Identification and Characterization of Human UDP- Glucuronosyltransferases responsible for the \textit{in vitro} Glucuronidation of Daphnetin

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Running title: Metabolism of daphnetin by human UGTs

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Abbreviations:
  UGT, UDP-glucuronosyltransferase; COMT, catechol-O-methyltransferase; SULT, sulfotransferase; UDPGA, uridine-5'-diphosphoglucuronic acid; HLM, human liver microsomes; HIM, human intestinal microsomes; PLM, minipig liver microsomes; RLM, rat liver microsomes; IS, internal standard; DAD, diode array detector; ESI, electrospray ionization; UFLC, Ultra-Fast liquid chromatography; NMR, nuclear magnetic resonance.
Abstract

Daphnetin has been developed as an oral medicine for treatment of coagulation disorders and rheumatoid arthritis in China, but its in vitro metabolism remains unknown. In the present study, the UDP-glucuronosyltransferase (UGT) conjugation pathways of daphnetin were characterized. Two metabolites, 7-O (M-1) and 8-O (M-2) mono-glucuronide daphnetin were identified by liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) when daphnetin was incubated respectively with liver microsomes from human (HLM), rat (RLM), minipig (PLM) and human intestinal microsomes (HIM) in the presence of uridine-5'-diphosphoglucuronic acid (UDPGA). Screening assays with 12 human recombinant UDP-glucuronosyltransferases (UGTs) demonstrated that the formations of M-1 and M-2 were almost exclusively catalyzed by UGT1A9 and UGT1A6, while M-1 was formed to a minor extent by UGT1A3, 1A4, 1A7, 1A8 and 1A10 at high substrate concentration. Kinetics studies, chemical inhibition, and correlation analysis were used to demonstrate that human UGT1A9 and UGT1A6 were major isoforms involved in the daphnetin glucuronidations in HLM and HIM. By in vitro-in vivo extrapolation of the kinetic data measured in HLM, the hepatic clearance and the corresponding hepatic extraction ratio were estimated to be 19.3 ml/min/kg body weight and 0.93, respectively, suggesting that human clearance of daphnetin via the glucuronidation is extensive. Chemical inhibition of daphnetin glucuronidation in HLM, RLM and PLM showed large species difference although the metabolites were formed similarly among the species. In conclusion, the UGT conjugation pathways of daphnetin were fully elucidated and its C-8 phenol group was more selectively catalyzed by UGTs than the C-7 phenol.
Introduction

Benzo-α-pyrone skeleton-containing coumarins are widely distributed in the plant kingdom and have a general application in food, drug and cosmetic products (Egan et al., 1990). As one of coumarin derivatives, daphnetin (7, 8-dihydroxycoumarin) has exhibited broad biological activities, such as anti-inflammatory (Fylaktakidou et al., 2004), antioxidant (Fylaktakidou et al., 2004), antimicrobial (Cottiglia et al., 2001) and anti-malarial (Huang et al., 2006) effects as well as the protein kinase inhibitory activities (Yang et al., 1999). Clinically, daphnetin not only has been developed as an oral medicine for treatment of coagulation disorders and rheumatoid arthritis in China since 1980s (http://app1.sfda.gov.cn/datasearch/face3/base.jsp), but also been used as a folk medicine to treat lumbago and reduce fever in Turkey (Yesilada et al., 2001). Moreover, recent studies demonstrated that daphnetin can act as a potent antiproliferative and differentiation-inducing agent in human renal cell carcinoma (Finn et al., 2004).

The pharmacokinetic study of daphnetin in rats after intravenous administration shows that the elimination of daphnetin is rapid with a $t_{1/2}$ of about 15 min and poor bioavailability; however, the responsible mechanisms for the pharmacokinetic properties are unclear (Qu et al., 1983). Structurally, daphnetin contains a catechol group on its basic skeleton, which could be susceptible to the metabolism by phase II enzymes, such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and catechol-O-methyltransferases (COMTs) (Antonio et al., 2002; Antonio et al., 2003). Many glucuronidation derivatives of catechol-containing drugs have been detected both in vitro and in vivo, e.g., L-3,4-dihydroxyphenylalanine, a therapeutic drug of Parkinson’s disease.
(Antonio et al., 2002; Antonio et al., 2003); protocatechuic aldehyde, a antasthmatic agent (Liu et al., 2008); and PH-302, an inhibitor of nitric oxide synthase (Loureiro et al., 2006). On the other hand, it is noted by the previous studies that 7-hydroxycoumarin and 4-methylumbelliferone, the structural analogs of daphnetin, are substrates for the UGTs with high affinity (Uchaipichat et al., 2004; Wang, 2006). Therefore, it is necessary to understand the role of glucuronidation in the metabolic elimination of daphnetin since the glucuronidation of most benzo-α-pyrone has been demonstrated as a primary route of clearance (Tukey and Strassburg, 2000).

Glucuronidation, the major phase II conjugation reaction, accounts for > 35% of all phase II drug metabolites (Kiang, 2005). Nineteen human UGT isoforms have been identified, and these UGTs have been classified into two families (UGT1 and UGT2) based on their sequence identity (Tukey and Strassburg, 2000; Mackenzie et al., 2005). Many UGTs have had broad and overlapping substrate specificities, though the specificity of UGT isoforms to certain substrates is known (e.g., UGT1A1 for bilirubin, UGT1A4 for trifluoperazine, UGT1A6 for serotonin, UGT1A9 for propofol, and UGT2B7 for 3’-azido-3’-deoxythymidine) (Court, 2005; Miners et al., 2006). In addition, human UGTs are tissue-specific due to their differences in expression. For example, UGT1A7, 1A8, 2A1, and 1A10 are mainly expressed in extrahepatic tissues, though liver is generally recognized as the major site of glucuronidation (Fisher et al., 2001). Notably, highly inter-individual variability and the differential UGT expression within different segments of the gut have been also reported (Strassburg et al., 1998). Identification of the human UGT isoform(s) responsible for drug glucuronidation is the pivotal information to understand the potential of
clinical drug drug interactions (DDIs) of drugs or drug candidates as the victims when co-administered with an inhibitor of the target UGT(s). Furthermore, the *in vivo* clearance prediction from *in vitro* enzyme kinetic parameters (IVIVE) can be employed to evaluate quantitative contribution of the UGT(s) to total clearance of a drug.

