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Establishment and Use of New MDCK II Cells Overexpressing Both UGT1A1 and MRP2 to Characterize Flavonoid Metabolism via the Glucuronidation Pathway

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Abstract

Scope—The purpose of this study is to characterize how overexpression of an efflux transporter and an UDP-glucuronosyltransferase (UGT) affects the cellular kinetics of glucuronidation processes.

Methods and Results—A new MDCK II cell line overexpressing both MRP2 and UGT1A1 (MDCKII-UGT1A1/MRP2 cells) was developed and used to determine how overexpression of an efflux transporter affects the kinetics of cellular flavonoid glucuronide production. The results showed that most model flavonoids (from a total of 13) were mainly metabolized into glucuronides in the MDCKII-UGT1A1/MRP2 cells and the glucuronides were rapidly excreted. Flavonoids with three or fewer hydroxyl group at 7, 3' or 6 hydroxyl group were also metabolized into sulfates. Mechanistic studies using 7-hydroxyflavone showed that its glucuronide was mainly (90%) effluxed by BCRP with a small (10%) but significant contribution from MRP2. Maximal velocity of glucuronide production MDCK-MRP2/UGT1A1 cells showed a fairly good correlation ($R^2 > 0.8$) with those derived using UGT1A1 microsomes, but other kinetic parameters (e.g., K_m) did not correlate.

Conclusion—Overexpression of a second efficient efflux transporter did not significantly change the fact that BCRP is the dominant transporter for flavonoid glucuronide nor did it diminish the influence of the efflux transporter as the “gate keeper” of glucuronidation process.

Keywords

UGT; Flavonoids; “Revolving Door”; Glucuronide; Efflux Transporters

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INTRODUCTION

Glucuronidation is among the most important systemic clearance mechanisms in humans, responsible for the elimination of a variety of compounds including drugs, hormones, and dietary phytochemicals such as polyphenols and flavonoids¹. Production of glucuronides by metabolically active cells is a two-step process, the formation of glucuronides, and subsequent excretion of glucuronides. Formation is mediated via the catalytic action of UDP-glucuronosyltransferases (UGT) whereas efflux is enabled by various efflux transporters such as BCRP and MRP2¹. Since glucuronides are too hydrophilic to passively traverse the cellular membrane with the lipid bilayer structure, efflux transporters were shown to act as a “Revolving Door” for facilitating and/or controlling the cellular glucuronide excretion in a simple cell model². Therefore, understanding of glucuronidation susceptibility/propensity will likely require us to separately predict glucuronidation and glucuronide efflux.

Many groups of investigators including this research team have worked extensively to enhance our understanding of the relationship between chemical structures and metabolic activity (SAR) using specific UGT isoforms^{3–9}. Together with the reports showing that glucuronidation rates in tissue microsomes (e.g., liver microsomes) are often directly correlated with the expression of various UGT isoforms and activity of each isoform^{10–12}, significant strides have been made in predicting glucuronidation susceptibility by major human UGT isoforms. However, we have made little progress with respect to the prediction of glucuronide efflux. The latter is important since development of a predictive algorithm useful to determine the amounts and distribution of glucuronides in vivo will require us to better predict how efflux transporter expression level could affect glucuronide formation and excretion, which in turn would allow us to find a way to increase the bioavailability of compounds susceptible to extensive glucuronidation.

Increasing the bioavailability of compounds undergoing extensive phase II metabolism such as glucuronidation is important for the pharmaceutical development of phenolics and related compounds because of the “bioavailability penalty” linked to the poorly bioavailable compounds. The penalty comes from the fact that poor bioavailability is always associated with inconsistent bioavailability with a broader range of in vivo exposure. Hence, when the basic dose response theory is applied, it is not difficult to understand why much larger trial population is needed, as subject with much lower than average bioavailability is not supposed to have response due to lack of exposure, whereas subject with much higher than average bioavailability is likely to experience toxicity (and thereby possibly drop out of study). This large trial population often meant that the study could not be conducted economically, impeding the development of drug candidates involved. Hence, there is an increasing need for us to predict more accurately which phenolic compound might have good bioavailability or what we could do to increase the bioavailability of poorly bioavailable phenolics.

As stated previously, prediction of glucuronidation involves both glucuronidation and glucuronide efflux. In a recent study, HeLa-UGT1A9 cells (which overexpress human UGT1A9 but only expressed moderate level of human BCRP)¹³, were used to measure

kinetic parameters of BCRP-mediated efflux of glucuronides without purified glucuronide standards². Using these cells, we were able to study the structure activity relationship for BCRP mediated efflux of 13 flavonoid glucuronides, and determined what was important for cellular production of flavonoid glucuronides². However, UGT1A9, an UGT isoform mainly expressed in liver and kidney, is not the main isoform for the metabolism of many flavonoids, which are often metabolized in intestine. Hence, additional UGT isoform may be needed if our goal is to study the efflux kinetics of other flavonoids.

Therefore, the current research overexpressed UGT1A1 in MDCKII-MRP2 cells. We changed a cell line from HeLa to MDCKII-MRP2 cells because the latter cells overexpress MRP2 but have constitutive BCRP expression, similar to HeLa cells. Derivation of a cell variant overexpressing both MRP2 and BCRP would allow us to ask an important follow-up question is: would BCRP remain the most important efflux transporter in such a cell when another transporter is also expressed and at a higher level? Also, MDCK II cells have sulfotransferase activities, which meant that the cell line variant we produced here are capable of both glucuronidation and sulfonation, which might allow its use as a screening tool for both phase II reaction processes. Our hypothesis was that overexpression of the additional efflux transporters will pump out the glucuronides and sulfates so efficiently that cell membrane will become “transparent” such that all metabolites formed will be efflux out of the cells. We also hypothesized that we would be able to determine the efflux transporter’s kinetic parameters even without the determination of intracellular glucuronide concentrations.

MATERIALS AND METHODS

Materials

Madin-Darby canine kidney (MDCK II) cells expressing human MRP2 were purchased from NKI-AVL (Netherlands cancer institute, Amsterdam, NL). UGT1A1 (NM_000463) human cDNA and pCMV-A- Hygro vector were purchased from Origene (Rochville, MD). Lipofectamine was purchased from Invitrogen (Gaithersburg, MD). Human UGT1A1 Supersome™ was purchased from BD Biosciences (Woburn, MA). 3-Hydroxyflavone (3HF), 3'-hydroxyflavone (3'HF), 6-hydroxyflavone (6HF), 7-hydroxyflavone (7HF), 4'-hydroxyflavone (4'HF), 3,3'-dihydroxyflavone (3,3'DHF), 3,7-dihydroxyflavone (3,7DHF), 3,5,7-trihydroxyflavone (3,5,7THF), 3,6,4'-trihydroxyflavone (3,6,4'THF), 3,7,4'-trihydroxyflavone (3,7,4'THF), 3,5,7,4'-tetrahydroxyflavone (3,5,7,4'QHF), 3,7,3',4'-tetrahydroxyflavone (3,7,3'4'QHF), and quercetin (3,5,7,3'4'PHF) were purchased from Indofine Chemicals (Hillsborough, NJ). All other chemicals and solvents (analytical grade or better) were used as received.

