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Tissue and species differences in the glucuronidation of glabridin with UDP-glucuronosyltransferases

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Abstract

Glabridin (GA) has gained wide application in the cosmetics and food industry. This study was performed to investigate its metabolic inactivation and elimination by glucuronidation by use of liver and intestine microsomes from humans (HLM and HIM) and rats (RLM and RIM), and liver microsomes from cynomolgus monkeys and beagle dogs (CyLM and DLM). Both hydroxyl groups at the C2 and C4 positions of the B ring are conjugated to generate two mono-glucuronides (M1 and M2). HIM, RIM and RLM showed the most robust activity in catalyzing M2 formation with intrinsic clearance values (Cl_{int}) above 2000 $\mu\text{L}/\text{min}/\text{mg}$, with little measurable M1 formation activity. DLM displayed considerable activity both in M1 and M2 formation, with Cl_{int} values of 71 and 214 $\mu\text{L}/\text{min}/\text{mg}$, respectively, while HLM and CyLM exhibited low activities in catalyzing M1 and M2 formation, with Cl_{int} values all below 20 $\mu\text{L}/\text{min}/\text{mg}$. It is revealed that UGT1A1, 1A3, 1A9, 2B7, 2B15 and extrahepatic UGT1A8 and 1A10 are involved in GA glucuronidation. Nearly all UGTs preferred M2 formation except for UGT1A1. Notably, UGT1A8 displayed the highest activity with a Cl_{int} value more than 5-fold higher than the other isoforms. Chemical inhibition studies, using selective inhibitors of UGT1A1, 1A9, 2B7 and 1A8, further revealed that

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Conflict of Interest

The authors declare that there are no conflicts of interest.

UGT1A8 contributed significantly to intestinal GA glucuronidation in humans. In summary, this *in vitro* study demonstrated large species differences in GA glucuronidation by liver and intestinal microsomes, and that intestinal UGTs are important for the pathway in humans.

Keywords

Glabridin; Glucuronidation; UDP-glucuronosyltransferases; Species differences

1 Introduction

Glabridin (GA), a polyphenolic isoflavonoid (Fig. 1) extracted from the licorice root, has been used for centuries in Asian and European countries as antidotes, demulcents, and expectorants, as well as flavoring and sweetening agents [21]. GA was reported to display various desired biological activities, including anti-oxidant, anti-inflammatory, anti-tumorigenic, anti-nephritic, anti-bacterial, anti-obesity, skin-whitening, estrogenic-like, and neuro-protective effects [21]. GA and extracts enriched in GA have gained wide application in the cosmetics and food industry [7,21].

Due to the wide range of applications of GA, several studies have been conducted on its metabolism. The oral availability of the current drug was very low in rats (about 7.5% of the oral dose), which was attributed to P-gp mediated efflux and extensive hepatic glucuronidation [3]. GA can also undergo oxidation by cytochromes P450 enzymes (CYP) with reactive metabolites generated and then inactivate CYP3A4 and CYP2B6 [12]. Since CYP3A4 and CYP2B6 are responsible for metabolism of more than half of all clinically-used drugs, inhibition of these two enzymes by drug–drug interactions may interfere in elimination of a broad spectrum of drugs and induce undesired side effects [12].

Structure–function relationship studies indicate that the two phenol hydroxyl groups in the B ring (Fig. 1) are essential for inactivation of CYP and pharmacological activities [21]. These two hydroxyl groups are potential sites for glucuronidation. Therefore, glucuronidation may exert an impact on the toxic and pharmacological effects of GA. However, detailed information of this metabolic pathway remains poorly understood.

Glucuronidation is mediated by a superfamily called UDP-glucuronosyltransferases (UGTs). UGT expression and even the function of the same homologous isoforms display significant species differences. For example, UGT1A4 and UGT1A9 are not expressed in rats [6], while human and cynomolgus monkey UGT1A6 display different enzymatic properties [8]. Elucidation of the species differences of the glucuronidation will be of great value in selecting an appropriate experimental animal model to conduct the pharmacokinetic, toxicological and pharmacodynamic studies.

