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Molecular modeling of flavonoids that inhibits xanthine oxidase

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Abstract

The inhibition of xanthine oxidase activity by various flavonoids was assessed. All of the tested flavonoids were competitive inhibitors, and from the kinetic analysis suggested that flavonoids bind to the reactive site. To further understand the stereochemistry between these flavonoids and xanthine oxidase, structure-based molecular modeling was performed. Apigenin was the most potent inhibitor which showed the most favorable interaction in the reactive site. The bicyclic benzopyranone ring of apigenin stacked with phenyl of Phe 914, and the phenolic group stretched to the space surrounding with several hydrophobic residues. Quercetin and myricetin composed a 3-hydroxyl group on benzopyranone which resulting in reduction of binding affinity. The phenolic group of genistein positioned in opposite orientation comparison with apigenin, and resulted in a weaker interaction with xanthine oxidase. Isovitexin showed the weakest inhibitory effect among the compounds tested. The bulky group of sugar in isovitexin may hamper its interaction with xanthine oxidase.

Keywords: Flavonoids; Xanthine oxidase; Molecular modeling

Phenolic compounds including flavonoids and flavanoids are widespread in plants and they are important in contributing the flavor and color of many fruits and vegetables. They are C_{15} compounds of 2 phenolic rings connected by a 3-carbon unit, and grouped according to presence of different substituents on the rings and the degree of ring saturation. They are frequently attached with sugars moiety to increase their water-solubility [1]. Many of them are known to possess biological and pharmacological activities, such as antioxidative, antibacterial, antiviral, and antimutagenic effects. They are also known to be potent inhibitors for several enzymes, such as xanthine oxidase, cyclooxygenase, lipooxigenase, and phosphoinositide 3-kinase [2–4]. Xanthine oxidase causes gout and is responsible for oxidative damage to living tissues. It catalyzes the oxidation of hypoxanthine and xanthine to uric acid yielded superoxide radical and raised the oxidation level in the organism. The active form of xanthine oxidase is that of a homodimer of molecular weight 290 Kd with each of the monomer independently catalyzing the reaction. Each subunit contains one molybdopterin cofactor, two distinct [2Fe–2S] centers, and one FAD cofactor. Enroth et al. [5] first reported the co-crystal structure of salicylate-xanthine oxidase complex. The substrate binding and oxidation take place at the molybdopterin center (Mo-pt). Salicylate had been shown to be a competitive inhibitor, and it bound to the Mo active site. Although salicylate itself does not bind to the Mo-pt cofactor, it blocks the approach of substrates toward the metal complex. Several amino acid residues, including Arg 880, Phe 914, Phe 1009, Thr 1010, and Glu 1261 contributed important hydrogen and electostatic interactions between salicylate and the binding site. Flavonoids' acting on xanthine oxidase inhibition has been reported, and the structure–function relationship of flavonoids interacting with this enzyme has also been

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discussed [6]. However, the stereochemistries of flavonoids binding on xanthine oxidase have not been characterized. In this study, the protection effect of flavonoids against ROS damaged living cells was presented. In addition, the molecular basis for binding to active site of xanthine oxidase by flavonoids is elucidated by computer aided molecular bind analysis.

Materials and methods

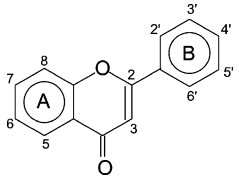
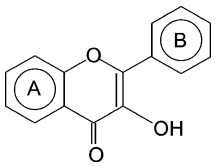
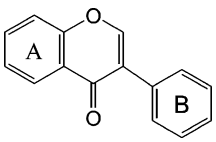
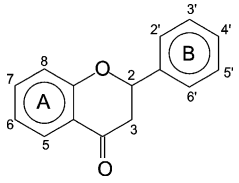
Materials. Xanthine oxidase (XO, EC 1.2.3.2.), xanthine (X), allopurinol, apigenin, genistein, kaempferol, myricetin, and quercetin were purchased from Sigma Chemical (St. Louis, MO). Isovitexin (Itx) was purified and identified from rice hulls in our laboratory [7]. The chemical structures are shown in Table 1.

