Trypanocidal activity of guaianolide obtained from *Tanacetum parthenium* (L.) Schultz-Bip. and its combinational effect with benznidazole

Juliana Cogo\(^a\), Angelo de Oliveira Caleare\(^b\), Tânia Ueda-Nakamura\(^a\), Benedito Prado Dias Filho\(^a\), Izabel Cristina Piloto Ferreira\(^a\), Celso Vataru Nakamura\(^a,b, *\)

\(^a\) Programa de Pós graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Maringá, Paraná, Brazil
\(^b\) Programa de Pós graduação em Biologia Celular, Universidade Estadual de Maringá, Maringá, Paraná, Brazil

**A R T I C L E   I N F O**

**Keywords:**
Trypanosoma cruzi
*Tanacetum parthenium*
Sesquiterpene lactone
Guaianolide

**A B S T R A C T**

In the present study, we evaluated the *in vitro* antiprotozoal activity of a guaianolide (11,13-dehydrocompressanolide) isolated from *Tanacetum parthenium* against *Trypanosoma cruzi* and investigated the possible combinational effect of guaianolide and benznidazole. The isolated compound was shown to be effective against *T. cruzi*, with IC\(_{50}\) values of 18.1 ± 0.8 and 66.6 ± 1.3 μM against the multiplicative epimastigote and amastigote forms, respectively. The best results were obtained against trypanomastigotes, with an EC\(_{50}\) of 5.7 ± 0.7 μM. The guaianolide presented no toxicity in LLCMK\(_2\) cells (CC\(_{50}\) of 93.5 μM) and was 16.4-fold more selective for trypromastigotes. The study of the combinational effect of benznidazole and guaianolide revealed the presence of a synergistic effect against the epimastigote form and marginal additive effect against the trypromastigote form. Striking morphological changes were observed in epimastigotes treated with guaianolide, such as thinning and stretching of the cell body and flagellum and changes in the format of the cell body with apparent leakage of the cytoplasmic content in trypromastigote forms. The ultrastructural analysis of epimastigotes revealed the presence of membranes that involved organelles and formation of myelin-like figures. Flow cytometry revealed a cell volume reduction and decrease in mitochondrial membrane potential. However, no major changes in cell membrane integrity were found in the epimastigote form treated with guaianolide.

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**Introduction**

Chagas’ disease is considered a major public health problem in the Americas. Presently, 10 million people are infected worldwide, with over 25 million at risk for infection, mostly in Latin America. This disease kills more than 10,000 people each year. Recently, new modes of transmission, including oral, have caused alarm in several countries, including Brazil, Colombia, and Venezuela (WHO 2010; Pereira et al. 2010).

The currently available treatment of infected patients is limited because the only drugs available, nifurtimox (Lampit) and benznidazole (Radanil, Rochagan), have limited efficacy and present systemic toxicity that causes several side effects (Maya et al. 2007; Soeiro et al. 2009). Therefore, the development of new effective and safe drugs is urgently needed for the treatment of Chagas’ disease.

Sesquiterpene lactones are a large group of molecules with several biological activities and uses for humans, including anti-inflammatory, anticancer, antimicrobial, and antiprotozoal (Tiuaman et al. 2005; Izumi et al. 2008; Ghantous et al. 2010; Chaturvedi 2011; Juliante et al. 2011). These different activities have been linked mainly to α-methylene-γ-lactone functionality, which can react with biological nucleophiles, such as sulfhydryl groups, through Michael-type addition. They can inhibit various thiol-containing enzymes involved in the synthesis and processing of proteins, RNA, and DNA (Kupchan et al. 1970; Picman 1986; Arantes et al. 2009).

These features make sesquiterpene lactones potent molecules for the treatment of human pathogenic protozoa species, including trypanosomatids. Among the bioactive sesquiterpenes, the guaianolide 11,13-dehydrocompressanolide recently isolated from *Tanacetum parthenium* deserves attention because of its activity against *Leishmania amazonensis* and low toxicity (Silva et al. 2010).

