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Mechanisms underlying the xanthine oxidase inhibitory effects of dietary flavonoids galangin and pinobanksin

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ABSTRACT

Xanthine oxidase (XOD) inhibitory activities of five dietary flavonoids pinobanksin, galangin, pinocembrin, pinocembrin-7-*O*-*β*-D-glucopyranoside and glabranin were evaluated. Enzyme kinetic studies and molecular docking simulation were conducted to investigate the mechanisms underlying the inhibitory activities. The results showed that these flavonoids exhibited excellent inhibitory activities (which were ranked in the order of pinobanksin > galangin > pinocembrin > pinocembrin-7-*O*-*β*-D-glucopyranoside > glabranin). Competitive inhibition and a mixed-type of competitive–noncompetitive inhibition were observed. The mode of inhibition was dependent on the type and concentration of the substrate and inhibitor. Fluorescence quenching data suggested that these flavonoids could interact with XOD at more than one binding site. The docking simulation revealed that galangin and pinobanksin could enter into the active site of XOD and form hydrogen bonding with amino acid residues (such as Ser-876, Asn-768, Glu-1261 and Thr-1010) and sandwiching aromatic interactions (π–π interactions) around the active site of XOD.

1. Introduction

Xanthine oxidase (XOD, EC 1.17.3.2) catalyses the oxidation of xanthine or hypoxanthine to uric acid, and usually induces the generation of superoxide radicals during the process (Chu, Chen, Wu, & Hsieh, 2014). Excess accumulation of uric acid in serum will lead to hyperuricaemia, which is a risk factor for gout, cardiovascular disease and other metabolic disorders (Wang et al., 2014). Thus, effective XOD inhibitors against XOD might be the perfect healthy functional component to prevent these metabolic disorders.

As an effective XOD inhibitor, allopurinol has been clinically used for the treatment of hyperuricaemia and gout (Wang, Zhang, Pan, & Gong, 2015; Wu et al., 2010). However, allopurinol can cause some side effects, including hepatitis, nephropathy, hypersensitivity and skin rash. Thus, it is important to search for the alternative XOD inhibitors with minimal side effects (Lin,

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Chemical compounds: Pinocembrin (PubChem CID: 68071); Glabranin (PubChem CID: 124049); Pinobanksin (PubChem CID: 73202); Galangin (PubChem CID: 5281616); Xanthine (PubChem CID: 1188); Hypoxanthine (PubChem CID: 790); Allopurinol (PubChem CID: 2094).

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Xie, Wu, Yang, & Wang, 2014). Dietary flavonoids have attracted growing attention due to their beneficial properties, including free radicals-scavenging capacity and anti-inflammatory activity (Marzocchella et al., 2011). Recently, flavonoids including quercetin, morin, myricetin, kaempferol and puerarin have been proposed as the effective XOD inhibitors (Mo et al., 2007; Moini, Guo, & Packer, 2000). Flavonoids are characterized by having two phenyl rings (A and B), a heterocyclic ring (ring C) and with hydroxyl groups (C-5 and C-7) on the A ring, and possess strong XOD inhibitory activities (Cos et al., 1998; Yan, Zhang, Hu, & Ma, 2013). Moreover, the hydroxyl groups on the B ring of flavonoids were found to contribute to the XOD inhibitory activity (Cos et al., 1998). Kaempferol, which could enter the hydrophobic cavity of XOD and interact with amino acid residues, was proven to be a competitive inhibitor of XOD (Wang et al., 2014). Epicatechin-(4β, 8)-epicatechin gallate and quercetin-3′-glucuronide were found as the mixed-type competitive inhibitors of XOD (Chu et al., 2014).

Propolis, a natural resinous mixture produced by honeybees, has been traditionally used in folk medicine to prevent hyperuricaemia and gout. Although polyphenols are believed to be the major active components in propolis (Yoshizumi, Nishioka, & Tsuji, 2005), there is little information available about its mechanisms underlying the XOD inhibitory effects of the flavonoids in propolis. Pinocembrin, pinobanksin and galangin are the typical flavonoids presented in propolis (Chang, Yang, Wen, & Chern, 2002; Rasul et al., 2013). Thus, it is of high interest to perform the corresponding experiments on this aspect.

