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Use of Glucuronidation Fingerprinting to Describe and Predict mono- and di-Hydroxyflavone Metabolism by Recombinant UGT Isoforms and Human Intestinal and Liver Microsomes

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Abstract

The present study aims to predict the regiospecific glucuronidation of three dihydroxyflavones and seven mono-hydroxyflavones in human liver and intestinal microsomes using recombinant UGT isoforms. Seven mono-hydroxyflavones (or HF), 2'-, 3'-, 4'-, 3-, 5-, 6-, and 7-hydroxyflavone, and three di-hydroxyflavones (or diHFs), 3,7-dihydroxyflavone (3,7-diHF), 3,5-dihydroxyflavone (3,5-diHF) and 3,4'-dihydroxyflavone (3,4'-diHF) were chosen and rates were measured at 2.5, 10 and 35 μ M. The results indicated that the position of glucuronidation of three diHFs could be determined by using the UV spectra of relevant HF. The results also indicated that UGT1A1, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 are the most important six UGT isoforms for metabolizing the chosen flavones. Regardless of isoforms used, 3-HF was always metabolized the fastest whereas 5-HF was usually metabolized the slowest, probably due to the formation of an intra-molecular hydrogen bond between 4-carbonyl and 5-OH group. Relevant UGT isoform-specific metabolism rates generally correlated well with the rates of glucuronidation in human intestinal and liver microsomes at each of the three tested concentrations. In conclusion, the glucuronidation "fingerprint" of seven selected mono-hydroxyflavones was affected by UGT isoforms used, positions of the -OH group, and the substrate concentrations, and the rates of glucuronidation by important recombinant UGTs correlated well with those obtained using human liver and intestinal microsomes.

Introduction

Flavonoids are widely distributed in a variety of natural plants and edible foods including fruits and vegetables. They appear to possess significant biological activities in the areas of anticancer, anti-inflammation, and in prevention of coronary heart diseases and other age-related illnesses ^{1,2}. Despite of these promises, no chemopreventive agent has been approved using this class of compounds, perhaps because their low bioavailabilities impede their development into chemopreventive agents ^{3,4}. Many studies including our own have demonstrated that extensive first-pass metabolism by phase II conjugating enzymes including UGTs and SULTs is the most important reasons for their low bioavailabilities ^{5,6}.

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Flavones and flavonols, two subclasses of flavonoids, are known to have low oral bioavailabilities⁷. It was reported that flavonols such as kaempferol and quercetin were mainly present as glucuronides in human plasma after oral administration^{8,9}, although significant amounts of sulfates were present as well⁹. In the rat intestine, a significant portion of the absorbed aglycones such as quercetin and apigenin was conjugated and the metabolites (mostly glucuronides) were excreted into the lumen¹⁰. Thus, the published studies provided strong evidence that rapid conjugation via glucuronidation and sulfation is the main reason for their low bioavailabilities *in vivo*.

Whereas it was possible to identify the main cause of poor bioavailability experimentally, a more desired course of action would be to predict glucuronidation and sulfation of flavonoids. Previously, it was shown that glucuronidation of hydroxyflavones in human jejunum S9 was affected by the structure of flavones, especially the positions of phenolic group^{11,12}. Our earlier study showed that glucuronidation of isoflavones are also affected by structural changes and the position of the phenolic group¹³, although the latter effect is distinctively different from that of flavones shown by other investigators^{11,12}. In any rate, we believed that the prediction can be achieved via several methods with increasing utility, degree of difficulty and broad structure features; (1) use of isoform-specific metabolism data (i.e., glucuronidation fingerprint) to predict organ specific glucuronidation; (2) use of the glucuronidation of a simpler flavone (e.g., 3-HF) to predict glucuronidation of more complex flavone (e.g., 3,5-diHF); (3) statistical modeling using experimental values and regression analysis of a large database set; (4) computational modeling using 3-D chemical structures incorporating conformations, again using large dataset. In the present study, our focus is on the first two areas as the latter two approaches are very different.

The first method of prediction uses glucuronidation fingerprint, which is useful because a flavone (e.g., eupatilin) was shown to be glucuronidated by specific UGT isoforms¹⁴. It is also possible to predict with high degree of success the glucuronidation of isoflavones using isoform-specific glucuronidation fingerprint¹³. Because fingerprinting the glucuronidation may be used to gauge the safety and efficacy concerns arisen from potential drug-drug interactions, this method is further expanded here to determine if they can be applied to flavones as well.

The second method of prediction is to use the data derived from simpler mono-hydroxyflavones to predict the metabolism behaviors of more complex flavones such as diHFs. To achieve this objective, the present investigation determined if it was possible to predict the regiospecific glucuronidation of diHFs via the use of multiple HFs, which had not been done previously.

The present study has chosen the following 7 mono-hydroxyflavones (or HFs): 2'-hydroxyflavone (2'-HF), 3'-hydroxyflavone (3'-HF), 4'-hydroxyflavone (4'-HF), 3-hydroxyflavone (3-HF), 5-hydroxyflavone (5-HF), 6-hydroxyflavone (6-HF) and 7-hydroxyflavone (7-HF), because they represent almost all HFs possible, although 8-hydroxyflavone was not used here because it was not commercially available. These seven HFs were used along with three di-hydroxyflavones: 3,5-diHF, 3,7-diHF and 3,4'-diHF to test the predictability of the first two prediction methods described previously.