Thus, the purpose of the present study was to evaluate the *in vitro* trypanocidal activity of guaianolide isolated from the aerial...
from parts of *T. parthenium* against *T. cruzi* and investigate the possible combinational effect of guianolide and benznidazole.

**Materials and methods**

*Isolation of guianolide*

The powder of the aerial parts of *T. parthenium* was kindly provided by the Laboratório Botânico Flores & Ervas (lot no. 029086; Piracicaba, São Paulo, Brazil). The general procedure for the isolation of guianolide (11,13-dehydrocompressanolide; Fig. 1A) was similar to the procedure described for parthenolide (Tiuman et al. 2005; Silva et al. 2010) with slight modifications. The compound was characterized by mass spectra data (Micromass Quatro Micro; API Waters) and nuclear magnetic resonance (NMR) spectra data (Gemini 2000BB; Varian).

Stock solutions were prepared aseptically in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA), with the final concentration of the experiments not exceeding 1%. Benznidazole (N-benzyl-2-nitro-1H-imidazole-1-acetamide; Fig. 1B), used as reference drug, was purchased from Laboratório Central de Medicamentos, Pernambuco, Brazil.

*Parasites and cells*

Epimastigote forms of *T. cruzi* (*Y* strain) were maintained at 28 °C by weekly transfers in live infusion tryptose medium (LIT) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco Invitrogen, New York, USA).

LLCMK₂ cells (epithelial cells from the kidney of the monkey *Macaca mulatta*) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Invitrogen) supplemented with 2 mM l-glutamine, 10% FBS, and 50 mg/l gentamicin at 37 °C in a humidified 5% CO₂ atmosphere.

Trypomastigote forms were obtained from the supernatant of a monolayer of infected LLCMK₂ cells in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO₂ atmosphere.

*Antiproliferative activity of guianolide on epimastigote forms*

Epimastigote forms harvested during the exponential growth phase were used for this assay. A cell density of 1.0 × 10⁶ cells/ml in LIT medium supplemented with 10% FBS was incubated in duplicate at 28 °C for 96 h in 24-well microplates in the absence or presence of increasing concentrations of guianolide (1–50 μM). Afterward, cell growth was estimated by counting the parasites using a Neubauer haemocytometer (Improved Double Neubauer), and IC₅₀ and IC₉₀ values (concentrations that inhibit cell growth in 50% and 90%, respectively) were determined. The results are expressed as a percentage of growth inhibition compared with control cultures. Benznidazole was used as a reference drug.

**Effect of guianolide on the viability of trypomastigote forms**

The tissue-culture-derived trypomastigote forms (1.0 × 10⁷ cells/ml) were resuspended in DMEM and added in duplicate to 96-well microplates in the absence or presence of different concentrations of guianolide (0.1–50 μM). Parasites were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. The results were obtained by observing motility, allowing the determination of the viability of the parasites, using the Pizzi–Brener method (Brener 1962). The EC₅₀ value (i.e., the concentration that lyser 50% of the parasites) was then calculated. Benznidazole was used as a reference drug.

**Activity of guianolide on intracellular amastigote forms**

To evaluate the effect on the intracellular amastigote forms, a suspension of 2.5 × 10⁵ LLCMK₂ cells/ml in DMEM supplemented with 10% FBS was seeded in 24-well microplates that contained round coverslips and then maintained at 37 °C in a 5% CO₂ atmosphere for 24 h until a confluent monolayer was obtained. Trypomastigotes were added to the wells at a concentration of 10 parasites per host cell. After 24 h, non-internalized parasites were removed by washing, and the infected LLCMK₂ cells were treated in duplicate with different concentrations of guianolide (50, 60, and 70 μM) for 96 h. The cells were fixed with methanol and stained with Giemsa, and the coverslips were permanently prepared with Entellan (Merck). By counting 200 cells under a light microscope (Olympus CX 31), we estimated the percentage of infected cells and number of intracellular amastigotes. The survival index (percentage of infected cells × number of amastigotes per cell) and IC₅₀ value were then determined.