UGTs are expressed in various tissues. In humans, since there is abundant expression of many UGTs, liver is usually a major glucuronidation site. However, there are several isoforms that are expressed in extrahepatic tissues. For example, UGT1A8 and UGT1A10 are expressed at the high levels in intestine, but not detected in liver [4,19]. When the

targeted chemical is mainly metabolized by extrahepatic UGTs, the *in vivo* elimination process may be different from compounds that are mainly metabolized in liver.

The present *in vitro* study was conducted to investigate GA glucuronidation in microsomes from human liver and intestine, and livers from experimental animals, as well as recombinant UGTs. This study will be helpful for a deeper understanding of human disposition of GA, and provide valuable information in selecting suitable animals for further studies.

2. Materials and methods

2.1. Chemical reagents

GA (>98%) and magnolol (>98%) were purchased from the Sichuan Victory company (Chengdu, SC, China). Uridine-5-diphos-phoglucuronic acid (trisodium salt) (UDPGA) and alamethicin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nilotinib (>98%) and fluconazole (99%) was obtained from Alfa Aesar (Shanghai, China). All other reagents were either of HPLC grade or of the highest grade commercially available.

2.2. Enzyme sources

Pooled human intestinal microsomes (HIM, $n = 10$, 7–83 years old, mean 46.7 years old, 20% female) and liver microsomes from cynomolgus monkeys (CyLM, $n = 2$) and beagle dogs (DLM, $n = 7$) were purchased from Research Institute for Liver Diseases (RILD, China). Human liver microsomes (HLM, $n = 24$, 16–77 years old, mean 47.5 years old, 29% female) and a panel of recombinant human UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) expressed in baculovirus-infected insect cells were purchased from BD Gentest Corp (Woburn, MA). Sprague-Dawley rats (RLM, $n = 20$, male; body weight, 180–220 g) were obtained from Dalian Medical University, and all animal experiments performed using protocols reviewed and approval by the Institutional Animal Care and Use Committee. The rats had free access to tap water and pellet diet. The rats and mice were killed by decapitation, and the livers and intestines rapidly excised and pooled for preparation of microsomes through centrifugal fractionation according to the methods described by previous studies [13]. Protein concentrations of RLM and RIM were determined by using bovine serum albumin as standards [14]. Protein concentrations of HLM, HIM, CyLM and DLM were provided by the manufactures (RILD or BD Gentest Corp.).

2.3. GA glucuronidation assays

Typical incubations were incubated with glucuronidation enzymes in a reaction mixture of 200 μ L of 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 4 mM UDPGA. For tissue microsomes from human and experimental animals, alamethicin (5% microsomal protein concentrations) were used to activate the microsomes in an ice bath for 20 min. For recombinant UGTs, no activation is required. In all experiments, GA (20 mM, dissolved in methanol) was serially diluted to the required concentrations, and the final concentration of methanol did not exceed 1% (v/v) in the reaction mixture. After pre-incubation at 37 °C for 3 min, the reactions were initiated by the addition of UDPGA. The reactions were

terminated by the addition of cold methanol (100 μ L). The mixture was kept on ice for 30 min and then centrifuged at 20,000g for 20 min at 4 °C. Aliquots of supernatants were stored at –20 °C until analysis on HPLC–UV or LC–MS.

2.4. Identification of GA glucuronidation

GA (100 μ M) was incubated with 0.25 mg/mL of HLM, HIM, CyLM, DLM, RLM, or RIM for 60 min, respectively. The control incubations were additionally performed without UDPGA, or substrate, or microsomes. After removal of protein, the Aliquots of supernatants (20 μ L) were analyzed by both UFLC–DAD and UFLC–MS.

2.5. Kinetic assays with HLM and HIM

To determine the initial velocities, preliminary experiments were performed to ensure that glucuronide was formed in the linear range of both reaction time and microsomal protein concentration. For determination of reaction kinetics HLM, GA (1, 2, 5, 10, 20, 30, 40 and 50 μ M) were incubated with microsomes (0.1 mg/mL) for 60 min. For reaction kinetics in HIM, GA (0.2, 0.5, 1, 2, 3.5, 5, 7.5, 10, 20 and 30.0 μ M) were incubated with microsomes (0.01 mg/mL) for 20 min. All incubations were conducted at 37 °C in triplicate experiments. After removal of protein, the supernatants (20 μ L) were analyzed on HPLC–UV. For HLM, kinetic data was fitting into the Michaelis–Menten equation (Eq. (1)); For HIM, kinetic data was fitting into the substrate inhibition model (Eq. (2)).

