Structural modifications at the C-4 position strongly affect the glucuronidation of 6,7-dihydroxycoumarins

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Abbreviations

ESI, electrospray ionization; HLM, human liver microsomes; NMR, nuclear magnetic resonance; RLM, rat liver microsomes; SPE, solid phase extraction; UDPGA, uridine 5'-diphospho-glucuronic acid; UFLC, ultra-fast liquid chromatography; UGT, UDP-glucuronosyltransferase; 4-AE, 4-acetic acid esculetin; 4-HME, 4-hydroxymethyl esculetin; 4-ME, 4-methyl esculetin; 4-PE, 4-phenyl esculetin; 6,7-dihydroxycoumarin, 6,7-DHC; 7,8-dihydroxycoumarin, 7,8-DHC.
Abstract

Esculetin (6,7-dihydroxycoumarin) and its C-4 derivatives have multiple pharmacological activities, but the poor metabolic stability of these catechols has severely restricted their application in the clinic. Glucuronidation plays important roles in catechols elimination, although thus far the effects of structural modifications on the metabolic selectivity and the catalytic efficacy of the human UDP-glucuronosyltransferase (UGT) enzymes remain unclear. This study was aimed at exploring the structure-glucuronidation relationship of esculetin and its C-4 derivatives, including 4-methyl esculetin, 4-phenyl esculetin, 4-hydroxymethyl esculetin as well as 4-acetic acid esculetin. It was achieved by identifying the main human UGTs responsible for the different reactions and by careful characterization of the reactions kinetics. These catechols, with the exception of 4-acetic acid esculetin, are selectively metabolized to the corresponding 7-O-glucuronides. UGT1A6 and UGT1A9 are the two major UGTs involved in the 7-O-glucuronidation of 4-methyl esculetin and esculetin. UGT1A6 was the major contributor for 7-O-glucuronidation of 4-hydroxymethyl esculetin, while UGT1A9 played a significant role in the 7-O-glucuronidation of 4-phenyl esculetin. The results of the kinetic analyses revealed that the $K_m$ values of the compounds, in both UGT1A9 and human liver microsomes (HLM), decreased with increasing hydrophobicity of the C-4 substitutions. The outcome of this was that, C-4 hydrophobic and hydrophilic groups on 6,7-dihydroxycoumarin exhibited contrasting effects on UGT affinity. All of these findings provide helpful guidance for further structural modification of 6,7-dihydroxycoumarins with improved metabolic stability.
Introduction

Coumarins are widely distributed in many medicinal plants, and plant-derived foodstuffs including soybeans and cruciferous vegetables. In the past half century, it was demonstrated that coumarins have various pharmacological activities including anti-inflammatory (Silván et al., 1996), antibacterial (Kayser and Kolodziej, 1999), antifungal (Sardari et al., 1996), anticoagulant (Arora and Mathur, 1963), anti-HIV (Spino et al., 1998) and antitumor activities (Weber et al., 1998). Due to several good properties including excellent safety, high solubility and good permeability, coumarins are good candidates of chemo-therapeutic agents, (Galkin et al., 2009). Currently, coumarins including warfarin, daphnetin and armillarisin A are used in clinic to treat cancer (Thornes et al., 1994), lymph edema (Casley-Smith and Casley-Smith, 1997), infection of biliary system (Wang et al., 2007), as well as disorders of the immune system (Thornes, 1983) and coagulation.

It has been reported that the presence and the position of phenolic groups attached on the coumarin skeleton are important for their pharmacological activities. Several studies on structure-activity relationships of various phenolic coumarins revealed that the catechol group is the key pharmacophore for chain-breaking antioxidant activity (Kancheva et al., 2010), radical-scavenging activity (Lin et al., 2008), and the inhibitory effects on lipid peroxidation (Thuong et al., 2010). Additionally, most of catecholic coumarins showed more potent cytotoxic activity than mono-hydroxycoumarins in several human tumor cell lines (Kostova et al., 2006; Weber et al., 1998; Kolodziej et al., 1997). Esculetin (6,7-dihydroxycoumarin, 6,7-DHC) and its C-4 derivatives, including 4-methyl esculetin (4-ME), 4-phenyl esculetin (4-PE) and 4-acetic acid esculetin (4-AE), are naturally occurring catecholic coumarins. They
are widely distributed in many medicinal plants such as *Artemisia capillaries*, *Citrus limonia*, and *Euphorbia lathyris* (Masamoto et al., 2003). Esculetin has various bioactivities including inhibitory activity on lipoxygenase extracted from ground soybean (Lee and Lillard, 1997), mushroom tyrosinase inhibitory activity, free radical scavenging activity (Payá et al., 1992), calf thymus DNA protective activity (Tahara et al., 2005), mammary carcinogenesis chemopreventive activity in female Sprague-Dawley rats (Matsunaga et al., 1998) and inhibitory activity on cell cycle progression of human leukemia HL-60 (Wang et al., 2002). 4-ME has exhibited enhanced intestinal anti-inflammatory activity in rat colitis (Witaicenis et al., 2010), while 4-PE is a potent rat lens aldose reductase 2 inhibitor (Kato et al., 2010). Recently, due to the easy accessibility of the structural modification in this position (Garazd et al., 2005), various substitution groups such as aryl groups and heterocyclic rings were introduced to C-4 position, to get products with potentially enhanced or new activities (Mashelka and Audi, 2006; Kini et al., 2012; Sokmen et al., 2013).

