1521-009X/41/8/1529–1537\$25.00 DRUG METABOLISM AND DISPOSITION Copyright © 2013 by The American Society for Pharmacology and Experimental Therapeutics

Glucuronidation of a Sarpogrelate Active Metabolite Is Mediated by UDP-Glucuronosyltransferases 1A4, 1A9, and 2B4^S

Hyo-Ji Kim, Eun Sook Jeong, Kyung-Ah Seo, Kye Jung Shin, Yeon Jae Choi, Su-Jun Lee, Jong Lyul Ghim, Dong-Ryul Sohn, Jae-Gook Shin, and Dong-Hyun Kim

Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, Busan, South Korea (H.-J.K., E.S.J., K.-A.S., S.-J.L., J.L.G., J.-G.S., and D.-H.K.); Integrated Research Institute of Pharmaceutical Sciences, College of Pharmacy, The Catholic University of Korea, Bucheon, South Korea (K.J.S. and Y.J.C.); and Department of Clinical Pharmacology, College of Medicine, Soonchunhyang University, Chonan, South Korea (D.-R.S.)

Received March 11, 2013; accepted May 23, 2013

ABSTRACT

Sarpogrelate is a selective serotonin 5-HT_{2A}-receptor antagonist used to treat patients with peripheral arterial disease. This drug is rapidly hydrolyzed to its main metabolite (R, S)-1-[2-[2-(3methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propanol (M-1), which is mainly excreted as a glucuronide conjugate. Sarpogrelate was also directly glucuronidated to an *O*-acyl glucuronide and a *N*glucuronide by UDP-glucuronosyltransferases (UGTs) in human liver microsomes (HLMs). Since M-1 is pharmacologically more active than sarpogrelate, we examined glucuronidation of this metabolite in HLMs and characterized the UGTs responsible for M-1 glucuronidation. Diastereomers of *O*-glucuronide (SMG1 and SMG3) and a *N*-glucuronide (SMG2) were identified by incubation of M-1 with HLMs in the presence of uridine 5'-diphosphoglucuronic acid (UDPGA), and their structures were confirmed by nuclear magnetic resonance and mass spectrometry analyses. Two

Introduction

Glucuronidation is one of the major phase II conjugation reactions catalyzed by a family of uridine 5'-diphospho-(UDP)glucuronosyltransferases (UGTs), which are expressed primarily in the liver and in extrahepatic tissues such as the intestine and kidney (Liston et al., 2001). UGTs are involved in the metabolism of a large number of drugs and endogenous substances by transferring glucuronic acid from its cofactor uridine 5'-diphosphoglucuronic acid (UDPGA) to substrates, thereby transforming them into hydrophilic glucuronides, which can be eliminated easily via biliary and renal routes (Guillemette, 2003).

Sarpogrelate, (R,S)-1-[2-[2-(3-methoxyphenyl)ethyl] phenoxy]-3 (dimethylamino)-2-propyl hydrogen succinate, is a competitive 5hydroxytryptamine (5-HT) receptor antagonist that inhibits responses to 5-HT mediated by 5-HT_{2A} receptors, including platelet aggregation and vasoconstriction (Hara et al., 1991; Rashid et al., 2003; Nishihira et al., 2006). This drug has been used to treat patients with peripheral

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST) [No. R13-2007-023-00000-0]. dx.doi.org/10.1124/dmd.113.051862.

S This article has supplemental material available at dmd.aspetjournals.org.

O-glucuronides were identified as chiral isomers: SMG1 as *R*-isomer and SMG3 as S-isomer. Using recombinant UGT enzymes, we determined that SMG1 and SMG3 were predominantly catalyzed by UGT1A9 and UGT2B4, respectively, whereas SMG2 was generated by UGT1A4. In addition, significant correlations were noted between the SMG1 formation rate and propofol glucuronidation (a marker reaction of UGT1A9; r = 0.6269, P < 0.0031), and between the SMG2 formation rate and trifluoperazine glucuronidation (a marker reaction of UGT1A4; r = 0.6623, P < 0.0015) in a panel of HLMs. Inhibition of SMG1, SMG2, and SMG3 formation by niflumic acid, hecogenin, and fluconazole further substantiated the involvement of UGT1A9, UGT1A4, and UGT2B4, respectively. These findings collectively indicate that UGT1A4, UGT1A9, and UGT2B4 are the major UGT isoforms responsible for glucuronidation of M-1, an active metabolite of sarpogrelate.