$$V = \frac{V_{\max} \times [S]}{K_m + [S]}, \quad (1)$$

where v is the rate of the reaction, $[S]$ is the substrate concentration, $V_{\max}$ is the maximum velocity estimate, K_m is the substrate affinity constant.

$$V = \frac{v_{\max} \times [S]}{K_s + [S] + \frac{[S]^2}{K_{SI}}}, \quad (2)$$

where K_S is the substrate affinity constant, K_{SI} is the substrate inhibition constant.

2.6. Screening of UGTs with GA glucuronidation activity

GA (2 μ M) was incubated with 0.1 mg/mL recombinant UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) for 60 min, respectively. HPLC–UV and LC–MS were used to detect the formation of glucuronides.

2.7. Kinetic assays with recombinant UGTs

Preliminary experiments were performed to ensure that glucuronides were formed in the linear range of both reaction time and microsomal protein concentration. Kinetic assays were conducted for the involved recombinant UGTs, including UGT1A1, 1A3, 1A8, 1A9, 1A10 and 2B7. Detailed incubation conditions for kinetic assays with recombinant UGTs

are displayed in Table 1. All incubations were conducted at 37 °C in triplicate experiments. Kinetic data was fitting into Michaelis–Menten equation (Eq. (1)) or substrate inhibition model (Eq. (2)).

2.8. Chemical inhibition studies on HIM

To reveal the roles of UGTs in human intestinal GA glucuronidation, chemical inhibition studies were performed with HIM. Nilotinib, magnolol and fluconazole were reported to be selective inhibitors of UGT1A1, 1A9 and 2B7, respectively [1,26,28]. Mycophenolic acid was reported to be a high affinity substrate of UGT1A8 [20]. Their inhibitory effects on GA glucuronidation in HIM were tested. According the inhibition potential, the concentrations of nilotinib, magnolol, fluconazole, and mycophenolic acid were set at 1, 1, 10, and 1000 μ M, respectively. GA concentration was set at 2 μ M, and other incubations were the same as kinetic assays in HIM. All incubations were conducted at 37 °C in triplicate experiments.

2.9. Kinetic assays with RIM and RLM

Preliminary experiments were performed to ensure that glucuronides were formed in the linear range of both reaction time and microsomal protein concentration. GA (0.2, 0.5, 1, 2, 5, 10 and 20 μ M) was incubated with RLM or RIM (0.01 mg/mL) at 37 °C for 20 min, respectively. All incubations were conducted in triplicate experiments. After removal of protein, the supernatants (20 μ L) were analyzed on HPLC–UV. Kinetic data was fitting into substrate inhibition model (Eq. (2)).

2.10. Kinetic assays with DLM and CyLM

Preliminary experiments were performed to ensure that glucuronides were formed in the linear range of both reaction time and microsomal protein concentration. For kinetics in DLM, GA (1, 2, 5, 10, 20, 30 and 40 μ M) was incubated with enzymes (0.05 mg/mL) for 30 min, respectively. For CyLM, GA (2, 5, 10, 20, 30, 40 and 50 μ M) was incubated with microsomes (0.1 mg/mL) for 60 min, respectively. All incubations were conducted at 37 °C in triplicate experiments. After removal of protein, 20 μ L of supernatants were analyzed on HPLC–UV. Kinetic data was fitting into Michaelis–Menten equation (Eq. (1)).

2.11. Analysis methods

The HPLC system was equipped with a CBM-20A communications bus module, an SIL-HTA autosampler, two LC-10AT pumps, a DGU-12A vacuum degasser, a CTO-20A column oven, and an SPD-M 20AVP diode array detector. A C18-ODS analytical column with an ODS guard column was used to separate GA and its metabolites. The mobile phase consisted of CH₃CN (A) and water containing 0.2% (v/v) formic acid (B), with the following gradient profile: 0–15 min, 70–10% B; 15–22 min, 10% B; 22–32 min, 70% B. The flow rate was 1 mL/min, and the column temperature was kept at 40 °C. GA and its metabolites were detected at 280 nm and quantified according to the calibration curves of the substrate with intra- and inter-day variance less than 5%.

