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Research paper

Novel dialkylphosphorylhydrazones: Synthesis, leishmanicidal evaluation and theoretical investigation of the proposed mechanism of action

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ABSTRACT

As part of a program to develop new drugs for the treatment of neglected diseases, new dialkylphosphorylhydrazones were synthesized and evaluated against the trypanosomatid parasites *Leishmania braziliensis* and *Leishmania amazonensis*. The synthesis of these compounds proved satisfactory with yields ranging from moderate to good. The most active compounds against *L. braziliensis* presented IC_{50} values in the 10^{-2} μM range, similar to that of the reference drug pentamidine. Two compounds, **4m** and **4n**, showed a significant dose dependent decrease in the infection index of *L. amazonensis* infected macrophages and caused a complete healing of nodules and ulcers when tested *in vivo* against *L. amazonensis*-infected mice, but the control of parasite burden at the inoculation site was statistically significant only in the case of treatment with **4n**. A target fishing (reverse docking) approach using molecular docking with 15 enzymes of *L. braziliensis* indicated that the probable target of the active compounds was hexokinase, the first enzyme of the glycolytic pathway.

1. Introduction

Leishmaniasis is one of the world's most neglected diseases, with a major impact among the poorest individuals, mainly in developing countries. The number of leishmaniasis cases is increasing worldwide [1,2]. Leishmaniasis transmission is endemic in 98 countries and 3 territories on 5 continents. According to the World Health Organization, 350 million people are considered at risk of contracting this disease, and some 2 million new cases occur each year [1,3]. Each year approximately 58,000 cases of visceral leishmaniasis and 220,000 cutaneous cases are officially reported. However, it is thought that only approximately two-thirds of countries actually report incidence data, with the sparsest data from Africa [3].

One of the main problems in leishmaniasis treatment is the limited number of available drug options, along with the adverse effects they can cause, including death [4]. In addition, there are reports of treatment failures due to increased parasite resistance to the drugs of first choice, the antimonials [5,6]. Second-choice drugs, such as amphotericin B, pentamidine, paromomycin, and more recently, miltefosine, also have toxic effects, and their use requires hospital supervision [4,7]. Therefore, there is an urgent need for the development of safer and more effective drugs against this parasite.

Screening tests implemented with a series of new dialkylphosphorylhydrazones (DAPH) synthesized by our group were indicative of promising activity profiles against *Trypanosoma cruzi* and *Leishmania amazonensis* [8]. New compounds were then synthesized and added to the series. In the present work, we show the synthesis of the entire DAPH series and a detailed evaluation of the compounds' leishmanicidal activity against *L. amazonensis* and *Leishmania braziliensis* in comparison with known leishmanicidal agents. Although the information available from these *in vivo* tests

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is essential for the identification of new leishmanicidal agents, an understanding of the observed effects is a prerequisite for improving the selectivity and potency of the investigated compounds. In an attempt to identify probable targets for the active compounds prepared in the present work, we also implemented a strategy based on molecular docking of the compounds into a set of candidate target enzymes.

The presence of the $(R'O)_2P(O)NHR$ group in DAPH suggests that these molecules could act as inhibitors of enzymes that have, as substrates, molecules containing the $(R'O)_2P(O)OR$ group. There are a huge number of such enzymes, so the target identification procedure should be based on some criteria to reduce the number of possibilities to be explored. Some parasite enzymes have been shown to be inhibited by phosphorous-containing molecules, such as farnesyl pyrophosphate synthase (FPPS) [9,10] and hexokinase (HK) [11], an enzyme of glycolysis metabolic pathway in which glucose is converted into pyruvate and the free energy released is used to form the high-energy compounds ATP and NADH. In addition to FPPS and the enzymes of the glycolytic pathway, promising targets also include the enzymes of the pentose phosphate pathway (PPP), which serves to convert glucose-6-phosphate to ribose-5-phosphate. The PPP has been proposed to have crucial roles in the protection of trypanosomatids against oxidative stress, as well as in the production of nucleotide precursors [12]. Each of the enzymes of the PPP has been identified and specific activities measured for one of the Leishmania species, *Leishmania Mexicana* [13].

2. Results and discussion

2.1. Synthesis of the dialkylphosphorylhydrazones

The synthesis of the dialkylphosphites **(1)** and dialkylphosphorylhydrazines **(2)** were performed using the synthetic route previously used by our research group [14–18], according to the synthetic route shown in Scheme 1.

The synthesis of the new DAPH **(4a-o)** occurred through a condensation reaction, catalyzed in an acidic medium, between the respective dialkylphosphorylhydrazines **(2)** and different aldehydes (**3**) at room temperature, as shown in Scheme 2.

