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Glucuronidation of flavonoids by recombinant UGT1A3 and UGT1A9

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ABSTRACT

Flavonoids are highlighted for their potential roles in the prevention of oxidative stress-associated diseases. Their metabolisms *in vivo*, such as glucuronidation, are the key points to determine their health beneficial properties. In this paper, we tested the glucuronidation of nineteen flavonoids by both recombinant human UGT1A3 and UGT1A9. Eleven compounds could be catalyzed by both enzymes. In general, both enzymes showed moderate to high catalyzing activity to most flavonoid aglycones, while the catalyzing efficiency changed with structures. Each flavonoid produced more than one monoglucuronide with no diglucuronide detected by liquid chromatography–mass spectrometry (LC–MS). Enzymatic kinetic analysis indicated that the catalyzing efficiency (V_{max}/K_m) of UGT1A9 was higher than that of UGT1A3, suggesting its important role in flavonoid glucuronidation. Both human UGT1A3 and UGT1A9 preferred flavonoid aglycone to flavonoid glycoside, and their metabolism to arabinoside was stronger than to other glycosides. Of the flavonoids studied, it is the first time to report isorhamnetin, morin, silybin, kaempferol, daidzein, quercetin-3′,4′-OCHO-, quercetin xylopyranoside and avicularin as substrates of UGT1A3. Apigenin, morin, daidzein, quercetin-3′,4′-OCHO-, quercetin xylopyranoside and avicularin were the newly reported substrates of UGT1A9.

1. Introduction

More than 4000 chemically different flavonoids have been identified to date [1]. Some have been highlighted for their potential health-beneficial properties in *in vitro* experiments [2], while it is impossible to translate *in vitro* observations into the reality of the human situation without understanding the forms in which flavonoids and their metabolites circulate *in vivo*. In this way, the key point to determine *in vivo* pharmacological activities of flavonoids is their bioavailabilities and metabolisms within target tissues [2]. During absorption, flavonoids are extensively conjugated in small intestine and later in liver [3]. The conjugation mechanisms are so efficient that unchanged forms of flavonoids are generally either absent or present in low concentrations in blood [4]. It indicates that flavonoids exert their functions at least in part via conjugates.

Glucuronidation is one of the three major flavonoid conjugations [4] and catalyzed by UDP-glucuronosyltransferases (UGTs), the microsomal enzymes encoded by a multigene family in human. To date, four UGT families have

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Abbreviations: UGTs, UDP-glucuronosyltransferases; UDPGA, UDP-glucuronic acid; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; FBS, fetal bovine serum; LC/ESI–MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry; TIC, total ion chromatograms; *m/z*, mass-to-charge ratio; MOI, multiplicity of infection.

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been identified. UGT1A is the major subfamily responsible for xenobiotics glucuronidations. Over the years, liver was found to be the major tissue for UGT activities, while microsomes and recombinant enzymes from the intestine were also reported to be effective *in vitro* for glucuronidation of flavonoids [5,6]. Hence the UGTs distributing in both liver and intestine, such as UGT1A3 [7] and UGT1A9, may be more important in flavonoid glucuronidation than other UGTs.

In the present study, catalyzing activities of UGT1A3 and UGT1A9, recombinantly expressed in Bac-to-Bac insect cell, to nineteen flavonoids were tested. Several new substrates were reported, and even more important, metabolism characteristics of UGT1A3 and UGT1A9 on flavonoids were presented.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Restriction endonucleases, DNA molecular marker and T4 ligase were obtained from MBI Fermentas (Amherst, NY, USA). Agarose LE was supplied by Roche Diagnostics (Mannheim, Germany). UDP-glucuronic acid, alamethicin (dissolved in methanol), D-saccharic acid 1,4-lactone, and β-glucuronidase from *E. coli* were provided by Sigma (St. Louis, MO, USA). Quercetin, isorhamnetin, baicalin, luteolin, puerarin, kaempferol, rutin, naringin, silybin, daidzein, hesperidin and morin were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and their purities were 98%. Avicularin, hirsutrin, apigenin, farrerol, quercetin-3′,4′-OCHO-, quercetin-3-*O*-xylopyranoside, hyperoside and isoformononetin were kindly provided by the Department of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University (Hangzhou, China), and their purities were 90%. PCR primers were obtained from Sangon (Shanghai, China). Bac-to-Bac expression system and all supplement reagents were from Invitrogen (Calsbad, CA, USA). All other reagents and solvents were commercially available.

2.2. Expression of UGT1A3 and UGT1A9 in Bac-to-Bac system

The recombinant expressions of UGT1A3 and UGT1A9 in a Bac-to-Bac system have been obtained previously [5,8]. In brief, *UGT1A3* or *UGT1A9* fragment with a C-terminal His-tag sequence was obtained by RT-PCR from human liver, transposed into bacmid and transfected into Sf9 cells to produce virus. After amplifications, a high-titer baculoviral stock was used for expression at a multiplicity of infection (MOI) value of 5. Infected cells were harvested at 72 h post-transfection, sonicated and quantitated by GeneQuant spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Expression was confirmed by western blot analysis using mouse anti-His primary antibody.