Downloaded from dmd.aspetjournals.org at ASPET Journals on March 7, 2015

arterial disease (Furukawa et al., 1991) and is equivalent to aspirin in preventing recurrence of cerebral infarction with significantly fewer bleeding events (Shinohara et al., 2008). Sarpogrelate is rapidly metabolized to its active metabolite (M-1) via enzymatic hydrolysis (Saini et al., 2004) and disappears from the plasma more rapidly in comparison with the duration of its pharmacological effect. Although plasma concentrations of M-1 are <1/10 of those of sarpogrelate, pharmacokinetic-pharmacodynamic modeling has demonstrated that M-1 is a more effective inhibitor of platelet aggregation than sarpogrelate (Shimizu et al., 1999). Sarpogrelate and its metabolites are primarily excreted into the bile as glucuronide conjugates, suggesting that glucuronidation is a critical step governing overall clearance of sarpogrelate and M-1 in humans. However, the isoforms involved in the glucuronidation of these compounds have not yet been characterized.

In this study, we identified the human UGTs responsible for the metabolism of sarpogrelate and M-1 into their glucuronide metabolites using in vitro systems. The structures of metabolites were tentatively characterized on the basis of mass and nuclear magnetic resonance (NMR) spectrometry, and metabolic screening was performed with a battery of recombinant human UGTs to identify the specific UGT isoforms involved in *N*- and *O*-glucuronidation. Kinetic enzymatic

ABBREVIATIONS: HLM, human liver microsome; HPLC, high-performance liquid chromatography; M-1, (R,S)-1-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propanol; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; RLM, rat liver microsomes; SG, sarpogrelate glucuronide; SMG, M-1 glucuronide; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase. studies were performed with human liver microsomes (HLMs) and with those UGT enzymes that catalyze glucuronidation of sarpogrelate active metabolites. Inhibition of UGT-glucuronidation activity and correlations with known marker activities in HLMs were determined using known high-affinity UGT substrates to facilitate identification of the UGT isoforms involved in M-1 glucuronidation. In addition, the feasibility of using M-1 as an isoform-specific probe substrate was evaluated because UGT isoform-specific substrates are not well characterized.

Materials and Methods

Chemicals and Reagents. Racemic sarpogrelate and racemic M-1 (Fig. 1) were kindly provided by Yuyu Pharma (Seoul, Korea). Alamethicin from *Trichoderma viride*, UDPGA, niflumic acid, hecogenin, propofol, and trifluoperzine were purchased from Sigma-Aldrich (St. Louis, MO). Fluconazole was obtained from DaeWoo Pharma (Seoul, Korea). Recombinant human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) were purchased from BD Gentest (Woburn, MA). All solvents were of high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Pittsburgh, PA).

Synthesis of M-1 Enantiomers. (S)-M-1 was prepared using the following procedure. 2-[2-(3-Methoxyphenyl)ethyl]phenol (200 mg, 0.876 mmol) was dissolved in 5 ml of ethanol and NaOH (42 mg, 1.051 mmol) was added. After stirring for 20 minutes at room temperature, (R)-epichlorohydrin (0.2 ml, 2.628 mmol) was added dropwise. The resulting mixture was stirred for 9 hours, solvent was evaporated, and then the residue was dissolved in ethylacetate. The resulting solution was washed with 1 N NaOH, dried over MgSO4, and concentrated in vacuo. The residue was purified by column chromatography (n-hexane/ethylacetate = 10: 1) to afford (S)-2-[2-(2-(3-methoxy)phenylethyl)]phenoxy]methyloxirane (190 mg). This compound (34 mg, 0.119 mmol) was dissolved in acetonitrile. LiClO₄ (19.0 mg, 0.179 mmol) and dimethylamine hydrochloride (14.6 mg, 0.179 mmol) were added gradually and the reaction mixture was heated for 20 hours at 65°C. After solvent evaporation, the residue was extracted with ethylacetate, washed with saturated NaHCO₃, dried over MgSO₄, and concentrated. The residue was purified by column chromatography (ethylacetate) to afford 15 mg (38%) (S)-1-[2-[2-(3methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propanol [(S)-M-1]. (*R*)-M-1 was also prepared using the same procedure except for the use of (S)-epichlorohydrin.

