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Uridine Diphosphate Glucuronosyltransferase Isoform-Dependent Regiospecificity of Glucuronidation of Flavonoids

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Supporting Information

ABSTRACT: The objective of this study was to determine the regiospecificity of the important uridine diphosphate glucuronosyltransferase (UGT) isoforms responsible for the glucuronidation of flavones and flavonols. We systematically studied the glucuronidation of 13 flavonoids (7 flavones and 6 flavonols, with hydroxyl groups at C-3, C-4′, C-5, and/or C-7 positions in flavonoid structure) at a substrate concentration of 10 μM by 8 recombinant human UGT isoforms mainly responsible for the metabolism of flavonoids, UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7. At 10 μM substrate concentration, different UGT isoforms gave different regiospecific glucuronidation patterns. UGT 1A1 equally glucuronidated 3-*O* (glucuronic acid substituted at C-3 hydroxyl group), 7-*O*, and 4′-*O*, whereas UGTs 1A8 and 1A9 preferably glucuronidated only 3-*O* and 7-*O* positions. UGT 1A1 usually showed no regiospecificity for glucuronidating any position, whereas UGT 1A8 and UGT 1A9 showed dominant, moderate, or weak regiospecificity for 3-*O* or 7-*O* position, depending on the structure of the compound. UGT 1A3 showed dominant regiospecificity for the 7-*O* position, whereas UGT 1A7 showed dominant regiospecificity for the 3-*O* position. We also showed that the glucuronidation rates of 3-*O* and 7-*O* positions in flavones and flavonols were affected by the addition of multiple hydroxyl groups at different positions as well as by the substrate concentrations (2.5, 10, and 35 μM). In conclusion, regiospecific glucuronidation of flavonols was isoform- and concentration-dependent, whereas flavones were dominantly glucuronidated at the 7-*O* position by most UGT isoforms. We also concluded that UGTs 1A3 and 1A7 showed dominant regiospecificity for only the 7-*O* and 3-*O* positions, respectively. UGTs 1A8 and 1A9 showed moderate or weak preference on glucuronidating position 3-*O* over the 7-*O* position, whereas other UGT isoforms did not prefer glucuronidating any particular positions.

KEYWORDS: regiospecificity, UGT isoforms, glucuronidation, flavonoids

INTRODUCTION

Uridine diphosphate glucuronosyltransferases (UDP-glucuronosyltransferases or UGTs) utilize uridine diphosphoglucuronic acid (UDPGA) as cofactor and transfer glucuronic acid to the usually lipophilic substrates, forming hydrophilic conjugates called glucuronides (G). Substrates of UGTs include endogenous compounds such as steroids, bile acids, bilirubin, hormones, dietary constituents such as flavonoids, xenobiotics such as morphine and valproic acid, the products of phase I metabolism, and environmental toxins and carcinogens such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).[1−3] UGT-catalyzed glucuronidation reactions are responsible for 35% of all drugs metabolized by phase II enzymes.[4] Despite the important role of UGTs in the metabolism of drugs, environmental chemicals, and endogenous compounds, the structural features of these enzymes responsible for substrate binding and formation of regioselective glucuronides remain poorly understood.[5,6]

Flavonoids are polyphenolic compounds with several hydroxyl groups on a heterocyclic skeleton of flavan(2-phenylbenzopyrone). Therefore, conjugations of glucuronic acid mainly occur at different hydroxyl groups and *O*-glucuronides are formed.[3] However, there is very limited information available in the literature on the regiospecificity of glucuronidation of compounds in general and flavonoids specifically. The regiospecific glucuronides of certain flavonoids have been shown to exhibit different pharmacological actions in vivo.[7,8] For example, quercetin-7-*O*-G had a stronger antioxidative property than quercetin-3-*O*-G.[8]

Also, it has been proposed that glucuronides in systemic circulation or after uptake into an organ such as the liver or intestine and neutrophils may hydrolyze back into aglycone by β-glucuronidase enzyme for pharmacological action. However, the rate of flavonoid glucuronide hydrolysis depends on the position of conjugation, which could affect the subsequent reconjugation rate.[9,10] Therefore, it becomes important to understand the regiospecificity of various UGT isoforms, as based on different expression levels of UGTs in various metabolic organs and mode of administration of drug, the exposure of regiospecific glucuronides to an organ may differ extensively.

Previously, we have shown that certain UGT isoforms were important for the glucuronidation of most flavonoids from different subclasses.[11−13] However, different UGT isoforms showed differences in their rate and position of glucuronidation of flavones and flavonols.[12] Among the various UGT isoforms expressed in the liver, UGTs 1A1, 1A3, 1A9, and 2B7 are of the

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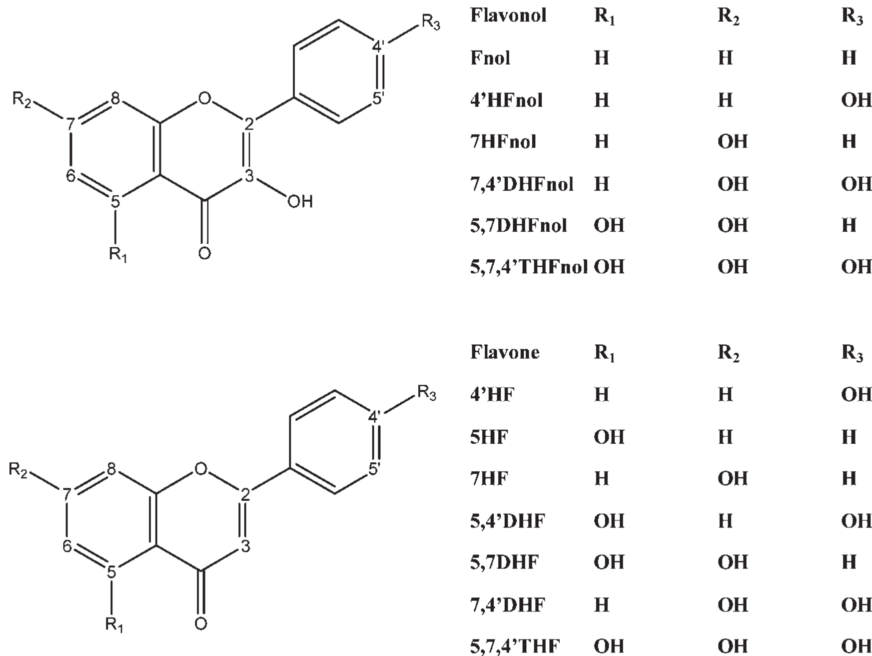

Flavonol	R_1	R_2	R_3
Fnol	H	H	H
4'HFnol	H	H	OH
7HFnol	H	OH	H
7,4'DHFnol	H	OH	OH
5,7DHFnol	OH	OH	H
5,7,4'THFnol	OH	OH	OH

Flavone	R_1	R_2	R_3
4'HF	H	H	OH
5HF	OH	H	H
7HF	H	OH	H
5,4'DHF	OH	H	OH
5,7DHF	OH	OH	H
7,4'DHF	H	OH	OH
5,7,4'THF	OH	OH	OH

Figure 1. Structures of flavonols and flavones used in the study.

greatest interest to us because of the published expression levels of UGT isoforms in the liver[14] as well as the rate of glucuronidation of various flavonoids by UGT isoforms.[11,12] UGT 1A6 is another hepatic isoform that is highly expressed in Caco-2 cell lines[15] and extensively used for disposition studies. Among extrahepatic isoforms, UGTs 1A7, 1A8, and 1A10 are the most important isoforms responsible for glucuronidation of flavonoids.[11,12]

Therefore, we decided to study all major UGT isoforms for the preference of position of glucuronidation using 13 flavonoids (7 flavones and 6 flavonols) (Figure 1) with hydroxyl groups at the C-3, C-4′, C-5, and C-7 positions. Compounds from flavone and flavonol subclasses were chosen for the study due to the commercial availability of congeneric compounds with hydroxyl group(s) at different positions on the same structural backbone in these subclasses of flavonoids. The purpose of this study was to give insight into the regiospecificity of various UGT isoforms and predict the relative occurrence of various regiospecific glucuronides of flavonoids based on expression levels of various metabolic organs such as the liver and intestine.

EXPERIMENTAL PROCEDURES

Materials. 3-Hydroxyflavone or flavonol (Fnol), 3,4′-dihydroxyflavone or 4′-hydroxyflavonol (4′HFnol), 3,7-dihydroxyflavone or 7-hydroxyflavonol (7HFnol), 5,4′-dihydroxyflavone (5,4′DHF), 5,7-dihydroxyflavone (5,7DHF), 7,4′-dihydroxyflavone (7,4′DHF), 3,7,4′-trihydroxyflavone or resokaempferol or 7,4′-dihydroxyflavonol (7,4′DHFnol), 3,5,7-trihydroxyflavone or galangin or 5,7-dihydroxyflavonol (5,7DHFnol), 5,7,4′-trihydroxyflavone or apigenin (Api or 5,7,4′THF), and 3,5,7,4′-tetrahydroxyflavone or kaempferol or 5,7,4′-trihydroxyflavonol (5,7,4′THFnol or Kamp) were purchased from Indofine Chemicals (Somerville, NJ). Eight commercially available recombinant human UGT isoforms (supersomes), UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7, were purchased from BD Biosciences (Woburn, MA). Uridine diphosphoglucuronic acid (UDPGA), alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (powder form) were purchased from Sigma-Aldrich (St. Louis, MO). All other materials (typically of analytical grade or better) were used as received.