Materials and Methods

Materials

Seven hydroxyflavones were all purchased from LC Laboratories (Woburn, MA). Expressed human UGT isoforms (Supersomes™), pooled female human liver and intestinal microsomes were purchased from BD Biosciences (Woburn, MA). Uridine

diphosphoglucuronic acid (UDPGA), β -glucuronidase, alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (HBSS, powder form) were purchased from Sigma-Aldrich (St Louis, MO). All other materials (typically analytical grade or better) were used as received.

Enzymatic Activities of Expressed UGTs

The incubation procedures for measuring UGTs' activities were essentially the same as published before ^{5,15}. Briefly, incubation procedures using microsomes or UGT SupersomesTM were as follows: (1) microsomes (final concentration $\approx$ in range of 0.0053~0.053 mg of protein per mL based on optimization of 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, add last) were mixed; (2) the mixture (final volume) 200 μ L 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 μ L of 94% acetonitrile/6% glacial acetic acid containing 100 μ M testosterone as the internal standard. For UGT profiling, three concentrations, 2.5, 10 and 35 μ M, were used.

UPLC Analysis of Hydroxyflavones and Their Glucuronides

We analyzed seven HF, three diHF and their respective glucuronides by using the following common method: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C18, 1.7 μ m, 2.1 $\times$ 50 mm; mobile phase B, 100% acetonitrile, mobile phase A, 100% aqueous buffer (2.5mM NH₄Ac, pH 7.4); flow rate 0.45 mL/min; gradient, 0 to 2.0 min, 10–35% B, 2.0 to 3.0 min, 35–70% B, 3.2 to 3.5 min, 70–10% B, 3.5 to 4.0 min, 10% B, wavelength, 254 nm for HF, 3,4'-diHF, 3,7-diHF and their respective glucuronides and testosterone; 263 nm for 3,5-diHF and its glucuronide, injection volume, 10 μ L. Linearity was established in the range of 0.78–50 μ M for all compounds. The LLOQ for all compounds was at least 0.78 μ M. The analytical method for each compound was validated for inter-day and intra-day variations using 6 samples at each of the three concentrations (50, 12.5 and 1.56 μ M). Precision and accuracy for all compounds were in acceptable range (<15%).

Confirmation of Hydroxyflavone and Dihydroxyflavone Glucuronide Structure by LC-MS/MS

Seven HF, three diHF and their respective glucuronides were separated by the same UPLC-MS/MS system but using slightly different chromatographic conditions because of mass spectrometer requirements. Here, mobile phase A was an ammonium acetate buffer (pH 7.4) and mobile phase B was 100% acetonitrile with the gradient as follows: 0 to 2.0 min, 10–35% B, 2.0 to 3.0 min, 35–70% B, 3.2 to 3.5 min, 70–10% B, 3.5 to 4.0 min, 10% B. The flow rate was 0.45 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 TurboIonSprayTM source. The mass spectrometer was operated in the negative ion mode to perform the analysis of six flavones and their glucuronides. The main working parameters for the mass spectrometers were set as follows: ion-spray voltage, -4.5 kV; ion source temperature, 400°C; nebulizer gas (gas1), nitrogen, 40 psi; turbo gas (gas2), nitrogen, 40 psi; curtain gas, nitrogen, 20 psi and minor adjustments were then made for each flavone. Flavone mono-glucuronides were identified by MS and MS2 full scan modes (Fig.2)

Determination of molar extinction coefficient ratio (ECR) for the glucuronides of seven HF and three diHFs

In order to provide more precise estimation on the concentrations and glucuronidation rates of flavones in the current study, a conversion factor (K), which represents the ratio between the molar extinction coefficient of the glucuronide and its aglycone, was determined for each flavone. To calculate the conversion factor K, glucuronides of HF were prepared using Caco-2 cell monolayers. The culture conditions for growing Caco-2 TC7 cells have been described previously^{16,17}. Briefly, the seeding density was 250,000 cells/ml (2 ml for each well in a 6-well plate), and the cells were grown in a regular growth medium (DMEM supplemented with 10% fetal bovine serum). Caco-2 TC7 cells were fed every other day, and the monolayer was ready for experiments at 11 days after seeding. On 12th day, the Caco-2 TC7 cells were washed with HBSS (pH7.4, 37°C) three times. After the HBSS buffer were aspirated out, 2.5ml solution of a HF (10 μ M, n=3) was added to each monolayer and allowed to incubate for 8 hr at 37°C. The supernatant was then removed and extracted with methylene chloride twice (sample/dichloromethane=1:3, v/v) to remove >95% of flavone aglycones. The resulting sample was then divided into two parts, one of which was analyzed directly and the other analyzed after being hydrolyzed with β -glucuronidase (100 units/ml) at 37°C for 2h. The difference in amount of aglycones found in these two samples was the amount of metabolite formed. The relationship between the peak areas of the metabolites before hydrolysis and the peak areas of aglycones after the hydrolysis is used to establish the conversion factor used to quantify the amounts of flavone conjugates as described previously^{16,18}. For diHFs, mono-glucuronides of dihydroxyflavones were prepared using selected UGT isoform (s) based on UGT fingerprint information (i.e., only one glucuronide was produced at each instance). The conversion factor (K) was then similarly calculated as described previously for HF. In quantification of metabolites, a previously published method in our labs was used¹³.