In this study, the relationships between the chemical structures of these three flavonoids (including pinocembrin, pinobanksin and galangin) and their XOD inhibitory activities, and the mechanisms underlying their XOD inhibitory effects were investigated. In addition, the XOD inhibitory effects of pinocembrin-7-*O*-*β*-D-glucopyranoside and glabranin, due to their structural similarities and differences compared to pinocembrin, pinobanksin and galangin, were also compared so as to elucidate the structure–activity relationship (as shown in Table 1). All these flavonoids are commonly found in fruits and vegetables (Kuo et al., 2010; Poncsak, Kocaefe, Simard, & Pichette, 2009; Xu, Xie, Hao, Jiang, & Wei, 2011). Hence, the research approaches and findings presented in this paper would be useful for screening natural resources rich in dietary flavonoids.

2. Materials and methods

2.1. Chemicals and materials

Xanthine oxidase (XOD, EC 1.17.3.2, 7.2 units mL^{-1}, from bovine milk), xanthine, hypoxanthine, allopurinol (>98%), pinocembrin, pinobanksin, galangin, glabranin and pinocembrin-7-*O*-*β*-D-glucopyranoside (>95%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals including reagents like dimethyl sulfoxide (DMSO) were of analytical grade.

2.2. Determination of in vitro XOD inhibitory activity

The XOD inhibitory activity was determined according to the previously published method with some modifications (de Araújo et al., 2013). Briefly, 50 μL of flavonoid solution at a specific concentration was mixed with 50 μL of XOD (0.1 U mL^{-1}) and 150 μL of xanthine or hypoxanthine solution (0.1 mM) with gentle stirring. The reaction was performed at 37 °C for 5 min. The absorbencies at 292 nm were recorded every 10 seconds from 0 to 5 min using a spectral scan multimode plate reader (Thermo Fisher Scientific, Thermo Electron Co., Waltham, MA, USA). Sodium phosphate buffer (200 mM, pH 7.4) was used as a negative control and allopurinol was set up as a positive control. All the determinations were carried out in triplicate.

$$\text{Inhibitory ratio}(\%) = (S_0 - S_A)/S_0 \times 100\% \quad (1)$$

S_0 is the slope of negative control reaction kinetics equation. S_A is the slope of tested sample reaction kinetics equation.

2.3. Lineweaver–Burk and Dixon plots

To determine the XOD inhibitory modes of pinocembrin, pinobanksin and galangin, Lineweaver–Burk (L-B) and Dixon plots were generated based on the data of the reaction rates versus the corresponding substrate concentrations. The absorbencies at 292 nm (according to Section 2.2) were recorded at an interval of 10 s. The slope of reaction kinetics equation was defined as the initial reaction velocity.

2.4. Fluorescence quenching assay

A flavonoid solution (50 μL) was mixed with XOD solution (50 μL, 0.1 U mL^{-1}) and sodium phosphate buffer (150 μL, 200 mM, pH 7.4) (details provided in Section 2.2). The reaction was performed at 37 °C for 30 min before fluorescence emission. The spectra were measured at 37 °C with the excitation wavelength at 280 nm and the emission wavelength ranging from 300 to 500 nm (Wang et al., 2015), using a spectral scan multimode plate reader (Thermo Fisher Scientific, Thermo Electron Co., Waltham, MA, USA). The obtained fluorescence data were corrected using the following equation (Wang et al., 2015):

$$F_{cor} = F_m \cdot e^{(A_{ex}+A_{em})/2} \quad (2)$$

F_{cor} and F_m are the corrected and measured fluorescence, respectively. A_{ex} and A_{em} represent the absorbencies of the test sample at the excitation and emission wavelengths, respectively.

2.5. Molecular simulation

The X-ray crystal structure of XOD (PDB ID: 3ETR) from bovine milk was obtained from RCSB Protein Data Bank (http://www.rcsb.org/pdb) (Nongonierma, Mooney, Shields, & FitzGerald, 2013). The water molecules in XOD were removed, while polar hydrogen atoms and Gasteiger charges were added to the macromolecule file using MGLTools before the docking program was run (Wang et al., 2015). The 3D structures of flavonoids were generated using Chem3D Ultra 8.0. The AutoDock VINA program was chosen for molecular docking and predicting the binding mode of the flavonoid–XOD complex. The model with the highest docking fitness score was chosen as the most favourable binding mode. During docking, the dimension of docking center