An LCMS-2010EV mass spectrometer system (Shimadzu, Kyoto, Japan) with an electrospray ionization (ESI) interface was used to identify GA and its metabolites (M-1 and M-2) operating in both the negative and positive ion modes from *m/z* 100 to 1000. The

detector voltage was set at +1.50 and 1.55 kV for positive and negative ion detection, respectively. The curved desolvation line temperature and the block heater temperature were both set at 250 °C. Other MS detection conditions were set as follows: voltage, 4 kV; interface voltage, 40 V; nebulizing gas (N₂) flow, tuned to be 1.5 L/min; and drying gas (N₂) pressure, 0.06 MPa. Data processing was performed using UFLC–MS Solution version 3.41 software (Shimadzu, Kyoto, Japan).

3. Results

3.1. Identification of GA Glucuronidation

Incubation of GA with HLM, HIM, RLM, RIM, DLM and CyLM, in the presence of UDPGA, all yielded two metabolite peaks (M1 and M2). In comparison with M2, the formation of M1 was negligible. These two metabolite peaks were absent in the control samples incubated without either microsomes, UDPGA or GA. GA and its glucuronides in assays with HLM and HIM are shown in Fig. 2. Mass spectrometry of M1 and M2 in the negative ionization mode both showed an m/z value of 409 for the deprotonated metabolite, a value that corresponding well to GA (324–1) with an m/z 176 representing the glucuronosyl substitution.

3.2. GA glucuronidation kinetics in HLM and HIM

To better understand the reaction mechanism, kinetic assays were conducted with HLM and HIM, respectively. HLM catalyzed M1 and M2 formation was simulated by the Michaelis–Menten equation, with K_m values of 6.3 ± 3.6 and 24.2 ± 5.7 μM , and V_{max} values of 0.044 ± 0.005 and 0.25 ± 0.03 nmol/min/mg protein, respectively for M1 and M2 formation. The intrinsic clearance values (Cl_{int} , V_{max}/K_m) for M1 and M2 formation were 7 and 10 $\mu\text{L}/\text{min}/\text{mg}$, respectively. Although HIM can also catalyzed formation of the two metabolites, the formation of M1 was too low to get. was too low to get reliable kinetic parameters. Unlike GA glucuronidation in HLM, the reaction in HIM followed substrate inhibition kinetics. The K_S , K_{SI} , and V_{max} values were demonstrated to be 8.3 ± 3.5 μM , 1.5 ± 0.7 μM , 16.7 ± 7.4 nmol/min/mg protein, respectively, and the Cl_{int} value was calculated to be 2.0 mL/min/mg. Kinetic plots for HLM and HIM are displayed in Fig. 3 and kinetic constants shown in Table 2.

3.3. UGTs with GA glucuronidation activity

To identify the UGT forms involved in GA glucuronidation, a panel of 12 recombinant human UGTs were analyzed and the results were displayed in Fig. 4. Multiple UGTs including UGT1A1, 1A3, 1A9, 2B7, and 2B15. and extra-hepatic UGT1A8 and 1A10 could convert GA to its glucuronides. At 2 μM substrate concentration, UGT1A8 displayed the highest catalytic activity, which was more than 5-fold higher than other isoforms. It should be noted that nearly all UGTs displayed higher activities in catalyzing the formation of M2. UGT1A1 was the exception, which preferred the formation of M1.

3.4. GA glucuronidation kinetics by recombinant UGTs

Since the GA glucuronidation activities of UGT1A9, 2B7 and 2B15 at the whole range of substrate concentrations were very low (<0.04 nmol/min/mg), no reliable kinetic constants