**Cytotoxicity assay**

LLCMK₂ cells were assayed for the determination of cell viability using a sulforhodamine B colorimetric assay. A suspension of 2.5 × 10⁵ cells was seeded in a 96-well microplate and maintained at 37 °C in a 5% CO₂ atmosphere for 24 h until a confluent cell monolayer was obtained. Thereafter, the medium was removed, and the cells were cultured with increasing concentrations of guianolide (37–500 μM) in triplicate for 96 h under the same conditions as above. Control wells without treatment were included. The cells were fixed with 10% trichloroacetic acid for 1 h at 4 °C and stained for 30 min with 0.4% sulforhodamine B (SRB) in 1% acetic acid. Bound SRB was dissolved with 10 mM unbuffered Tris-base (tris[hydroxymethyl]aminomethane) solution. Absorbance was read in a 96-well plate reader (BIO-TEK FL-600 Microplate Fluorescence Reader) at 530 nm. The results were calculated as the ratio between the optical density of untreated and treated cells. Dose–response curves were plotted, and CC₅₀ values (50% cytotoxic concentration) were estimated. The selectivity index (SI) was used to compare cytotoxicity between LLCMK₂ cells and trypomastigote forms of *T. cruzi* (ratio: CC₅₀ on LLCMK₂ divided by EC₅₀ of the compound in the protozoa).

**Evaluation of the combinatorial effect between guianolide and benznidazole**

Briefly, epimastigote forms (1.0 × 10⁶ cells/ml) in the exponential growth phase were resuspended in LIT medium supplemented with 10% FBS. Afterward, different concentrations of the drugs (guianolide and benznidazole) were added alone or in combination to the cell suspension in 24-well microplates. Cell growth was determined by counting the parasites with a Neubauer haemocytometer after 96 h. Trypomastigote forms (1.0 × 10⁷ cells/ml) were
**Fig. 2.** Effect of guianolide (11,13-dehydrocompressanolide) in epimastigote forms. (A) Antiproliferative activity of guianolide and benznidazole for 96 h at 28 °C. The data are expressed as mean ± SD of three independent experiments performed in duplicate. The results are expressed as a percentage of inhibition compared with control. Morphological alterations were observed by SEM. (B) Untreated parasite that shows typical elongated morphology. (C and D) Parasites treated with IC_{50} and IC_{90}, respectively, for 96 h, showing thinning and stretching of the cell body and flagellum. Ultrastructural alterations were observed by TEM. (E) Untreated parasites, showing no plasma alterations and organelles with normal morphology. (F–I) Parasites treated with IC_{50} of guianolide for 96 h, k, kinetoplast; f, flagellum; fp, flagellar pocket; n, nucleus. Arrow indicates membranes that envelope reservosomes and nucleus. Star indicates the formation of myelin-like figures. Scale bars = 1 μm.

Resuspended in DMEM and added to 96-well microplates that contained different concentrations of the drugs alone or combined and incubated for 24 h at 37 °C. The results were obtained by observing motility under a light microscope (Brener 1962). Cytotoxicity was assessed by exposing the LLCMK2 cell monolayer to different concentrations of the drugs alone or combined for 96 h. Cell growth was assessed using the SRB colorimetric assay as described above. To evaluate the combinational effect of guianolide and benznidazole, we adopted the fractional inhibitory concentration index (FICI) and used isobolograms. The FICI was the sum of the fractional inhibitory concentration (FIC) of drug A plus the FIC of drug B. The FIC of drug A is the IC_{50} of the drug when it is used in combination with drug B, divided by the IC_{50} of the drug when it is used alone (Hallander et al. 1982).