To date, information on the metabolism of daphnetin still remains unclear. The purposes of the present study are (i) to characterize the *in vitro* metabolites of daphnetin, (ii) to identify the human UGT isoform(s) responsible for the formation of the metabolites, (iii) to determine the kinetics of daphnetin-glucuronides in microsomes from human liver (HLM), human intestine (HIM), rat liver (RLM) and minipig liver (PLM) and recombinant UGTs, respectively and (iv) to compare the species difference by inhibition analyses.
Materials and Methods

Chemicals and reagents

Daphnetin (≥ 97%), alamethicin, Brij 58, magnesium chloride, D-saccharic acid 1,4-lactone, β-glucuronidase (EC No. 3.2.1.31), uridine 5’-diphospho-glucuronic acid trisodium salt (UDPGA), serotonin, propofol, mafenamic acid and phenylbutazone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Microcon® YM-30 centrifugal filter devices were obtained from Millipore (Bedford, MA, USA). HLM, RLM and PLM were prepared according to the methods described by Guengerich (1989) and previous reports (Zhang et al., 2008). Protein concentration was determined by using bovine serum albumin as the standard (Lowry et al., 1951). Pooled HIM containing equal amounts of microsomes was prepared from both the duodenum and jejunum section of each of the five donors (one female and four males of Caucasian and African American race, with ages ranging from 16 to 64 years) and a panel of recombinant human UGT Supersomes expressed in baculovirus-infected insect cells were purchased from BD Gentest Corp. (Woburn, MA). The glucuronidation activity of Supersomes using different substrates, estradiol for UGT 1A1 and UGT1A3, trifluoperazine for UGT1A4, eugenol for UGT2B17, and 4-methylumbelliferone for UGT1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7 and 2B15, were substantiated by the supplier, with the values of 1000, 250, 1300, 2200, 6900, 12000, 630, 8700, 86, 330, 1300, and 2100 pmol/min/mg, respectively. Millipore water (Millipore, Bedford, MA, USA) and HPLC grade methanol and acetonitrile (Tedia, USA) were used throughout; other reagents were of HPLC grade or of the highest grade commercially available.
Identification of daphnetin glucuronidation

The incubation mixture (200 μl) contained HLM (0.5 mg of protein/ml), 5 mM UDPGA, 5 mM MgCl₂, 25 μg/ml alamethicin, 10 mM d-saccharic acid 1,4-lactone, 200 μM daphnetin and Tris-HCl buffer (pH 7.4). After 10 min of incubation at 37°C, the reaction was terminated by the addition of 0.1 ml of methanol containing 0.3 μg of internal standard (7-hydroxycoumarin), followed by centrifugation at 20,000g for 10 minutes to obtain the supernatant for Ultra-Fast liquid chromatography spectrometry (UFLC) analysis. Control incubations without UDPGA or without substrate or without microsomes were performed to ensure that the metabolites produced were microsome- and UDPGA-dependent. A Shimadzu LC/MS-2010EV mass spectrometer (Kyoto, Japan) with an ESI interface was used to identify daphnetin and its metabolites (M-1 and M-2) operating in both negative and positive ion modes from m/z 100 to 1000. The detector voltage was set at +1.50 kV, and −1.55 kV for positive and negative ion detection, respectively. The curved desolvation line temperature (CDL) 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 was tuned to be 1.5 l/min and the drying gas (N₂) pressure was set at 0.06 MPa. Data processing was performed using the UFLC-MS Solution version 3.41 software.

In order to confirm these two metabolites are glucuronide compounds, 200 μl glucuronidation incubation mixture (see above, but without d-saccharic acid 1,4-lactone) was incorporated into an equal volume of 0.15 M acetate buffer (pH 5.0) containing 1800 Fishman units of β-glucuronidase. After 2 h incubation at 37°C, the hydrolysis reaction was terminated by addition of 0.1 ml of cold methanol, followed by centrifugation at 20,000g for
10 minutes, then 10 μl supernatant was injected for UFLC-DAD-ESI-MS analysis. Control incubations without β-glucuronidase were performed simultaneously.

