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UDP-Glucuronosyltransferases 1A6 and 1A9 are the Major Isozymes Responsible for the 7-O-Glucuronidation of Esculetin and 4-Methylesculetin in Human Liver Microsomes

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Running Title Page

Running title: UGT metabolism of esculetin and 4-methylesculetin in vitro.

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Number of text pages: 33

Tables: 1

Figures: 4

Supplemental Figures: 4

References: 32

Words in the abstract: 228

Words in the introduction: 638

Words in the discussion: 813

Abbreviations: AIC, Akaike’s information criterion; ET, esculetin; ET-G, 7-O-monoglucuronide esculetin; 4-ME, 4-methylesculetin; 4-ME-G, 7-O-monoglucuronide 4-methylesuletin; KPI, potassium phosphate buffer; LC-MS, liquid chromatography–mass spectrometry; HLM, human liver microsomes; HIM, human intestinal microsomes; HPLC, high pressure liquid chromatography; HRMS, high-resolution mass spectrometry; 1HNMR, 1H-nuclear magnetic resonance; RLM, rat liver microsomes; UDPGA, uridine diphosphate glucuronic acid; UGT, UDP-glucuronosyltransferase; UHPLC, ultra high pressure liquid chromatography.
Abstract

Esculetin (6,7-dihydroxycoumarin, ET) and 4-methylesculetin (6,7-dihydroxy-4-methylcoumarin, 4-ME) are typical coumarin derivatives that are attracting considerable attention because of their wide spectrum of biological activities, but their metabolism remains unknown. This study aimed to elucidate the in vitro UDP-glucuronosyltransferase (UGT) metabolism characteristics of ET and 4-ME. 7-O-monoglucuronide esculetin (ET-G) and 7-O-monoglucuronide 4-methylesuletin (4-ME-G) were identified by liquid chromatography–mass spectrometry (LC-MS) and 1H-nuclear magnetic resonance (1H-NMR) when ET or 4-ME was incubated with human liver (HLM) in the presence of UDP-glucuronic acid. Screening assays with 12 human expressed UGTs demonstrated that the formations of ET-G and 4-ME-G were almost exclusively catalyzed by UGT1A6 and UGT1A9. Phenylbutazone and carvacrol (UGT1A6 and UGT1A9 chemical inhibitors, respectively) at different concentrations (50, 100, and 200 μM) significantly inhibited the formation of glucuronidates of ET and 4-ME in HLM, UGT1A6, and UGT1A9 when the concentrations of ET and 4-ME ranged from 10 to 300 μM (P < 0.05). Clearance rates of ET in HLM, HIM, UGT1A6, and UGT1A9 were 0.54, 0.16, 0.69, and 0.14 mL/min/mg, respectively. Corresponding clearance rates values of 4-ME were 0.59, 0.03, 0.14, and 0.04 mL/min/mg, respectively. In conclusion, 7-O-monoglucuronidation by UGT1A6 and UGT1A9 was the predominant UGT metabolic pathway for both ET and 4-ME in vitro. The liver is probably the major contributor to the glucuronidation metabolism of ET and 4-ME. ET showed more...
rapid metabolism than 4-ME in glucuronidation.
Introduction

Coumarins (2H-1-benzopyran-2-one) and their derivatives are potentially useful leading compounds for the development of new chemical entities because of their wide spectrum of biological activities, such as anti-inflammatory, anticoagulant, anticancer, anti-HIV, antiviral, antifungal, antioxidant, anti-Alzheimer, and neuroprotective properties (Venugopala et al., 2013). From 2008 to 2011, around 42 patent publications on biologically active coumarin derivatives have emerged, suggesting that they are a promising source of new candidate drugs for multiple diseases (Kontogiorgis et al., 2012). However, the real mechanism of action of coumarins and their derivatives, as well as their pharmacokinetic properties, is unclear, which may explain why only a few new coumarins have been advanced to the clinical trial stage. Thus, studies on their pharmacokinetic profiles in humans may provide important information on their discovery and development.

Esculetin (6,7-dihydroxycoumarin, ET) is a derivative of coumarin that is present in various plants used as folk medicines, such as *Fraxinus rhynchophylla*, *Rehmanniae glutinosa*, and *Artemisia capillaries* (Prabakaran and Ashokkumar, 2013). ET is a proven antioxidant that protects hamster lung fibroblasts from lipid peroxidation, protein carbonylation, and DNA damage induced by hydrogen peroxide (Kim et al., 2008; Lin et al., 2000; Paya et al., 1992). ET has also been shown to promote analgesia (Tubaro et al., 1988), immunomodulatory function (Leung et al., 2005), and the apoptosis of various tumor cells by inhibiting signaling pathways or inducing apoptotic pathways (Park et al., 2008; Kok et al., 2009). In addition, ET protects DNA
against oxidative stress (Kaneko et al., 2003); inhibits the synthesis of leukotriene B4, thromboxane B2 (Hoult et al., 1994), and platelet aggregation (Okada et al., 1995); hinders the growth of human leukemia cells; and prevents the production of IL-6 and IL-8 (Hu et al., 2009). The chemopreventive activity of ET has further been demonstrated in benzo[a]pyrene induced lung carcinogenesis in mice (Jay R et al., 2013). 4-Methylesculetin (6,7-dihydroxy-4-methylcoumarin, 4-ME) is a synthetic derivative of coumarin, also displays anti-inflammatory, potent reactive oxygen species scavenging, and neuroprotective properties. As a metal-chelating agent, 4-ME can be a promising candidate in the management of Alzheimer’s disease (Shirole et al., 2013). A recent study has shown the significant antigenotoxic effect of 4-ME in mouse cells, suggesting that it might be beneficial for cancer prevention (Edson and Rafael, 2012).

