Identification of the UGT isozyme involved in senecionine glucuronidation in human liver microsomes

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Running title: UGT1A4, the major isozyme involved in senecionine glucuronidation

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Abbreviations

HPA, hepatotoxic pyrrolizidine alkaloid; SEN, senecionine; DHPE, dehydrogenation pyrrolic ester; DHR, dehydrogenation retronecine; UDPGA, uridine 5'-diphosphate glucuronic acid; HLM, human liver microsome; UGT, UDP-glucuronosyl transferase; FMO, flavin-containing monooxygenase; UPLC, ultra performance liquid chromatography; MS, mass spectrometry; MS/MS, a tandem mass spectrometry consists of two sets of quadruple MS; Q-TOF, a quadruple MS coupled with a time of flight MS.
Abstract

Senecionine (SEN) is a representative of hepatotoxic pyrrolizidine alkaloids (HPAs). Although phase I metabolisms in particular for P450-mediated metabolic activation of SEN were investigated extensively, the phase II metabolisms in particular for glucuronidation of this compound have not been investigated until now. In our present study, one unique glucuronidation product of SEN in human liver microsomes (HLM) was identified as SEN N-glucuronide using an authentically synthesized product of which the structure was identified via $^1$H and $^{13}$C NMR analysis. Subsequently, kinetics indicated that SEN N-glucuronidation followed the typical Michaelis-Menten model and only one major isozyme participated in it. Finally, this isozyme was demonstrated to be UGT1A4, with the direct evidence that recombinant UGT1A4 exhibited the predominant and exclusive activity on SEN N-glucuronidation. This result was confirmed by other experiments including chemical inhibition by selective inhibitors and correlation study between activities of SEN N-glucuronidation and various UGT isozymes. The exclusive role of UGT1A4 on SEN N-glucuronidation was strengthened additionally by its inhibitory kinetic study in which the selective inhibitor of UGT1A4 showed the similar inhibition pattern and $K_i$ values in both HLM and recombinant UGT1A4 systems. As UGT2B10 activity failed to correlate with SEN N-glucuronidation in 10 HLMs, it became impossible that UGT2B10 played the important role on this metabolism.
Introduction

Hepatotoxic pyrrolizidine alkaloids (HPAs) widely distribute in high plants around the world (Roeder, 1995), and are highly toxic to wildlife (Hartmann, 2008), livestock (Nobre et al., 2005; Stegelmeier et al., 2009), experimental animals (Tang et al., 2007) and humans (Stegelmeier et al., 1999), by multiple pathways such as consumption of foods (Crews et al., 1997; Prakash et al., 1999) and traditional herbs (Roeder, 1995; Roeder, 2000) containing HPAs.

Generally, HPAs induce liver intoxication through the metabolic activation by liver P450 enzymes especially by CYP 3A4 to form reactive dehydrogenated pyrrolic ester (DHPE) (Couet et al., 1996) which can covalently conjugate with proteins and nucleic acids to evoke various toxicities such as cell necrosis, veno-occlusive diseases (VOD) and carcinogenicity (Fu et al., 2004). Whereas the detoxification pathways of HPAs were also observed such as non-enzymatic DHP-GSH conjugation (Lin et al., 2000), N-oxidation (Huan et al., 1998) catalyzed by flavin-containing monooxygenase (FMO) and hydrolysis catalyzed by esterase GPH1 (Dueker et al., 1992).

As the most important phase II drug metabolizing enzymes, UDP-glucuronosyl transferases (UGTs) play a very important role on the elimination of xenobiotic and endogenous substances and nearly half of known phase II metabolisms of top 200 prescribed drugs in United States are catalyzed by them (Williams et al., 2004). However whether UGTs participate in the metabolism of HPAs is still not clear.

In our preliminary experiment, senecionine (SEN, Figure 1) – a representative HPA isolated
from *Senecio vulgaris*, has undergone the glucuronidation by UGTs in human liver microsome (HLM). As a competitive metabolic pathway of P450-mediated metabolic activation, glucuronidation is promising to be a new detoxification pathway of SEN and the UGT isozyme(s) participating in SEN glucuronidation needs to be clarified.

