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Primary targets of the sesquiterpene lactone deoxymikanolide on *Trypanosoma cruzi*

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Abstract

Background

Deoxymikanolide is a sesquiterpene lactone isolated from *Mikania micrantha* and *M. variifolia* which, has previously demonstrated *in vitro* activity on *Trypanosoma cruzi* and *in vivo* activity on an infected mouse model.

Purpose

Based on these promising findings, the aim of this study was to investigate the mechanism of action of this compound on different parasite targets.

Methods

The interaction of deoxymikanolide with hemin was examined under reducing and non-reducing conditions by measuring modifications in the Soret absorption band of hemin; the thiol interaction was determined spectrophotometrically through its reaction with 5,5'-dithiobis-2-nitrobenzoate in the presence of glutathione; activity on the parasite antioxidant system was evaluated by measuring the activity of the superoxide dismutase and trypanothione reductase enzymes, together with the intracellular oxidative state by flow cytometry. Superoxide dismutase and trypanothione reductase activities were spectrophotometrically tested. Cell viability, phosphatidylserine exposure and mitochondrial membrane potential were assessed by means of propidium iodide, annexin-V and rhodamine 123 staining, respectively; sterols were qualitatively and quantitatively tested by TLC; ultrastructural changes were analyzed by transmission electron microscopy. Autophagic cells were detected by staining with monodansylcadaverine.

Results

Deoxymikanolide decreased the number of reduced thiol groups within the parasites, which led to their subsequent vulnerability to oxidative stress. Treatment of the parasites with the

compound produced a depolarization of the mitochondrial membrane even though the plasma membrane permeabilization was not affected. Deoxymikanolide did not affect the intracellular redox state and so the mitochondrial dysfunction produced by this compound could not be attributed to ROS generation. The antioxidant defense system was affected by deoxymikanolide at twenty four hours of treatment, when both an increased oxidative stress and decreased activity of superoxide dismutase and trypanothione reductase (40 and 60% respectively) were observed. Both the oxidative stress and mitochondrial dysfunction induce parasite death by apoptosis and autophagy.

Conclusion

Based on our results, deoxymikanolide would exert its anti-*T cruzi* activity as a strong thiol blocking agent and by producing mitochondrial dysfunction.

Keywords: deoxymikanolide, sesquiterpene lactone, *Trypanosoma cruzi*, oxidative stress, mitochondrial dysfunction, ultraestructural damage.

Abbreviations: DCF: Dichlorofluorescein, **H2DCFDA**: 2',7'-dichlorodihydrofluorescein diacetate; **DCIP**: 2,6-dichlorophenolindophenol, **DMSO**: dimethyl sulfoxide, **DTNB:** 5,5'-dithiobis-2-nitrobenzoate, **GSH**: glutathione, **PBS**: phosphate buffered saline, **PI**: propidium iodide, **Rh123**: rhodamine 123, **ROS**: reactive oxygen species, **SCR**: succinate cytochrome c reductase, **SOD**: superoxide dismutase, **TryR**: trypanothione reductase

Introduction

Sesquiterpene lactones (STLs) are known natural compounds that exhibit promising biological activities. Activity against *Trypanosoma cruzi*, the causative agent of Chagas disease, has been demonstrated for several members of this family such as parthenolide, helenalin, dehydroleucodine, cynaropicrin, psilostachyin, psilostachyin C, and cumanin, among others (Sülsen and Martino, 2018).

Trypanosoma cruzi is a hemoflagellate parasite that belongs to the Trypanosomatidae family. Over the last years, significant progress has been achieved in understanding the parasite biology and biochemistry and identifying the main chemotherapeutic targets. Among them, the antioxidant defense system and certain organelles such as the mitochondrion, which plays an important role in the bioenergetic pathways of the parasite, can be mentioned (Menna-Barreto and de Castro, 2017). Although possible targets for the trypanocidal activity of STLs have been studied, most of their mechanisms of action still remain unclear.

Deoxymikanolide (**Fig. 1)** isolated by our group from *Mikania micrantha* and *M. variifolia* in a previous research, appeared as a highly potent compound with *in vitro* activity against *T. cruzi* epimastigotes and trypomastigotes and *in vivo* activity in a mouse model (Laurella et al., 2017).

Insert Fig. 1

Therefore, in this paper we report the investigation of several biochemical approaches to determine the mode of action of deoxymikanolide on *T. cruzi*. The interaction of the compound with hemin and thiol groups, the evaluation of oxidative stress, the effect on the mitochondrial state, cellular viability and sterol biosynthesis, as well as its effect on *T. cruzi* ultrastructure will be presented.

Materials and methods

Test compound

The deoxymikanolide used in the assays was isolated and identified from *Mikania micrantha* Kunth (Asteraceae) (LIL609699). The purity of the compound (95.8%) was determined by HPLC/DAD (Laurella et al., 2017).

