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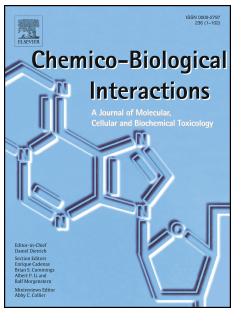

The terpenic diamine GIB24 inhibits the growth of *Trypanosoma cruzi* epimastigotes and intracellular amastigotes, with proteomic analysis of drug-resistant epimastigotes

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Author Satement

The authors contributed equally to the present work. They were divided into two groups: Mauricio Frota Saraiva, Marcus Vinicius Nora de Souza, Mauro Vieira de Almeida, Gisele Barbosa, Maristela Ribeiro de Oliveira, who worked on the synthesis and characterization of this series of compounds. And the second group: Camila Maria Azeredo and Maurílio José Soares, who tested and made the proteomic analysis of the molecule.

The terpenic diamine GIB24 inhibits the growth of *Trypanosoma cruzi* epimastigotes and intracellular amastigotes, with proteomic analysis of drug-resistant epimastigotes

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ABSTRACT

The effect of *N*-geranyl-ethane-1,2-diamine dihydochloride (GIB24), a synthetic diamine, was assayed against different developmental forms of the parasitic protozoan *Trypanosoma cruzi* (strain Dm28c). The compound was effective against culture epimastigote forms (IC_{50}/24h=5.64 μM; SI=16.4) and intracellular amastigotes (IC_{50}/24h=12.89 μM; SI=7.18), as detected by the MTT methodology and by cell counting, respectively. Incubation of epimastigotes for 6h with 6 μM GIB24 (IC_{50}/24h value) resulted in significant dissipation of the mitochondrial membrane potential, prior to permeabilization of the plasma membrane. Rounded epimastigotes with cell size reduction were observed by scanning electron microscopy. These morpho-physiological changes induced by GIB24 suggest an incidental death process. Treatment of infected Vero cells did not prevent the intracellular amastigotes from completing the intracellular cycle. However, there was a decrease in the number of

released parasites, increasing the ratio amastigotes/trypomastigotes. Proteomic analysis of 15 μM GIB24 resistant epimastigotes indicated that the compound acts mainly on mitochondrial components involved in the Krebs cycle and in maintaining the oxidative homeostasis of the parasites. Our data suggest that GIB24 is active against the main morphological forms of *T. cruzi*.

Key words: Diamine, terpenoid, geraniol, *Trypanosoma cruzi*.

HALLMARKS

- The diamine GIB24 (IS=7.18) showed high selective activity against intracellular amastigotes of *T. cruzi*.
- Flow cytometric analysis showed that GIB-24 induced dissipation of the mitochondrial membrane potential reduced the cell size and led to phosphatidylserine exposure in treated epimastigotes. The data suggest that GIB-24 cause incidental cell death in *T. cruzi*.
- Analysis of the proteome of 15 μM GIB-24 resistant epimastigotes indicated that GIB-24 acts preferentially on proteins involved in metabolism, mitochondrial functions, and oxidative stress control. Further experiments are needed to clarify how differentially expressed proteins communicate.
- GIB-24 appears as a promising compound for further evaluation against *T. cruzi*. Additional studies on synthetic derivatives of GIB-24 could produce a compound with reduced cytotoxicity and increased activity against *T. cruzi*.

1. INTRODUCTION

Chagas disease is a chronic systemic parasitosis caused by the protozoan *Trypanosoma cruzi* (Euglenozoa: Kinetoplastea). It estimated that there are currently six to seven million people infected in the world, most of them in Latin America, where the disease is endemic (WHO, 2016). This disease is considered a zoonosis since the natural reservoirs are marsupials and placental mammals that occur in the American continent. In humans, the disease results from the invasion of natural ecotypes, as well as the establishment of the insect vectors in human dwellings due to the socio-economic conditions of poor rural populations, where the disease is endemic (Urbina, 2010). Due mainly to the emigration of chronically infected people from Latin America, in the last decades, Chagas disease has been found more frequently in non-endemic countries, such as the United States of America, Canada, countries of the European continent and some Western Pacific countries (Basile et al., 2011; Blumental et al., 2015; Requena-Méndez et al., 2015; Urbina, 2010;

WHO, 2016). Two empirically determined nitro-heterocyclic derivatives emerged in the late 1960s to be the only drugs used to treat Chagas disease: Benznidazole and Nifurtimox. Both currently used in the acute phase, and their efficacy is limited and variable during the chronic phase of the illness (Bermudez et al., 2016; Maya et al., 2007; Urbina, 2010; Urbina, 2015). Furthermore, both drugs are toxic, with several side effects. An effective, single-dose, non-toxic, and low-cost drug with use in both the treatment of patients and the prevention of Chagas disease is still a goal to be achieved. Therefore, there is a demand for the research and evaluation of new compounds that may be active on *Trypanosoma cruzi* (Romanha et al., 2010; Alviano et al., 2012). The search for biologically active substances more effective and less toxic than benznidazole, coming from different natural and synthetic sources, such as compounds based on polyamines (diamines). Natural polyamines, such as putrescine, spermidine, and spermine, are organic cations in eukaryotic and prokaryotic cells. These compounds are essential for cell growth and differentiation (Reigada et al., 2016; Yamanaka et al., 2013). Therefore, compounds based on this class could interfere with the metabolism or function of polyamines being an excellent strategy to find new drugs against this disease (Yamanaka et al., 2013). Polyamines can be obtained by de novo synthesis from ornithine and, in some cases, arginine, or transported from the extracellular medium (Colotti and Ilari, 2011). In contrast to other protozoan parasites, *T. cruzi* is auxotrophic for polyamines. The reason for that is due to the inability to synthesize putrescine. This synthesis does not occur due to the lack of arginine (ADC) and ornithine (ODC) decarboxylase (Carrillo et al., 1999; Carrillo et al., 2003). Therefore, the intracellular availability of polyamines in *T. cruzi* depends exclusively on the transport processes (Reigada et al., 2016). Some polyamine biosynthesis inhibitors have already produced clinically useful tools, particularly for the treatment of parasitic diseases (Yamanka et al., 2013). In this context, it can be mention pentamidine used to treat leishmaniasis. This drug inhibits the transport of polyamines in *L. infantum* and *T. cruzi* (O'Sullivan et al., 2015). Synthetic diamines have been evaluated as possible inhibitors of trypanothione synthase (TryS) activity. This enzyme catalyzes the binding of glutathione and spermidine to form trypanothione being essential for the redox system of trypanosomatids. For example, 6-arylpyrido [2,3-d] pyrimidine-2,7-diamine derivatives were mild inhibitors of *T. cruzi* TryS (Benitez et al. 2016). Therefore, the study of diamine derivatives is a strategy that targets the action on natural polyamines. The main target would be trypanothione and/or components of its metabolic pathway (Flohe, 2012). Considering that, several groups demonstrated the trypanocidal activity of lipidic (Sales Júnior et al., 2014), aliphatic (Yamanaka et al., 2013), lipophilic long chain diamines (Legarda-Ceballos et al., 2015). Intracellular amastigotes of *T. cruzi* were highly susceptible

to lipidic diamines (Sales Junior et al., 2014) and aliphatic diamines (Yamanaka et al., 2013). Such data indicate that diamine derivatives could be useful prototypes for the development of more effective and more specific new drugs against *T. cruzi*. Therefore, in this work, we tested the effect of a new synthetic terpenic diamine derivative (GIB24) on the different developmental forms of *T. cruzi*. Our data show that GIB24 is active against the main morphological forms of *T. cruzi*.