Expression Vector of Human UGT1A1

UGT1A1 (NM_000463) human cDNA was cloned into pCMV-A- Hygro vector. Preparation of purified plasmid was achieved using a standard molecular biology kit, and correct sequence was confirmed through sequencing.

Development of MDCK II Cells Overexpressing Both UGT1A1 and MRP2

MRP2 overexpressing MDCK II cells or MDCKII-MRP2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C under 5% CO₂. The transfection of purified vector expressing human UGT1A1 into MDCK II-MRP2 cells was performed using Lipofectamine. At 50% confluence, cells in 150-mm dishes were exposed to serum-free DMEM containing the UGT1A1 plasmid (1 µg/ml) and Lipofectamine (1 µg/ml). At 5 hours after the initiation of transfection, the plasmid-Lipofectamine complex was removed, and the medium, consisting of DMEM supplemented with 10% fetal bovine serum, was added. After 2–3 weeks of hygromycin (200 µg/mL) selection, single colonies were screened for the expression of UGT1A1 by Western blot (WB) analysis. Stable expression was verified by a functional test. Level of UGT1A1 expression was also determined using a recently published LC-MS/MS method¹⁴.

UGT1A1 and MRP2 overexpressing MDCK II cells (MDCKII-MRP2/UGT1A1 cells) without alteration were cultured in DMEM with 10% fetal bovine serum at 37°C under 5% CO₂ for use. The cells from 3 days post seeding were used for the glucuronide excretion experiments and cell lysate experiments described below. The numbers of cells were correlated to protein concentrations of harvested cells, which is determined using Micro BCA ProteinTM Assay kit using bovine serum albumin as the standard.

Cell Culture

MDCKII-MRP2/UGT1A1 cells were then cultured in DMEM with 10% fetal bovine serum at 37°C under 5% CO₂ for use after successful construction. Cells from 3 days post-seeding were used for the glucuronide excretion experiments described below. The numbers of cells were correlated to protein concentrations of harvested cells, which were determined using Pierce BCA Protein Assay kit (Thermo) using bovine serum albumin as the standard.

Excretion Experiments Using MDCKII-UGT1A1/MRP2 Cells

Before experiments, the MDCKII-MRP2/UGT1A1 cells were washed twice with pre-warmed (37°C) HBSS buffer (Hank's balanced salt solution, PH=7.4). The cells adapted itself to the condition in 37°C HBSS buffer for 30 minutes. Then the cells were incubated with a HBSS buffer containing these flavones(3HF, 3'HF, 6HF, 7HF, 4'HF, 3,3'DHF, 3,7DHF, 3,5,7THF, 3,6,4'THF, 3,7,4'THF, 3,5,7,4'QHF, 3,7,3'4'QHF and Quercetin) (defined as the "loading solution"). The aglycone substrate concentrations in the range of 0.3125–20 µM were used unless method sensitivity or substrate solubility necessitated otherwise. In the loading solution, the proper concentration was diluted from 1000X concentrated stock solutions in organic solvent (DMSO/Ethanol=1:4), so that the final concentration of organic solvents were 0.1% (v/v) of the experimental media. The sampling times were selected to ensure that the amounts excreted vs. time plots stay in the linear range. At each time point, 200 µl of incubating media from each well was collected and the same volume of loading solution was used to replenish each well. The collected incubating media were mixed with 50 µl of "Stop Solution" consisted of 94% acetonitrile and 6% acetic acid containing 100 µM testosterone as the internal standard. Supernatants were ready for UPLC analysis after centrifugation (15 min at 15,000 rpm). All experiments were performed in triplicates.

Preparation of MDCK II-UGT1A1/MRP2 Cell Lysates for Glucuronide Studies

Cell lysates were prepared using freshly collected overexpressing MDCK II-UGT1A1/MRP2 cells, and used for measuring rates of glucuronide formation. For cell lysate preparation, cells were first washed in pre-warmed (37°C) Hank's Balanced Salt Solution or HBSS (pH 7.4) and scraped off and put into in a centrifuge tube. After it is centrifuged at 3000rpm for 5 minutes, the supernatant was removed, and cell pellets were mixed with ice-cold 50mM pH 7.4 potassium phosphate buffer. The cell suspension was sonicated using Aquasonic 150D sonicator (VWR Scientific, Bristol, CT) for 30 min in short pulses at the maximum power (135 average watts) in an ice-cold water bath (the temperature is 4°C). The resulting cell lysate was then harvested and pooled. It was used fresh or frozen at -80°C until use, for measuring the rates of conjugate formation (sulfonation activities were maintained at -80°C with a single defrosting action). The protein concentration of the cell lysate was determined using the BCA protein assay, using the bovine serum albumin as the standard.

Glucuronidation of Flavonoids by MDCK II-UGT1A1/MRP2 Cell Lysates

Cell lysates harvested from approximately 10 cm² cells (grown in the flask and is equivalent to one well's surface area in the six-well culture plate) in a 50 mM potassium phosphate buffer (pH 7.4) were mixed with magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), 0.5 to 20 µM concentration of flavonoid (3,3'DHF, 3,7,3',4'QHF, and 3574'QHF respectively) and UDPGA (3.5 mM, add last). And incubation procedure was exactly the same as that of Human UGT1A1 Supersome™. Supernatants were ready for UPLC analysis after centrifugation (15 min at 15,000 rpm). All experiments were performed in triplicates.

Glucuronidation of Flavonoids by Human UGT1A1 Supersome™

UGT1A1 was used to generate glucuronides of 7HF, 6HF, 3,3'DHF, 3,7,3',4'QHF, and Quercetin. The incubation procedure was exactly the same as our previous publications^{10,15,16}. Briefly, UGT1A1 Supersomes™ final concentration 52.9µg/ml as optimum for the reaction, magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), 0.5 to 20 µM concentration of flavonoid 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 µL) was incubated at 37°C for 10–30min so as to generate sufficient amounts of flavonoid glucuronides; and (3) the reaction was stopped by the addition of 50µL of 94% acetonitrile/6% glacial acetic acid containing 10 µM internal standard (testosterone was used as an internal standard for all these compounds). Supernatants were ready for UPLC analysis after centrifugation (15 min at 15,000 rpm). All experiments were performed in triplicates.