Parasites

Trypanosoma cruzi epimastigotes (RA) were grown in a culture medium containing 3.3% brain-heart infusion (Difco); 0.3% tryptose (Difco); 0.3% disodium phosphate (Merck); 0.04% potassium chloride (Merck); 0.03% dextrose (Merck), and 20 µg/ml hemin (Sigma Aldrich). At the time of inoculating the parasite, this medium was supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% v/v heat-inactivated fetal calf (Natocor). Cultures were routinely maintained in the exponential phase by weekly passages at 28°C.

Hemin binding assay

The interaction of deoxymikanolide with hemin was examined spectrophotometrically under reducing and non-reducing conditions, following the methodology previously reported (Sülsen et al., 2016). The assay system under non-reducing conditions consisted of 0.23 M sodium phosphate buffer of pH 7.4, 1% SDS, 7.5 µM hemin and different concentrations of deoxymikanolide (7.5 to 45 µM). Under reducing conditions, 14 mM sodium dithionite was added to the assay system. Modifications in the Soret absorption band of hemin were recorded

using a Hewlett Packard 8452 – Diode Array spectrophotometer. The absorbance ratio at 430 and 400 nm (A430/A400) was used to quantify the changes caused by this interaction. Chloroquine was used as reference drug.

Thiols interaction assay

Sesquiterpene lactones interaction with thiol groups was determined by quantifying the level of free thiol groups using Ellman's reaction (Ellman, 1959). This assay is based on the reaction of free thiols with the chromogenic compound 5,5'-dithiobis-2-nitrobenzoate (DTNB) (Sigma-Aldrich), whereby the formation of the yellow dianion 5-thio-2-nitrobenzoate is measured. Briefly, deoxymikanolide (25 µM) was pre-incubated for 10 min with 65 mM sodium phosphate buffer of pH 8.0, containing 1.25 mM EDTA and different concentrations of glutathione (25 to 50 µM). After this time period, DTNB (0.2 mg/ml) was added and the absorbance was measured at 410 nm after 15 min of reaction.

Effect on trypanothione reductase activity

Partially purified trypanothione reductase (TryR) was obtained from a cell-free extract of *T. cruzi* epimastigotes as previously described (Ciccarelli et al., 2007). The enzymatic activity was determined spectrophotometrically, in the absence/ presence of deoxymikanolide (ranging from 5 to 50 µM), following NADPH oxidation at 340 nm (Ciccarelli et al., 2007). The corresponding non-enzymatic conversion controls were carried out.

Evaluation of deoxymikanolide on the antioxidant system of T. cruzi

- *Reduced thiol groups levels*

The thiol groups content was determined using the chromogenic compound DTNB, as described above (Ciccarelli et al., 2007).

- *Antioxidant enzyme activity*

The activity of superoxide dismutase (SOD) and TryR was assayed, as previously described (Ciccarelli et al, 2012). Briefly, parasites were harvested by centrifugation at 12,000 x g for 10 min, washed once, and re-suspended in either sodium phosphate buffer of pH 7.4 or potassium phosphate buffer of pH 7.2, for SOD or TryR activity, respectively. Suspended cells were disrupted by sonication in an MSE Soniprep 150 ultrasonic disintegrator for 45 s. The resulting homogenate was centrifuged at 5,000 x g for 15 min, and the supernatant was employed as the enzyme source. SOD activity was assayed by a spectrophotometric method based on the inhibition of superoxide-driven NADH oxidation (Ciccarelli et al., 2012). The EU (enzyme unit) for SOD is defined as the amount of enzyme required to inhibit 50% of NADH oxidation. TryR activity was determined following NADPH oxidation at 340 nm at 25°C. The activity was calculated using an extinction coefficient of $6.22x10^3 M^{-1} cm^{-1}$. The enzyme unit (EU) for TryR is defined as the amount of enzyme forming 1 nmol of product per min under standard incubation conditions. Both, SOD and TryR activity were expressed as specific activity (EU/mg of protein). Protein concentration was determined according to the method described by Lowry et al (1951)

- *Intracellular oxidative state*

The intracellular oxidative activity was assessed by flow cytometry using the oxidant-sensitive fluorescent probe H_2DCFDA. The parasites were harvested, suspended in PBS at

a concentration of $1x10^6$ parasites/ml, and stained for 30 min in the dark at 37°C with 10µM H_2DCFDA. Parasites treated with 0.2 mM H_2O_2 were used as positive control. Stained epimastigotes were then analyzed by a FACSCalibur flow cytometer (BectonDickinson). A total of 20,000 events were acquired in the region previously established as that corresponding to the parasites. Results were expressed as Gm_t/Gm_c, where Gm_t and Gm_c represent the geometric mean of the histograms corresponding to treated and untreated (control) parasites, respectively.