Preparation of Microsomes. HLMs were prepared from human liver tissues (20 different donors) from patients undergoing partial hepatectomy for removal of metastatic tumors at the Department of General Surgery, Busan Paik Hospital (Busan, Korea). The samples were of nontumor-bearing parenchymal tissue and were confirmed to be histopathologically normal. The liver tissues and their clinical information were obtained from Inje Biobank at the Inje University Busan Paik Hospital and were in accordance with the policies of the institutional review board of the hospital. For the preparation of rat liver microsomes, male Sprague-Dawley rats (6-7 weeks of age, 200-220 g body weight) were obtained from Orient Bio (Seongnam, Korea) and were acclimated for one week. The animal quarters were maintained at 23 \pm 3°C and at 50% \pm 10% relative humidity. A 12-hour light and dark cycle was used with an intensity of 150-300 Lux. The use of animals was approved by institutional animal care and use committees at the Inje University School of Medicine. HLMs were individually prepared by differential centrifugation of the liver homogenate, as described previously (Shin et al., 1999). Ten different HLMs were pooled and used for the experiments. In the case of rats, liver tissues from six rats were pooled for the preparation of liver microsomes.

Glucuronidation of Sarpogrelate and M-1 in HLMs or Recombinant UGT Isoforms. The incubation mixtures consisted of 0.25 mg/ml HLMs or recombinant UGTs, 25 μ g/ml alamethicin, Tris-HCl buffer (50 mM, pH 7.5), 10 mM MgCl₂, and substrate (sarpogrelate or M-1) in a total volume of 125 μ l. The final volume of the organic solvents in the incubation mixture was 1% (v/v). After preincubation on ice for 15 minutes, the reaction was initiated by the addition of 5 mM UDPGA and incubated for 20 minutes at 37°C in a shaking water bath. The reaction was terminated by the addition of 125- μ l acetonitrile containing 10 μ M 7-hydroxycoumarin glucuronide as an internal standard and centrifuged at 10,000g for 5 minutes at 4°C. Aliquots of the supernatant were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of glucuronide conjugates of sarpogrelate and M-1.

LC-MS/MS Analysis of Sarpogrelate and M-1 Glucuronides. LC-MS/ MS was performed on an API 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA), coupled with an 1100 series HPLC system (Agilent, Santa Clara, CA). The separation was performed on a Synergi Fusion-RP 80A column (150 \times 2.0 mm, 4 μ m; Phenomenex, Torrance, CA) using a mobile phase of acetonitrile and 5 mM ammonium formate (pH 3.0) (40:60, v/v) at a flow rate of 0.2 ml/min. Electrospray ionization (ESI) was performed in the



Fig. 1. Postulated in vitro metabolic pathways of sarpogrelate and its active metabolite M-1 in human liver microsomes. An asterisk denotes a chiral carbon.

positive ion mode with nitrogen as the nebulizing, turbo spray, and curtain gases with the optimum values set at 50, 50, and 25 (arbitrary units). The turbo ion spray interface was operated at 5000 V and 500°C. The multiple-reaction monitoring mode using specific precursor/product ion transitions was employed for quantification. Detection of the ions was performed by monitoring the m/z transitions of $606\rightarrow430$ for sarpogrelate glucuronide (SG), $506\rightarrow330$ for M-1 glucuronide (SMG), $339\rightarrow163$ for the internal standard, 7-hydroxycoumarin glucuronide. The collision energies were 30, 40, and 20 eV for SGs, SMGs, and the internal standard, respectively. Peak areas for all compounds were integrated automatically using the Analyst software (v. 1.3; Applied Biosystems).