Sample Analysis by UPLC

The conditions for UPLC analysis of these flavones (3HF, 6HF, 7HF, 4'HF, 3,3'DHF, 3,7DHF, 3,5,7THF, 3,6,4'THF, 3,7,4'THF, 3,5,7,4'QHF, 3,7,3'4'QHF and quercetin) and their glucuronides were modified based on a previously published method^{12,16}. The conditions were: system, Waters Acquity with a binary pump and a 2996 DPA diode array

detector (DAD, Waters, Milford, MA); column, Acquity UPLC BEH C18 column (50X2.1mm I.D.1.7 μ m (Waters, Milford, MA); mobile phase A, 2.5mM ammonium acetate (pH 6.4); mobile phase B, 100% acetonitrile; gradient, 0–2.0min, 10–20% B, 2.0–3.0min, 20–40% B, 3.0–3.5min 40%–50% B, 3.5–4min, 50–90% B, 4–4.5min, 90% B, 4.5–5min, 90%–10% B; injection volume, 10 μ l; monitoring wavelength was 342nm (for 3HF), 306nm (for 6HF), 312nm (for 7HF), 333nm (for 4'HF), 345nm (for 33'DHF), 340nm (for 37DHF and 357THF), 361nm (for 373'4'QHF), 354nm (for 364'THF and 374'THF), 366nm (for 3574'QHF), 372nm (for Quercetin), and 244nm (for internal standard), respectively. Their representative chromatograms were shown in Fig. 1 and Fig. 2, respectively. The precision and accuracy is typically within acceptable range (<15%). The detection limits were at least 0.2 μ M. Quantitation of the glucuronide was based on the standard curve of the parent compound and further calibrated using the conversion factor (see below) as described earlier^{17,18}.

The structures of glucuronides were further confirmed by the “UV spectrum maxima ($\lambda_{\max}$) shift method” and UPLC-MS/MS as previously shown^{11,13,19}. Quantitation of the glucuronide was based on the standard curve of the parent compound and further calibrated using the conversion factor as described earlier^{5,13,20}. Conversion factors of 13 flavone glucuronides were also showed in Table S1 (Supplemental Data).

Data Analysis

Plots between efflux rates and intracellular concentrations would allow the determination of the kinetic parameters of MRP2-mediated efflux using a variety of kinetic equations (see later). However, we could not determine the intracellular concentrations of most model flavonoid glucuronides because of rapid glucuronide efflux by the MDCKII-UGT1A1/MRP2 cells. Instead, we were able to measure the glucuronide concentration in culture medium as a function of time, and this was used as apparent glucuronide formation rates and plotted against substrate concentration outside of the cells. This is not totally unreasonable since we assumed that efflux transporter MRP2 was overexpressed here and all formed glucuronides were effluxed out right away. In any rate, cellular glucuronide excretion rates were expressed as amounts of glucuronides effluxed per mg protein per min (pmole/mg/min). Rates of metabolism in expressed UGT1A1 (or UGT1A1 SupersomeTM) or MDCK II-UGT1A1/MRP2 cell lysates were expressed as amounts of glucuronides formed per mg protein per min (nmol/mg/min). If the Eadie-Hofstee plot was linear, formation rates (V) or excretion rates of a flavonoid glucuronide at various substrate concentrations (C) were fit to the standard Michaelis-Menten equation:

$$V = \frac{V_{\max} C}{K_m + C} \quad \text{equation (1)}$$

where K_m (substrate concentration at which half maximal velocity is achieved) represents the Michaelis constant, and $V_{\max}$ is the maximum excretion rate. To distinguish between UGT1A1-mediated glucuronidation using expressed UGT1A1 or MDCKII-UGT1A1/MRP2 cell lysates from glucuronide formation in the MDCKII-UGT1A1/MRP2 cells, we used K_m , $J_{\max}$ and CL_{int} for glucuronide efflux (excretion) rates in intact cells and K_m' , $V_{\max}'$ and

CL_{int}' for UGT-1A1-mediated glucuronidation in microsomes. If Eadie-Hofstee plots showed characteristic profiles of atypical kinetics such as auto-activation or biphasic kinetics^{21,22}, the data were fit to the corresponding equations as described previously²³. Kinetics parameters for glucuronide excretion were performed with Microsoft Excel add-in program for modeling steady-state enzyme kinetics¹³, the goodness of fit was evaluated on the basis of coefficient of determination (R^2), Akaike's information criterion (AIC)²⁴, weighted sum of squared residual (WSS), and the rule of parsimony was applied. After the kinetic parameters were determined, the intrinsic clearance ($CL_{int}=V_{max}/K_m$) was calculated, and used to estimate the efficiency of the efflux transporter. Previously, intrinsic clearance is often used to predict the in-vivo pharmacokinetics of a drug using in vitro data.

Statistical Analysis

One-Way ANOVA was used to determine if the difference was statistically significant. The significance value was set at 0.05 or $p < 0.05$.

RESULTS

Confirmation of Stable UGT1A1 Expression in MDCKII-MRP2 Cells

UGT1A1 expression was confirmed by Western blot using expressed human UGT1A1 as the positive control (Fig. 3A). We also measured the expression level of UGT1A1 using a recently published method¹⁴ and found the cellular expression level of 26.73 ± 0.42 pmol per mg of MDCKII-UGT1A1/MRP2 cells. This expression level is approximately 16% of the expression level of expressed UGT1A1 or UGT1A1 SupersomesTM, which was reported to be nearly 160 pmol per mg protein. We then determined the relative activities of the expressed UGT1A1 against 3'HF, a preferred substrate for UGT1A1, and found that the activities stayed leveled (but fluctuated at times) for a substantial period of time (nearly passages) (Fig. 3B). We also determined the kinetic parameters of glucuronidation for 373'4'QHF in both expressed UGT1A1 and in MDCKII-UGT1A1/MRP2 cell lysates, and found the K_m values to be similar (2.7 vs. 3.4 μ M, details later).

Metabolisms of Model Flavonoids by MDCK II Cells Overexpressing Both UGT1A1 and MRP2 (MDCKII-MRP2/UGT1A1)

We studied the UGT1A1-mediated metabolism of 4 mono-hydroxyflavonoids (with 1 -OH group at 3, 7, 3', or 4' position) and 8 polyphenolic flavonoids (with 2 to 5 -OH groups at positions 3, 5, 6, 7, 3' or 4') in MDCKII-UGT1A1/MRP2 cells. Among the four mono-hydroxyflavonoids, glucuronides were the main or only metabolites for 3HF and 4'HF, whereas sulfate was the main metabolite for 7HF (Table 1).

Among the 8 polyphenolic flavonoids, all formed glucuronides as the major metabolites, with main metabolite at 3', 4', or 7 -OH position. Among the 8 polyphenolic flavonoids, 3 compounds with 4 or 5 -OH groups (3,7,3',4'-QHF, 3,5,7,4'-QHF or 3,5,7,3',4'-PHF) did not form sulfates, 3 compounds (33'DHF, 3,7DHF, and 3,5,7 THF) formed minor amount of sulfates, whereas 2 compounds (3,7,4'THF and 3,6,4'THF) formed significant amount of sulfate although the sulfate metabolite was not the major metabolite (Table 1). Similar to glucuronidation, position of sulfate formation is usually at 3' or 7 -OH position (Table 1).