Identification of Position of Glucuronidation in the Structure of Flavones and Flavonols by UV Shift Method. The sites of glucuronic acid substitutions in flavones and flavonols were established on the basis of the online UV spectral shift method, optimized and validated in our laboratory on the basis of the published literature.[16,17] Briefly, substitution of a single hydroxyl group by glucuronic acid in the structure of flavone at a specific position would result in diagnostic shifts or no shift in λ_{max} of band I (300−380 nm) and/or band II (240−280 nm) in the UV spectrum of the resulting glucuronide as compared to the UV spectrum of the parent compound. On the basis of these differentiating diagnostic shifts for each position of glucuronidation, the structure of a glucuronide can be estimated. Details of the method and its validation can be found in Singh et al.[18]

Methods. *Solubility and Stability of the Tested Flavonoids.* The solubility and stability of the tested flavonoids were established under the experimental conditions. The results showed that these compounds were stable and soluble at the tested concentrations (not shown).

Glucuronidation Activities of Recombinant Human UGTs. The incubation procedures for measuring UGTs' activities were essentially the same as published before.[19,20] Briefly, incubation procedures using supersomes were as follows: (1) supersomes (final concentration in range of ≈0.013−0.053 mg of protein/mL as optimum for the reaction), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4), and UDPGA (3.5 mM, added last) were mixed; (2) the mixture (final volume), 200 μL, was incubated at 37 °C for a predetermined period of time (30 or 60 min); and (3) the reaction was stopped by the addition of 50 μL of 94% acetonitrile/6% glacial acetic acid containing 100 μM testosterone or 50 μM 5-hydroxyflavone or formononetin as internal standard. Testosterone was used as internal standard for 4′HFnol, 7,4′THF, 7,4′DHFnol, 5,7,4′THF, and 5,7,4′THFnol; formononetin for Fnol, 5,4′DHF, 5,7DHF, and 5,7DHFnol; and 5-hydroxyflavone for 7HFnol. Three substrate concentrations, 2.5, 10, and 35 μM, were used to study the UGT isoforms.

UPLC Analysis of Flavonoids and Their Glucuronides. We analyzed flavonoids and their respective glucuronides by using the following common method: system, Waters Acquity UPLC with photodiode array

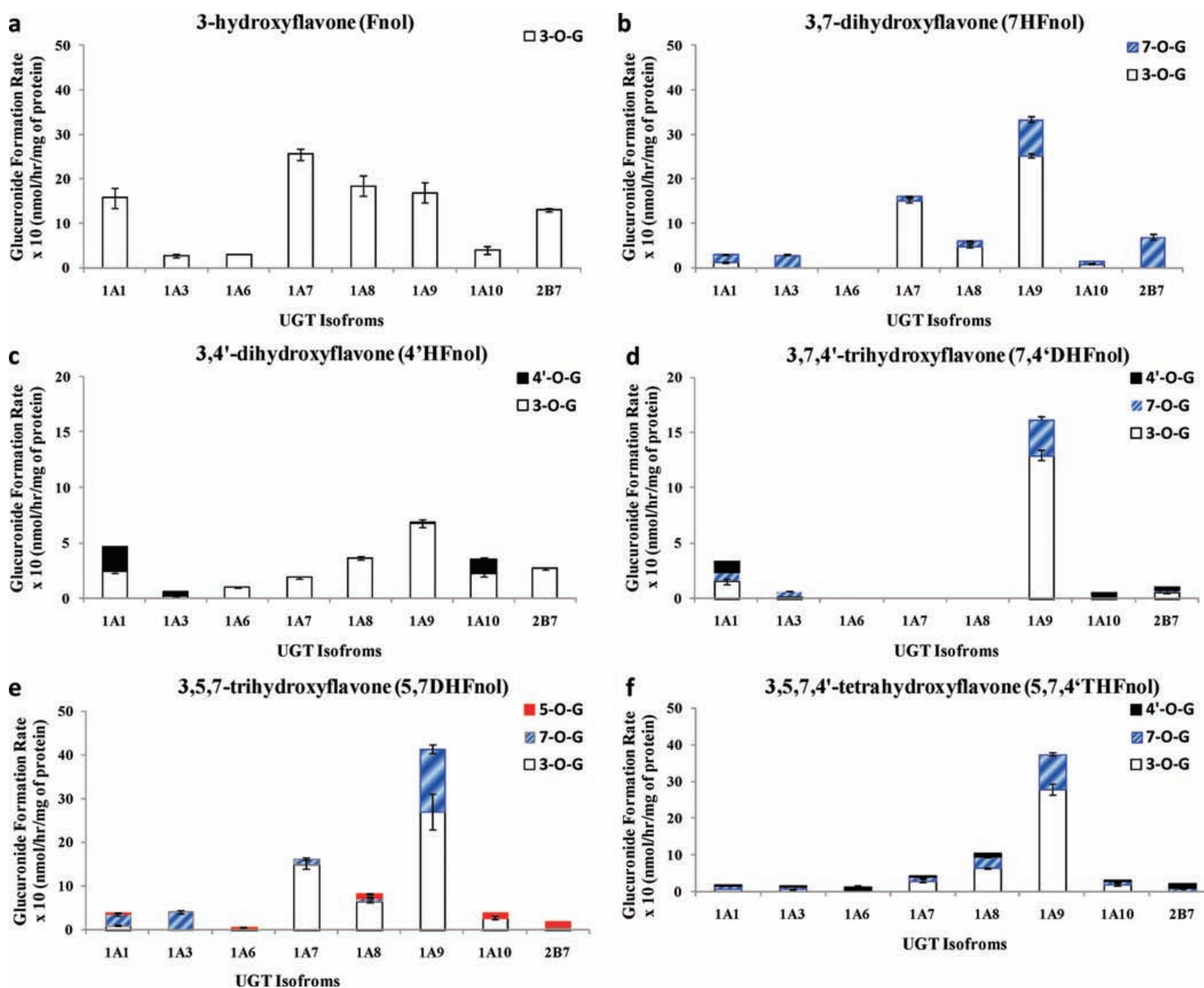

Figure 2. Regiospecific glucuronidation of flavonols by uridine diphosphate glucuronosyltransferases (UGTs): rate of glucuronidation of regiospecific glucuronides of Fnol (a), 4′HFnol (b), 7HFnol (c), 7,4′DHFnol (d), 5,7DHFnol (e), and 5,7,4′THFnol (f) with UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7. Flavonols (at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGTs (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3).

detector and Empower software; column, BEH C_{18}, 1.7 μm, 2.1 × 50 mm; and injection volume, 10 μL. Representative chromatograms are shown in Figure S1 of the Supporting Information.

Quantification of Glucuronides of Flavonoids. The quantification of glucuronides of flavonoids was done using the standard curve of the parent compound with a correction factor for the difference in extinction coefficient of the compound and its metabolites as shown in our previous publication.[18] The correction factors were in the range from 0.5 (for 3-*O*-G of Fnol) to 2.5 (for 3-*O*-G of 7HFnol), as reported previously.[18]

Confirmation of Flavonoid Glucuronide Structures by LC-MS/MS. Flavonoids and their respective glucuronides were separated by the same UPLC system but using slightly different chromatographic conditions because of mass spectrometer requirements. Here, mobile phase A was ammonium acetate buffer (pH 7.5), and mobile phase B was 100% acetonitrile with the gradient as follows: 0−2.0 min, 10−35% B; 2.0−3.0 min, 35−70% B; 3.2−3.5 min, 70−10% B; 3.5−3.7 min, 10% B. The flow rate was 0.5 mL/min. The effluent was introduced into an API 3200 Qtrap triple-quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) equipped with a TurboIonSpray source.

The mass spectrometer was operated in negative ion mode to perform the analysis of flavonoids and their respective glucuronides. The main working parameters for the mass spectrometers were set as follows: ion source temperature, 600 °C; nebulizer gas (gas 1), nitrogen, 40 psi; turbo gas (gas 2), nitrogen, 40 psi; curtain gas, nitrogen, 20 psi; DP, −50 V; EP, −10 V; CE, −30 V; CXP, −3 V; and IS, −4.5 kV. Minor adjustments were then made for each flavonoid. Flavonoid mono-*O*-glucuronides were identified by MS and MS^2 full scan modes.

The glucuronides were extracted by solid phase extraction from the glucuronidation experiment samples and reconstituted in a small amount of 30% acetonitrile in water. The concentrated samples were then used to identify the glucuronides in UPLC-MS/MS.

