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In vitro evaluation of cytotoxicity and leishmanicidal activity of phthalimido-thiazole derivatives

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ABSTRACT

It is estimated that the worldwide prevalence of leishmaniasis is around 12 million individuals in 80 countries, with 400,000 new cases per year. In the search for new leishmanicidal agents, the hybrid phthalimido-thiazoles have been identified as an important scaffold for drug design and discovery. The present study thus reports the *in vitro* activity of a series of phthalimido-thiazole derivatives. Cytotoxicity against a strain of *L. infantum*, Vero cells, J774 macrophages and peritoneal macrophages was evaluated, as well as nitric oxide (NO) production. Activity against amastigote and promastigote forms of *L. infantum* and microscopic changes in the parasite and intracellular targets of the parasite were achieved. The results show that the compounds arising from hybridization of phthalimide and 1,3-thiazole exhibit promising leishmanicidal activity. Compounds **2j** and **2m** were the most potent of the series tested and the parasites treated with these compounds exhibited ultrastructural changes, such as cell body shrinkage, loss of cellular membrane integrity, vacuolization of cytoplasm, membrane profiles surrounding organelles and swelling of mitochondria. The data showed that these compounds reduced the survival of intracellular amastigotes and presented low toxicity for mammalian cells. The compounds produced increased NO production compared to untreated cells in non-infected macrophages. Treated promastigote forms showed an increase in the number of cells stained with propidium iodide. The compounds brought about significant changes in mitochondrial membrane potential. According to the present study, phthalimido-thiazole compounds exhibit leishmanicidal activity and could be used to develop novel antileishmaniasis drugs and explore potential molecular targets.

1. Introduction

Leishmania infantum is the etiological agent of Visceral Leishmaniasis in the Americas, Europe and parts of Africa. The disease affects individuals of all age-groups and can lead to death if not treated (da Silva et al., 2012).

The first treatment of choice for leishmaniasis has been based on pentavalent antimonials (Marín et al., 2015). Second choice drugs include Amphotericin B, Pentamidine and Miltefosine (Cavalli and Bolognesi, 2009; Espuelas et al., 2012; Santos et al., 2008; Srividya et al., 2012). Most treatments are inadequate owing to factors such as low therapeutic index leading to high toxicity and side effects, emergence of resistant parasites, high costs beyond the means of affected countries, and others. These disadvantages, together with the lack of an effective vaccine, indicate the need to investigate new drugs (Cavalli and Bolognesi, 2009; Espuelas et al., 2012).

The literature reports that the 1,3-thiazole nucleus is widely found in a variety of pharmacologically active substances and in some

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naturally occurring compounds, constitutes a versatile building-block for lead compound generation and allows easy access of various derivatives for subsequent optimization (Ayati et al., 2015). In recent years, many thiazole derivatives have been synthesized and subjected to various biological activities (Ayati et al., 2015). Phthalimide derivatives have also been shown to exhibit a broad range of pharmacological properties: analgesic (Alanazi et al., 2015), anticonvulsant (Obniska et al., 2011), antitubercular (Abdel-aziz et al., 2011; Berk and Akgün, 2012), hypolipidemic (Abdel-aziz et al., 2011), anxiolytic (Alanazi et al., 2015), anti-inflammatory (Alanazi et al., 2015; José et al., 2016), antimicrobial (Berk and Akgün, 2012; Elumalai and Ashraf, 2013; Singh et al., 2015) and antipsychotic (Williams et al., 2011).

The process of obtaining phthalimido-triazole derivatives identified as potent antiparasitic agents has been described by various studies. De Farias Santiago et al. (2014) reported on biomolecules with powerful schistosomicide activity, some of which brought about significant ultrastructural changes, including destruction of the integument in both male and female worms. De Moraes Gomes et al. (2016) described a number of compounds that exhibited potent anti-*T. cruzi* (trypomastigote form) properties, with various changes in parasite cells.

Compounds with a thiazole ring and phthalimide nucleus were chosen, in view of the promising results achieved with these and the fact that they are common pharmacophores present in diverse drug classes. Our ongoing research into new leishmanicidal agents found that phthalimido-thiazoles and their effect on *Leishmania* spp. have not yet been explored. The present study thus describes the effect of a previously described series of phthalimido-thiazole derivatives (De Moraes Gomes et al., 2016) on *L. infantum* promastigotes and intracellular amastigotes. It also evaluates the effect of compounds on mammalian cells and measures the production of NO by macrophages infected with *L. infantum*. The study also investigates the effect of these compounds on mitochondrial membrane potential and plasma membrane integrity in promastigotes.