Despite of a variety of claimed activities, the poor metabolic stability severely restricted the development of these catechol coumarins into therapeutic agents. To date, esculetin (Tsai et al., 1999) and its analog daphnetin (7,8-dihydroxycoumarin, 7,8-DHC) (Qu et al., 1983) exhibited quite short *in vivo* half-lives in the rat, due to the extensive first-pass metabolism in the liver. As the existence of phenolic hydroxyl groups, catechol coumarins should be the expected substrates of phase II metabolizing enzymes. For example, 4-methylumbelliferone (Tukey and Strassburg, 2000), daphnetin (Liang et al., 2010) and fraxetin (Xia et al., 2014) are quickly metabolized by various human UDP-glucuronosyltransferases (UGTs) with high hepatic clearance. Although most UGT1A isoforms are involved, previous studies
demonstrated that UGT1A6 and UGT1A9 were the major enzymes catalyzing 7-O- and 8-O-glucuronidation of 7,8-DHCs (Liang et al., 2010; Xia et al., 2014). However, glucuronidation positions of 6,7-DHCs and the involved human enzymes have not been characterized yet, which is necessary for structural modifications to improve their metabolic stability. Since all previous studies only focused on the characterization of metabolic positions on a given phenolic coumarin, the structure-metabolism relationship of a series of coumarin analogues remain unclear.

In the present study, a series of 6,7-DHCs (Figure 1) were selected as the model substrates to investigate the potential effects of C-4 substitutions on the glucuronidation in human. The present study provided helpful guidance for the structural modification of 6,7-DHCs with improved metabolic stability.
Materials and Methods

Chemicals and reagents

Esculetin (purity>98%) was purchased from the Boyle company (Shanghai, China), 4-methyl esculetin (4-ME) (purity>97%) was purchased from Alfa Aesar (Ward Hill, MA, USA), 4-phenyl esculetin (4-PE) (purity>98%) was purchased from ImagineChem company (Hangzhou, China) and magnolol (purity>98%) was purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, Sichuan, China). 4-acetic acid esculetin (4-AE) (purity>98%) was purchased from Santa Cruz Biotechnology and 4-hydroxymethyl esculetin (4-HME) (purity>98%) was synthesized by the author (Ping Wang) and fully characterized by LC-MS, $^1$H-NMR and $^{13}$C-NMR. Alamethicin, Brij 58, magnesium chloride, D-saccharic acid, 1,4-lactone, β-glucuronidase isolated from Helix pomatia (EC No. 3.2.1.31), uridine 5′-diphospho-glucuronic acid trisodium salt (UDPGA), and niflumic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pooled male human liver microsomes (HLM) (n=11) (Lot. DXOV) were purchased from Research Institute for Liver Diseases (Shanghai, China). A panel of recombinant human UGT isoforms (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, USA). All other reagents were of HPLC grade or of the highest grade commercially available.

Incubation conditions for glucuronidations of 6,7-DHCs

The incubation mixture (200 μl) contained HLM (0.5 mg protein/ml), 5 mM UDPGA, 5 mM MgCl$_2$, 25 μg/ml alamethicin, 10 mM D-saccharic acid 1,4-lactone, 100 μM substrate and Tris-HCl buffer (pH 7.4). After 60 min of incubation at 37°C, the reaction was terminated.
by the addition of 200 μl of acetonitrile, followed by centrifugation at 20,000 g for 20 min to
obtain the supernatant for analysis by using ultra-fast liquid chromatography spectrometry
(UFLC) chromatography–diode array detector (UFLC-DAD) or UFLC-Mass spectrometry.
Control incubations without UDPGA or without substrate or without microsomes were also
performed to ensure that the formed metabolites were microsome- and UDPGA-dependent.