**Biosynthesis metabolite and NMR spectrometry**

The glucuronidation metabolites (M-1 and M-2) of daphnetin were biosynthesized and purified for structure elucidation and quantitative analysis. Enzymatic biosynthesis of M-1 and M-2 was conducted using RLM, because they resemble HLM in daphnetin metabolism. In brief, 600 μM daphnetin was incubated with RLM (0.5 mg protein/ml), 0.1 M Tris-HCl pH 7.4, 10 mM MgCl₂, Brij 58 (0.5 mg/mg protein), 10 mM D-saccharic acid 1,4-lactone, and 5 mM UDPGA in 100 ml of final incubations for 2 h at 37°C. The stock solution of daphnetin (60 mM) was prepared in methanol. The concentration of organic solvent in the final incubation was 1%. The reaction was terminated by transferring the vessel to an ice bath and cooling for 20 min. After the removal of protein by centrifugation at 20,000 g for 15 min at 4°C, the combined supernatants were loaded on a SPE cartridge (C₁₈, 1000 mg, Agela Technologies, USA), which was preconditioned by sequential washing with 5 ml methanol and 5 ml water containing 0.2% formic acid. After loading on the incubation material, the cartridge was washed with 15 ml water containing 0.2% formic acid. Then the trapped compounds were eluted with 5 ml methanol, and blown dry with nitrogen gas at 20°C. Finally, the residual was redissolved in 1ml methanol, and separated by HPLC (Shimadzu, Kyoto, Japan) equipped with an SCL-10A system controller, two LC-10AT pumps, an SIL-10A autoinjector, an SPD-10AVP UV detector, and Shim-pack (Shimadzu) C₁₈ column (4.6 × 150 mm, 5μm). The mobile phase consisted of CH₃CN (A) and 0.2% (v/v) formic
acid (B) with linear gradient from initially 5 to 80% A over 15 min. The flow rate was 1.4 ml/min. The purity was greater than 99% for both metabolites by using UFLC-DAD detection.

The structures of metabolites M-1 and M-2 were determined by spectral methods including 2D-NMR such as HSQC, HMBC and NOESY. All experiments were recorded on a Bruker AV-600 (Bruker, Newark, Germany). The purified metabolites of M-1 (2.6 mg) and M-2 (2.3 mg) were stored at –20°C before dissolving in DMSO-d6 (Euriso-Top, Saint-Aubin, France) for NMR analysis. Chemical shifts were given on δ scale and referenced to tetramethylsilane (TMS) at 0 ppm for 1H- (600 MHz) and 13C-NMR (150 MHz).

**Assay with recombinant UGTs**

Daphnetin glucuronidation was measured in reaction mixtures containing recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17. The incubations were carried out as shown above for the HLM study. Three substrate concentrations (10, 100 and 600 μM) were used in this study: 600 and 100 μM were the approximate concentration at \( V_{\text{max}} \) and \( K_m \) value for HLM, respectively; 10 μM was used to evaluate the catalytic activity of the UGT isoform(s) with the high affinity for daphnetin glucuronidation. All assays were conducted at 37°C for 60 min with the final protein concentration of 0.5 mg protein/ml. UFLC with DAD detection was used to monitor possible metabolites.
**Chemical inhibition studies**

Daphnetin glucuronidation in pooled HLM, HIM, UGT1A6, and UGT1A9 were measured in the absence or presence of inhibitor (phenylbutazone or mefenamic acid). Phenylbutazone is the potent inhibitor for UGT1As (Uchaipichat et al., 2006) and mefenamic acid is the potent inhibitor for UGT1A9 (Tachibana et al., 2005). Daphnetin (100 μM) was incubated in the absence or presence of either phenylbutazone (10-500 μM) or mefenamic acid (1-50 μM). All incubations were performed for 10 min using a protein concentration of 0.1 mg of protein/ml. The IC₅₀, representing the inhibitor concentration that inhibits 50% of control activity, was determined by nonlinear curve fitting with Origin (Orgin Lab Corporation, Northampton, MA), as described previously (Zhang et al., 2009).

For comparison of the inhibitory effects of phenylbutazone and mefenamic acid among different species, several concentrations of phenylbutazone (50 μM and 500 μM) and mefenamic acid (10 μM and 100 μM) in pooled liver microsomes from human, rat and minipig were conducted to investigate their inhibitory effects on daphnetin glucuronidation.

**Correlation analysis in HLM**

Linear regression analysis was used to assess the correlation between the glucuronidation activity toward daphnetin and serotonin or propofol in microsomes from 14 individual human livers. The activity of UGT1A6-catalyzed serotonin glucuronidation and UGT1A9-catalyzed propofol glucuronidation was 1.27 to 3.05 nmol/min/mg and 0.25 to 2.20 nmol/min/mg of protein, respectively. The daphnetin and HLM concentrations were 100 μM and 0.1 mg of protein/ml, respectively. The reaction mixture was incubated for 10 min at
37°C. The glucuronidation activities of serotonin and propofol were provided by the manufacturer as typical reference activities for UGT1A6 and UGT1A9, respectively. \( P < 0.05 \) was considered statistically significant.

**Kinetic study**

The formation rates of daphnetin metabolites were linear over 30 min of incubation and 0.05 to 0.3 mg of microsomal protein. To ensure that less than 10% of substrate was metabolized in all incubations, the kinetic determinations were performed using a microsomal protein concentration of 0.1 mg/ml (HLM, HIM, PLM, PLM or Supersomes) with 10 min incubation. For estimating kinetic parameters, daphnetin (5-600 \( \mu \)M) was incubated with different sources of pooled microsomes (HLM, HIM, PLM or RLM). For recombinant human UGT1A9 and UGT1A6, daphnetin (5-400 \( \mu \)M) was incubated with UGT1A9 and UGT1A6 for kinetic analysis. Kinetic constants for daphnetin glucuronidation by HLM, HIM, UGT1A9, or UGT1A6 were obtained by fitting with the Michaelis-Menten equation using Origin. The Michaelis-Menten equation is \( v = (V_{\text{max}} + [S])/(K_m +[S]) \), where \( v \) is the rate of reaction, \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is the Michaelis constant (substrate concentration at 0.5 \( V_{\text{max}} \)), and [S] is the substrate concentration. Results are expressed as mean ± computer-calculated S.E. of the estimate.