**Electron microscopy**

Epimastigote and trypomastigote forms were treated as described above with concentrations of guianolide based on the IC_{50} and IC_{90} values and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C. For transmission electron microscopy (TEM), the cells were postfixed in a solution that contained 1% OsO_{4}, 0.8% potassium ferrocyanide, and 10 mM CaCl_{2} in 0.1 M cacodylate buffer, dehydrated in an increasing acetone
gradient, and embedded in Epon resin. Ultrathin sections were then obtained and observed on a Zeiss 900 TEM. For scanning electron microscopy (SEM), epimastigote and trypomastigote forms were placed on a specimen support with poly-l-lysine. The samples were dehydrated in a graded series of ethanol, critical-point-dried with CO₂, coated with gold, and observed on a Shimadzu SS-550 SEM. The epimastigote forms were measured for the quantification of morphological changes.

Flow cytometry analysis

After the treatment described above with concentrations based on the IC₅₀ and IC₉₀ values, epimastigote forms were washed, and the pellet was resuspended in phosphate-buffered saline (PBS). To evaluate the mitochondrial membrane potential (ΔΨₘ), the cells were incubated with 13.1 μM rhodamine 123 (Rh123; Sigma–Aldrich) for 15 min at 37 °C. The carbonyl cyanide m-chlorophenylhydrazone (100 μM) was used as a positive control. To estimate plasma membrane integrity, the cells were incubated with 3 μM propidium iodide (PI; Invitrogen) for 5 min at 37 °C. Antimycin (40 μM) and actinomycin D (50 μM) were used as positive controls for plasma membrane integrity and cell volume, respectively. Thereafter, the cell volume, ΔΨₘ, and plasma membrane integrity of individual cells were analyzed with a flow cytometer (FACSCalibur; Becton-Dickinson, Rutherford, NJ, USA). CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA) was used for the data analyses. A total of 10,000 events were acquired for each sample in the region previously established as the one that corresponded to the parasites.

Statistical analyses and reproducibility of the results

All of the experiments were repeated at least three times independently, and the data are expressed as mean±standard deviation. The statistical analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA, USA). Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s or Dunnnett’s tests was used. Values of p < 0.05 were considered statistically significant.

Results

We initially evaluated the activity of guaianolide in epimastigote forms. This compound caused a dose-dependent progressive inhibition of protozoa growth, exhibiting an IC₅₀ of 18.1 ± 0.8 μM and IC₉₀ of 44.1 ± 0.7 μM (Fig. 2A). The IC₅₀ of the reference drug benznidazole was 8.1 ± 0.3 μM.

To investigate and identify the organelles that might be the potential targets of guaianolide on T. cruzi, SEM, TEM, and flow cytometry techniques were employed. Scanning electron microscopy was used to further investigate morphological alterations in the protozoa treated with guaianolide (Fig. 2C and D). Epimastigotes treated with the IC₅₀ concentration of guaianolide showed an increase of approximately 11.9% in cell length and decrease of approximately 14.3% in cell body width (Table 1). These changes were observed in greater proportions when the cells were treated with the IC₉₀ concentration of 44.1 μM, with an increase of approximately 30.6% in cell length and decrease of approximately 30% in cell body width compared with untreated cells (Fig. 2B). The biggest change was observed in the flagellum, with an increase of approximately 46% and 68% in cells treated with 18.1 and 44.1 μM, respectively.

To confirm the volume reduction observed by SEM, epimastigotes treated with 18.1 and 44.1 μM guaianolide were analyzed using flow cytometry. The histogram analysis revealed reductions in the average cell size of 9.7% and 15.5%, respectively, compared with the mean cell size of untreated parasites. Treatment with 50 μM actinomycin D (standard drug) reduced cell size by 22.9%.