2. MATERIAL AND METHODS

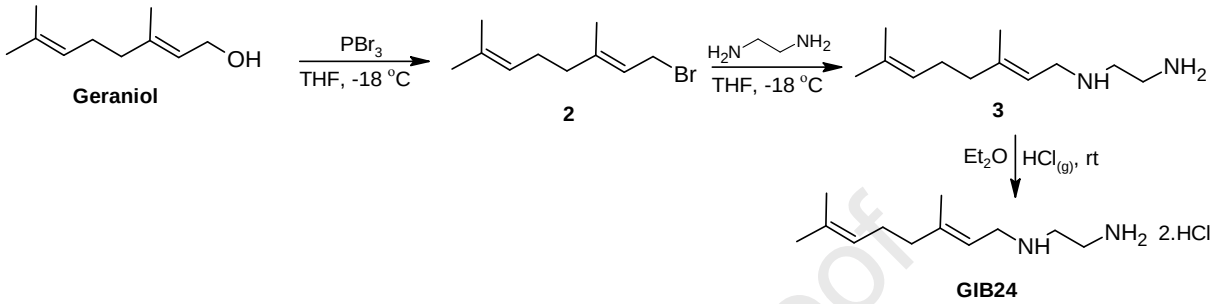
2.1 Chemistry

All reagents and solvents were reagent grade and were used without prior purification. All reactions were monitored by thin layer chromatography (TLC, Sigma-Aldrich® 60). The Geranyl bromide (compound **2**) were purified by liquid-liquid extraction and the geranyldiamine was purified by liquid-liquid extraction followed by flash chromatography on Sigma-Aldrich® silica gel 60 (230-400 mesh) using CH_2Cl_2:CH_3OH:NH_4OH (80:18:2) as eluent. The IR spectra were acquired on a Perkin Elmer Spectrum 100 FTIR spectrophotometer with an Attenuated Total Reflectance (ATR) attachment and only significant peaks were recorded. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra of the final compound (**4**) was recorded in $CDCl_3$ on a Bruker Avance ACX300 (300MHz). The chemical shifts (δ) are quoted in parts per million (ppm) downfield from the internal reference standard, tetramethylsilane (TMS), and the coupling constants (*J*) were recorded in Hertz. Splitting pattern abbreviations are as follows: s = singlet, brs = broad singlet, d = doublet, t = triplet and m = multiplet. ESI-HRMS mass spectra were carried out on a Bruker MicroTOF spectrometer.

2.1.1 Synthesis of GIB24

Sixteen terpenic diamines were previously screened against intracellular amastigotes of T. *cruzi* (data not shown). From these, only GIB24 presented a satisfactory Selectivity Index (SI) and therefore it was selected for further analyses. The synthesis of terpenic diamine *N*-geranyl-ethane-1,2-diamine dihydochloride (**GIB24**) was made according to the literature (dos Reis et al., 2016) (Scheme 1). The terpenic alcohol, (*E*)-3,7-dimethylocta-2,6-dien-1-ol **1** was converted into their respective bromide **2** by treatment with phosphorus tribromide (PBr_3) in tetrahydrofuran (THF). The allyl bromide **2** was then reacted with an excess of the ethane-1,2-diamine in dichloromethane at -18 ºC for 24 hours to provide the intermediate compound **3** in 58% yield. The transformation of terpenic diamine **3** in your respective hydrochloride salt was prepared by using a solution containing **3** in diethyl ether. In another

flask, sulfuric acid was added dropwise over sodium chloride with vigorous stirring. The gaseous hydrochloric acid was bubbled in diamine **3** solution. After twenty minutes, the formation of a precipitate was observed. The solution remained under agitation and bubbling for more 10 minutes. Finally, the precipitate was filtered under reduced pressure to furnish a white solid, compound **GIB24** in 95% of yield. This final compound was confirmed using ^{1}H NMR, ^{13}C NMR, and ESI-HRMS.



Scheme 1: Synthesis of *N*-geranyl-ethane-1,2-diamine dihydochloride (GIB24).

2.1.2 Characterization of GIB24

(*E*)-*N*¹-(3,7-dimethylocta-2,6-dien-1-yl)ethane-1,2-diamine dihydrochloride (GIB24)

white solid (yield: 1.6 g, 95%). **FTIR** (ATR, cm^{-1}): 3220 (υ, N-H_3^+), 2937 (υ_{as}, CH_2), 2886 (υ_s, CH_3), 2704, 2592 and 2435 (υ, NH^+), 1674 (υ, C=C), 1606 (δ_{as}, NH_3^+), 1532 (δ_s, NH_3^+), 1454 (δ_s, CH_2), 1378 (δ_s, CH_3), 1027 (υ, C-N), 809 (γ, =C-H),. **^{1}H NMR** ($(CD_3)_2SO$, 300 MHz) δ (ppm): 1.57 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 1.70 (s, 3H, CH_3), 2.04 (m, 4H, 2xCH_2), 3.17 (brs, 4H, 2xCH_2), 3.58 (d, 2H, CH_2, *J* = 7,3 Hz), 5.09 (m, 1H, CH), 5.28 (t, 1H, CH, *J* = 7.0 Hz), 8.51 (brs, 3H, NH_3), 9.50 (brs, 2H, NH_2). **^{13}C NMR** ($(CD_3)_2SO$, 75 MHz) δ (ppm): 16.5 (CH_3), 17.6 (CH_3), 25.5 (CH_3), 25.8 (CH_2), 35.3 (CH_2), 39.1 (CH_2), 43.2 (CH_2), 44.2 (CH_2), 114.4 (CH), 123.6 (CH), 131.2 (C), 144.1 (C). **HRMS**: *m/z* calculated for $C_{12}H_{24}N_2$. $[M+H]^+$ 197.2012, found 197.2004.

2.2 Biology

The terpenic diamine GIB24 was synthesized at the Institute of Physic and Chemistry of the Federal University of Itajubá (UNIFEI, Itajubá, MG, Brazil). Stock solutions of GIB24 and benznidazole (positive control) were solubilized at 100 mM in DMSO and stored at 4°C.

2.2.1 Reagents

Fetal calf serum was purchased from Cultilab (Campinas, SP, Brazil). Rhodamin-123 and propidium iodide were purchased from Invitrogen (Carlsbad, CA, USA). Trypsin was purchased form Promega (Madison, WI, USA). Giemsa stain, L-glutamine, MTT, DMEM medium, benznidazole and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Vero Cells

Vero cells (ATCC: CCL-81) were used in the assays to determine cytotoxicity and trypanocidal activity against intracellular amastigote forms. Cell cultures were maintained at 37°C in a humidified CO_2 atmosphere, in 75 cm^2 culture flasks containing DMEM medium (pH 7.4) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL streptomycin and 100 IU/mL penicillin. The cell cultures were kept by weekly subcultures in this same medium.