2.3. Glucuronidation assay

A typical incubation mixture (100 μL of total volume) contained 100 mmol/L Tris–HCl buffer, pH 8.0, 10 mmol/L $MgCl_2$, 2 mmol/L UDPGA [9], 5 mmol/L D-saccharic acid 1,4-lactone, 20 μg/mg protein alamethicin, 0.5 mg/mL recombinant protein and flavonoid (Fig. 1). Quercetin, isorhamnetin, luteolin, puerarin, kaempferol, silybin, morin, apigenin and farrerol were dissolved in methanol and added to the reaction mixture to make 0.3% of the final organic solution. Baicalin, rutin, naringin, daidzein, hesperidin, avicularin, hirsutrin, quercetin-3′,4′-OCHO-, quercetin-3-*O*-xylopyranoside, hyperoside and isoformononetin were dissolved in *N*,*N*-dimethylformamide and added to the reaction mixture to make 0.5% of the final organic solution. Incubations were carried out at 37 °C. UDPGA was added to start the reaction. The reaction was stopped by 300 μL of methanol. After centrifugation, 25 μL of supernatant was injected into the HPLC system. Controls without UDPGA, protein or substrate were treated in parallel. All incubations were performed in triplicates.

2.4. HPLC analysis

HPLC analysis was performed on a Shimadzu LC-10A (Kyoto, Japan) system, equipped with two LC-10AD pumps, a SHIMADZU SPD-10A UV detector and a Diamonsil C18 column (5 μm particle size, 250 mm × 4.6 mm). Mobile phase A (0.2% phosphoric acid in water, v/v, pH 2.0) and mobile phase B (methanol) were run at a total flow rate of 1 mL/min (0.5 mL/min for avicularin). Chromatographic condition for each flavonoid is listed in Table 1. Data acquisition and integration were performed using a HS 2000 chromatographic workstation.

2.5. Hydrolysis with β-glucuronidase

β-Glucuronidase was used to identify glucuronides. For this purpose, an aliquot of glucuronidation incubates was heated at 90 °C for 5 min, centrifuged at 10,000 rpm for 5 min and divided into two parts equally. One part was treated with 0.2 mL of KH_2PO_4 buffer (0.1 mol/L, pH 5.0) containing 200 U of β-glucuronidase for 2 h at 37 °C. The other part containing no β-glucuronidase was treated in parallel. After incubation, both parts were centrifuged at 10,000 rpm for 5 min. An aliquot of 25 μL of each supernatant was analyzed by HPLC as described above.

2.6. Analysis of glucuronides by liquid chromatography–mass spectrometry (LC–MS)

The LC–MS was also used to identify glucuronides. HPLC was performed on a Survegor system (Thermo Finnigan, San Jose, CA, USA) and a Diamonsil C18 column (5 μm particle size, 250 mm × 4.6 mm). Mobile phase consisted of 0.67% formic acid, pH 2.0 and methanol at different ratios. Samples were separated at a rate of 0.5 mL/min into a LCQ Deca ion traps mass spectrometer (Thermo Finnigan) without split. Mass spectrometric analysis was performed in the negative electrospray mode using a spray voltage of 5 kV and a capillary voltage of −14.9 kV with nitrogen (99.999%) as sheath and auxiliary gas. MS and MS/MS spectra were obtained in the range of mass-to-charge ratio (*m*/*z*) 150–800. Collision-induced dissociation was performed using helicon as collision gas, and data were processed using an Xcalibur software with license. PDA detection was carried out to facilitate peak assignment.