The purpose of this study was to authenticate the conjugated site of SEN by glucuronic acid, and to identify the UGT isozyme(s) participating in SEN glucuronidation in HLM. Both recombinant UGT isozymes and the pooled HLM were used in the present study and multiple methods were adopted to examine the contribution of various UGT isozymes on SEN glucuronidation.
Methods

Chemicals and Reagents. Uridine 5′-diphosphate (UDPGA), alamethicin, azidothymidine (AZT), estradiol, propofol, trifluoperazine (TFP), serotonin, fluconazole, androsterone, phenylbutazone, hecogenin and cotinine were purchased from Sigma-Aldrich (St. Louis, MO, USA). SEN was isolated from Senecio vulgaris in our laboratory and identified via NMR and mass spectrometry data. The purity of SEN was more than 99 % as assayed based on peak area normalization by the high performance liquid chromatography (HPLC) coupled with a diode array detector and HPLC coupled with a mass spectrometer. Recombinant UGTs expressed in baculovirus-infected insect cells were purchased from BD Gentest Corp. (Woburn, MA, USA). Tris base and MgCl₂ were purchased from Majorbio biotech Corp., Ltd. (Shanghai, China). Pooled HLM (prepared from human livers of 13 donors, Mongoloid Race) were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). Experiments involving human objects followed the ethical approval of local governments and Helsinki Declaration. All other reagents were of HPLC grade or of the highest grade commercially available.

Chromatography parameters. An ultra performance liquid chromatography (UPLC) (Acquity, Waters, USA) system included an UPLC pump, a column temperature controller and an autosampler. A Waters BEH C₁₈ column (2.1 × 50 mm, 1.7 μM) was used for separation. Acetonitrile and 0.1 % formic acid were used as mobile phase A and B, respectively. Eluting gradient was set as follows: 0 – 1 min, 5 % of A; 1 – 2.5 min, 5 % - 17 % of A; 2.5 – 3 min, 17 % - 20 % of A; 3 – 4 min, 20 % - 35 % of A; 4 – 6 min, 35 % - 50 % of A. Flow rate was set at 0.3 mL/min and all mobile phase was introduced to mass spectrometry...
without split.

**Mass spectrometry parameters.** For qualitative determination of the glucuronidation metabolite of SEN, a tandem mass spectrometry system (Micromass, Waters, USA) consisting of a single quadrupole and a time of flight mass spectrometer (Q-TOF) was used to test the exact molecular weight and m/z values of secondary order mass (MS/MS) fragment ions. Positive scan mode was used and parameters of electronic spray ion source was set as follows: capillary voltage, 4.2 kV; cone voltage, 45 V; extractor voltage, 3 V; RF lens, 0; source temperature, 120 °C; desolvation temperature, 300 °C; cone and desolvation gas flow, 60 and 600 L/h, respectively. A mass scan method was used to test the exact molecular weight of the product, a daughter scan of the [M+H]⁺ ion of the metabolite was used to get its MS/MS spectrum.

For quantitative determination of SEN and its metabolite, a tandem mass system (Premier, Waters, USA) consisting of two sets of triple quadrupole mass spectrometer (TQ) was used. The parameters for the electronic spray ion source of the TQ were completely the same as that of the Q-TOF. Multiple reaction monitor methods were used to quantify the metabolite with ion transition from m/z 512 to 336.

**NMR analysis.** ¹H-NMR and ¹³C-NMR, and two-dimensional spectra of NOESY, HSQC and HMBC were obtained from a Bruker NMR analyzer (AV-400, Bruker, Newark, DE) at 400 or 100 MHz. Deuterated dimethyl sulfoxide (d-DMSO) was used as the solvent. The tetramethylsilane and the solvent were used as the internal standard for ¹H and ¹³C signals, respectively. Chemical shift change of ¹H and ¹³C in metabolite from that in SEN, and the
H-H correlation between the anomeric H and the substrate H in NOESY spectrum were used to determine the conjugated site.

