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THE TRYPANOCIDAL ACTIVITY OF THE ALKALOID OLIVERINE INVOLVES INHIBITION OF DNA SYNTHESIS

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Abstract – The *Trypanosoma cruzi* parasite is an etiologic agent of the American trypanosomiasis called Chagas disease. This pathology affects more than 24 million persons and represents one of the most important public health problems in Latin America. Taking into account this, it is necessary the search of new antitrypanosomal agents that show a major level of efficacy and minor indexes of toxicity in affected patients. Vast source of them are the natural products from plants with enormous structural diversity. A particular type of these compounds is represented by aporphinoid alkaloids. In our experiments, anonaine (**2**), oliverine (**3**) and guatterine (**5**) displayed antitrypanosomal activity. The compound **3** showed the most important activity with an IC_{50} = 12.00 ± 0.36 μM. Its mechanism of action may include inhibition of DNA synthesis.

Key words: Aporphinoid alkaloids, *Trypanosoma cruzi*, natural products, oliverine, biological activity.

INTRODUCTION

The *Trypanosoma cruzi* parasite is the etiologic agent for the American trypanosomiasis called Chagas disease. This pathology affects more than 24 million persons, representing the principal public health problem in Latin America. Nowadays this disease is slightly controlled by the elimination of the insect vector (*Triatoma infestans*). The development of vaccines has been ineffective due to the enormous variability of antigens presented by these parasites. This variability is product of complex molecular mechanisms of recombination, transcription and translation of proteins. The chemotherapy of Chagas disease is not only inadequate, but also limited, with only two drugs available: nifurtimox and benznidazole (10). Unfortunately, these drugs are not only toxic, but also their efficiency is modest, even in acute infections (1). Taking all this into account, it is necessary to make more efforts in the search of new trypanocidal agents that show a major level of efficacy and minor indexes of toxicity in affected patients. Vast source of them, are the natural products of plants, which present an enormous structural diversity. A particular type of these compounds are aporphinoid alkaloids such as actinodafnine, casitine and dicentrine (Fig. 1), that exhibited moderate activity against *T. cruzi* in "*in vitro*" experiments (6). Aporphinoid alkaloids form an important group of plant secondary metabolites. Some of these compounds have been used for a long time in traditional medicine for the treatment of various diseases, ranging from benign syndromes to more severe illnesses. More than 500 aporphinoid alkaloids have been isolated from various plant families and many of these compounds display potent biological activities (15). The objective of this work was to evaluate the trypanocidal activity of the alkaloids: reticuline (**1**), anonaine (**2**), oliverine (**3**), lanoginosine (**4**), guatterine (**5**) and liriodenine (**6**) (Fig. 2), which were isolated from different vegetable species.

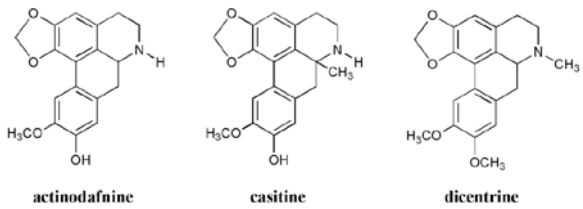

Figure 1. Chemical structures of actinodafnine, casitine and dicentrine. Compounds with moderate activities against *T. cruzi* "*in vitro*".