For the bioassays, cell monolayers were washed with phosphate buffered saline (PBS, pH 7.2), trypsinized and collected by centrifugation at 100 *g* for 4 minutes. Cell concentration was adjusted to 10^6 cells/mL in DMEM medium + 10% FBS and the cells were then seeded in 96 well plates ($2x10^4$ cells/well) for the cytotoxicity assays, or in 24 well plates ($6x10^4$ cells/well) for the antiparasitic activity assays. After 24h the cell cultures were used in the bioassays.

2.2.3 Parasites

Trypanosoma cruzi clone Dm28c was used in all experiments. Culture epimastigotes were kept at 28°C in LIT medium containing 10% FBS, with passages at every three days.

Cell-derived trypomastigotes were obtained from previously infected Vero cell cultures. After 3-4 days of infection the trypomastigotes present in the culture supernatant were collected, centrifuged for 10 min at 3000 *g*, suspended in DMEM + 10% FBS and used either for a new infection or for the drug assays.

In order to obtain intracellular amastigotes, Vero cells cultures were infected with cell derived trypomastigotes (ratio 10 parasites per cell). After four hours of interaction the monolayers were washed twice with PBS for removal of non-internalized parasites. The infected cultures were kept for 12h at 37°C and 5% CO_2 atmosphere in culture flasks containing DMEM medium + 10% FBS, and then used in the drug assays.

2.2.4 Cytotoxicity Assays

Vero cell cultures were incubated for 24h at 37°C with different concentrations (20 to 500 μM) of GIB-24. Cell viability was then determined by the MTT colorimetric assay, by incubation with 50 μL MTT (at 2 mg/ml in PBS). The plates were wrapped in foil and then for 3h incubated at 37°C. The medium was then removed by abrupt reversal of the plate and 100 μL DMSO was added. After solubilization of the formazan crystals the optical density (O.D.) reading was carried out at 550 nm in EL800 ELISA reader (Biotek, Winooski, VT, USA). The CC_{50}/24h value (cytotoxic concentration for 50% of the population) was estimated from the mean values of technical triplicates by non-linear regression using GraphPad Prism 5.0 software.

2.2.5 Effect of GIB-24 on epimastigotes

Three-day-old culture epimastigotes were suspended in LIT medium at $1x10^7$ cells/mL and then incubated at 28°C in 96-well plates containing different concentrations of GIB24 (1 to 500 μM). Benznidazole and 1% DMSO were used as positive and negative controls, respectively. The antiparasitic activity was evaluated by the MTT technique. After the incubation period, 50 μl MTT (at 10 mg/mL in PBS) was added to all wells, the plate was wrapped in foil was incubated at 37°C. After 3h the plate was centrifuged for 10 min at 500g and the medium was removed by abrupt reversal of the plate. The parasite pellet was resuspended in 20 μL of 10% SDS in 0.01 M HCl and this solution was incubated at 37°C for 1 hr, or until lysis of all parasites. Then 80 μL of DMSO was added to all wells and the plate was again incubated at 37°C with manual shaking at every 15 min until complete solubilization of the formazan crystals. The O.D. reading was carried out at 550 nm in a Biotek EL800 ELISA reader.

The IC_{50} value (inhibitory concentration for 50% of the parasite population) was estimated from the mean values obtained from triplicate experiments, with GraphPad Prism 5.0 software. The Selectivity Index (SI) was calculated by dividing the cytotoxic concentration obtained in Vero cells (CC_{50}/24h) by the inhibitory concentration in epimastigote forms (IC_{50}/24h).

2.2.6 Effect of GIB24 on intracellular amastigotes

Infected Vero cells containing intracellular amastigotes were incubated with different concentrations (3.75 to 100 μM) of GIB24. Benzonidazole and 1% DMSO were used as

controls. After 24 hours the cell culture plates were washed with PBS, fixed with methanol and stained for 30 minutes with Giemsa diluted in PBS pH 7.2.

Evaluation of trypanocidal activity was made by random counting of 100 host cells per plate well with the aid of ImageJ software, from images obtained in an inverted microscope. The number of infected cells and the number of intracellular amastigotes were evaluated in an experiment performed in technical triplicate. The values were expressed as percent inhibition (PI), as calculated using the following formula: PI = 100 - (T / C × 100), where T is the mean number of total intracellular amastigotes in treated cells and C is the mean number of total intracellular amastigotes in control cells. The IC_{50}/24h value was then estimated from the PI values with the CompuSyn software, by using the concentration-effect data.

2.2.7 Effect of GIB24 on cell-derived trypomastigotes

Cell-derived trypomastigotes were washed in PBS (by centrifugation at 3000 *g* for 10 min) and then incubated for 4h at 37°C in a 5% CO_2 atmosphere with GIB24 diluted at different concentrations (3 to 25 μM) in DMEM + 10% FBS. After this period the number of trypomastigotes was counted in Neubauer chamber and the percentage of parasite inhibition in treated wells relative to untreated wells (control with 1% DMSO) was calculated. The concentration-effect data were analyzed with CompuSyn software to calculate the IC_{50}/4h for trypomastigotes.

2.2.8 Effect of GIB24 on the release of intracellular parasites

Vero cells previously seeded in 24 well plates ($1x10^5$ cells per well) were infected with $1x10^6$ cell-derived trypomastigotes. After incubation for 4h the cell monolayers were washed with PBS for removal of non-internalized parasites and then 1 ml of DMEM + 10% FBS was added to each well. After incubation for 12h at 37°C and 5% CO_2 atmosphere the compound GIB24 was added at different concentrations (5 to 13 μM). After 72 hours the trypomastigotes and amastigotes released into the supernatant were collected and counted in Neubauer chamber. The percent inhibition of total released cells (trypomastigotes + amastigotes) relative to the control was used to calculate the IC_{50}/72h value, by using the CompuSyn software. Furthermore, the relative percentage of trypomastigotes and amastigotes released in treated and untreated cultures was also quantified, by using the Excel software.

2.2.9 Effect of GIB24 on medium pH

A 24-well plate was prepared containing pure DMEM medium, as well as infected and uninfected Vero cell cultures in DMEM medium. Then, 5 to 13 μM GIB-24 was added to the

wells, in order to evaluate whether GIB24 would modify the pH of the culture medium. Measurements were performed after 48h of incubation using an ORION 520A-X pH meter. The experiment was performed in technical duplicate. Statistical analyzes were performed using GraphPad Prism 5.0 software.

2.2.10 Flow Cytometry

Culture epimastigotes ($1x10^6$ cells) were pre-treated for 24h with 3,6 (IC_{50}/24h) or 12 ($2xIC_{50}$/24h) μM of GIB24, centrifuged for 1 min at 7000 *g*, washed with PBS and then processed for flow cytometry as described below.

For cell membrane permeability assays, the parasites were resuspended in PBS containing 5 μg/ml propidium iodide (PI). After 15 min at 28°C the cells were immediately quantified without washing in a FACSCanto II cytometer. Parasites were considered dead when positively labeled, using a 585/42 nm filter. As a positive control, epimastigotes were permeabilized with 0.125% saponin, which was added to the sample after the PI labeling. About collected 20,000 events (SSC×FSC scatter, ungated) were collected and the data were analyzed using the FlowJo software (Treestar, Ashland, OR, USA).