Parasite cell viability and mitochondrial status

Cell viability and mitochondrial membrane potential were assessed using staining with propidium iodide (PI) plus rhodamine 123 (Rh123). After treatment, parasites were harvested by centrifugation at 12,000 x g for 10 min, re-suspended in PBS (2×10^6 cells/ml), and incubated for 15 min at 37°C with 30 µg/ml (PI) plus 10 µg/ml Rh123. Samples were then immediately placed and kept on ice until analysis. Trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) (250 nM), a depolarizing agent, was used as positive control. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton Dickinson). A total of 20,000 events per sample were acquired in the region previously established as that corresponding to parasites. Alterations in the fluorescence for Rh123 were quantified using an index of variation (IV) obtained by the equation $(Gm_t - Gm_c)/Gm_c$, where Gm_t and Gm_c correspond to the geometric mean of treated and untreated (control) cells histograms, respectively. Negative IV values correspond to the depolarization of mitochondrial membranes.

The effect of deoxymikanolide on mitochondria was also evaluated by measuring activity of

succinate-cytochrome c reductase (SCR). This enzyme, which catalyzes the oxidation of succinate together with the reduction of cytochrome c, can also oxidize succinate using 2,6-dichlorophenolindophenol (DCIP) as an electron acceptor, instead of cytochrome c.
The activity of SCR was measured in a reaction mixture (1.0 ml) containing 20 mM succinate, 0.2 mM EDTA, 0.1 M KH_2PO_4 buffer of pH 7.4, and 50 μM DCIP (Balbaa et al., 2010). The reaction was carried out at 30°C and was started by the addition of 100 - 200 μl of enzyme source (the supernatant employed to measure TryR activity). The activity was followed by the decrease of the absorbance at 600 nm (ΔA/s), which corresponds to the consumption of DCIP, using an ε value of 21 mM^{-1} cm^{-1}. The EU is defined as the amount of enzyme forming 1 nmol of product per min under the standard incubation conditions.

Sterol biosynthesis analysis

After 24 h of treatment, control parasites (untreated and terbinafine-treated) and deoxymikanolide-treated parasites were harvested and the total lipids were extracted and analyzed as previously described (Sülsen et al., 2016). Qualitative and quantitative analyses were carried out using TLC. Ergosterol, lanosterol, and squalene were run in parallel. The relative intensities of the bands were established by densitometry using Scion Image software (Scion). Results were expressed in arbitrary units.

Phosphatidylserine exposure

Exposure of phosphatidylserine (PS) on the parasite surface was assessed by double staining with propidiumiodide (PI) and AnnexinV-fluorescein isothiocyanate (FITC), according to the manufacturer's instructions (Invitrogen). Epimastigotes exposed to 30%

fresh human serum for 2 h at 28°C were used as positive control. Parasites were analyzed by flow cytometry acquiring 20,000 events per sample.

Staining with monodansylcadaverine

Parasites were fixed in 2% formaldehyde, adhered in poly-lysine-coated coverslips and stained with 0.05 mM monodansylcadaverine (MDC) to detect autophagic cells. Percentage parasites displaying positive staining was determined by examination of over 300 cells per experiment. Samples were analyzed in an Olympus BX51fluorescence microscope.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed using a Siemens Elmiskop I microscope. *T. cruzi* epimastigotes were treated with 0.1 and 2.5 μg/ml of deoxymikanolide at 28 °C for 24 h. Parasites were fixed with 3% glutaraldehyde, washed three times with PBS, and post-fixed with 2% Osmium tetroxide (OsO_4) overnight. After washing the parasites twice with PBS, cells were stained with 1% uranyl acetate. Samples were dehydrated sequentially in ethanol and acetone and were embedded in Epon 812. Ultrathin sections were examined under the microscope (Sülsen et al., 2011).

Statistical analysis

The results shown are representative of three separate experiments, which were carried out in duplicate or triplicate depending on the assay reproducibility. The significance of any differences was evaluated using Student'*t* tests, considering a value of $p < 0.05$ as significant. Flow cytometry data were analyzed by WinMDI 2.9 software.

Results

Hemin binding

To evaluate whether the heme prosthetic group could be a target for deoxymikanolide, its affinity to hemin was tested. As shown in **Fig. 2**, no interaction with hemin was observed at the assayed hemin: drug ratios. Regarding chloroquine used as a reference drug, such interaction was evident under reducing and non-reducing conditions as well.

Insert Fig. 2

Reduced thiol group binding

Sesquiterpene lactones bind bond covalently to SH- groups of biological molecules via Michael addition to α, β-unsaturated carbonyl structures. The Michael addition reaction with either reduced low molecular weight thiols, involved in the intracellular redox balance, or thiol groups of sulfhydryl-bearing enzymes, could seriously affect the cell function. Since glutathione (GSH) is an important physiological peptide that participates in vital reactions for the parasite, the interaction between deoxymikanolide (25 μM) and different GSH concentrations (25 – 50 μM) was investigated (**Fig. 3**). Curves parallelism shows an absorbance decrease which is the same for all GSH concentrations and corresponds to a reduction of 13.34 μM in the GSH concentration. This decrease accounts for approximately 50% of the deoxymikanolide concentration, indicating that not all the potentially active sites for the Michael addition reaction (two sites per molecule) have reacted under the testing conditions.