**Quantitative analysis**

The separation and quantification of daphnetin glucuronides by UFLC were carried out using a Shimadzu (Kyoto, Japan) Prominence ultra-fast liquid chromatography (UFLC)
system, which equipped with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, and a CTO-20AC column oven, as well as a diode array detector (DAD). A Shim-pack XR-ODS column (100 mm × 2.0 mm, 2.2 µm, Shimadzu) was kept at 40°C. Elution was carried out by the following gradient program with solvent A (CH₃CN) and solvent B (0.2% formic acid): 0-2 min, 95-83% B; 2-7 min, 85-76% B; 7-9.5 min, 10% B; 9.5-14.5 min, 95% B. The flow rate was 0.3 ml/min, and the detection wavelength was set at 320 nm. The biosynthesized glucuronidation metabolites M-1 and M-2 were dissolved in methanol and used as the calibration standards. The calibration curves were generated by peak area ratios (calibration standard/internal standard) over the concentration range of 0.5-30 μM. The standard curves of the two metabolites were linear over this concentration range, with r² values > 0.99. The limits of detection and quantification were determined at signal to noise ratios of 3 and 10, respectively. The accuracy and precision of the back-calculated values for each concentration were less than 15%.

Prediction of tissue clearances from in vitro data

The in vivo intrinsic clearances (CL'_{int}) of daphnetin glucuronidation in liver and intestine were scaled-up by the equation 1 (Obach et., al 1997):

\[
CL'_{int} = \frac{V_{max}}{K_m \times f_{u,m}} \times \frac{mg \ of \ microsomal \ protein}{g \ of \ tissue} \times \frac{g \ of \ tissue}{kg \ of \ weight}
\]  \hspace{1cm} (1)

where \( V_{max} \) is the maximum velocity, \( K_m \) is the Michaelis constant and \( f_{u,m} \) is the free fraction of daphnetin in the microsomes. The scaling factors for human liver (40 mg/g and 21.4 g/kg body weight) and intestine (3 mg/g and 30 g/kg body weight) used in these calculations were
obtained from Cubitt et al. (2009) and Soars et al. (2002), respectively. $f_{u,m}$ was predicted to be 0.98 according to equation 2 (Austin et al. 2002):

$$f_{u,m} = \frac{1}{(C_{mic} \times 10^{0.56 \log P - 1.41}) + 1}$$  \hspace{1cm} (2)

where $C_{mic}$ is the microsomal protein concentration used in the incubation, the value of which is 0.1 mg/ml for both HIM and HLM, and LogP is the log of the octanol buffer (pH = 7.4) partition (P) coefficient of the daphnetin, the value of which is 1.2 according to the website (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=5991). Therefore, the bound fraction of daphnetin to microsomal protein can be negligible in this study.

For evaluation of the magnitude of UGT conjugation pathways to the elimination of daphnetin, the hepatic clearance ($CL_H$) was predicted from the resulting estimated in vivo hepatic intrinsic clearance ($CL'_{int,liver}$) using equation 3 based on the well-stirred model (Miner et al. 2006):

$$CL_H = \frac{Q_H \times f_{u,b} \times CL'_{int,liver}}{Q_H + f_{u,b} \times CL'_{int,liver}}$$  \hspace{1cm} (3)

where $f_{u,b}$ is the fraction unbound of daphnetin in blood, $Q_H$ is hepatic blood flow, and $Q_H$ values of 20.7 ml/min/kg weight (Cubitt et al. 2009) were used. Fraction unbound of daphnetin in blood and plasma ($f_{u,p}$) were considered equivalent for extrapolation purposes, although it is acknowledged that some difference may occur. $f_{u,p}$ was obtained from the separate experiments using the ultrafiltration method (Taylor and Harker, 2006) at two concentrations (17.8 and 178 ng/ml) of daphnetin.
Results

Identification of daphnetin metabolites

Two new peaks were eluted at 4.0 (M-1) and 4.5 min (M-2) by UFLC, respectively when daphnetin (200 μM) was incubated with HLM, HIM (data not shown), RLM or PLM (0.1 mg/ml) in the presence of UDPGA (Fig. 1). The peaks were not detected in the control samples in the absence of UDPGA, daphnetin or microsomes (data not shown). The negative-ion mode was adopted for chemical identification because it is more sensitive than the positive-ion mode for daphnetin and its metabolites. Mass spectra were dominated by [M-H]⁻. M-1 and M-2 were identified by UFLC-ESI-MS to show identical product ions at m/z 353 in negative-ion mode with characteristic m/z 176, corresponding to the glucuronide. In addition, these two metabolites were hydrolyzed by β-glucuronidase to the parent (daphnetin), indicating that M-1 and M-2 are the monoglucuronide metabolites. The two metabolites were further purified and characterized for structural elucidation by ¹H-NMR, ¹³C-NMR, HSQC and HMBC spectral technology (Table. 1). Compared with that of daphnetin, the ¹³C-NMR spectrum of metabolite M-1 showed that C-7 of M-1 shifted upfield to δ 147.8 (Δδ –1.9) due to the glycosidation shift of phenolic compound, while C-6 and C-8 shifted downfield to 114.5 (Δδ +1.9) and 134.1 (Δδ +1.8), respectively (Table. 1). All these evidence indicated that the presence of glucuronosyl substitution should be at the C-7 phenolic group. Similarly, M-2 identified to be 8-O mono-glucuronide daphnetin by comparing with ¹³C-NMR spectrum of daphnetin and regulation of glycosidation shift. Moreover, all the ¹H- and ¹³C-NMR spectral data of metabolites M-1 and M-2 were assigned by the 2D-NMR spectra (data not shown).
Assay with recombinant human UGTs

With the substrate concentrations of 10 or 100 μM, UGT1A6 and UGT1A9 were the only isoforms involved in the glucuronidation of daphnetin. At the high substrate concentration (600 μM), UGT1A6 and UGT1A9 were still the major isoforms involved in daphnetin glucuronidation, though trace amount of M-1 was formed by UGT1A3, 1A4, 1A7, 1A8 and 1A10 (< 20 pmol/min/ mg protein, Fig. 2).