**Standard incubation system in HLM.** A standard incubation system for UGT reaction included HLM (0.5 mg/mL), alamethicin (25 μg/mg protein), UDPGA (5 mM), MgCl₂ (4 mM), Tris-HCl (50 mM, pH 7.4) and SEN. Microsomes were pre-incubated with alamethicin for 5 min at 0 °C prior to incubation. Total incubation volume was 200 μL. Reaction was started by adding UDPGA at 37 °C for 30 min and terminated by adding ice bathed 10 % trichloroacetic acid (100 μL). The incubation mixtures were then centrifuged at 20,000 g and 4 °C for 10 min to obtain the supernatant. Aliquots (2 μL) were used for analysis. SEN solution was prepared as follows: dissolved the SEN in 5 % HCl first, and then adjusted the pH value of the solution to pH 7.4 using NaOH (1 M). A blank solution without SEN was prepared according to the preparation procedure of SEN solution. The activity of several major UGT isozymes including UGT 2B7, 1A1, 1A4, 1A6 and 1A9 were not inhibited or induced by the blank solution (Data not shown).

**Identification of glucuronidation product of SEN.** Three experimental groups were set to characterize the UDPGA-dependent product of SEN: a reaction group with all reaction factors described in the standard incubation system, a negative control without UDPGA and a blank control without SEN. The UDPGA-dependent product (detected in reaction group but not in negative control and blank control) was introduced to the Q-TOF to obtain its exact molecular weight and MS/MS spectrum.

The N-glucuronidation metabolite of SEN was synthesized followed an established method
(Luo et al., 1992) with the brief procedure as follow: protect all the hydroxyls of the glucuronic acid via acetylation and then substitute the anomeric acetyl-hydroxyl group with a bromine atom, the brom-acetyl-glucuronic acid was used to react with pure SEN to prepare the sugar-acetylated conjugation product. The SEN N-glucuronide was obtained after remove the protect group from the hydroxyl. The structure of synthesized product was identified by analyzing its high resolution mass spectrum, one-dimensional and two-dimensional $^1$H and $^{13}$C NMR spectra. Subsequently, the retain behavior of the synthesized product on C18 column and its mass spectrum were compared with that of the microsome-generated metabolite.

**Kinetics of SEN glucuronidation in HLM.** The kinetic experiment was performed in pooled HLM at a set of SEN concentrations from 50 to 1250 μM. Microsome protein concentration and reaction time were set at 0.5 mg/mL and 30 min to ensure the formation of product in the linear range according to the preliminary data. Other parameters for the incubation were completely the same as those in described standard incubation system. Substrate concentrations and reaction velocities were dealt with Eadie-Hofstee plots to determine the kinetic type. $V_{max}$ and $K_m$ values were obtained from a non-linear regression of substrate concentrations and reaction velocities from corresponding kinetic equation. Ratio of the $V_{max}$ and $K_m$ values was used to calculate the intrinsic clearance (CL$_{int}$) for evaluating the eliminating capacity of human liver on SEN via glucuronidation pathway.

**Chemical inhibition on SEN glucuronidation in HLM.** Fluconazole (Liu et al., 2008a), phenylbutazone (Uchaipichat et al., 2006a; Liu et al., 2008b), androsterone (Uchaipichat et al., 2006a; Liu et al., 2008b) and hecogenin (Uchaipichat et al., 2006a) were used to inhibit SEN
glucuronidation in HLM, respectively. A set of concentrations including 1, 10, 50 and 200 μM were for all of the four inhibitors. Concentration of SEN was set at 500 μM. The reaction time and protein concentration were set at 30 min and 0.5 mg/mL, respectively. All other reaction parameters were the same as those in the standard incubation system described above. The concentration of organic solvent dissolving inhibitors was less than 0.5 % of the total incubation volume. An experimental group without inhibitors but solvents was set as the solvent control, in which the activities of SEN glucuronidation were normalized to 100 %. The activities of SEN glucuronidation in the inhibited samples were compared with the solvent control to calculate the remaining enzyme activity (% activity remaining).

Hecogenin was used as the selective inhibitor of UGT 1A4 as its specific inhibitory effect on UGT 1A4 but not other UGT isozymes including UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 (Uchaipichat et al., 2006a). However, whether hecogenin can inhibit the activity of UGT 2B10 is still not clear. Thus, the inhibitory effect of hecogenin on UGT 2B10 was tested prior the formal experiments. As reported, cotinine was a potent substrate of UGT 2B10 (Chen et al., 2007), thus, cotinine glucuronidation was used as the probe reaction to determine the activity of UGT 2B10. In this experiment, hecogenin with concentrations of 1, 10, 50 and 200 μM were used to inhibit the glucuronidation of cotinine with system concentration of 5 mM closed to its K_m value (Chen et al., 2007). Inhibition of TFP glucuronidation by hecogenin was set as the positive control. Other incubation parameters, setting of the solvent control and the method for calculation of enzyme activity were identical to those described above.