Data Analysis. Rates of metabolism in recombinant human UGT isoforms were expressed as amounts of metabolites formed per hour per milligram of protein (or nmol/h/mg).

Table 1. Degree of Regiospecificity (Dominant, Moderate, Weak or No) of Various Uridine Diphosphate Glucuronosyltransferase (UGT) Isoforms for Glucuronidating Flavones and Flavonols[a]

UGT Isoforms	Substrate Concentration (μM)	4'HFnol	7HFnol	5,4'DHF	5,7DHF	7,4'DHF	5,7DHFnol[1]	7,4'DHFnol[1]	5,7,4'THF[2]	5,7,4'THFnol[1]
1A1	2.5	No (3-*O*, 4'-*O*)	Weak (7-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Weak (7-*O*)	Weak (7-*O*)	No (3-*O*, 7-*O*)	Dominant (7-*O*)	Weak (7-*O*)
	10	No (3-*O*, 4'-*O*)	No (3-O,7-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Moderate (7-*O*)	Weak (7-*O*)	No (3-*O*, 7-*O*)	Dominant (7-*O*)	No (3-*O*, 7-*O*)
1A3	2.5	Dominant (3-*O*)	Moderate (7-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	No (3-*O*, 7-*O*)
	10	No (3-*O*, 4'-*O*)	Dominant (7-*O*)	Moderate (4'-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Weak (7-*O*)	Dominant (7-*O*)	No (3-*O*, 7-*O*)
1A6	2.5	Dominant (3-*O*)	*	*	Moderate (7-*O*)	*	-	*	Dominant (7-*O*)	Moderate (3-*O*)
	10	Dominant (3-*O*)	*	*	Moderate (7-*O*)	Dominant (7-*O*)	#	*	Dominant (7-*O*)	##
1A7	10	Dominant (3-*O*)	Dominant (3-*O*)	*	Dominant (7-*O*)	*	Dominant (3-*O*)	*	Dominant (7-*O*)	Weak (3-*O*)
1A8	2.5	Dominant (3-*O*)	Moderate (3-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (3-*O*)	*	Dominant (7-*O*)	Weak (3-*O*)
	10	Dominant (3-*O*)	Moderate (3-*O*)	Moderate (4'-*O*)	Dominant (7-*O*)	Moderate (7-*O*)	Moderate (3-*O*)	*	Dominant (7-*O*)	Weak (3-*O*)
1A9	2.5	Dominant (3-*O*)	Moderate (3-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Moderate (7-*O*)	Weak (3-*O*)	Moderate (3-*O*)	Dominant (7-*O*)	Moderate (3-*O*)
	10	Dominant (3-*O*)	Moderate (3-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Moderate (7-*O*)	No (3-*O*, 7-*O*)	Moderate (3-*O*)	Dominant (7-*O*)	Weak (3-*O*)
1A10	2.5	No (3-*O*, 4'-*O*)	Weak (3-*O*)	Dominant (4'-*O*)	Dominant (7-*O*)	Dominant (7-*O*)	Dominant (3-*O*)	*	Dominant (7-*O*)	Moderate (3-*O*)
	10	Dominant (3-*O*)	No (3-*O*, 7-*O*)	Dominant (4'-*O*)	Moderate (7-*O*)	No (7-*O*, 4'-*O*)	#	No (3-*O*, 7-*O*)	Dominant (7-*O*)	Weak (3-*O*)
2B7	10	Dominant (3-*O*)	Dominant (7-*O*)	Dominant (4'-*O*)	Moderate (7-*O*)	*	#	Weak (3-*O*)	Dominant (7-*O*)	##

[a] The position shown in parentheses stands for major glucuronide. In the case of dihydroxyflavones and hydroxyflavonols, the degree of regiospecificity was determined on the basis of ratio of rates of formation of two glucuronides. In the case of trihydroxyflavones, dihydroxyflavonols, and trihydroxyflavonols, the degree of regiospecificity was determined on the basis of the ratio of rates of formation of two faster glucuronides. [1] stands for the ratio of rates of formation of 3-*O*- and 7-*O*-glucuronides. [2] stands for the ratio of rates of formation of 5-*O*- and 7-*O*-glucuronides. * No glucuronidation was detected at any hydroxyl group. # Two faster glucuronides were 3-*O* and 5-*O*, instead of 3-*O* and 7-*O*. ## Two faster glucuronides were 4′-*O* and 7-*O*, instead of 3-*O* and 7-*O*. The highlighted cells in the table represent any change in regiospecificity with change from lower (2.5μM) to higher (10μM) substrate concentration.

Statistical Analysis. One-way ANOVA or an unpaired Student's *t* test (GraphPad Prism, GraphPad Software Inc., San Diego, CA) with or without Tukey−Kramer multiple-comparison (post hoc) tests was used to analyze the statistical significance among various data. The prior level of significance was set at 5%, or $p < 0.05$.

RESULTS

Confirmation of Flavones and Flavonols Glucuronides Structure by LC-MS/MS. LC-MS/MS studies of the glucuronides of flavonoids revealed that only monoglucuronide was formed and a position-specific monoglucuronide is identified below.

Position of Glucuronides in the Structure of Flavones and Flavonols by UV Shift Method. We determined the position of glucuronidation on the basis of the diagnostic shift in λ_{max} of bands I and II of the UV spectra of glucuronides as shown in Figure S1 of the Supporting Information.

For 4′HFnol, the first glucuronide was 4′-*O*-G, whereas the second glucuronide was 3-*O*-G; for 7HFnol, the first glucuronide was 3-*O*-G, whereas the second glucuronide was glucuronidated at position C-7 (Figure S1 of the Supporting Information). In the case of 5,7DHFnol, the positions of glucuronidation of the first, second, and third glucuronides were 3-*O*-G, 7-*O*-G, and 5-*O*-G, respectively (Figure S1 of the Supporting Information). In the case of both 7,4′DHFnol and 5,7,4′THFnol (kaempferol), the positions of glucuronidation of the first, second, and third glucuronides were 7-*O*-G, 4′-*O*-G, and 3-*O*-G, respectively (Figure S1 of the Supporting Information).

The first and second glucuronides of 5,4′DHF were 5-*O*-G and 4′-*O*-G, respectively. Similarly for 5,7DHF, the first glucuronide

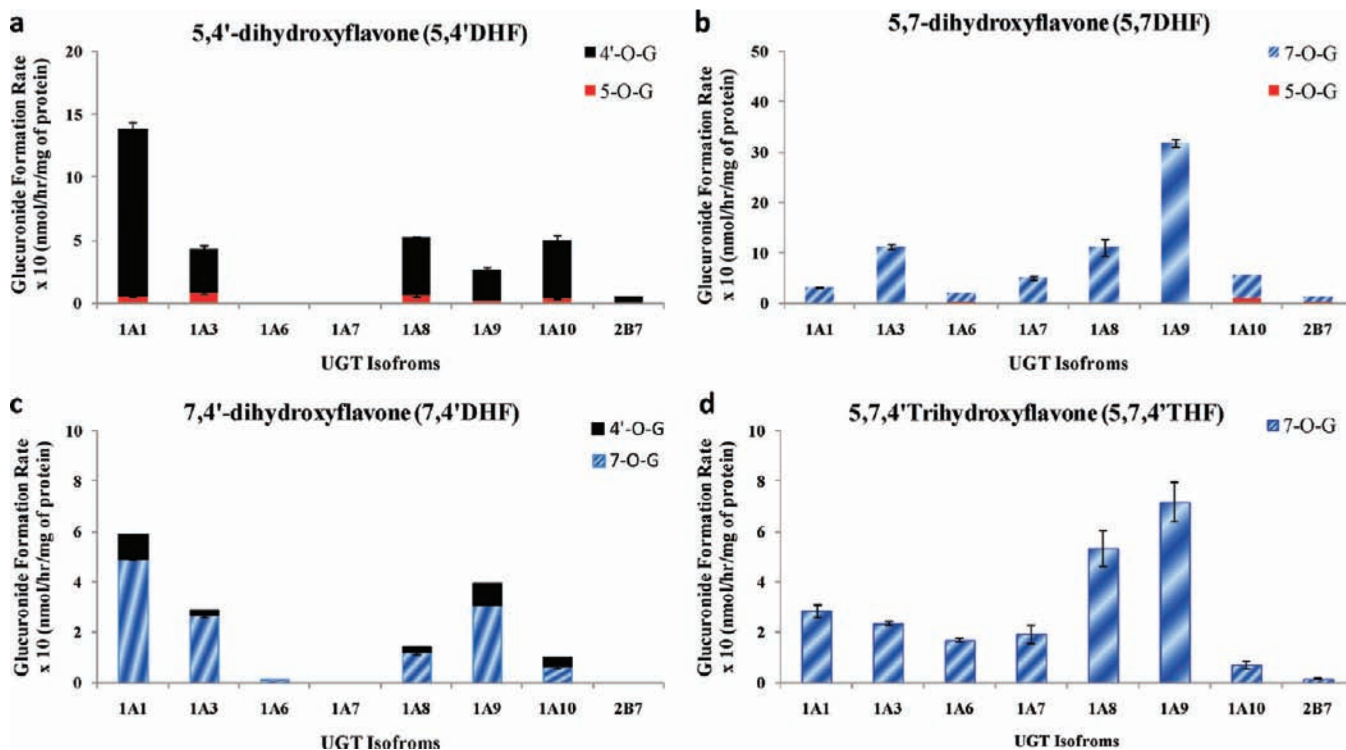

Figure 3. Regiospecific glucuronidation of flavones by uridine diphosphate glucuronosyltransferases (UGTs): rate of glucuronidation of regiospecific glucuronides of 5,4′DHF (a), 5,7DHF (b), 7,4′DHF (c), and 5,7,4′THF (d) with UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7. Flavones (at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGTs (using optimum final protein concentrations of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3).