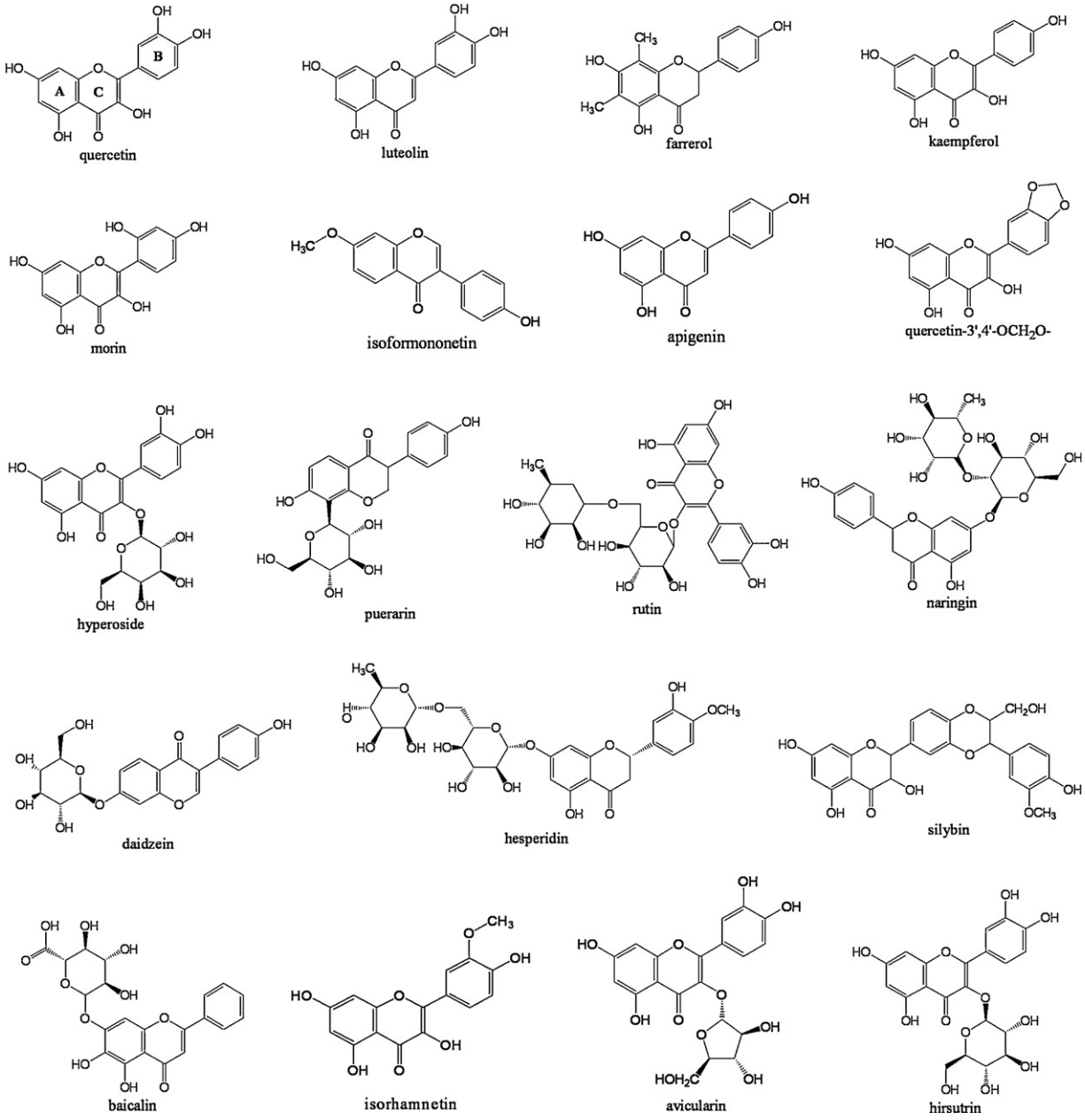

Fig. 1 – Structures of tested flavonoids.

2.7. *Kinetics parameters analysis*

The apparent K_m and V_{max} of flavonoid glucuronidation by recombinant UGT1A3 or UGT1A9 were calculated based on the total amount of substrates depletion. Standard curves were prepared by analyzing enzymes-free reaction mixtures fortified with 5, 10, 50, 100, 150, 200, 300 μmol/L flavonoids and plotting S_{sub}/S_{int} (peak area of substrate/peak area of internal standard) versus substrate concentrations. The acceptance standard for the calibration curves was a regression coefficient (r^2) > 0.99. The limit of quantification (LOQ) was defined as a signal to noise ratio of 10:1, with an acceptable level of variation (<10%).

The kinetic analysis for each substrate was performed using optimal protein concentration (0.5 mg/mL) and reaction time (10 min). The enzyme preparations were used for all tested flavonoids at the same concentrations.

Substrate inhibition of UGT1A3 or UGT1A9 was generally observed. For example, the glucuronidation of avicularin by UGT1A3 reached a maximum at 100 μmol/L and then declined (Fig. 2). Kinetic constants were obtained by fitting untransformed experimental data to the following kinetic model:

The Michaelis–Menten equation in substrate inhibition [10],

$$v = \frac{V_{max}S}{K_m + S + (S^2/K_s)},$$

where v is the rate of reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant, K_s is the inhibition constant, and

Table 1 – HPLC condition for each flavonoid

Flavonoids		Mobile phase (B%, v:v)	Detection wavelength (nm)
Flavones	Luteolin	52%	350
	Apigenin	57%	334
Flavonols	Isorhamnetin	0–22 min: 50%; 22–35 min: 50–80%	371
	Morin	50%	355
	Quercetin	50%	368
	Kaempferol	0–20 min: 50%; 20–30 min: 50–80%	365
	Quercetin-3′,4′-OCHO-	70%	339
Flavonones	Farrerol	60%	293
Isoflavones	Isoformononetin	50%	290
	Daidzein	50%	300
Biflavonoids	Silybin	0–30 min: 42%; 30–35 min: 42–54%; 35–70 min: 54%	286
Monoglycosides	Avicularin	48%	355
	Hirsutrin	45%	355
	Hyperoside	45%	257
	Baicalin	52%	276
	Puerarin	50%	250
	Quercetin xylopyranoside	45%	358
Biglycosides	Rutin	45%	380
	Naringin	45%	282
	Baicalin	52%	276

S is the substrate concentration. While at the low substrate concentration, the equation can be simplified as:

$$v = \frac{V_{max}S}{K_m + S}$$

Each experiment was carried out in triplicate. The apparent K_m values and V_{max} values for substrates were indicated as the mean of three independent experiments.

3. Results

3.1. *Glucuronidation assay*

In the present study, a series of flavonoids was investigated for glucuronidation reactivities with recombinant UGT1A3 or UGT1A9. Among nineteen tested flavonoids, 11 compounds were catalyzed by recombinant UGT1A3 or UGT1A9. For example, luteolin (Fig. 3a and a′) or apigenin (Fig. 3b and b′), two tested flavones, were catalyzed into three metabolites or one metabolite. The representative chromatograms are shown in Fig. 3. All hypothesized glucuronide peaks disappeared after β-glucuronidase hydrolysis (Fig. 3j′).

