Trypanosoma rangeli: growth in mammalian cells *in vitro* and action of a repositioned drug (17-AAG) and a natural extract (*Artemisia sp.* essential oil)

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Abstract: *Trypanosoma rangeli* and *T. cruzi* are both parasitic unicellular species that infect humans. Unlike *T. cruzi*, the causative agent of Chagas disease, *T. rangeli* is an infective and non-pathogenic parasite for humans, but pathogenic for vectors from the *Rhodnius* genus. Because both species can coexist in different hosts and overlap their infective cycles but very little is known about the infection of *T. rangeli* in mammalian cells, we decided to characterize both the development of this parasite in cell culture and the effect of therapeutic agents with potential trypanocidal action on it. We found that *T. rangeli* exhibits a cycle of infection in Vero cells similar to that for *T. cruzi* and that the repurposed drug, 17-AAG, and the natural extract *Artemisia* sp. essential oil produce a toxic effect on epimastigotes showing a trypanocidal action from the fifth day of culture. Both treatments also affected the infection of trypomastigotes and reduced the capacity of replication of amastigotes of *T. rangeli*. Since *T. cruzi* / *T. rangeli* coinfection cases have been reported, the finding of drugs with potential activity against both species could be significant in the future. Furthermore, studies of susceptibility of both species to drugs could also help to know the different mechanisms of pathogenicity in humans displayed by *T. cruzi* that are absent in *T. rangeli*.

Introduction

Tr y panosoma rangeli is a protozoan parasite that infects mammals and triatomines, causing different levels of pathogenicity in its invertebrate vectors, particularly those from the genus *Rhodnius* (Ferreira *et al.*, 2018; Grewal, 1957; Añez *et al.*, 1985). Unlike *T. cruzi*, the causative agent of Chagas disease, *T. rangeli* infects humans without causing pathologies.

Morphologically corresponds to a trypanosome that measures about 31 μm in length and has a more developed undulating membrane than *T. cruzi*. Its kinetoplast is subterminal and small, features that allow its morphological differentiation with *T. cruzi*.

The development of *T. rangeli* in triatomine insects begins when the trypomastigote forms are ingested with the blood of infected mammals. When these forms reach the midgut of the vector (hemolymph), they differentiate into epimastigotes that become capable of replicating. After 10 to 15 days they invade the salivary glands, where they differentiate into metacyclic trypomastigotes. Rounded forms are also found as well as short and long epimastigotes, which can be transformed into trypomastigotes in the hindgut (D'Alessandro and Hincapie, 1986). After inoculation of man, the parasites enter the circulation (Urdaneta-Morales and Tejero, 1985). Some groups have shown the presence of amastigote nests in tissues (Urdaneta-Morales and Tejero, 1985; Osorio *et al.*, 1995; Eger-Mangrich *et al.*, 2001), while others have not observed them (Tanoura *et al.*, 1999).

The majority of reported human cases of *T. rangeli* infections correspond to Venezuela, Colombia, Paraná, Guatemala, El Salvador and Brazil. The laboratory procedures used for the diagnosis are based on the direct search, in fresh or stained material, which serves for the morphological study. Its differential diagnosis is important, because *T. rangeli* and *T. cruzi* can coexist, producing mixed infections. More specific methods, including immunofluorescence, immunoprecipitation and ELISA techniques, have been used

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to confirm *T. rangeli* misdiagnosed infections (Hudson *et al.*, 1988) Norte de Santander, Columbia (n = 327. Recently, a conventional PCR and a loop-mediated isothermal amplification (LAMP) assay were developed to differentiate *T. rangeli* and *T. cruzi* in samples extracted from the vector bugs (Thekisoe *et al.*, 2010).

Regarding treatment little is known about the performance of drugs with trypanocidal action. The first report of drug test on *T. rangeli* was that of nifurtimox (Lampit) in infected mice (Marinkelle, 1982). The ability of 54 different derivatives of pyrazolo[3,4-*d*]- or pyrazolo[4,3-*d*]-pyrimidine have also been studied to inhibit the multiplication of *T. rangeli* forms in culture. Among them, 4-aminopyrazolo-[3,4-*d*]-pyrimidine (APP) is the most active (Avila *et al.*, 1981). Growth inhibitory effects of six guanine and guanosine analogs, 3-desazaguanine; 3-deazaguanosine; 6-aminoallopurinol; 9-beta-xylofuranosyl guanine; a ribosyl derivative of the compound 3,6-aminopyrazolo[3,4-*d*]-pyrimidin-4-one; and 5-aminoformycin B, have also been tested against *T. rangeli* (Avila *et al.*, 1987). Idarubicin (a topoisomerase inhibitor) has shown a promising trypanocidal activity with a maximum mean growth inhibition value in the submicromolar range (Jobe *et al.*, 2012).