could be determined. The kinetic parameters were obtained for UGT1A1 (M1 and M2), 1A3 (M2), 1A8 (M2) and 1A10 (M2), and kinetic plots for recombinant UGTs displayed in Fig. 5. GA glucuronidation by UGT1A1 and 1A3 can be described by the Michaelis–Menten model (Eq. (1)). For UGT1A1, K_m values for M1 and M2 formation were 7.6 ± 1.8 and $24 \pm 14 \mu\text{M}$, and $V_{\max}$ values were 0.17 ± 0.01 (M1) and 0.11 ± 0.03 (M2) nmol/min/mg, respectively. The Cl_{int} value for M1 formation was calculated to be $22 \mu\text{L}/\text{min}/\text{mg}$, which was higher than that for M2 formation ($5 \mu\text{L}/\text{min}/\text{mg}$). For UGT1A3, due to the low activity, kinetic assays were only performed for M2 formation, with the K_m and $V_{\max}$ values of $11 \pm 4 \mu\text{M}$ and 0.32 ± 0.06 nmol/min/mg, respectively. The Cl_{int} value was further calculated to be $29 \mu\text{L}/\text{min}/\text{mg}$. UGT1A8 and 1A10 catalyzed GA glucuronidation yielded only M2 formation, following the substrate inhibition model (Eq. (2)). The K_m , $V_{\max}$, K_{SI} values of UGT1A8 were $64 \pm 9 \mu\text{M}$, 11 ± 3 nmol/min/mg, $1.7 \pm 0.4 \mu\text{M}$, while the kinetic constants of UGT1A10 were $28 \pm 8 \mu\text{M}$, 0.50 ± 0.10 nmol/min/mg, $36 \pm 11 \mu\text{M}$, respectively. The Cl_{int} values of UGT1A8 and 1A10 were 172 and $18 \mu\text{L}/\text{min}/\text{mg}$, respectively. Kinetic constants for GA glucuronidation by recombinant UGTs are displayed in Table 3.

3.5. Chemical inhibition studies in HIM

As displayed in Fig. 6 and $10 \mu\text{M}$ mycophenolic acid can inhibit GA glucuronidation activity by HIM, with the remaining activity was 28% of the control. The other inhibitors (nilotinib, magnolol and fluconazole) showed little inhibition of HIM activity, with remaining activity all above 85% of the control.

3.6. GA glucuronidation kinetics in RLM and RIM

As M1 formation rates in RLM and RIM were too low to get reliable kinetic constants, kinetic analysis was only performed for M2 formation. M2 formation kinetics by RLM and RIM are displayed in Fig. 7 and Table 2. The reaction in RLM and RIM both obeyed substrate inhibition model. The K_S , $V_{\max}$, and K_{SI} values for RLM were $3.9 \pm 1.9 \mu\text{M}$, 16 ± 5 nmol/min/mg, and $7.6 \pm 3.0 \mu\text{M}$, and the constants for RIM are $3.8 \pm 1.1 \mu\text{M}$, 19 ± 6 nmol/min/mg, and $5.3 \pm 2.5 \mu\text{M}$, respectively. The Cl_{int} values of RLM and RIM were further calculated to be 4 and $5 \text{ mL}/\text{min}/\text{mg}$, respectively.

3.7. GA glucuronidation kinetics in DLM and CyLM

GA glucuronidation kinetics (M1 and M2 formation) kinetics by DLM and CyLM are displayed in Fig. 8 and Table 2. M1 and M2 formation by DLM and CyLM can be described by the Michaelis–Menten model (Eq. (1)). For CyLM, the K_m and $V_{\max}$ values of M1 formation were $10 \pm 3 \mu\text{M}$ and 0.051 ± 0.005 nmol/min/mg, while the constants for M2 formation were $35 \pm 11 \mu\text{M}$ and 0.51 ± 0.09 nmol/min/mg, respectively. The Cl_{int} values for M1 and M2 formation were 5 and $15 \mu\text{L}/\text{min}/\text{mg}$, respectively. For DLM, the K_m and $V_{\max}$ values of M1 formation are demonstrated to be $34 \pm 12 \mu\text{M}$ and 2.4 ± 0.5 nmol/min/mg, whereas the constants of M2 formation were $29 \pm 12 \mu\text{M}$ and 6.2 ± 1.3 nmol/min/mg, respectively. The Cl_{int} values for M1 and M2 formation were calculated to be 71 and $214 \mu\text{L}/\text{min}/\text{mg}$, respectively.