Protection of HL-60 cells from ROS injury. Human promyelocytic leukemic HL-60 cells were maintained in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 in air at 37 °C. 2×10^5/ml of HL-60 cells was incubated with serum-free medium and pretreated with various compounds for 30 min, then treated with 50 μM xanthine (X) and 5.0 U/L xanthine oxidase (XO) for 4 h. The treatment HL-60 cells were harvested and lysed with 100 μl lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 0.5% *N*-lauroyl-sarcosine, 0.5 mg/ml proteinase K, and 0.5 mg/ml RNase A) at 50 °C for 3 h. The cell lysate was extracted with phenol/chloroform and the DNA fraction was subjected to a 1.8% agarose gel electrophoresis for DNA fragmentation as described [8].

Xanthine oxidase activity assay. The enzyme activity was measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as substrate [9]. The xanthine oxidase assay consisted of a 500 μl reaction mixture containing 7.5 mM phosphate buffer, 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 38 μM EDTA (pH = 7.0), 3 U/L xanthine oxidase, and 10–60 μM xanthine as substrate. The assay was started by addition of the enzyme to the reaction mixture without or with inhibitors. The assay mixture was incubated for 3 min at 37 °C and absorbency readings were taken every 5 s. The substrate concentration was less than 60 μM to avoid substrate inhibition. All data obtained for enzyme kinetic assays and plotting were proceeded using the Excel of Microsoft Office 2000.

3D computational docking modeling. Modeling building, ligand docking, energy minimization of the complexes, and molecular dynamics calculations were performed using the software DOCK 4.0.1

Table 1
Chemical structures of the various flavonoids tested for the xanthine oxidase inhibition

Chemical formula	Name	Substitution							
		5	6	7	8	2′	3′	4′	5′
Flavone	Apigenin	OH	H	OH	H	H	H	OH	H
									
Flavonol	Quercetin	OH	H	OH	H	H	OH	OH	H
									
	Myricetin	OH	H	OH	H	H	OH	OH	OH
Flavone glycoside	Isovitexin	OH	Sugar	OH	H	H	H	OH	H
Isoflavone	Genistein	OH	H	OH	H	H	H	OH	H
									
Flavanone	Naringenin	OH	H	OH	H	H	H	OH	H
									

(University of California, San Francisco) [10–12]. The favorable binding orientations of the competitive inhibitors of xanthine oxidase were focused in the active site. The three-dimensional structure of bovine milk xanthine oxidase complexed with salicylate (Protein Data Bank entry 1fiq) was used to prepare the target site for docking calculations. The A-chain, B-chain, and all small molecules were removed from the protein except for the two structural waters (Wat176, Wat196) in the active site. The inhibitor binding region was defined by selecting a subset of residues within 8 Å of the location of salicylate in the crystal structure. A Connolly molecular surface of this subset was generated using a probe radius of 1.4 Å. The negative image of the docking site was then constructed on the molecular surface. This image was represented by a set of overlapping spheres whose centers are putative locations for ligand atoms. The size of the sphere cluster was reduced to 16 spheres by removing all sphere centers farther than 3.0 Å from the crystal orientation of salicylate. The docking experiments were performed for flavonoids and allopurinol. The conformer databases of the former three inhibitors were created by using Grid Search module of Sybyl 6.5. The conformations were searched by rotating (15–45° in 1° intervals) the single bond connecting the bicyclic and the phenolic rings, and the energy minimization used the Tripos force field with Gasteiger–Huckel charges. Docking calculations were carried out on an SGI Octane R12000 270 MHz dual processor workstation. Compounds were docked using rigid-body docking to search for favorable binding orientations based on the interaction force field scoring that included Van der Waals and electrostatic terms. The highest scoring orientation for each ligand proposed the feasible binding mode of the inhibitor in the active site of xanthine oxidase.

