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Short communication

Ex vivo trypanocidal activity of 1-(2-hydroxybenzylidene)thiosemicarbazide against *Trypanosoma equiperdum*

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ABSTRACT

Trypanosoma equiperdum is the causative agent of dourine, a venereal disease in horses and donkeys. This parasite has a widely distribution, is found in Africa, Asia, Southern and Eastern Europe, Russia, Mexico and Venezuela. The *T. equiperdum* is morphologically indistinguishable to other *Trypanozoon* species, however differs from other mammalian trypanosomes due to the fact that it is primarily a tissue parasite, generating cutaneous plaques, swelling of genitalia and neurological signs. The aim of this study was to evaluate the trypanocidal effectiveness of a set of derivatives of thiosemicarbazones on a *T. equiperdum* ex vivo culture. All compounds appeared to have trypanocidal activity, however one of them shown better solubility and a dose-dependent effect. The median inhibitory concentration (IC_{50}) was 1.2 μM. The selected compound exhibits a greater inhibitory activity than diminazene aceturate, a common drug for animal trypanosomosis treatment.

1. Introduction

Livestock trypanosomoses caused by parasites belonging to the *Trypanozoon* subgenus has a significant socio-economic impact reducing productivity. *Trypanosoma equiperdum*, a flagellate pathogenic protozoon, is the causative agent of dourine in horse and donkeys, and is morphologically indistinguishable from other *Trypanozoon* species (Verducci et al., 1989; Brun et al., 1998). Dourine is endemic in part of Africa, Asia and Russia. Currently outbreaks of dourine have been reported in the Middle East and Europe (OIE, 2009). Recently, a *T. equiperdum* strains isolate has been molecularly characterized for first time in Latin América, particularly in Venezuela (Sanchez et al., 2015). Dourine is a venereal disease, whose transmission occurs animal to animal during coitus and no arthropod vector is involved. Chemotherapy is probably the main form to control infections caused by trypanosome, however at the present time, there are not official approved drugs to treat dourine; instead are applied a reduce number of trypanocidal drugs used in *T. evansi* infections, as: Diminazene aceturate, suramin or quinapyramine, which are widely used in Africa, and imidocarb dipropionate, which is officially marketed in Brazil (Gressler et al., 2015; Silva et al., 1986; Brun et al., 1998).

However, the toxicity of these drugs and the emergence of parasites resistance due to drug inappropriate use, have led to research in order to find more effective compounds to treat the disease, diminishing toxic effects, and reducing cost. In this sense, several studies have shown promising results using compound derived from thiosemicarbazones on trypanosomatids, Pervez et al. (2014) reported IC_{50} of 1.78 μM to derivatives of thiosemicarbazone evaluated on *Leishmania sp.,* likewise, Caputto et al. (2012) and Magalhaes Moreira et al., (2014), reported IC_{50} values of 4.36 and 6.2 μM respectively to derivatives of thiosemicarbazone evaluated on *T. cruzi*, meanwhile, Soares et al., 2011 and Moreno-Rodríguez et al. (2014) reported LD_{50} values of 1.8 and 5.1 μM respectively of thiosemicarbazones evaluated on *T. cruzi*. It should be noted that there are no recent researches for the development of new biologically active compounds against *T. equiperdum* in order to develop new safer active molecules as chemotherapeutic alternative. In this context, the aim of this study is to evaluate the antiparasitic activity in vitro of a 1-(2-hydroxybenzylidene)thiosemicarbazone derivates on a Venezuelan *T. equiperdum* strain.

2. Materials and methods

2.1. Chemistry

Aldehyde and hydrazine used for synthesis of all derivatives are sold by Aldrich™, USA. A mixture of hydrazine (1 mmol), aldehyde

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(1 mmol), in a mixture water/ethanol (1:1) was placed in a reactor of Teflon inside a microwave (closed vessel) reactor in multivessel rotor (Synthos 3000, Anton Paar GmbH). Microwave irradiation of 300W was used, was irradiated for 5 min at 80 °C. Once ended the time of the reaction it was cooled and filtered, washing with cold water to finally dry (Restrepo et al., 2015). The compound purity was confirmed by high performance liquid chromatography in high performance liquid chromatography LC-20AB HPLC (Shimadzu) with an Allure® AK 5 µ 200 × 4.6 mm column. The melting points were taken on a fusiometer Electrothermal MEL-TEMP® and are not corrected.

2.2. in vivo expansion of trypanosomes

In this study one strain of *T. equiperdum* (TeAp-N/D1) (Sánchez et al., 2015) was used. Parasites were expanded into a Sprague Dawley rat. When parasitemia reached 1×10^6 parasites/ml, trypanosomes were harvested aseptically from rat by cardiac puncture and purified from blood under sterile conditions (laminar flow hood) by chromatography on DEAE cellulose as describe by Lanham and Godfrey (1970). All procedures were approved by the Institutional Committee of the Venezuelan Institute for Scientific Research.

2.3. Methods of culture

The culture medium was formulated according to a modified method of Kaminsky and Brun (1998). Purified parasites were grown ex vivo in a minimum essential medium (MEM) with Earle's salts supplemented with 1 mg/ml of glucose, 1% MEM nonessential amino acids (100 ×), 2.2 mg/ml of NaHCO3 and 10 mM HEPES. The medium was further supplemented with 2 mM sodium pyruvate and 0.1 mM hypoxanthine (dissolved in NaOH, 0.1 M). pH was adjusted to 7.3 with NaOH. The culture medium was then sterilized by filtration (at 0.22 µm) and store in a refrigerator. In the day of the testing, the medium was supplemented with 2-mercaptoethanol (0.2 mM) and 15% heat-inactivated horse serum. The parasites in culture medium was distributed (1×10^6 trypomastigotes/ml) in a 12-well culture plates, with a final volume of 1000 µl, and store at 37 °C with 5% CO2 until the growth inhibition assay.