Insert Fig. 3

Effect on the TryR activity

Trypanothione reductase enzyme is present in the parasite but not in the host and besides being an attractive target for anti-*T. cruzi* drug designs, it plays a very important role for maintaining the intracellular redox balance. Concentrations of deoxymikanolide ranging from 5 to 50 µM, did not show any effects on the TryR activity (35.12 ± 4.20 nmoles/min.mg) of a cell-free extract from *T. cruzi* epimastigotes (data not shown).

Effect of deoxymikanolide on the T. cruzi antioxidant system

To determine if deoxymikanolide affects the redox state of treated parasites, the thiol groups content, together with the activity of the SOD and TryR antioxidants enzymes were evaluated (**Table 1**). The remarkable decrease (70% of the control corresponding to untreated parasites) in the level of reduced SH groups, clearly detected after only 3 h of treatment, is noteworthy. This behaviour could possibly express the aforementioned addition reaction between the STL and thiol groups present inside the parasite. The SOD activity did not show significantly different values compared to the control after 3 and 8 h of treatment; while at 24 h of treatment, a 40% decrease was observed. On the other hand, TryR showed a significant and progressive activity decrease after 3 to 24 h of treatment.

Insert Table 1

To evaluate whether these modifications in the antioxidant system could affect the intracellular redox state, a flow cytometry assay using the fluorescent probe H_2DCFDA was performed. This probe is non-fluorescent until it is hydrolyzed by intracellular esterase and

readily oxidized to the highly fluorescent dichlorofluorescein (DCF) in the presence of ROS.

We were not able to find a significant increase in the oxidative state of epimastigotes after 8 h of treatment with deoxymikanolide (**Table 2**). On the other hand, after 24 h of treatment, the increase was significant.

Insert Table 2

Cell viability and mitochondrial function

As with other targets of deoxymikanolide activity, the mitochondrial state was evaluated. Figure 4 shows the density plots via flow cytometry assay of parasites treated and untreated with deoxymikanolide, which were labeled with either PI alone (A) or PI + Rh123 (B). No significant permeabilization of the plasma membrane was observed with PI either for the 3 or 8 h treatment (**Fig. 4A**). Even though the percentage of PI+ parasites increased only by 8% at 8 h, 4 and 10-fold increases higher than the control after 24 and 48 h of treatment were recorded, respectively. On the other hand, a significant decrease in labeling with Rh123 was observed for the 3-h treatment, which decreased progressively until the end of treatment (**Table 3** and **Fig. 4B**). During treatment with deoxymikanolide, a reduction ranging from 75 to 90% in the activity of the SCR respiratory chain enzyme was observed (**Table 3**).

Insert Fig. 4

Insert Table 3

Sterol biosynthesis

Sterol biosynthesis is essential for the survival of parasite. Sterols cannot be supplied by either the vector or the host cell (Lombardo and Batlle, 2018). Lipids were extracted and analyzed by TLC, after treating epimastigotes for 24 h in the presence and absence of deoxymikanolide (9 µM). Even though parasites were treated with the compound, no significant modification was observed either in the chromatographic profile or in the Sq/Erg ratio. Such ratio yielded values of 0.78 for the parasites that were treated with deoxymikanolide, and 0.82 for those that were not (the control group). Terbinafine-treated parasites (30 µM, positive control) showed a Sq/Erg ratio 3-fold higher than that obtained for untreated parasites (data not shown).

Evaluation of cell death by apoptosis

Treatment with deoxymikanolide 9 µM for 24 h leads to death of the parasite (**Fig. 4A**). Both the intracelullar oxidative stage (**Table 2**) and mitochondrial damage (**Fig. 4**) showed alterations that could be indicating apoptotic events. By labeling with Annexin V FITC/PI, that form of death was evident (Fig 5). Results demonstrated that the number of apoptotic cells increased during the treatment with deoxymikanolide in a time-dependent manner. Level of early apoptotic cells did not show significant variation with time of treatment, while, the percentage of late apoptotic cells increased around 2.5 times at 24 and 48 h.

Insert Fig. 5

Transmission electron and fluorescence microscopy

To further characterize the effect of deoxymikanolide on the parasite, a TEM analysis was performed in order to observe the ultrastructural alterations induced by the compound.