Inhibitory kinetics of SEN N-glucuronidation by hecogenin in HLM and recombinant
UGT 1A4. Dilutions of hecogenin were used to inhibit SEN N-glucuronidation at a set of SEN concentrations from 60 to 1000 μM in HLM and recombinant UGT 1A4 incubation system. Hecogenin concentrations were set at 1, 5, 10 and 25 μM, respectively. One group without inhibitors but the same volume of solvent was used as control. Protein concentration was set at 0.5 mg/mL in HLM and 0.2 mg/mL in recombinant UGT 1A4 incubation system. The activity of UGT 1A4 in recombinant enzyme incubation system has been adjusted to be identical to that in HLM incubation system. Other incubation parameters were identical to those in the standard incubation system described above. Lineweaver-Burk plots were used to determine the inhibitory type. $K_m$ values were obtained by the nonlinear regression for kinetic data of the control group from the typical Michaelis-Menten equation and the $K_i$ values were obtained by the nonlinear regression from the corresponding equation (competitive, non-competitive, uncompetitive or mixed type inhibition).

Correlation study. 10 human liver individuals were adopted in the correlation study. SEN N-glucuronidation rates were compared with activities of various UGT isozymes in these 10 HLMs via linear regressions. The isozyme, of which the activities are highly correlated with that of SEN N-glucuronidation in 10 HLMs, would be assigned to be the most responsible participator. Assays of activities of UGT isozymes were conducted with AZT, TFP, estradiol, serotonin, propofol and cotinine glucuronidation as the probe reactions for UGT 2B7 (Liu et al., 2008a), 1A4 (Uchaipichat et al., 2006b), 1A1 (Hanioka et al., 2001), 1A6 (Hanioka et al., 2001), 1A9 (Soars et al., 2004) and 2B10 (Kaivosaari et al., 2007), respectively. As UGT 1A4 also slightly participates in cotinine N-glucuronidation, 10 μM of hecogenin was added to inhibit UGT 1A4 in the incubation system for determination of UGT 2B10 activity.
SEN N-glucuronidation by recombinant UGT isozymes. To confirm the results obtained from the chemical inhibition study and correlation study, the SEN N-glucuronidation was measured in recombinant UGT incubation system at 100, 500 and 1000 μM. 12 recombinant UGTs including UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 (0.2 mg protein/mL) were adopted in this experiment. All other incubation parameters such as bath temperature and reaction time were identical to those described in standard incubation system in HLM.

Data analysis. All results were obtained from three replicates in different microsomal incubations. Reaction velocities and remain enzyme activities are expressed as mean ± SD, and kinetic parameters are expressed as estimate ± SE. Reaction velocities were calculated based on the product formation. To estimate the apparent kinetic parameters, formation of metabolite versus SEN concentrations was fit to Michaelis-Menten enzyme kinetic model (equation 1). CL\text{int} was calculated based on the ratio of V\text{max} and K\text{m} values (equation 2). To obtain the K\text{i} value, reaction velocities versus substrate concentrations at various inhibitor concentrations were fit to the non-competitive inhibition type enzymatic kinetic model (equation 3). Both the fitting to equation 1 and 3 were performed via non-linear regression with Origin software (OriginLab Corporation, Northampton, MA).

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

\[ CL_{\text{int}} = \frac{V_{\text{max}}}{K_{\text{m}}} \]  

\[ V = \frac{[S]/V_{\text{max}}/(1+[I]/K_{\text{i}})}{K_{\text{m}} + [S]} \]
where $V_{\text{max}}$ was the maximum reaction velocity, $K_m$ was the Michaelis-Menten constant that represented the substrate concentration at which the velocity was half of the $V_{\text{max}}$, $V$ was the reaction velocity, $[S]$ was the substrate concentration, $[I]$ was the inhibitor concentration and $K_i$ was the inhibitory constant.
Results

Identification of metabolite structure. A unique UDPGA-dependent metabolite labeled as SEN-M (Figure 2, A) was detected after incubation of SEN with HLM. The Q-TOF gave an exact value for the [M+H]$^+$ ion of the metabolite at $m/z$ 512.2138 (Figure 2, B) which corresponded to that of SEN mono-glucuronide (calc. 512.2131). Subsequently, the neutral loss (176) of a glucuronic acid residue was observed from $m/z$ 512.2138 to 336.1806 in the MS/MS spectrum, additionally confirming the existence of a glucuronic acid residue. Thus, SEN-M was putatively deduced to be SEN-glucuronide.