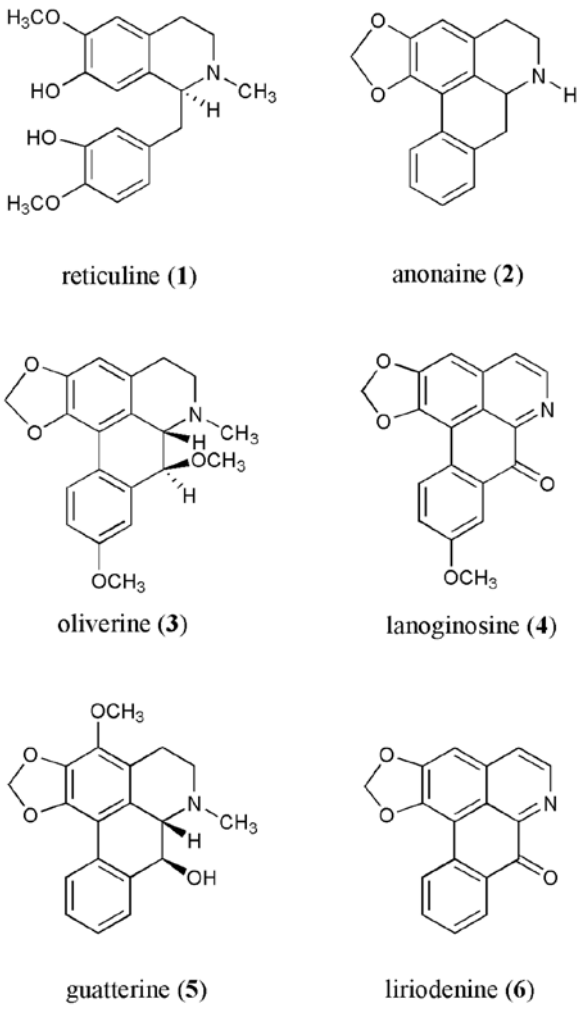

Figure 2. Chemical structure of alkaloids (compounds 1-6). Compounds evaluated against *T. cruzi* in this work.

MATERIALS AND METHODS

Chemicals and reagents

All starting materials were commercially available research-grade chemicals and used without further purification. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) was from Gibco (Grand Island, NY), trichloroacetic acid (TCA), glutamine and gentamicin were from Merck (Darmstadt, Germany), dimethyl sulfoxide (DMSO), bovine serum albumine (BSA), phosphate buffered saline (PBS), and cycloheximide were from Sigma (St Louis, MO) and sodium dodecyl sulfate (SDS) was from Biopack. EC and Saboureau broth were from Merck.

Compounds

The compounds (**1-6**) were isolated from vegetable species, such as *Rollinia marginata* (Schlecht) from Chaco, Argentine; *Enantia pilosa* (Exell); *Xylopia lemúrica* (Diels) and *Guatteria psilopus* from Republic of the Congo, Africa (2-5, 8-9, 11).