Ultrastructural alterations were not observed at a concentration of 0.4 µM. Approximately 30% of epimastigotes displayed double (or strechted) kinetoplast and / or double flagellum (**Fig. 6B**). At a concentration of 9 µM, the compound induced an intense vacuolization and autophagosome-like structures were also observed (**Fig. 6C, asterisk**). The presence of these structures was confirmed by fluorescence microscopy staining with monodansylcadaverine (**Fig. 7**). We observed the untreated parasites poorly labeled (**Fig 7A)** while the treaties were strongly labelled by the probe (**Fig. 7B**). Treatment with deoxymikanolide 9 µM increased approximately 25% the number of parasites stained with monodansylcadaverine compared to control (**Fig, 7C**).

Insert Fig. 6

Insert Fig. 7

Discussion

Based on a previous report about the *in vitro* and *in vivo* trypanocidal activity of deoxymikanolide, this compound was selected to carry out a biochemical characterization of its mechanism of action. Even though the presence of an α-β unsaturated lactone moiety is a common structural feature among STLs to which most of their biological activities are attributed, different antiprotozoal mechanisms of action have been described for them (Sülsen et al., 2010; Sülsen et al., 2016; Wulsten et al., 2017).

In this study, different assays were carried out to get an insight about the possible mechanism of action of deoxymikanolide. The assays included hemin and free SH-groups interaction, oxidative stress evaluation, effect on mitochondrial state, cellular viability, and

sterol biosynthesis. Finally, parasites ultrastructure was assessed by transmission electron microscopy.

Since parasites lack a heme biosynthetic pathway and based on the importance of this cofactor for parasite survival and growth (Choi et al., 2013), we evaluated if this could be a molecular target for deoxymikanolide. In view of the results presented herein, the compound has no affinity for hemin, so hemin would not be a target for this compound. Nevertheless, deoxymikanolide showed interaction with thiol groups, in consonance with the presence of a O=C-C=CH_2 moiety in its molecule (Zimmermann et al., 2014). The methylene group of this structural arrangement is known to react via a Michael-type addition reaction with various bio- nucleophiles, especially SH groups of cysteine residues in proteins and in free intracellular low molecular weight thiols such as glutathione (Gach et al. 2015). The fact that deoxymikanolide has two potentially active sites, an exocyclic methylene group and an endocyclic double bond, where a Michael-type addition reaction could occur, would explain the decrease of reduced thiol groups within the parasites and the subsequent vulnerability of parasites to oxidative stress. When we evaluated the effect of this compound on epimastigotes, a decrease in the level of free thiols, a gradual decrease in Rh123 fluorescence, and a decrease in SCR activity were observed after three hours treatment. The reduced retention of Rh123 observed up to eight hours of treatment could be due to a depolarization of the mitochondrial membrane, and cannot be attributed to permeabilization of the plasma membrane, as shown by PI labeling. This sesquiterpene lactone after eight hours of treatment does not unbalance the intracellular redox state, so the mitochondrial dysfunction produced by deoxymikanolide cannot be attributed to ROS generation. The antioxidant defense system is affected at twenty four hours of treatment,

when both an increased oxidative stress and decreased activity of antioxidant enzymes were observed. Both SOD and TryR enzymes showed a decrease in their activity of 40 and 60%, respectively. According to our results, deoxymikanolide would not act as an inhibitor of TryR enzyme, so both antioxidant enzymes would decrease their activity due to the damage produced by the intracellular oxidative state. Both oxidative stress and mitochondrial damage, produced by deoxymikanolide, would induce programmed cell death in *T. cruzi*, as other STLs (Barrera et al., 2013; Sülsen et al., 2016).

An analysis of the free sterols resulting from the parasites incubated with deoxymikanolide did not show that the biosynthesis of ergosterol is a possible target for this STL, unlike STL psilostachyin C which was able to inhibit this pathway causing the accumulation of squalene (Sülsen et al., 2016). Based on the parasites structural changes observed via transmission electron microscopy, deoxymikanolide could induce a delay in the cell cycle of the parasites at low concentrations, whereas at higher concentrations the compound induced intense vacuolization and the appearance of autophagosome-like structures. It is possible that autophagy processes could be triggered as a result of mitochondrial injury, as observed in mitochondrial membrane potential and on succinate cytochrome c reductase activity assays. The treatment with smaller amounts (9 µM) of the deoxymikanolide, beyond 24 hours, would kill the parasite by both types of death, apoptosis and autophagy.

Conclusions

This study provides new insights to understand the mechanism of action of the sesquiterpene lactone deoxymikanolide on *T. cruzi*. In summary, this compound would

exert its anti-*T cruzi* activity as a strong thiol blocking agent and by producing mitochondrial dysfunction.

Acknowledgments

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Conflict of interest

The authors do not have any conflicts of interest.

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Table legends

Table 1. Effect of deoxymikanolide on *T. cruzi* antioxidant system. The experimental conditions were as described in Materials and Methods. For each period of time, the values obtained in the absence of deoxymikanolide were considered as the control value (100%). * Significant differences ($p<0.05$) were found when compared to the control as assessed by Student´ t test.