Statistical Analysis

One-way ANOVA with or without Tukey-Kramer multiple comparison (posthoc) tests were used to evaluate statistical differences. Differences were considered significant when *p* values were less than 0.05 (or *p*<0.05).

Results

Confirmation of Glucuronide Formation by LC-MS/MS

We conducted LC-MS/MS studies of the metabolites of all flavones (Fig.1) used in the present studies to show that all glucuronides formed using the expressed human UGTs were mono-glucuronides (Fig.2, 3). Although no di-glucuronide of dihydroxyflavones was found, a second but minor mono-glucuronide was found for all three diHFs.

Determination of the position of glucuronidation for 3,7-diHF, 3,5-diHF and 3,4'-diHF

All three diHFs have two glucuronides. Because there is a lack of commercial standards of flavone glucuronides, it was necessary to determine the position of mono-glucuronide using the UV spectra. The site of glucuronic acid substitutions in flavonol(s) was established using UV peak shift method¹⁹. In general, the UV spectrum of flavones have two λ_{max} 's, one at 300 nm plus region (λ_{max1}), and the other at 240 nm region (λ_{max2}). For 3-HF, glucuronidation at the 3-OH position severely diminished the peak at 343.7 nm, causing λ_{max1} to blue shift (i.e., move λ_{max1} to a shorter wavelength) approximately 34 nm. For 7-HF and 4'-HF, the blue shift was no change or only 10 nm around λ_{max1} and shift around λ_{max2} was minimal. Glucuronidation of 5-hydroxyl group for 5-HF resulted in major change in UV spectrum with blue shift in both λ_{max1} (a large 35 nm shift) and λ_{max2} (a smaller 8 nm

shift), suggesting glucuronidation at this site significantly altered electron conjugation of the flavone ring structure. Identification of glucuronide position using the shift in $\lambda_{\max 1}$ and $\lambda_{\max 2}$ values was shown in Figure 4, and the extinction coefficients ratios of all HFs and diHFs were listed in Table 1.

For diHFs, a similar approach was used based on the shift at $\lambda_{\max 1}$ and $\lambda_{\max 2}$ regions. For 3,7-diHF, when the $\lambda_{\max 1}$ region of the UV spectrum of a glucuronide showed a diminished peak and a blue shift of nearly 28 nm, this metabolite was assigned to 3-O-glucuronide. Similarly, because $\lambda_{\max 1}$ and $\lambda_{\max 2}$ regions of the second mono-glucuronide showed no change when compared to 3,7-diHF, this metabolite was assigned to 7-O-glucuronide (Figure 4, E1~E3).

For 3,4'-diHF, metabolite 1 showed a large $\lambda_{\max 1}$ shift of 21 nm, it was assigned to 3-O-glucuronide of 3,4'-diHF, whereas metabolite 2 showed a hypsochromic (or red) shift at $\lambda_{\max 2}$ region of 7.1 nm and nearly no shift at $\lambda_{\max 1}$ region when compared to aglycone, this metabolite was assigned as 4'-O-glucuronide of 3,4'-diHF (Figure 4, F1~F3). Using a similar methodology for 3,5-diHF, metabolite 1 that caused a major blue shift at $\lambda_{\max 1}$ region was assigned to 3-O-glucuronide whereas the other one was assigned to 5-O-glucuronide (Figure 4, G1~G3). This identification of glucuronide position is important because it provided the basis for determine molar extinction coefficient ratios as shown in Table 1.

Glucuronidation “fingerprints” of 4 HFs by 12 expressed human UGTs

We incubated the selected four mono-hydroxyflavones (3-HF, 4'-HF, 5-HF and 7-HF) at three concentrations (2.5, 10, 35 μ M) with 12 human UGT isoforms (Figure 5). It was clear that each HF had a distinctive UGT isoform fingerprint and this fingerprint often changed with the concentration of the substrate used, sometimes drastically as in the case of 3-HF.

By examining the contributions of individual UGT isoform, UGT1A1 was found to be one of the main isoforms for 4 selected HFs at all concentrations. For 4'-HF, UGT1A10 contributed to its glucuronidation the most, UGT1A1 contributed at 10 μ M and 35 μ M, and UGT1A3 generated a detectable glucuronide of 4'-HF at 35 μ M (Fig.5A). For 3-HF, UGT1A7, 1A9, 1A8, 1A1, 2B7 and UGT1A10 were the top 6 isoforms at 10 and 35 μ M, whereas UGT1A3, 1A6 and 2B15 all made small and variable contributions (Fig.5D). For 7-HF, UGT1A1 was the top UGT isoform, followed by UGT1A9 and UGT1A8 at 2.5 μ M. The rank-order changed to 1A3, 1A1, 1A9, 1A8, 1A7 and 1A10 at 10 μ M, and to 1A3, 1A9, 1A8, 1A1, 1A10, 1A7 at 35 μ M (Fig.5B). For 5-HF, UGT1A1, 1A8, 1A9, 1A10 and 2B7 generated a detectable glucuronidation at three concentrations, whereas the other UGT isoforms did not metabolize 5-HF (Fig.5C). Taken together, the glucuronidation of HFs was compound-, concentration-, and isoform-dependent.

