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The 2′,4′-dihydroxychalcone could be explored to develop new inhibitors against the glycerol-3-phosphate dehydrogenase from *Leishmania* species

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ABSTRACT

The enzyme glycerol-3-phosphate dehydrogenase (G3PDH) from *Leishmania* species is considered as an attractive target to design new antileishmanial drugs and a previous in silico study reported on the importance of chalcones to achieve its inhibition. Here, we report the identification of a synthetic chalcone in our in vitro assays with promastigote cells from *Leishmania amazonensis*, its biological activity in animal models, and docking followed by molecular dynamics simulation to investigate the molecular interactions and structural patterns that are crucial to achieve the inhibition complex between this compound and G3PDH. A molecular fragment of this natural product derivative can provide new inhibitors with increased potency and selectivity.

The Leishmaniasis is a complex of diseases caused by a protozoan of the genus *Leishmania*, which is spread over 98 countries around the world and affects about 12 million people, endangering other 350 million.[1] It is one of the most neglected diseases due to the absence of interest from pharmaceutical industry for developing new drugs against it.[2] This parasitic disease has been classified into three groups, visceral, cutaneous, and mucocutaneous Leishmaniasis[3], which have different clinical manifestations and degree of morbidity and mortality.[4] Transmission is caused by the flying vector Phlebotomines,[5] and two morphological forms are present during the life cycle of this parasite, promastigotes and amastigotes.[6] Promastigotes are injected into the mammalian host during the insect blood meal,[7] and phagocytised by macrophages. After, they transform into amastigotes form that survive and multiply within the macrophage phagolysosome.[8] The current therapy of Leishmaniasis relies upon few drugs[9,10] that show toxicity and/or inefficacy,[5,7] which evidence the high necessity of more efficacious drugs. In this sense, natural products can be a valuable alternative to provide huge diversity of chemical structures for biological screening campaigns against *Leishmania* species to develop new antileishmanial drugs.

We have identified a 2′,4′-dihydroxychalcone (DHC) inhibitor against promastigotes of *Leishmania amazonensis* during our cellular assays. Accordingly, chalcones have been reported that are promising inhibitors of glycerol-3-phosphate dehydrogenase,[11] a glycosome enzyme that plays a critical role in the parasite to keep its essential metabolism, and it is considered as an attractive target to develop new antileishmanial drugs. The simple structure of chalcones and their ease of preparation make them an attractive scaffold to attach functional groups for enhancing their biological activities.[12] Basically, these molecules are open-chain flavonoids (Fig. 1) in which the two aromatic rings join by a three-carbon α,β-unsaturated carbonyl system.[13]

Based on these good results we extend the investigation with DHC and the G3PDH enzyme by docking and dynamics simulation to reveal the molecular interactions that could drive the formation of this inhibition complex. A deep understanding of such

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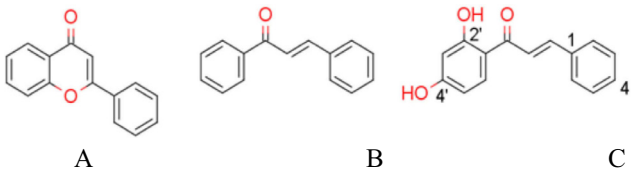

Figure 1. 2D basic structure of a flavonoid (A), a chalcone (B), and the leishmanicidal DHC identified in our screening assays with atoms numbered accordingly (C). Structures sketched with Marvin.[14]

interactions is a crucial step to address the synthesis of new derivatives with increased affinity for G3PDH.

The DHC (MW 240.26) was synthesized following the Claisen Schmidt condensation using the described protocol by Zeraik and co-workers,[15] with minor modifications. Briefly, in a 200 mL vial, 2′,4′-dihydroxyacetophenone (20 mmol) and potassium hydroxide (0.8 mol) were dissolved in ethanol (80 mL), and the mixture was stirred at 5 °C for 10 min followed by addition of benzaldehyde (20 mmol in 10 mL of ethanol). The reaction mixture was stirred at room temperature and monitored by TLC using hexane:ethyl acetate as the mobile phase (7:3). The reaction was quenched after 24 h by pouring the mixture into 500 mL of ice-cold water with stirring. A precipitate formed after quenching with cold water, which was then filtered. The crude product was purified by flash chromatography with hexane:ethyl acetate in increasing order of polarity. The compound was identified using ^{1}H and ^{13}C NMR spectral data obtained from a Varian DRX-500 spectrometer (11.7 T). Chemical shifts (δ) were expressed in ppm. Coupling constants (J) were expressed in Hz, and splitting patterns are described as follows: s = singlet; br s = broad singlet; d = doublet; t = triplet; m = multiplet; and dd = doublet of doublets. The white solid was obtained in 20% yield. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (d; J = 9.0 Hz, H6′), 7.93 (d; J = 15.5 Hz, Hβ), 7.86 (dd; J = 7.0, 2.0 Hz, H2 and H6), 7.75 (d; J = 15.5 Hz, Hα), 7.43 (m, H3, H5), 6.36 (dd; J = 9.0, 2.0, H5′), 6.22 (d; J = 2.0 Hz, H3′); ^{13}C NMR (125 MHz, DMSO-d_6): δ 190.7 (Cβ′), 168.0 (C4′), 166.1 (C2′), 143.1 (Cβ), 134.7 (C1), 132.9 (C6′), 130.5 (C4), 128.9 (C2, C3, C5, C6), 121.5 (Cα), 112.3 (C1′), 109.1 (C5′), 102.7 (C3′).