Isolation and NMR Analysis of M-1 Glucuronides. M-1 glucuronides were isolated from large microsomal incubations for NMR analysis. The incubation mixture (total volume 80 ml) consisted of 50 mM Tris-HCl buffer (pH 7.5), 25 µg/ml alamethicin, 10 mM MgCl₂, 200 µM M-1, and 0.5 mg/ml HLMs. After preincubation for 15 minutes, the reaction was initiated by the addition of 5 mM UDPGA and incubated for 2 hours at 37°C. The reaction mixtures (2 ml each) were then loaded through preactivated Sep-Pac C18 cartridges (Waters, Milford, MA). Each cartridge was washed with 1 ml distilled water and M-1 glucuronides were eluted with 1 ml methanol. The final methanol eluents were pooled and evaporated under a N2 evaporator. The residue was then reconstituted in 50% acetonitrile. M-1 glucuronides were separated by an HPLC column (Symmetry C8, 150×4.6 mm, 5 μ m; Waters) using a mobile phase of 25% acetonitrile containing 0.1% formic acid at a flow rate of 1.2 ml/min. The fractions containing M-1 glucuronides were collected and the organic solvent was evaporated under nitrogen stream. The remaining fractions were lyophilized to obtain white powder. The final amounts of M-1 glucuronides obtained after purification were 0.5 mg of SMG1, 0.18 mg of SMG2, and 1.06 mg of SMG3. Overall M-1 glucuronide yield was 21.5% after purification. The ¹H-NMR spectra were acquired using a JNM ECP 400-MHz spectrometer (JEOL, Tokyo, Japan) with CD₃OD as the solvent. Chemical shifts are expressed in parts per million downfield from tetramethylsilane.

Characterization of Hydrolysis Products of Sarpogrelate Glucuronides. To characterize the hydrolysis products of sarpogrelate glucuronides, two SGs were first isolated using a Synergi Fusion-RP 80A column (150 mm \times 2.0 mm, i.d., 4 μ m; Phenomenex). The mobile phase consisted of eluent (A), 0.1% formic acid, and eluent (B), acetonitrile containing 0.1% formic acid at a flow rate of 0.2 ml/min. The gradient program (B) was as follows: 30% (0–4 minutes), 38% (4–9 minutes), 30% (9–10 minutes), 30% (10–13 minutes). The peaks were monitored by UV detector at 254 nm. The fractions containing SG1 or SG2 were collected, dried, and reconstituted in 500 mM Tris-HCl buffer (pH 7.5). The samples were then kept at room temperature for 48 hours and analyzed by LC-MS/MS.

Chemical Inhibition. The inhibitory effects of known UGT isoformselective inhibitors on the formation of M-1 glucuronides were evaluated to identify the UGT isoforms involved in the metabolic pathway. Inhibitors used in this study were as follows: hecogenin (1 and 10 μ M) for UGT1A4, niflumic acid (0.1 and 1 μ M) for UGT1A9, and fluconazole (1 and 3 mM) for UGT2B4 and 2B7. The formation rates of M-1 glucuronides from M-1 (50 μ M) were determined from reaction mixtures incubated in the presence or absence of inhibitors. These inhibitors included niflumic acid for UGT1A9, hecogenin for UGT1A4, and fluconazole for UGT2B4/2B7. With the exception of adding UGT-isoform-specific inhibitors, all other incubation conditions were similar to those described above.