To confirm the structure and the substitution position of glucuronic acid, the proposed metabolite was synthesized by following a reported method. NMR analysis were conducted to identify the structure of the synthesized product. The proton and carbon signals were assigned by $^1$H and $^{13}$C NMR, NOESY, HSQC and HMBC spectra and summarized in Table 1. Compared to SEN (Roeder, 1990), the large downfield shifts were observed for C-3 (4.07 ppm), C-5 (8.34 ppm) and C-8 (7.59 ppm) respectively, which indicated that the glucuronic acid substituted at N-4 position. The correlation between H-1’ (anomeric proton of glucuronic acid) and H-8 on the pyrrole cycle in NOESY experiment further confirmed the assignment (Figure 1). Analyzed by the UPLC-MS/MS, the synthesized product showed the unambiguously identical retain time and MS/MS spectrum to the microsome-generated product, proving the metabolite generated by HLM was SEN N-glucuronide (Figure 1).

Kinetic study of SEN N-glucuronidation in HLM. A straight trend line of the Eadie-Hofstee plots (Figure 3, B) indicated that SEN N-glucuronidation followed the typical
Michaelis-Menten model, and only one isozyme or more than one isozymes with the same $K_m$ value were involved in this metabolism. Non-linear regression of kinetic data to Michaelis-Menten equation showed a good correlation with a coefficient of 0.99, and gave $K_m$ and $V_{max}$ values of $(5.6 \pm 0.46) \times 10^2 \mu M$ and $2.1 \pm 0.080$ nmol/min/mg, respectively. The intrinsic clearance was calculated to be $3.7 \mu L/min/mg$.

**Chemical inhibition on SEN N-glucuronidation in HLM.** Four potent chemical inhibitors including fluconazole, androsterone, phenylbutazone and hecogenin for UGT isozymes were adopted to inhibit the SEN N-glucuronidation at concentrations of four levels (1, 10, 50 and 200 $\mu M$). As a result, hecogenin showed the most potent and concentration-dependent inhibitory effects (Figure 4) on SEN N-glucuronidation by more than 75% compared to the solvent control at only 10 $\mu M$. Fluconazole showed no inhibition while phenylbutazone and androsterone showed weak inhibition on SEN N-glucuronidation even at the highest concentration of 200 $\mu M$. The above result indicated that UGT 1A4 played the predominant role on SEN N-glucuronidation.

As a specific inhibitor of UGT 1A4 as previously reported (Uchaipichat et al., 2006a), hecogenin showed significant inhibitory effect on TFP glucuronidation (Figure 5). In contrast, no inhibitory effect on cotinine N-glucuronidation was observed for hecogenin. This result indicated that hecogenin did not inhibit UGT 2B10 and additionally demonstrated the selectivity of hecogenin on inhibition of UGT 1A4. Thus, the inhibition on SEN N-glucuronidation by hecogenin could be completely attributed to the inhibition on UGT 1A4. In addition, at the concentration of 200 $\mu M$, hecogenin showed strong inhibition on SEN N-glucuronidation by more than 80%. That meant, even if UGT 2B10 also participated in
SEN N-glucuronidation, its contribution rate would not exceed 20% of total glucuronidation.

**Inhibitory kinetics of SEN N-glucuronidation by hecogenin in HLM and recombinant UGT 1A4.** According to the Lineweaver-Burk plots, hecogenin showed non-competitive inhibition on SEN N-glucuronidation in both HLM and recombinant UGT 1A4 incubation system (Figure 6). Fitting the data to the enzymatic non-competitive inhibition equation (equation 3), the similar $K_i$ values of $3.3 \pm 0.37$ and $3.9 \pm 0.20 \, \mu M$ were given for hecogenin in HLM and recombinant enzyme incubation system, respectively. Fitting the data of the control group to the Michaelis-Menten equation, $K_m$ value of SEN N-glucuronidation in recombinant UGT 1A4 incubation system was calculated to be $(5.1 \pm 0.37) \times 10^2 \, \mu M$ via the non-linear regression. This $K_m$ value was consistent with that in HLM incubation system. All of these data for SEN N-glucuronidation showed high similarity in HLM and recombinant UGT 1A4.

