1803

Prata A (1994) Chagas' Disease. Infect Dis Clin North Am 8:61–76

Raay B, Medda S, Mukhopadhyay S, Basu MK (1999) Targeting of piperine intercalated in mannose-coated liposomes in experimental leishmaniasis. Indian J Biochem Biophys 36: 248–251

Ribeiro TS, Freire-de-Lima L, Previato JO, Mendonça-Previato L, Heise N, de Lima MEF (2004) Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi*. Bioorg Med Chem Letters 14:3555–3558

Robinson DR, Sherwin T, Ploubidou A, Byard EH, Gull K (1995) Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the Trypanosome cell cycle. J Cell Biol 128:1163–1172

Romeiro A, Solé-Cava A, Sousa MA, de Souza W, Attias M (2000) Ultrastructural and biochemical characterization of promastigotes and cystic forms of *Leptomonas wallacei* n. sp. isolated from the intestine of its natural host *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). J Eukaryotic Microbiol 47:208–220

Saraiva VB, Gibaldi D, Previato JO, Mendonça-Previato L, Bozza MT, Freire-de-Lima CG, Heise N (2002) Proinflammatory and cytotoxic effects of hexadecylphosphocholine (miltefosine) against drug-resistant strains of *Trypanosoma cruzi*. Antimicrob Agents Chemother 46:3472–3477

Silva JS, Vespa GNR, Cardoso MAG, Aliberti JCS, Cunha FQ (1995) Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect Immun 63:4862–4867

Stöhr JR, Xiao PG, Bauer R (2001) Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis in vitro. J Ethnopharmacol 75:133–139

Stoppani AO (1999) The chemotherapy of Chagas disease. Medicina (Buenos Aires) 59:147–165

Szallasi A (2005) Piperine: researchers discover new flavor in an ancient spice. Trends Pharmacol Sci 26:437–439

Urbina JA, Docampo R (2003) Specific chemotherapy of Chagas disease: controversies and advances. Trends Parasitol 19:495–501

Woo HM, Kang JH, Kawada T, Yoo H, Sung MK, Yu R (2007) Active spice-derived components can inhibit inflammatory responses of adipose tissue in obesity by suppressing inflammatory actions of macrophages and release of monocyte chemoattractant protein-1 from adipocytes. Life Sci 80:926–931

World Health Organization, 2002 World Health Organization (2002) Chagas Disease. Tropical Disease Research, 18th Program Report, UNDP/WB/TDR, Geneva