Time of treatment (h)	SH-Groups (%)	SOD activity (%)	TryR activity (%)
3	30.73 ± 2.70*	78.79 ± 5.25	72.91 ± 4.20*
8	21.96 ± 2.30*	82.98 ± 4.70	66.80 ± 2.25*
24	10.50 ± 1.85*	60.40 ± 3.60*	40.15 ± 1.90*

Table 2. Intracellular oxidative stress on *T.* cruzi after treatment with the deoxymikanolide. Intracellular oxidative activity was measured by flow cytometry using H2DCFDA as an oxidation-sensitive fluorescent probe. Results are expressed as the Gm_t/Gm_c ratios, which were calculated as indicated in Materials and Methods. * $p<0.05$ significance of differences between treated and control parasites.

Time of treatment (h)	Control Gm_c	Treated Gm_t	Gm_t/Gm_c
3	12.9	14.3	1.11
8	10.9	14.0	1.28
24	11.2	26.2*	2.34

Table 3. **Effect of deoxymikanolide on the mitochondrial membrane potential and on succinate cytochrome C reductase activity.** Alterations in the fluorescence for Rh123 and SCR activity were quantified as indicated in Materials and Methods. The variation index (IV) was calculated as IV = $(Gm_t - Gm_c)/Gm_c$, where Gm_t and Gm_c correspond to the geometric mean of histograms obtained for treated and untreated (control) cells, respectively. The enzymatic unit (EU) of SCR is defined as the amount of enzyme forming 1 nmol of product per min under standard incubation conditions. * p values < 0.05 were considered as significant.

Time of treatment (h)	Rh123 Control Gm_c	Rh123 Treated Gm_t	IV	SCR activity Control EU/mg	SCR activity Treated EU/mg	%
3	1157.53	814.30*	- 0.30	13.71±0.25	3.33±0.40*	24.29
8	1124.18	760.50*	- 0.32	11.63±0.33	2.74±0.22*	23.63
24	1186.58	626.54*	- 0.47	11.20±0.15	2.18±0.25*	19.46
48	1144.65	401.15*	- 0.65	10.30±0.28	0.88±0.21*	8.54

Figure legends

Figure 1. Chemical structure of deoxymikanolide.

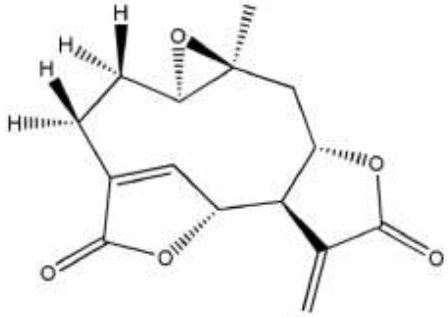


Figure 2. Interaction of deoxymikanolide with hemin. Hemin interaction was evaluated spectrophotometrically under reducing and non-reducing conditions by monitoring the Soret absorption band of hemin. * Significant differences ($p<0.05$) were found when compared to the control (hemin:drug ratio 1:0), as assessed by Student's *t*-test.

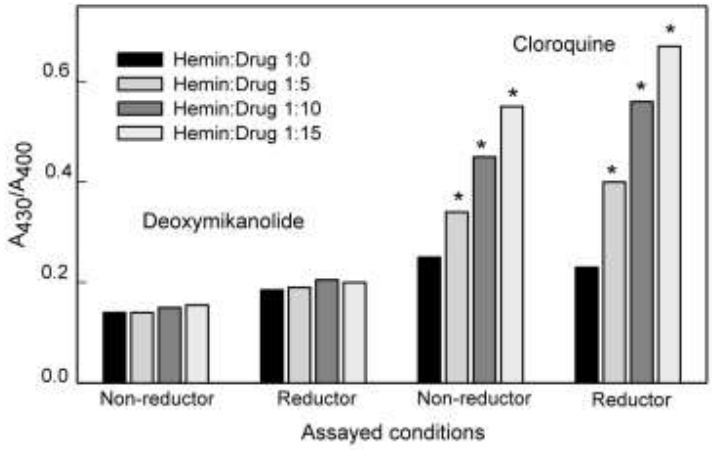


Figure 3. Interaction of deoxymikanolide with thiol groups. The reaction of dexymikanolide (25 µM) with different concentrations of GSH (25 – 50 µM) was evaluated spectrophotometrically by quantifying the level of free thiol groups with DTNB. *Significant

differences ($p<0.05$) were detected when compared to the control (without deoxymikanolide), as assessed by Student's *t*-test.