The position of the glucuronidation or sulfation was assigned the same as described, where a combination of change in $\lambda_{\max}$ ¹⁸ and metabolic susceptibility (e.g., 7-OH position is more prone to rapid sulfation^{25–28}) was used to assign position of conjugation.

Whereas we were able to identify and measure phase II metabolites of these 13 flavonoids in the cellular media, we were usually unable to accurately measure their concentrations inside the cells due to low concentration using a protocol that we published previously², indicating that the glucuronides were mostly effluxed due to overexpressed MRP2.

Mechanisms of Glucuronide and Sulfate Efflux in MDCKII-MRP2/UGT1A1 Cells

Because MDCKII-MRP2/UGT1A1 cells overexpress both an efflux transporter (MRP2) and a UGT enzyme (UGT1A1), it was different from our previously characterized HeLaUGT1A9 cells, where only one UGT enzyme (UGT1A9) was expressed. In those cells, BCRP was the dominating efflux transporter. We determined if the overexpression of another efflux transporter MRP2 would change the main transporter for cellular glucuronide efflux, using 7-hydroxyflavone (7HF) as a model compound.

Our results showed that the efflux transporter for the glucuronide remained to be BCRP as BCRP inhibitor Ko143 (5 μ M) decreased the 7-HF-glucuronide efflux by more than 90%. In contrast, the BCRP inhibitor MK-571 (20 μ M) only decreased efflux by 56%. Additional functional knockdown with siRNA (2 μ g per monolayer) did not result in further decrease in the efflux. On the other hand, the residual efflux ($\approx$ 10%) in the presence of BCRP inhibitor was probably due to the action of functional MRP2, which was efficiently enough to eliminate any cellular glucuronide in our study such that no 7-HF-glucuronide was found inside the cells (i.e., below LLOQ). We also determined how these inhibitors affected the f_{met} values, which reflects cellular enzyme activities and is derived from amounts of metabolites (in this case glucuronides) divided by sum of metabolites (glucuronides and sulfates) and parent at the end of the experiment, and found that Ko143 decreased glucuronide f_{met} values by more than 90% whereas siRNA knockdown only decreased it by less than 35% (Fig. 5E). In contrast, MK-571 decreased glucuronide f_{met} values by more than 60% whereas siRNA knockdown only decreased it by less than 35% (Fig. 5F).

Unlike the efflux of glucuronides, the efflux of 7-HF-sulfate was only mildly affected by the BCRP and MRP2 chemical inhibitors, but addition of siRNA against these transporter was additive. The maximal effects were 64% for MRP2 inhibition (chemical inhibitor plus siRNA) and 47% for BCRP inhibition (chemical inhibitor plus siRNA), respectively. Because flavonoid glucuronides were predominantly via BCRP, we focused the rest of our study on glucuronidation of 13 model flavonoids. We also determined how these inhibitors affected the f_{met} values, and found that Ko143 and siRNA knockdown each decreased sulfate f_{met} values by approximately 20% (Fig. 5G). In contrast, MK-571 and siRNA knockdown decreased sulfate f_{met} values by approximately 35% (Fig. 5H).

We also determined if these two chemical inhibitors will inhibit the glucuronidation of 7-HF in the cell lysate by directly incubating the inhibitors with 7HF. We found that at lower concentration (2.5 μ M for MK-571 and 0.5 μ M for Ko143, both at 1/10 of the extracellular concentration used), neither compound significantly affected the formation of 7-HF

metabolite (Table S2, Supplemental Data). But at the concentrations of 5 μ M Ko143 and 25 μ M MK-571, 7HF glucuronidation was reduced 46% and 81%, respectively (Table S2, Supplemental Data). We believe that the intracellular concentrations of these two inhibitors were less than 10% of the extracellular concentrations, since these two inhibitors at the same concentrations (25 μ M for MK-571 and 5 μ M for Ko143) did not decrease cellular excretion of genistein glucuronide (Fig. S1, Supplemental Data), even though both inhibitors also significantly decreased the glucuronide formation in the same cell lysate assay (Fig. S2, Supplemental Data). In fact, 2.5 μ M for MK-571 also significantly decreased the formation of genistein glucuronide in cell lysate (Fig. S2, Supplemental Data), suggesting that the intracellular concentration maybe even lower in these cells.

Kinetics of Glucuronide Formation by expressed UGT1A1 Supersomes™

Many of the flavonoids used here have been used previously for determination of UGT1A1-mediated metabolism and their K_m and V_{max} values were published. Therefore, only five flavonoids (7HF, 6HF, 33'DHF, 373'4'QHF and quercetin) were used in the study of expressed UGT1A1-mediated metabolism, and the results showed that only 7HF displayed typical Michaelis-Menten kinetics, whereas the rest displayed other kinetics. Among these four compounds, glucuronidation of 6HF displayed biphasic kinetics, 33'DHF and 373'4' displayed substrate inhibition pattern, whereas quercetin displayed unknown kinetic patterns, showing a strong inhibitory effects on its own glucuronidation (Fig. 5). Since some of the compounds have more than one –OH group available for glucuronidation, and we were able to identify the position of the major metabolite, and found that the major metabolite was usually at the 7 or 3' –OH position. Furthermore, there was general agreement between expressed UGT1A1-generated glucuronide profile and MDCKII-UGT1A1/MRP2 cell glucuronide profile with respect to the major metabolite (Fig. 1). However, some of the minor glucuronides found when using the expressed UGT1A1 were not found in the MDCKII-UGT1A1/MRP2 cells (Fig. 1).

Kinetics of Flavonoid-7-O-Glucuronide Efflux in MDCKII-UGT1A1/MRP2 Cells

Among the 12 flavonoids, there were 6 flavonoids capable of forming a glucuronide at the 7 –OH position, allowing us to determine if change in structure affect their apparent excretion rates in the intact cells (Fig. 6). This apparent excretion rates measure how fast a flavonoid is glucuronidated and then its glucuronide excreted by the cells, and therefore is a hybrid rate of both formation and excretion of a glucuronide. As expected, the excretion rates of 7HF versus substrate concentration followed a typical Michaelis-Menten kinetics. Addition of 3 –OH group to the flavonoid (as in 37DHF) drastically changed the profile of rates vs. concentration plot, and enhanced the glucuronidation at concentrations higher than 2.5 μ M. Addition of a 5 –OH group (as in 357THF) also substantially changed the profile of rates vs. concentration plot although the rates at higher concentration were similar. Addition of 4' –OH group significantly and substantially slowed down the maximal observed glucuronidation rates (4–6 folds). Interestingly, Eadie-Hofstee plots of 374'THF and 3574'QHF were really similar even though the Eadie-Hofstee plots of 37DHF and 357THF were highly different, suggesting the importance of the 4' –OH group. More importantly, we did not found measurable amount of 7-O-glucuronide in quercetin, which also has a –OH group in the 3' –OH position, consistent with the UGT1A1 glucuronidation data which

showed quercetin as a good inhibitor of UGT1A1 (Fig. 5). Lastly, in the cellular model system, the apparent K_m values varied four times, whereas the apparent J_{max} values varied a bit less than 5 times (Table 2).