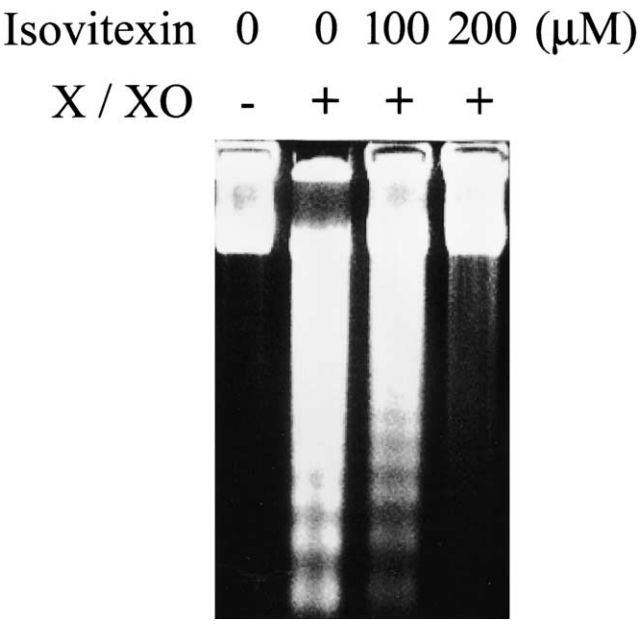


Fig. 1. Flavonoid protected HL-60 cells from ROS induced damage in the xanthine/xanthine oxidase reaction. HL-60 cells treated with or without isovitexin in the presence of X/XO for 4 h, and DNA fragmentation was shown.

Results

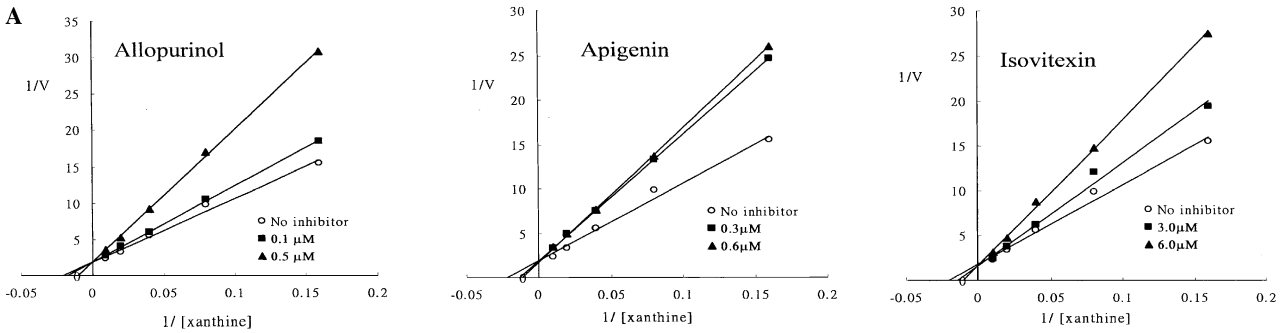
Protection of HL-60 cells from ROS induced DNA damage by flavonoids

Flavonoids are known to inhibit xanthine oxidase activity and scavenge ROS. Therefore, they might be useful for the suppression of living cell death or prevention of DNA damage induced by X/XO reaction. The isovitexin prevention of the living cells death induced by external X/XO reaction was analyzed. The HL-60 cells were treated with 50 μM xanthine and 5.0 U/L xanthine oxidase, and cell apoptosis was characterized by DNA fragmentation in agarose gel. The DNA ladder was prevented when cells were treated with 100 and 200 μM of isovitexin (lanes 3 and 4, Fig. 1). In addition, isovitexin significantly protected cells from ROS induced apoptosis in the X/XO reaction in a dose-dependent manner. These results suggested that isovitexin was a potent inhibitor of xanthine oxidase and was able to prevent cell damage induced by X/XO reaction, but with very low cytotoxicity ($IC_{50} > 400\,\mu M$).