The main objective of this work was to characterize the *in vitro* development of *T. rangeli* at different times of its life cycle; mainly at the time of infection of trypomastigotes into mammalian cells, and the time of the intracellular replication of amastigotes. Additionally, we studied the possible effect of potential trypanocidal compounds on the different stages of *T. rangeli* cycle.

Two classes of treatments were studied: (1) the geldanamycin derivative 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a drug used in the treatment of certain classes of cancers, and (2) the *Artemisia* sp. (Asterales, Asteraceae) essential oil. The first one is the less toxic analog of geldanamycin and a potent inhibitor of heat shock protein 90 (Hsp90). It was the first Hsp90 inhibitor to enter clinical trials as an antineoplastic drug due to its significant anti-tumor activity (Jhaveri *et al.*, 2014); 17-AAG was repurposed as a drug against *Leishmania* spp. (Kinetoplastida, Trypanosomatidae), because of its demonstrated *in vitro* and *in vivo* action on this parasite (Petersen *et al.*, 2012), and we thought it could also have trypanocidal activity on *T. rangeli*. We also explored the action of *Artemisia* essential oil because there was evidence of its action against *T. brucei*, *T. cruzi* and *T. congolense* (Naß and Efferth, 2018)the safety and efficacy of current synthetic drugs are limited due to the development of drug resistance and adverse side effects. PURPOSE Artemisia annua and artemisinin are not only active against Plasmodia, but also other protozoa. Therefore, we reviewed the literature on species of the genus Artemisia and their phytochemicals regarding their activity against trypanosomes. STUDY DESIGN A PubMed search for \"Artemisia/Artemisinin and Trypanosoma\" has been conducted for literature until December 2017. RESULTS Interestingly, not only A. annua L. and its active principle, artemisinin revealed inhibitory activity towards trypanosomes. Other Artemisia species (*A. absinthium, A. abyssinica, A. afra, A. douglasia, A. elegantissima, A. maciverae, A. mexicana, and A. roxburghiana*). It has also been proposed as a therapeutic agent in malaria (Pellicer *et al.*, 2018).

Material and Methods

Reagents
The Diamond medium contains 6.25 g/l tryptose (Sigma), 6.25 g/l tryptone (Sigma), 6.25 g/l yeast extract (Sigma), 7.16 g/l KH_2PO_4 (Biopack) (pH 7.2) and 6.66 mM hemin (Calbiochem) prepared in 3 ml of 1N NaOH (Tetrahedron) and 20 ml of 1M Tris/HCl (Tetrahedron) (pH 6.8).

Mammalian cells culture
Epithelial cells (Vero cell line) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in an atmosphere of 5% CO_2.

Epimastigotes
We used the LDG (Colombian) strain of *T. rangeli*. Epimastigotes of *T. rangeli* were cultured in Diamond medium with 10% fetal bovine serum (Natocor) at 28°C. The culture contain hemin (20 mg/l), 10% inactivated fetal bovine serum, and streptomycin (100 mg / ml; Gibco) and penicillin (100 U / ml; Gibco).

Trypomastigotes
T. rangeli trypomastigotes (LDG Colombian strain) were obtained by *in vitro* metacyclogenesis of epimastigotes, as described elsewhere (Contreras *et al.*, 1986), and maintained in a culture of Vero cells in DMEM supplemented with 3% FBS and antibiotics at 37°C in an atmosphere of 5% CO_2.

AlamarBlue assay
Cell viability was estimated by the AlamarBlue assay (Invitrogen), according to the manufacturer's instructions. Vero cells grown in 96-well plates were washed three times with PBS and incubated in control medium (DMSO) in presence or absence of 0.1 µM 17-AAG and 0.5 µg / ml *Artemisia* essential oil (stalks, leaves, and flowers, were distilled in water steam stills for extracting the essence) at 37ºC for 24 h. After that, cells were washed and 10% of the AlamarBlue reagent was added to the medium and incubated for 6 h at 37ºC before measurement of absorbance at 540 nm.