To evaluate the mitochondrial membrane potential, the parasites were incubated for 15 min at 28°C with 10 μg/mL rhodamine 123, washed 3 times in PBS and quantitated in a FACSCanto II cytometer using the 530/30 nm filter. About 20,000 events (SSC×FSC scatter, ungated) were collected. As a control, epimastigotes were treated with 200 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) after the rhodamine labeling. The data were analyzed using the FlowJo software.

Apoptosis was evaluated by quantification of the phosphatidylserine exposure with the BD PharMingen Annexin V-FITC Apoptosis Detection kit, according to manufacturer's instructions. Briefly, the cells were stained for 30 min at 28°C with annexin-V-FITC and then stained for 15 min at 28°C with propidium iodide. In all analyzes, 20,000 events were collected at the gate corresponding to epimastigotes (FSCxSSC scatter). Annexin-V labeling was observed with the 530/30 nm filter and propidium iodide with the 585/42 nm filter. The data were analyzed using the FlowJo software.

2.2.11 Scanning electron microscopy (SEM)

After incubation in LIT medium containing the IC_{50}/24h value of GIB24, epimastigotes were collected by centrifugation for 1 min at 9300 *g* and washed in PBS. The cells were fixed for 40 minutes with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, washed in 0.1 M cacodylate buffer pH 7.2 and adhered on coverslips coated with 0.1% poly-

L-lysine. It followed post-fixation for 10 min with 1% osmium tetroxide, dehydration in increasing acetone series (30, 50, 70, 90 and 100%) and critical point drying. The coverslips were adhered in SEM stubs and coated with a 20 nm-thick gold layer. The sample was observed in a Jeol JSM 6010PLUS-LA scanning electron microscope.

2.2.12 Selection of GIB24 reistant epimastigotes

Epimastigotes were selected in LIT medium containing increasing concentrations of GIB24 (3 to 15 μM), with passages at every 4-5 days. The drug concentration was increased at every 15 days, until reaching 15 μM. Such selection was performed in biological duplicate. The parasites were maintained at this concentration for 5 months and then a growth curve was daily evaluated (starting with $1x10^6$ epimastigotas/ml) for both biological replicates by countings in Neubauer chamber, until to the 8th day. Furthermore, it was made a flow cytometric evaluation of plasma membrane integrity and mitochondrial membrane potential of 3-day-old resistant cultures, as described above. Untreated epimastigotes and wild epimastigotes treated for 3 days with 15 μM GIB24 were used as a control.

2.2.13 Proteomics of GIB-24 resistant epimastigotes – Protein extract preparation

Two biological replicates of wild epimastigotes and two biological replicates of 15 μM GIB-24 resistant epimastigotes were cultured for 3 days. Then, $3x10^8$ cells were centrifuged for 5 min at 5000 *g* and washed twice with PBS. It followed addition of 240 μl of lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl, pH 7.5) and the samples were heated for 3 min at 95°C. The samples were left for 1 h in a sonicator bath to break the DNA and then centrifuged for 5 min at 20000 *g* at 20°C. The supernatant was collected and stored at -70°C until use.

The samples were thawed and processed for FASP (Filter-Aided Sample Preparation) as previously described (Wisniewski et al., 2009). Each sample was first packed in an Amicon Ultra-15 10K centrifugal filter unit (Merck Millipore, Barueri, SP, Brazil), about 5 mg protein per filter. An UA solution (8 M Urea in 100 mM Tris-HCl pH, 8.8 + 10 mM dithiothreitol) was then added to each Amicon filter. The samples were homogenized and centrifuged for three times at 4000 *g* for 30 minutes at 20°C. It followed alkylation for 1 min with 50 mM iodoacetamide in UA, incubation in the dark for 20 min and centrifugation for 40 min at 4000 *g* at 20°C. Two wash steps were then performed with UA, followed by two wash steps with 50 mM ammonium bicarbonate (ABC), with centrifugation for 30 min at 4000 *g*.

The extracts were then dosed in a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Trypsin (1 μg trypsin per 100 μg protein), and ABC solution were then added,

with incubation for 16h at 37°C. After elution of the digested peptides with ABC, it followed homogenization and centrifugation for 40 min at 4000 *g* at 20°C. The samples were then resuspended in 0.5 M NaCl, followed by homogenization and centrifugation for 40 min at 4000 *g* at 20°C. Finally, the peptides were dosed at 280 nm in a NanoDrop ND-1000 equipment (Thermo Fisher Scientific, Waltham, MA, USA) and acidified with 0.5% trifluoroacetic acid.

2.2.14 Proteomics of GIB24 resistant epimastigotes - Peptide fractionation

After protein digestion the peptides were fractionated by basic reverse phase liquid chromatography, using Sep Pack C18 130 mg columns. The columns were activated with 5 mL methanol and equilibrated with 5 mL of 20 mM ammonium formate (FA: 0.8% ammonium hydroxide + 0.16% formic acid). About 10 mL for each peptide sample was passed through the column and the column was washed twice with 5 mL of 20 mM FA. Peptide elution was performed with different concentrations of acetonitrile (ACN) into different flasks: 1 mL of 10% ACN/20 mM FA for the first, 1 mL of 14% ACN/20 mM FA for the second, 1 mL of 18% ACN/20 mM FA for the third flask and 1 ml of 60% ACN/20 mM FA for the last flask. The elutions were then dosed in a Qubit fluorometer.

All fractions were dried in Speed Vac (~ 10 hours) and then stored in Stage-tips manufactured by inserting a piece of C18 activated membrane into a P200 pipette tip. The C18 membrane was activated with 100 µL methanol and equilibrated with 200 µL of solution A (1% trifluoroacetic acid in water), with centrifugations for 6 min at 1000 *g*. The Speed-Vac dried peptides were resuspended in 1 mL of solution A and then passed through the C18 membrane by centrifugation at 1000 *g*. About 10 µg peptide was added per Stage-tip, which were washed twice with 200 µL solution A, centrifuged for 6 min at 1000 *g* and then stored at 4°C until elution.

2.2.15 Proteomics of GIB24 resistant epimastigotes – Analysis by Nano LC-MS/MS

Peptides in each Stage-tip were eluted by washing with 200 µL of solution, and collected by centrifugation for 6 minutes at 1000 *g*. The peptides were transferred to new tubes and eluted twice with 20 µL of solution B (1% trifluoroacetic acid in 80% acetonitrile). Acetonitrile was removed by Speed-Vac drying (approximately 30 minutes, without heating). The peptides were then resuspended in 12.5 µL of solution ATD (5% acetonitrile, 1% trifluoroacetic acid, 5% DMSO) for injection in the chromatography, where they were separated and entered into the mass spectrometer for analysis.

The samples were analyzed in duplicate, by injection of 4 µg peptides per run. The peptide mixtures were separated by reversed phase liquid nanocromatography and analyzed by nano ESI MS/MS. The experiments were performed with an EASY-nLC 1000 chromatograph (Thermo Scientific) coupled to the Orbitrap XL ETD (Thermo Scientific) LTQ mass spectrometer equipped with a Phoenix ST ionization source. Peptide separation occurred with a flow of 250 nL/min in mobile phase with acetonitrile (ACN)/0.1% formic acid/5% DMSO. A linear gradient of 5 to 40% ACN was used in 120 minutes. Chromatography was performed on a 30 cm silica analytical column with internal diameter of 75 µm and C18 particles (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) with a diameter of 1.9 µm heated to 60°C. The peptides were ionized by nano electro spray (at 2.7 kV) and injected into the MS. The acquisition mode was determined by Data Dependent Analysis (DDA) as follows: initial scan in Orbitrap with resolution of 15.000, followed by selection of the 10 most intense ions, which were fragmented by ICD and analyzed in the ion trap.