Chemical inhibition

The inhibitory effects of phenylbutazone and mefenamic acid on daphnetin glucuronidation in pooled HLM, HIM, UGT1A6 and UGT1A9 were evaluated. As shown in Table. 3, phenylbutazone inhibited potently the UGT activity for the formation of the metabolites in HLM, HIM, UGT1A6 and UGT1A9. Mefenamic acid appeared generally to be more potent in inhibition than phenylbutazone except for UGT1A6. Similar IC_{50} values of phenylbutazone for HLM, HIM, UGT1A6 and UGT1A9 were measured. However, mefenamic acid showed differential inhibitory effects on the enzyme activity, where the inhibition of UGT1A6 (IC_{50} > 50 μM) was poorer than that observed from other enzyme preparations. As shown in Fig. 3, the inhibitory profiles of phenylbutazone and mefenamic acid on daphnetin glucuronidation in HLM, RLM and PLM were illustrated to show the species difference. The inhibition rank order of daphnetin glucuronidation among the species was HLM > PLM > RLM for phenylbutazone, and HLM > RLM > PLM for mefenamic acid, respectively.
Correlation analysis

The velocities for daphnetin glucuronidation in liver microsomes from 14 human donors were obtained to range from 1.2 to 10.6 nmol/min/mg of protein for M-1, and from 1.3 to 7.8 nmol/min/mg of protein for M-2, respectively. The mean ± S.D. for rate of the metabolite formation of M-1 and M-2 were 6.0 ± 3.3 and 4.8 ± 2.8 nmol/min/mg of protein, with a coefficient of variation (CV) of 57.9 % for M-1 and 59.3 % for M-2, respectively. Correlation analyses were performed between the daphnetin glucuronosyltransferase activities and serotonin (UGT1A6) or propofol (UGT1A9) glucuronosyltransferase activities in 14 human liver microsomes. As shown in Fig. 4, the daphnetin glucuronosyltransferase activities were significantly correlated with the propofol glucuronosyltransferase activities \( (r = 0.91, P < 0.001 \) for M-1; \( r = 0.94, P < 0.001 \) for M-2) and serotonin glucuronosyltransferase activities \( (r = 0.64, P = 0.011 \) for M-1; \( r = 0.69, P = 0.006 \) for M-2).

Kinetic characterization

The kinetic parameters of M-1 and M-2 production in HLM, HIM, RLM, PLM and the potent UGT isoforms (Table 3) were determined. It was unrealistic to evaluate the kinetic parameters in UGT 1A3, 1A4, 1A7, 1A8 and 1A10, since the metabolite (M-1) formation rate was around the limits of quantification. The ranges of substrate concentration for the kinetic analyses in liver and intestine microsomal assays were 5 to 600 μM and 5 to 400 μM in UGT1A9 and UGT1A6, respectively. In the concentration ranges tested, daphnetin glucuronidation exhibited the Michaelis-Menten kinetic characteristics, as evidenced by a linear Eadie-Hofstee plot (Fig. 5). The \textit{in vitro} total intrinsic clearances (the sum of the M-1
and M-2; calculated as \( V_{\text{max}}/K_{\text{m}} \) of the glucuronidation of daphnetin in human liver and intestinal microsomes were 402, and 108 \( \mu \text{l/min/mg} \) protein, respectively. By scaling-up, the \textit{in vivo} intrinsic clearances in liver and intestine were estimated to be 344 and 9.7 ml/min/kg body weight, respectively. Based on the detected \( f_{u,p} \) (0.8), the \textit{in vivo} hepatic intrinsic clearance was further extrapolated to the hepatic clearance with the value of 19.3 ml/min/kg body weight using well-stirred model equation (see Methods). The hepatic extraction ratio, calculated from the ratio of the predicted hepatic clearance and the hepatic blood flow, was 0.93. Kinetic parameters for M-1 and M-2 formation by HLM, HIM, RLM, PLM and recombinant UGTs are shown in Table 2. The intrinsic clearance values (\( V_{\text{max}}/K_{\text{m}} \)) for the two metabolites among the species were: M-1 > M-2 in HLM, M-2 > M-1 in PLM and M-2 \( \geq \) M-1 in RLM. Intrinsic clearance values (M-1 and M-2) in species were PLM > RLM > HLM.
Discussion

Most of hydroxycoumarin derivatives sharing their benzo-α-pyrone skeleton are good substrates of human UGTs (Tukey and Strassburg, 2000). For example, 4-methylumbelliferone has been widely used as non-selective probes for evaluation of glucuronidation activities (UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15 and 2B17) due to its high affinity for UGTs and turnover for the formation of the metabolite (Uchaipichat et al., 2004). With respect to the catechol compounds, UGT1A6, UGT1A9 and UGT2B7 can be potentially involved in their glucuronidation (Antonio et al., 2003; Loureiro et al., 2006). Daphnetin is the hydroxycoumarin derivative as well as the catechol-containing compound. In the present study, UGT1A9 and UGT1A6 were the most capable of catalyzing the glucuronidation of daphnetin at three substrate concentrations, but trace amount of daphnetin 7-O-glucuronide could be formed by UGT1A3, 1A4, 1A7, 1A8 and 1A10 (<20 pmol/min/mg protein) at the high substrate concentrations (600 μM) (Fig. 3). The findings indicated that the C-8 phenolic group of daphnetin was more selectively catalyzed by UGTs compared with the C-7 phenol.