We also examined the kinetic parameters of flavonoid glucuronidation by UGT1A1, and found that there were large discrepancies in CL_{int} values between the two model systems (Table 2). The major difference was that there were much larger difference (>40 folds) in the K_m values when using the expressed UGT1A1. However, there was good agreement with respect to the fact that quercetin did not produce 7-O-glucuronide in either model system.

Kinetics of Other Flavonoid Glucuronide Efflux in MDCKII-UGT1A1/MRP2 Cells

We also determined the apparent efflux rates of other flavonoid glucuronides, primarily those at 3, 3' or 4' -OH position since UGT1A1 is known to glucuronidate 3' and 4' flavonoids²⁹. It appeared that CL_{int} value for 3HF in the cells was relatively small, and addition of a 7 -OH or 6 -OH group appear to increase CL_{int} value, whereas addition of 4' -OH position appears to decrease it (Table 2). However, the compound number was small and additional studies may be necessary to make a reliable prediction. In contrast, CL_{int} values for most 3'-O-glucuronide appear to be rapid, especially so when the flavonoid has four or more -OH groups. Efflux of 4'-O-glucuronide and 6-O-glucuronide also appeared to vary with structural changes but the sample number was too small to make a definitive predicament.

When examining the rate of efflux (or excretion) versus loading concentration plots (Fig. 7), we found that efflux of 4'-O-glucuronide always (n=2) followed that of the Michaelis-Menten kinetics, whereas those of 6-O-glucuronide (n=2) or 3'-O-glucuronide (n=3) never followed the Michaelis-Menten kinetics. On the other hand, efflux of 3-O-glucuronide followed classical Michaelis-Menten kinetics some of the time (50% here, n=4). Whereas these observations are interesting, more structural variations are needed to ensure the trend observed here is meaningful.

In general, there were a lot of discrepancies between the “kinetic type” for glucuronidation of these 13 flavonoids when using expressed UGT1A1 microsomes vs MDCKII-UGT1A1/MRP2 cells (Table 2, last two columns). We expect the “kinetic type” to be more similar if the UGT1A1-mediated glucuronidation was able to dominate the cellular glucuronide production process.

Kinetics of Glucuronidation of Selected Flavonoids in MDCKII-UGT1A1/MRP2 Cell Lysate

Previous studies in HeLa-UGT1A9 cells have shown that the K_m values tend to be well conserved between the cell lysate and expressed isoform (i.e., difference usually less than 2 fold), even though the concentration of protein present is very different. We found that two of the K_m values determined using the cell lysates were similar to those determined using UGT1A1 Supersomes, but one was different (Table 3). The reason for this difference was not apparent.

Correlation between Kinetic Parameters Generated from Expressed UGT1A1 and MDCKII-UGT1A1/MRP2 Cells

It has been assumed routinely in published literatures that glucuronidation rates are primarily driven by activities of the UGTs involved in the metabolism. Here, we plotted various kinetic parameters associated with flavonoid glucuronidation in two model systems using data shown in Table 2. We plotted K_m' values obtained from expressed UGT1A1-mediated metabolism experiments versus K_m values obtained from intact MDCKII-UGT1A1/MRP2 cell experiments (Fig. 8A). We also plotted V_{max}/J_{max}' values and CL_{int}/CL_{int}' values, just as we have done for the K_m/K_m' values (Fig. 8B, 8C). We then tried to derive positive correlation by forcing the regression line through the origin (i.e., no intercept), and found moderately good correlation between V_{max}/J_{max}' values ($R^2=0.83$), which were derived without using kinetic parameters for 3'-OH-3'-O-glucuronide or 3'-OH-4'-O-glucuronide as they appeared to be outliers. Including these outliers would have diminished the correlation coefficients but not the trend. On the other hand, we found no correlation between $\log CL_{int}/\log CL_{int}'$ values, weak correlation between the K_m/K_m' values. Therefore, UGT1A1 driven enzyme kinetic process could dominate at high substrate concentrations, allowing its use as a screening model.

DISCUSSION

A central paradigm in the understanding of phase II metabolic process, which produces a highly hydrophilic metabolite, is that efflux transporter will act as a “gate keeper” or “Revolving Door” to control the cellular production of these highly hydrophilic metabolites. In earlier investigations, we showed that BCRP, not overexpressed and present at a moderate level, could act as the “Revolving Door” in a HeLa-UGT1A9 cell model system^{2,30}. An important follow up question is whether BCRP is unique in this capability, and our result showed, as expected, that BCRP is not a unique transporter with this function and that MRP2 can perform as a “Revolving Door” for the glucuronide (Fig. 4). Because MDCKII-MRP2/UGT1A1 cells expressed UGT1A1 (Fig. 3) and were capable of excreting flavonoid sulfates (Fig. 1, Fig. 2, Table 1), our results also showed that these efflux transporters can also act as the “gate keeper” for sulfate efflux (Fig. 4C, Fig. 4D). These “gate keeper” could potentially affect the apparent or effective cellular enzyme activities (Fig. 4E–4H). In fact, MRP2 appeared to be more important for sulfate efflux than for glucuronide efflux, at least in the case of 7HF-sulfate (Fig. 4). Together, these observations are consistent with our earlier study, which also showed that BCRP is important for the intestinal efflux of apigenin and genistein glucuronides and sulfates³¹. Hence the current cell variant may be used to study the efflux of both glucuronides and sulfates, provided that the substrate could be both glucuronidated and sulfonated in these cells. This makes the current cell variant more useful than HeLa-UGT1A9 cells we developed earlier (which favors mostly glucuronidation) for phase II metabolism studies.

Another important follow-up question is if an efflux transporter is overexpressed in a system such that all the formed glucuronides are effluxed out of the cells right away, would efflux transporter still have the same impact on the cellular glucuronide production? In other word, if an efflux transporter is overexpressed on the cell surface, will cellular membrane become

less of a barrier to the cellular glucuronide production process? If the cellular membrane is not a barrier, then do we expect to have similar $V_{\max}$ and K_m values between cell lysate and whole cells or between whole cells and expressed UGT1A1, after adjusting for amount of UGT1A1 protein between these two systems?.

The answer to the last important question is yes if we mainly focus on the maximal metabolite production ($J_{\max}$ and $V_{\max}$ values). This is because our data showed that there was a very decent correlation (through origin) between $V_{\max}/J_{\max}$ values ($r^2=0.83$), and the slope of this correlation was 0.119 (Fig. 8), consistent with the fact that the expression level in these cells were 16% of that of expressed UGT1A1 SupersomesTM. By adjusting for relative abundance, the slope of this correlation would have been 0.754. This suggests that at higher substrate concentration, at which metabolic stability is typically tested, the two model systems provided answers that were largely the same. For example, 2 of the top 3 flavonoids with the highest $V_{\max}$ values in UGT1A1 system were also among the top 3 with the highest $J_{\max}$ values in the MDCKII-UGT1A1/MRP2 cells (Table 2), and the third one was very close to be the top 3 in the other system (Table 2). On the other hand, only 1 of bottom 3 compounds showed up in both model systems. Taken together, these results tend to suggest that the MDCKII-UGT1A1/MRP2 cells may be useful as a screening tool to determine metabolic (i.e., glucuronide) susceptibility at high substrate concentrations, situation that might occur in the intestine following oral ingestion of phenolics.