2. Materials and methods

2.1. Compounds

The compounds were obtained and purified as described by De Moraes Gomes et al. (2016). All were chemically characterized using nuclear magnetic resonance, infrared spectroscopy, mass spectroscopy and elemental analysis and exhibited > 95% purity (De Moraes Gomes et al., 2016).

For the *in vitro* assays, 14 phthalimido-triazole derivatives (**2a-n**) and their precursor compound (**1**) were solubilized in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, USA). This solution was then dissolved in a suitable culture medium for each assay (RPMI for macrophage/amastigotes and Schneider's medium for promastigotes), in order to obtain different test concentrations for the experiments. The preparation of the solutions used was always carried out at the time of the experiments to ensure the stability of the compounds.

Parasites incubated only with culture medium or Amphotericin B (Sigma Aldrich, St. Louis, USA) were used as negative and positive controls respectively. Incubation was performed with the same DMSO concentration range (0.01 a 1%) present in the dilution of compounds.

2.2. Parasites

Leishmania infantum (strain MHOM/BR/BH 46) promastigotes were maintained at 26 °C in Schneider's medium (Sigma Aldrich, St. Louis, USA) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution (Sigma Aldrich, St. Louis, USA). Parasites at the exponential growth phase were used in all experiments. The intracellular amastigotes forms were obtained by inoculation of infective promastigotes in a culture of peritoneal macrophages and used in the biological activity assays of compounds as described below.

2.3. Biological activity on Leishmania infantum promastigotes

To perform the initial screening of the compounds with leishmanicidal activity, *L. infantum* promastigote forms were collected, counted and diluted in Schneider's medium supplemented with 10% FBS at a concentration of 1×10^6 parasites/mL. After dilution, the cells were incubated in the presence of test concentrations (6.25; 12.5; 25; 50 and 100 μg/mL) of phthalimido-thiazoles for 72 h. Cells incubated with Schneider's medium alone were used as a control. Cell culture growth was accompanied by daily counts in a Neubauer chamber (iNCYTO C-Chip DHC-N01, Cheonan-Si, South Korea). The IC_{50} (a concentration that inhibits 50% of parasite growth) value was determined after 72 h of culture by linear regression analysis. Each experiment was performed in biological duplicate and technical quadruplicate.

2.4. Mammalian cell culture

Vero cells, J774 macrophages and peritoneal macrophages were used for the cytotoxicity assay. These cells were cultured in a RPMI-1640 medium (Sigma Aldrich, St. Louis, USA) supplemented with 10% FBS and maintained at 37 °C in 5% CO_2. Peritoneal macrophages were obtained after injection of 5 mL of RPMI-1640 medium containing 20% FBS (Sigma Aldrich, St. Louis, USA) into the peritoneal cavity of female BALB/c mice.

2.5. Cytotoxicity assay

Cytotoxicity in mammalian cells was evaluated by way of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Dos Santos Aliança et al., 2014). The cells were counted in a Neubauer chamber, seeded in a 96-well plate at a concentration 6×10^4 cells/well and incubated in an atmosphere of 5% CO_2 at 37 °C. After 24 h, the medium was removed and cells were incubated at concentrations of 6.25, 12.5, 25, 50 and 100 μg/mL of the compounds for 48 h. The absorbance readings for soluble formazan crystals were performed using a Benchmark Plus ELISA reader (Bio-Rad, California, EUA) at a wavelength of 490 nm. The concentration capable of causing 50% loss of cell viability was determined by linear regression analysis. The Selectivity Index (SI) was calculated as the ratio between the CC_{50} and IC_{50} for each compound. Each experiment was performed in biological duplicate and technical quadruplicate.

2.6. In vitro biological activity on intracellular amastigote forms

To evaluate the effect of the compounds on intracellular amastigote forms, peritoneal macrophages, in RPMI-1640 medium (Sigma Aldrich, St. Louis, USA) containing 10% of FBS (Sigma Aldrich, St. Louis, USA), were distributed in culture plates with round coverslips at a concentration of 1×10^6 cells/mL and incubated for 3 h at 37 °C in an atmosphere of 5% CO_2. The macrophages were infected with *L. infantum* promastigotes for 3 h, at a multiplicity of infection of 10:1. After this period of infection, the cells were treated with 4.9, 9.8 and 19.6 μM of **2j** and 6.9, 13.9 and 27.8 μM of **2m**, and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. The cells were then washed with PBS, fixed with methanol and Giemsa stained. The percentage of infected macrophages was determined by counting 100 cells in triplicate. The IC_{50} (a concentration that inhibits 50% of parasite growth) was determined after 24 h of culture by linear regression analysis. The survival index was determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell. Amphotericin B was used as a control.