UGT1A6 and UGT1A9, which exhibited the highest activity towards daphnetin, are expressed in both the liver and intestine (Strassburg et al., 1998; Basu et al., 2004; Miners et al., 2006). Many phenol-containing xenobiotics are substrates of UGT1A6 and UGT1A9, and significant overlap of substrate selectivity occurs (Ebner and Burchell, 1993; Uchaipichat et al., 2004). The kinetic studies of daphnetin glucuronidation by UGT1A9 and UGT1A6 showed the similar $V_{\text{max}}/K_m$ values between these two isoforms for M-1 formation, though the $V_{\text{max}}/K_m$ values with UGT1A9 was higher than that of UGT1A6 for M-2 formation, which could be attributed to the relative low $K_m$ value with UGT1A9 (Table 2). In addition, the $K_m$
values in recombinant UGT1A9 and UGT1A6 were lower than those in HLM and HIM, it may due to 1) the difference of the single/multiple UGT nature between cDNA-expressed and microsomal samples; 2) the relative low fatty acid content in expression systems, since long-chain unsaturated fatty acids can act as potent competitive inhibitors of UGT1A9 (Rowland et al., 2008); 3) the involvement of other UGTs for the formation of M-1 at high substrate concentrations (UGT1A3 and UGT1A4 for HLM; UGT1A3, 1A4, 1A7, 1A8 and 1A10 for HIM; See Fig. 2). The comparable kinetic profiles of UGT1A9 and UGT1A6 were also confirmed by the evidence of monophasic Eadie-Hofstee plots observed in HLM and HIM, which suggested that UGT1A9 and UGT1A6 play major roles in formation of daphnetin glucuronides in these two organs, though UGT1A9 showed marginally higher affinity than that of UGT1A6 (Table 2). Moreover, the findings were also supported by the results of correlation analysis and the subsequent inhibition studies, high correlation between daphnetin glucuronides formation with propofol O-glucuronidation as well as serotonin O-glucuronidation was displayed in Fig. 4, and the comparable inhibitory effects of phenylbutazone and mfenamic acid on human liver and intestine microsomes with similar IC₅₀ values can be seen from Table. 3. It should be noted that mfenamic acid is a known potent inhibitor of UGT1A9 and UGT2B7 in the literature (Tachibana et al., 2005; Gaganis et al., 2007). Since UGT2B7 was not found to be involved in daphnetin glucuronidation, mfenamic acid can be served as selective inhibitors to UGT1A9 in this study. Altogether, in vitro UGT conjugation pathways of daphnetin in human liver and intestine were proposed in Fig. 6. As reported, UGT1A7, UGT1A8, and UGT1A10 are exclusively expressed in gastrointestinal tract (Fisher et al., 2001), and thus these isoforms can not be involved in the formation of 7-O mono-glucuronide daphnetin (M-1) in HLM.
In the current study, the large inter-individual variability (57.9% CV for M-1 and 59.3% for M-2) was observed in the liver samples from 14 donors, which would be attributed to the effect of age, sex, enzyme inducers (e.g., drugs, tobacco, and alcohol), and genetic polymorphism on the enzyme sources from different donors (Court, 2009). In agreement with our data, the work by Court (2005) showed that UGT1A9-mediated propofol glucuronidation (CV = 55%) and UGT1A6-mediated serotonin glucuronidation (CV = 76%) were high variability within the human liver bank containing 54 individualities. Suffering from the large inter-individual variability in the hepatic glucuronidation of daphnetin, hence the different response to the pharmacokinetic and clinical outcomes within people after administration of daphnetin, can be also forecasted. Furthermore, interactions induced by daphnetin with dietary components and other compounds primarily catalyzed by UGT1A9 and UGT1A6 should be given attention in clinical applications.

IVIVE of the kinetic data for daphnetin glucuronidation in HLM and HIM was performed, and results showed that the in vivo intrinsic clearance in liver was markedly larger than that of in intestine (344 vs. 9.7 ml/min/kg body weight), which suggested that the liver might play a major role for daphnetin metabolic clearance via glucuronidation. The well-stirred model was constrained to predict the hepatic clearance in this study, considering that gut is a not single homogenous tissue like liver since intestinal UGTs are mainly expressed in intestinal mucosa, and the UGT activities are also not same in different region of intestine (Strassburg et al., 1998). The predicted results showed that daphnetin was a high clearance molecule in vivo with the hepatic extraction ratio larger than 0.7, and most of the orally administered daphnetin might not reach the systemic circulation due to the significant first-pass hepatic metabolism. Therefore, given the high clearance of daphnetin by
glucuronidation, how to retain the plasma concentration of daphnetin achieving the therapeutic effect remains to be known. In addition, whether the metabolites (M-1 and M-2) can play a role in pharmacological activity needs to be understood.

Two mono-glucuronide metabolites (M-1 and M-2) were detected in liver microsomes of all species, and HIM. However, no di-glucuronide was detected in all the microsomal samples, which may be caused by the steric hindrance of conjugation at one catecholic hydroxyl group from the conjugation at the other group as previously reported (Lautala et al., 2000). The metabolic profiles and enzyme kinetic parameters can be used to evaluate the variation among species (Shiratani et al., 2008). The inhibitory patterns are also very important for selection of animal model(s) suitable to investigation of the pharmacodynamics, toxicology, and pharmacokinetics of new chemical entities (Mano et al., 2008). For further evaluation of daphnetin glucuronidation in HLM, RLM and PLM, the inhibition assays were conducted. The inhibition studies by phenylbutazone and mefenamic acid showed significant species difference (Fig. 3), indicating that different UGT isozymes may afford to the glucuronidation of daphnetin among these species.