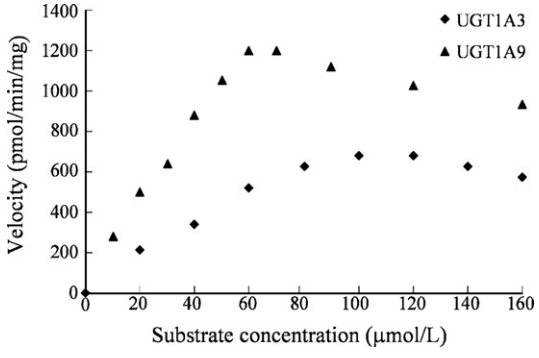

Fig. 2 – Correlation between the velocity of the glucuronidation reaction and avicularin concentration.

Morin is an isomeric antioxidant with quercetin, with the different distribution of hydroxyl groups in ring B. In the present study, quercetin was metabolized by either UGT to produce four glucuronides (Fig. 3c and c′), while only two morin glucuronides were detected in UGT1A3 incubate (Fig. 3d), and three in UGT1A9 incubate (Fig. 3d′). It is in agreement with previous studies in rats that the difference in hydroxylation pattern on ring B of flavonols markedly affected their fates [11].

Few reports on glucuronidation of isohamnetin (Fig. 3e and e′) have been published, while 3-isohamnetin-glucuronide was widely found in human plasma after administration of quercetin. In this study, one more glucuronide was produced by recombinant UGT1A3 (Fig. 3e) than UGT1A9 (Fig. 3e′), indicating other UGTs besides UGT1A9 may participate in some flavonoid metabolisms. In addition, isorhamnetin is a metabolite of quercetin. The metabolisms of UGT1A3 and UGT1A9 to both quercetin and isohamnetin support that UGTs not only metabolize quercetin directly, but are also involved in the subsequent glucuronidation of quercetin metabolites.

Four kaempferol monoglucuronides have been identified in human plasma, while only two monoglucuronides were detected in the present study in both UGT1A9 and UGT1A3 incubates (Fig. 3f and f′). One of them was speculated as 3-conjugate since this position is much more acidic than the other positions [12]. Other glucuronides found *in vivo* may be formed by other UGTs (even unidentified) or conversion from other flavonoids.

Silybin is a rather complex target for glucuronidation, offering two native diastereomeric forms, 10*S*, 11*S* and 10*R*, 11*R* in a 1:1 ratio [13]. The metabolism of silybin with bovine liver microsomes produced two major glucuronides, 20-OH or