In parallel to the MS/MS a full scan was performed in Orbitrap with a resolution of 60.000. In the selection of ions for MS2, a dynamic exclusion list of 90 seconds was used. The lock mass option was used to obtain better accuracy of mass (error below 0.5 ppm) of tryptic peptide precursors detected by MS.

2.2.16 Analysis of proteomic data

The generated files (raw) were analyzed with the MaxQuant algorithm, version 1.5.5.1. Searches were made against a database of *Trypanosoma cruzi* protein sequences plus common contaminants, such as keratins and trypsin, together with a database composed by the reverse of the sequences used in the search. In the search parameters, a tolerance up to 20 ppm (first search) and 4.5 ppm (main search) was allowed for the MS1 spectra, with tolerance of 0.5 Da for the MS2 spectra. Analysis was specific for tryptic peptides, allowing up to two missed cleavages, and peptides with a minimum size of 7 aminoacid residues. The standard MaxQuant parameters were used, as follows: *Fixed modifications*: Carbamidomethyl (C); *Variable modifications*: Acetyl (Protein N-term); *Oxidation* (M). The database used was from *Trypanosoma cruzi* CL Brener with 19,242 entries, downloaded on 08/10/2016 from Uniprot (https://www.uniprot.org/). The LFQ tool was used for protein quantification and the match between runs option was used to increase identifications by comparing spectra between runs.

The identified proteins were analyzed using Perseus software (https://maxquant.net/perseus). Protein classified as only identified by site, pollutants and reverse were excluded from the data structure. The remaining data were transformed to Log2

scale to continue the analyses (LNBio, 2014). Afterwards, the analysis was performed in two approaches:

1) Statistical analysis of the data was performed using the Student's t-test with p = 0.05 for the evaluation of differences in protein quantity (LFQ intensity) between control samples (untreated epimastigotes) and samples resistant to 15 µM GIB24. Among the proteins with statistical difference, only those with a quantitative difference (GIB24 - Log2 scale control ≥ +1 or ≤ -1) were analyzed.

2) The data were divided into two groups (Control and Resistant to 15 µM GIB24) and were filtered only those proteins found in one group and absent in the other. For the analyzes were considered only those proteins that obtained LFQ intensity close to 24, an average intensity value found in all the proteins in this identification. Samples close to this mean value were more reliable as correctly identified, because low LFQ values represent poorly abundant proteins that may not be detected at all runs. Therefore, in proteins with LFQ intensity close to the average of all proteins, the observed difference is due to the difference in expression and not to difference in detection.

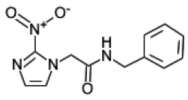
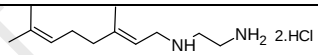
A Gene Ontology Consortium terms/class enrichment search was performed using TriTrypDB for all selected proteins. The Gene Ontology (GO) project provides controlled vocabulary of defined terms that represent the properties of genetic products. These cover three domains: Cell Component (GOCC, the parts of a cell or its extracellular environment), Molecular Function (GOMF, the elementary activities of a gene product at the molecular level, such as binding or catalysis), and Biological Processes (GOBP, operations or sets of molecular events with a defined beginning and end, relevant to the functioning of integrated life units: cells, tissues, organs and organisms).

The TriTrypDB and Pfam platforms were searched with proteins with mean intensity difference ≥+1 or ≥-1 (in Log2), and with proteins found in only one group (control or GIB24) in order to evaluate their possible biological functions from the identification of functional domains.

3. RESULTS

The effect of the diamine GIB24 was first evaluated on *T. cruzi* epimastigotes, by using the MTT methodology. The compound GIB24 was highly effective against these developmental forms (CI_{50}/24h=5.64 µM), but its selectivity index (SI) was lower than that of benznidazole (Table 1). Similar results were obtained for intracellular amastigotes in Vero cells, with SI=7.18 (Table 1).

Table 1. Effect of benzonidazol (BZN) and the terpenic diamine GIB24 on culture epimastigotes (Epi) and intracelular amastigotes (Ama) of *T. cruzi*, with cytotoxicity to Vero cells.

Compound	IC_{50}/24h Epi (µM)	IC_{50}/24h Ama (µM)	CC_{50}/24h Vero (µM)	SI Epi	SI Ama	Structure
BZN	12.16	6.86	2720	223.68	396.5	 (Oliveira et al., 2008a)
GIB-24	5.64	12.89	92.6	16.4	7.18	

In order to confirm the MTT colorimetric assay, epimastigotes were treated with 3, 6 or 10 µM GIB24 and the number of cells were evaluated 24h after treatment. It was possible to observe a significant reduction in the number of cells as compared to the untreated control, with about 50% less parasites in the treatment with 6 µM GIB24 (Fig. 1).

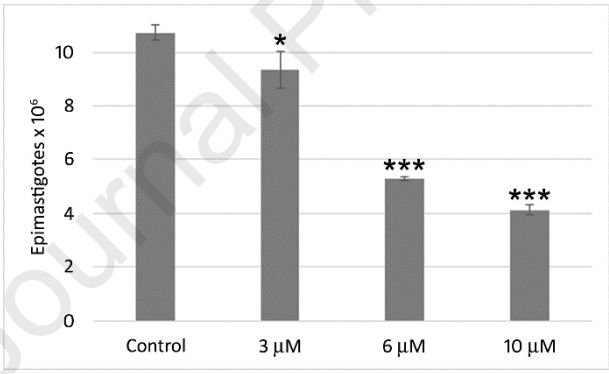


Figure 1. Total number of *T. cruzi* epimastigotes in cultures incubated for 24h with GIB24. *** $p < 0.0001$.

Incubation of cell-derived trypomastigotes for 4h with different GIB24 concentrations (3 to 25 µM) resulted in no significant lysis of the treated parasites (Fig. 2A). However, there was a dose-dependent increase in the percentage of amastigotes found in the supernatant, with a consequent decrease in the number of trypomastigotes (Fig. 2B). This difference was significant ($p < 0.05$) in the treatment with 25 µM, suggesting that GIB-24 could be increasing the rate of parasite differentiation.

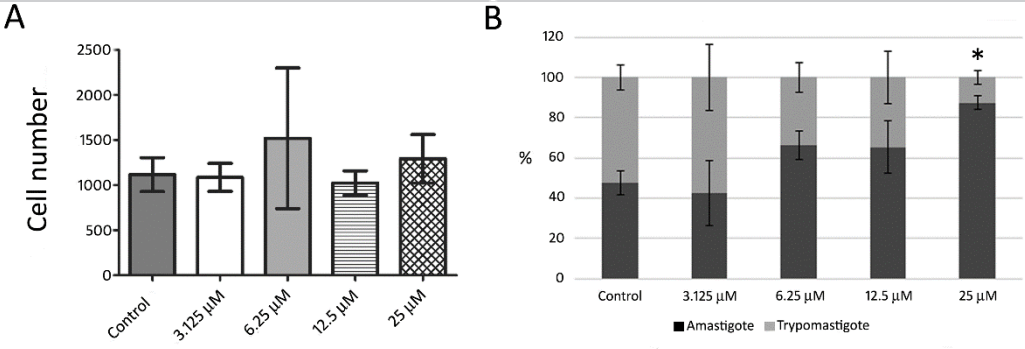


Figure 2. Number of trypomastigotes after incubation for 4h with GIB24. **A**. Mean the number of total parasites (amastigotes + trypomastigotes). **B**. Percentage of amastigotes and trypomastigotes. *p< 0.05.