was 5-*O*-G, whereas the second glucuronide was 7-*O*-G (Figure S1 of the Supporting Information). In the case of 7,4′DHF, the positions of conjugation of the first and second glucuronides were 7-*O*-G and 4′-*O*-G, respectively, whereas only one quantifiable glucuronide was observed in the case of 5,7,4′THF (apigenin), glucuronidated at 7-*O* position (Figure S1 of the Supporting Information).

Regiospecificity of Flavonoid Glucuronidation by UGT Isoforms. We studied the regiospecificity of flavonol and flavone glucuronidation, that is, the preference of glucuronidating a particular hydroxyl group position, by UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7. For the ease of understanding the data, the regiospecificity here is randomly defined into four categories: dominant, moderate, weak, and none. *Dominant regiospecificity* means that one hydroxyl group position in the structure is dominantly glucuronidated, such that the ratio of the most prevalent glucuronide to other glucuronide(s) is ≥9:1. *Moderate regiospecificity* means the same ratio is ≥3:1 but <9:1. *Weak regiospecificity* means that the same ratio is ≥2:1 but <3:1, whereas *no regiospecificity* means that the same ratio is <2:1. All UGT isoforms were studied for their regiospecificity for each compound on the basis of these random categories.

Flavonols. In general, most isoforms preferably glucuronidated the 3-*O* position in the structure of flavonols, followed by glucuronidation of the 7-*O* position, except 1A3, which exclusively preferred glucuronidation of the 7-*O* position (Figure 2; Table 1). However, different isoforms might display different regiospecificities depending upon the structure of compounds. Fnol was a universal substrate and could be metabolized by any of the eight UGT isoforms tested (Figure 2a). For 7HFnol, different isoforms showed great differences in their regiospecificity. UGTs 1A3 and 2B7 were dominantly regiospecific by glucuronidating the hydroxyl group only at the C-7 position, whereas UGT 1A7 dominantly glucuronidated the hydroxyl group at the C-3 position. UGTs 1A8 and 1A9 showed moderate regiospecificity for the 3-*O* position, whereas UGTs 1A1 and 1A10 showed no preference (Figure 2b; Table 1).

For 4′HFnol, most isoforms showed dominant regiospecificity by glucuronidating almost only the 3-*O* position, except UGTs 1A1 and 1A3, which showed no regiospecificity and glucuronidated both hydroxyl groups at the C-4′ and C-3 positions comparably (Figure 2c; Table 1).

In the case of 7,4′DHFnol, UGTs 1A1, 1A10, and 2B7 glucuronidated all three hydroxyl groups, whereas UGTs 1A3 and 1A9 glucuronidated the hydroxyl groups at the C-3 and C-7 positions only (Figure 2d). UGTs 1A1 and 1A10 showed no regiospecific preference for any position, whereas UGTs 1A9 and 2B7 showed moderate and weak regiospecific preferences of glucuronidating 3-*O* and 7-*O* positions, respectively. On the other hand, UGT 1A3 showed weak regiospecific preference for the 7-*O* over the 3-*O* position (Figure 2d; Table 1).

In the case of 5,7DHFnol, position(s) and regiospecificity of glucuronidation varied significantly for different isoforms (Figure 2e). UGT 1A3 was dominantly regiospecific for 7-*O*, whereas UGT 1A7 was dominantly regiospecific for 3-*O* (Figure 2e; Table 1). UGT 1A1 weakly preferred the glucuronidation of the 7-*O* position over the 3-*O* position, whereas UGTs 1A8 and 2B7 moderately preferred the glucuronidation of 3-*O* and 5-*O*, respectively (Figure 2e; Table 1). UGTs 1A9 and 1A6 did not show any regiospecific preference for any position,

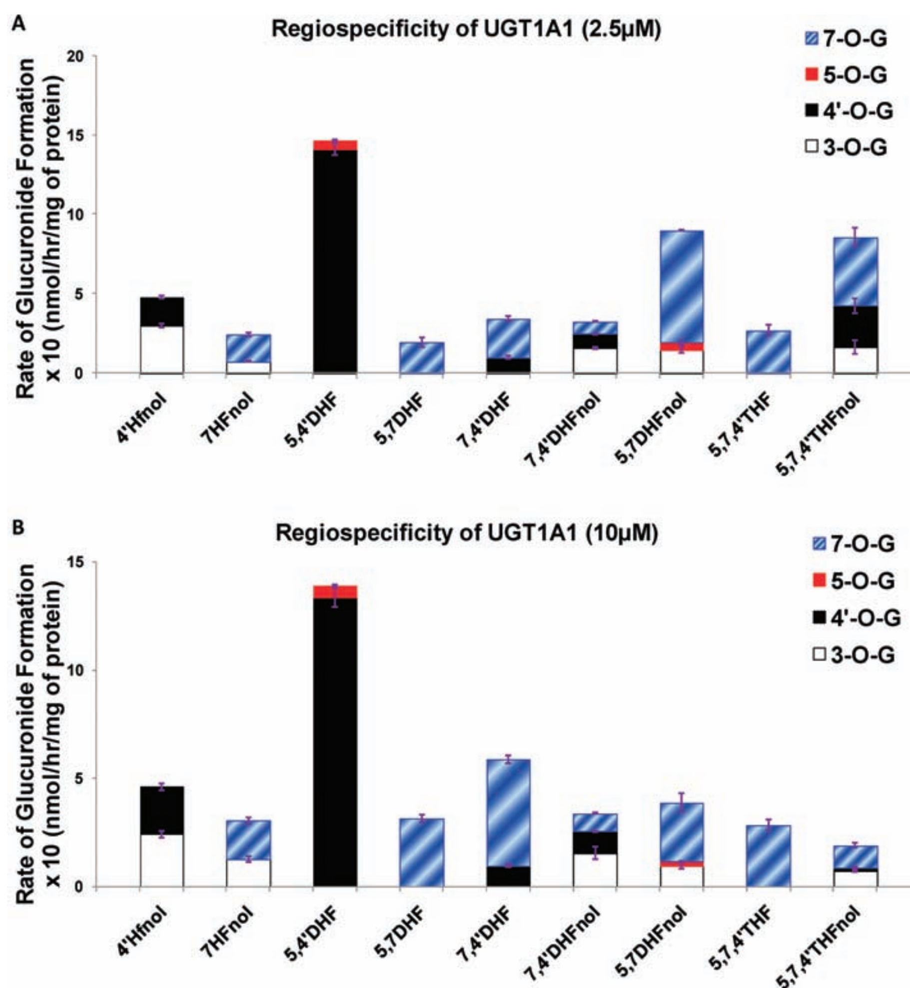

Figure 4. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A1 at 2.5 μM (A) and 10 μM (B). Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A1 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids at 10 μM have been replotted from Figures 2 and 3.

whereas UGT 1A10 weakly preferred 3-*O* over 5-*O* (Figure 2e; Table 1).

In the case of 5,7,4′THFnol, the 5-*O* position was not significantly glucuronidated by any isoform. UGTs 1A1, 1A3, 1A7, 1A8, and 1A10 glucuronidated 3-*O*, 7-*O*, and 4′-*O*, whereas UGTs 1A6 and 2B7 glucuronidated only 7-*O* and 4′-*O* and UGT 1A9 glucuronidated only the 3-*O* and 7-*O* positions (Figure 2f). No isoforms showed any dominant or moderate regiospecificity for any position, except UGT 1A6, which moderately preferred regiospecific glucuronidation of the 4′-*O* position over the 7-*O* position (Figure 2f; Table 1). UGTs 1A1 and 1A3 showed no regiospecific preference between 3-*O* and 7-*O*, whereas UGTs 1A7, 1A8, 1A9, and 1A10 only weakly preferred the 3-*O* position (Figure 2f; Table 1).