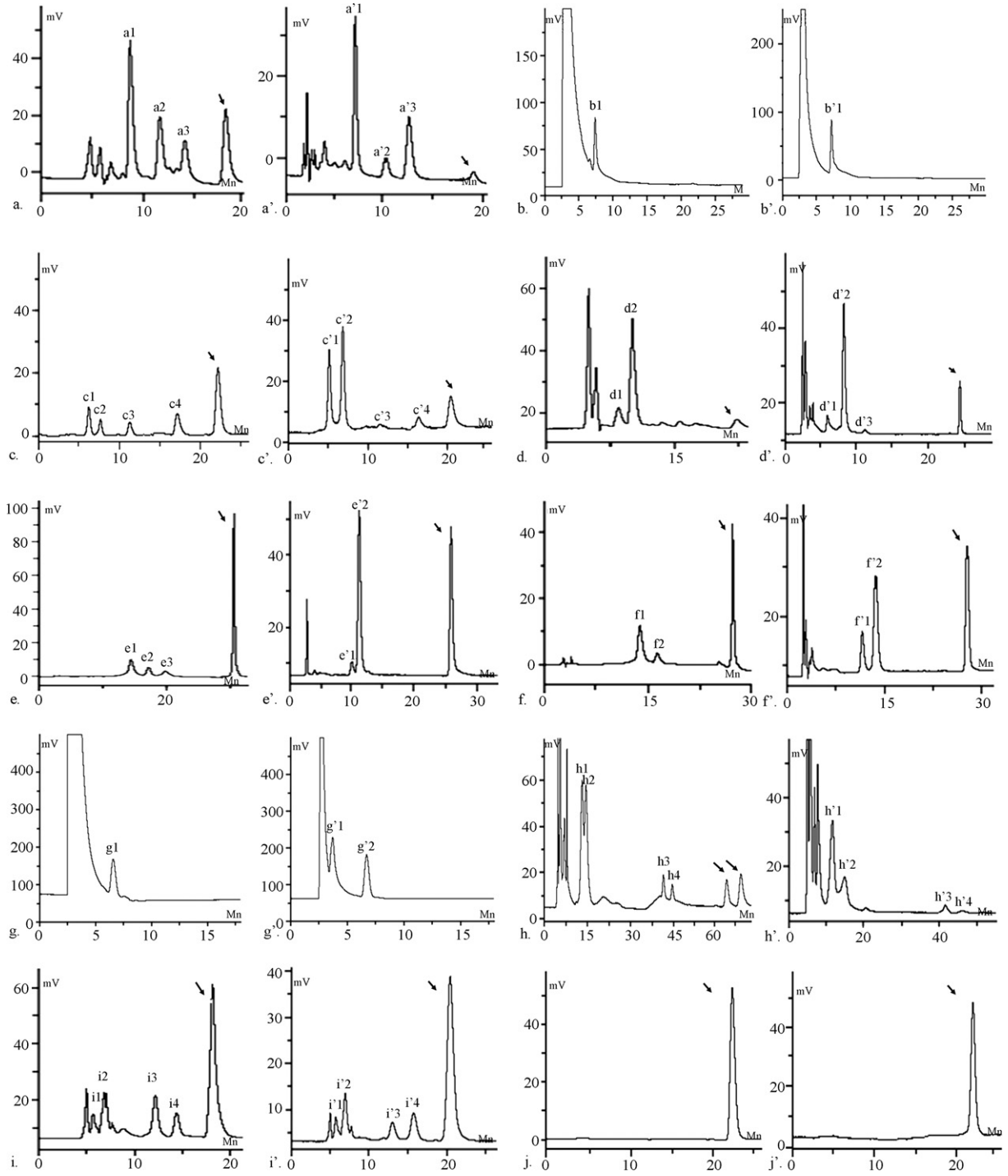

Fig. 3 – Typical HPLC chromatograms of flavonoids incubate with recombinant UGT1A3 or UGT1A9 enzyme. (a) Luteolin metabolized by UGT1A3; (a′) luteolin metabolized by UGT1A9; (b) apigenin metabolized by UGT1A3; (b′) apigenin metabolized by UGT1A9; (c) quercetin metabolized by UGT1A3; (c′) quercetin metabolized by UGT1A9; (d) morin metabolized by UGT1A3; (d′) morin metabolized by UGT1A9; (e) isorhamnetin metabolized by UGT1A3; (e′) isorhamnetin metabolized by UGT1A9; (f) kaempferol metabolized by UGT1A3; (f′) kaempferol metabolized by UGT1A9; (g) quercetin-3′,4′-OCHO- metabolized by UGT1A3; (g′) quercetin-3′,4′-OCHO- metabolized by UGT1A9; (h) silybin metabolized by UGT1A3; (h′) silybin metabolized by UGT1A9; (i) avicularin metabolized by UGT1A3; (i′) avicularin metabolized by UGT1A9; (j) negative control of quercetin glucuronidation without recombinant UGTs; (j′) quercetin chromatogram after hydrolysis with β-glucuronidase. The *x*-axis indicates retention time, and *y*-axis indicates absorbance at detection wavelength. Numbered peaks represent glucuronides. Substrate peaks are indicated with black arrow. Silybin has two substrate peaks corresponding to its two diastereomers. Unlabelled peaks in chromatograms are the background of incubates.

Table 2 – Glucuronidations of nineteen tested flavonoids by recombinant UGT1A3 or UGT1A9

Flavonoids		The number of glucuronides	
		UGT1A3	UGT1A9
Flavones	Luteolin	3	3
	Apigenin	1	1
Flavonols	Isorhamnetin	3	2
	Morin	2	3
	Quercetin	4	4
	Kaempferol	2	2
	Quercetin-3′,4′-OCHO-	1	2
Flavonones	Farrerol	nd[a]	nd
Isoflavones	Isoformononetin	nd	nd
	Daidzein	1	1
Biflavonoids	Silybin	4	4
Monoglycosides	Avicularin	4	4
	Hirsutrin	nd	nd
	Hyperoside	nd	nd
	Puerarin	nd	nd
	Quercetin xylopyranoside	3	3
Monoglucuronide	Baicalin	nd	nd
Biglycosides	Rutin	nd	nd
	Naringin	nd	nd
	Baicalin	nd	nd

[a] nd, No metabolite was detected.

7-OH glucuronide, and a 5-OH glucuronide in a low efficiency [14]. Here, after the incubation with recombinant UGT1A3 or UGT1A9, only two monoglucuronides of each diastereomer were detected. Significant stereoselectivity was observed in the glucuronidation of silybin by UGT1A9 (Fig. 3h′) but not by UGT1A3 (Fig. 3h). Such a selection was also reported in bovine liver microsomes [14].

Few cases of *in vitro* glucuronidation of avicularin have been reported to date. In the present experiment, UGT1A3 (Fig. 3i) or UGT1A9 (Fig. 3i′) was found to catalyze avicularin to form four glucuronides. Among all other tested monoglycosides, quercetin xylopyranoside could also be weakly metabolized by both UGT1A3 and UGT1A9 (chromatogram not shown).

In addition, the only tested flavonone, farrerol, could not be metabolized by either UGT1A3 or UGT1A9. Both UGT1A3 and UGT1A9 showed weak activities to isoflavones (chrogram not shown). All tested biglycosides did not produce detectable metabolites. A summary on glucuronidations of flavonoids with UGT1A3 and UGT1A9 is listed in Table 2.

3.2. *Analysis of glucuronides by LC–MS*

To further confirm the structures of flavonoids glucuronides, samples from the recombinant UGT incubations were also analyzed by LC–MS. Total ion chromatograms (TICs) were collected by full scanning ions over a mass range of *m*/*z* 150–800, encompassing the *m*/*z* values of both diglucuronides and monoglucuronides for each flavonoid. Major daughter ions transitions of peaks identified as flavonoids glucuronides are described in Table 3. All of them were monoglucuronides. No diglucuronide was detected in either UGT1A3 or UGT1A9 incubates. The metabolites peaks of daidzein and quercetin xylopyranoside were too weak to be detected by LC–MS in current experimental condition.