Growth of epimastigotes
We started with a solution of 1×10^6 epimastigotes per milliliter in control (DMSO), 0.1 µM 17-AAG and 0.5 µg / ml *Artemisia* essential oil condition. Then, we counted the epimastigotes at different times, using a Neubauer chamber.

T. rangeli infection assays on Vero cells
We collected the trypomastigotes from the infected Vero cell cultures, by centrifugating samples for 15 min at 4000 rpm. The supernatant was discarded and 1 ml of fresh medium was added. Two hous later, we collected the supernatant rich in swimming trypomastigotes. We then treated the trypomastigotes with control medium (DMSO), 0.1 µM 17-AAG and 0.5 µg / ml *Artemisia* essential oil for 30 min before seeding them on Vero cell monolayers, at a proportion of 10 parasites per cell. After 24 h, the cells were washed with PBS, fixed with 4% paraformaldehyde, quenched with 50 mM ammonium chloride and mounted with Mowiol containing Hoechst. The percentage

of infected cell was quantified by confocal microscopy using a FV1000 Confocal Olympus microscope.

Growth of amastigotes

Vero cells were infected with *T. rangeli* tripomastigotes for 24 hours, then they were washed with PBS to eliminate the trypomastigotes that did not infect and fresh medium was placed in control condition (DMSO), 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil for additional 24 h. Then the cells were fixed with 4% paraformaldehyde for 15 min, quenched with 50 mM ammonium chloride for 15 min, treated with albumin saponin for 20 min, and then the amastigotes were detected by immunoluorescence. The number of parasites in each cell was quantified by confocal microscopy using a FV1000 Confocal Olympus microscope.

Statistics

Multigroup comparisons were made by ANOVA followed by the Tukey test (using Kyplot®).

Results

T. rangeli infection course in Vero cells

As mentioned above, we wanted to study the capacity of *T. rangeli* to infect and to develop into mammalian cells. To asses this, we performed an *in vitro* assay using Vero cells as host cells. These kidney-derived epithelial cells are broadly used for growing viruses and eukaryotic parasites, especially the trypanosomatids. We also used these cells to generate the parasites used for infection assays. In the experiment, cells were incubated in the presence of trypomastigotes of *T. rangeli* (MOI = 10) for 24 h (interaction period) and, after washing, they were fixed and stained with the DNA marker Hoechst 33342 for microscopy analysis. Other samples were washed and incubated in medium without parasites for an additional time of 24 h before fixation (chase period) and then processed as described before (Fig. 1A). Microscopy studies showed the presence of parasite nuclei and kinetoplasts inside Vero cells after 24 h incubation (Fig. 1B). Interestingly, samples maintained for 48 h showed a higher number of amastigote-like parasites surrounding Vero cell nuclei (Fig. 1C), denoting that parasites can replicate inside the cells. Quantitative data showed that more than 50% of cells became infected after 24 h of interaction (Fig. 3D) and approximately 10 amastigote-like parasites/cell developed at 48 h (Fig. 4C). Seven to 10 days after interaction, new trypomastigotes were released into the culture medium (data not shown), indicating that *T. rangeli* had completed its intracellular cycle by this time. Taking together, these results revealed that *T. rangeli* can infect and replicate in mammalian cells and that its intracellular cycle can be studied *in vitro*.
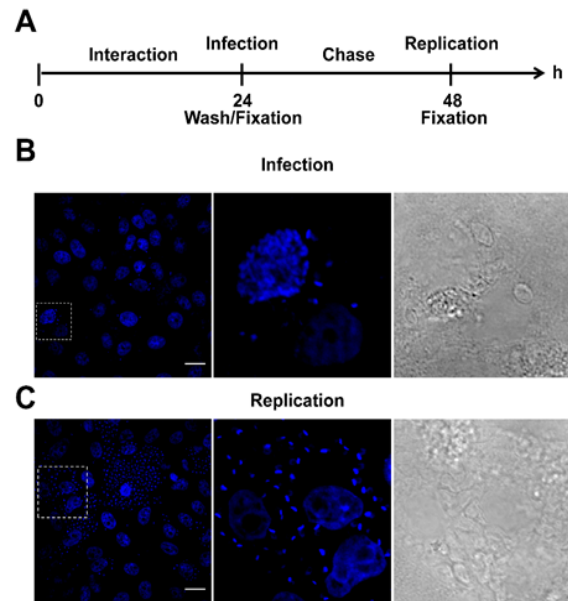