Structural-Specific Metabolic Patterns of Seven Hydroxyflavones by Six UGT isoforms

As shown in Figure 5, human recombinant UGT1A1, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 isoforms were the most important isoforms for metabolizing HFs. Additionally, UGT1A1, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 are highly expressed in human liver and/or intestinal microsomes. Therefore, these six UGT isoforms probably play the most important role in metabolizing all the HFs. To highlight the importance of these six isoforms, the data from the previous 4 HFs were re-plotted together with glucuronidation rates of 3 additional HFs to first show how changes in structures affected glucuronidation. The results showed that at all concentrations, 3-HF was metabolized the fastest and at all concentrations by all isoforms among the seven mono-hydroxyflavones studied, whereas 5-HF or 4'-HF was usually metabolized the slowest (Fig.

6). On the other hand, the middle of the rank order often changed with isoform or concentration, sometimes drastically. In fact, formation of glucuronidation metabolite of 4'-HF was not detected when UGT1A7, UGT1A8 UGT1A9 or UGT2B7 was used, even though the same compound was fairly rapidly metabolized by UGT1A1. More detailed accounts for each UGT isoform-specific structural effects were presented below.

For **UGT1A1**, at 2.5 μ M substrate concentration, glucuronidation of 3-HF was the fastest (2.68 ± 0.47 nmol per min per mg of protein), followed by 7-HF (1.73 ± 0.21), 3'-HF (1.59 ± 0.26), 6-HF (1.50 ± 0.18), 4'-HF (1.34 ± 0.21), 2'-HF (1.28 ± 0.12) and 5-HF (0.28 ± 0.00). At 10 μ M and 35 μ M substrate concentrations, the trends changed somewhat but 3-HF was still glucuronidated the fastest (3.80 ± 0.21 and 3.85 ± 0.12) and 5-HF the slowest (0.28 ± 0.01 and 0.308 ± 0.02).

For **UGT1A7**, at 2.5 μ M substrate concentration, the rank order of glucuronidation for seven hydroxyflavones was 3-HF (8.27 ± 0.15) > 3'-HF (1.44 ± 0.20) > 2'-HF (1.13 ± 0.11) > 7-HF = 5-HF = 4'-HF = 6-HF (0 nmol per min per mg of protein). At 10 μ M and 35 μ M, the rank order of glucuronidation was approximately the same, and the glucuronidation rates were mostly the same as well.

For **UGT1A8**, at 2.5 μ M, glucuronidation rates difference between various HF's were large since some was not metabolized at all. The rank order was 3-HF (2.19 ± 0.27 nmol per min per mg of protein, the fastest), followed by 2'-HF (1.18 ± 0.26), 6-HF (0.87 ± 0.12), 5-HF (0.28 ± 0.04), 3'-HF (0.27 ± 0.02), 7-HF (0.23 ± 0.03), and 4-HF (0.00 ± 0.00). At 10 μ M, the rank order in the middle changed and the magnitude of the difference also increased. At 35 μ M, there was an even bigger difference between the slowest and the fastest glucuronidation rates, which were also accompanied by a big change in the middle of the rank order.

For **UGT1A9**, at 2.5 μ M substrate concentration, 3-HF was still glucuronidated at the fastest rate (3.03 ± 0.04 nmol per min per mg of protein), followed by 7-HF (1.30 ± 0.09), 3'-HF (0.48 ± 0.13), 2'-HF (0.43 ± 0.06), 5-HF (0.29 ± 0.01), and 6-HF (0.10 ± 0.00), whereas metabolism of 4'-HF was not detectable. At 10 μ M substrate concentrations, the rank order did not change much. At 35 μ M, 4'-HF still showed no detectable metabolism, but the compound with the slowest glucuronidation rate changed from 6-HF to 5-HF.

For **UGT1A10**, there was no detectable metabolism at 2.5 μ M due to slow rate of glucuronidation for any of the seven HF's. At 10 μ M substrate concentration, the rank order of the UGT1A10-mediated glucuronidation rates (nmol per min per mg of protein) was 3-HF (1.37 ± 0.19) > 3'-HF (0.86 ± 0.08) > 6-HF (0.62 ± 0.07) > 4'-HF (0.30 ± 0.05) > 7-HF (0.17 ± 0.03) > 5-HF (0.12 ± 0.01) > 2'-HF (0.09 ± 0.01). At 35 μ M, the rank order in the middle changed somewhat but the maximum and minimum were maintained.

For **UGT2B7**, only glucuronidation of 3-HF and 5-HF were measurable at all three tested concentrations (2.5 μ M, 10 μ M and 35 μ M). The glucuronidation rates of 3-HF were always much higher than 5-HF, which were 1.39 ± 0.14 , 5.35 ± 0.91 and 2.19 ± 0.07 (nmol per min per mg of protein) compare to 0.20 ± 0.01 , 0.40 ± 0.02 and 0.11 ± 0.00 (nmol per min per mg of protein, $p < 0.05$) at 2.5 μ M, 10 μ M and 35 μ M, respectively. This isoform did not metabolize other five HF's.