Peaks in MS 1 scan showing *m*/*z* values corresponding to possible flavonoids and glucuronides were further investigated using negative ion mode electrospray ionization–tandem mass spectrometry (LC/ESI–MS/MS). Diagnostic ions corresponding to flavonoid aglycons formed by the loss of a glucuronic acid moiety were observed in MS/MS analysis (Table 3) after collisions. Fragment characteristics of flavonoid aglycons were also observed (Table 4). It seemed monoglucuronides of flavonoids were unstable. Glucuronic acid lost from monitoring ions with 23% collision energy. In contrast, flavonoid aglycons had the stable parent structures (a common structure consists of two aromatic rings (A and B) that are bound together by three carbon atoms that form an oxygenated heterocycle (ring C)). No daughter ions were detected in MS/MS analysis with 30% collision energy except isorhamnetin, silybin and avicularin. Isorhamnetin had a [M−H]- at *m*/*z* 300 in MS/MS analysis, 15 mass units lower than the isorhamnetin aglycon, identifying it as a fragment losing a methyl unit (moiety) of isorhamnetin. Avicularin had a [M−H]- at *m*/*z* 301 in MS/MS analysis, identifying it as a fragment losing an arabinoside unit. Silybin showed a fragment losing an H_2O fragment, and a broken biflavonoid fragment. MS/MS spectra of these pseudomolecular ions contained fragments corresponding mainly to the aglycone part and thus did not allow a determination of the sites of glucuronidation. Fragmentation patterns of each monoglucuronide differed in intensities of minor ions and in their LC retention times, indicating that they represent different conjugates on the flavonoid ring. Although the relative abundances of key

Table 3 – LC/ESI–MS/MS analysis of flavonoids glucuronides

Peak	Compound	$[M-H]^-$ (*m*/z)	Major fragment ions in MS/MS (*m*/z)
a1	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA[a]
a2	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA
a3	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA
a′1	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA
a′2	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA
a′3	Luteolin monoglucuronide	461	285$[M-H]^-$, GlucUA
b1	Apigenin monoglucuronide	445	269$[M-H]^-$, GlucUA
b′1	Apigenin monoglucuronide	445	269$[M-H]^-$, GlucUA
c1	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA[a]
c2	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c3	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c4	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c′1	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c′2	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c′3	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
c′4	Quercetin monoglucuronide	477	301$[M-H]^-$, GlucUA
d1	Morin monoglucuronide	477	301$[M-H]^-$, GlucUA
d2	Morin monoglucuronide	477	301$[M-H]^-$, GlucUA
d′1	Morin monoglucuronide	477	301$[M-H]^-$, GlucUA
d′2	Morin monoglucuronide	477	301$[M-H]^-$, GlucUA
d′3	Morin monoglucuronide	477	301$[M-H]^-$, GlucUA
e1	Isorhamnetin monoglucuronide	491	315$[M-H]^-$, GlucUA
e2	Isorhamnetin monoglucuronide	491	315$[M-H]^-$, GlucUA
e3	Isorhamnetin monoglucuronide	491	315$[M-H]^-$, GlucUA
e′1	Isorhamnetin monoglucuronide	491	315$[M-H]^-$, GlucUA
e′2	Isorhamnetin monoglucuronide	491	315$[M-H]^-$, GlucUA
f1	Kaempferol monoglucuronide	461	285$[M-H]^-$, GlucUA
f2	Kaempferol monoglucuronide	461	285$[M-H]^-$, GlucUA
f′1	Kaempferol monoglucuronide	461	285$[M-H]^-$, GlucUA
f′2	Kaempferol monoglucuronide	461	285$[M-H]^-$, GlucUA
g1	Quercetin-3′,4′-OCHO-monoglucuronide	489	313$[M-H]^-$, GlucUA
g′1	Quercetin-3′,4′-OCHO-monoglucuronide	489	313$[M-H]^-$, GlucUA
g′2	Quercetin-3′,4′-OCHO-monoglucuronide	489	313$[M-H]^-$, GlucUA
h1	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h2	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h3	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h4	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h′1	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h′2	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h′3	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
h′4	Silybin monoglucuronide	657	481$[M-H]^-$, GlucUA
i1	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA
i2	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA
i3	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA; 477$[M-H]^-$, arabinoside; 301$[M-H]^-$, GlucUA, arabinoside
i4	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA; 477$[M-H]^-$, arabinoside
i′1	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA; 590$[M-H]^-$, H_2O
i′2	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA
i′3	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA; 477$[M-H]^-$, arabinoside; 301$[M-H]^-$, GlucUA, arabinoside
i′4	Avicularin monoglucuronide	609	433$[M-H]^-$, GlucUA; 477$[M-H]^-$, arabinoside

[a] GlucUA, Glucuronic acid. Peaks are corresponding to that in Fig. 3.

fragment ions may provide information on the position of conjugation, further studies employing nuclear magnetic resonance (NMR) are needed to understand identities of flavonoid metabolites.