The release of trypomastigotes forms was evaluated in Vero cell cultures incubated for 72h with different GIB24 concentrations (5 to 13 µM). The treatment led to a significant decrease in the number of parasites (trypomastigotes + amastigotes) present in the supernatant at all concentrations evaluated (Fig. 3A). Treatment with GIB24 also induced a significant increase in the relative number of amastigotes, with a consequent decrease of trypomastigotes in the supernatant (Fig. 3B).

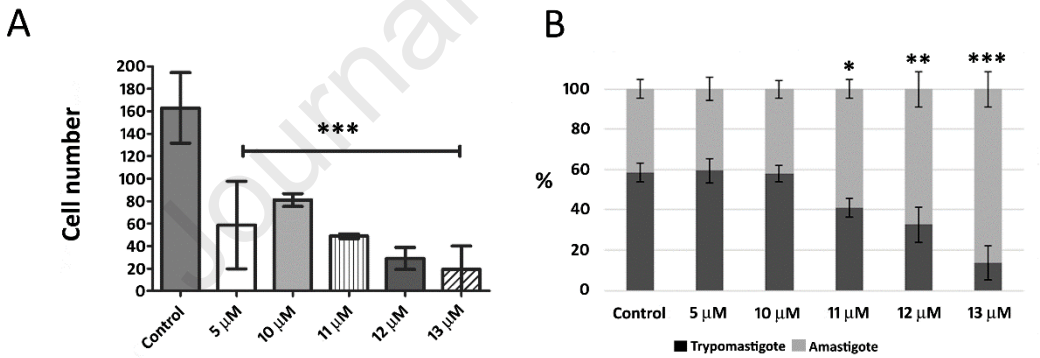


Figure 3. Effect of incubation for 72h with GIB24 on the release of parasites from Vero cells. **A**. Mean number of parasites (amastigotes + trypomastigotes) in the supernatant. **B**. Percentage of amastigotes and trypomastigotes in the supernatant. *p <0.05; **p<0.01; ***p< 0.0001.

To exclude the possibility that GIB24 was altering the pH of treated cultures, with consequent effect on the trypomastigote-to-amastigote differentiation, uninfected Vero cells, infected Vero cells, and pure DMEM medium were incubated for 48 hours with 5, 10, 11, 12 or 13 µM GIB24. It was observed a tendency in alkalinization in pure DMEM and the DMEM medium in infected Vero cell cultures, but the changes were not statistically significant (Fig.

4). Since GIB24 does not significantly alter the medium pH, the difference observed in the treated cultures could be due to cellular changes promoted by the treatment.

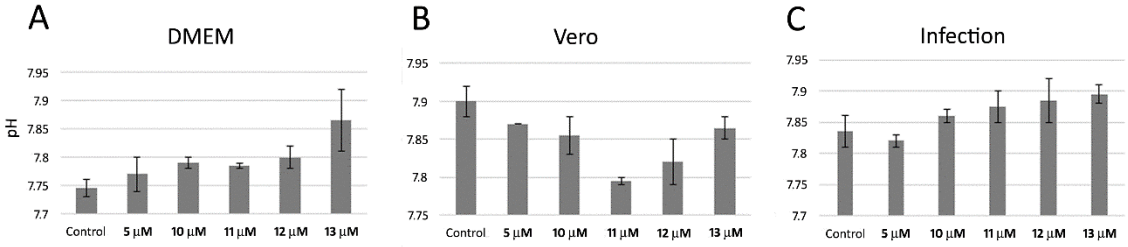


Figure 4. Effect of GIB24 on the pH of culture medium after incubation for 48h. **A**. Pure médium; **B**. Uninfected Vero cell culture; **C**. Infected Vero cell culture.

Culture epimastigotes were incubated for 24h with 3,6 (IC_{50}/24h) or 12 µM GIB24, and the plasma membrane integrity was evaluated by flow cytometry using propidium iodide (Fig. 5). Treatment for 24h with 6 µM GIB24 led to a significant increase (about 20%) in PI labeling, as related to the negative (untreated) control (Fig. 5A). Damage to the plasma membrane was significantly higher in parasites treated with 12 µM GIB24, when most cells were permeable to PI and showed positive labeling. Changes in mitochondrial membrane potential were analyzed by quantification of rhodamine-123 labeling, with non-viable cells showing a reduction in fluorescence. GIB24-treated epimastigotes showed a significantly reduced mitochondrial membrane potential after 24h of treatment at concentrations of 6 and 12 µM, with a significant increase in the number of negatively stained cells when compared to the untreated control (Fig. 5B).

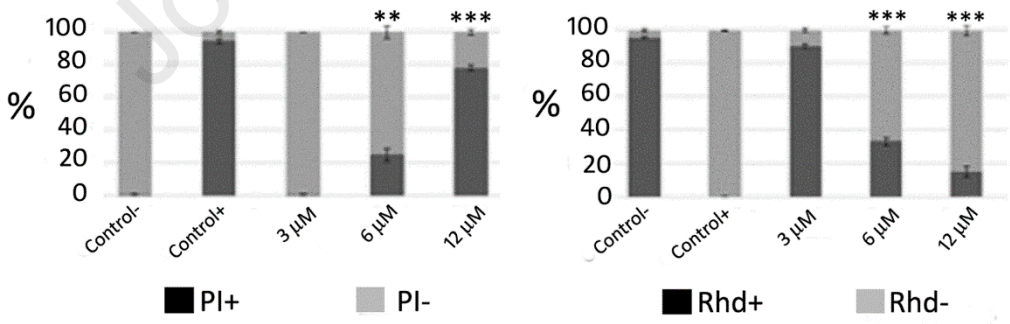


Figure 5. Flow cytometry analysis of *T. cruzi* epimastigotes after incubation for 24h with GIB24. **Left Panel**. Percentage of viable epimastigotes as assessed after propide iodide labeling. PI+= cells with damaged plasma membrane; PI-= cells with intact cell membrane. Positive control: parasites permeabilized with 0.125% saponin. **Right Panel**. Percentage of viable *T. cruzi* epimastigotes as assessed after rhodamine-123 labeling. Rhd-= cells with no labeling (reduced mitochondrial membrane potential). Rhd+= cells with positive labeling (normal mitochondrial membrane potential). Positive control: parasites treated with 200 µM

CCCP. Significance relative to untreated control (Control-). *p<0.05, **p<0.01, ***p<0.0001.