Concentration-dependent glucuronidation of seven hydroxyflavones by expressed human UGT1A1, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7

To highlight the importance of concentration on the metabolism of 7 HF's by six UGT isoforms, the data from the previous 4 HF's were re-plotted together with glucuronidation

rates of 3 additional HF to first show the effects of concentration change on the rates of metabolism for each flavone (Fig. 7). It was shown that most glucuronidation rates increased with an increase in concentration but a few did not change or even decreased as the concentration increased.

Glucuronidation of HF by Human Liver Microsomes

The rates of glucuronidation of the selected hydroxyflavones by human liver microsomes were determined at three concentrations of 2.5 μ M, 10 μ M and 35 μ M (Fig.8A). The results of this study showed that the rate of glucuronidation of 3-HF was always the fastest of all hydroxyflavones at all of the three substrate concentrations, while 5-HF was glucuronidated the slowest by human liver microsomes, which were consistent with the UGT isoform data.

At 2.5 μ M substrate concentration, the rank order of the glucuronidation rates (nmol per min per mg of protein) was 3-HF (5.04 $\pm$ 0.48) > 3'-HF (3.14 $\pm$ 0.01) > 7-HF (1.39 $\pm$ 0.01) > 2'-HF (0.97 $\pm$ 0.03) > 6-HF (0.91 $\pm$ 0.04) > 4'-HF (0.80 $\pm$ 0.05) > 5-HF (0.00 $\pm$ 0.00). At 10 μ M substrate concentration, the rank order in the middle changed and the same order was observed at 35 μ M substrate concentration.

The results clearly indicated that at different concentrations, there were significant differences in the glucuronidation of the seven hydroxyflavones by human liver microsomes ($p < 0.05$, one way ANOVA) (Fig. 8A). In addition, the effects of concentration also showed the trend as we observed in UGT isoforms that glucuronidation rates increased with concentrations.

Lastly, we plotted rates of glucuronidation by liver microsomes against rates of glucuronidation by UGT1A1 and UGT1A9. UGT1A1 and UGT1A10 were chosen for the plot because they are known to be expressed at high level in human liver²⁰ and these two isoforms also metabolized most if not all seven flavones albeit at different rates (Fig.6 and Fig.7). In contrast, UGT1A7, 1A8 and 1A10 are poorly expressed in human liver, and UGT2B7 only prefers 3-HF. The results indicated that isoform-specific metabolism rates correlated well with liver microsome metabolism rates at each of the three tested concentrations with the exception of 3'-HF (Fig.8 B1~B3). The slopes of all three correlative curves were close to 1.

Hydroxyflavone Glucuronidation by Human Intestinal Microsomes

When compared to glucuronidation by human liver microsomes, human intestinal microsomes usually metabolized the hydroxyflavones much faster in a per mg microsomal protein basis (Fig.9A). The HF-specific profiles look like a “right-triangle” at all three concentrations, which is different from the HF glucuronidation profile in human liver microsomes.

At all three substrate concentrations (2.5 μ M, 10 μ M and 35 μ M), when metabolism rates of seven hydroxyflavones in intestine were compared, we found that 3-HF were always metabolized the fastest (12~18 nmol per min per mg of protein), followed by 3-HF, 6-HF, 3'-HF, 4'-HF, 7-HF, 2'-HF and 5-HF. At 2.5 μ M substrate concentration, the magnitude of the difference was 25 folds (12.35 $\pm$ 1.32 vs. 0.49 $\pm$ 0.06), and this magnitude of difference was essentially maintained at all three concentrations (Fig.9A).

To determine the level of correlation, intestinal glucuronidation rates were plotted against the rates of metabolism via UGT1A1+UGT1A8+UGT1A10 at three different concentrations (Fig.9B1~B3). UGT1A1, UGT1A8 and UGT1A10 were chosen for the plot because they are known to be expressed at high level in human intestine²⁰ and these three isoforms also metabolized most if not all seven flavones albeit at different rates (Fig.6 and Fig.7). In

contrast, UGT1A7 and 1A9 are poorly expressed in human intestine, and UGT2B7 only prefers 3-HF. The results of correlation analysis indicated that the slope is shown to be highly variable, and only slope at 35 μ M was close to 1, whereas the slopes at the other two lower concentrations were much bigger (2 and above).

Isoform-Specific Glucuronidation of 3 diHF's at 3 Concentrations

The selected diHFs (3,4'-HF, 3, 5-HF and 3, 7-HF) were incubated at three concentrations (2.5, 10, 35 μ M) with 12 human UGT isoforms. The formation rates of two mono-glucuronides at three concentrations were quantified (Figure 10). First of all, the formation rates of 3-O-G glucuronide was almost always higher than formation rates at other -OH position for all three tested concentrations, but there were occasional exceptions. For example, UGT1A3 and UGT1A4 were shown unexpectedly to form 7-O-glucuronide rapidly, which occurred at 10 and 35 μ M. For all three HF's, the fingerprints for different phenolic positions of the flavones were always different from each other. Moreover, more isoforms appeared to become active when an extra -OH group was added to the main backbone, regardless whether the -OH group being glucuronidated is at the 3-OH or other position. A detailed description for each diHF followed.