Most coumarins are poorly bioavailable (2 to 6%) because of extensive metabolism and rapid urine excretion although they are completely absorbed after oral administration (Lake, 1999). The important pathway of coumarin metabolism is 7-hydroxylation mediated by hepatic cytochrome P450 (CYP) 2A6, and then further extensively conjugated with D-glucuronic acid and/or sulfate at 7-OH (Egan et al., 1990). ET and 4-ME are containing two hydroxyl groups at 6-C and 7-C, respectively, indicating that UDP-glucuronosyltransferase (UGT) conjugation is probably the main factor affecting their bioavailability, biological activities, and toxicity in vivo. Glucuronidation is the primary phase II conjugation reaction that accounts for more than 35% of all phase II drug metabolites (Kiang et al., 2005). Many UGT isoforms
(UGTs) have had broad and overlapping substrate specificities; and human UGTs are tissue specific because of their differences in expression (Liang et al., 2010). Identification of human UGTs responsible for the metabolism of ET and 4-ME can provide pivotal information to their pharmacokinetic properties.

Accordingly, this study aimed to elucidate the \textit{in vitro} glucuronidation characteristics of ET and 4-ME using human microsomes and expressed UGTs. Glucuronidation metabolites of ET and 4-ME in human liver microsomes (HLM) have been identified by chromatography–mass spectrometry (LC-MS) and \textsuperscript{1}H-nuclear magnetic resonance (\textsuperscript{1}HNMR). Glucuronidation pathways of ET and 4-ME were identified and determined by using incubation with a panel of 12 expressed recombinant UGTs (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) and HLM in the presence of specific inhibitors of UGTs. Enzyme kinetics of ET and 4-ME in HLM, human intestinal microsomes (HIM) and UGTs were also analyzed.
Materials and Methods

Chemicals and Reagents

Esculetin (ET), 4-Methylesculetin (4-ME), and propiophenone (internal standard, IS) (purity >98%, HPLC grade, confirmed by LC-MS) were purchased from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, China). Expressed human UGT isoforms (Supersomes™ Enzymes), pooled human liver/intestinal microsomes (HLM and HIM), and rat liver microsomes (RLM) were purchased from BD Biosciences (Woburn, MA, USA). Uridine diphosphate glucuronic acid (UDPGA), alamethicin, magnesium chloride, D-saccharic-1,4-lactone monohydrate, and β-glucuronidase (Type HP-2 from helix pomatia) were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were analytical grade or better.

Identification of Glucuronidation of ET and 4-ME

The incubation mixture (200 μL) contained HLM (0.5 mg/mL), magnesium chloride (0.88 mM), alamethicin (0.022 mg/mL), saccharolactone (4.4 mM), substrates (80 μM), UDPGA (3.5 mM), and 50 mM potassium phosphate buffer (KPI) (pH 7.4). After 20 min of incubation at 37 °C, the reaction was terminated by the addition of 100 μL of 94% acetonitrile 6% glacial acetic acid containing 90 μM propiophenone as an internal standard, followed by centrifugation at 17,900 g for 30 min to obtain the supernatant for LC-MS analysis. Control incubation without UDPGA or without substrates or without microsomes were performed to ensure that the metabolites produced were microsome- and UDPGA-dependent. A quadrupole-time of flight
A (Q-TOF) tandem mass spectrometer (Bruker, Daltonics, USA) with an Agilent 1260 high pressure liquid chromatography (HPLC) (Agilent Technologies, CA, USA) method was used to analyze the molecular weight of the glucuronides of ET and 4-ME. The glucuronides of ET and 4-ME were separated using Agilent ZORBAX SB-C18 column (4.6×150 mm, 5 µm). The mobile phase B was 100% acetonitrile, where the mobile phase A was 100% aqueous buffer (2.0 mM CH$_3$COONH$_4$, pH 6.8) with a flow rate of 1 mL/min. The gradient elution method was as follows: 0 to 4 min, 5 to 35% B, 4 to 8 min, 35 to 80% B, 8 to 10 min, 80% B, 10 to 12 min, 80 to 5% B. The detection wavelength was 350 nm. The injection volume was 200 µL. Ionization was achieved using electro spray ionization in the positive mode at a capillary voltage of +4500 V. The temperature of the dry heater was maintained at 200 °C, and the nebulizer voltage was set at 1.5 bars. The dry gas was set at a flow rate of 8.0 L/min. The MS/MS spectra were produced by collision-induced dissociation of the selected precursor ions. Data were collected and analyzed using Bruker Daltonics software (version 4.0, Bruker, USA).