Another answer to the above mentioned important question is, unfortunately, less obvious. If we were to examine K_m vs K_m' values, although we really did not expect them to be highly correlated. The reasons we did not expect the K_m values to be highly correlated is that we used extracellular concentration in the calculation of the cellular kinetic parameter, K_m (Table 2). Assuming that these compounds are well bound to the protein, which has been shown repeatedly previously, extracellular concentration is unlikely to be the same as the intracellular free concentration. In addition, the relationship between extracellular and intracellular is not going to be a constant ratio since binding is concentration-dependent, with higher percent bound at lower concentration. Therefore, we are unable in this study to determine the free flavonoid concentration inside the cells, which drives the metabolism. On the other hand, in cell lysate or expressed UGT1A1 system, the concentrations measured usually reflect free drug concentration or is close to the free drug concentration. Since these are two sets of different concentrations, we did not expect the similar K_m values. Indeed, K_m and K_m' values only displayed moderate poor correlation, suggesting that these values are not well conserved between the two models systems. Moreover, since CL_{int} is related to both $V_{\max}$ and K_m values, CL_{int} is also not expected to be well correlated (Fig. 8). Furthermore, the kinetic profiles were also expected to be different for most flavonoid glucuronide efflux (Fig. 5–Fig. 7), displaying different kinetic patterns (Table 2, Table 3).

Besides its use as a tool to determine glucuronidation, the current model system may be used to determine the sulfonation of flavonoids. However, UGT1A1 appears to have much broader substrate specificity than the SULT isoforms expressed by the cells, because all 13 flavonoids used were glucuronidated by UGT1A1, several forming multiple metabolites (Table 1, Fig. 1, Fig. 2), although not always the same on as those found using expressed UGT1A1 (Table 1). It appears that only 7, 3' and 6 hydroxyl group was sulfated with fastest

rate at the 7 position, followed by 3' and then 6 position (Table 1). However, sulfonation of flavonoids was affected by the presence of other hydroxyl groups on the flavonoid structures. For example, flavonoids with 4 or more hydroxyl groups were not sulfonated even though there was a presence of one or more of the susceptible hydroxyl groups (e.g., 7-OH group) in their structures (Table 1).

In conclusion, we have demonstrated clearly that the current cell variant MDCKII-MRP2/UGT1A1 cells could pump out all the conjugates formed within the cells by the relevant phase II enzymes such that the amount of metabolites generated are the same regardless if the cells were intact or not (after adjusting for amount of enzymes in the reactions). This is especially true at high substrate concentrations. Remarkably the presence of a second transporter did not significantly alter the major efflux mechanism for flavonoid glucuronides, which remains to be BCRP. Both BCRP and MRP2 can act as the “Revolving Door” or “gate keeper” for flavonoid glucuronides and sulfates. Taken together, overexpression of efflux transporters will not significantly diminish the influence of “Revolving Door” on glucuronidation process and inhibition of the efflux transporters result in major reduction in cellular glucuronide production. Thus, transporter continues to significantly impact the rate and extent of a substrate’s metabolism, and as such future work to predict a compound’s susceptibility to glucuronidation must target both enzymes and efflux transporters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

3HF	3-hydroxyflavone
3'HF	3'-hydroxyflavone
6HF	6-hydroxyflavone
7HF	7-hydroxyflavone
4'HF	4'-hydroxyflavone
33'DHF	3,3'-dihydroxyflavone
37DHF	3,7-dihydroxyflavone
357THF 3	5,7-trihydroxyflavone
364'THF	364'-trihydroxyflavone

374'THF	3,7,4'-trihydroxyflavone
3574'QHF	3574'-tetrahydroxyflavone
373'4'QHF	3,7,3',4'-tetrahydroxyflavone
DMSO	dimethyl sulfoxide
BCRP	breast cancer resistance protein
MRP2	multidrug resistance related protein 2
CL_{int}	Intrinsic Clearance
O-G	O-glucuronide
UPLC	ultra performance liquid chromatography
UGT	UDP-glucuronosyltransferases
UGPDA	uridine diphosphoglucuronic acid
AIC	Akaike's information criterion
WSS	Weighted Sum of Squared Residual
SAR	structure activity relationship
HBSS	Hank's balanced salt solution
DMEM	Dulbecco's modified Eagles medium
FBS	fetal bovine serum
MM	Michaelis-Menten equation
BL	Biphasic linear portion model
SI	Substrate inhibition equation

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Fig. 1. Representative UPLC chromatograms of flavones and their metabolites in expressed UGT1A1 or UGT1A1 Supersomes (left) or UGT1A1 and MRP₂ overexpressing MDCK II cells or MDCKII-UGT1A1/MRP2cells (right)

Chromatograms were derived from 7HF (at 312nm, Panels A), 6HF (at 306nm, Panel B), 33'DHF (at 345nm, Panel C), 373'4'QHF (at 361nm, Panel D) and Quercetin (at 372nm, Panel E), respectively. Left panel samples were from enzyme assay in which 5 μ m substrate was incubated with 52.9 μ g/ml UGT1A1 SupersomeTM for 30mins. Right panel samples were from MDCKII-UGT1A1/MRP2cells assay in which the substrate concentration was 5 μ m and the incubation time was 4h. These chromatograms were chosen because glucuronidation of these five flavonoids by UGT1A1 microsomes were done the first time in our lab. Other results of expressed UGT1A1-mediated metabolism of flavonoids were published already.

Fig. 2. Representative UPLC chromatograms of flavones and their metabolites in MDCKII-UGT1A1/MRP2 cells that were not shown in Fig. 1

Chromatograms were derived from 3HF (at 342nm, Panel A), 4'HF (at 333nm, Panel B), 37DHF (at 340nm, Panel C), 357THF (at 340nm, Panel D), 374'THF (at 354nm, Panel E), 364'THF (at 354nm, Panel F), and 3574'QHF (at 366nm, Panel G), respectively. Samples were from MDCKII-UGT1A1/MRP2 cells assay in which the substrate concentration was 5 μ m and the incubation time was 4h.

Fig. 3. Western blot and activity analysis of UGT1A1 expression in MDCKII-UGT1A1/MRP2cells

Fig. 3A; Top stripe, UGT1A1 antibody results, and Bottom stripe, β-actin antibody results. Lane 1 was MDCK II MRP2 cells, lane 2 was the MDCK II MRP2 cells overexpressing UGT1A1, and lanes 3 was commercially available human recombinant UGT1A1 microsomes (approximately 2 ug) used as positive antibody control. Fig. 3B; Relative formation rates of 3'HF glucuronides in MDCKII-UGT1A1/MRP2cells, when 10 uM of 3'HF was used as a substrate.