Ultrastructural changes in epimastigotes treated with guaianolide are illustrated in Fig. 2. Untreated parasites showed organelles with normal morphology and no plasma alterations (Fig. 2E). Epimastigote forms treated with guaianolide (IC₅₀ and IC₉₀) for 96 h showed membranes that enveloped reservosomes and the nucleus and the formation of myelin-like figures (Fig. 2F–I).

Based on the morphological and ultrastructural data, treated parasites were or were not incubated with fluorescent markers to evaluate alterations using flow cytometry. The mitochondrial function of the parasites was investigated using the fluorescent probe Rh123, which is collected in energized and respiring mitochondria. Guaianolide dose-dependently decreased the percentage of Rh123 fluorescence in the upper-right quadrant from 97% in untreated cells (Fig. 3A) to 76.6% and 47.8%, indicating a decrease in the ΔΨₘ in epimastigotes treated with 18.1 and 44.1 μM, respectively (Fig. 3C and D). Treatment with the standard drug CCCP at 100 μM reduced fluorescence to 0.3% (Fig. 3B). Epimastigotes treated with guaianolide (IC₅₀ and IC₉₀) and labeled with PI showed no significant alterations in the permeability of the plasma membrane. The gated percentages (upper-left quadrant) of PI-stained parasites after treatment were 3.86% and 12.36%, respectively, very close to the value obtained with untreated cells. Treatment with 40 μM digitonin increased the number of parasites marked with PI by 68.5% (data not shown).

The activity of guaianolide in trypomastigotes, representing the main infective stage of T. cruzi, also presented a dose-dependent effect, in which all concentrations tested showed significant activity compared with controls (p < 0.05). After 24 h of treatment, we observed a decrease in viability, with EC₅₀ and EC₉₀ values of 5.7 ± 0.7 μM and 44.9 ± 2.0 μM, respectively (Fig. 4A). The EC₅₀ of the reference drug benznidazole was 3.4 ± 0.9 μM. No significant differences were found between the activity of benznidazole and guaianolide against the trypomastigote form.

Morphological changes induced by guaianolide in trypomastigotes are illustrated in Fig. 4C and D. Trypomastigotes treated with 5.7 and 44.9 μM showed changes in body shape, with rounding and shortening of the parasite and apparent leakage of cytoplasmic contents, compared with untreated cells (Fig. 4B).

Because of the trypanocidal activity of guaianolide against epimastigote and trypomastigote forms, the efficacy against intracellular amastigotes in LLCMK₂ cells was evaluated using non-toxic concentrations. When the parasites were treated with guaianolide for 96 h, we observed a dose-dependent decrease in intracellular amastigotes. Fig. 5 shows survival indices of 39.5% after treatment with 70 μM guaianolide, 65% after treatment with 60 μM, and 80% after treatment with 50 μM. The IC₅₀ was observed at a concentration of 66.6 ± 1.3 μM guaianolide.

The cytotoxicity assays against mammalian host cells (LLCMK₂) incubated for 96 h showed that guaianolide displayed low toxicity, exhibiting a CC₅₀ value of 93.5 ± 1.3 μM (Table 2). Based on the CC₅₀

<table>
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<th>Table 1</th>
<th>Cell body size variation observed on epimastigote forms cultivated in the presence of guaianolide (11,13-dehydrocompressanolide).</th>
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</thead>
<tbody>
<tr>
<td>Epimastigote</td>
<td>Control</td>
</tr>
<tr>
<td>Cell body</td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>8.04 ± 1.5</td>
</tr>
<tr>
<td>Width</td>
<td>1.89 ± 0.24</td>
</tr>
<tr>
<td>Flagellum</td>
<td>8.15 ± 1.63</td>
</tr>
</tbody>
</table>

The results are expressed as the mean±SD from 50 parasites. The IC₅₀ and IC₉₀ values represent the concentrations that inhibit cell growth in 50% and 90% after 96 h treatment at 28 °C.
and EC50 values, the SI was determined. This compound was at least 16.4-fold more potent against the trypomastigote form compared with mammalian cells.