**Hydrolysis of metabolites by β-glucuronidase**

In order to further characterize whether the formed metabolites of esculetin and its
derivatives are glucuronides, the corresponding metabolite of each 6,7-DHCs was isolated
and then hydrolyzed by β-glucuronidase. The hydrolysis mixture included 200 μl glucuronide
solution (containing 100 μM glucuronide) and 200 μl 0.15 M acetate buffer (pH 5.0)
with/without β-glucuronidase (2,000 Fishman units), and then the incubation was carried out
at 37 °C for 60 min. The reaction was stopped by the addition of 200 μl acetonitrile and
centrifuged at 20,000 g for 20 min. The aliquots (10 μl) of the supernatant were then analyzed
by UFLC-UV and UFLC-ESI-MS.

**Analysis of glucuronidation samples**

6,7-DHCs and their glucuronides were analyzed by a ultra-fast liquid chromatography
spectrometry system (Shimadzu, Kyoto, Japan), equipped with two LC-20AD pumps, a
DGU-20A3 vacuum degasser, a SIL-20ACHT auto-sampler, a CTO-20AC column oven, an
SPD-M 20A diode-array detector (DAD), a CBM-20A communications bus module, a mass
detector (2010EV) with an electrospray ionization (ESI) interface, and a computer equipped
with UFLC-MS Solution version 3.41 software. A Hedera C18 (150.0 mm×2.1 mm, 3 μm)
analytical column was used to separate 6,7-DHCs and their glucuronides. Column
temperature was kept at 40 °C. The mobile phase was acetonitrile (A) and 0.2% formic acid water (B) at a flow rate of 0.4 ml/min, with a gradient: 0-6.0 min, 95% B-65% B; 6.0-9.0 min, 5% B; 9.0-15min, balance to 95% B. The maximum absorption wavelengths for esculetin, 4-ME, 4-PE, 4-HME and 4-AE were 349 nm, 337 nm, 300 nm, 343 nm and 348 nm, respectively.

Mass detection was performed on a Shimadzu LCMS-2010EV instrument with an ESI interface both in positive (ESI+) and negative ion mode (ESI-) from m/z 100 to 500. The detector voltage was set at +1.55 kV and -1.55 kV, for positive and negative ion detection, respectively. The curved desolvation line (CDL) temperature and the block heater temperature were both set at 250 °C, while the CDL voltage was set at 40 V. Other MS detection conditions were as follows: interface voltage, +4.5 kV and -4.0 kV for respective positive and negative ion detection; nebulizing gas (N2) flow was 1.5 L/min and the drying gas (N2) pressure was set at 0.06 MPa. Data processing was performed using the LC/MS Solution version 3.41 software (Shimadzu, Kyoto, Japan).

**Biosynthesis of glucuronides and structural characterization**

The major glucuronide of each substrate including esculetin, 4-ME, 4-PE and 4-HME was biosynthesized using mixed liver microsomes from rat and human (90% RLM and 10% HLM) and purified for structure elucidation and quantitative analysis. In brief, substrate (1 mM) was incubated with mixed liver microsomes (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 1 ml of final incubations for 4 h at 37°C. This analytical-scale reaction was scaled up to a volume of 48 ml for each substrate. The stock solution of substrate (100 mM)
was prepared in DMSO. The concentration of organic solvent in the final incubation was 1%.

The reaction was terminated by the addition of ice-cold methanol, and then the vessels were transferred to an ice bath and cooling for 20 min. After the removal of protein by centrifugation at 20,000 g for 30 min at 4°C, the combined supernatants were loaded on a solid phase extraction (SPE) cartridge (C18PN, 1000 mg, Acchrom Technologies, Beijing, China), which was preconditioned by sequential washing with 6 ml methanol and 6 ml Millipore water. After sample loading, the SPE cartridge was sequentially eluted with 12 ml Millipore water, 12 ml methanol and 12 ml methanol containing 5% formic acid. The entire process was monitored by UFLC-UV, and the glucuronide was collected in methanol containing 5% formic acid. After vacuum evaporation, the glucuronide of each 6,7-DHCs was obtained by powder and the purity was greater than 96%. The starting amounts, yields of the reaction, and final amounts of products obtained for biosynthesis of each glucuronide were listed in Supplemental Table 1. The structure of each glucuronide was characterized by NMR technique including ¹H-NMR and ¹³C-NMR. All experiments were carried on a Bruker Avance-500 NMR spectrometer (Bruker, Switzerland). Each glucuronide was stored at -80°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-NMR (500 MHz) and ¹³C-NMR (150 MHz).