For 3,7-diHF, its isoform fingerprint at 7-OH position was maintained at three concentrations but its isoform-specific fingerprint at 3-OH position was suddenly changed at 35 μ M (Fig.10A), when two additional isoforms unexpectedly became active. This effect was observed in two independent experiments done at different time.

For 3,4'-diHF, the isoform-specific fingerprints were usually the same across three concentrations for each glucuronidation position (Fig.10B). Hence, metabolism at 3-OH position of the diHF followed similar trend as did the metabolism at the 4'-OH position.

For 3,5-diHF, metabolism at the 3-OH position dominated and isoform-specific fingerprint were similar for all three concentrations although the rates at 35 μ M were unexpected high (rate increased exceeded proportional increases in concentrations) (Fig.10C). Metabolism at the 5-OH position was not measurable at most instances, except at 35 μ M.

To more clearly display how position of phenolic group in a simple mono-hydroxyflavone may be used to predict the glucuronidation of a flavone with two phenolic groups, we re-plotted data shown in Fig.5 and Fig.10 to make Fig.11. The re-plotted data were used to demonstrate how metabolic fingerprint discovered using simple HF's may be used to predict the glucuronidation behaviors of more complex flavones. The results showed that almost all the patterns were predictable qualitatively. However, additional isoform became active in metabolizing diHFs when compared to those that metabolized HF's.

Discussion

Our results clearly shown that there is an isoform-specific glucuronidation fingerprint for each of the tested HF's (Fig.5) and diHF's (Fig.10). Additionally, simpler (i.e., six-isoform) fingerprints of HF's, with exception of 3'-HF, may be used to predict their metabolic rates in human intestinal and liver microsomes (Fig.9, Fig.10). Both of these conclusions are novel for flavones, although our earlier investigation of isoflavone metabolism using the same approach but with 12 isoforms also showed good correlation between rates predicted based on glucuronidation fingerprints of isoflavones and their organ-specific glucuronidation rates in human intestinal and liver microsomes¹³. Therefore, this present study demonstrated once again the value of isoform-specific fingerprinting in predicting the organ-specific glucuronidation rates of flavonoids. Since expressed UGT isoforms are widely available commercially, our approach may be used broadly to predict metabolism of a particular

flavonoid or drug at a target organ, assuming the UGT expression level is known via quantitative RT-PCR. However, further refinement of our approach is needed in at least two areas: incorporation of additional UGT isoform including the newly cloned UGT3A family²¹ and quantitation of UGT isoform levels of the target tissues using a proteomic approach²² since isoform-specific UGT antibodies are generally unavailable and quantitative-PCR do not directly measure the protein expression level.

Our approach of using UGT isoform fingerprint to predict metabolism of flavonoids in a target organs/tissues is rather novel although other research groups have successfully predicted organ-specific metabolism of flavonoids using microsomes made from the same organ^{11,23-25}. Our approach is probably more labor intensive as more studies have to be run to cover the number of isoforms included and three different substrate concentrations. The three substrate concentrations are often necessary since fingerprint of any of the six flavones could change with concentration, especially in the case of UGT1A8, UGT1A10 and UGT2B7 (Fig.6-7). However, our approach should have greater utility since this fingerprint can be used to predict metabolism of flavonoids in organs whose microsomes are not readily available. This is highly valuable since it would be possible to use this approach to predict the metabolism of flavonoids at other organs, especially the target organs of flavonoids' actions. Future investigation of this potential utility will be warranted to validate this hypothesis.

In addition to the utilities of UGT fingerprinting as discussed previously, our data showed that the fingerprints obtained from using HF were useful for describing the fingerprints of more complex diHFs. As shown in Fig.11, the fingerprint of more slowly metabolized phenolic group in a diHF was always more similar to that of the more slowly metabolized HF than the more rapidly metabolized HF. However, addition of an additional phenolic group, which makes the overall flavone structure more electron rich, generally resulted in the participation of additional UGT isoforms, or higher rates of metabolism or both.

By using the structural-specific metabolic profiles (Figure 6, 7), the structure-activity relationship (SAR) for selected HF may be elucidated. Since all HF share the same structural backbone, the SAR studies mainly examined how changes in the position of hydroxyl group (the site of glucuronidation) affected its glucuronidation by UGT1A1, 1A7, 1A8, 1A9, 1A10 and 2B7. These six UGT isoforms appeared to be the most important expressed human UGT isoforms for metabolism of flavones (Fig.5 and Fig.10) and isoflavones¹³. Our analysis indicated that UGT2B7 only glucuronidated hydroxyflavones with 3-hydroxyl or 5-hydroxyl groups. Therefore, 3-HF, 5-HF, 3,4'-diHF, 3,5'- diHF and 3,7'- diHF, which has 3- or 5-hydroxyl group, were its substrates, and flavones without the 5-hydroxyl group were not. Additionally, the glucuronidation of 3-HF was always the fastest and that of 5-HF was always among the bottom two among the six chosen UGTs (Figure 7). We believed that the slow or lack of metabolism of 5-HF is due to the position of its phenolic group, which favors the formation of an intra-molecular hydrogen bond with the carbonyl group at C-4 position. C-4 carbonyl group may also represent a significant steric hindrance for glucuronidation reaction at the 5-phenolic group.