To confirm that the metabolites are glucuronide compounds, 200 µL of glucuronidation incubation mixture containing glucuronide and aglycone was extracted three times with dichloromethane (sample/dichloromethane=2:5, v/v) to remove aglycone. The extracted aqueous sample was subsequently divided into two equal portions, one portion was analyzed after hydrolysis by β-glucuronidase (40 U/mL) at 37 °C for 60 min, and the other portion was analyzed by ultra high pressure liquid chromatography (UHPLC) directly.
Biosynthesis Metabolites and $^1$HNMR Spectrometry

The glucuronidation metabolites of ET and 4-ME were biosynthesized and purified for structure elucidation and quantitative analysis. Enzymatic biosynthesis of ET-G and ME-G was conducted using rat liver microsome (RLM), because they resemble in HLM in the metabolism of ET and 4-ME. In brief, 800 μM ET or 4-ME was respectively incubated with RLM (3 mg/mL), 5 mM UDPGA, 5 mM MgCl₂, 25 μg/mL alamethicin, 10 mM D-saccharic acid 1,4-lactone and 50 mM KPI solution (pH 7.4) in 10 mL final incubation for 6 h at 37 °C. The reaction was terminated by the addition of 2 mL of methanol in an ice bath. The supernatant was collected after being centrifuged at 17,900 g for 30 min. Then, the supernatant was separated by Agilent 1200 HPLC (Agilent Technologies, CA, USA) equipped with an Agilent 1200 system controller, four G1310A LC pumps, a G1329B auto injector, a G1314B UV detector, and Agilent ZORBAXSB-C18 column (4.6×150 mm, 5 μm). The mobile phase consisted of methanol (A) and 0.1% (v/v) formic acid (B) with a linear gradient from initially 10 to 60% A over 11 min, then constantly 60% A, 11 to 15 min. The flow rate was 1 mL/min. The purity was greater than 99% for both metabolites by using HPLC-diode array detection. The glucuronic acid substitution positions of ET and 4-ME were determined by $^1$HNMR. All experiments were recorded on Bruker AVANCE III system (Bruker, Daltonics, USA). The purified metabolites of ET-G (1.6 mg) and 4-ME-G (1.3 mg) were stored at –20°C before dissolving in deuterated acetone (Sigma-Aldrich, MO, USA) for $^1$HNMR analysis. Chemical shifts are given on a δ scale and referenced to tetramethylsilane at 0 ppm for $^1$HNMR (500 MHz).
Assay with Expressed UGTs

Human expressed UGTs (Supersomes™ Enzymes) are prepared from baculovirus-transfected insect cells with very high levels of catalytic activities (typically 6-fold higher than an average HLM sample). This is ideal for identifying the metabolic pathways of drugs, screening high throughput drug interactions, studying slowly metabolized chemicals, or manufacturing large-scale production of metabolites for structural identification (FDA, 2006). The glucuronidation of ET and 4-ME were measured in reaction mixtures containing expressed human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17. The incubations were conducted as shown above for the HLM study. Three substrate concentrations (10, 30, and 100 μM) were performed in this study. All assays were conducted at 37 °C for 30 min with the final protein concentration in range of ≈0.25~1 mg/mL based on catalytic activities of individual expressed UGTs. All samples were pretreated as described above for HLM study and analysis by using UHPLC.

Chemical Inhibition Studies

The glucuronidation activities of ET and 4-ME in microsomes (HLM and HIM) and expressed UGTs (UGT1A6 and UGT1A9) were determined in the absence or presence of phenylbutazone (UGT1A6 inhibitor) or cavacrol (UGT1A9 inhibitor). Glucuronidation of ET and 4-ME by HLMs and UGT isoforms were measured at three concentrations of 10, 100, and 300 μM, and were incubated in the absence or presence of phenylbutazone or cavacrol at low (50 μM), medium (100 μM), and high
(200 μM) concentrations. (final concentration in range of ≈ 0.013~0.053 mg protein/ml based on optimization of the reaction) All protein concentrations were 0.5 mg/mL, incubation time for ET and 4-ME in microsomes and UGTs were range from 15 to 60 min.