**Correlation study.** As shown in Figure 7 (E), formation rates of SEN N-glucuronide correlated significantly with the activity of UGT1A4-mediated TFP glucuronidation ($r=0.92$, $P<0.05$). In contrast, SEN glucuronidation did not correlate with the activities of other UGT isozymes including UGT 2B10 (Figure 7, A, B, C, D and F) ($P>0.05$), which further demonstrated that UGT 1A4 plays the predominant role in SEN N-glucuronidation.

**SEN N-glucuronidation by recombinant UGTs.** Twelve recombinant UGTs including UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 were used to catalyze SEN N-glucuronidation to identify which isozymes participated in this metabolism. At all of the three SEN concentrations, UGT 1A4 showed the highest and predominant
catalytic activity (0.30, 1.0 and 1.4 nmol/min/mg at 100, 500 and 1000 μM of SEN, respectively) towards SEN N-glucuronidation (Figure 8). Besides UGT 1A4, UGT 1A3 also exhibited slight activity (3.5, 12, 17 pmol/min/mg at 100, 500 and 1000 μM of SEN, respectively). Other UGT isozymes investigated did not catalyze this reaction. This result indicated that UGT 1A4 is the most important isozyme involved in N-glucuronidation of SEN.
Discussion

The selective inhibitors for most UGT isozymes have not been identified as extensively as P450 isozymes (Bjornsson et al., 2003). Until now, only two selective inhibitors have been identified for UGT 2B7 (fluconazole) (Liu et al., 2008a) and UGT 1A4 (hecogenin) (Uchaipichat et al., 2006a) respectively. These two inhibitors were also applied in the present study and demonstrated that UGT 1A4 but not UGT 2B7 participated in the SEN N-glucuronidation (Figure 4). Besides these two selective inhibitors, other two potent inhibitors named androsterone and phenylbutazone were also used in the present study. Although they show significant inhibitory effects on several UGT isozymes (Uchaipichat et al., 2006a), their selectivity are rather low. However, the using of these two inhibitors also strengthened the purpose of the present study. Androsterone had been demonstrated to be a potent inhibitor for UGT 1A9, 2B7 and 2B15 (Uchaipichat et al., 2006a), however this inhibitor showed only slight inhibition on SEN glucuronidation even at a high concentration. Thus, these three isozymes may not be involved in glucuronidation metabolism of SEN. Androsterone also inhibited UGT 1A4 slightly (Uchaipichat et al., 2006a), which may be the reason for its slight inhibition on SEN glucuronidation. Phenylbutazone shows significant inhibition on UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10 and 2B15 (Uchaipichat et al., 2006a), while it can not inhibit the activity of UGT 1A4, suggesting that SEN N-glucuronidation may be not catalyzed by these isozymes.

Human UGT1 gene complex has been shown to encode at least 12 isozymes, which share the second through fifth exons but each of them has its own unique first exon (Green et al., 1998). The amino-terminal protein of UGT 1A4 is encoded by the fourth exon 1 of UGT1 gene
complex (Ritter et al., 1991). This isozyme shows activity on N-glucuronidation of primary,
secondary, tertiary and aromatic amine, and also shows activity on O-glucuronidation of
steroids sapogenins (King et al., 2000). Especially for N-glucuronidation of tertiary amine,
UGT 1A4 shows far higher activity than other isozymes such as UGT 1A3 (King et al., 2000).
This isozyme exists in several species such as humans, monkeys and rabbits, however, is not
expressed in rodent animals such as rats and mouse (Chiu and Huskey, 1998), as the fourth
exon 1 in UGT1 gene complex of them is a pseudogene (King et al., 2000). That means
rodent animals such as rats, mouse and guinea pigs maybe not suitable for further in vivo
study of SEN and other HPAs because their different metabolic network from humans.