Combinational effects can be demonstrated by calculating the FICI (Chou 2006). The evaluation of the combinational effect of different concentrations of both drugs on epimastigotes revealed synergistic activity with a FICI of 0.6. As shown previously, the IC50 values of the reference drug benznidazole and guianolide for the epimastigote form were 8.1 and 18.1 µM, respectively. The same effect was observed with lower concentrations of both drugs. The addition of low concentrations of guianolide (5.4 µM) resulted in a reduction of the IC50 value of benznidazole to 3 µM. However, when guianolide was combined with benznidazole against the trypomastigote form, the IC50 values slightly decreased from 5.7 and 3.4 µM to 2.1 and 1.87 µM, respectively. The FICI was 0.93, indicating marginal additive activity. When assessing the effect of the combination with regard to cytotoxicity in LLCMK2 cells, was obtained a FICI of 1.06, i.e., no increase in the levels of toxicity was observed. The CC50 values of guianolide and benznidazol were 93.5 and 1218.2 µM, respectively. The graphical representation of this interaction is shown in the isobolograms. The isobolograms formed from the combinations of different concentrations of the guianolide and benznidazol (Fig. 6) confirmed the FICI results (Wagner and Ulrich-Merzenich 2009).

**Discussion**

The large number of patients who die from Chagas' disease and safety and efficacy limitations associated with available treatments emphasize the need for novel trypanocidal drugs (Urbina 2010). Sesquiterpene lactones have been successfully used to combat a wide variety of parasites that can cause human infections (Chaturvedi 2011; Juli antic et al. 2011). In this context, the present study sought to investigate the in vitro trypanocidal activity of 11,13-dehydrocompressanolide isolated from Tanacetum parthenium against T. cruzi.

Our data showed that this compound displayed significant activity against the epimastigote, trypomastigote, and amastigote forms of T. cruzi and was effective at low micromolar concentrations that did not affect mammalian cell viability. The highest activity was against the trypomastigote form. This result is especially interesting because this form is found in the bloodstream of infected vertebrates.

Significant antiprotozoal activity of sesquiterpene lactones was reported in previous studies conducted by our group. When tested against L. amazonensis, guianolide influenced the growth of the amastigote form and showed strong activity against the promastigote form, similar to the results obtained for the epimastigote form of T. cruzi (Silva et al. 2010). High activity of the hydroalcoholic extract, fractions, and parthenolide isolated from the aerial parts of

### Table 2

<table>
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<tr>
<th>Trypanocidal activity against epimastigote and trypomastigote forms, cytotoxicity in LLCMK2 cells, and selectivity index of guianolide (11,13-dehydrocompressanolide) isolated from Tanacetum parthenium.</th>
<th>Guianolide (µM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epimastigote</td>
<td>IC50</td>
<td>18.1 ± 0.8</td>
</tr>
<tr>
<td>Trypanomastigote</td>
<td>EC50</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>LLCMK2 cells</td>
<td>EC50</td>
<td>93.5 ± 1.3</td>
</tr>
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</table>

The results are expressed as the mean ± SD from three independent experiments. The IC50 value represents the 50% cytotoxicity in LLCMK2 cells (96h at 37°C). The IC50 value represents the concentration that inhibits cell growth in 50% (96h at 28°C), and the EC50 value represents the concentration that lyses 50% of the parasites (24h at 37°C) compared with controls. SI: CC50 LLCMK2/IC50.
Fig. 4. Effect of guaianolide on the viability, morphology and ultrastructure of trypomastigote forms treated for 24 h at 37 °C. (A) Viability assay of trypomastigotes treated with guaianolide and benznidazole. The data are expressed as mean ± SD from three independent experiments performed in duplicate. The results were analyzed as percentages of viable cells compared with untreated parasites. Morphological alterations were observed by SEM. (B) Untreated parasite, showing typical elongated shape. (C and D) Parasites treated with IC50. (E) Parasites treated with IC90, showing changes in body shape with apparent leakage of cytoplasmic contents. Scale bars = 1 μm.