In general, UGTs 1A1, 1A6, 1A10, and 2B7 did not show any dominant regiospecificity for any position; however, in some cases, moderate or weak regiospecificity can be seen for glucuronidation of different positions of flavonols. UGT 1A3 showed dominant regiospecificity for the 7-*O* position, whereas UGT 1A7 showed dominant regiospecificity for the 3-*O* position. UGTs 1A8 and 1A9 preferably glucuronidated the 3-*O* and 7-*O* positions only, with moderate or weak regiospecificity for the 3-*O* position in most cases.

Flavones. In general, most isoforms preferably glucuronidated the 7-*O* position in the structure of flavones, followed by glucuronidation of the 4′-*O* position to some extent (Figure 3). In the case of 5,4′DHF, except for UGT 2B7, all metabolizing isoforms glucuronidated hydroxy groups at both C-5 and C-4′ positions, although 4′-*O* was the preferred position of glucuronidation in all cases. UGTs 1A1, 1A9, 1A10, and 2B7 showed dominant regiospecificity, whereas UGTs 1A3 and 1A8 showed moderate regiospecificity for the 4′-*O* position (Figure 3a; Table 1).

In 5,7DHF, UGTs 1A1, 1A3, 1A7, 1A8, and 1A9 glucuronidated the 7-*O* position only and hence showed dominant regiospecificity toward the 7-*O* position (Figure 3b; Table 1). On the other hand, UGTs 1A6, 1A10, and 2B7 glucuronidated both the 7-*O* and 5-*O* positions and showed only moderate regiospecificity toward the 7-*O* position (Figure 3b; Table 1).

In the case of 7,4′DHF, except for UGT 1A6, all metabolizing isoforms glucuronidated both hydroxyl groups at the C-7 and C-4′ positions (Figure 3c). UGTs 1A3 and 1A6 showed dominant regiospecificity for glucuronidation of the 7-*O* position,

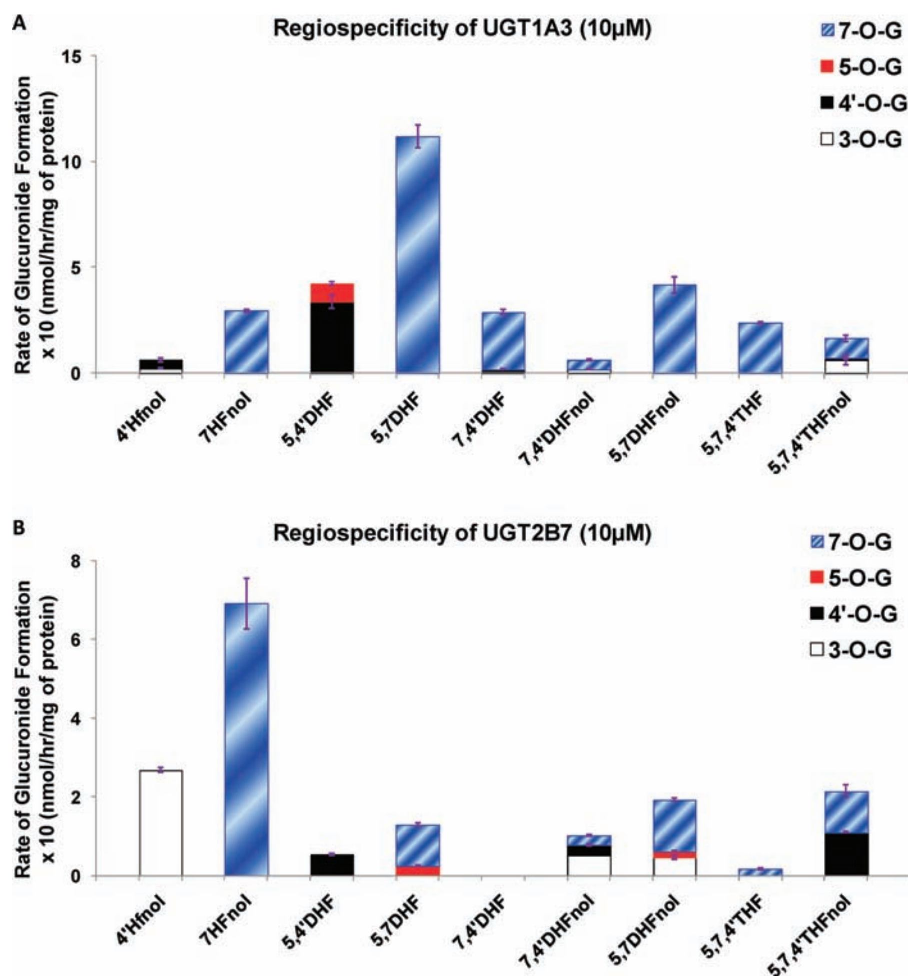

Figure 5. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A3 (A) and UGT 2B7 (B). Flavonoids (flavones and flavonols at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A3/UGT 2B7 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids have been replotted from Figures 2 and 3.

whereas UGTs 1A1, 1A8, and 1A9 showed only moderate regiospecificity for glucuronidation of the 7-*O* position (Figure 3c; Table 1). UGT 1A10 did not show any regiospecificity and glucuronidated both positions comparably (Figure 3c; Table 1). In the case of 5,7,4′THF, all isoforms showed dominant regiospecific glucuronidation of the hydroxyl group at the C-7 position only (Figure 3d; Table 1).

In summary, for flavones with the hydroxyl group at the C-7 position, most UGT isoforms showed either dominant or moderate regiospecificity for glucuronidating the 7-*O* position over the 4′-*O* and 5-*O* positions. In the absence of a hydroxyl group at the C-7 position, most UGT isoforms showed either dominant or moderate regiospecificity for glucuronidating the 4′-*O* position over the 5-*O* position.

Regiospecificity of UGT Isoforms for Flavonoid Glucuronidation. We studied in detail the regiospecific glucuronidation of flavonoids by hepatic (UGTs 1A1, 1A3, 1A9, 2B7, and 1A6) and extrahepatic (UGTs 1A7, 1A8, and 1A10) UGT isoforms using flavonoids (at 2.5 and 10 μM) with hydroxyl group(s) at different position(s), that is, 3-*O*, 4′-*O*, 5-*O*, and 7-*O*.

UGT 1A1. UGT 1A1 was able to effectively glucuronidate the 3-*O*, 4′-*O*, and 7-*O* positions in selected flavones and flavonols, but the 5-*O* position was not favored at either 2.5 or 10 μM substrate concentration (Figure 4). In the case of monohydroxyflavonols (4′HFnol and 7HFnol), UGT 1A1 did not show any regiospecific preference of glucuronidation between the 3-*O* and 4′-*O* (at 2.5 and 10 μM) or 3-*O* and 7-*O* positions (at 10 μM), but weak regiospecificity for the 7-*O* position at 2.5 μM (Figure 4; Table 1). In dihydroxyflavones, UGT 1A1 showed moderate regiospecificity at 10 μM, but weak regiospecificity at 2.5 μM for the 7-*O* position in 7,4′DHF. It showed dominant regiospecificity for the 7-*O* position in 5,7DHF and for the 4′-*O* position in 5,4′DHF at both 2.5 and 10 μM substrate concentrations (Figure 4; Table 1).

UGT 1A3. At 10 μM, UGT 1A3 dominantly preferred glucuronidation of the 7-*O* position in flavonols and flavones, wherever a free hydroxyl group was present at C-7, except for 5,7,4′THFnol, where UGT 1A3 did not show dominant regiospecificity for the 7-*O* position (Figure 5A; Table 1). When 7-OH was absent, dominant regiospecificity was absent (Figure 5A; Table 1).

UGT 2B7. At 10 μM, UGT2B7 did not show strong preference at any particular position in flavonols (Figure 5B, Table 1). However, number and position of hydroxyl group in the structure of flavonols affected which one (3-*O* or 7-*O*) or both (3-*O* and 7-*O*) are preferred position (Figure 5B, Table 1). For flavones, moderate and dominant