Competitive inhibition of xanthine oxidase by flavonoids

It has been demonstrated that xanthine oxidase inhibitors are potential therapeutic agent for hyperuricemia that causes gout, renal stone, ischemic myocardiam, and ROS induced diseases. Allopurinol, a potent inhibitor of xanthine oxidase, is clinically used for gout treatment to prevent urate from accumulating in the joint. Several reports have indicated that flavonoids were able to inhibit xanthine oxidase activity [3,6,7]. To further characterize the bind region on xanthine oxidase, the kinetic assay was performed and Lineweaver–Burk double reciprocal plots were shown in Fig. 2A. Allopurinol competitively inhibited xanthine oxidase activity with K_i value of 0.34 μM. Apigenin and isovitexin also displayed competitive inhibition with K_i value of 0.61 and 5.22 μM, respectively. Quercetin, myricetin, and genistein also exhibited the competitive inhibition. The K_i values of selected compounds on xanthine oxidase were listed in Fig. 2B. Isovitexin displayed the weakest inhibitory effect toward xanthine oxidase compared to other selected flavonoids. Apigenin had the comparable inhibitory effect as allopurinol. Genistein was a weaker inhibitor than quercetin and myricetin with K_i value of 3.23 μM. Naringenin did not show xanthine oxidase inhibition activity with IC_{50} value $> 50\,\mu M$. These results indicated that all selected flavonoid analogs are competitive inhibitors of xanthine oxidase. The flavonoid with C3 hydroxyl substitution exhibited slightly weaker inhibitory activity, while flavonoid with sugar moiety, such as isovitexin, or phenolic group with a different substitute site to benzopyranone ring of genistein, significantly deteriorates binding to the active site.

A



B **Inhibition of xanthine oxidase by various flavonoids.**

Compounds	Inhibitory type	Ki value (μM)
Apigenin	competitive	0.61 ± 0.31
Quercetin	competitive	1.40 ± 0.78
Myricetin	competitive	2.17 ± 1.13
Isovitexin	competitive	5.22 ± 2.02
Genistein	competitive	3.23 ± 1.01
Allopurinol	competitive	0.34 ± 0.22
Naringenin	$IC_{50} > 50$ μM	

Fig. 2. Kinetic assays of xanthine oxidase inhibition by flavonoids. (A) Lineweaver–Burk double reciprocal plots for the inhibition of xanthine oxidase by allopurinol (left panel), apigenin (middle panel), and isovitexin (right panel). Plots are expressed 1/velocity vs. 1/xanthine (μM^{-1}) without or with inhibitors in reaction solution. (B) Inhibitory type and K_i values of various flavonoids on xanthine oxidase.

3D modeling docking of flavonoids on xanthine oxidase

To provide a further insight on the observed activities, a molecular model was created for flavonoid docking on xanthine oxidase. From kinetic assay results, the active site of xanthine oxidase is specific for selected flavone analogs; and the main focus will be the flavonoid dockings in molybdopterin domain of xanthine oxidase. Allopurinol docking in molybdopterin domain of xanthine oxidase showed the same position and interaction as salicylate binding except that Phe 1009 was apart from the bicyclic ring, and a hydrogen bonding to Glu 802 (Fig. 3D). We superimposed apigenin (green) with salicylate (bold yellow) revealing that bicyclic benzopyranone ring overlapped with salicylate ring, and the phenolic group of apigenin stretched to the space surrounding with several hydrophobic residues including Phe 1076, Phe 649, Leu 648, Leu 873, and Leu 1014. It could be inferred that the hydrophobic forces stabilize the compound in this pocket. Several hydrogen bonds and electrostatic interactions were revealed including C7 hydroxyl bound to Glu 1261 via water, C5 hydroxyl close to the guanidium group of Arg 880, and C4 carbonyl interacting with hydroxyl of Thr 1010 (Fig. 3A). Quercetin and myricetin positioned in Mo-pt center in the same orientation as apigenin with weaker interaction than apigenin. The polar C3 hydroxyl of quercetin and myricetin stretched to the space surrounded by nonpolar Phe 1009, Val 1011, and Leu 1014. Genistein is a constitutional isomer of apigenin with difference on the phenolic group substitution on benzopyranone ring. The binding of genistein was in opposite orientation to apigenin, with the phenolic group stacking with Phe 914, and benzopyranone bicyclic ring stretching to the hydrophobic surrounding pocket (Fig. 3B). Isovitexin inserted into molybdopterin domain with the same orientation as genistein and displayed fairly low affinity to enzyme. The bulky sugar moiety located in the space between hydrophobic Phe 649 and Phe 1013 also destabilize its binding (Fig. 3C).