Spectroscopic data

Identities of (**1-6)**, were confirmed by comparison of both spectroscopic and physical data with previously published values (2-5, 8-9, 11). Reticuline (**1**): ^{1}H-NMR (60 MHz, $CDCl_3$) δ 6.91-6.84 (2 H, H_5 and $H_{5'}$), δ 6.60-6.40 (3 H, H_8, $H_{6'}$ and $H_{2'}$), δ 4.32 (*t*, 1 H, H_1), δ 3.82 and 3.74 (*s*, 6 H, 2 -O-CH_3, positions C_6 and $C_{4'}$) and δ 3.01-2.59 (6 H, Hs_3, Hs_4 and αs). Anonaine (**2**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 8.00 (*m*, 1 H, H_8), δ 7.05-7.40 (3H, H_{11}, H_9 and H_{10}), δ 6.54 (*s*, 1H, H_3) and δ 5.96-5.82 (*d*, 2H, *J*=1.5 Hz, -O-CH_2-O-). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 146.8 (C_2), 142.5 (C_1), 135.4 (C_{7a}), 131.4 (C_{11a}), 128.7 (C_{3a}), 116.3 (C_{1a}), 108.0 (C_3), 100.6 (-CH_2-), 53.6 (C_{6a}), 43.6 (C_5), 37.4 (C_7) and 29.6 (C_4). Oliverine (**3**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.90 (*d*, *J*=9 Hz, 1 H, H_{11}), δ 7.10 (*d*, *J*=2.8 Hz, 1 H, H_8), δ 6.78 (*dd*, *J*=2.8 Hz y 9 Hz, 1 H, H_{10}), δ 6.43 (*s*, 1 H, H_3), δ 5.98-5.88 (*d*, *J*= 1.8Hz, 2 H, -O-CH_2-O-), δ 4.30 (*d*, *J*=12.5Hz, 1 H, H_7), δ 3.81 (*s*, 3 H, -O-CH_3, position C_9), δ 3.68 (*s*, 3 H, -O-CH_3, position C_7), δ 3.66 (*d*, *J*=12.5 Hz, 1 H, H_{6a}), and δ 3.55 (*s*, 3 H, N-CH_3). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 159.0 (C_1), 146.4 (C_2), 141.4 (C_1), 139.4 (C_{7a}), 128.0 (C_{11}), 127.4 (C_{3a}), 123.5 (c_{1b}), 122.2 (C_{11a}), 116.0 (C_{1a}), 112.2 (C_{10}), 109.3 (C_8), 106.5 (C_3), 81.5 (C_7), 63.4 (C_{6a}), 52.0 (C_5), 40.7 (N-CH_3) and 25.3 (C_4). Lanoginosine (**4**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 8.86 (*d*, *J*= 5.18 Hz, 1 H, H_5), δ 8.48 (*d*, *J*=8.85 Hz, 1 H, H_{11}), δ 7.98 (*d*, *J*=2.44 Hz, 1 H, H_8), δ 7.73 (*d*, *J*=5.18 Hz, 1 H, H_4), δ 7.24 (*dd*, *J*=8.85 and 2.24 Hz, 1 H, H_{10}), δ 7.08 (*s*, 1 H, H_3), δ 6.33 (*s*, 2 H, -O-CH_2-O-) and δ 3.99 (*s*, 3 H, -O-CH_3). Guatterine (**5**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.94-7.08 (4 H, H_8, H_9, H_{10} and H_{11}), δ 6.73 (*s*, 2 H, -O-CH_2-O-), δ 4.45 (*d*, *J*=12 Hz, 1 H, H_7), δ 4.30 (*d*, *J*=1.2 Hz, 1 H, H_{6a}), δ 3.92 (*s*, 3 H, -O-CH_3) and δ 2.49 (*s*, 3 H, N-CH_3). ^{13}C-NMR (50 MHz, $CDCl_3$), δ 143.1 (C_1), 139.5 (C_3), 138.7 (C_{7a}), 134.9 (C_2), 128.7 (C_{11a}) 126.9 (C_{10}), 126.9 (C_9), 125.7 (C_{11}), 124.1 (C_{11a}), 123.6 (C_8), 119.3 (C_{3a}), 110.7 (C_{1a}), 69.7 (C_7), 64.2 (C_{6a}), 49.3 (C_5), 39.0 (N-CH_3) and 17.2 (C_4). Liriodenine (**6**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 8.87 (*d*, *J*=5.19 Hz, 1 H, H_5), δ 8.60 (*d*, *J*=7.94 Hz, 1 H, H_{11}), δ 8.57 (*dd*, *J*= 7.93 Hz y 1.22 Hz, 1 H, H_8), δ 7.74 (*d*, *J*= 5.19 Hz, 1 H, H_4), δ 7.74 (*s*, 1 H, H_3), δ 7.72 (*dt*, *J*= 7.94 Hz y 1.22 Hz, 1 H, H_{10}), δ 7.57 (*dt*, *J*= 7.94 Hz y 1.22 Hz, 1 H, H_9), δ 7.16 (*s*, 1 H, H_3) and δ 6.36 (*s*, 2 H, -O-CH_2-O-). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 179.9 (C_7), 165.3 (C_2), 163.2 (C_1), 161.4 (C_{6a}), 140.0 (C_5), 135.4 (C_{10}), 135.2 (C_{11a}), 132.4 (C_9), 131.2 (C_8), 130.4 (C_{11}), 129.8 (C_{7a}), 129.0 (C_4), 124.5 (C_{1a}) 107.6 (O-CH_2-O) and 105.6 (C_3).