It was shown earlier that investigation into glucuronidation of HF allowed us to project glucuronidation of di-HF to certain degrees. However, application of this approach to more complex flavones was not done here. An analysis of the literatures suggested that 3-OH group appeared to be the most reactive group even in the more complex flavones with more than 2 phenolic groups. For example, after oral administration of quercetin and kaempferol, quercetin-3-glucuronide and kaempferol-3-glucuronide were found to be the major glucuronides in human plasma^{26,27}, suggesting that our approach of using simple HF to predict the metabolic patterns has significant potentials, although additional studies are

needed to better define the algorithm necessary for a more accurate and quantitative prediction.

In conclusion, we demonstrated for the first time that the glucuronidation of seven HF's were UGT isoform- and concentration- dependent and significantly impacted by the position of the phenolic group. We also showed for the first time that glucuronidation fingerprints of more complex flavones (e.g., diHFs) could be reasonably predicted by the structurally simpler flavones (e.g., HF's). In addition, it was shown again that glucuronidation fingerprint of seven selected HF's could be used to predict the glucuronidation rates in both human liver and intestinal microsomes. We believe that the approach developed here may be of general values in the prediction of glucuronidation of drugs and phenolic compounds in specific organs and tissues. Further studies are needed to refine the prediction processes, by defining the levels of UGT isoforms in a particular organ and by modeling the interactions between UGTs and their substrates.

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Abbreviations

3-HF	3-hydroxyflavone
5-HF	5-hydroxyflavone
6-HF	6-hydroxyflavone
7-HF	7-hydroxyflavone
2'-HF	2'-hydroxyflavone
3'-HF	3'-hydroxyflavone
4'-HF	4'-hydroxyflavone
3, 7-diHF	3,7-dihydroxyflavone
3, 5-diHF	3,5-dihydroxyflavone
3, 4'-diHF	3,4'-dihydroxyflavone
HBSS	Hank's balanced salt solution
UGT	UDP-glucuronosyltransferase
UDPGA	uridine diphosphoglucuronic acid
UPLC	ultra performance liquid chromatography
SAR	structure-activity relationship

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Fig.1. Structures of Model Flavones

Shown in the scheme are structures of aglycone forms of hydroxyflavone analogs.

Glucuronidated metabolites may be formed at the 5, 6, or 7 phenolic group on the A ring, or 2'-OH, 3'-OH or 4'-OH phenolic group on the B ring, or 3-OH group on the C ring. 2'-

Hydroxyflavone(2'-HF) 3'-Hydroxyflavone(3'-HF) 4'-Hydroxyflavone(2'-HF) 3-Hydroxyflavone(3-HF) 5-Hydroxyflavone(5-HF) 6-Hydroxyflavone(6-HF) 7-Hydroxyflavone(7-HF) 3,4'-Dihydroxyflavone(3,4'-diHF) 3,7-Dihydroxyflavone(3,7-diHF) 3,5-Dihydroxyflavone(3,5-diHF)

3,7-diHF

3,4'-diHF

3,5-diHF

Fig.2. UPLC and LC-MS/MS Chromatographic Profiles of diHFs and Their Glucuronides
Each panel showed one diHF (e.g., 3,7-diHF, 3,5-diHF or 3,4'- diHF) and its two mono-glucuronides. Conditions for generating these profiles were presented in the Method section.

Fig.3. UPLC and LC-MS/MS Chromatographic Profiles of HF and Their Glucuronides
 Each panel showed a single HF (e.g., 2'-HF, 3'-HF, 4'-HF, 3-HF, 5-HF, 6-HF, or 7-HF) and its mono-glucuronide. Conditions for generating these profiles were presented in the Method section.

A: 7-HF and metabolite

B: 4'-HF and metabolite

C: 3-HF and metabolite

D: 5-HF and metabolite

Fig.4. UV Spectra of Seven Model Flavones and Their Respective Mono-glucuronide

The seven flavones are 4'-HF, 3-HF, 5-HF, 7-HF, 3,4'-diHF, 3,5-diHF and 3,7-diHF. There were also 10 mono-glucuronides in this figure since each of the diHF generated two monoglucuronides. Each of the UV spectra has two regions, marked by a peak near the 330 nm region ($\lambda_{\max 1}$) and another one near the 250 nm region ($\lambda_{\max 2}$). Shifts in $\lambda_{\max 1}$ and/o $\lambda_{\max 2}$ values or a lack of shift were used to determine the position of glucuronidation in a diHF.

Fig.5. Glucuronidation of 4'-HF, 3-HF, 5-HF and 7-HF at Three Concentrations by 12 Expressed Human UGTs

Experiments were conducted at three concentrations (2.5, 10, and 35 μM) at 37 °C for 1 (or 0.5) hr and the amounts of mono-glucuronides formed were measured using UPLC. Rates of glucuronidation were calculated as nmol/min/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3).

Fig.6. Concentration-dependent Glucuronidation of Seven Hydroxyflavones by Expressed Human UGT1A1, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7

Experiments were conducted at three concentrations (35 μM, 10 μM and 2.5 μM). The experiments were conducted at 37 °C for 1 (or 0.5) hr and the amounts of glucuronides formed were measured using UPLC. Rates of glucuronidation were calculated as nmol/min/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3).