3.3. *Kinetics analysis*

The limits of quantification were 2 pmol/100 μL mixture for puerarin, naringin and hesperidin; 3 pmol/100 μL mixture for rutin and daidzein; 6 pmol/100 μL mixture for hyperoside, hirsutrin and quercetin-3-*O*-xylopyranoside; 8.5 pmol/100 μL mixture for querctin, morin, kaemperfol, luteolin, silybin, apigenin, farrerol and isoformononetin; 10.2 pmol/100 μL mixture for isorhamnetin, avicularin and quercetin-3′,4′-OCHO-; and 24.8 pmol/100 μL mixture for baicalin (CV $< 10\%$, $n = 5$).

Before calculating kinetic parameters, some preliminary experiments were performed to optimize reaction conditions.

Table 4 – LC/ESI–MS/MS analysis of flavonoids

Compound	$[M-H]^-$ (*m*/*z*)	Major fragment ions in MS/MS (*m*/*z*)
Luteolin	285	N[a]
Apigenin	269	N
Quercetin	301	N
Morin	301	N
Isorhamnetin	315	300$[M-H]^-$, Met[b]
Kaempferol	285	N
Silybin	481	463$[M-H]^-$, H_2O; 301$[M-H]^-$, flavonoid
Avicularin	433	301$[M-H]^-$, arabinoside

[a] N, No fragment is detected under the conditions used.
[b] Met, Methyl.

Glucuronidation was proportional to the concentration of cell homogenate up to 1.2 mg/mL. At 0.5 mg/mL, the reaction was linear with time up to 100 min. Stabilities of flavonoids in incubation were also evaluated. Quercetin, isorhamnetin, baicalin, kaempferol, morin and avicularin were stable incubates with time. Silybin and luteolin decomposed at 37 °C for 3 h 16.6 and 17.5%, respectively. Considering the short incubation time (10 min) and 2% analysis precision, the decomposition of each compound was not considered in calculating rates.

Substrate inhibitions of UGT1A3 or UGT1A9 were generally observed among flavonoids. Kinetic constants for flavonoids glucuronidation by each enzyme were obtained by Michaelis–Menten equation described in Section 2.7. The kinetic values are described in Tables 5 and 6.

Both enzymes could catalyze all tested flavonoids aglycones but farrerol, while UGT1A9 showed a higher catalyzing efficiency (V_{max}/K_m) than UGT1A3 to all tested flavonoids. Kinetic analysis showed that the glucuronidation efficiencies of UGT1A3 or UGT1A9 for flavonoid aglycones were moderate or strong except for two isoflavones.

4. Discussion

Flavonoids have attracted increasing attention in the past several decades for their great abundance and antioxidant properties [15–17]. Some researches pointed out that flavonoids were generally recovered in plasma and urine as conjugated derivatives, with no or trace amounts of native forms detected [18–22]. As one of the three major conjugation pathways, glucuronidation plays an important role in biological effects of flavonoids. In the past, many studies on the glucuronidation of flavonoids were performed on animal models or human liver microsomes [23,24]. They are efficient to provide panoramic *in vivo* metabolism but are limited in mechanism research by the complexity of mixed enzymes. In the present study, the *in vitro* recombinant human enzyme provided a good model for research on single enzyme characteristics, which was a complementary view to the comprehensive *in vivo* study. Some UGTs were previously reported as glucuroindation catalysts on flavonids. Among these UGTs, UGT1A9 has been suggested as the major enzyme contributing to glucuronidation of flavonoids [25], while other UGTs cannot be neglected because of unclear substrate binding characteristics and potential preference to produce glucuronides with high antioxidant and cardiovascular protective effects [5,26]. In the present study, a series of flavonoids was investigated for their glucuronidation reactivities with recombinant UGT1A3 or UGT1A9.

Table 5 – Kinetic parameters of each flavonoid glucuronidation with recombinant UGT1A3 (n =9)

Substrate	K_m (μmol/L)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)
Luteolin	36 ± 0.9	1.63 ± 0.04	46 ± 2.1
Apigenin	nq[a]	nq	nq
Quercetin	39 ± 2.1	2.02 ± 0.09	51 ± 1.8
Kaempferol	37 ± 1.6	1.02 ± 0.05	28 ± 2.5
Morin	39 ± 3.5	1.57 ± 0.04	41 ± 3.7
Isorhamnetin	33 ± 0.4	1.11 ± 0.03	34 ± 1.1
Farrerol	nd[b]	nd	nd
Isoformononetin	nd	nd	nd
Daidzein	nq	nq	nq
Silybin[c]	57 ± 2.5	0.84 ± 0.08	15 ± 0.9
Quercetin-3′,4′-OCHO-	nq	nq	nq
Avicularin	89 ± 2.2	1.35 ± 0.05	15 ± 0.9
Quercetin-3-O-xylopyranoside	nq	nq	nq
Hirsutrin	nd	nd	nd
Hyperoside	nd	nd	nd
Baicalin	nd	nd	nd
Puerarin	nd	nd	nd
Hesperidin	nd	nd	nd
Rutin	nd	nd	nd
Naringin	nd	nd	nd

All assays were performed at pH 8.0 and incubated at 37 °C for 10 min, using total proteins of sf9 cells expressing UGT1A3 (0.5 mg/mL in final concentration).
[a] nq, No quantitation.
[b] nd, Indicates glucuronides were not detected.
[c] Parameters of silybin were measured assuming that there was only one substrate peak.