**UHPLC Analysis of ET and 4-ME and Their Glucuronides**

The UHPLC condition used to quantify glucuronides of ET and 4-ME were as follows. Agilent 1290 UHPLC system coupled a photodiode array detector and the ChemStation software program (Agilent, CA, USA) was used with a SB C18 column (3.0×100 mm, 1.8 μm). The mobile phase B was 100% acetonitrile, where the mobile phase A was 100% aqueous buffer (2.0 mM CH₃COONH₄, pH 6.8) with a flow rate of 0.3 mL/min. The gradient program was as follows: 0 to 2 min, 5% to 35% B, 2.0 to 4.0 min, 35% to 80% B, 4.0 to 5 min, 80% B, 5.0 to 6.0 min, 80% to 5% B. The detection wavelength was 350 nm for ET, 4-ME and their respective glucuronides and 250 nm for propiophenone (IS). The injection volume was 10 μL. The test linear response range for ET and 4-ME were 1.5625 μM to 800 μM.

**Enzymes Kinetic Studies**

For estimating kinetic parameters, serial concentrations of ET or 4-ME were incubated with different enzymes (UGT1A6, UGT1A9, HLM, or HIM). The kinetic studies were conducted using a microsomes or Supersomes at the concentrations in range from 0.013 to 0.053 mg/mL based on optimization of the reaction. Rates of the glucuronidation of ET and 4-ME by microsomes and expressed UGTs were expressed
as amounts of glucuronides formed per milligram protein per minute (nmol/mg/min).

Kinetic parameters were then obtained according to the profile of Eadie-Hofstee plots (Liu et al., 2007). If the Eadie-Hofstee plot was linear, Formation rates \( (V) \) of glucuronides at different substrate concentrations \( (C) \) were fit to the standard Michaelis-Menten equation:

\[
V = \frac{V_{\text{max}} \times C}{K_m + C}
\]  

(1)

Where \( K_m \) is the Michaelis–Menten constant and \( V_{\text{max}} \) is the maximum rate of glucuronidation.

If Eadie–Hofstee plots showed characteristic profiles of atypical kinetic (autoactivation and biphasic kinetics) (Wang et al., 2006), the data from these atypical profiles were fit to equations 2 and 3, using the ADAPT II program (D’Argenio and Schumitzky, 1997). To confirm the best-fit model, the model candidates were discriminated using the minimum Akaike’s information criterion (AIC) value (Yamaoka et al., 1978), and the rule of parsimony was employed.

The following equation (2) describes enzyme reactions with auto activation:

\[
V = \frac{V_{\text{max}-0} + V_{\text{max-d}}(1-e^{-CR})}{K_m + C} \times C
\]  

(2)

Where \( V_{\text{max-0}} \) is the intrinsic enzyme activity and \( V_{\text{max-d}} \) is maximal induction of enzyme activity. \( R \) is the rate of enzyme activity induction, \( C \) is concentration of substrate, and \( K_m \) is concentration of substrate needed to achieve 50% of \( (V_{\text{max-0}}+V_{\text{max-d}}) \).

The following equation (3) describes enzyme reactions with biphasic kinetics:
\[
V = \frac{V_{\text{max1}} \times C}{K_{m1} + C} + \frac{V_{\text{max2}} \times C}{K_{m2} + C}
\]

(3)

Where \(V_{\text{max1}}\) is the maximum enzyme velocity of the high-affinity phase, \(V_{\text{max2}}\) is the maximum velocity of the low-affinity phase, \(K_{m1}\) is concentration of substrate to achieve half of \(V_{\text{max1}}\) for high-affinity phase, and \(K_{m2}\) is concentration of substrate to achieve half of \(V_{\text{max2}}\) for low-affinity phase.

When Eadie–Hofstee plots revealed substrate inhibition kinetics, the reaction rate \((V)\) were fit to equation (4):

\[
V = \frac{V_{\text{max1}}}{1 + (K_{m1}/C) + (C/K_{si})}
\]

(4)

Where \(C\) is the substrate concentration, \(V\) is the initial reaction rate, \(V_{\text{max1}}\) is the maximum enzyme velocity, \(K_{m}\) is the substrate concentration required to achieve 50\% of \(V_{\text{max}}\), and \(K_{si}\) is the substrate inhibition constant.

**Statistical Analysis**

One-way ANOVAs with or without Tukey–Kramer multiple comparison (post hoc) tests were used to evaluate statistical difference. Differences were considered significant when \(P<0.05\).
Results

Identification of Metabolites of ET and 4-ME

LC-MS analysis showed that only mono-glucuronides were formed in HLM incubations with 80 μM ET or 4-ME in the presence of UDPGA. Retention times of ET, its metabolite (ET-G), and IS were 4.41, 4.13, and 5.11 min, respectively (Figure 1A). The peak eluting at 4.59 and 4.39 min corresponded with 4-ME and its metabolite (4-ME-G) (Figure 1B).