Adult male Swiss albino mice (20–35 g) were used in the experiments. They were housed in single-sex cages under a 12 h light/12 h dark cycle (lights on at 06:00) in a controlled room temperature (22 °C), and free access to food and water. Groups of two animals were used in each test group. The experiments were performed after the protocol was approved by the local Institutional Ethics Committee (protocol number 53/2012), which follows the Legislation for the protection of animals as described by the Directives from the European Commission.

L. amazonensis promastigotes (MPRO/BR/1972/M1841-LV-79) freshly isolated from mice were kept at 28 °C in liver infusion tryptose (LIT) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (Sigma–Aldrich®).

Flat-bottom plates (TPP®) were seeded with cultured promastigotes from *L. amazonensis* at the end of exponential growth phase (7 days) to achieve 8×10^6 parasites/mL per well. DHC was dissolved in DMSO, added to each well in dilution series ranging from 0.195 μg/mL to 100.0 μg/mL, and incubated at 28 °C for 72 h. The assays were carried out in triplicate. Leishmanicidal effect was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method modified accordingly.[16] Absorbance was read at 490 nm (Robonik®). The drug concentration corresponding to 50% of parasite growth inhibition was expressed as the inhibitory concentration (IC_{50}).

To evaluate the cytotoxicity, thioglycolate-stimulated mice were used to collect peritoneal macrophages. Murine peritoneal macrophages were seeded in 96 well flat-bottom plates (TPP®) at a density of 1×105 cells/well (100 μL/well) in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 25 mM HEPES and 2 mM L-glutamine, and incubated during 4 h at 37 °C in a 5% CO_2–air mixture. The medium was removed and a new one was added to the cells which were treated with different concentrations of DHC and pentamidine. Cells without drugs were used as negative control. After that, plates were incubated for 24 h at 37 °C in a 5% CO_2–air mixture. Subsequently, the MTT colorimetric assay was carried out as described above. Absorbance was read in a 96-well plate reader (Robonik®) at 540 nm. The drug concentration corresponding to 50% of cell growth inhibition was expressed as the cytotoxic concentration (CC_{50}).

Previous study reported that chalcones can exhibit strong interaction with G3PDH from *Leishmania*.[11] In that in silico investigation the crystal structures deposited in Protein Data Bank[17] with codes 1EVZ, 1M66, 1N1E and 1N1G were used, all of them with identical residue sequences. A rapid comparison of the aminoacid sequences of G3PDH from *Leishmania mexicana*, *Leishmania infantum*, *Leishmania major*, *Leishmania brasiliensis*, and *Leishmania donovani* through an alignment procedure in the UniProt[18] (www.uniprot.org) corroborated the high conservation degree of their binding site (data not shown). These evidences prompted us to choose 1N1E[19] (1.90 Å of resolution) as the based target structure for our computational studies. The crystal structure of human G3PDH (PDB ID 1X0X)[20] was chosen for structural superposition and comparison. The steps of reconstruction, stereochemical corrections and energy minimization of the G3PDH structure 1N1E were performed by the KoBaMIN server.[21] The 3D structure of DHC was sketched through Maestro[22] and docking was performed with AutoDock v4.2 and AutoDock Tools v1.5.6[23] following the default steps. Briefly, merged non-polar hydrogens were set to DHC structure and in the modeled 1N1E, flexibility of the DHC was considered by the number of torsions set automatically, and Gasteiger charges were applied for both. The map of the region for docking was assigned through Autogrid considering the binding site of the adenosine analogue in the 1N1E structure. The grid was set to 70 Å in all dimensions taking the coordinates for the center as 29, 25 and 22 for x, y and z, respectively. Rigid docking was carried out, using Lamarckian genetic algorithm as search parameter, keeping the other default options. After, we performed a structural comparison through a superposition of 1N1E with docked DHC and 1X0X to identify differences between the binding sites for useful subpockets to be explored for selectivity.

The best pose out of 10 conformations in terms of energy scoring was used as the initial complex to set up the system for molecular dynamics simulation with Desmond v3.8[24] with graphical user interface from Maestro.[22] The system was neutralized with adequate counter ions, NaCl in a concentration of 150 mM was added, and the complex of G3PDH-DHC was surrounded by single point charge (SPC) model of water molecules. The force field assigned for the system was OPLS-AA[25] in orthorhombic box shape with the distance of 10 Å from the complex to the box wall in all directions. The SHAKE algorithm was used for geometrical constrains of the molecules and bond lengths with hydrogens, and long-range electrostatic interactions treated by Smooth Particle Mesh Ewald while a cutoff radius of 9 Å was used to calculate short-range coulombic interactions. Minimization was carried out using steepest descent method until 1.0 kcal/mol/Å of convergence and equilibration following a multistep protocol. Basically, these were the steps: (i) system relaxation with Brownian dynamics using NVT ensemble at 10 K with position restraints for protein and ligand, except for hydrogens; (ii) simulation with NVT ensemble and Berendsen thermostat with temperature kept at 10 K; (iii) simulation with NPT ensemble and Berendsen for both thermostat and barostat in the same temperature with 1 atm of pressure, with