In vitro trypanocidal activity assay

Trypanosoma cruzi epimastigotes, strain CL Brener, were grown at 28ºC in rich medium containing brain heart

infusion and tryptose (BHT) and supplemented with 10 % of heat inactivated FBS. The cultures were grown in the presence of one of the tested compounds or DMSO (1 %) as control. Trypanocidal activity was evaluated by the incorporation of [^{3}H]-thymidine, after 48 hr of incubation. The positive control was performed using the protein synthesis inhibitor cycloheximide.

Effect on the synthesis of different macromolecules

In order to evaluate a possible direct effect of drugs on the synthesis of different macromolecules, the incorporation of radiolabelled precursors ([^{3}H]-thymidine, [^{3}H]-uridine and [^{35}S]-methionine) was measured after a short pre-incubation of the parasites with the drugs. With this goal, parasites at the logarithmic growth phase (1-2 x 10^6 parasites/ml) were washed in PBS and resuspended in PBS supplemented with 10 mM glucose. Aliquots of 100 µl were incubated with different compounds or DMSO as control for 4 hr at 30°C in water-bath. After that, radiolabelled precursors were added to each tube followed by incubation for 60 min. Incorporation of [^{35}S]-methionine was stopped by adding 150 µl of 1.5 M NaOH, 1 mM methionine, BSA 170 µg/ml, and incubation for 30 min at 37°C. Finally, proteins were precipitated with 1 ml of 25% TCA and incubated for 60 min on ice. The samples were filtered using glass fiber filters, washed with cold 10% TCA and ethanol and finally the radioactivity on the filters was counted. In the case of [^{3}H]-thymidine and [^{3}H]-uridine, reactions were stopped by the addition of SDS 0.075%. After that, precipitation with TCA, filtration and counting was performed as it was for [^{35}S]-methionine.

Effect of the growth phase on the DNA synthesis inhibition by oliverine

A culture was grown and aliquots were taken at different times (days 1, 2, 3 and 7). Each aliquot was subdivided and treated by 4 hr with oliverine 50 µM or DMSO as control. After that, the rate of DNA synthesis was measured by [^{3}H]-thymidine incorporation as described**.**

In vitro translation assay using T. cruzi polysomes

Purification of *T. cruzi* polysomes and *in vitro* protein synthesis assays were performed as described (7). Protein synthesis inhibition by tested compounds was determined from decreased incorporation of [^{35}S]-methionine in the presence of each compound.

Biological activity of the compounds against prokaryotic (Escherichia coli) and eukaryotic (Saccharomyces cerevisae) cells

E. coli was incubated in EC broth for 24 hr at 37°C and *S. cerevisae* in Saboureau broth for 24 hr at 28°C. After the period of incubation the cell densities were adjusted to OD_{580} = 0.1. One hundred and fifty microliters of each culture were added to 100 ml of the respective medium containing one drug at a concentration of 250 µM or DMSO as negative control. Aliquots of 100 µl were used to check the OD_{580} at each time; the cultures were incubated for 72 hr. The inhibition positive controls were performed with streptomycin and amphotericin B at a concentration of 250 µM each.

Statistical analysis

Dose-response curves and IC_{50} values were obtained by fitting the data to a four-parameter logistic equation using non-linear regression. Significance of differences between control and treated experiments were tested by the t-test. Values of $p<0.05$ were considered significantly different. Error bars represent standard deviation.

RESULTS

After four days of culture, it was observed that the compounds anonaine (**2**), oliverine (**3**) and guatterine (**5**) had a trypanocidal effect at a concentration of 250 μM. Besides, after 48 hr of growth in presence of these compounds, DNA synthesis decreased significantly (Fig.3). The most important decrease was observed with oliverine (**3**). Taking into account these results, we proceeded to evaluate the effect of oliverine (**3**) on DNA, RNA and protein synthesis processes at a concentration of 50 μM. In this experiment, we observed a significant 52 % inhibition of DNA synthesis on four days cultures (Fig. 4). In contrast, RNA and protein synthesis were not affected by oliverine (**3**). The absence of activity of this compound on protein synthesis was further confirmed using *in vitro* translation assay by *T. cruzi* polysomes (Fig. 5).

