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In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis*

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ABSTRACT

Studies on the anti-proliferative activity *in vitro* of seven ternary nickel (II) complexes with a triazolopyrimidine derivative and different aliphatic or aromatic amines as auxiliary ligands against promastigote and amastigote forms of *Leishmania infantum* and *Leishmania braziliensis* have been carried out. These compounds are not toxic for the host cells and two of them are effective at lower concentrations than the reference drug used in the present study (Glucantime). In general, the *in vitro* growth rate of *Leishmania* spp. was reduced, its capacity to infect cells was negatively affected and the multiplication of the amastigotes decreased. Ultrastructural analysis and metabolism excretion studies were executed in order to propose a possible mechanism for the action of the assayed compounds. Our results show that the potential mechanism is at the level of organelles membranes, either by direct action on the microtubules or by their disorganization, leading to vacuolization, degradation and ultimately cell death.

1. Introduction

Leishmaniasis is one of the so-called neglected diseases, considered by WHO as one of the seven primary sicknesses that affect to all continents and usually located in tropical and subtropical regions, including 22 countries in the New World and 66 in the Old World [1]. The etiologic agents are different species of protozoa of the genus *Leishmania*, transmitted by dipteral insects that belong to the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World.

Pentavalent antimonials, including sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), have been used in the treatment of leishmaniasis for over 50 years. The molecular structure of antimonials, their metabolism and mechanism of action are still being investigated [2,3]. Biochemical studies over the past two decades have indicated a number of potential targets, for example glycolysis, particularly inhibition of ADP phosphorylation and beta-oxidation of fatty acid [4,5]. On the other hand, some more recent studies suggest that pentavalent antimony acts as a prodrug that is reduced within the organism into more active and toxic trivalent antimony. Among the potential molecular targets of Sb(III) evidence was obtained that trypanothione reductase [6] or zinc-finger protein [7] may be involved. Even though antimonials are still the fist-line drugs, they exhibit several limitations like severe side effects including anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy [8,9], the need of daily parenteral administration and drug resistance [10,11]. Thus, there is an urgent need for new and more efficient therapies to fight leishmaniasis and minimize its impact in society.

Previous studies have indicated that newly synthesised triazolopyrimidine metal complexes are promising chemotherapeutic drugs in the treatment of diseases caused by members of Trypanosomatidae [12–16]. Following this research line, we have started to explore the coordination possibilities and biological applications of another triazolopyrimidine family: 1,2,3-triazolo[4,5-*d*]pyrimidines which possess three contiguous N atoms in the imidazole ring and may also be regarded as purine mimetics, replacing the external C atom of the imidazole ring with an N atom (Scheme 1). Previously reported studies describe some of their potential biological applications [17–20].

In view of the above findings and, as a continuation of our effort to identify new candidates that are more potent, selective and less toxic antitrypanosomals, we report in this article the anti-proliferative *in vitro* activity of seven nickel complexes bearing the anionic form of 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidine-5,7-dione against promastigote and amastigote forms of *Leishmania infantum* and *Leishmania*

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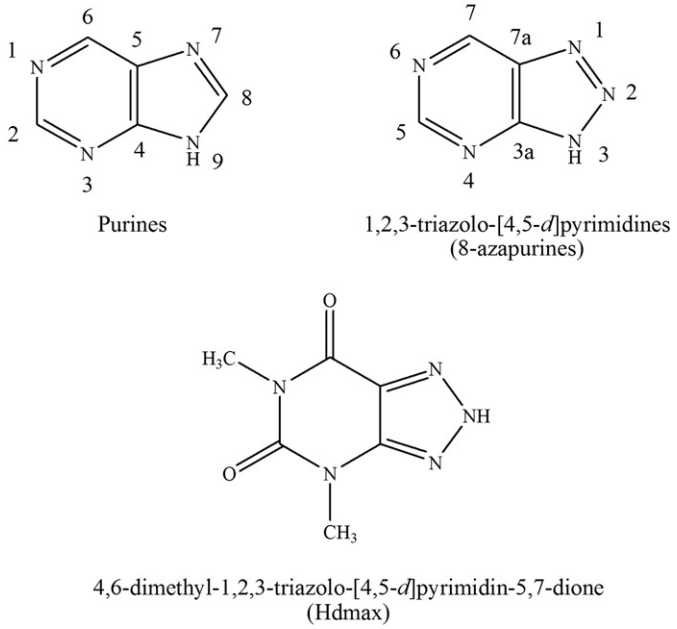

Scheme 1. Purine and 1,2,3-triazolo[4,5-*d*]pyrimidine skeletons displaying the biochemical and IUPAC numbering schemes respectively and molecular structure of Hdmax, the main ligand of this work.

braziliensis. Furthermore, unspecific mammal cytotoxicity was evaluated *in vitro*, and the non-toxic derivatives were thoroughly studied in order to elucidate their possible mechanisms of action. For this purpose, we have included ^{1}H-NMR studies about the nature and percentage of the excreted metabolites, in order to gather information about the inhibitory effect of our compounds over the glycolytic pathway which is the primary energy source for the parasite, and we have analyzed the effect of these compounds on the ultrastructure of *Leishmania* spp. by transmission electronic microscopy studies (TEM).