Epimastigotes incubated for 24h with 6 or 12 µM GIB24 were washed and incubated with annexin-V-FITC and PI. Annexin-V is a protein that binds to phosphatidylserine exposed on the surface of cells that entered the process of apoptosis (Jiménez-Ruiz et al., 2010). Propidium iodide (PI) will only label the DNA of cells that have some membrane discontinuity, as occurs in both late apoptosis and necrotic cells. The treatment induced a significant increase of cells in apoptosis (Annexin+/ PI-) and late apoptosis or necrosis (Annexin+/PI+) when compared to control cultures (Table 2). When the parasites were treated with 12 µM GIB24 ($2xIC_{50}$/24h) there was a significant increase in the percentage of epimastigotes in early apoptosis (20.54 times) and in the percentage of epimastigotes in late apoptosis/necrosis (87.72 times) when compared to control cultures (Table 2).

Table 2. Percentage of labeled epimastigotes after incubation for 24h with GIB-24, followed by incubation with annexin-V-FITC and PI. Early Apoptosis: Anexina+/PI- labeled cells. Late apoptosis/necrosis: Annexin+/PI+ labeled cells. Viable cells: Anexina-/PI-.

GIB-24	**Early Apoptosis**	**Late apoptosis \ Necrosis**	**Viable cells**
Untreated control	0.23%	0.67%	99.13%
6 µM	0.59%	2.80%	96.62%
Ratio Treated/control	2.6	4.18	0.97
12 µM	4.62%	57.96%	37.42%
Ratio Treated/control	20.54	87.72	0.377

Analysis by scanning electron microscopy shows that untreated *T. cruzi* epimastigotes present an elongated, fusiform body (Fig. 6A). It is possible to observe the spiral torsion of the parasite body due to the cytoskeleton of subpellicular microtubules (Fig. 6B). Incubation for 24h with 6 µM GIB24 induced drastic morphological change in the parasite body, with many parasites showing a rounded and wrinkled appearance (Fig. 6C, D).

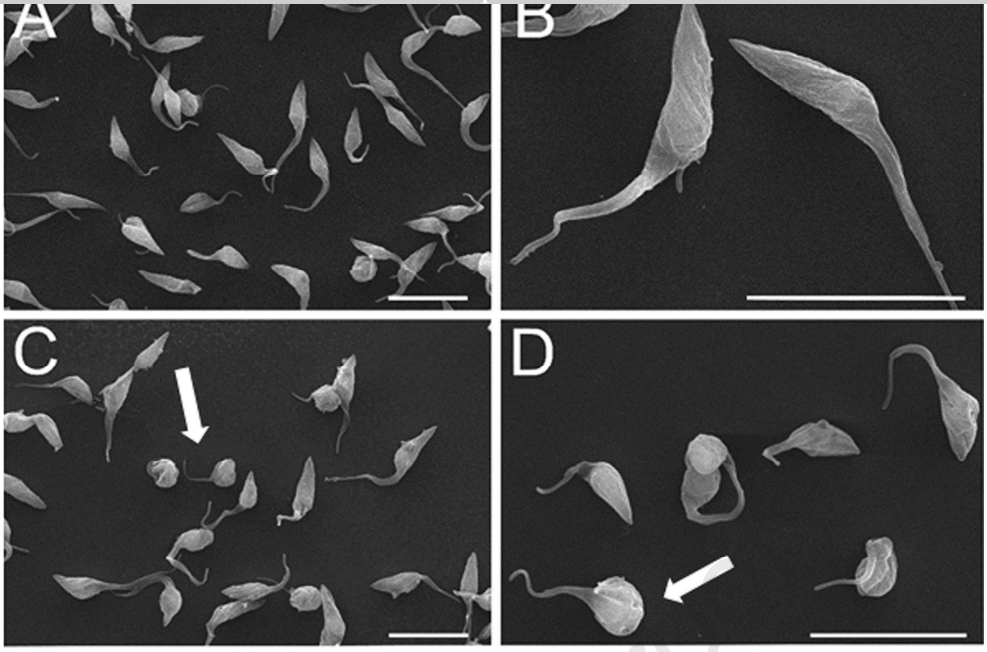


Figure 6. Scanning electron microscopy of epimastigotes treated for 24h with 6 µM GIB24. **A**, **B**. Untreated, control cells. **C**, **D**. Epimastigotes treated with 6 µM GIB24. Note the rounding of the cell body.

Resistant epimastigotes were selected by growing in cultures with increasing concentrations of GIB24 until reaching 15 µM (approximately 2.5xIC_{50}/ 24h). The selection time was 2 months, followed by a further 5 months of culture in LIT medium containing 15 µM GIB24. A growth curve showed that the resistant cultures had a growth rate similar to that of an untread culture (Fig. 7).

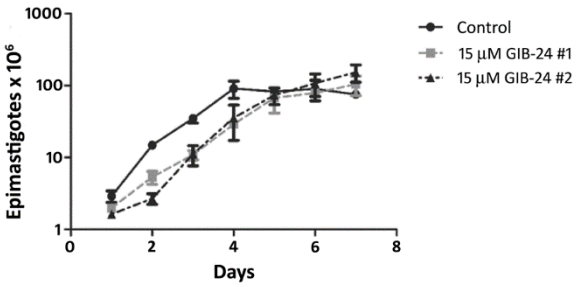


Figure 7. Growth curve of 15 µM GIB24 resistant epimastigotes in LIT medium at 28°C, as compared to a control, untreated culture.

Plasma membrane integrity (PI) and mitochondrial membrane potential (Rhd-123) of epimastigotes resistant to 15 µM GIB24 were evaluated by flow cytometry. Our data show that membrane integrity was not significantly altered in the resistant parasites (Fig. 8A). Still, there was a substantial decrease in viability of control, wild type epimastigotes incubated with

15 µM GIB24. A similar result was observed when evaluating the mitochondrial membrane potential (Fig. 8B).

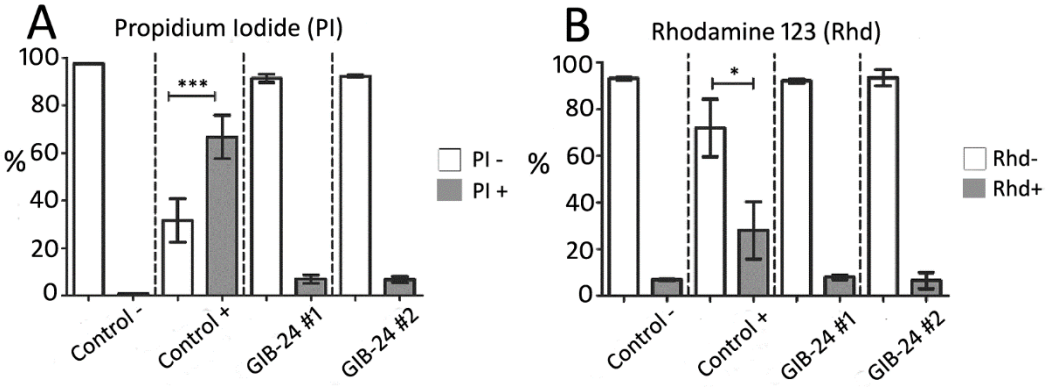


Figure 8. Evaluation of plasma membrane integrity (A) and mitochondrial membrane potential (B) in *T. cruzi* epimastigotes. Negative control: untreated cells. Positive control: wild type parasites incubated for 3 days with 15 µM GIB24. GIB24#1 and #2: duplicate cultures of resistant epimatigotes. **A**. Percentage of parasites with undamaged (PI-) or damaged (PI+) plasma membrane. **B**. Percentage of parasites with no alteration (Rhd+) or alteration (Rhd-) in the mitochondrial membrane potential.