UGT 1A3 is a UGT isozyme transcribed from UGT1 gene complex of human, the third exon
1 of which encoded its amino-terminal protein (Ritter et al., 1992). This isozyme is 93 %
identical with UGT 1A4 in primary amine acid sequence (King et al., 2000). Substrates of
UGT 1A4 such as steroid sapogenins and primary, secondary, tertiary and aromatic amines are
also catalyzed by UGT 1A3 (Green et al., 1998). However, the catalysis of these substrates by
UGT 1A3 are far less efficient than by UGT 1A4 (Green et al., 1998). In the present study,
both recombinant UGT 1A3 and 1A4 were adopted to catalyze the SEN glucuronidation. The
result showed that UGT 1A4 generated SEN N-glucuronide significantly but UGT 1A3
showed slight effect, which was consistent with the known activity of UGT 1A4 and 1A3 on
glucuronidation of tertiary amines in humans.

Besides UGT 1A4, UGT 2B10 is another UGT isozyme showing high efficiency on
N-glucuronidation of some compounds with tertiary amine groups especially for
tobacco-specific alkaloids such as nicotine and cotinine (Chen et al., 2007). This isozyme
received more and more attention in recent years. However, this isozyme may not be involved, or at least not be the major participator in SEN N-glucuronidation. At first, in the HLMs, SEN N-glucuronidation showed no correlation to UGT 2B10-mediated cotinine glucuronidation; second, hecogenin inhibited SEN N-glucuronidation by more than 80% at 200 μM, but showed no inhibitory effect at any concentrations on UGT 2B10, indicating that UGT 2B10 did not or only slightly participate in SEN N-glucuronidation; finally, kinetics of SEN N-glucuronidation showed a mono-phasic feature, indicating only one major isozyme participates in this metabolism. Thus, UGT 2B10 may not participated in the SEN N-glucuronidation, or at least participated in this metabolism with weak activity. Different types of tertiary amine groups on SEN and the tobacco-specific alkaloids may be used to explain why SEN was not significantly catalyzed by UGT 2B10. UGT 2B10 catalyzes the N-glucuronidation of nicotine and cotinine on their aromatic tertiary amine groups (Chen et al., 2008), however, this kind of structure is absent in molecule of SEN but only an aliphatic amine group exists on it. As reported, UGT 2B10 showed significantly high activity than UGT 1A4 on N-glucuronidation of aromatic tertiary amines (Kaivosaari et al., 2007), but lack of evidence to prove its efficient catalysis on N-glucuronidation of aliphatic tertiary amines. In the recent study (Kaivosaari et al., 2008), UGT 2B10 showed activity on the N-glucuronidation of the aliphatic tertiary amine group of dexmedetomidine, however, the efficiency was rather low. Thus, it is understandable that UGT 2B10 does not participate in SEN N-glucuronidation.

In our present study, the glucuronidation of SEN in HLM was investigated and the product was identified as SEN N-glucuronide by comparing with a synthesized product of which the
structure was identified via high resolution mass, one-dimensional and two-dimensional NMR analyses. In conclusion, the major isozyme involved in SEN N-glucuronidation is UGT 1A4. Besides, UGT 1A3 also showed slight effect. As the deficiency of UGT 1A4 in several animals such as rats, mouse and guinea pigs, these animals will not be suitable for metabolism and toxicology study of HPAs in vivo. Finally, considering the competition of this metabolic pathway with metabolic activation of SEN, it is essential to investigate whether this metabolism can decrease the SEN-induced toxicity in the further study
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Footnotes

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Legends to Figures:

**Figure 1.** Structures of SEN and its glucuronidation metabolite. The selected HMBC is used to assistant the assignment of $^1$H and $^{13}$C which are summarized in Table 1. The key NOESY correlation between H-1' and H-8 is the direct evidence to prove the conjugated site to be N-4.

**Figure 2.** Chromatograms (A) and high resolution mass spectrum (B) of SEN glucuronide. GluA represents glucuronic acid residue. The differences between the exactly tested and the calculated m/z values are less than 2 ppm for [M+H]$^+$ and [M+H-GluA]$^+$ ions.

**Figure 3.** Michaelis-Menten (A) and Eadie-Hofstee (B) plots of SEN N-glucuronidation in human liver microsome incubations. SEN (50 – 1250 μM) was incubated with HLMs (0.5 mg/ml) at 37 °C for 30 min with UDPGA. “m” in the x axis represents “10$^3$”. Data was obtained from three replicates.