4. Discussion

This study investigated the glucuronidation of GA by using recombinant UGTs and microsomes from different tissues of humans and experimental animals. The results indicated that GA glucuronidation could be conjugated by multiple UGTs with remarkable tissue and species variability. GA glucuronidation activity of HIM was demonstrated to be much higher than that of HLM, with the Cl_{int} value of more than 100-fold higher (Table 2). Earlier studies revealed that GA glucuronidation occurs at a low rate in the human colon adenocarcinoma cell line Caco-2 [10]. This may result from the low UGT activity in Caco-2 cells compared to intestinal epithelial cells as previously documented [27].

An earlier study demonstrated that GA was a mechanism-based inhibitor of CYP3A4, which was thought to likely result in interactions with dietary chemicals and drugs, and further induce the corresponding toxic effects [12]. This study suggests that due to the low liver glucuronidation activity, the inactivation of CYP may be dependent on the activity of intestinal UGTs rather than hepatic enzymes. The low GA glucuronidation activity in liver additionally indicates that GA escaping from intestinal glucuronidation may be eliminated slowly, which is supported by pharmacokinetic studies in human [2].

To reveal the mechanism behind the higher intestinal glucuronidation activity, assays with recombinant UGTs were conducted. Multiple UGTs, including UGT1A1, 1A3, 1A9, 2B7, 2B15 and intestinal UGT1A8, 1A10, were able to carry out GA glucuronidation. Among these, UGT1A8 displayed the highest GA glucuronidation activity, with a Cl_{int} value of more than 5-fold higher than other forms. In comparison with liver, UGT1A1, 1A3, 1A9, 2B7 and 2B15 are expressed in the intestine at very low levels [9]. Considering that HLM displays low GA glucuronidation activity, it is conceivable that intestinal activity is mainly catalyzed by UGT1A8 and 1A10.

Kinetic assays and chemical inhibition study also showed that UGT1A8 and 1A10 played important roles in intestinal GA glucuronidation. The reaction in HIM exhibited substrate inhibition kinetics, distinct from the Michaelis–Menten kinetics observed in HLM. The difference resulted from intestinal UGT1A8 and 1A10 that displayed substrate inhibition kinetics, while liver abundant UGT1A1 and 1A3 showed Michaelis–Menten kinetics. Nilotinib, magnolol, and fluconazole serve as selective inhibitors of UGT1A1, 1A9 and 2B7, respectively [1,26,28]). Their lack of inhibition against HIM (Fig. 6) excludes UGT1A1, 1A9 and 2B7 from having important roles in intestinal GA glucuronidation. Mycophenolic acid showed a high affinity towards UGT1A8 with a K_m value of 1.39 μ M [20]. It potently inhibited GA glucuronidation in HIM, thus indicating that UGT1A8 has an important role in intestinal glucuronidation. However, it should be noted that UGT1A10 may also be important in HIM. A previous study demonstrated that there are some activity problems in the commercial UGT1A10 preparations, and its contribution to glucuronidation pathway might be under-estimated when this isoform is involved [29].

The intestine acts as the first protective barrier and UGT1A8/1A10 is the first enzyme exposed to the food chemicals and clinical drugs in the gut. Several compounds, such as bakuchiol, isoliquiritigenin and stilbene glucoside, were reported to be inhibitors of UGT

activity or mRNA expression for these two isoforms [5,15,16,25]. When the inhibitors are co-administered, GA may possibly escape the intestinal glucuronidation and then enter the liver to potentially inhibit CYP3A activity and further induce undesired effects.

This study also tested GA glucuronidation in rat intestinal and liver microsomes. RLM and RIM both displayed the robust GA glucuronidation activity, greatly higher than HLM. These data are consistent with the finding UGT1A8 is expressed in both liver and intestine in rats [22]. Since GA glucuronidation occurs at high efficiency in rats, results from pharmacokinetic or toxicity studies conducted in this animal model may differ from those in humans. However, it should be noted that the observation of effective GA glucuronidation in RIM differs from a previous study in which RIM displayed very low glucuronidation activity (1/15–1/20 of RLM) [3]. The different GA glucuronidation activity of RIM may possibly result from the different incubation conditions in the two studies. In the study by Cao et al., low oral bioavailability was mainly attributed to P-glycoprotein mediated efflux and hepatic glucuronidation. This study demonstrates that in rats, the contribution of intestinal glucuronidation to low oral bioavailability cannot be excluded.