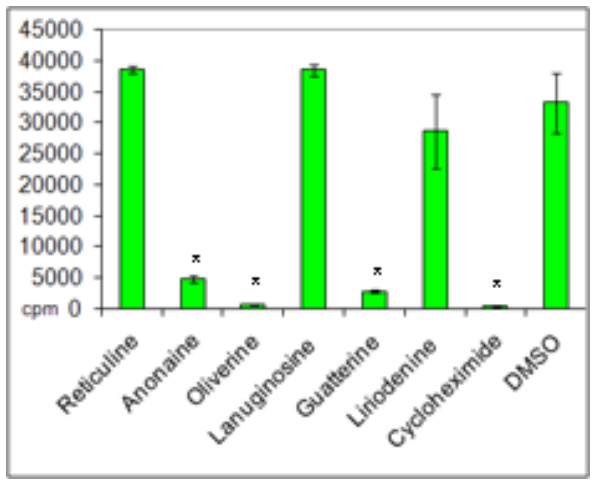

Figure 3. *In vivo* trypanocidal activity assay. *T. cruzi* epimastigotes were cultured in the presence of different compounds at 50 µM, cycloheximide 50 µg/ml (positive control) or DMSO (1 %, negative control) for 2 days. After that, parasites were labeled with [^{3}H]-thymidine as described in Methods. (*); significantly different from negative control ($p<0.05$); N=3.

Later, we evaluated the inhibition of DNA synthesis at different stages of cellular growth. In this assay we found that oliverine (**3**) showed more significant inhibitory activity on 1-day-old culture and its potency decreased in *T. cruzi* cultures at later growth stages (Fig. 6)

This result suggests that the inhibition is more effective on actively dividing parasites. Also, we determinated the IC_{50} value of oliverine (**3**) for the DNA synthesis inhibition after 48 hr

incubation and we got a value of 12.00 ± 0.36 μM (IC_{50} ±SD) (Fig. 7).

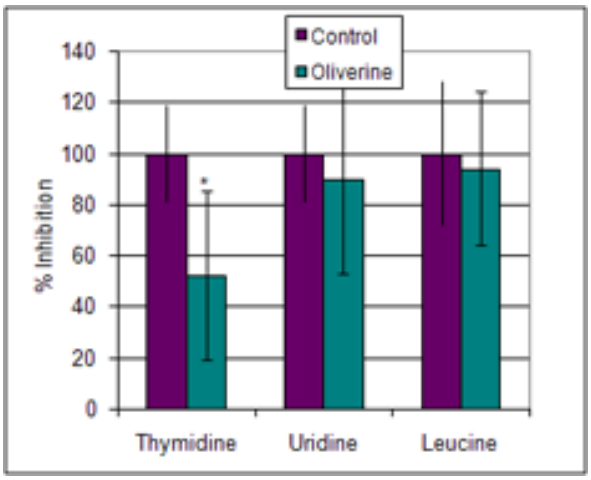

Figure 4. Effect of oliverine on the synthesis of different macromolecules. Parasites were treated in the presence or in the absence of oliverine 50 µM for 4 hr and labeled with [^{3}H]-thymidine, [^{3}H]-uridine or 35S-methionine as described in Methods. (*); significantly different from control ($p<0.05$); N=5.