Reaction phenotyping assays with recombinant UGTs

Reaction phenotyping assays of each substrate were measured in the reaction mixtures containing one of recombinant human UGTs, including UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17. The incubation conditions were used as above.
mentioned in glucuronidation in HLM. For esculetin, 4-ME and 4-HME, three substrate concentrations (10, 100 and 1000 μM) were used in these assays. For 4-PE, two substrate concentrations (10 and 100 μM) were used, which were the approximate concentration at \( K_m \) and \( V_{max} \) value for HLM, respectively. All assays were conducted at 37°C for 60 min with the final protein concentration of 0.1 mg protein/ml. UFLC with DAD detection was used to monitor possible metabolites.

**Kinetic characterization**

Kinetic analyses were performed in HLM and major involved recombinant UGT enzymes. Preliminary experiments were carried out to make sure that the formation of metabolite was in the linear range of both reaction time (0-30 min) and the concentration of microsomes (0.01-0.1 mg/ml). A protein concentration in which appropriate amount of product could be generated and less than 10% of substrate was consumed was selected for each compound. For esculetin, substrate (10-1000 μM in HLM and UGT1A6, and 5-1000 μM in UGT1A9) was incubated with pooled HLM (0.02 mg/ml), UGT1A6 (0.02 mg/ml), or UGT1A9 (0.05 mg/ml) at 37 °C for 20 min. For 4-ME, substrate (2.5-1000 μM in HLM, and 5-1000 μM in UGT1A6 and UGT1A9) was incubated with pooled HLM (0.02 mg/ml), UGT1A6 (0.02 mg/ml), or UGT1A9 (0.05 mg/ml) at 37 °C for 20 min. For 4-PE, substrate (1-400 μM) was incubated with pooled HLM (0.02 mg/ml), or UGT1A9 (0.02 mg/ml) at 37 °C for 20 min. For 4-HME, substrate (10-1000 μM) was incubated with pooled HLM (0.05 mg/ml), or UGT1A9 (0.05 mg/ml) at 37°C for 20 min. All of these incubations were conducted in triplicate and all reactions were terminated by adding ice-cold acetonitrile (200 μl). The concentration of the glucuronide in each incubation was determined by using a
standard curve of the corresponding metabolite. Kinetic constants for glucuronidations of 6,7-DHCs by HLM, UGT1A6 and UGT1A9 were obtained by fitting the Michaelis-Menten equation to experimental data using GraphPad Prism 6.0 (GraphPad Software, San Diego, USA). The Michaelis-Menten equation is \( v = \frac{V_{\text{max}} [S]}{K_m + [S]} \), 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 were expressed as mean ± S.E. of triplicate independent determinations.

**Chemical inhibition assays**

Two selective UGT1A9 inhibitors (niflumic acid or magnolol) were used to explore the contribution of UGT1A9 in glucuronidation of each 6,7-DHCs (Miners et al., 2011; Zhu et al., 2012). Each substrate (esculetin, 4-ME, 4-PE or 4-HME) was incubated with pooled HLM, UGT1A6 and UGT1A9, in the absence or presence of niflumic acid (10 μM) or magnolol (1 μM). The final concentration of esculetin, 4-ME, 4-PE, or 4-HME was 350 μM, 100 μM, 10 μM, or 750 μM, respectively, which was near to the \( K_m \) value of glucuronidation of each substrate in HLM. The incubation time and the protein concentrations were same to the section of kinetic characterization, as mentioned above.
Results

Metabolic profiles of 6,7-DHCs

As shown in Figure 2, a major metabolite was detected by UFLC-DAD-ESI-MS when each of esculetin, 4-ME, 4-PE and 4-HME (100 μM) was incubated with HLM (0.5 mg/ml) in the presence of UDPGA, while no metabolite detected in the incubation system containing 4-AE (100 μM) (Supplemental Figure 1). In sharp contrast, these products could not be detected in control incubations without UDPGA or enzyme sources, indicating the formation of these metabolites was UDPGA and microsome dependent (data not shown). Mass spectra were dominated by \([M-H]^-\) for esculetin, 4-ME, 4-PE and 4-HME. Mass spectrometry in the negative ion mode showed an \(m/z\) value for the deprotonated metabolite, a value that corresponds well to the parent with \(m/z\) 176 of the glucuronosyl substitution (Supplemental Figure 2-5). In addition, all of these metabolites can be hydrolyzed by β-glucuronidase to the corresponding parent, indicating that they were the monoglucuronides. The metabolites were further purified and characterized for structural elucidation by \(^1\)H-NMR, \(^{13}\)C-NMR, HSQC and HMBC spectral technology (Supplemental Figure 6-13). The \(^1\)H-NMR and \(^{13}\)C-NMR spectral data of four mono-glucuronides were listed in Supplemental Table 2-5. Compared with NMR data of the substrate, the \(^{13}\)C-NMR spectrum of mono-glucuronide of esculetin displayed the carbon signal of C-7 (δ 152.0) shifted upfield to δ 150.2 (Δδ -1.8), while the signals of C-6 and C-8 shifted downfield to 145.7 (Δδ +1.1) and 105.5 (Δδ +1.9), respectively, implying that the mono-glucuronide should be the esculetin-7-O-glucuronide. It was equally clear that the mono-glucuronides of 4-ME, 4-PE and 4-HME were also 7-O-glucuronides, which could be readily seen from the shifts of the carbon signals of C-7 as well as C-6 and
C-8. Moreover, the coupling constants for the anomeric protons of 7-O-glucuronides of esculetin (J=7.2), 4-ME (J=7.3), 4-PE (J=7.2), 4-HME (J=7.2) were characteristic of the β-anomers (Natsume et al., 2003).

**Reaction phenotyping assays with recombinant UGTs**

Three substrate concentrations (10, 100, and 1000 μM) were used for isoform screening to assign the involved human UGT isoforms in glucuronidations of esculetin and its derivatives. UGT1A6 and 1A9 were demonstrated to be the two most important isoforms for glucuronidations of 6,7-DHCs at all three selected concentrations. For esculetin, UGT1A6 and UGT1A9 catalyzed the formation of 7-O-glucururonide at low, medium and high substrate concentrations, while trace amounts of 7-O-glucururonide could be formed by UGT1A1, 1A3, 1A7 and 1A8 (Figure 3) at the high substrate concentration (1000 μM). UGT1A6 displayed relatively higher activities compared to other isoforms. For 4-ME, the involved UGT isoforms were very similar to that involved in esculetin-7-O-glucururonidation, while UGT1A6 and UGT1A9 showed relatively high activity toward 4-ME-7-O-glucururonidation. For 4-PE, UGT1A9 displayed the highest activity in the formation of 7-O-glucururonide, while UGT1A1, 1A3, 1A6, 1A7 and 1A8 also catalyze 4-PE-7-O-glucururonidation at high substrate concentration. For 4-HME, four human UGT isoforms including UGT1A1, 1A3, 1A6, and UGT1A9 were involved in the formation of 7-O-glucururonide. UGT1A6 exhibited the highest activity towards this biotransformation at three tested substrate concentrations, while other involved isoforms displayed limited activities toward 4-HME-7-O-glucururonidation.

**Kinetic characterization**
The kinetic parameters including \( K_m \), \( V_{\text{max}} \) and the intrinsic clearance (\( V_{\text{max}}/K_m \)) for glucuronidations of 6,7-DHCs in HLM and in major human enzymes involved in these glucuronidations were determined and listed in Table 1. Over the whole concentration range tested, glucuronidations of each substrate in HLM and in human UGTs isoforms including UGT1A6 and UGT1A9, followed Michaelis-Menten kinetics, as evidenced by the Eadie-Hofstee plots (Figure 4, Supplemental Figure 14). The kinetic parameters of esculetin and 4-ME in UGT1A1, 1A3, 1A7 and 1A8, 4-PE in UGT1A1, 1A3, 1A6, 1A7 and 1A8, and 4-HME in UGT1A1, 1A3 and 1A9 could not be well characterized, due to the very limited activities of these isoforms.

**Contribution of UGT1A9 in glucuronidation of 6,7-DHCs**

Although UGT1A6 is the predominant isoform in glucuronidation of esculetin, 4-ME and 4-PE, the lack of highly specific inhibitors of this isoform hinders the direct determination of its contribution in metabolism of each compound. Considering that UGT1A9 is also a major metabolizing enzyme participating in glucuronidation of 6,7-DHCs and is highly expressed in human liver (Court et al., 2012), a series of inhibition assays were performed to reveal the contribution of UGT1A9 in the formation of 7-O-glucuronides of esculetin and its derivatives. Two highly selective UGT1A9 inhibitors (niflumic acid and magnolol) were used, due to their potent inhibitory effects on UGT1A9 mediated glucuronidations in both recombinant enzymes and in human tissue preparations, as well as their minor effects on the activities of other UGTs (Miners et al., 2011; Zhu et al., 2012). The inhibitory effects of niflumic acid (10 \( \mu \text{M} \)) and magnolol (1 \( \mu \text{M} \)) on glucuronidations of esculetin and its derivatives in pooled HLM, UGT1A6 and UGT1A9 were evaluated. As
shown in Figure 5, for the formation of 7-O-glucuronide of esculetin, the remaining activity of HLM was about 62% in the presence of niflumic acid or magnolol, indicating that the contribution of UGT1A9 in the formation of 7-O-glucuronide was about 40%. For 4-ME, the remaining activity of HLM was about 57% when UGT1A9 was completely inhibited in the presence of niflumic acid or magnolol, implying that the contribution of UGT1A9 to the glucuronidation of 7-O-glucuronide was slightly larger than 40%. For 4-PE, the remaining activity of HLM was about 18% in the presence of niflumic acid or magnolol, demonstrating that the contribution of UGT1A9 to the glucuronidation of 4-PE was about 80%. For 4-HME, both of niflumic acid and magnolol displayed minor inhibitory effects on the formation of 4-HME-7-O-glucuronide in HLM, suggesting that UGT1A9 played little contribution in 4-HME glucuronidation in human liver.
Discussion

It is well known that phenolic coumarins have a varied range of bioactivities and display excellent safety, while these compounds also have a small molecular weight and exhibit good permeability (Galkin et al., 2009). Unfortunately, our preliminary study and several previous literatures have demonstrated that phenolic coumarins have relatively high metabolic liability, which may leads to metabolic instability. For catechol coumarins, phase II metabolisms was confirmed to be the primary elimination pathways. We found that 7-O-glucuronides and methylated 7-O-glucuronides were major the metabolites of esculetin and 4-ME in rats, respectively (Ge et al., 2010). Considering the important role of UGT in the metabolism of catechol coumarins, structure-glucuronidation relationship of catechol coumarins should be done to guide structure modification for metabolic stability improvement. In the present study, the potential effects of C-4 substitution on the glucuronidation of 6,7-DHCs were investigated, using a series of 6,7-DHCs including esculetin, 4-ME, 4-PE, 4-AE and 4-HME as the model compounds.

Our results demonstrated that most of the tested 6,7-DHCs including esculetin, 4-ME, 4-PE and 4-HME were UGT substrates in human liver microsomes. The major metabolite was identified as 7-O-glucuronide by NMR. Although trace amount of 6-O-glucuronide was observed, 7-O-glucuronide was still the predominant one. It is also noted that 6-O-glucuronides of these substrates were also hardly detectable in rat liver microsomes, indicating the high regioselectivity of UGTs-mediated glucuronidation of 6,7-DHCs in both human and rat liver. These findings suggested that the C-7 phenol group should receive more attention in further structure modification aimed to increase the metabolic stability of
To date, UGT1A6 and 1A9 have been identified as the major enzymes catalyzing C-7 glucuronidation of coumarins including 4-MU, daphnetin and fraxetin (Tukey and Strassburg, 2000; Liang et al., 2010; Xia et al., 2014). In the present study, UGT1A6 and UGT1A9 are also effective in 7-O-glucuronidation of 6,7-DHCs. It has been reported that UGT1A6 catalyzed simple or planer phenols, and UGT1A9 showed much greater proficiency in bulky substrates glucuronidations (Ethell et al., 2002). In this study, we also found that 4-PE is a weak substrate (10 folds less Cl_int of 4-PE in UGT1A6 than that of serotonin, a specific UGT1A6 probe) (Krishnaswamy et al., 2003) of UGT1A6 that is considered for being limited to small-size substrate, due to its relatively large molecular size. It also should be noted that the expression of these two isoforms varies considerably with the expression of UGT1A9 in human liver more pronounced than UGT1A6 in both mRNA and protein (Court et al., 2012; Harbourt et al., 2012; Fallon et al., 2013; Sato et al., 2014). Furthermore, UGT1A9 typically displays relatively higher affinity towards substrates compared to UGT1A6 (Ethell et al., 2002). The high protein level and the high catalytic efficacy of UGT1A9 implied that UGT1A9 plays an important role in glucuronidation of 6,7-DHCs in human liver. The kinetic parameters further reinforced the high UGT1A9 contribution in 6,7-DHCs glucuronidation. In addition, all of these findings suggested that UGT1A9 can serve as a major target for improving the phase II metabolic stability of these catechols.

It has been reported that lipophilicity was the most important factor for affecting the catalytic efficacy of UGT1A9, whereas the presence of polar groups is negatively correlated with activity (Taskinen et al., 2003). In this study, we found that UGT1A9 catalyzed the
glucuronidation of 6,7-DHCs with hydrophobic groups at the C-4 position, i.e., 4-ME or 4-PE, exhibiting improved apparent substrate affinity and high conjugation rate, with the increasing hydrophobicity of C-4 substituents, indicating the preference for UGT1A9 to more lipophilic substrates. In contrast, the glucuronidation activity of 4-HME in UGT1A9 was very low and 4-AE could not be glucuronidated by UGT1A9, confirming that UGT1A9 prefers to hydrophilic substituted compounds. As shown in Figure 3, at the same substrate concentration (100 μM), a 160-fold variation in the catalytic activities of UGT1A9 towards 4-PE and 4-HME was observed, where the formation rates were 20.8, and 0.13 nmol/min/mg protein, respectively. Furthermore, the kinetic analysis of UGT1A9-mediated 4-HME glucuronidation could not be well characterized because the velocity of this reaction cannot be saturated at the maximum 4-HME concentration. These results implied that 4-PE was a good substrate (10 folds greater Clint in UGT1A9 than that of propofol, a specific UGT1A9 probe) (Korprasertthaworn et al., 2012) of UGT1A9 but 4-HME was a weak substrate (10 times less Clint in UGT1A9 than that of propofol) of this enzyme.

Despite the effects on enzymatic selectivity and the kinetic parameters in UGT1A9, the effects of varied substituents at the C-4 position of 6,7-DHC on the kinetic behaviors could also be observed in HLM. For example, the Km and Vmax values for 4-PE in HLM were 7.7 μM and 12.0 nmol/min/mg, respectively, while the corresponding values for 4-HME in HLM were 748.8 μM and 10.3 nmol/min/mg, respectively. As a result, the metabolic clearance (Vmax/Km) of 4-PE displayed a hundred times higher value than that of 4-HME. The significant difference in metabolic clearance between 4-PE and 4-HME can be partly attributed to the different role of UGT1A9 in their glucuronidations, by considering that
UGT1A9 displayed good catalytic activity toward 4-PE while UGT1A9 played very minor role in 4-HME glucuronidation.

It was also noted that extrahepatic UGTs including UGT1A8 and UGT1A10 displayed only a minor contribution to the glucuronidations of 6,7-DHCs, indicating that metabolism of these compounds by intestinal UGTs should be relatively slow. Considering that 6,7-DHCs have good permeability and low molecular weight, it is conceivable that these type of compounds could be absorbed into the blood rapidly in the intestinal border after oral administration, and then hepatic probably as well as kidney metabolism may participate in their metabolic clearance in human body. Taking into account the fact that UGT1A9 is the key determinant in the glucuronidation of 6,7-DHCs and its abundant expression in both liver and kidney (Knights et al., 2013), decreasing the apparent substrate affinity and catalytic activity in UGT1A9 by structural modification or co-administration with UGT1A9 inhibitor(s) could be helpful for improving the metabolic stability towards glucuronidation of these catechol compounds.

It has been reported that the dihydroxyl moiety of 6,7-DHCs are important to their biological activities, such as anti-oxidant and the anti-cancer activities (Kancheva et al., 2010; Lin et al., 2008; Thuong et al., 2010; Kostova et al., 2006; Weber et al., 1998; Kolodziej et al., 1997). The direct modification of the catechol group would potentially diminish their bioactivity and structural changes on the non-pharmacophore groups are more reasonable strategies for the structural modification of 6,7-DHCs. During the past half century, many coumarin derivatives including a lot of C-4 derivatives were synthesized and reported, due to the fact that these derivatives can be readily synthesized and various substituents can be easily
introduced to the C-4 position by total synthesis or semi-synthesis (Garazd et al., 2005). Several previous studies have also found that C-4 hydrophilic modification does not affect the anti-oxidant and the anti-cancer activities of 6,7-DHCs (Lin et al., 2008; Kostova, 2005). Therefore, C-4 hydrophilic modification, such as C-4 hydroxy methyl or acetic acid substitution, may be a potential strategy to balance the activity and the metabolic stability.

In summary, the structure-glucuronidation relationship of 6,7-DHCs has been systematically explored for the first time, at the level of both HLM and recombinant UGTs using a series of C-4 coumarin derivatives. Our findings demonstrated that the C-7 phenol group is the major metabolic site of 6,7-DHCs metabolized by human UGTs, while UGT1A6 and UGT1A9 are the two major enzymes participating 7-O-glucuronidation of these compounds. Furthermore, we also found that the lipophilicity of the C-4 substituents on 6,7-DHCs strongly affected the selectivity of involved human UGTs, and the enzyme kinetics including apparent substrate affinity and the catalytic activity. All of these findings are very helpful to guide the further structural modification of 6,7-DHCs with improved metabolic stability.
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Authorship Contributions

Participated in research design: Xia, Ge, and Yang

Conducted experiments: Xia, Wang, Liang, and Qian.

Contributed new reagents or analytic tools: Xia, Ge, and Wang.

Performed data analysis: Xia, Ge, Liang, He, Ning and Li.

Wrote or contributed to the writing of the manuscript: Xia, Ge, He, and Yang.
References


Structure-cytotoxicity relationships of a series of natural and semi-synthetic simple coumarins as assessed in two human tumour cell lines. *Z Naturforsch C* **52**: 240-244.


Footnotes

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Figure legends

**Figure 1.** Structures of esculetin and its C-4 derivatives.

**Figure 2.** Chromatograms of incubation systems with esculetin (A), 4-methyl esculetin (B), 4-phenyl esculetin (C), and 4-hydroxymethyl esculetin (D). Each substrate was incubated in human liver microsomes with (+UDPGA) and without (-UDPGA) UDPGA for 60 min.

**Figure 3.** Glucuronidations of esculetin (A), 4-methyl esculetin (B), 4-phenyl esculetin (C), 4-hydroxymethyl esculetin (D) by recombinant human UGTs. Substrates were incubated with recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 for 60 min. Substrate concentrations of 10, 100 and 1000 µM and protein concentrations of 0.1 mg/ml were used. Data was expressed as mean±S.D.

**Figure 4.** Glucuronidation kinetics of esculetin and its C-4 derivatives in HLM and related recombinant UGTs.

**Figure 5.** Inhibitory effects of two selective inhibitors on glucuronidations of esculetin (A), 4-methyl esculetin (B), 4-phenyl esculetin (C) and 4-hydroxymethyl esculetin (D) in HLM, recombinant UGT1A6 and UGT1A9. Substrates (348 µM, 178 µM and 115 µM of esculetin, and 86 µM, 69 µM and 31 µM of 4-ME in HLM, UGT1A6 and UGT1A9, respectively; 8 µM and 4 µM of 4-PE in HLM and UGT1A9, respectively; and 749 µM and 790 µM of 4-HME in HLM and UGT1A6, respectively) were incubated with pooled HLMs and UGTs for 20 min in the absence and presence of inhibitors (1 µM magnolol and 10 µM niflumic acid). Data was expressed as mean±S.D.
Table 1. Kinetics of esculetin, 4-methylesculetin, 4-phenylesculetin and 4-hydroxymethyl esculetin in HLM and UGTs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_m ) (μM)</th>
<th>( V_{\text{max}} ) (nmol/min/mg)</th>
<th>( C_{\text{int}} ) (μl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLM</td>
<td>1A6</td>
<td>1A9</td>
</tr>
<tr>
<td>esculetin</td>
<td>347.9±19.0</td>
<td>178.1±8.9</td>
<td>115.1±10.3</td>
</tr>
<tr>
<td>4-ME</td>
<td>85.5±3.9</td>
<td>69.1±4.9</td>
<td>30.5±1.8</td>
</tr>
<tr>
<td>4-PE</td>
<td>7.7±0.5</td>
<td>-</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>4-HME</td>
<td>748.8±177.0</td>
<td>789.9±85.1</td>
<td>-</td>
</tr>
</tbody>
</table>

\( K_m \) and \( V_{\text{max}} \) values are expressed as best fit values ± S.E..
Figure 1

R

H

CH₃

C₆H₅

CH₂OH

CH₂COOH

Compounds

esculetin

4-methylesculetin

4-phenylesculetin

4-hydroxymethyl esculetin

4-acetic acid esculetin
Figure 5

(A) Esculetin

(B) 4-ME

(C) 4-PE

(D) 4-HME

Residual activity (%)