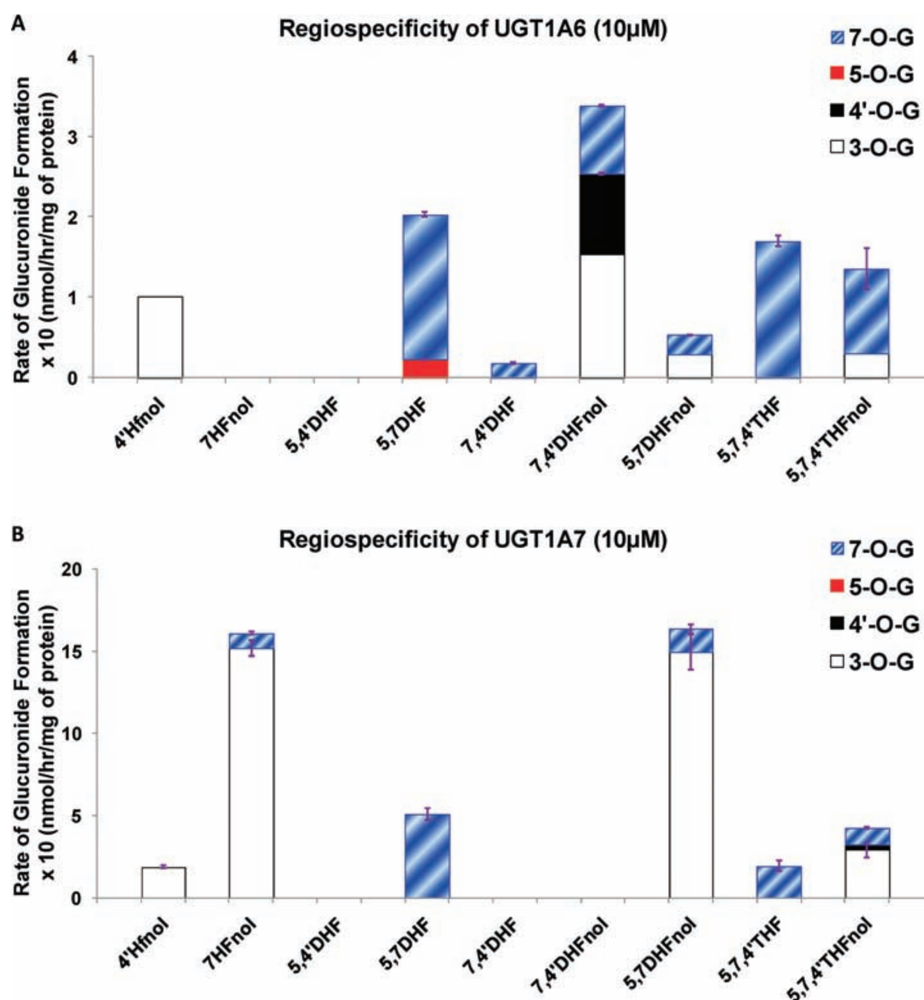

Figure 6. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A6 (A) and UGT 1A7 (B). Flavonoids (flavones and flavonols at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A6/UGT 1A7 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids have been replotted from Figures 2 and 3.

regiospecificity occurred at 7- hydroxyl group in 5,7DHF and 5,7,4′THF respectively (Figure 5B, Table 1).

UGT 1A6. At 10 μM, for flavonols, UGT 1A6 did not show any pattern for glucuronidating any particular position. In 4′HFnol, UGT 1A6 showed dominant regiospecificity for the 3-*O* position, whereas in 7HFnol and 7,4′DHFnol, no position was glucuronidated (Figure 6A; Table 1). However, UGT 1A6 showed dominant or moderate regiospecificity for 7-*O* position in flavones (Figure 6A; Table 1). The position of glucuronidation appeared to be highly affected by the number and position of the hydroxyl group in the structure of flavonoids.

UGT 1A7. At 10 μM, UGT 1A7 dominantly preferred glucuronidation of the 3-*O* position over the 7-*O* position in all flavonols (Figure 6B; Table 1), except for 5,7,4′THFnol, which showed weak preference for the 3-*O* position. However, in flavones, the 7-*O* position was dominantly favored (Figure 6B; Table 1). UGT 1A7 did not glucuronidate the 4′-*O* and 5-*O* positions at all (Figure 6B).

UGT 1A8. The glucuronidation of 4′-*O* and 5-*O* was not much favored by UGT 1A8 at 10 μM and not at all favored at 2.5 μM (Figure 7). At both 2.5 and 10 μM, UGT 1A8 showed dominant or moderate regiospecificity for glucuronidating the 3-*O* position in flavonols except in 5,7,4′THFnol, which showed only weak regiospecificity, and 7,4′DHFnol, which was not glucuronidated (Figure 7; Table 1). UGT 1A8 showed dominant or moderate regiospecificity for glucuronidating the 7-*O* position in flavones at both concentrations (Figure 7; Table 1).

UGT 1A9. UGT 1A9 mainly glucuronidated the 3-*O* and 7-*O* positions in flavonols at both 2.5 and 10 μM; 3-*O* was the more favored position than 7-*O* for glucuronidation, except for 5,7THFnol, at 10 μM, whereas 3-*O* and 7-*O* were glucuronidated at comparable rates (Figure 8; Table 1). 7-*O* was the dominantly preferred position of glucuronidation in flavones at both concentrations (Figure 8; Table 1). Glucuronidation at the 4′-*O* or 5-*O* position was not favored at all by UGT 1A9 at both concentrations (Figure 8).

UGT 1A10. UGT 1A10 was the only isoform that rapidly glucuronidated all of the positions (3-*O*, 4′-*O*, 5-*O*, and 7-*O*) in selected flavonoids. However, UGT 1A10 did not show any preference for glucuronidating any particular position (Figure 9).

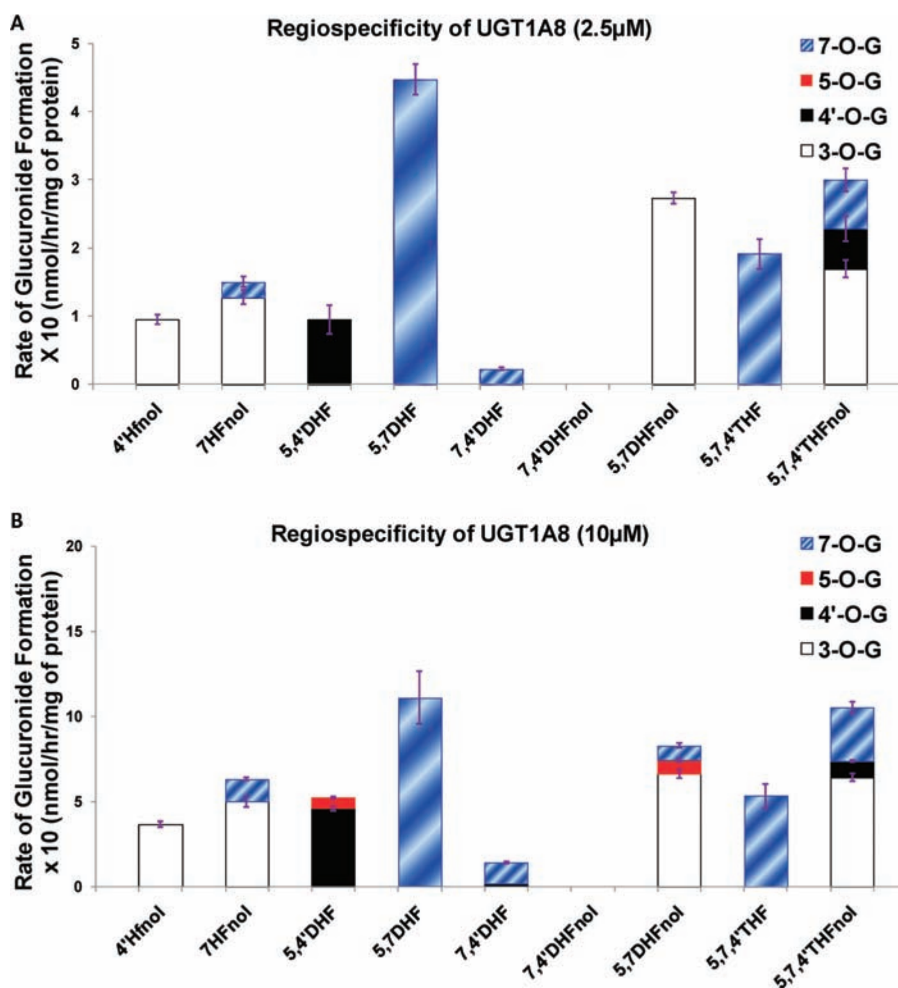

Figure 7. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A8 at 2.5 μM (A) and 10 μM (B). Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A8 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids at 10 μM has been replotted from Figures 2 and 3.

Also, the degree of regiospecificity and the position of preference of glucuronidation varied significantly for both flavonols and flavones at both 2.5 and 10 μM substrate concentrations (Figure 9; Table 1).

Effect of Higher Substrate Concentration on the Regiospecificity of UGTs 1A1, 1A8, and 1A9. We also studied the effect of higher substrate concentration (35 μM) on the regiospecificity of the three isoforms UGTs 1A1, 1A8, and 1A9. Figure 10 presents the data of two flavanols (7HFnol and 5,7,4′THFnol) as examples. We found that the ratio of rates of glucuronidation of the 3-*O* and 7-*O* positions in 7HFnol and 5,7,4′QHFnol by UGTs 1A1, 1A8, and 1A9 significantly changed with the concentration ($p < 0.05$) (Figure 10).

For 7HFnol, UGT 1A1 weakly preferred the glucuronidation of the 7-*O* position at 2.5 μM, but showed no regiospecificity at 10 and 35 μM substrate concentrations (Figure 10A). UGT 1A8 showed moderate regiospecificity for the 3-*O* position at all three concentrations (Figure 10A). UGT 1A9 showed moderate regiospecificity for the 3-*O* position at 2.5 and 10 μM, but no regiospecificity at 35 μM substrate concentration (Figure 10A).