Table 6 – Kinetic parameters of each flavonoid glucuronidation with recombinant UGT1A9 (n =9)

Substrate	K_m (μmol/L)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)
Luteolin	41 ± 1.5	14.1 ± 0.42	343 ± 19.7
Apigenin	nq[a]	nq	nq
Quercetin	38 ± 2.4	10.2 ± 0.50	271 ± 9.9
Kaempferol	39 ± 1.1	9.26 ± 0.09	237 ± 9.3
Morin	41 ± 1.7	3.57 ± 0.07	88 ± 3.6
Isorhamnetin	33 ± 1.5	15.2 ± 0.61	459 ± 38.6
Farrerol	nd[b]	nd	nd
Isoformononetin	nd	nd	nd
Daidzein	nq	nq	nq
Silybin[c]	55.7 ± 1.4	2.98 ± 0.07	54 ± 2.0
Quercetin-3′,4′-OCHO-	nq	nq	nq
Avicularin	56 ± 1.7	2.25 ± 0.06	40 ± 0.4
Quercetin-3-O-xylopyranoside	nq	nq	nq
Hirsutrin	nd	nd	nd
Hyperoside	nd	nd	nd
Baicalin	nd	nd	nd
Puerarin	nd	nd	nd
Hesperidin	nd	nd	nd
Rutin	nd	nd	nd
Naringin	nd	nd	nd

All assays were performed at pH 8.0 and incubated at 37 °C for 10 min, using total proteins of sf9 cells expressing UGT1A9 (0.5mg/mL in final concentration).

[a] nq, No quantitation.

[b] nd, Indicates glucuronides were not detected.

[c] Parameters of silybin were measured assuming that there was only one substrate peak.

Avicularin could form monoglucuronides after being catalyzed by either UGT1A3 or UGT1A9 (Fig. 3). It was reported that no avicularin glucuronide was detected *in vivo* after the direct intake of avicularin [27]. It is highly possible that avicularin is hydrolyzed first by intestinal enzymes or colonic microflora before its absorption. This result supports the controversial hypothesis that hydrolysis is the requisite for the absorption of quercetin glycosides. Further investigation of this point is required, especially because other authors [28,29] found that arabinosides appeared to be absorbed or metabolized differently from the corresponding glucosides or galactosides in the stomach of anesthetized rats or human. Our additional *in vitro* tests on other monoglycosides also showed that UGT1A3 and UGT1A9 metabolized other monoglycosides less efficiently than arabinosides. Only quercetin xylopyranoside can be metabolized by both enzymes weakly (Tables 5 and 6).

Flavonoids are multi-hydroxyl compounds. UGTs have more than one choice to conjugate them at O-positions. This study showed neither UGT1A3 nor UGT1A9 had the ability to form diglucuronides, although diglucuronides of some flavonoids have been detected in human plasma [6,30]. The observation that baicalin (the monoglucuronide of baicalein) could not be catalyzed by UGT1A3 or UGT1A9 further supported this conclusion. Other UGTs (even unidentified) or the combinations of several UGTs may catalyze the formation of diglucuronides in human.

For UGT1A3, flavonoids with two hydroxyl groups on ring B showed higher metabolism efficiencies than those with one hydroxyl: luteolin, quercetin and morin > kaempferol and isorhamnetin. To UGT1A9, the number of hydroxyl groups on flavonoids ring B showed different effects on metabolism efficiency. Morin with a hydroxyl at 2′ site in ring B, had less glucuronidation activity compared with luteolin, quercetin, kaempferol and isorhamnetin. B2′ hydroxyl may hinder flavonoids glucuronidation by UGT1A9 [31].

It is interesting to note that flavonoids containing quercetin structure followed a decreased glucuronidation activity order with UGT1A3 or UGT1A9: quercetin > avicularin > rutin. Avicularin, a quercetin-3-alpha-L-arabinofuranoside, had a smaller catalyzing efficiency than quercetin with UGT1A3. Rutin, substituted with a rutinose at the same position, had no detectable glucuronide catalyzed by UGT1A3. These results suggest that human UGT1A3 and UGT1A9 catalyze glucuronidation preferentially at flavonoid aglycones to glycosides. This may be explained by hindered enzyme–substrate interactions and poor lipophilic properties of glycosides. The same results were observed in two other glycosides. They reacted with expressed UGT1A3 or UGT1A9 at an undetectable rate.

In summary, UGT1A3 and UGT1A9 catalyzed most tested flavonoids at a moderate to high rate, while their activities to flavonoid glycosides were much less. Their catalyses to arabinosides were different from other monoglycosides. Eight compounds: isorhamnetin, morin, silybin, kaempferol, daidzein, quercetin-3′,4′-OCHO-, quercetin xylopyranoside and avicularin, are reported for the first time as new substrates of UGT1A3. Six compounds: apigenin, morin, daidzein, quercetin-3′,4′-OCHO-, quercetin xylopyranoside and avicularin are reported as substrates of UGT1A9. They both could catalyze glucuronidations at different hydroxyls while such conjugations only happened once in each flavonoid. To all tested compounds, UGT1A9 showed higher catalyzing ability than UGT1A3, which further confirmed the importance of UGT1A9 in flavonoid glucuronidations. UGT1A3 formed several unique glucuronides in some flavonoids. Considering the locations of UGT1A3 in both liver and intestine, the role of UGT1A3 in

flavonoids glucuronidation should not be neglected in the future.

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