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Old drug, new indication: Olsalazine sodium reduced serum uric acid levels in mice via inhibiting xanthine oxidoreductase activity

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ABSTRACT

Hyperuricemia, a long-term purine metabolic disorder, is a well-known risk factor for gout, hypertension and diabetes. In maintaining normal whole-body purine levels, xanthine oxidase (XOD) is a key enzyme in the purine metabolic pathway, as it catalyzes the oxidation of hypoxanthine to xanthine and finally to uric acid. Here we used the protein-ligand docking software idock to virtually screen potential XOD inhibitors from 3167 approved small compounds/drugs. The inhibitory activities of the ten compounds with the highest scores were tested on XOD *in vitro*. Interestingly, all the ten compounds inhibited the activity of XOD at certain degrees. Particularly, the anti-ulcerative-colitis drug olsalazine sodium demonstrated a great inhibitory activity for XOD (IC_{50} = 3.4 mg/L). Enzymatic kinetic studies revealed that the drug was a hybrid-type inhibitor of xanthine oxidase. Furthermore, the drug strikingly decreased serum urate levels, serum/hepatic activities of XOD at a dose-dependent manner *in vivo*. Thus, we demonstrated a successful hunting process of compounds/drugs for hyperuricemia through virtual screening, supporting a potential usage of olsalazine sodium in the treatment of hyperuricemia.

1. Introduction

Gout results from hyperuricemia, a metabolic disorder where excessive concentrations of uric acid in the blood lead to deposition of monosodium urate crystals in and around the joints and other connective tissues.[1] Hyperuricemia is defined as a serum urate level of >6.5 or 7.0 mg/dl in men and >6.0 mg/dl in women,[2,3] and it is a common disease caused by long-term purine metabolic disorders. Because hyperuricemia is the *sine qua non* of gout, strategies to lower serum urate (sUA) levels have been a mainstay of chronic gout therapy for many years.

Xanthine oxidase (XOD), a successful and safe drug target for lowering sUA levels clinically, plays an important role in the catabolism of purine. Gout patients are primarily treated with XOD inhibitors, for example allopurinol. However, allopurinol has side effects including skin rash, diarrhea, abdominal pain fever and leucopenia.[4] Currently another XOD inhibitor, Febuxostat, has been administered clinically to achieve rapid and substantial reductions of sUA levels. However, 10% of gout patients suffer liver function abnormalities, nausea, arthralgias, and rash by febuxostat.[5]

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Therefore, searching for new compounds with stronger XOD inhibitory activities and less side effects is necessary for future development of new drugs.

With the development of biology and technology, virtual screening allows us to quickly narrow down potential compounds specifically targeting to the proteins, including XOD. In particular, the docking-based virtual screening allows us to filter compound candidates with desired binding strength and elaborate putative physiochemical interactions.[6] Promisingly, the powerful synergy of drug repositioning coalesced with ensemble docking by the idock software[7] has been demonstrated in two previous publications,[8,9] where, by targeting cyclin-dependent kinase 2 (CDK2), two FDA-approved drugs (fluspirilene and adapalene) were rediscovered as anticancer agents *in vitro* and *in vivo* for the treatment of hepatocellular and colorectal carcinoma, respectively. More recently, the same in silico docking and repositioning approach was employed to search for inhibitors of fibroblast growth factor receptor 3 (FGFR3) and managed to identify a potential new indication of the acaricide drug fluazuron in treating bladder carcinoma.[10] Encouraged by these successful case studies, here we employed the same overall computational workflow but advanced it in some key aspects, attempting to find potential inhibitors of XOD out of approved drugs worldwide for the treatment of gout. *In vitro* and *in vivo* assays were then conducted to validate the result.

2. Materials and methods

2.1. Materials

Xanthine oxidase (EC 1.1.3.22) from bovine milk, xanthine, sodium pyrophosphate, potassium oxonate, febuxostat and allopurinol were purchased from Sigma and Aldrich Chemical Co. (St. Louis, MO, USA). The biochemical kits used in the experiments were products of Nanjing Jiancheng Bioengineering Institute. All other reagents were commercially available and of analytical grade.

2.2. Animals and ethical considerations

Male mice (♂) weighing 18–22 g were obtained from Beijing HFK Bioscience CO., LTD (Certificate No. SCXK 2014-0004). The animals were housed on a constant cycle of 12-h light/dark in a temperature- and humidity-controlled room. Animals were given ad libitum access to food and water throughout the study.