This study additionally investigated GA glucuronidation in DLM and CyLM, and the results indicated that DLM displayed considerable GA glucuronidation activity, higher than HLM, but lower than RLM, RIM, and HIM. For CyLM, the catalytic activities were low and comparable to those for HLM. This is consistent with data from cynomolgus monkeys that UGT1A8 is also abundant in intestine but not expressed in liver [18]. Although GA glucuronidation was not tested in intestinal microsomes in cynomolgus monkeys (CyIM), it is likely that the reaction may also occur efficiently due to the high expression of UGT1A8. While little information is available for UGT expression in beagle dogs, this study suggests that in liver, UGT1A8 may be expressed at significant levels.

One interesting finding in this study is that the two hydroxyl groups at C2 and C4 in the GA B ring display very different glucuronidation potential. Indeed, M2 formation is a preferred conjugation pathway for nearly all UGTs. Previous studies indicated that the hydroxyl group at C4 in the B ring is subject to glucuronidation by intestinal UGTs [24]. One notable example is prunetin, in which the C4 hydroxyl group in the B ring can be effectively conjugated with the glucuronosyl group by UGT1A8 and 1A10 rather than liver UGTs [11]. Assays with chalcones (a group of flavones), indicated that C2 hydroxyl group in the B ring can be conjugated by intestinal UGTs, however, presence of the C4 hydroxyl group can quench glucuronidation at this site [17]. Therefore, it is suspected that M2 may possibly result from glucuronidation at the C4 hydroxyl group in the B ring, which still remains to be elucidated by additional studies.

Similar with recombinant UGTs, tissue microsomes of human and experimental animals also displayed regioselectivity. M2 formation was a dominant metabolic pathway in all assays with microsomes, and HIM, RLM, and RIM lost most of the M1 formation activity. Different from the other microsomes, DLM exhibited considerable activity towards M1 formation. Among the isoforms involved in GA glucuronidation, only UGT1A1 displayed relatively high capacity to generating M1. Thus, it seems that UGT1A1 activity in dog liver is much higher than human, rats and monkeys. Interestingly, an earlier study supports this

notion, demonstrating that ethylestradiol-3-O-glucuronidation, a probe reaction for UGT1A1, occurs 10-fold more rapidly by DLM than HLM [23].

5. Conclusions

The current study indicates that intestinal UGTs, notably UGT1A8 and 1A10, play important roles in GA glucuronidation in humans. Thus, any pharmacological and toxicological of GA effects may depend on activities of these two isoforms. This study also revealed that GA glucuronidation activity varies among species. The rank order of microsomal GA glucuronidation activity is RIM and RLM > HIM > DLM > CyLM and HLM. However, caution should be exercised in attempting to determine the pharmacokinetics and toxicity of GA in rats, due to the effective glucuronidation in both liver and intestine of this experimental animal model.

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Abbreviations:

GA	glabridin
UGT	UDP-glucuronosyltransferases
HLM and HIM	liver and intestinal microsomes from humans, respectively
RLM and RIM	liver and intestinal microsomes from rats, respectively
CyLM and DLM	liver microsomes from cynomolgus monkeys and beagle dogs, respectively
UDPGA	uridine-5-diphosphoglucuronic acid
Cl_{int}	intrinsic clearance.

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Fig. 1.
Molecular structure of glabridin (GA).

Fig. 2. Representative LC profiles of GA and its glucuronides produced by HLM and HIM. GA, M1, and M2 were eluted at 15.7, 10.8 and 11.2 min, respectively.

Fig. 3. Kinetics of GA glucuronidation of HLM (A) and HIM (B). Data points represent the mean of triplicate determinations, and error bars are the calculated S.D. value.

Fig. 4. Formation of M1 and M2 by recombinant UGT isoforms. 2 μ M GA was incubated with individual recombinant human UGTs (0.1 mg/mL) at 37 $^{\circ}$ C for 60 min. Data column represented the mean of triplicate determinations and error bar was the calculated S.D. value.

Fig. 5. Kinetics of GA glucuronidation by recombinant UGT1A1 (A), 1A3 (B), 1A8 (C), and 1A10 (D). Data points represent the mean of triplicate determinations, and error bars are the calculated S.D. values.