In conclusion, daphnetin is extensively glucuronidated in *in vitro* incubation systems in the presence of UDPGA. UGT1A9 and UGT1A6 are identified as the major isoforms for daphnetin glucuronidation in HLM and HIM. More studies on the biological activities of these glucuronide metabolites remain to be investigated. Our results confirmed the crucial role of UGT1A9 and UGT1A6 in the glucuronidation of catechol-containing compounds in human and further suggested that daphnetin might serve as a probe substrate for UGT1A6 and UGT1A9 to phenotype the UGTs in liver microsomes from human and animals.
Acknowledgements

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Footnotes

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Legends to the Figures

FIG. 1. Representative UFLC-DAD profile of daphnetin and its metabolites. 0.1 mg/ml PLM (1), RLM (2) or HLM (3) incubate with daphnetin (200 μM) at 37°C for 30 min with UDPGA (5 mM) as described under Materials and Methods. Metabolites M-1 and M-2, daphnetin and internal standard (7-hydroxycoumarin) were eluted at 4.0, 4.5, 5.0, and 6.3 min, respectively.

FIG. 2. The formation of two metabolites, M-1 (A) and M-2 (B) catalyzed by various recombinant human UGTs. Daphnetin (10, 100 and 600 μM) was incubated with various recombinant human UGTs (0.5 mg/ml) at 37°C for 60 min. Data represent the mean of duplicate incubations.

FIG. 3. Inhibitory effects of phenylbutazone and mefenamic acid on the formation of daphnetin glucuronidated metabolites (M-1 and M-2) in HLM, RLM and PLM. Daphnetin (200 μM) was incubated with HLM, RLM and PLM (0.1 mg of protein/ml) with either phenylbutazone (50 and 500 μM) or mefenamic acid (10 and 100 μM) at 37°C for 10 min. Data are expressed as mean ± S.D. of duplicate incubations.

FIG. 4. Correlation analysis between the formation rate of metabolite (M-1 or M-2) and UDP-glucuronosyltransferase isozyme-catalyzed glucuronidation in microsomes from 14 individual human livers. Daphnetin (100 μM) was incubated with microsomes (0.1 mg of protein/ml) for 10 min. Data represent the mean of duplicate determinations. For the formation rate of M-1, the x-axis represents the activity for propofol glucuronidation (A), serotonin glucuronidation (C). For the formation rate of M-2, the x-axis represents the
activity for propofol glucuronidation (B), serotonin glucuronidation (D).

FIG. 5. Enzyme kinetics of daphnetin glucuronidation by HLM (A), HIM (B), UGT1A9 (C), and UGT1A6 (D). Daphnetin was incubated with pooled HLM and HIM (5-600 μM, 0.1 mg of protein/ml) or UGT1A9 and UGT1A6 (5-400 μM, 0.1 mg of protein/ml) at 37°C for 10 min. An Eadie-Hofstee plot was shown as an inset to illustrate monophase kinetics.

FIG. 6. In vitro UGT conjugation pathways of daphnetin in human liver and intestine microsomes.
**TABLE 1. Proton and carbon NMR chemical shift assignments for daphnetin and its metabolites**

<table>
<thead>
<tr>
<th>Position</th>
<th>Daphnetin</th>
<th>M-1</th>
<th>M-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta^1\text{H Mult} (\text{J in Hz}))</td>
<td>(\delta^{13}\text{C})</td>
<td>(\Delta^1\text{H Mult} (\text{J in Hz}))</td>
</tr>
<tr>
<td>2</td>
<td>_</td>
<td>160.4</td>
<td>_</td>
</tr>
<tr>
<td>3</td>
<td>6.19 t (9.6)</td>
<td>111.2</td>
<td>7.09 d (8.4)</td>
</tr>
<tr>
<td>4</td>
<td>7.90 d (9.0)</td>
<td>143.7</td>
<td>7.95 d (9.6)</td>
</tr>
<tr>
<td>5</td>
<td>7.01 d (8.4)</td>
<td>118.8</td>
<td>7.13 d (9.0)</td>
</tr>
<tr>
<td>6</td>
<td>6.80 t (7.8)</td>
<td>112.5</td>
<td>6.32 d (9.6)</td>
</tr>
<tr>
<td>7</td>
<td>9.33 brs</td>
<td>149.7</td>
<td>_</td>
</tr>
<tr>
<td>8</td>
<td>10.01 brs</td>
<td>132.1</td>
<td>_</td>
</tr>
<tr>
<td>9</td>
<td>_</td>
<td>145.1</td>
<td>_</td>
</tr>
<tr>
<td>10</td>
<td>_</td>
<td>112.1</td>
<td>_</td>
</tr>
<tr>
<td>1'</td>
<td>_</td>
<td>5.04 d (7.8)</td>
<td>101.2</td>
</tr>
<tr>
<td>2',</td>
<td>_</td>
<td>3.38(1H, m)</td>
<td>72.9</td>
</tr>
<tr>
<td>3',</td>
<td>_</td>
<td>3.34(1H, m)</td>
<td>75.2</td>
</tr>
<tr>
<td>4',</td>
<td>_</td>
<td>3.41(1H, m)</td>
<td>71.4</td>
</tr>
<tr>
<td>5'</td>
<td>_</td>
<td>3.90(1H, d, (J = 9.6\text{ Hz}))</td>
<td>75.3</td>
</tr>
<tr>
<td>6'</td>
<td>_</td>
<td>_</td>
<td>170.1</td>
</tr>
</tbody>
</table>

brs = broad singlet, d = doublet, t = triplet, m = multiplet
**TABLE 2. Kinetic parameters of daphnetin glucuronidation in microsomes obtained from humans, experimental animals (n = 5), and recombinant UGT1A9 and UGT1A6**

Daphnetin was incubated with microsomes (0.1 mg/ml) and UDPGA (5 mM) for 10 min. The kinetic parameters were calculated with Origin Lab software.