2. Materials and methods

2.1. Synthesis of the compounds

The assayed compounds are seven nickel(II) complexes bearing the anionic form of 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidin-5,7-dione (dmax) and different aliphatic amines [ethylenediamine (en), 1,3-diaminopropane (dap), bis(3-aminopropyl)-amine (bapa)] or aromatic amines [1,10-phenanthroline (phen), 2,2′-bipyridyl (bpy), 2,2′-dipyridylamine (dpyamin)] as auxiliary ligands. The synthesis of the ligand 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidine-5,7-dione (Hdmax) and its nickel salt (Nidmax) were carried out according to previously published procedures [21,22]. Other reagents were obtained from commercial sources and used without further purification.

Detailed synthesis and structural characterization of $[Ni(dmax)_2(en)_2]\cdot 3H_2O$ (Nidmax-en), $[Ni(dmax)_2(dap)_2](H_2O)_{1.5}$ (Nidmax-dap) and $[Ni(dmax)_2(bapa)(H_2O)]$ (Nidmax-bapa) [23] and $[Ni(dmax)_2(phen)_2]$ (Nidmax-phen), $[Ni(phen)_3](dmax)_2\cdot 7H_2O$ (Nidmax-3phen), $[Ni(dmax)_2(bpy)_2](H_2O)_{0.5}$ (Nidmax-bpy) and $[Ni(dmax)_2(dpyamin)_2]\cdot H_2O$ (Nidmax-dpyamin) have been recently reported by our group [20]. The three metal complexes including aliphatic amines in their structures were obtained by mixing three aqueous solutions containing $Ni(NO_3)_2\cdot 6H_2O$, the triazolopyrimidine ligand and the corresponding aliphatic amine in 1:2:2 molar ratio. On the other hand, those including aromatic amines were obtained by mixing two organic solutions containing $Ni(H_2O)_6(dmax)_2$ (Nidmax) and the corresponding aromatic amine in 1:2 (Nidmax-phen and Nidmax-bpy) or 1:3 (Nidmax-3phen and Nidmax-dpyamin) molar ratio.

2.2. Parasite strain, culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultured *in vitro* in medium trypanosomes liquid (MTL) plus 10% inactivated foetal bovine serum (IFBS) and were kept in an air atmosphere at 28 °C in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González et al. [24].

2.3. Extracellular forms. Promastigotes assay

The synthesized compounds were dissolved in the culture medium with a final DMSO concentration not exceeding 0.1%, this percentage of DMSO was previously assayed as nontoxic and without inhibitory effects on the parasite growth according to Magán et al. [13]. The dosages of the compounds used in this study were 100, 50, 25, 10 and l μM. The effect of each compound against promastigote forms, at different concentrations, was evaluated at 72 h using a Neubauer hemocytometric chamber. The leishmanicidal effect on promastigotes is expressed as IC_{50} values, i.e. the concentration required to give 50% inhibition, calculated by linear-regression analysis from the *Kc* values at the concentrations employed.

2.4. Cell culture and cytotoxicity tests

J774.2 macrophages (ECACC number 91051511) were originally obtained from a tumour in a female BALB/c rat in 1968. The cytotoxicity tests on macrophages were performed according to a previously published methodology [24]. After 72 h of treatment, the cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 μg/mL) was added and incubated for 10 min at 28 °C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubated under the same aforementioned conditions. Finally, the cells were recovered by centrifugation at 400*g* for 10 min and the precipitate was washed with PBS. Flow cytometric analyses were performed with a FACS Vantage™ flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison to the control culture. The IC_{50} was calculated using linear-regression analysis from the *Kc* values at the employed concentrations.

2.5. Intracellular forms: amastigote-macrophage assay

Two experimental procedures were designed for this study. In all cases, J774.2 macrophages were grown in minimum essential medium (MEM) plus glutamine (2 mM) and 20% IFBS in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.5.1. Experimental model no. 1 ([Mϕ + Leishmania spp.] + drug)

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 48 h. Afterwards, adherent macrophage cells were infected with promastigotes in stationary growth phase at a 10:1 ratio and kept for 24 h at 37 °C in 5% CO_2. Non-phagocytosed parasites were removed by washing, and afterwards the infected cultures were incubated with the compounds (1, 10, 25, 50 and 100 μM concentrations) and cultured for 72 h. The activity of the compounds, expressed as amastigote IC_{50} values, was determined from the percentage of the amastigote number reduction in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the average of four separate determinations [24].

2.5.2. Experimental model no. 2 (Mϕ + Leishmania spp. + drug)

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 48 h. Afterwards, the cells were infected *in vitro* with promastigote