The molecular formula of ET-G was established to be \( \text{C}_{15}\text{H}_{14}\text{O}_{10} \) through high-resolution mass spectrometry (HRMS) (m/z = 355.0657 [M+H]\(^+\), calcd. for 355.0659) (Figure 1C), which indicated that a \( \text{C}_{6}\text{H}_{8}\text{O}_{6} \) moiety was added by comparing the molecular formula of ET \( \text{C}_{9}\text{H}_{7}\text{O}_{4} \) (m/z = 179) (Figure 1D). The MS\(^2\) spectrum of ET-G provided predominant characteristic fragment ions at m/z 179, which confirmed the pseudo-molecule ion [M+H]\(^+\) of ET. Inspection of \(^1\)HNMR spectroscopic data suggested that the moiety belongs to glucuronic acid, revealing that a proton of OH in ET was substituted by glucuronic acid (Supplemental Figures 1 and 3). The substitution position of the proton of 7-OH can be confirmed by the nuclear overhauser effect spectroscopy (NOESY) correlation between Glu-H-1\(^{\prime}\) (\(\delta_H\) 5.24, 1H, d, \(J = 7.0\) Hz) and H-8 (\(\delta_H\) 7.18, 1H, s) (Figure 1G).

The molecular formula of 4-ME-G was established to be \( \text{C}_{16}\text{H}_{16}\text{O}_{10} \) through HRMS (m/z = 369.0821 [M+H]\(^+\), calcd. for 369.0816), which also indicated that a \( \text{C}_{6}\text{H}_{8}\text{O}_{6} \) moiety was added by comparing the molecular formula of 4-ME \( \text{C}_{10}\text{H}_{9}\text{O}_{4} \) (m/z = 193) (Figure 1F). In addition, the MS\(^2\) spectrum of 4-ME-G provided predominant...
characteristic fragment ions at m/z 193, which confirmed the pseudo-molecule ion [M+H]+ of 4-ME (Figure 1F). Inspection of 1HNMR spectroscopic data also suggested that the moiety belongs to glucuronic acid, revealing that a proton of OH in 4-ME was substituted by glucuronic acid (Supplemental Figures 2 and 4). The substitution position of the proton of 7-OH can be confirmed by the disappearance of broad singlet for 7-OH at $\delta_H 9.09$ (1H, s) (Supplemental Figures 2 and 4).

**Main UGTs Responsible for the Glucuronidation of ET and 4-ME in vitro**

Twelve expressed human UGTs including UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 were used to catalyze the glucuronidation of ET and 4-ME and to identify UGTs involved in their glucuronidation metabolism. Substrates of ET and 4-ME ranged within 10 $\mu$M to 100 $\mu$M in this experiment. We found that UGT1A6 produced the most rapid glucuronidation rates of ET (1.49 ± 0.12 to 2.98 ± 0.15 nmol/mg/min), followed by UGT1A9 (0.97 ± 0.06 to 1.81 ± 0.19 nmol/mg/min) (Figure 2A). UGT1A7 and UGT2B15 also generated a detectable metabolite of ET (ET-G). Similarly, UGT1A6 produced the most rapid glucuronidation rates of 4-ME (4.10 ± 0.14 to 12.06 ± 0.51 nmol/mg/min), followed by UGT1A9 (2.46 ± 0.20 to 2.62 ± 0.05 nmol/mg/min) (Figure 2B). UGT1A7 and UGT1A1 also generated a detectable metabolite of 4-ME (4-ME-G).

**Effects of Chemical Inhibitors on the Glucuronidation of ET and 4-ME in HLM and Expressed UGTs**

To further confirm that UGT1A6 and UGT1A9 were the main UGT isoforms
involved in ET and 4-ME glucuronidation in vitro, inhibitory effects of phenylbutazone (UGT1A6 inhibitor) and carvacrol (UGT1A9 inhibitor) on the glucuronidation of ET (Figures 3A–3D) and 4-ME (Figures 3E–3H) in pooled HLM, UGT1A6, and UGT1A9 were investigated (Aprile et al., 2010; Dong et al., 2012). About 100 and 200 μM of phenylbutazone and carvacrol significantly inhibited >50% ET-G formation in HLM using ET at a low concentration (10 μM) (Figures 3A and 3B) (P < 0.05). Results for UGT1A6 and UGT1A9 (Figures 3C and 3D) were highly consistent to those in HLM (P < 0.05). Similar results were also observed in the formation of 4-ME-G in microsomes and UGTs (Figures 3E–3H). In addition, both phenylbutazone and carvacrol also significantly inhibited the glucuronidation of ET and 4-ME in almost all experiments, although the inhibition effects did not surpass 50% in some samples (P < 0.05).

**Kinetics of ET and 4-ME Glucuronidation by Microsomes and Expressed UGT1A6 and UGT1A9**

Glucuronidation rates of ET and 4-ME by UGTs and microsomes were determined at different substrate concentrations. Within the tested concentration ranges, both UGTs and microsome-mediated glucuronidation of ET (Figures 4A–4D) and 4-ME (Figures 4E–4H) exhibited classic Michaelis–Menten kinetic characteristics, as evidenced by a linear Eadie–Hofstee plot (Figures 4a–4h). All kinetic parameters of UGTs and microsome-catalyzed glucuronidation of ET and 4-ME are shown in Tables 1.