*T. parthenium against T. cruzi have been reported, with a progressive increase in the antitrypanosomal effect during the course of the purification process (Izumi et al. 2008). The results obtained with guaianolide in this study, showed that parthenolide is not solely responsible compound present in T. parthenium, for this activity against T. cruzi.

Several sesquiterpene lactones have been reported to have trypanocidal activity, including parthenolide, dehydroeleucodine, psilostachyin C, α-cyclocostunolide, costunolide, and dehydrocostuslactone (Brengio et al. 2000; Izumi et al. 2008; Sülsen et al. 2010; Julianti et al. 2011). Dehydrocostuslactone and zalunin D are also guaianolide-type sesquiterpene lactones with potent

Fig. 5. Effect of guaianolide on Trypanosoma cruzi-LLCMK2 cell interaction. (A) LLCMK2 cells infected with trypomastigotes and treated with guaianolide for 96 h at 37 °C. The survival index (SI) was calculated by multiplying the percentage of infected cells by the number of amastigotes per cell, and the SI (%) was calculated using the following equation: (SI control/SI treated cells) × 100. The data are expressed as mean values obtained in duplicate from three independent experiments. *p<0.05, significant difference of each group from control. Light microscopy of Trypanosoma cruzi-infected LLCMK2 cells treated with guaianolide for 96 h at 37 °C. (B) Untreated cells showed high infection with intracellular amastigotes. (C) Cells treated with 60 μM presented low infection with amastigotes. (D) Cells treated with 50 μM. 100× magnification.
antitrypanosomal activity against epimastigote and trypomastigote forms (Uchiyama et al. 2002).

The trypanocidal activity of this group of compounds relies on the covalent bond formation of the unsaturated γ-lactone moiety with nucleophiles, such as the thiol and amino groups of enzymes, that are essential for the life of the parasite and affects the trypanothione (1,8-bis-glutathionyl spermidine) reductase system, inactivating the defense system and exposing the parasite to oxidative damage (Uchiyama et al. 2002; Otoguro et al. 2011).

Another explanation for the marked activity displayed against the multiplicative forms of T. cruzi can be found in the high cytotoxicity against cancer cells, acting mainly on the telomerase (Huang et al. 2005; Wang et al. 2007; Kanno et al. 2008). Multiple stages need to divide very quickly. Given the tight connection between cell division and telomeric extension, the blockade of telomerase activity by sesquiterpene lactones could provide a way to control parasite proliferation and therefore disease formation (Muñoz and Collins 2004; Campelo et al. 2011).

The activity of this class of compounds has usually been associated with high cytotoxicity. However, some sesquiterpene lactones, mainly of the guaianolide type, are considerably more toxic to parasites than to mammalian cells, as observed in the present study. The SI for guaianolide was 16.4 for trypomastigotes, showing good selectivity for this infective form. Recently, the cytotoxicity of this guaianolide was also assessed on J774G8 macrophages, with no toxicity at CC50 > 4000 μM (Silva et al. 2010). Cytotoxicity assays in LLCMK2 cells and J774G8 macrophages showed that parthenolide had higher cytotoxicity, with CC50 values of 12.9 and 56.4 μM, respectively (Tiuman et al. 2005; Izumi et al. 2008). These data indicate that guaianolide presents lower cytotoxicity than the precursor parthenolide. The lower cytotoxicity of this guaianolide compared with other guaianolides and sesquiterpene lactones may be directly related to the alkalylation of fewer structural elements (Scotti et al. 2007; Ghanous et al. 2010; Silva et al. 2010).