E: BCRP Inhibitors on Glucuronide **F: MRP2 Inhibitors on Glucuronide** **G: BCRP Inhibitors on Sulfate** **H: MRP2 Inhibitors on Sulfate**

*, $p < 0.05$; **, $p < 0.01$

Fig. 4. Effects of Efflux Transporter Inhibitors on the Efflux of 7HF-O-G and 7HF-O-S from MDCKII-MRP2/UGT1A1 cells

Experiments were conducted in six-well plates, which meant a surface area of approximately 20 cm² and protein amount of 0.8 mg/monolayer. Effects of BCRP inhibitors Ko143 (5 μ M) and siRNA (2 ug per monolayer) on glucuronide (Fig. 4A) and sulfate (Fig. 4C) efflux was determined using 10 μ M 7HF, which was both glucuronidated and sulfated in these cells. Effects of MRP2 inhibitors MK-571 (20 μ M) and siRNA (2 ug per monolayer) on glucuronide (Fig. 4B) and sulfate (Fig. 4D) efflux was also determined using 10 μ M 7HF. 7HF was loaded onto the cells simultaneously with chemical inhibitors in chemical inhibitor (alone) study. In siRNA knockdown study, cells were treated with siRNA (2 ug per monolayer) in the presence of Lipofectamine (1 μ l per monolayer) per manufacturer's instruction for 48 hr prior to loading 7HF. In experiments where both chemical inhibitors and siRNA was used, siRNA was used first and then 7HF along with chemical inhibitors were then used.

Fig. 5. Kinetics profiles of expressed UGT1A1-mediated glucuronidation of five flavones (7HF, 6HF, 33'HF, 373'4'QHF and Quercetin)

The concentrations versus rates plots were on the left panels and the corresponding Eadie-Hofstee plots were present on the right panels. Profiles were for 7-O-glucuronidation of 7HF (Panel A), 6-O-glucuronidation of 6HF (Panel B), 3'-O-glucuronidation of 3,3'-DHF (Panel C), 7-O-glucuronidation of 3,7,3',4'-QHF (Panel D), 4'-O-glucuronidation of 3,7,3',4'-QHF (Panel E), 3'-O-glucuronidation of 3,7,3',4'-QHF (Panel F), 7-O-glucuronidation of quercetin (Panel G), 4'-O-glucuronidation of quercetin (Panel H), and 3'-O-glucuronidation of quercetin (Panel I), respectively. Each data represents the average of triplicates, and error bars are the standard deviations of the mean (n=3)

Fig. 6. Kinetics profiles of MRP2-mediated efflux of 7-O-glucuronides of five flavonoids (7HF, 37DHF, 357THF, 374'THF, and 3574'QHF) in MDCKII-UGT1A1/MRP2 cells

The concentrations (in extracellular incubating media) versus rates plots were on the left panels and the corresponding Eadie-Hofstee plots were on the right panels. Profiles were for the cellular excretion of 7-O-glucuronide of 7HF (Panel A), 7-O-glucuronide of 3,7DHF (Panel B), 7-O-glucuronide of 3,5,7THF (Panel C), 7-O-glucuronide of 3,7,4'-THF (Panel D), and 7-O-glucuronide of 3,5,7,4'-QHF (Panel E), respectively. Each data represents the average of triplicates, and error bars are the standard deviations of the mean (n=3).

Fig. 7. Kinetics profiles of MRP2-mediated efflux of non 7-O-glucuronides of nine flavones (3HF, 6HF, 4'HF, 33'DHF, 37DHF, 364'THF, 374'THF, 373'4'QHF and Quercetin) in MDCKII-UGT1A1/MRP2 cells

Profiles were for the cellular excretion of 3-O-glucuronide of 3HF (Panel A), 6-O-glucuronide of 6HF (Panel B), 4'-O-glucuronide of 4' HF (Panel C), 3'-O-glucuronide of 3,3'-DHF (Panel D), and 3-O-glucuronide of 3,7-DHF (Panel E), 3-O-glucuronide of 3,6,4'THF (Panel F), 6-O-glucuronide of 3,6,4'THF (Panel G), 4'-O-glucuronide of 3,6,4'-THF (Panel H), 3-O-glucuronide of 3,7,4'-THF (Panel I), 3'-O-glucuronide of 3,7,3',4'-QHF (Panel J), and 3'-O-glucuronide of quercetin (Panel K), respectively. The concentrations (in extracellular incubating media) versus efflux rates plots were on the left panels and the corresponding Eadie-Hofstee plots were on the right panels. Each data represents the average of triplicates, and error bars are the standard deviations of the mean (n=3).

A

B

C

Fig. 8. Scatter plots of kinetic parameters derived from UGT1A1 and MDCKII-UGT1A1/MRP2cells

Kinetic parameters (K_m , J_{max} , and $\text{Log}(\text{CL}_{\text{int}})$) derived from MDCKII-UGT1A1/MRP2whole cell experiments were plot against those derived from UGT1A1-mediated glucuronidation (K_m' , V_{max}' , and $\text{Log}(\text{CL}_{\text{int}})'$). Values derived from cells were always presented as the X values and those from the microsomes as Y values. A, the K_m vs K_m' plot; B, J_{max} vs V_{max}' ; C, $\text{Log}(\text{CL}_{\text{int}})$ vs $\text{Log}(\text{CL}_{\text{int}})'$. Two data points not used in the calculation of correlation coefficients were circled, and represented the kinetic parameters associated with 3'-O-glucuronides of 33'DHF (red circle) and 373'4QHF (blue circle).

Table 1

Differences in metabolism of the flavonoids between UGT1A1 Supersome™ and UGT1A1 and MRP₂ overexpressing MDCKII or MDCKII-UGT1A1/MRP₂ cells.