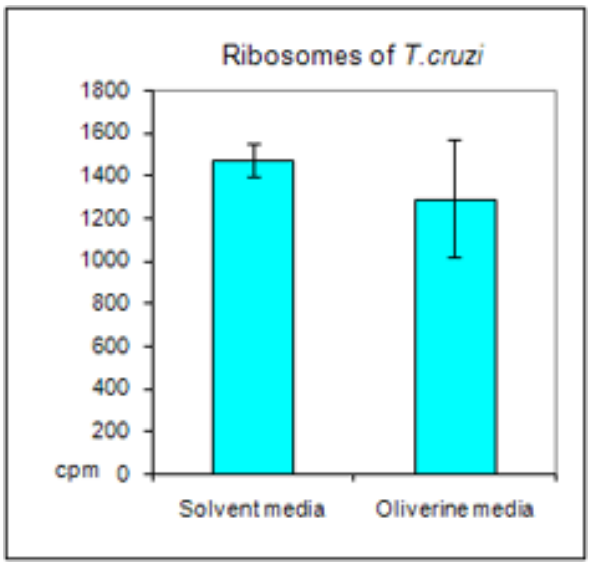

Figure 5. *In vitro* translation assay using *T. cruzi* polysomes. *In vitro* translation assays were performed as described in Methods, in the presence or in the absence of oliverine 100 µM. N=3.

In order to analyze its activity against different cell types as *E. coli*, a prokaryotic organism, and *S. cerevisiae*, the compound (**3**) was added to the culture medium before adding the cells. As it is showed in Fig. 8 and Fig. 9, not inhibition was observed.

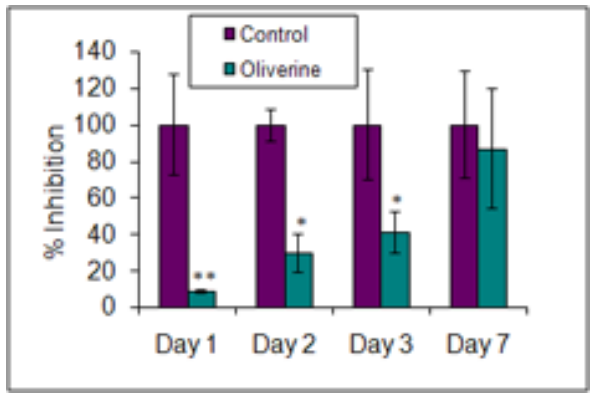

Figure 6. Activity of oliverine on DNA level in different stages of cellular growth. Parasites were grown and aliquots were taken at different times (days 1, 2, 3 and 7). Each aliquot was subdivided and treated for 4 hr with oliverine 50 µM or DMSO as control. After that, the rate of DNA synthesis was measured by [^{3}H]-thymidine incorporation as described. (**); significantly different from control, $p<0.001$. (*); significantly different from control, $p<0.05$. N=4.

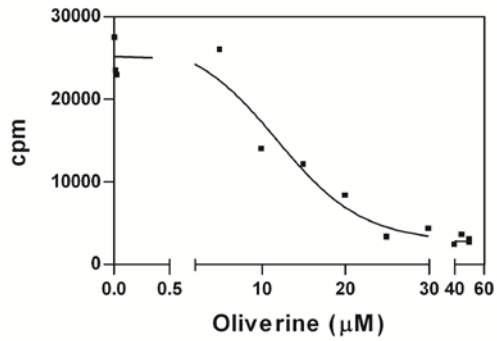

Figure 7. Determination of oliverine (3) IC_{50} value for inhibition of DNA synthesis. Parasites were grown in the presence of different concentrations of oliverine, and DNA synthesis was measured by incorporation of [^{3}H]-thymidine. IC_{50} were calculated by fitting the data to a four-parameter logistic equation and expressed as IC_{50} ± SD.

DISCUSSION

The tested compounds can be divided into non-aporphinoid alkaloids such as reticuline (**1**) and aporphinoid alkaloids (compounds **2-6**) with methylenedioxy substituent. It is known that this substituent plays a role in modulating biological activities of aporphinoids (12). Also, the aporphinoid group can be subdivided into two groups depending on the presence or the absence of carbonyl groups. The first group includes lanoginosine (**4**) and liriodenine (**6**). They exhibited a weak activity against *T. cruzi*. The second group includes anonaine (**2**), oliverine (**3**) and guatterine (**5**). These three molecules, -