For 5,7,4′THFnol, UGT 1A1 weakly preferred the glucuronidation of the 7-*O* position at 2.5 μM, but showed no regiospecificity at 10 and 35 μM substrate concentrations (Figure 10B). UGT 1A8 showed only weak regiospecificity at all three concentrations; however, 3-*O* was the preferred position of glucuronidation at 2.5 and 10 μM, whereas 7-*O* was the preferred position at 35 μM substrate concentration (Figure 10B). UGT 1A9 showed moderate regiospecificity for glucuronidation of the 3-*O* position at 2.5 μM, weak regiospecificity for glucuronidation of the 3-*O* position at 10 μM substrate concentrations, but no regiospecificity at the 35 μM substrate concentration (Figure 10B).

In conclusion, UGT 1A1 showed similar regiospecificities at 10 and 35 μM for both compounds but behaved differently at 2.5 μM substrate concentration. UGT 1A9 showed similar regiospecificities at 2.5 and 10 μM for both compounds but behaved differently at 35 μM substrate concentration. UGT 1A8 also showed a behavior toward 5,7,4′THFnol similar to that of UGT 1A9 but regiospecificity was independent of concentration for 7HFnol.

DISCUSSION

We concluded that regiospecific glucuronidation of flavonols was isoform- and concentration-dependent, whereas flavones

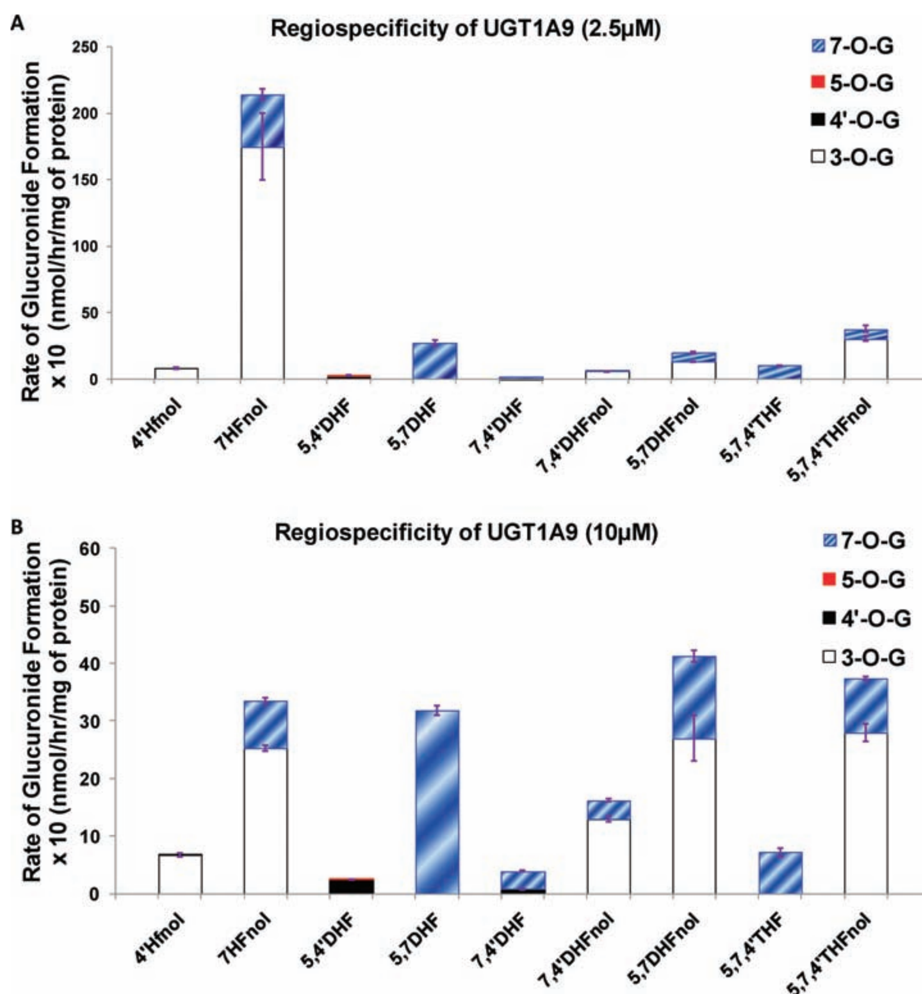

Figure 8. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A9 at 2.5 μM (A) and 10 μM (B). Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A9 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids at 10 μM have been replotted from Figures 2 and 3.

were dominantly glucuronidated at the 7-*O* position by most UGT isoforms (Figures 2−10). This is the first time that a study has demonstrated a UGT isoform-specific regiospecificity in the glucuronidation of flavonoids, although the pattern of regiospecificity may be concentration-dependent. We showed that different UGT isoforms had differences in their regiospecificity of glucuronidation of flavonoids; however, most isoforms preferred glucuronidation of the 3-*O* position followed by the 7-*O* position in flavonols, 7-*O* position in flavones, and the 5-*O* position was hardly glucuronidated (Figures 2−10). Our findings on regiospecificity are consistent with conclusions of several published studies that investigated the glucuronidation of a particular or a few flavonoids.[21−23]

We also concluded that UGTs 1A3 and 1A7 showed dominant regiospecificity for only the 7-*O* and 3-*O* positions, respectively (Figures 5A and 6B, Table 1). UGTs 1A8 and 1A9 showed moderate or weak preference on glucuronidating position 3-*O* over the 7-*O* position (Figures 7 and 8; Table 1), whereas other UGT isoforms, UGTs 1A1, 1A6, 1A10, and 2B7, showed either no positional preference or no regiospecific pattern in most cases (Figures 4, 5B, 6A, and 9; Table 1). The regiospecificity patterns for UGTs 1A3 and 1A6 were also confirmed at 2.5 μM substrate concentration (data not shown).

The dominant and moderate regiospecificity of glucuronidation shown by different UGT isoforms can be useful in predicting the major glucuronide(s) formed in human plasma. For example, chrysin (5,7DHF) is shown to predominantly form 7-*O*-glucuronide in human volunteers.[24] Chen et al. (2005) showed that the catalytic efficiency order for regiospecific glucuronidation of quercetin (3,5,7,3′,4′-pentahydroxyflavone or 5,7,3′,4′-tetrahydroxyflavonol) by UGT 1A9 was 3- > 7- > 3′- > 4′-*O*-glucuronide.[21] The fact that 3-*O*-glucuronide of quercetin was also the main glucuronide found in bioavailability studies done in humans[8,25,26] showed that in vitro regiospecificity studies done using recombinant human UGT isoforms could give a reasonable prediction of the main glucuronide(s) to be found in vivo.

Day et al. (2000) showed that a hydroxyl group on the C-5 position did not glucuronidate,[22] which was confirmed our findings. This along with the fact that there has been no published report of detecting in vivo 5-*O*-glucuronides of flavonoids supported our prediction that 5-*O* was not a favored site for glucuronidation of

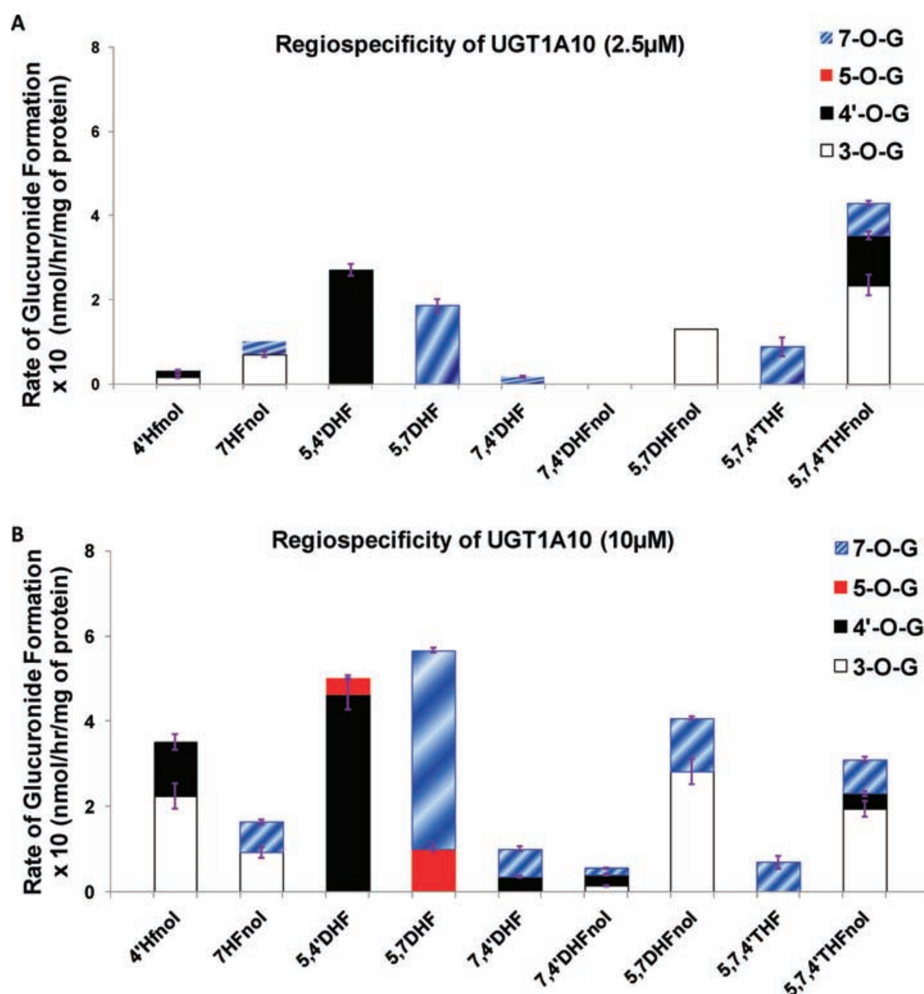

Figure 9. Regiospecific glucuronidation of flavonoids by uridine diphosphate glucuronosyltransferase (UGT) 1A10 at 2.5 μM (A) and 10 μM (B). Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A10 (using optimum final protein concentration of ~0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3). The regiospecific data of the flavonoids at 10 μM have been replotted from Figures 2 and 3.