The intrinsic clearance (CL; V\text{max}/K\text{m}) of ET-G in UGT1A6, UGT1A9, HLM, and
HIM were 0.69, 0.14, 0.54, and 0.16 mL/min/mg, respectively, accompanying the large variation in $V_{max}$ values (127.40 μM for UGT1A6, 19.23 μM for UGT1A9, 72.61 μM for HLM, and 30.45 μM for HIM). No large difference was observed among the $K_m$ values of ET glucuronidation in UGTs and microsomes (185.59 μM for UGT1A6, 134.75 μM for UGT1A9, 134.19 μM for HLM, and 189.33 μM for HIM).

$CL$ of 4-ME-G in UGT1A6, UGT1A9, HLM, and HIM were 0.14, 0.04, 0.59, and 0.03 mL/min/mg, respectively, also accompanying the large variation in $V_{max}$ values (24.70 μM for UGT1A6, 0.99 μM for UGT1A9, 69.91 μM for HLM, and 3.88 μM for HIM). However, $K_m$ values of 4-ME in different enzymes also showed larger fluctuation: 172.51 μM for UGT1A6, 25.20 μM for UGT1A9, 117.81 μM for HLM, and 129.12 μM for HIM.
Discussion

Glucuronidation pathway is responsible for metabolism clearances of drugs that contain phenolic hydroxyl groups, such as coumarins and flavonoids. Most coumarin and flavonoid derivatives exert pleiotropic biological activities and are also good substrates of human UGTs. ET and 4-ME are coumarin derivatives that contain two hydroxyl groups at 6-C and 7-C, respectively. Accordingly, the \textit{in vitro} glucuronidation metabolism of ET and 4-ME in HLM, HIM, and human-expressed UGTs were investigated first in this study.

Mono-glucuronidation metabolites (ET-G and 4-ME-G) of ET and 4-ME were detected and identified in HLM. The glucuronidation position of ET and 4-ME was 7-OH. These results were consistent with a previous report demonstrating that 7-hydroxyl coumarins are easily metabolized by substitution with glucuronic acid at 7-OH (Egan et al., 1990). No diglucuronide was detected in any microsomal and UGT samples. Human-expressed UGTs and chemical inhibition assays were used to determine the main UGTs involved in the glucuronidation metabolism of ET and 4-ME. For both ET and 4-ME, UGT1A6 and UGT1A9 were the most capable of catalyzing their glucuronidation at tested substrate concentrations. In addition, a small amount of ET-G could be produced by UGT1A7 and UGT2B15, whereas a small quantity of 4-ME-G could be formed by UGT1A1 and UGT1A7. Different concentrations (50 to 200 \(\mu\text{M}\)) of UGT1A6 (phenylbutazone) and UGT1A9 (carvacrol) inhibitors were further used to confirm these results in inhibition assays with HLM or UGTs. Both phenylbutazone and carvacrol at different concentrations significantly
inhibited the formation of glucuronidates of ET and 4-ME in HLM, UGT1A6, and UGT1A9 at ET and 4-ME concentrations of 10 to 300 μM (Figure 3). These results demonstrated that the UGTs primarily responsible for the glucuronidation of ET and 4-ME in vitro were UGT1A6 and UGT1A9. It was demonstrated that UGT1A6, UGT1A9, and UGT2B7 can be involved in the glucuronidation of hydroxycoumarins (Antonio et al., 2003; Loureiro et al., 2006). However, UGT2B7 was not found to be involved in glucuronidating ET and 4-ME in our study.

To further elucidate the characteristics of glucuronidation metabolism of ET and 4-ME, rates of glucuronidation metabolism of ET and 4-ME by UGT1A6, UGT1A9, HLM, and HIM were determined at different substrate concentrations. Both UGTs and microsomes mediated ET and 4-ME glucuronidation following classic Michaelis–Menten kinetics. The comparable kinetic profiles of UGT1A6 and UGT1A9 were confirmed by evidence from Eadie-Hofstee plots observed in HLM and HIM, suggesting that UGT1A6 and UGT1A9 played primary roles in the formation of glucuronides of ET and 4-ME in these two organs, although 4-ME showed markedly higher affinity to UGT1A9 than to those of any other substrate and enzyme (Table 1). In addition, CL values of ET-G and 4-ME-G in HLM were 3.4-fold and 19.7-fold more than those in HIM, respectively (Table 1). UGT1A6 and UGT1A9, which showed the highest activity toward ET and 4-ME, were primarily expressed in the liver (Rowland et al., 2013). Therefore, the liver is probably the major contributor to the glucuronidation of ET and 4-ME. Moreover, CL and V_max values of ET in UGTs and HIM were greater than those of 4-ME, suggesting that ET
may possess more rapid excretion rate and less residence time in vivo than 4-ME.