2.4. Assays

In order to select whether of the 20 thiosemicarbazone analogues have activity against *T. equiperdum* in *ex-vivo* culture, a preliminary screening was done. Parasites in culture medium (1×10^5 trypomastigotes/ml) were cultured in presence of the 20 thiosemicarbazone at 30 µM for 72 h. The compounds were first solubilized in DMSO and then in culture medium for working stocks preparation. The growth inhibition ability was evaluated by counting the number of parasites/ml in a Neubauer chamber in presence of Trypan blue stain (0.4%). For the count only the motile parasites and not stained were taking into account, these two conditions constitute the evidences of the viability of the observed parasites. Parasite survival (%) at 72 h was determinate respect the control no treated. Once selected the compound which showed high growth inhibition of *T. equiperdum*, parasites in culture medium (1×10^6 trypomastigotes/ml) were cultured with different concentrations of the compound for 72 h. There was a negative control (DMSO, 0.1%) and a positive control (diminazene aceturate, 9 µM). Each concentration was tested in triplicate. The parasites density was determinate as describe above. Parasites growth curves were constructed, the percentage of inhibition (%I) was calculated according to the formule: % I = {1-(CDft/CDfc)} x 100, where CDft is cellular density of treated parasites at 72 h; CDfc is cellular density of untreated parasites (control) at 72 h (Yao et al., 2007). The median inhibitory concentration (IC_{50}) was estimated by linear regression analysis from the% I values and the decimal logarithm (log) of drug concentration, using GraphPad Prism 5.0 (Caputto et al., 2012).

2.5. Cytotoxicity assay

The evaluation of the toxic effects of the compound on mammalian cells was based on the reduction of MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Salomao et al., 2010). In brief, vero cells were maintained in MEM, 10% fetal bovine serum, 37 °C and 5% CO_2. Cells were seed at 1×10^6 cell/ml in a 96-well plate, after 24 h plate was washed and added the compound at different concentration in a wide range between 0,5–60 µM. The optical density was measured at 490 nm with a spectrophotometer. All experiments were performed in triplicate. Cell viability was expressed with respect to the absorbance of the control wells (untreated cells), which were considered as 100% of absorbance. The 50% cytotoxic concentration (CC50) was defined as the compound concentration required for the reduction of cell viability by 50%, which were calculated by regression analysis.

3. Results and discussion

Thiosemicarbazones have a wide range of biological activities due to their capacity to inhibits DNA replication and proteases activities, and its have been reported activities such as: antiviral (Garcia et al., 2003), antibacterial (Sau et al., 2003; Rebolledo et al., 2003; Kasuga et al., 2003), antimalarial (de Oliveira et al., 2008), trypanocidal (Du et al., 2002; Fujii et al., 2005; Moreno-Rodríguez et al., 2014). Thiosemicarbazones have been extensively studied on *T. cruzi*; however, it is unknown their activity on trypanosomes of veterinary interest as *T. equiperdum,* this reason make sense to evaluate these compounds. On this way, the antiparasitic activity of twenty hydrazone, thiosemicarbazones and hydrazine derivatives was determined against trypomastigotes ex vivo of *T. equiperdum.* A preliminary test performed to define the solubility of these compounds before addition to the parasites was carried out. The survival of the parasites in culture medium was followed for 48 h in order to evaluate the antiparasitic effect of the compounds. All the compounds were found to be active against *T. equiperdum* at 30 µM (Fig. 1). From this screening, the compounds 18 and 19 showed high inhibition of the culture growing, however compound 18 was select for further analysis due to it showed better inhibition of the parasites growth and solubilization in culture medium. In spite of being completely solubilized in culture medium, compound 19 showed formation of crystals upon observing the culture under the light microscope. These crystals remained even solubilizing the stock of the compound with DMSO.

The thiosemicarbazone 18 is analogues from 16 and 17, varying in the substitution in of the groups of the aromatic ring A (Fig. 2A). A pyridine replacement (16) in position 4 could be more favorable for trypanocidal activity of the compound than a substitution of hydroxyl group in position 4 and a metoxi group in position 3 (17). However, even when this compound are analogues, the inhibition of the parasites growth was not significative comparing to 18, where a substitution of a hydroxyl group in position 2 in the aromatic ring increased surprisingly the antiparasitic activity. The importance of the substitution of a hydroxyl group in position 2 in the aromatic ring for the activity against *T. equiperdum* is revealed when comparing compound 19 and 20 (Fig. 2B), were the hydrazone derivate has more inhibitory activity than the derivate 20. Comparing structurally the compound 18 and 19, both belong to the same family since display the same fragment showed in Fig. 2C. However, compound 19 have a carbonyl C═O, while compound 18 have a thyocarbonyle C = S, this suggest that the presence of thyocarbonile group could be the responsible of the antiparasitic activity.

The compound 18 exhibit a dose-dependent effect (Fig. 3A) with an IC_{50} of 1.26 µM; higher concentration led to parasites dead. This IC_{50} value is much lower than reported for other subgenus *Trypanozoon* members as *T. brucei,* IC_{50} = 9.52 µM on in vitro assays (Fatondji et al., 2013). Considering that Du et al., 2002, on ex vivo *Trypanosoma cruzi* assays had established that thiosemicarbazones are trypanocidal when