FIGURE 1. *T. rangeli infection and replication in epithelial cells in vitro.* Interaction of trypomastigotes of *T. rangeli* with Vero cells were evaluated by adding trypomastigotes (MOI = 10) to cell monolayers for 24 h (interaction period) before washing and fixation. Other samples were washed and left for additional 24 h to evaluate the replication of amastigotes (chase period). After fixation, both samples were stained with Hoechst 33342 to label kinetoplast DNA from parasites and nuclear DNA from parasites and host cells. **(A)** Experimental scheme. **(B)** Images show Vero cells infected with *T. rangeli* after the interaction period. The nuclei of Vero cells and the nuclei and kinetoplasts of *T. rangeli* are labeled in blue. **(C)** Images show amastigotes generated in the host cytoplasm during the chase period. All cell nuclei are shown in blue. Scale bar: 10 μm.
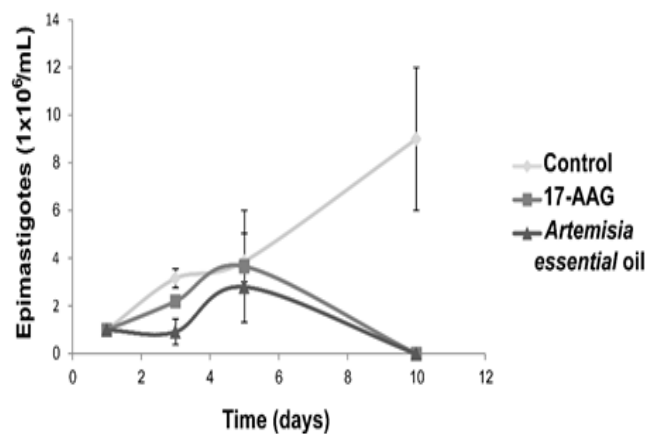

FIGURE 2. *Effect of drugs on the growth of epimastigotes of T. rangeli.* We started with a culture of 1×10^6 *T. rangeli* epimastigotes /ml in Diamond medium under control conditions (with DMSO), 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil. A small aliquot of each sample was taken on days 3, 5, and 10 to quantify the number of parasites by counting them in a Neubauer chamber. The graph shows the mean ± SE of each treatment at the different times.
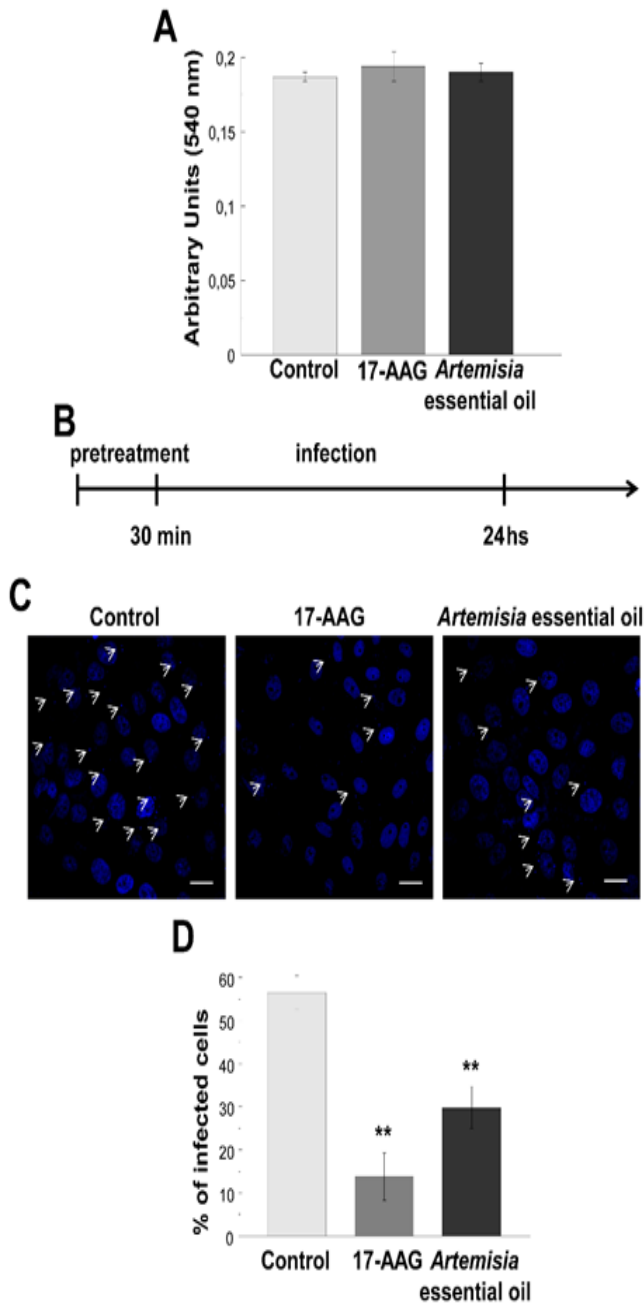
FIGURE 3. *Effect of drugs on T. rangeli infection of epithelial cells.* First, we evaluated the cell viability by the AlamarBlue test. Vero cells grown in 96-well plates were incubated in control medium in the presence or absence of 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil at 37°C for 24 h. After washing, the AlamarBlue reagent was added to each sample and incubated for 6 h at 37°C before absorbance measurement at 540 nm. Cell growth, based on the detection of cell metabolic activity, was proportional to these value. In other set of experiments, trypomastigotes were incubated in control condition (DMSO), 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil for 30 min at 37°C. After that, parasites were placed on Vero cell monolayers for 24 h before washing and fixation. **(A)** The graph indicates the mean ± SE of absorbances at 540 nm at the different conditions. **(B)** Scheme of the infection experiment. **(C)** Images show the level of infection under the different treatments. Scale bar: 10 μm. **(D)** Percentage of infected cells. Bars show the mean ± SE of two independent experiments. Tukey test ** *P* < 0.01.