Correlation Experiments. M-1 (50 μ M) was incubated with microsomes from 20 different human livers to examine the formation of SMG1, SMG2, and SMG3 from M-1, relative to specific UGT isoform activity. The activity of each UGT isoform was assessed by LC-MS/MS as described previously for propofol glucuronidation (UGT1A9) (Court, 2005) and trifluoperazine glucuronidation (UGT1A4) (Uchaipichat et al., 2006a) with slight modifications; 10 μ M concentrations were used for both compounds and the incubation time was 30 minutes. The correlation coefficients between the formation rates of M-1 glucuronides and the activities of each UGT isoform in a panel of 20 HLMs were calculated by Spearman's rank correlation coefficient analysis using the



Fig. 2. LC-MS/MS chromatograms of sarpogrelate glucuronides (A, B) and M-1 glucuronides (C, D) after incubation with rat liver microsomes (RLM) and human liver microsomes (HLM) in the presence of UDPGA. Chromatograms for parent sarpogrelate and M-1 obtained after microsomal incubation are shown as an inserted chromatogram.

SAS software (SAS Institute, Cary, NC). P values <0.05 were considered to be statistically significant.

Data Analysis. In the microsomal incubation studies, the apparent kinetic parameters of M-1 biotransformation ($K_{\rm m}$ and $V_{\rm max}$) were determined by fitting a one-enzyme Michaelis–Menten equation or a Hill equation. The calculated parameters included the maximum rate of formation ($V_{\rm max}$), the Michaelis–Menten constant (apparent $K_{\rm m}$), and the intrinsic clearance ($Cl_{\rm int} = V_{\rm max}/$ apparent $K_{\rm m}$). Calculations were performed using the WinNonlin software (Pharsight, Mountain View, CA). The percentages of inhibition were calculated by means of the ratio of the quantities of metabolites formed in the absence and presence of the appropriate specific inhibitor.

Results

Glucuronidation of Sarpogrelate and M-1 in Human and Rat Liver Microsomes. Two metabolites (SG1 and SG2) were generated after incubation of sarpogrelate with HLMs in the presence of UDPGA, whereas one metabolite (SG1) was generated with rat liver microsomes (RLMs) (Fig. 2). These metabolites were identified as glucuronide conjugates by full-scan mass analysis, which showed the addition of a 176-Da glucuronic acid to the parent sarpogrelate (m/z 430). Incubation of M-1 with HLMs in the presence of UDPGA resulted in the formation of three metabolites (SMG1, SMG2, and SMG3). However, only two metabolites (SMG1 and SMG3) were identified when M-1 was incubated with RLMs (Fig. 2). M-1 metabolites were also confirmed as glucuronide conjugates as judged by addition of 176 Da to the parent M-1 (m/z 506). The subsequent product ion mass spectra of the metabolites generated identical fragment ion patterns, which showed the major fragment ion at m/z 330, indicating loss of a glucuronide moiety (Supplemental Fig. 1).

Structure of M-1 Glucuronides. SMG1, SMG2, and SMG3 were isolated and their structures determined by comparing their proton NMR spectra to that of M-1 (Fig. 3). SMG1 and SMG3 were tentatively



Fig. 3. ¹H NMR spectra of M-1 (A) and its glucuronide metabolites, SMG1 (B), SMG2 (C), and SMG3 (D) in CD₃Cl.

assigned as O-glucuronides and SMG2 was postulated to be a quaternary *N*-glucuronide. The ¹H chemical shifts of the anomeric proton of SMG1 and SMG3 were 4.45 and 4.64 ppm, respectively. The ¹H chemical shifts of the anomeric proton of SMG2 were farther downfield at 4.89 ppm, as evidenced by other quaternary N-glucuronides (Alonen et al., 2008; Zhu et al., 2008). The racemic mixture of M-1 and the similarity of ¹H anomeric proton chemical shifts of SMG1 and SMG3 suggest that these compounds are diastereomers of O-glucuronide. To assign the chirality of SMG1 and SMG3, an LC-MS/MS analysis was performed after incubation of individual enantiomer of M-1 with HLMs. The SMG1 peak was only shown in (R)-M-1, whereas the SMG3 peak was observed in (S)-M-1, suggesting that SMG1 was (R)-M-1 O-glucuronide and SMG3 was (S)-M-1 O-glucuronide (Fig. 4). Sarpogrelate can be enzymatically transformed to M-1 by ester hydrolysis of succinic ester. Furthermore, sarpogrelate can be spontaneously hydrolyzed to M-1 in the aqueous phase (Kamatsu et al., 1992). To further characterize the nature of SMG2, the hydrolysis products of sarpogrelate glucuronides were characterized. It is reasonable to assume that N-glucuronide of M-1 can be generated only from a sarpogrelate N-glucuronide via hydrolysis of the succinic ester. HPLC fractions containing SG1 and SG2 were left



