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In silico identification and experimental validation of diuresis compounds from *Euphorbia lathyris* for potential UT-B inhibitors

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ABSTRACT

Euphorbia lathyris (*E. lathyris*), a traditional Chinese medicine, rich in diterpenoids, has been widely used for the treatment of hydropsy, especially the ascites in China, with its mechanism is still unclear. For exploring possible diuresis mechanism of *E. lathyris*, in this study, a potential diuresis related target, urea transporter B (UT-B), and its potential ligands were discovered by using bioinformatics method of "Inverse Docking". Then, homology modeling, molecular docking and ligand-based pharmacophore methods were applied to research the possible mechanism of diuresis. Bioinformatics analysis showed that most of diterpenoids in *E. lathyris* were optimal ligands for the UT-B protein and these predicted compounds also exhibited stable affinity to UT-B binding site, a hydrophobic region in urea passage. The HPLC-TOF-MS method was used to characterize the main diterpenoids content of *E. lathyris* extracts and three out of eight proposed diterpenoids were detected and assigned. In vitro UT-B inhibition experiment of erythrocyte lysis assay was then applied to confirm the diuretic effects of the diterpenoids concentrated extracts of *E. lathyris*. The half erythrocyte lysis rate of 23.89 μg/ml of diterpenoids concentrated extracts showed moderate UT-B inhibition efficacy. All these performances might also raise an alternative approach for the design of new diuretic drugs.

1. Introduction

Ascites is an abnormal syndrome of fluid accumulation in the abdomen, survival rate of human who caught this disease is proved to be poor [1]. During ascites, the risk of acquiring additional complications for patients increases significantly. Accordingly, considerable research efforts have been made by numerous groups to unearth drugs to combat its severe threat. Currently, therapies on this disease can be separated into following methods: (1). Using of diuretic agent such as Aldactone [2], Furosemide [3], Hydrochlorothiazide [4], Vasopressin receptor inhibitors (tolvaptan) [5]; (2). Abdominal paracentesis [6]; (3). Peritoneovenous shunting [7]. Among all currently available methods, drug therapy is frequently used because of its better effect and lower pain.

E. lathyris is a glabrous herb belonging to the *Euphorbia* genus, widely distributed in Europe, Central Asia and most regions in China. Its seeds, commonly known as "Qian-Jin-Zi" in China, have long been recognized as folk medicine for the treatment of hydropsy, ascites, schistosomiasis and snakebites [8]. Chemical research on this plant results in the discovery of a variety of constituents including diterpenoids, coumarins, flavonoids, fatty acids, and so on. Other *Euphorbia* plants (*i.e. E. kansui* [9] and *E. pekinensis* [10]) were also well known for the strong effect of relieving edema and used as diuresis drugs in clinic. Major hydragogue compounds in *E. lathyris* referred to diterpenoid derivatives [11]. As there are still no reports revealing the diuresis mechanism of *E. lathyris*, a possible mechanism elucidation experiment is of significant.

Urea plays an important role in the urine concentrating mechanism, and the process is mainly mediated by the urea transporters (UTs), a family of channel proteins which are expressed in the renal epithelial cells and some vascular endothelial cells. The UTs family

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consists of two members: UT-A and UT-B, are two kinds of channel proteins which mediate passive diffusion of urea down the concentration gradient and involve in facilitating urinary concentration in kidney [12], promoting the development of early spermatocyte [13], and regulating blood volume [14]. UT-A, Encoded by SLC14A2 gene, is expressed on kidney tubules [15]. UT-A subunits, including UT-A1, UT-A2, UT-A3, UT-A4, UT-A5 and UT-A6, are mainly distributed on inner medullary collecting duct (IMCD), medullary loop descending limb and cytoplasm of IMCD in mammals' kidney [16–18]. The UT-B protein expressed on kidney microvessels as well as erythrocytes surface, testis, brain, heart, and urinary bladder, which encoded by SLC14A1 gene, is mainly involved in intrarenal urea recycling and water reabsorption. Two UT-B substructures of UT-B1 and UT-B2 were identified till now [19]. UTs expressed in the inner medullary collecting ducts allow the rapid equilibration of urea between the lumen and the interstitium, preventing water loss driven by the high concentration of urea present in the urine [20]. UT-B knockout mice exhibited decreased urinary concentrating ability suggesting a theoretical likely diuresis target for the development of UT-B inhibitors [21]. Current available UT-B inhibitors included nonselective membrane intercalating agent phloretin and urea analogues [22]. Since their low efficacy (millimolar degree of IC_{50}), new potent and high specificity UT-B inhibiting agents are required.