All procedures were carried out in accordance with the Institute Ethical Committee for Experimental Use of Animals. This study was approved by the laboratory animal ethics committee of Kunming Medical University.

2.3. In silico molecular docking-based virtual screening

Popular proteins often have more than one structure available from the PDB.[6] Some guidelines on how to select appropriate structures for ensemble docking were recently proposed,[11] with one main conclusion being that holo structures with large ligands bound, even though they might not be of the highest resolution determined by X-ray crystallography, should be preferred over holo structures bearing small ligands or apo structures. Such selection criterion also has the benefit of conveniently defining a conformational sampling space for the screening compounds to be docked, as it can be easily calculated from the coordinates of the bound ligand. Following this guideline, we selected five X-ray crystallographic structures of XOD (PDB codes: 1N5X, 1V97, 1VDV, 3AM9, 3NVY) for ensemble docking, as these are holo structures of XOD in complex with a ligand of moderate molecular size bound at the ATP binding site. Notably, the ligand of the 1N5X entry is febuxostat.

Next, the XOD structures and the co-crystallized ligands were manually extracted from their corresponding complexes with water molecules deleted, and then converted from PDB format to PDBQT format with AutoDockTools[12] for use by the docking software. The cubic search space was located at the geometrical center of the bound ligand, with the length, width and height set to be 30% greater than that of the bound ligand, according to the observation that the geometry of the binding site is typically proportional to that of the bound ligand. The search space was further expanded by 4 Å in all three dimensions to spare sufficient room for the screening compounds to translate and rotate within.

The structures of approved drugs worldwide were obtained from three catalogs of the ZINC database,[13] which are DrugBank-approved,[14] FDA-approved drugs (via DSSTOX), and the NCGC Pharmaceutical Collection (NPC).[15] These compounds, after deduplication, constituted a non-redundant set of 3167 drugs that have been approved for clinical use by US (FDA), UK (NHS), EU (EMA), Japanese (NHI), and Canadian (HC) authorities. Similarly, these compounds in Mol2 format were also converted to PDBQT format for use by the docking software.

The free and open source docking software idock[7] v2.2.1 was invoked to predict the binding conformations as well as the binding affinities of the 3167 compounds upon docking against the 5 XOD structures. Program settings were tweaked to make the conformational searching procedure more exhaustive than the default settings in order to reduce the probability of missing the optimal binding conformation. In detail, for each protein structure, grid maps of free energy with a fine granularity of 0.08 Å were constructed in parallel; and for each compound, 256 Monte Carlo conformational optimization tasks were distributed to multiple CPU cores for parallel execution.

After docking, up to nine putative conformations were outputted for each input compound. Only the docked conformation with the best idock score was considered. The compounds were sorted in the ascending order of their predicted binding free energy averaged across the 5 XOD structures. Meanwhile, the more accurate scoring function RF-Score v3[16] was used to re-score all the compounds and thus provided an alternative but more reliable estimation of intermolecular binding strength, given the assumption that the compounds had been correctly docked. Therefore, the best-scoring compounds would be those with both a low idock score (in terms of binding free energy) and a high RF score (in terms of binding affinity). The best-scoring compounds were visually inspected using our in-house web-based visualizer iview[17] in the context of XOD using the crystal structure of the highest resolution, i.e. PDB code 1N5X in this case (which happened to feature febuxostat as its bound ligand), so as to derive putative physiochemical intermolecular interactions. Finally, commercially available compounds were purchased and subsequently validated *in vitro*.

2.4. In vitro inhibitory activity of the compounds on xanthine oxidase

The inhibitory activity of the compounds on xanthine oxidase *in vitro* was assayed spectrophotometrically by monitoring uric acid formation from xanthine, using a previously described method.[16–20] The reaction mixture contained 50 mM sodium pyrophosphate buffer (pH 7.4) and 5.0 U/L xanthine oxidase, with or without the test compounds. Allopurinol or febuxostat was used as a positive control. After preincubation at 25 °C for 15 min, the formation of uric acid in the reaction mixture was initiated by addition of 120 μM xanthine, and the increase of absorption of uric acid at 295 nm was monitored. In the enzyme kinetics tests, the concentrations of xanthine used were 10, 20, 30, 40, 50, 60, 70,

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