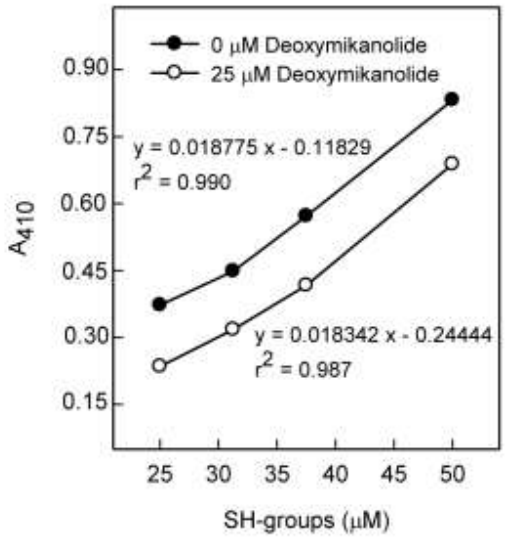


Figure 4. Effect of deoxymikanolide on cell viability and mitochondrial status. **E**pimastigotes from a 4 day culture were treated with 9 μM deoxymikanolide during 8, 24, or 48 h. Labeling was performed with PI (A) or PI+Rh123 (B): cell viability and mitochondrial membrane status were evaluated by flow cytometry as indicated. Density plots correspond to treated and untreated cells (control) with 9 μM deoxymikanolide for 8, 24, and 48 h.

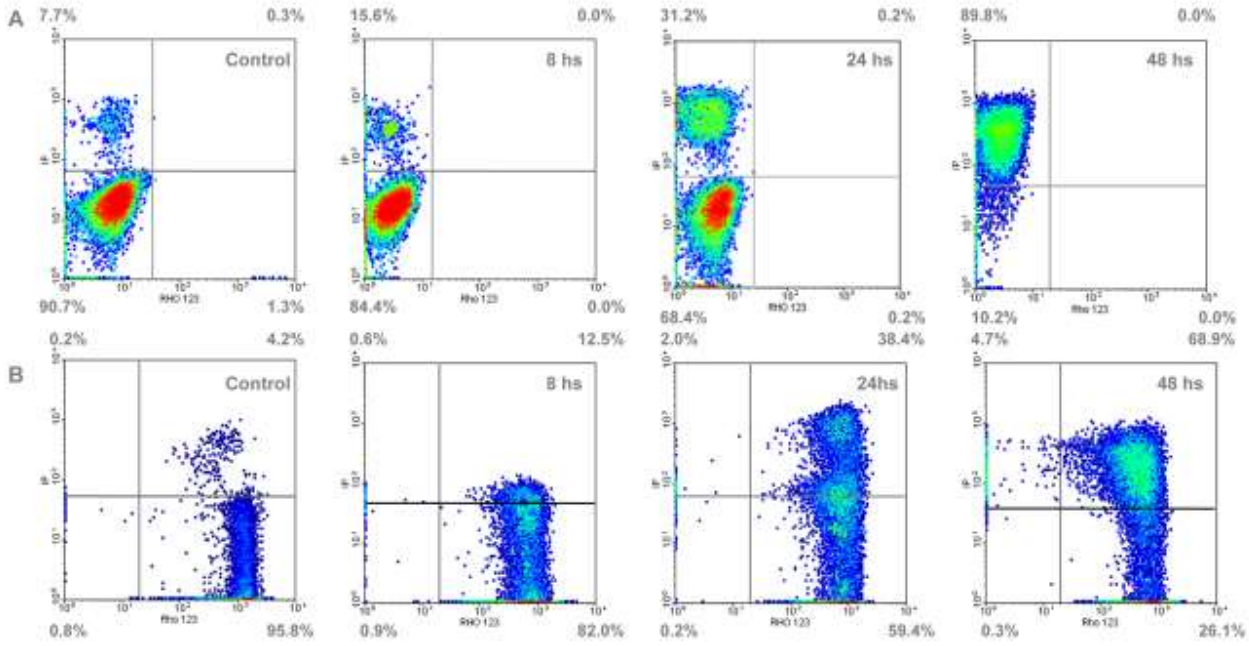


Figure 5. Effect of deoxymikanolide on phosphatidylserine exposure. *T. cruzi* epimastigotes were treated with deoxymikanolide 9 µM during 24 and 48 h and stained with Annexin V-FITC/PI. Bars correspond to $AV^{-}PI^{-}$: viable cells, $AV^{+}PI^{-}$: early apoptotic cells, $AV^{+}PI^{+}$: late apoptotic cells and $AV^{-}PI^{+}$: necrotic cells. Epimastigotes exposed to 30% fresh human serum for 2 h at 28°C were used as positive control. * p values < 0.05 were considered significant