Flavonoid	UGT1A1 Supersome™	MDCKII-UGT1A1/MRP2 cells	
		Glucuronides	Sulfate
7HF	7HF-O-G	7HF-O-G	Yes, the main metabolite *
3HF	3HF-O-G	3HF-3-O-G	No
4'HF	4'HF-O-G	4'HF-O-G	No
3'HF	3'HF-O-G	3'HF-O-G	Yes, one of the main metabolite
6HF		6HF-O-G	Yes but not the main metabolites
33'DHF	33'DHF-3'-O-G (1) [#] 33'DHF-3-O-G	33'DHF-3'-O-G	Yes, but not the main metabolites
37DHF	37DHF-7-O-G (1) 37DHF-3-O-G (2)	37DHF-7-O-G (1) 37DHF-3-O-G (2)	Yes, but not the main metabolites
357THF	357THF-7-O-G (1) 357THF-3-O-G (2)	357THF-7-O-G (1) 357THF-3-O-G	Yes, but not the main metabolites
374'THF	374'THF-7-O-G (1) 374'THF-3-O-G (2)	374'THF-7-O-G (1) 374'THF-3-O-G (2)	Yes, one of the main metabolites
364'THF	364'THF-4'-O-G (1) 364'THF-3-O-G (2) 364'THF-6-O-G (3)	364'THF-4'-O-G (1) 364'THF-3-O-G (2) 364'THF-6-O-G (3)	Yes, one of the main metabolites
3574'QHF	3574'QHF-7-O-G	3574'QHF-7-O-G	No
373'4'QHF	373'4'QHF-3'-O-G (1) 373'4'QHF-4'-O-G (2) 373'4'QHF-7-O-G (3)	373'4'QHF-3'-O-G (1) 373'4'QHF-4'-O-G 373'4'QHF-7-O-G	No
Quercetin	Quercetin-3'-O-G (1) Quercetin-4'-O-G (2) Quercetin-7-O-G (3)	Quercetin-3'-O-G	No

[#] Number in “()” means the relative abundance of glucuronides. The symbol “(1)” means it is the most abundant glucuronide, or the main glucuronide. The symbol “(2)” and “(3)” means they are the second and third most abundant glucuronides.

^{*} If sulfate was not present, it was marked as “No” in this column. If the amount present of sulfates was much lower than the main glucuronide (<50%), it was marked as “Yes, but not the main metabolites.” If the amount present was similar but no more than the main glucuronide (50%~100%), it was marked as “Yes, but one of the main metabolites.” If the amount present was more than the main glucuronide (>100%), it was marked as “Yes, the main metabolite.”

The “ ” symbol means the metabolite was very low concentration that we cannot calculate its enzymatic kinetics parameters accurately.

Table 2

Kinetic parameters (K_m , J_{max} , CL_{int} and kinetic type) of MRP₂-mediated efflux of glucuronides of flavones with UGT1A1 and MRP₂ overexpressing MDCK cells and those derived from UGT1A1 SupersomeTM-mediated glucuronidation (K_m' , V_{max}' , CL_{int}' and kinetic type').

Compound	K_m (μ m)	K_m' (μ m)	J_{max} (pmol/mg/min)	V_{max}' (nmol/mg/min)	$CL_{int}(J_{max}/K_m)$ (μ L/mg/min)	$CL_{int}'(V_{max}'/K_m')$ (μ L/mg/min)	Kinetic type	Kinetic type'
7HF-G	2.79±0.57	5.28±1.12	16.50±0.98	1.23±0.11	5.92	232.29	MM	MM
37DHF-7-O-G▼	1.14±0.47	1.04±0.19	25.59±1.01	3.04±0.42	22.45	2923.08	BL	SI
357THF-7-O-G▼★	1.34±0.36	0.51±0.08	31.42±2.10	4.59±0.46	23.43	9000.00	BL	SI
374'THF-7-O-G▼	1.73±1.26	0.43±0.10	6.86±2.72	1.08±0.13	3.96	2511.63	SI	SI
3574'THF-7-O-G▼	0.73±0.39	0.13±0.03	12.03±2.93	1.38±0.14	16.57	10615.40	SI	SI
3'HF-G								
33'DHF-3'-O-G	1.70±1.36	6.03±7.44	23.98±9.70	7.43±7.80	14.10	1231.93	SI	SI
373'4'QHF-3'-O-G★	0.29±0.54	2.70±2.95	15.18±1.06	3.26±2.50	53.08	1209.79	BL	SI
Quercetin-3'-O-G◆	0.56±0.46	1E-06±0.21◆	15.98±5.53	1.77±0.71◆	28.54	1.8E+09◆	SI	SI
3HF-G▼	2.21±0.61	1.46±0.24	11.04±0.76	1.74±0.03	5.01	1196.16	MM	MM
37DHF-3-O-G▼	0.91±0.77	1.57±0.49	6.07±1.94	1.02±0.34	6.70	649.68	SI	SI
364'THF-3-O-G▼	0.96±0.21	1.50±0.40	8.20±0.37	0.34±0.05	8.51	226.67	MM	SI
374'THF-3-O-G▼	1.29±0.80	0.30±0.05	4.8±1.4	0.68±0.05	3.68	2266.67	SI	SI
4'HF-G▼	1.17±0.26	2.24±0.11	13.82±1.37	0.85±0.01	11.82	381.31	SI	MM
364'THF-4'-O-G▼	0.67±0.15	0.76±0.18	8.47±0.33	0.34±0.03	12.62	447.37	MM	SI
364'THF-6-O-G▼	0.59±0.34	0.68±0.13	2.70±0.27	0.12±0.01	4.56	176.47	MM	SI

The “▼” symbol means the kinetic parameters derived from UGT1A1 SupersomeTM (K_m' , V_{max}' , CL_{int}' and kinetic type') were previously published. The “★” symbol means metabolites of 357THF-3-O-G, 3734'QHF-4'-O-G and 3734'QHF-7-O-G derived from UGT1A1 and MRP₂ overexpressing MDCK cells were very low concentration that we cannot calculate its enzymatic kinetics parameters accurately and show it here for comparison. The “◆” symbol means there are not suitable enzymatic kinetic model for Quercetin-3'-O-G, Quercetin-4'-O-G and Quercetin-7-O-G derived from UGT1A1 SupersomeTM.

Table 3

Kinetic parameters (K_m , J_{max} , CL_{int} and kinetic type) of MRP₂-mediated efflux of glucuronides of flavones with UGT1A1 and MRP₂ overexpressing MDCK cells and those derived from UGT1A1 SupersomeTM - mediated glucuronidation (K_m' , V_{max}' , CL_{int}' and kinetic type') and MDCKII-UGT1A1/MRP2 cell lysate (K_m'' , V_{max}'' , CL_{int}'' and kinetic type'').

Kinetic Parameters	Flavonoid		
	33'DHF-3'-O-G	373'4'QHF -3'-O-G	3574'QHF -7-O-G
$K_m(\mu\text{M})$	1.70±1.36	0.29±0.54	0.73±0.39
$K_m'(\mu\text{M})$	6.03±7.44	2.70±2.95	0.13±0.03
$K_m''(\mu\text{M})$	14.03±41.47	3.41±1.40	2.17±2.3
J_{max} (pmol/min/mg)	23.98±9.70	15.18±1.06	12.03±2.93
V_{max}' (pmol/min/mg)	7430±7800	3260±2500	1338±140
V_{max}'' (pmol/min/mg)	5123±128	4020±590	1450±180
$CL_{int}(J_{max}/K_m)$ (μL/mg/min)	14.10	53.08	16.57
$CL_{int}'(V_{max}'/K_m')$ (μL/mg/min)	1231.93	1209.79	10615.40
$CL_{int}''(V_{maxc}'/K_{mc}')$ (μL/mg/min)	365	118	460
kinetic type	SI	BL	SI
kinetic type'	SI	SI	SI
kinetic type''	SI	MM	BL