17-AAG and Artemisia *essential oil affected the growth and caused death on epimastigotes of T. rangeli*

Next we studied the effect of the two potential trypanocidal treatments on the different stages of the *T. rangeli* life cycle. Initially, we analyzed the action of treatments on axenic epimastigote cultures, by incubating the parasites in Diamond medium alone (control) or supplemented with 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil for 10 days. We selected these concentrations from previously published works, which have shown the effect of these compounds on other trypanosomatids (Santos *et al.*, 2014; Pellicer *et al.*, 2018). The number of parasites counted at different times of incubation showed that epimastigotes increased exponentially in the control condition from day 0 to 10 (Fig. 2). No significant differences were observed in the 17-AAG and *Artemisia* essential oil conditions at the initial times of growth compared to controls. Interestingly, from the fifth day both 17-AAG and *Artemisia* essential oil curves decreased and reached to value 0 on day 10. These data suggest a trypanocidal action of both treatments from this day onwards.

17-AAG and Artemisia *essential oil reduce the infective capacity of trypomastigotes of T. rangeli*

Since those results were encouraging, we decided to test the effect of both treatments on the infective forms of *T. rangeli*. To control for a possible toxic effect of compounds on Vero cells, we subjected cells to the treatments for 24 h at 37°C and then analyzed cell viability using the AlamarBlue assay. Both 17-AAG and *Artemisia* essential oil did not affect cell viability (Fig. 3A). Next, we tested the effects of these compounds on trypomastigote-infected Vero cells. For such purpose, *T. rangeli* trypomastigotes were exposed to control medium (with DMSO), 0.1 μM 17-AAG or 0.5 μg / ml *Artemisia* essential oil for 30 min and then placed on Vero cell monolayers for 24 h in the same conditions (Fig. 3B). After fixation, we stained with the fluorescent DNA dye, Hoechst, as explained above, to evaluate the level of host cell infection. As shown in the images depicted in Figure 3C the amount of parasites in cells was lower under both treatments than in control cells., The percentage of infected cells was, in consequence, significantly reduced at these conditions compared to cells under control medium (Fig. 3D). We concluded that both drugs significantly reduced the infective capacity of trypomastigotes of *T. rangeli*, having 17-AAG a more marked effect.