**Figure 4.** Chemical inhibition of SEN N-glucuronidation at SEN concentration of 500 μM by four potent inhibitors (1 – 200 μM) including fluconazole, hecogenin, phenylbutazone and androsterone in human liver microsome (0.5 mg/mL) incubations. The incubations without inhibitors but the same volume of solvent were set as the solvent control in which the activity of SEN N-glucuronidation was normalized to 100 %. Data was obtained from three replicates.

**Figure 5.** Chemical inhibition of cotinine glucuronidation and TFP glucuronidation by hecogenin (0 – 200 μM) in human liver microsome incubations. The incubations of cotinine and TFP glucuronidation without hecogenin (0 μM) were set to be the controls of which the metabolic activities were normalized to 100%. Inhibition of TFP glucuronidation by hecogenin was the positive control for that of cotinine glucuronidation. Data was obtained from three replicates.
Figure 6. Lineweaver-Burk plots of inhibitory kinetic data of hecogenin on glucuronidation of SEN (60 – 1000 μM) in human liver microsome (A, 0.5 mg/mL) and recombinant UGT 1A4 (B, 0.2 mg/mL) incubations. Preliminary experiments indicated that the activity of human liver microsome on SEN N-glucuronidation at protein concentrations of 0.5 mg/mL was equivalent to that of recombinant UGT 1A4 at protein concentrations of 0.2 mg/mL. Data was obtained from three replicates.

Figure 7. Correlation study between SEN N-glucuronidation and 6 UGT isozymes in 10 HLM individuals. Activities of UGT 1A1 (A), 1A6 (B), 1A9 (C), 2B7 (D), 1A4 (E) and 2B10 (F) were characterized by estradiol, serotonin, propofol, AZT, TFP and cotinine glucuronidation. Correlation was performed via linear regressions from activity of SEN N-glucuronidation and these UGT isozymes mentioned here.

Figure 8. Assays of glucuronidation activities of SEN (100, 500 and 1000 μM) catalyzed by various recombinant UGT isozyme. Data was obtained from three replicates.
Table 1. $^1$H (400 MHz) and $^{13}$C(100 MHz) NMR data of SEN and its glucuronidation metabolite

<table>
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<th>Position</th>
<th>$^1$H of SEN</th>
<th>$^1$H of Metabolite</th>
<th>$^{13}$C of SEN</th>
<th>$^{13}$C of Metabolite</th>
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1 Reported by Roeder et. al. (Roeder, 1990)
2 Tested by the present study. Assignment confirmed by HSQC, HMBC and NOESY
3 Chemical shift values expressed as ppm
4 Coupling constant expressed as Hz
5 s, singlet; d, doublet; dd, double doublet; t, triplet; tt, triple triplet; q, quadruplet; br s, broad singlet; m, multiplet.
Figure 1

Selected HMBC \[ H \rightarrow C \]

Key NOESY \[ H \leftrightarrow H \]

SEN N-glucuronide
Figure 2

A

Time (min)

B

$336.1806$

$[\text{M+H-GluA}^+]$

$[\text{M+H}^+]$

$512.2138$

$m/z$
Figure 4

The figure shows a bar graph representing the percentage activity remaining for different compounds across various concentrations. The x-axis represents different compounds: Fluconazole, Hecogenin, Phenylbutazone, and Androsterone. The y-axis represents the percentage activity remaining, ranging from 0% to 120%. The bars are color-coded to represent different concentrations: Solvent control, 1 µM, 10 µM, 50 µM, and 200 µM. The graph visually compares the activity remaining across these compounds and concentrations.
Figure 6
Figure 7

A. UGT 1A4 Activity vs. SEN N-glucuronidation (R = 0.37)

B. UGT 1A6 Activity vs. SEN N-glucuronidation (R = 0.45)

C. UGT 1A8 Activity vs. SEN N-glucuronidation (R = 0.17)

D. UGT 2B7 Activity vs. SEN N-glucuronidation (R = 0.61)

E. UGT 1A4 Activity vs. SEN N-glucuronidation (R = 0.92)

F. UGT 2B10 Activity vs. SEN N-glucuronidation (R = 0.34)