Discussion

From the structure–activity relationship (SAR) combined with computer aided structural modeling data in this study reveal several important factors on the flavoind binding in the active site of xanthine oxidase. The olefins at C2 and C3 that maintain a planar struc-

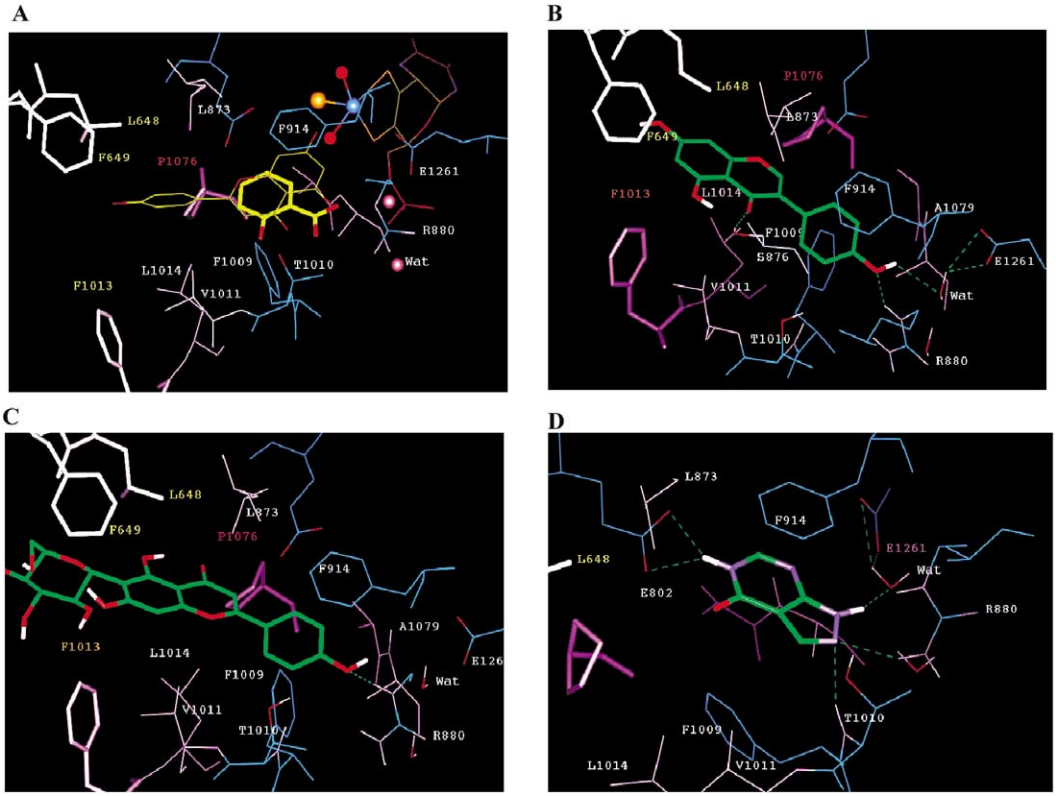


Fig. 3. Molecular model of flavonoids binding to active site of xanthine oxidase. Three-dimensional model of apigenin (green) superimposed with salicylate (yellow) (A), genistein (B), isovitexin (C), and allopurinol (D).

ture of flavonoids are essential for potent inhibitory activity on xanthine oxidase. The non-planar flavanoids, such as naringenin, exhibited no xanthine oxidase inhibition activity. Since apigenin was the most potent inhibitors among tested flavoinds, the molecular modeling revealed that hydroxyl moiety at C7 and C5 and the carbonyl group at C4 contribute favorable hydrogen bonds and electrostatic interactions between inhibitors and the active site. However, 3-substituted hydroxyl benzopyranone ring exhibited weaker inhibitory effect, which could be explained by the destabilization of polar hydroxyl stretching into the hydrophobic region of active site and resulting in lowering binding affinity. The glycosyl substitution on C6 benzopyranone ring flavonoid also hinders the binding of inhibitors into the active site of xanthine oxidase. These results provide the basis for flavonoid interaction with xanthine oxidase that may develop new potential drugs for xanthine oxidase blockade. Moreover, it also accounts for the molecular basis of various natural products containing flavonoids for the prevention of ROS damage.

Acknowledgments

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