17-AAG and Artemisia *essential oil affect replication of amastigotes of* T. rangeli *long after infection*

Next we analyzed the effect of treatments on amastigote replication. Vero cell monolayers were infected with trypomastigotes of *T. rangeli* (MOI = 10) for 24 h. Then they were washed to eliminate free trypomastigotes and incubated in control (DMSO), 0.1 μM 17-AAG and 0.5 μg / ml *Artemisia* essential oil conditions for a chase period of 24 h (Fig. 4A). After fixation, samples were subjected to an indirect immunofluorescence method to detect the parasites. Remarkably, we observed that samples treated with 17-AAG and *Artemisia* essential oil displayed a fewer number of amastigote-like forms than that in controls (Fig. 4B). Further quantification showed an important reduction in the number of amastigotes per cell as compared to controls,

indicating that both treatments affect amastigote replication of *T. rangeli* (Fig. 4C).
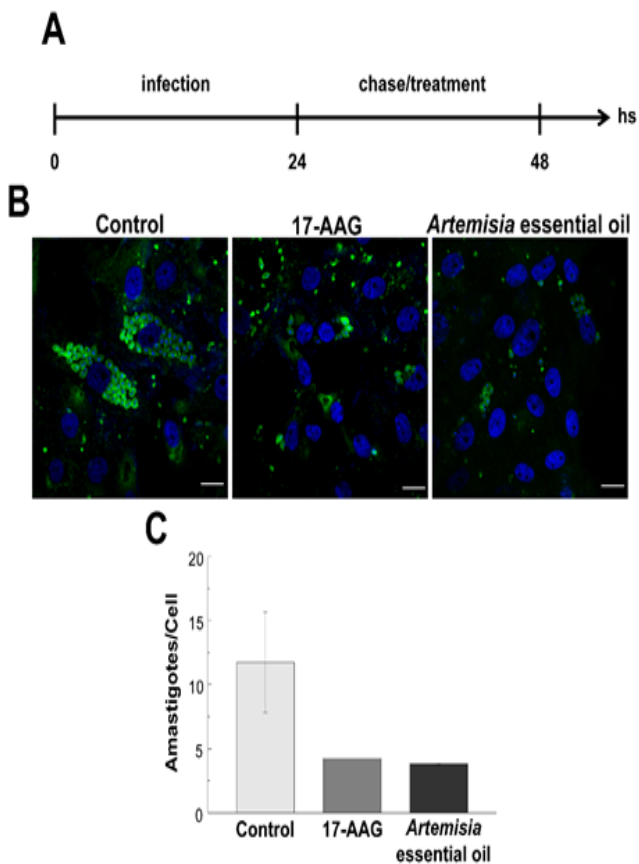

FIGURE 4. *Effect of drugs on the growth of T. rangeli amastigotes.* Vero cells were infected with trypomastigotes at a ratio of 10 parasites per cell for 24 h, and then were washed and incubated for an additional period of 24 h in control condition (DMSO), 0.1 µM 17-AAG or 0.5 µg / ml *Artemisia* essential oil at 37°C. **(A)** Experimental scheme. **(B)** Images show the level of replication of amastigotes under the different treatments. Scale bar: 10 µm. **(C)** Quantification of the number of amastigotes per cell. Bars show the mean ± SE of two independent experiments.

Discussion

The main interest in the study of *Trypanosoma rangeli*, a trypanosomatid initially described by Tejera in 1920 (Borzone *et al.*, 1950), is that it has the same geographical distribution, is frequently transmitted by the same vectors, and infects the same vertebrates as *T. cruzi*, the etiologic agent of Chagas disease. This explains the number of morphological, biochemical and molecular studies pursuing its differentiation from *T. cruzi*, which have been based in the morphology and DNA of kinetoplasts, the electron microscopy study of epimastigotes in many trypanosomatid species under standardized conditions (Mühlpfordt, 1975), the identification of carbohydrates as beta-D-galactose and alpha or beta-N-acetyl-D-galactosamine on the cell surface (Marinkelle *et al.*, 1986; Chung *et al.*, 2003; Bretting and Schottelius, 1978; González *et al.*, 1996; de Miranda Santos and Pereira, 1984), and the isoenzyme patterns (Kreutzer and Sousa, 1981; Tibayrenc and Le Ray, 1984). The use of

restriction enzymes that divide the mini circles of DNA of the kinetoplast in its variable region (Frasch e*t al.*, 1981) and the amplification of the mini-exon gene repeated in tandem (Murthy *et al.*, 1992) or DNA fingerprints (Pires *et al.*, 2008) are also some of the molecular methods that make possible the differentiation of *T. cruzi* from *T. rangeli*. Some metabolic differences between both species have also been observed (Avila *et al.*, 1981; Nosei and Avila, 1985; Holguín *et al.*, 1987), as well as the resistance of epimastigotes to lysis by serum (Schottelius, 1982; Marinkelle *et al.*, 1986). Regarding infection and intracellular cycle in mammalian cells, this study showed that *T. rangeli* shows a behavior similar to *T. cruzi*. We observed that trypomastigotes of *T. rangeli* can infect cells, replicate intracellularly as amastigote-like forms and exit the cells as trypomastigotes. Although we have not studied the complete intracellular cycle displayed by *T. rangeli*, our observations suggest that this parasite can differentiate from trypomastigote to amastigote-like forms and viceversa. More experiments will be needed to elucidate the mechanisms implicated in these processes.