The infrared spectra of the DAPH synthesized show the characteristic absorption bands. The main absorption bands correspond to the stretching frequencies of the P=O, P–O–C and C=N bonds. In pentavalent phosphorus compounds containing a bond between a phosphorus atom and a nitrogen atom, the stretching frequency range of the P=O bond is generally from 1198 to 1274 cm^{-1} [19], the P–O–C bond absorbs in the 950-1018 cm^{-1} range, and the C=N bond has a stretching frequency in the 1580-1690 cm^{-1} range [20]. These frequencies were observed for all products, which confirm the expected reactions.

In the ^{1}H-NMR analyses, two characteristic signals confirm that the DAPH were obtained. These signals correspond to the iminic hydrogen –NHN=C<u>H</u>-Ar, with a chemical shift (δ) in the range of 7.88–8.26 ppm; and to the phosphoramidic hydrogen, P(O)N<u>H</u>, with a chemical shift in the 9.74–10.21 ppm range and showing a doublet signal with a coupling constant ranging between 27 and 31 Hz. The literature reports that this coupling occurs between 23 and 53 Hz [21]. Compounds **4i** and **4j** also presented an additional doublet in the region approximately 12.8 ppm, with a coupling constant of approximately 34 Hz, which is characteristic of an intramolecular hydrogen bond.

NOEDIF experiments were used to determine the configuration of the synthetized compounds. The results clearly show, according to ^{1}H-NMR spectroscopy, that all molecules have the *E* configuration, except for compounds **4i** and **4j** which were obtained as a diastereoisomeric mixture with *E*/*Z* ratio of 80:20 and 85:15, respectively.

In the ^{13}C NMR spectrum of DAPH, the signal that characterizes these compounds is related to the iminic carbon, (–NHN=C<u>H</u>–Ar), which has a chemical shift in the 136–145 ppm range and is observed as a doublet because it is coupled with the phosphorus atom, with a coupling constant in the 18–21 Hz range. The same feature can be observed with the alkoxide groups, $(RCH_xO)_2P(O)$-, where the methylene hydrogens, neighbors to the ester oxygen atoms, have chemical shifts in the range from 3.7 to 4.5 ppm.

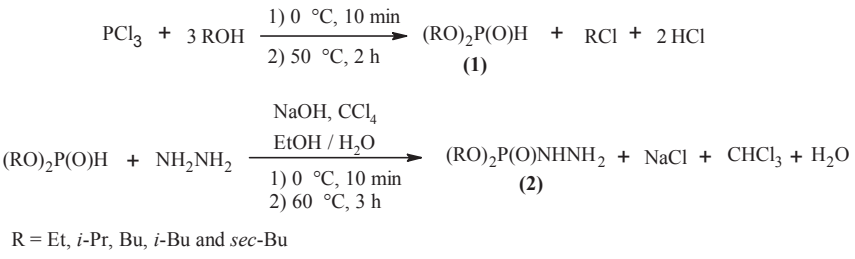
2.2. Biological evaluation

An initial screening was carried out to evaluate and compare the in vitro leishmanicidal profiles of the 18 DAPH and 2 standard drugs, miltefosine and pentamidine, against the promastigote forms of *L. braziliensis* and *L. amazonensis*. The maximum effects and the IC_{50} values (concentrations causing 50% inhibition of growth of the promastigotes) were used as the parameters for leishmanicidal activity (Table 1).

After 48 h of incubation, most of the compounds were significantly active against *L. braziliensis*. Among these, those that showed efficacy greater than 70% were as follows: **4b** (IC_{50}: 5.1 ± 0.5 μM), **4d** (IC_{50}: 0.06 ± 0.0 μM), **4f** (IC_{50}: 5.4 ± 0.2 μM), **4g** (IC_{50}: 0.4 ± 0.3 μM), **4h** (IC_{50}: 40.7 ± 3.5 μM), **4j** (IC_{50}: 0.06 ± 0.0 μM), **4m** (IC_{50}: 3.6 ± 0.3 μM), **4n** (IC_{50}: 5.2 ± 0.7 μM) and **4o** (IC_{50}: 0.03 ± 0.02 μM).

However, not all of these compounds were active against *L. amazonensis* as they were for *L. braziliensis*. The compounds **4b**, **4d**, **4g** and **4h** showed high specificity against the *L. braziliensis* species. The compounds were active against *L. amazonensis* with efficacies greater than 70% were as follows: **4f** (IC50: 53.3 ± 2.9 μM), **4j** (IC50: 6.2 ± 1.7 μM), **4m** (IC50: 26.3 ± 2.0 μM), **4o** (IC50: 26.0 ± 7.8 μM) and **4n** (IC50: 0.001 ± 0.1 μM). Compound **4n** was as effective as miltefosine (IC50: 3.4 ± 0.4 μM) and pentamidine (IC50: 1.8 ± 1.1 μM) and was approximately 2000 times more potent than these standard drugs.

An important criterion in the search for new substances with leishmanicidal activity is that they should not be toxic to mammalian cells, a requirement for further clinical development. Therefore, the cytotoxic potential of these substances on J774



Scheme 1. Synthetic route for dialkylphosphites **(1)** and dialkylphosphorylhydrazines **(2)**.