Thanks to the development of bioinformation method, fast screen drug target and precisely predict the drug therapy mechanisms in right ways were made realistic. Combined with truly validation experiments, the reliability of prediction results could be guaranteed. For clarifying potential diuresis target functioned effect by *E. lathyris* compounds, in this study, "Inverse Docking", a program of automatically mapping compounds with protein binding pocket and sorted on fit value, was used. The UT-B protein, related to diuretic effects and belonged to top 5 high fitness values, was picked out. Since there were no available X-ray structures of human UT-B, the homology modeling was used to construct 3D structure of human UT-B. Molecular docking and ligand-based pharmacophore were then applied to simulate possible interaction mechanisms of human UT-B active sites with *E. lathyris* compounds. The erythrocyte lysis assay was finally carried out to confirm the potential UT-B inhibition compounds of *E. lathyris*.

2. Materials and methods

2.1. Materials

The seeds of *E. lathyris* were purchased from Anguo, Hebei Province, People's Republic of China, in April 2010. The plant material was identified by Professor Jimin Xu (National Institutes for Food and Drug Control). A voucher specimen (No. 20100420) has been deposited at the Research Department of Natural Medicine, Shenyang Pharmaceutical University. Phloretin (UT-B inhibitor) was purchased from Aladdin® (Shanghai, China). Acetamide and glucose were purchased from Kermel® (Tianjin, China). Rat whole blood was collected into heparinized tubes, stored at 4 °C, and used within 24 h.

2.2. Preparation for diterpenoid concentrated extracts of E. lathyris

Seeds of *E. lathyris* were refluxed with 95% ethanol for three times (each time for 2 h), Solvents were removed in a vacuum to yield the crude extract, then, the extract was dispersed in distilled water and partitioned with petroleum ether (boring point 60–90 °C) to furnish the P.E. part. Then, the P.E. part extracts were kept in sealed vials and stored at 4 °C for further use.

2.3. Mechanism of the Inverse Docking protocol

The "Inverse Docking" is an assistant tool designed by our research group to simplify the targets screening process. In this protocol, the PDB database recorded aggregation of receptor proteins as well as the docking method of LibDock in Discovery Studio (DS) 3.5 is needed. When it is ready to predict the potential target for ligands, offering the "mol2" format of all ligands is simply needed. When processing, the ligands binding sites of all protein crystal structures are recognized immediately by this tool. Then, all proteins binding sites are prepared to match with the ligands. Those proteins with small volume of binding pocket that are unable to accommodate the ligands are abolished. Then, genetic algorithm based LibDock was called to evaluate the fitness between ligands and receptors. The fitness was finally determined via the LibDock score and a higher score indicates a better matching.

The advantage of the "Inverse Docking" protocol is easy operation and time saving. Moreover, multi-compounds can be packaged into one "mol2" format document and submitted to predict their appropriate target in parallel.

2.4. Homology modeling and global energy minimization

The amino acid sequence of Human UT-B was searched and downloaded from NCBI database (accession code: CAB60834.1) with 389 residues imbedded. By submission of FASTA sequence to Swiss-Model server and using BLAST tool, 3D structures of both *Desulfovibrio vulgaris* urea transporter (PDB code: 3M6E) and lately disclosed *Bos Taurus* urea transporter 1 (PDB code: 4EZC) were confirmed and could be used as modeling templates to construct human UT-B conformation. All the next modeling procedures including sequence alignment, homology modeling were performed by utilizing Discovery Studio (DS) 3.5 (Accelrys) package [23].

Global energy minimization of modeled human UT-B protein was performed under CHARMm force field. Initially, 3D model of UT-B was solvated with SPC water molecules with 0.145 mol/L of counter ions of sodium and chloride added within an orthorhombic solvent box. In case of poor contacts, 2000 steps of steepest descent and 2000 steps of conjugate gradient energy minimization were selected with RMS gradient of 0.05 and 0.001 set up. A method of restraint of D/F of protein and water molecules and relief of the restraint gradually was applied. Firstly, protein was constrained to optimize solvent molecules. Secondly, heavy atoms of protein were still constrained and hydrogen atoms were all released to be optimized. Lastly, all atoms in the whole system were all relaxed and optimized.

2.5. Molecular dynamics simulations

The energy minimized 3D structure of human UT-B protein was further criticized the stability by using 10 ns molecular dynamics (MDs) simulations. MDs simulation was performed with Gromacs version 4.5 under GROMOS96 43a1 force field. Since energy minimization in MDs simulation was of important, the former optimization methods used in "*global energy minimization*" was repeated. During MDs simulation studies, equilibration and production phases were also included. To equilibrate the system, the solute was subjected to the position-restrained dynamics simulation of NPT at 300 K for 300 ps. Particle-Mesh Ewald (PME) methods was used in dynamics production to calculate the electrostatic interactions. Finally, the full system was subjected to MDs production run at 300 K temperature and 1 bar constant pressure for 5000 ps. For analysis, the atom coordinates were recorded at every 0.5 ps during the MDs simulation.

2.6. Molecular docking

The DS CDOCKER protocol [24] was employed as docking approach to conduct semi-flexible docking. This protocol is powerful in studying the precise interactions between ligands and proteins based on the grid algorithm, simulated annealing optimization and CHARMM force field. Preliminary modeled and refined 3D human