Fig.7. Impact of Structural Changes on Flavone Glucuronidation by Six Major UGT Isoforms
Experiments were conducted at three concentrations as designated previously in Fig.6. Each column represents the average of three determinations and the error bar represents the standard deviation of the mean. Significant differences at each concentration by each UGT were found among the seven HF's ($p < 0.05$, One-way ANOVA, marked by *).

Fig.8. Glucuronidation of Seven HF by Pooled Human Liver Microsomes (Fig.8A) and Correlation Between Glucuronidation Rates Derived from UGT Isoforms and Those from Human Liver Microsomes at Concentrations of 35 μM (Fig.8 B1), 10 μM (Fig.8 B2) or 2.5 μM (Fig.8 B3)

Three different concentrations (2.5, 12.5 and 35 μM) of substrates were used to determine the rates of metabolism. In Fig.8A, each column represents the average of three determinations and the error bar represents the standard deviation of the mean. Significant differences at each concentration by each UGT were found among the seven hydroxyflavones ($p < 0.05$, One-way ANOVA, marked by *). In Fig.8B1~B3, the glucuronidation rates by human liver microsomes (X-axis) was plotted against expressed

UGT isoform (UGT1A1+UGT1A9) rates. The linear regression was used to derive the apparent correlation coefficient. The blank lines are the ones with all seven hydroxyflavones, whereas the pink lines are the one without 3'-hydroxyflavone.

Fig.9. Glucuronidation of Seven HFIs by Pooled Human Intestinal Microsomes (Fig.9A) and Correlation Between Glucuronidation Rates Derived from UGT Isoforms and Those from Human Intestinal Microsomes at Concentrations of 35 μ M (Fig.9B1), 10 μ M (Fig.9B2) or 2.5 μ M (Fig.9B3)

In Fig.9A, each column represents the average of three determinations and the error bar represents the standard deviation of the mean. Significant differences at each concentration by each UGT were found among the seven HFIs ($p < 0.05$, One-way ANOVA, marked by *). In Fig.9B1~B3, the glucuronidation rates by human intestinal microsomes (X-axis) was plotted against expressed UGT isoform (UGT1A1+UGT1A8+UGT1A10) rates. The linear regression was used to derive the apparent correlation coefficient. The blank lines are the

ones with all seven hydroxyflavones, whereas the pink lines are the one without 2'-hydroxyflavone.

Fig.10. Glucuronidation of 3, 7-diHF, 3, 5-diHF and 3, 4'-diHF by 12 Human UGTs

Experiments were conducted at three concentrations (2.5, 10, and 35 μM). Glucuronidation of 3,7-diHF and 3'4'-diHF produced either one or two mono-glucuronide (M1 and M2) depending on which UGT was used. Glucuronidation of 3, 5-diHF only produced one metabolite at 2.5 and 10 μM, and two metabolites at 35 μM. The experiments were conducted at 37 °C for 1 (or 0.5) hr and the amounts of glucuronides formed were measured using UPLC. Rates of glucuronidation were calculated as nmol/min/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3).

Fig.11. Comparison of Isoform-Specific Fingerprints of diHFs with Those of HFs

The fingerprints of glucuronide formation of 3-O-glucuronide from 3-HF (labeled as 3-HF), 7-O-glucuronide from 7-HF (labeled as 7-HF), 3-O-glucuronide from 3,7-diHF (labeled as 3-O-G(3,7-diHF)), and 7-O-glucuronide from 3,7-diHF (labeled as 7-O-G (3,7-diHF)) were shown in Figure.11A. Similarly, those for the fingerprints of glucuronide formation of 3-HF, 4'-HF, 3-O-G derived from 3,4'-diHF, and 4'-O-G derived from 3,4'-diHF were shown in Figure. 11B, and those for 3-HF, 5-HF, 3-O-G derived from 3, 5-diHF, and 5-O-G derived from 3, 5-diHF were shown in Figure.11C.

Table.1

The conversion factors (*K*) are showed at different wavelengths for all seven glucuronides of HFs, and all six mono glucuronides of diHFs.

Glucuronide	wavelength (λ_{max} , nm)	<i>K</i> (conversion factor)
2'-Hydroxyflavone 2'-O-G	254	1.26±0.08
3'-Hydroxyflavone 3'-O-G	254	0.93±0.02
4'-Hydroxyflavone 4'-O-G	254	1.16±0.02
3-Hydroxyflavone 3-O-G	254	0.96±0.06
5-Hydroxyflavone 5-O-G	254	0.84±0.13
6-Hydroxyflavone 6-O-G	254	0.99±0.03
7-Hydroxyflavone 7-O-G	254	0.96±0.03
3,4'-Hydroxyflavone 3-O-G	254	1.02±0.03
3,4'-Hydroxyflavone 4'-O-G	254	0.87±0.03
3,5-Hydroxyflavone 3-O-G	263	1.38±0.03
3,7-Hydroxyflavone 3-O-G	254	1.29±0.03
3,7-Hydroxyflavone 7-O-G	254	0.86±0.09