<table>
<thead>
<tr>
<th>UGT Source</th>
<th>M-1</th>
<th></th>
<th></th>
<th>M-2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{\text{max}})</td>
<td>(K_m)</td>
<td>(V_{\text{max}}/K_m)</td>
<td>(V_{\text{max}})</td>
<td>(K_m)</td>
<td>(V_{\text{max}}/K_m)</td>
</tr>
<tr>
<td>HLM</td>
<td>25.3 ± 0.3</td>
<td>122 ± 4.0</td>
<td>219</td>
<td>18.8 ± 0.3</td>
<td>103 ± 4.2</td>
<td>183</td>
</tr>
<tr>
<td>RLM</td>
<td>54.9 ± 3.1</td>
<td>165 ± 14</td>
<td>333</td>
<td>19.0 ± 0.5</td>
<td>56.6 ± 5.4</td>
<td>335</td>
</tr>
<tr>
<td>PLM</td>
<td>114 ± 18</td>
<td>217 ± 21</td>
<td>526</td>
<td>152 ± 13</td>
<td>175 ± 17</td>
<td>866</td>
</tr>
<tr>
<td>HIM</td>
<td>13.5 ± 0.2</td>
<td>199 ± 6.2</td>
<td>65.5</td>
<td>6.9 ± 0.2</td>
<td>161 ± 8.3</td>
<td>42.5</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>12.4 ± 0.3</td>
<td>60.5 ± 3.9</td>
<td>207</td>
<td>8.1 ± 0.2</td>
<td>44.3 ± 4.1</td>
<td>183</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>15.6 ± 0.3</td>
<td>74.4 ± 4.1</td>
<td>208</td>
<td>7.4 ± 0.3</td>
<td>74.4 ± 8.8</td>
<td>99</td>
</tr>
</tbody>
</table>

Units are: \(V_{\text{max}}\); nmol/min/mg protein; \(K_m\); \(\mu\)M; \(V_{\text{max}}/K_m\); \(\mu\)l/min/mg protein.
TABLE 3. Inhibition of daphnetin glucuronidation in HLM, HIM and recombinant UGTs with mefenamic acid and phenylbutazone

Daphnetin was incubated with microsomes (0.1 mg/ml) and UDPGA (5 mM) for 10 min in the presence of mefenamic acid (1-50 μM) or phenylbutazone (10-500 μM). The IC₅₀ values are given as mean ± computer-calculated S.E. of the estimate.

<table>
<thead>
<tr>
<th>UGT Source</th>
<th>Mefenamic acid</th>
<th>Phenylbutazone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-1</td>
<td>M-2</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>8.1 ± 1.6</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>HLM</td>
<td>10.4 ± 2.2</td>
<td>10.5 ± 2.6</td>
</tr>
<tr>
<td>HIM</td>
<td>8.8 ± 0.9</td>
<td>7.8 ± 2.7</td>
</tr>
</tbody>
</table>
Figure 1

The figure shows chromatograms with peaks labeled M-1 and M-2, as well as an internal standard (IS). The y-axis represents the absorbance at 320 nm (mV), and the x-axis represents time in minutes. The chromatograms are labeled 1, 2, and 3.
Figure 2

(A) M-1 formation rate (nmol/min/mg protein)

(B) M-2 formation rate (nmol/min/mg protein)

Control, Supersome, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, UGT2B17

Legend:
- Black: 0 μM
- Gray: 100 μM
- Light gray: 600 μM
Figure 3

- Open bars: phenylbutazone 50 μM
- Grey bars: phenylbutazone 500 μM
- Dark grey bars: mefenamic acid 10 μM
- Black bars: mefenamic acid 100 μM

Y-axis: Residual Activity (%)
X-axis: Enzyme types: HLM, PLM, RLM
Enzyme samples: M-1, M-2
Figure 4

(A) Propofol glucuronidation (nmol/min/mg protein) vs. M-1 formation rate (nmol/min/mg protein).

(B) Propofol glucuronidation (nmol/min/mg protein) vs. M-2 formation rate (nmol/min/mg protein).

(C) Serotonin glucuronidation (nmol/min/mg protein) vs. M-1 formation rate (nmol/min/mg protein).

(D) Serotonin glucuronidation (nmol/min/mg protein) vs. M-2 formation rate (nmol/min/mg protein).

Correlation coefficients:
- (A) $r = 0.91$, $P < 0.001$
- (B) $r = 0.94$, $P < 0.001$
- (C) $r = 0.64$, $P = 0.011$
- (D) $r = 0.69$, $P = 0.006$
Figure 5

(A) Graph showing the relationship between substrate concentration (μM) and V (nmol/min/mg protein) for M-1 and M-2.

(B) Graph showing the relationship between substrate concentration (μM) and V (nmol/min/mg protein) for M-1 and M-2.

(C) Graph showing the relationship between substrate concentration (μM) and V (nmol/min/mg protein) for M-1 and M-2.

(D) Graph showing the relationship between substrate concentration (μM) and V (nmol/min/mg protein) for M-1 and M-2.
**Figure 6**

Daphnetin → 7-O-glucuronide daphnetin (M-1)

Daphnetin → 8-O-glucuronide daphnetin (M-2)