It was demonstrated that carvacrol was a specific inhibitor of UGT1A9. Chemical inhibition experiments revealed that carvacrol dose-dependently inhibited the formation of ET-G and 4-ME-G in both HLM and UGT1A9 at three tested substrate concentrations (Figures 3B, 3D, 3F, and 3H). Different from the inhibition effect of carvacrol, phenylbutazone showed attenuated inhibition on the formation of ET-G and 4-ME-G in both HLM and UGT1A9 with increased substrate concentration (Figures 3A, 3C, 3E, and 3G). Phenylbutazone is not a selective inhibitor for UGT1A6 because it exhibited an inhibition effect on UGT1As. In addition, the inhibition kinetics of phenylbutazone and carvacrol has been previously described to be substrate dependent (competitive and noncompetitive), but the mechanism of inhibition of drug-metabolizing enzymes is still unclear (Dong et al., 2012; Uchaipichat et al., 2006). Thus, the mechanism of inhibition of phenylbutazone and carvacrol require further studies, and in vitro data on the inhibition of phenylbutazone and carvacrol should be interpreted with caution.

Glucuronidation is the primary phase II conjugation reaction for most coumarin derivatives. Besides the liver, UGT1A6 and UGT1A9 are also expressed in the intestine, kidney, and stomach (Rowland et al., 2013). Their activity and expression regulation are vulnerable to genetic polymorphism, hepatocyte nuclear factor 1, aryl hydrocarbon receptor, as well as chemical inhibitors and inducers (Kiang et al., 2005). Thus, in vivo metabolism characteristics of ET and 4-ME in human subjects, as well as drug–drug interactions related to glucuronidation metabolism, require further study.
In conclusion, this study demonstrated that 7-O-glucuronidation, the main metabolic pathway of ET and 4-ME \textit{in vitro}, was mainly catalyzed by UGT1A6 and UGT1A9 isoforms. The liver is probably the major contributor to the glucuronidation of ET and 4-ME. ET showed more rapid metabolism than 4-ME in glucuronidation.
Authorship Contributions

Participated in research design: Zhongqiu Liu, Lijun Zhu, Linlin Lu.

Conducted experiments: Shan Zeng, Feifei Luo, Peimin Dai.

Contributed new reagents or analytic tools: Ming Hu and Liang Liu.


Wrote or contributed to the writing of the manuscript: Lijun Zhu, Ming Hu, Linlin Lu, Zhongqiu Liu, Liang Liu.
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Footnotes

This work was supported by the Projects of National Natural Science Foundation of China [81120108025, 81473410] and the Platform Project of Department of Education of Guangdong Province.

There is no financial conflict of interests with the authors of this paper. Publication of this paper will not benefit or adversely affect the financial situations of the authors.

Dr. Lijun Zhu and Dr. Linlin Lu contributed equally to this paper.
Figure legends

Figure 1 Identification of glucuronides of ET and 4-ME. UHPLC, HRMS, and MS\textsuperscript{2} scans were used to separate and identify ET, 4-ME, and their glucuronides in the experimental samples. \textsuperscript{1}HNMR was conducted to elucidate the glycosylation site of ET and 4-ME. Figures 1A and 1B show the chromatogram of ET, 4-ME and their glucuronides, as well as internal standard (IS), respectively. About 80 μM ET (or 4-ME) was incubated with HLM (final concentration = 0.5 mg/mL) in a phase II reaction system at 37 °C for 30 min. Figures 1C and 1E represent HRMS scans for glucuronides of ET and 4-ME, respectively. Figures 1D and 1F represent MS\textsuperscript{2} scan for glucuronides of ET and 4-ME, respectively. Analysis of \textsuperscript{1}HNMR data also revealed that the added moiety belonged to glucuronic acid (Supplemental Figures 1 to 4). The verified location of glucuronic acid at 7-OH position of ET was based on NOESY correlation between Glu-H-1' (δ\textsubscript{H} 5.24, 1H, d, J = 7.0 Hz) and H-8 (δ\textsubscript{H} 7.18, 1H, s) (G).

Figure 2 Glucuronidation of ET (A) and 4-ME (B) by 12 recombinant expressed human UGTs. Three concentrations (10, 30, and 100 μM) of ET (or 4-ME) were incubated with 12 commercially available recombinant expressed human UGTs (0.25 –1 mg/mL) at 37 °C for 30 min. Glucuronides formed in the incubation samples were quantified by UHPLC-MS\textsuperscript{2}, and glucuronidation rates were calculated and expressed as nmol/min/mg of protein. Each bar is the average of three determinations, and error bars are standard deviations of the mean (n = 3).