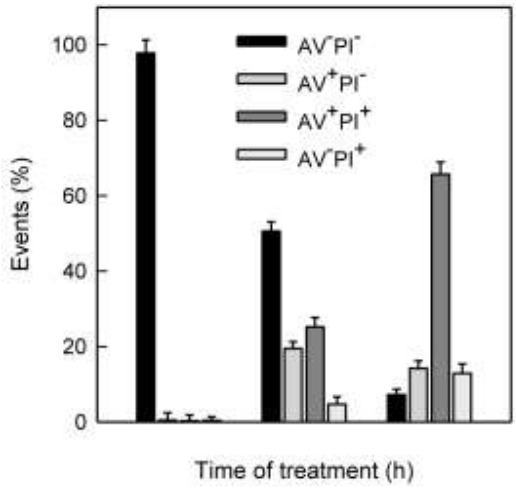


Figure 6. Effect of deoxymikanolide on the ultrastructure of *T. cruzi* epimastigotes. Parasites were grown for 24 h in a liquid culture medium, either in the absence (A) or presence of 0.4 µM (B) or 9 µM deoxymikanolide (C), as described in Materials and Methods. N: nucleus, K: kinetoplast, F: flagellum. Arrows indicate intense vacuolization and the asterisk indicates an autophagic structure. Magnification: x 2500.

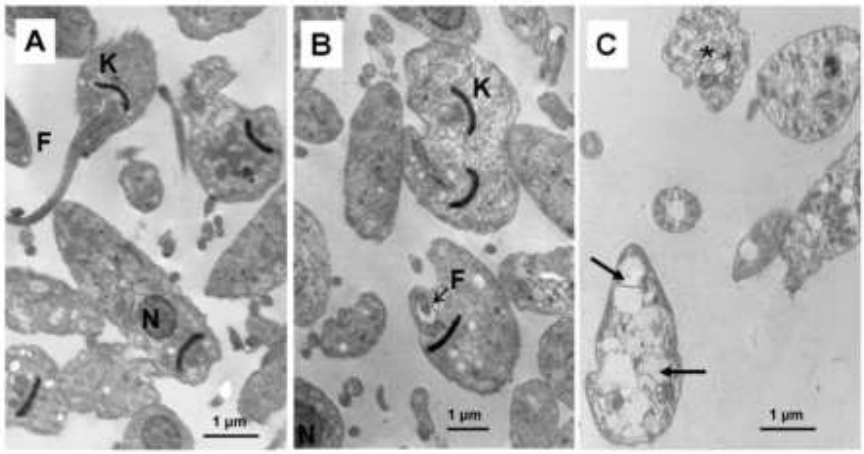
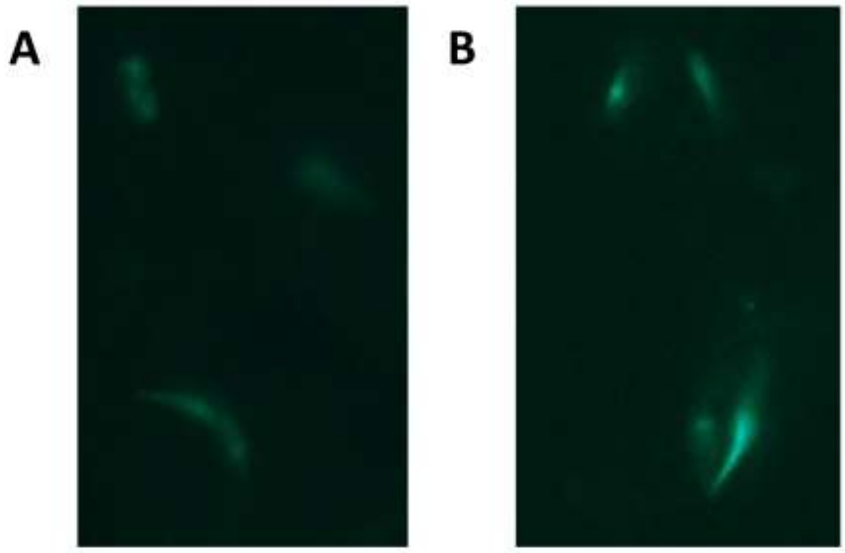
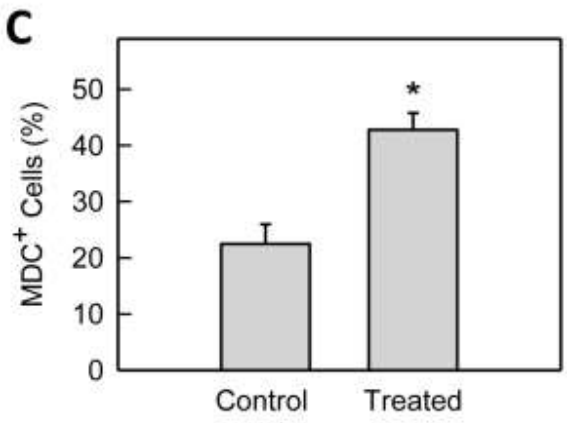


Figure 7. Detection and quantitation of autophagic cells by the probe monodansylcadaverine. Epimastigotes were grown during 24 h either in the absence (A) or presence of 9 µM deoxymikanolide (B). Percentage of parasites displaying positive

staining was determined by examination of over 300 cells per experiment, by fluorescence microscopy (C).





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