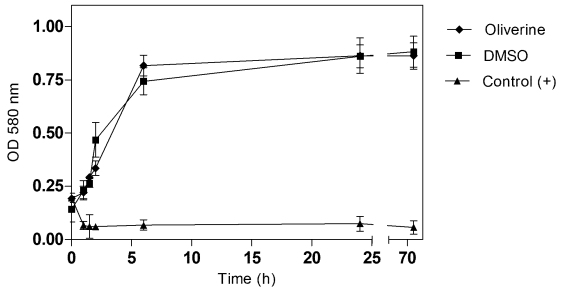

Figure 8. Evaluation of oliverine growth-inhibitory activity on prokaryotic cell (*E. coli*). The assay was performed during 72 hr at 37° C. The concentration of oliverine, DMSO (negative control) and streptomycin (positive control) was 250 µM each. N=3.

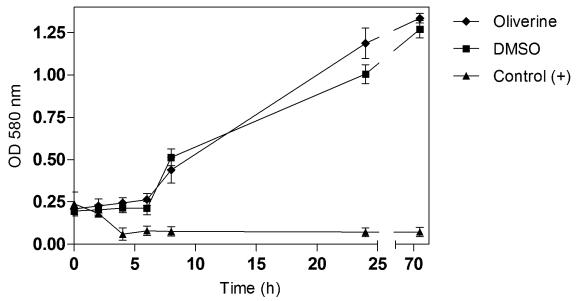

Figure 9. Evaluation of oliverine growth-inhibitory activity on eukaryotic cell (*S. cerevisiae)*. The assay was performed during 24 hr at 28° C. The concentration of oliverine, DMSO (negative control) and amphotericin B (positive control) was 250 µM each. N=3.

bearing a methylenedioxy group but without carbonyl groups, exhibited more prominent antitrypanocidal activity. Taking into account these results, it is possible to conclude that the aporphinoid structure, in absence of carbonyl groups, is necessary for this inhibitory activity.

We focused in oliverine (**3**) because its activity was the most important and we made different assays to elucidate the mechanism of action for this compound. First, we evaluated the activity of the compound **3** in replication, transcription and translation events and we determined a selective inhibition of DNA synthesis. From these results, we determined that the activity of compound (**3**) likely involves direct inhibition of DNA synthesis.

There are two possible explanations for the mechanism by which oliverine (**3**) could inhibit DNA synthesis in *T. cruzi*. First, it may act via topoisomerases because it has been demonstrated that some aporphines can inhibit these enzymes (16-18). Taking into account the flat and rigid structure of oliverine (**3**), and its typical intercalating function, topoisomerases can be considered as potential molecular targets of this compound, probably by an indirect mechanism involving a strong interaction with DNA. Second, trypanosomas are characterized by a prominent network of interconnected circular DNA molecules present in a specialized region of their single mitochondrion, called the kinetoplast. Treatment of trypanosomatids with DNA intercalating agent, such as ethidium bromide and acriflavin has been shown to lead to the loss of the kinetoplast DNA networks and the subsequent formation of so called dyskinetoplastic strain (14). Therefore such compounds have been used as successful drugs against cattle trypanosomiasis in Africa. The loss of kinetoplast DNA networks may also be induced by DNA intercalating aporphinoids. Therefore, more investigation is needed to understand the proper (or exact) mechanism of action of oliverine (3).

Interestingly, we also determined some specificity in the trypanocidal activity of oliverine (**3**) because this compound did not show cytotoxic activity against bacterium and yeast.

In summary, these results suggest that the alkaloids anonaine (**2**), oliverine (**3**) and guatterine (**5**) have trypanocidal activity. The most active compound was oliverine (**3**) and the mechanism of its action likely includes inhibition of DNA synthesis. Its IC_{50} value is similar to commercial drugs as benznidazole (IC_{50} = 7.8 ± 1.4 µM) (13), and for this reason the compound **3** could be considered as a lead molecule for the development of drugs against *T. cruzi*.

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