Fig. 6. Inhibitory effects of nilotinib (1 μ M), magnolol (1 μ M), fluconazole (1 mM) and mycophenolic acid (10 μ M) on GA (2 μ M) glucuronidation (formation of M2) in HIM. Incubation without chemical inhibitors is set as the control. Data columns and error bars represent the means and S.D. values of triplicate determinations.

Fig. 7.
Kinetics of GA glucuronidation (M2 formation) by RLM (A) and RIM (B). Data points represent the mean of triplicate determinations, and the error bars are the calculated S.D. values.

Fig. 8.
Kinetics of GA glucuronidation by DLM (A) and CyLM (B). Data points represent the mean of triplicate determinations, and error bars are the calculated S.D. values.

Table 1

Incubation conditions for kinetic assays in recombinant UGTs

Isoforms	GA concentrations (μM)	Enzyme levels (mg/mL)	Incubation time (min)
UGT1A1	1, 2, 5, 7.5, 10, 20, 50	0.1	60
UGT1A3	0.5, 1, 2, 5, 7.5, 10, 20	0.1	60
UGT1A8	0.2, 0.5, 1, 2, 5, 7.5, 10, 15, 20	0.02	40
UGT1A9	2, 5, 10, 20, 50, 100, 150, 200	0.1	60
UGT1A10	2, 5, 10, 20, 50, 75	0.1	60
UGT2B7	1, 2, 5, 10, 20, 50, 75, 100	0.1	60
UGT2B15	1, 2, 5, 10, 20, 50, 75, 100	0.1	60

Kinetic constants for GA glucuronidation in liver and intestinal microsomes from experimental animals. Data represent as mean $\pm$ S.E. of computer calculation. K_m (K_S) and K_{SI} values are in μ M, V_{max} values are nmol/min/mg, and Cl_{int} and Cl_{total} values are in lL/min/mg.

Table 2

Enzymes	Metabolites	K_m (K_S)	V_{max}	K_{SI}	Cl_{int}	Cl_{total}
HLM	M1	6.3 ± 3.6	0.044 ± 0.005	N.A.	7	17
	M2	24.2 ± 5.7	0.25 ± 0.03	N.A.	10	
HIM	M2	8.3 ± 3.5	16.7 ± 7.4	1.6 ± 0.7	2010	2010
RLM	M2	3.9 ± 1.9	16 ± 5	7.6 ± 3.0	4100	4100
RIM	M2	3.8 ± 1.1	19 ± 6	5.3 ± 2.5	5000	5000
DLM	M1	34 ± 12	2.4 ± 0.5	N.A.	71	285
	M2	29 ± 12	6.2 ± 1.3	N.A.	214	
CyLM	M1	10 ± 3	0.051 ± 0.005	N.A.	5	20
	M2	35 ± 11	0.51 ± 0.09	N.A.	15	

Due to the low activity, kinetic assays with HIM, RLM and RIM were only conducted for M2 formation. For the other enzymes, kinetic assays were performed for both M1 and M2 formation. Kinetic data for HLM, DLM and CyLM were fitted into the Michaelis–Menten equation, while data for HIM, RLM and RIM were fitted into substrate inhibition equation.

Table 3

Kinetic constants for GA glucuronidation by recombinant UGTs. Data are shown as mean $\pm$ S.E. K_m (K_S) and K_{SI} values are in μM , $V_{\max}$ values are nmol/min/mg, and Cl_{int} values are in lL/min/mg

UGTs	Metabolites	K_m (K_S)	$V_{\max}$	K_{SI}	Cl_{int}
1A1	M1	7.6 ± 1.8	0.17 ± 0.01	N.A.	22
	M2	24 ± 14	0.11 ± 0.03	N.A.	5
1A3	M2	11 ± 4	0.32 ± 0.06	N.A.	29
1A8	M2	64 ± 9	11 ± 3	1.7 ± 0.4	172
1A10	M2	28 ± 8	0.5 ± 0.1	36 ± 11	18

Due to the low activity, kinetic assays were mainly conducted for M2 formation; kinetic assays with UGT1A1 also included M1 formation. Data for GA glucuronidation by UGT1A1 and 1A3 were fitted into the Michaelis–Menten equation, while data for UGT1A8 and 1A10 were fitted into substrate inhibition equation.