The proteomic analysis resulted in the identification of a total of 3954 proteins in both wild type and GIB24 resistant parasites. This number was reduced to 3810 proteins after the exclusion of contaminants and possible false identifications (Reverse/Only Identified by site). Of these, 71 proteins (Supplementary Table 3) were considered with a significant difference in expression (Student's t-test). In addition, there were five proteins found only in resistant epimastigotes (Supplementary Table 4) and five present only in the control epimastigotes (Supplementary Table 5).

GO (Gene Ontology) gene enrichment analysis of these selected 81 proteins was performed on the TritrypDB, which identified a significant enrichment (p-value and Benjamini $\leq$ 0.05) in proteins involved with general metabolic biosynthetic processes, in mitochondrial transport and cellular homeostasis, including cellular redox homeostasis (Supplementary Table 6, GOBP Terms). The analysis also pointed to the enrichment of proteins that act as components of the plasma membrane, organelles, and mitochondrion (Supplementary Table 7, GOCC Terms). According to the enrichment of GOMF terms (molecular functions), the molecular functions are mainly involved in the transport of molecules (Supplementary Table 8, GOMF Terms).

Among the 71 proteins with a significant difference in quantity, 24 proteins were selected with a fold change $\geq$2 or $\geq$-2 (or $\geq$1 and $\geq$-1 when on Log2 scale). Of these 24

proteins (Supplementary Table 9) only one is an adequately characterized protein, the KAP6. Thirteen are hypothetical proteins, and 10 are putative proteins. After searching the TriTrypDB and pFAM databases for protein identification and assignment of functional domains, the vast majority of these proteins can be grouped into the following three major groups:

A) Mitochondrion and Krebs cycle: KAP6 (Tc00.1047053509791.120), putative aspartate Aminotransferase (Tc00.1047053503679.10), Putative glutamate dehydrogenase (Tc00.1047053508111.30) and a cytochrome c oxidase copper chaperone (COX17; Tc00.1047053508153.994);

B) Generation or control of oxidative stress: hypothetical with thioredoxin domain (Tc00.1047053511181.20), hypothetical with heme-peroxidase domain (Tc00.1047053507011.130), hypothetical with thiosulfate sulfurtransferase domain (Tc00.1047053506753.130), putative alcohol dehydrogenase (Tc00.1047053511277.60);

C) Amino acid metabolism: Putative cytosolic leucyl aminopeptidase (Tc00.1047053509859.40), putative glutamate dehydrogenase (Tc00.1047053508111.30), hypothetical with class IV aminotransferase domain (Tc00.1047053506559.410), putative aspartate aminotransferase (Tc00.1047053503679.10).

When analyzing the five proteins present only in control epimastigotes and the five proteins present only in GIB24 resistant epimastigotes, it could be noted that a putative fatty acid elongase (Tc00.1047053511245.150) is present only in the resistant epimastigotes (Supplementary Table 4). while the peroxin PEX-12 (Tc00.1047053503641.19), involved with the import of proteins into the matrix of glycosomes, it is present only in the control epimastigotes (Supplementary Table 5).

4. DISCUSSION

The terpene diamine GIB24 was effective to inhibit the growth of *Trypanosoma cruzi* culture epimastigotes and intracellular amastigotes, with IC_{50}/24h values of 5.64 µM (SI=16.4) and 12.89 µM (SI=7.18), respectively.

Incubation of infected Vero cells with GIB24 led to a reduction in a number of intracellular amastigotes. As a result, there was a significant decrease in the number of released parasites (trypomastigotes + amastigotes) into the extracellular medium. Interestingly, our data showed that incubation of cell-derived trypomastigotes with GIB24 resulted in no significant lysis of the parasites. However, our data showed a dose-dependent increase in the percentage of amastigotes in the supernatant, indicating that GIB24 somehow affected the amastigogenesis process (differentiation of trypomastigotes into amastigotes).

This event results from a relationship between environmental changes and adjustment of gene expression, which triggers this process, and can be mimicked in vitro by parasite exposure to low pH (Burchmore and Barrett, 2001; Hernandez-Osório et al., 2010; Pucci, 2009; Queiroz et al., 2014). However, no significant change in pH was observed in the culture medium used to maintain infected Vero cells treated with GIB24, excluding the hypothesis of amastigogenesis induced by pH change. This information indicated that the significant increase in number of amastigotes occurred due to a direct action of the compound on the released trypomastigotes.

When *T. cruzi* epimastigotes were treated with GIB24 and then analyzed by flow cytometry and scanning electron microscopy, it was possible to observe the dissipation of mitochondrial membrane potential, cell rounding (with a reduction in cell size), positive annexin V and propidium iodide labeling. These data indicated cell death by an apoptosis-like process. However, there are indications that phosphatidylserine is absent, or below the level of detection, in trypanosomatids, raising doubts about the specificity of annexin V in these parasitic protozoa. Thus, increased binding of annexin V may not necessarily be indicative of programmed cell death/apoptosis in Trypanosomatids (Proto et al., 2013).

Although several groups have shown the existence in trypanosomatids of phenotypes characteristic of programmed cell death/apoptosis-like/autophagy (Anjos et al., 2016; Jiménez-Ruiz et al., 2010; Sandes et al., 2004; Smirlis and Soteriadou, 2011), the existence of regulated cell death still under debated. Mainly because the biochemical pathways that precede its appearance have not yet been elucidated (Jiménez-Ruiz et al., 2010; Proto et al., 2013; Smirlis and Soteriadou, 2011). For these reasons, it has been suggested that cellular deaths identified in parasitic protozoa, such as trypanosomatids, should be classified as unregulated deaths, with two types: necrosis and incidental death (Proto et al., 2013). Incidental cell death is one that includes the cell death events that have some morphological and biochemical characteristics typical of defined (regulated) cell death processes. Therefore, the cell death identified in *T. cruzi* treated with GIB24 should be classified as incidental cell death.

In summary, the proteomic analysis suggests that treatment with GIB24 causes changes in mitochondrial function, generates oxidative stress, and influences the metabolism of amino acids, and lipids. Further studies are needed to demonstrate that the other identified proteins are involved in these pathways or response to stress caused by these changes. Our proteomic analysis of the differential protein expression in *T. cruzi* epimastigotes resistant to 15 μM GIB24 begins to elucidate the pathways by which the compound acts. This proteomic analysis is indicating that mitochondrial functions, amino acid metabolism, and control of

oxidative stress are targets of this compound. However, we can not yet claim that the compound acts specifically on the pathways involving trypanothione, as advocated for diamines in general. Further studies on the proteins differentially expressed due to the action of the compound open a perspective that should improve the understanding of how GIB24 acts and how the pathways affected by the compound may be communicating. More broadly, they can support the understanding of the mechanism of action of diamines on *T. cruzi*.

5. ACKNOWLEDGEMENTS

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Highlights

- A diamine showed high selective against intracellular amastigotes of *T. cruzi*
- Geraniol derivative reduces the cell size of *T. cruzi*. epimastigotes forms
- Geraniol derivative can cause incidental cell death in *T. cruzi*
- Terpenoid affects the mitochondrial membrane potential in epimastigote *T. cruzi*
- A Geraniol derivative as potential lead compound against *T. cruzi*

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: