Identification and Characterization of Human UDP-glucuronosyltransferases Responsible for the In Vitro Glucuronidation of Salvianolic Acid A

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Summary: Glucuronidation is an important pathway in the elimination of salvianolic acid A (Sal A); however the mechanism of UDP-glucuronosyltransferases (UGTs) in this process remains to be investigated. In this study, the kinetics of Sal A glucuronidation by pooled human liver microsomes (HLMs), pooled human intestinal microsomes (HIMs) and 12 recombinant UGT isozymes were investigated. The glucuronidation of Sal A can be shown both in HLMs and HIMs with $K_m$ values of 39.84 ± 3.76 and 54.04 ± 4.36 µM, respectively. Among the 12 human UGTs investigated, UGT1A1 and UGT1A9 were the major isoforms that catalyzed the glucuronidation of Sal A ($K_m$ values of 29.72 ± 2.20 and 24.40 ± 2.60 µM). UGT1A9 showed the highest affinity of Sal A glucuronidation. Furthermore, a significant correlation between Sal A glucuronidation and propofol glucuronidation (a typical UGT1A9 substrate) was observed. The chemical inhibition study showed that the IC$_{50}$ for phenylbutazone inhibition of Sal A glucuronidation was 50.3 ± 4.3 and 39.4 ± 2.9 µM by HLMs and UGT1A9, respectively. Mefenamic acid inhibited Sal A glucuronidation in UGT1A1 and HLMs with IC$_{50}$ values of >200 and 12.4 ± 2.2 µM, respectively.

Keywords: salvianolic acid A; UDP-glucuronosyltransferases; human liver microsomes; UGT1A1; UGT1A9

Introduction

The glucuronidation reaction catalyzed by the enzyme UDP-glucuronosyltransferases accounts for over thirty-five percent of all phase II drug metabolism. Human UGTs have been identified and classified into two subfamilies (UGT1 and UGT2) based on gene sequence. Most of the human UGTs exhibited distinct, overlapping substrates and selective inhibitors. A few specific reactions of certain UGT isoforms have been presented, such as the glucuronidation for UGT1A4 of trifluoperazine, UGT1A1 of bilirubin, UGT1A9 of propofol, UGT1A6 of serotonin, and UGT2B7 of 3′-azido-3′-deoxythymidine. The contribution of different isoforms to glucuronidation is based on the relative expression levels in different tissues. For example, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7 and UGT2B17 have been detected in liver, while UGT1A7, UGT1A8, UGT1A10, and UGT2A1 are mainly present in extrahepatic tissues, although liver is generally considered as the major site of glucuronidation. Glucuronides have more polarity than the parent drugs, and are thus more readily excreted into bile and urine. Salvia miltiorrhiza Bunge (Danshen), a well known Chinese herbal medicine, has been widely used in the treatment of coronary heart disease, cerebrovascular disease, hepatic cirrhosis and hepatitis. The water-soluble components, extracted from Salvia miltiorrhiza Bunge by water, are phenolic acids. These components are likely to undergo phase II conjugation metabolisms, which result in the formation of a covalent linkage between a functional group on the parent compound. Salvianoic acid (Sal A) (Fig. 1) has been identified as one of the major water-soluble active
constituents. Many pharmacological studies have demonstrated that Sal A possesses obvious biological activities such as decreasing the infarct area and inhibiting cerebral edema in ischemic rats by improving regional cerebral blood flow in the ischemic hemisphere, inhibiting platelet aggregation and scavenging oxygen free radicals in rats. The pharmacokinetics of Sal A in rats has been reported after intravenous administration of Danshen; Pei et al. have researched the pharmacokinetics of Sal A in rats after a single intragastric administration.

Glucuronidation of Sal A has been identified in rats and the majority of metabolites appeared in the plasma as conjugated forms. Although the pharmacokinetics and metabolites of Sal A have been studied, the metabolic characterization of glucuronidation was still sparse. Identification of isoforms responsible for drug metabolism is important to understand the possible effect of enzymes on the pharmacokinetics of the administered drugs. In addition, the identification of drug-metabolizing enzymes will be useful for characterizing the role of these particular enzymes in potential clinical herbal and chemical drug interactions and estimating the impact of genetic polymorphisms of interested enzymes on drug disposition.

This study characterized the enzyme kinetics of Sal A glucuronidation in human liver (HLMs) and intestinal microsomes (HIMs). The recombinant human UGTs and chemical inhibition study were also employed to identify the human UGT isoforms responsible for Sal A glucuronidation.

Materials and Methods

Chemicals and reagents: Sal A was purchased from Sichuan Victory Biotechnology Co., Ltd. (Sichuan, China). Alamethicin, magnesium chloride, D-saccharic acid 1,4-lactone, β-D-glucuronoside, UDPGA, propofol, phenylbutazone and mafenamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bilirubin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pool HLMs, HIMs and a panel of recombinant human UGT Supersomes™ (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) expressed in baculovirus-infected insect cells were purchased from BD Biosciences (Bedford, MA, USA). All other reagents were of HPLC grade or of the highest grade commercially available.

Identification of Sal A glucuronide by HPLC-MS analysis: The total volumes of 200 µl incubation mixture comprised 50 mM Tris-HCl buffer (pH 6.5), 5 mM MgCl₂, 25 µg/ml alamethicin, 0.1 mg/ml human liver microsomes, and 50 µM Sal A. After preincubation at 37 °C for 10 min, the reaction was initiated by the addition of UDPGA. At 10 min, the reaction was terminated with 200 µl of cold acetonitrile containing 1% formic acid and 0.3 µg of internal standard (nateglinide). After removal of the protein by centrifugation at 16,000 × g for 10 min, a HPLC-MS-2010EV mass spectrometer system (Shimadzu, Japan) with an electro-spray ionization interface was used to identify Sal A glucuronide operating in both negative and positive ion modes from m/z 100 to 800. The detector voltage was set at +1.70 and −1.85 kV for positive and negative ion mode, respectively. The block heater and the curved desolvation line temperature were both put at 250°C. Other MS detection conditions were set as follows: interface voltage, 40 V; voltage, 4 kV; drying gas (N₂) pressure, 0.06 MPa; and nebulizing gas (N₂) flow, tuned to be 1.5 L/min. Data processing was performed using HPLC-MS Solution version 3.0 software (Shimadzu, Japan).

Glucuronidation assay of Sal A: The incubation of Sal A with HLMs and HIMs were performed as depicted above. Detection of Sal A glucuronide was obtained with injection of 20 µl of the centrifugal supernatant into the HPLC-UV (model 2010C, Shimadzu, Japan) with the wavelength of 286 nm. Separation was performed on a Thermo Scientific BDS HYPERSIL C18 column (150 mm × 2.1 mm, i.d., 1.7 µm particle sizes) maintained at 30°C and a flow rate of 1 ml/min. The gradient, shown as changes in mobile phase A (acetonitrile) and B (0.1% formic acid water), was as follows: 0–2 min, hold at 80% B; 2–4 min, a linear decrease from 80% to 20% B; 4–7 min, hold at 80% B.

Quantification of the glucuronide in the incubation mixtures was established using standard curves of Sal A because of the absence of Sal A glucuronide standards. The outflows by HPLC from the incubation mixture with HLMs were obtained for the quantification of Sal A glucuronide. Aliquots of the outflows were hydrolyzed by β-glucuronidase performed as below. The thoroughly hydrolyzed Sal A glucuronide was quantified as Sal A by HPLC-UV. The standard curve for Sal A was linear from 0.2 to 200 µM, and the correlation coefficient was >0.99. The accuracy and precision of the back-calculated values for each concentration were less than 15%.

Hydrolysis with β-glucuronidase: The eluate corresponding to Sal A glucuronides was dried and redissolved in 1.0 ml of 0.05 M sodium acetate buffer (pH 5.0). Each sample was incubated in the absence (control) and presence of 100 units of β-glucuronidase at 37°C for 24 h. Parts (100 µl) of each incubation were terminated with 200 µl of ice-cold acetonitrile containing 1% formic acid. The supernatant was injected to HPLC-UV as described above after removal of the protein by centrifugation.

Glucuronidation by recombinant UGTs: Incubations in recombinant UGTs were conducted as described
above for liver microsome incubations. Human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 were used to investigate the glucuronidation of Sal A. Incubation mixtures (total volume 200 µl) were prepared containing 50 mM Tris-HCl buffer (pH 6.5), 5 mM MgCl2, 25 µg/ml alamethicin, 0.1 mg/ml human liver microsomes, and Sal A. The final concentration series of Sal A were 50, 200, and 400 µM, yielding a pH of 6.5 for the whole UGT incubation system.

**Kinetic analysis:** Sal A concentration series from 1 to 200 µM were performed in HLMs, HIMs, recombinant UGT1A1 and UGT1A9. Kinetic parameters were evaluated from the suitable curves using Graphpad Prism (GraphPad Software Inc., San Diego, CA, USA), followed by nonlinear regression analysis. The following equation was used:

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

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.

**Correlation analysis:** The glucuronidation of Sal A at a concentration of 50 µM was conducted in HLMs to assess the individual differences of Sal A glucuronidation and to correlate with the specific UGT substrates (the final concentration also 50 µM). Pearson’s product-moment correlation coefficient (r) was applied to estimate the relationship between glucuronidation activities for Sal A in HLMs, bilirubin glucuronidation activity for UGT1A1,4,23 and propofol glucuronidation activity for UGT1A9.24

**Chemical inhibition studies:** Sal A glucuronidation activity in HLMs, HIMs, UGT1A1 and UGT1A9 were determined in the absence or presence of phenylbutazone or mefenamic acid, known UGT inhibitors. Phenylbutazone is an effective inhibitor for UGT1As25 and mefenamic acid is an effective inhibitor for UGT1A9.26 Sal A (50 µM) was incubated in the absence or presence of either mefenamic acid (1–50 µM) or phenylbutazone (10–500 µM). All incubations were carried out for 10 min at a concentration of 0.1 mg/ml protein. The glucuronidation activities were calculated as a percentage of control concentration, and the IC50, indicating the concentration that inhibits 50% of the control concentration, and were calculated by nonlinear curve fitting with Graphpad Prism (GraphPad Software Inc.).

**Results**

**Identification of Sal A glucuronide formed by HLMs:** Sal A glucuronidation by HLMs was first identified by HPLC-ESI-MS (Figs. 2A and 3). Figure 2A indicated representative chromatograms of enzymatic Sal A glucuronide incubated with HLMs in the presence of UDPGA. The Sal A glucuronide was eluted at 4.8 min in the reaction mixture. The negative ion mode was adopted for chemical identification because it is more sensitive than the positive ion mode for Sal A and its metabolites. The mass spectrum was dominated by \([M – H]^-\) ion at m/z 669.19, corresponding to Sal A glucuronides and followed by an ion at m/z 493.38, corresponding to the parent drug Sal A – H with characteristic m/z 176 loss of the glucuronic acid moiety. The same metabolite, Sal A glucuronide, was formed in HIMs (figures not shown).
Hydrolysis with β-glucuronidase: Sal A glucuronide was hydrolyzed by β-glucuronidase and converted to parent Sal A (Fig. 2B). The increase of Sal A was equivalent to the decrease of Sal A glucuronide after incubation with β-glucuronidase, while no changes were detected in the control samples without β-glucuronidase.

Kinetics of Sal A glucuronidation in HLMs and HIMs: Kinetic analysis of Sal A glucuronidation was investigated in HLMs and HIMs. The substrate concentration glucuronidation velocity curves showed typical Michaelis-Menten kinetics (Figs. 4A and 4B). Moreover, an Eadie-Hofstee plot was monophasic. The kinetic parameters fitting the data points to the Michaelis-Menten equation are displayed in Table 1. Incubation of Sal A from 1 to 200 µM with HLMs revealed that the K_m and V_max values for Sal A glucuronide were 39.84 ± 3.76 µM, and 23.59 ± 0.52 µmol/min/mg protein, respectively. Simultaneously, incubation of appropriate Sal A concentrations with HIMs revealed that the K_m and V_max values for Sal A glucuronide were 54.04 ± 4.36 µM, 9.28 ± 0.19 µmol/min/mg protein, respectively. The in vitro total intrinsic clearances (calculated as V_max/K_m) of the glucuronidation of Sal A in HLMs and HIMs were 0.59 and 0.17 µl/min/mg protein, respectively.

Sal A glucuronidation in recombinant UGT isoforms: Twelve recombinant UGTs including UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 were applied to catalyze Sal A glucuronidation and to identify the isozymes involved in the metabolism. At all of the three Sal A concentrations, UGT1A1 and UGT1A9 displayed the highest and most prominent catalytic activity to Sal A glucuronidation (Fig. 5). Kinetics analyses of the Sal A glucuronidation in recombinant UGT1A1 and UGT1A9 were carried out (Figs. 4C and 4D). Sal A glucuronidation by recombinant UGT1A1 and UGT1A9 also revealed typical Michaelis-Menten kinetics, similar to that by HLMs and HIMs. Moreover, each Eadie-Hofstee plot was monophasic. The kinetic parameters fitting the data points to the Michaelis-Menten equation are displayed in Table 1.
Incubation of Sal A from 1 to 200 µM with recombinant UGT1A1 and UGT1A9 revealed that the $K_m$, $V_{max}$, and $CL_{int}$ values for Sal A glucuronide were 29.72 ± 2.20 and 24.40 ± 2.60 µM, 13.65 ± 0.22 and 12.71 ± 0.28 µmol/min/mg protein, and 0.46 and 0.52 µl/min/mg protein, respectively. UGT1A3, UGT1A7 and UGT1A8 also showed slight activity, while other UGT isozymes did not catalyze this reaction. This result demonstrates that UGT1A1 and UGT1A9 are the most important isoforms involved in the glucuronidation of Sal A.

Correlation study: Correlation analyses were performed between the Sal A glucuronosyltransferase activity and UGT1A1 probe substrate bilirubin and UGT1A9 probe substrate propofol glucuronosyltransferase activities in HLMs. The Sal A glucuronosyltransferase activities were less extent related with the bilirubin ($R^2 = 0.637$, $p < 0.001$) glucuronosyltransferase activities (Fig. 6A), but the Sal A glucuronosyltransferase activities were significantly related with the propofol glucuronosyltransferase activities ($R^2 = 0.961$, $p < 0.001$) (Fig. 6B).

Chemical inhibition analysis: The inhibitory effects of mefenamic acid and phenylbutazone on Sal A glucuronidation in HLMs, HIMs, and human recombinant UGTs were investigated. As shown in Table 2, the UGT activity for the formation of the metabolites in HLM, HIM, UGT1A1, and UGT1A9 was prominently inhibited by phenylbutazone. Similar $IC_{50}$ values for phenylbutazone inhibition of HLMs, HIMs, UGT1A1, and UGT1A9 were displayed as follows: 50.3 ± 4.3 µM, 53.2 ± 4.6 µM, 43.1 ± 5.2 µM, and 39.4 ± 2.9 µM, respectively. Mefenamic acid seemed generally to be more effective in inhibition than phenylbutazone except for that of UGT1A1. Moreover, mefenamic acid displayed a differential inhibitory profile on the enzyme activity; however, the inhibition of UGT1A1 ($IC_{50}$ over 200 µM) was weaker than that investigated for other enzyme preparations. The $IC_{50}$ values for mefenamic acid inhibition of HLMs, HIMs, and UGT1A9 were calculated to be 12.4 ± 2.2 µM, 9.8 ± 1.1 µM, and 9.1 ± 1.6 µM, respectively.

Discussion

In this study, we examined Sal A glucuronidation in HLMs, HIMs, and human recombinant UGTs and compared the enzyme kinetic parameters. Shen et al. had found that glucuronidation was an elimination pathway of Sal A in rats. In this study, the same metabolite was detected in human microsomal incubations. Based on the screening experiment conducted with baculosome of 12 recombinant UGTs, UGT1A1, UGT1A3, UGT1A7, UGT1A8 and UGT1A9 were isozymes responsible for the glucuronidation of Sal A. Due to the relatively pronounced catalytic
activity, kinetic experiments were carried out in recombinant UGT1A1 and UGT1A9.

It is noticeable that UGT1A9 has been involved in the metabolic elimination of many types of endogenous compounds (fatty acids) and xenobiotic compounds (phenols, primary amines, and flavones). The kinetic studies of Sal A glucuronidation by UGT1A1 and UGT1A9 revealed that these two isoforms had similar apparent intrinsic clearance (\(V_{\text{max}}/K_m\)) values, although the apparent intrinsic clearance value of UGT1A9 was higher than that of UGT1A1, which could be attributed to the relatively low \(K_m\) value of UGT1A9 (Table 1). Moreover, the \(K_m\) values in recombinant UGT1A9 and UGT1A1 were lower than those in HLMs and HIMs. It has been reported that long-chain unsaturated fatty acids could be considered as potent competitive inhibitors of UGT1A1, UGT1A6, and UGT1A9. In addition, correlation analysis and chemical inhibition study were applied to distinguish the relative contributions of the UGT isoforms. The high correlation between Sal A glucuronide formation and propofol O-glucuronidation is shown in Fig. 6B, and the inhibitory effects of phenylbutazone and mefenamic acid on HLMs and HIMs with similar IC\(_{50}\) values are listed in Table 2. Mefenamic acid is a known potent inhibitor of UGT1A9 and UGT2B7 and was considered as a specific inhibitor of UGT1A9 in this study, because UGT2B7 was not involved in Sal A glucuronidation.

The intrinsic clearance value in HIMs was about one-third of that in HLMs, which suggested that the intestine might not be the main glucuronidation metabolic site but the intestine could still contribute to presystemic elimination after oral administration of Sal A. Therefore, the liver might play an important role in Sal A metabolic clearance via glucuronidation. However, because no HLMs or HIMs were obtained from the same donor, we could not detect their exact contribution. Therefore, considering the intrinsic clearance of Sal A by glucuronidation, how to maintain the therapeutic plasma concentration of Sal A remains to be studied. In addition, the role of Sal A glucuronidation activity in the intestine needs to be further investigated.

In conclusion, Sal A is glucuronidated in the presence of UDPGA in vitro incubation systems. UGT1A1 and UGT1A9 are identified as the major isoforms responsible for Sal A glucuronidation.

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