Figure 3 Effect of chemical inhibitors of UGT1A6 (phenylbutazone) and UGT1A9
(carvacrol) on the glucuronidation of ET (A, B, C, and D) and 4-ME (E, F, G, and H) in HLM, UGT1A6, and UGT1A9. Glucuronidation of ET and 4-ME by HLM and UGT isoforms were measured at three concentrations (10, 100, and 300 μM) and incubated in the presence of phenylbutazone or cavacrol at low three concentrations (50, 100, and 200 μM). All protein concentrations were 0.5 mg/mL, incubation time for ET and 4-ME in microsomes and UGTs were range from 15 to 60 min. Control experiments were incubated without chemical inhibitors. Samples were processed and analyzed by UHPLC-MS². All incubations were performed in triplicate. Each column represents mean percentage control of formation rates of these three metabolites, and error bars are standard deviations of the mean. An asterisk (*) indicates a statistically significant difference \( P < 0.05 \) compared with the control according to Student’s \( t \)-test.

**Figure 4** Kinetics of ET (A–D) and 4-ME (E–H) by UGT1A6, UGT1A9, HLM, and HIM. Kinetic assays were conducted using a microsomes or Supersomes at the concentrations in range from 0.013 to 0.053 mg/mL based on optimization of the reaction. Glucuronidation rates of ET by UGT1A6 and UGT1A9 were determined at concentration ranges of 100 to 800 μM and 15 to 250 μM, respectively, within 60 min of reaction time. Glucuronidation rates of ET by HLM and HIM were determined at concentration ranges of 30 to 400 μM and 20 to 400 μM, respectively, within 30 min of reaction time. Glucuronidation rates of 4-ME by UGT1A6 and UGT1A9 were determined at concentration ranges of 30 to 400 μM and 4 to 80 μM, as well as reaction times of 30 and 60 min, respectively. Glucuronidation rates of 4-ME by HLM
and HIM were determined at concentration ranges of 40 to 500 µM and 30 to 400 µM, respectively, within 30 min of reaction time. Curves were estimated based on fitted parameters generated using the Michaelis–Menten equation. An Eadie–Hofstee plot is shown in the inset to illustrate kinetics. Each data point was the average of three determinations, with error bars representing standard derivations. The apparent kinetic parameters are listed in Tables 1.
Table legend

Table 1. Kinetic parameters of esculetin (ET) and 4-methylesculetin (4-ME) glucuronidation obtained from recombinant UGT1A6 and UGT1A9 and human liver and intestinal microsomes (HLM and HIM).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Kinetic Parameters</th>
<th>UGT1A6</th>
<th>UGT1A9</th>
<th>HLM</th>
<th>HIM</th>
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<tr>
<td>ET</td>
<td>$K_m$ (µM)</td>
<td>185.59</td>
<td>134.75</td>
<td>134.19</td>
<td>189.33</td>
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<td></td>
<td>$V_{max}$ (nmol/min/mg)</td>
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<td>19.23</td>
<td>72.61</td>
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<td></td>
<td>$CL(V_{max}/K_m$, ml/min/mg)</td>
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<td>0.14</td>
<td>0.54</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.9823</td>
<td>0.9775</td>
<td>0.9833</td>
<td>0.9738</td>
</tr>
<tr>
<td></td>
<td>AIC</td>
<td>40.98</td>
<td>14.05</td>
<td>29.53</td>
<td>26.69</td>
</tr>
<tr>
<td>4-ME</td>
<td>$K_m$ (µM)</td>
<td>172.51</td>
<td>25.20</td>
<td>117.81</td>
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<tr>
<td></td>
<td>$V_{max}$ (nmol/min/mg)</td>
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<td>$CL(V_{max}/K_m$, ml/min/mg)</td>
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<td>0.59</td>
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<tr>
<td></td>
<td>$R^2$</td>
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<td>0.9879</td>
<td>0.9918</td>
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<tr>
<td></td>
<td>AIC</td>
<td>15.21</td>
<td>-41.49</td>
<td>34.75</td>
<td>-26.16</td>
</tr>
</tbody>
</table>

Kinetic parameters were obtained from using simple Michaelis–Menten models as described under “Material and Methods” section.
Figure 4

(A) Glucuronidation Rate by UGT1A6 (nmol/min/mg) vs. ET Conc. (μM)
(B) Glucuronidation Rate by UGT1A9 (nmol/min/mg) vs. ET Conc. (μM)
(C) Glucuronidation Rate by HLM (nmol/min/mg) vs. ET Conc. (μM)
(D) Glucuronidation Rate by HIM (nmol/min/mg) vs. ET Conc. (μM)
(E) Glucuronidation Rate by UGT1A6 (nmol/min/mg) vs. 4-ME Conc. (μM)
(F) Glucuronidation Rate by UGT1A9 (nmol/min/mg) vs. 4-ME Conc. (μM)
(G) Glucuronidation Rate by HLM (nmol/min/mg) vs. 4-ME Conc. (μM)
(H) Glucuronidation Rate by HIM (nmol/min/mg) vs. 4-ME Conc. (μM)