There is controversy about the course of infection of *T. rangeli* in vertebrate hosts. The first case of human infection was discovered in Brazil (de Lucena and Marques, 1954). In white male NMRI mice, a high (up to 7 times the original inoculum in the peak) and persistent parasitemia (for up to 2 weeks) have been observed (Urdaneta-Morales and Tejero, 1985). The parasites disappeared completely from the circulation after 20-25 days. Using a similar infection model, these and other authors have also observed numerous nests or intracellular pseudocysts containing amastigotes and trypomastigotes in the heart, liver, and spleen (Scorza *et al.*, 1986; Urdaneta-Morales and Tejero, 1985; Osorio *et al.*, 1995; Eger-Mangrich *et al.*, 2001). The above characteristics, as well as the location of the pseudocysts in tissues, are similar to those of *T. cruzi*. However, there are other works in which the histological examination could not detect any form of *T. rangeli* in several organs of mice (Tanoura *et al.*, 1999). Our *in vitro* results tend to support the findings of the first group of authors, because of the intracellular formation of amastigote nests in cultured cells.

It is interesting to observe that, among the trypanosomes that can infect humans, *T. cruzi* seems to be pathogenic for vertebrates but not for invertebrates (Chagas, 1909), whereas on the contrary, *T. rangeli* seems to be pathogenic for invertebrates but not for vertebrates (Tejera, 1920). Moreover, it has been demonstrated that both trypanosomatid species can coexists in the host (Araújo *et al.*, 2013) mixed infections and their consequences for the host's health and parasite transmission are still a poorly known phenomenon. The mini-exon multiplex PCR characterization detected the infection by *T. rangeli* and *T. cruzi* (TcI genotype and even this coinfection could be benefitial for them. New investigations are needed to clarify these aspects.

Considering the similar intracellular cycle of *T. rangeli* as compared with that of *T. cruzi*, as well as the non-pathogenic profile of this parasite, we next focused our study to the analysis of the effect of two new treatments against *T. rangeli* that could be tested against *T. cruzi* in the future. We used 17-AAG, a repurposed drug that were fistly used in the therapy against cancer (Menden *et al.*, 2018), and with good

results in the treatment of *Leishmania* infections (Petersen *et al.*, 2018; Santos *et al.*, 2014; Petersen *et al.*, 2012). Our data showed that 17-AAG was effective to reduce the infection of trypomastigotes on Vero cells and the replication of epimastigotes and amastigotes. Similar results were obtained with the *Artemisia* essential oil, although this extract could show toxic effects at large doses and after prolonged periods of treatment (Ribnicky *et al.*, 2004) a common medicinal and culinary herb with centuries of use. Artemisia dracunculus is a close relative of the French or cooking tarragon and contains components common to many herbs that are routinely consumed without reported adverse effects. Since safety information of Artemisia dracunculus and its extract is limited to historical use, TARRALIN was examined in a series of toxicological studies. Complete Ames analysis did not reveal any mutagenic activity either with or without metabolic activation. TARRALIN was tested in an acute limit test at 5000 mg/kg with no signs of toxicity noted. In a 14 day repeated dose oral toxicity study, rats appeared to well tolerate 1000 mg/kg/day. Subsequently, TARRALIN was tested in an oral subchronic 90-day toxicity study (rat. This is why we are interested in testing the effect of the specific component, artemisinin, which is effective in the treatment of malaria and is not toxic (Desrosiers and Weathers, 2016).

We consider that our work makes a contribution in the field of therapies against trypanosomatid parasites. Future experiments will be conducted with *T. cruzi* to confirm the possible antichagasic action of these treatments. Furthermore, the comparative study of these therapies on both species could be useful to elucidate the mechanisms for the different pathogenicity of them in vertebrate and invertebrate hosts.

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