The use of drugs derived from natural products for the control of microorganisms is growing (Wagner and Ulrich-Merzenich 2009). In the present study, we found a combinational effect of guaianolide and benznidazole. This combination displayed a synergistic and nearly additive effect against the epimastigote and trypomastigote forms, respectively. Recently, a combinational effect of parthenolide and benznidazole against T. cruzi was also tested. Strong synergistic activity against the epimastigote form, an additive effect against the trypomastigote form, and antagonist effect on cytotoxicity were observed with this drug combination (Pelizzaro-Rocha et al. 2010). These results demonstrate that drug combinations can be an alternative for the treatment of Chagas’ disease because it allows the use of lower concentrations of benznidazole that may reduce side effects in patients.

Electron microscopy is a powerful tool for the study of morphological alterations and target organelles in protozoa treated with compounds from natural products. The effect of guaianolide on the epimastigote and trypomastigote forms should be determined to evaluate the possible therapeutic use of these compounds against Chagas’ disease. Despite the large number of reports that have shown the trypanocidal activity of sesquiterpene lactones, few studies have found changes in the parasite caused by these compounds. In the present study, we found that guaianolide caused different morphological alterations in the epimastigote and trypomastigote forms. Trypomastigotes treated with guaianolide showed changes in body shape, similar to the effect of parthenolide, such as rounding and shortening of the parasite with apparent leakage of cytoplasmic contents (Pelizzaro-Rocha et al. 2010). The promastigotes form of L. amazonensis treated with guaianolide presented different morphological alterations in size and shape (i.e., rounded and smaller), and structural alterations, such as the number of flagellum (Silva et al. 2010). The changes in the structure and shape of the plasma membrane may be caused by an action of sesquiterpene lactones on submembrane microtubules, stimulating tubulin polymerization (Bocca et al. 2004; Miglietta et al. 2004; Pelizzaro-Rocha et al. 2010).

An increase in cell length was observed in the epimastigote form, followed by a decrease in cell body width. The most significant change was the increase in flagellum length. This is the first time that such an elongation has been observed in parasites treated with natural products. The mean flagellar length of the population is modulated by variations in the concentrations of substances in the culture medium. Glucose depletion may signal an increase in the gene expression of the flagellar components required for the

Fig. 6. Isobolograms that illustrate the effect of guaianolide, benznidazole, and their combination on the epimastigote and trypomastigote forms of Trypanosoma cruzi and LLCMK2 cells. (A) Synergistic effect between guaianolide and benznidazole on epimastigote forms treated for 96 h at 28 °C (concave curve); (B) Marginal additive effect of guaianolide and benznidazole on trypomastigote forms treated for 24 h at 37 °C (line over the isobole). (C) Additive effect of guaianolide and benznidazole on LLCMK2 cells incubated for 96 h at 37 °C (line over the isobole). The dashed line represents the isobole of additivity.
construction of a longer flagellum (Tyler and Engman 2000). Therefore, we suggest that guaianolide may interfere with the absorption of glucose from the medium, leading to an increase in the flagellum.

The slight changes in mitochondria observed by TEM and flow cytometry in the epimastigote forms treated with guaianolide showed that this organelle is not the main target of this compound. This result differs from other studies of sesquiterpene lactones, in which mitochondrial swelling was found (Izuimi et al. 2008; Sülser et al. 2010). Transmission electron microscopy also revealed the presence of myelin-like figures and formation of membranes that enveloped reservosomes and the nucleus in epimastigote forms treated with guaianolide. Similar alterations were found in the epimastigote form treated with parthenolide (Izuimi et al. 2008).

In conclusion, guaianolide exerted actions on trypanosomatids at low concentrations, with low cytotoxicity in mammalian cells. These results suggest that this compound can recognize different targets in parasites and act through multiple mechanisms. Further investigations are needed to evaluate the underlying mechanism of action of this compound against parasites.

Conflict of interest

The authors declare that there are no conflicts of interest.

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