flavonoids. Even the weak regiospecificity can give useful prediction, if most of the UGT isoforms favor the glucuronidation of only a particular position. For example, after oral intake of kaempferol (5,7,4′THFnol) in humans, the 3-*O*-glucuronide of kaempferol was found to be the predominant form in plasma,[27] which matched with our prediction based on the regiospecific glucuronidation of 5,7,4′THFnol in our study.

We also showed that the pattern and degree of regiospecificity of glucuronidation of flavonols was significantly affected by the substrate concentrations (Figures 4 and 7−9). For flavones, the preferred site of glucuronidation was independent of concentration used; however, the degree of regiospecificity could change in certain cases (Figures 4 and 7−9). The differences in the in vivo cellular substrate concentration and the UGT isoform expression in various organs[14] could significantly affect the various regiospecific glucuronide(s) formed in an organ. Yang et al. (2010) showed that the C_{max} of 7-*O*-glucuronide was 4 times higher than that of 4′-*O*-glucuronide during intravenous administration of genistein, when the liver was the first-pass metabolic organ. On the other hand, the C_{max} of 7-*O*-G was only 1.85 times higher than that of 4′-*O*-glucuronide during oral administration of genistein, when the intestine was the first-pass metabolic organ.[28]

This could have therapeutic implications in the case of localized treatment, as the total concentration of a particular flavonoid available in an organ will be dependent on the rate of glucuronidation of the flavonoid and the rate of hydrolysis of various regiospecific glucuronide(s) back into the flavonoid in that organ.[9,10] For example, O'Leary et al. (2001) showed that the activity of β-glucuronidase found in human liver extract in hydrolyzing quercetin glucuronides was twice the activity of β-glucuronidase found in human intestinal extract.[29] Also, the ratio of k_{cat}/k_m of the human recombinant β-glucuronidase against hydrolysis of 3-*O*-glucuronide of quercetin was highest, followed by 7-*O*-G and 4′-*O*-G.[29]

In conclusion, the regiospecificity of glucuronidation of flavonols was an isoform- and concentration-dependent phenomenon, whereas most UGT isoforms showed dominant to moderate regiospecificity for the 7-*O* position in glucuronidating flavones. For flavonols, certain hepatic and extrahepatic UGT isoforms

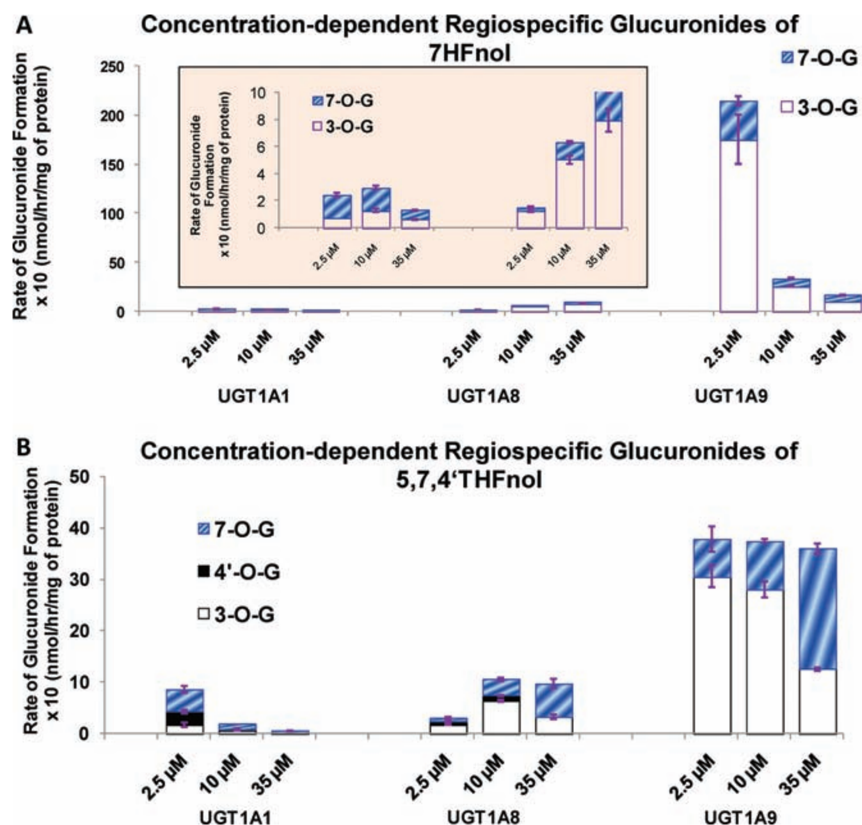

Figure 10. Concentration-dependent regiospecific glucuronidation of 7HFnol (A) and 5,7,4′THFnol (B) by uridine diphosphate glucuronosyltransferases (UGTs) 1A1, 1A8, and 1A9. 7HFnol and 5,7,4′THFnol (at 2.5, 10, and 35 μM concentrations) were incubated at 37 °C for 1 (or 0.5) h with UGT 1A1, 1A8 or 1A9 (using optimum final protein concentration of ∼0.25, 0.5, or 1 mg/mL). The amounts of each regiospecific monoglucuronide formed were measured using UPLC. Rates of monoglucuronide formation were calculated as nmol/h/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n = 3).

showed dominant regiospecificity for one position such as UGT 1A3 for the 7-*O* and UGT 1A7 for the 3-*O* position, whereas isoforms such as UGTs 1A8 and 1A9 mostly preferred glucuronidation at the 3-*O* position followed by the 7-*O* position. The rest of the UGT isoforms did not prefer glucuronidating any particular phenolic group. We believe that these insights about isoform-specific regiospecificity will be a great help in isoform-specific future in silico modeling of the structure−metabolism relationship of glucuronidation of flavonoids/substrates by UGT enzymes. In a recent publication, Wu et al. (2011) successfully developed a pharmacophore-based CoMFA model for the prediction of UGT 1A9-mediated formation of 3-*O*-glucuronide of flavonols using the recombinant human UGT 1A9 isoform.[30] Further studies are required to delineate the protein structural differences among the UGT isoforms that are responsible for the dominant or moderate regiospecificity for a particular OH group in the flavonoids.

ASSOCIATED CONTENT

S Supporting Information. UPLC chromatograms and UV spectras of 3-hydroxyflavone or flavonol (Fnol), 3,4′-dihydroxyflavone or 4′-hydroxyflavonol (4′HFnol), 3,7-dihydroxyflavone or 7-hydroxyflavonol (7HFnol), 5,4′-dihydroxyflavone (5,4′DHF), 5,7-dihydroxyflavone (5,7DHF), 7,4′-dihydroxyflavone (7,4′DHF), 3,7,4′-trihydroxyflavone or resokaempferol or 7,4′-dihydroxyflavonol (7,4′DHFnol) and 3,5,7-trihydroxyflavone or galangin or 5,7-dihydroxyflavonol (5,7DHFnol), 5,7,4′-trihydroxyflavone or apigenin (Api or 5,7,4′THF), 3,5,7,4′-tetrahydroxyflavone or kaempferol or 5,7,4′-trihydroxyflavonol (5,7,4′THFnol or Kamp) and their glucuronides. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

UGT, uridine diphosphate glucuronosyltransferases; UDP, uridine diphosphate; UDPGA, uridine diphosphoglucuronic acid; UPLC, ultra performance liquid chromatography; MS, mass spectroscopy; UV, ultraviolet; HF, hydroxyflavone; DHF, dihydroxyflavone; THF, trihydroxyflavone; Fnol, flavonol; HFnol, hydroxyflavonol; DHFnol, dihydroxyflavonol; THFnol, trihydroxyflavonol; and OH, hydroxyl.

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