Fig. 4. LC-MS/MS chromatograms of M-1 glucuronides after incubation of racemic M-1 (A), *R*-enantiomer (B), and *S*-enantiomer (C) of M-1 with human liver microsomes in the presence of UDPGA.

at room temperature for 48 hours and reinjected into an HPLC column. Only SMG2 was detected after partial hydrolysis of SG2, whereas SMG1 and SMG3 were not detected in the hydrolysis products of SG1 or SG2 (Supplemental Fig. 2).

Identification of UGT Isoforms Involved in Glucuronidation of M-1. The relative activities of 12 recombinant human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) expressed in insect cells were determined in terms of the formation of M-1 glucuronides at 15 and 150 μ M. The formation rates of M-1 glucuronides after incubation of M-1 with UGT isoforms are shown in Fig. 5. SMG1 formation was catalyzed primarily by UGT1A9 with minor contributions by UGT2B4 and 2B7, whereas SMG3 formation was mediated predominantly by UGT2B4 and 2B7. In contrast, N-glucuronide SMG2 was generated only by UGT1A4. The formation of SMG1, SMG2, and SMG3 from M-1 was assessed using UGT-isoform-selective inhibitors or substrates. SMG1 formation was inhibited by the UGT1A9 inhibitor niflumic acid, while formation of SMG2 and SMG3 were not affected (Fig. 6). The formation of SMG2 was preferentially inhibited by hecogenin, a known UGT1A4 inhibitor, whereas SMG3 formation was inhibited by fluconazole, a known inhibitor of UGT2B4 and 2B7. Correlation analyses with activities of known marker UGT isoforms were performed to confirm the involvement of UGT1A4 and 1A9 in the formation of M-1 glucuronides. As shown in Fig. 7, significant correlations were found between SMG1 formation rates and UGT1A9-mediated propofol glucuronidation (r = 0.6269, P < 0.0031) and between SMG2 formation rates and UGT1A4-medated trifluoperazine glucuronidation (r = 0.6623, P < 0.0015).

Enzyme Kinetic Analysis. Kinetic analyses of formation of M-1 glucuronides were performed with pooled HLMs using 10–1000 μ M M-1 (Fig. 8A). The formation of M-1 glucuronides by the HLMs and recombinant UGT isoforms was fitted by Michaelis-Menten oneenzyme model. Fitting of the data to a two-enzyme model did not significantly improve the regression compared with fitting of the data to one-enzyme model. The kinetic parameters estimated from the pooled HLMs and recombinant UGT isoforms are shown in Table 1. Intrinsic clearance (Clint) of SMG1, SMG2, and SMG3 in HLMs were 4.80, 1.16, and 8.72 ml/min•mg protein, respectively. Substrate inhibition of O-glucuronides (SMG1 and SMG 3) formation was noted at the highest concentration (1000 μ M) used. Next, the kinetic parameters of formation of M-1 glucuronides were determined using 10-1000 μ M M-1 after incubation with recombinant UGT1A4, 1A9, 2B4, and 2B7 (Fig. 8, B-D). UGT1A9-mediated formation of SMG1 showed 17-fold higher V_{max} than did UGT2B4-mediated formation of SMG1. The Clint of SMG1 by UGT1A9 was 3-fold greater than that by UGT2B4. However, the Clint of SMG3 by UGT2B4 was similar to that of SMG1 formation by UGT2B4. Based on the Clint data, UGT2B7 was considered to make a greater contribution to SMG3 formation did than UGT2B4.

Discussion

The glucuronidation of sarpogrelate and its active metabolite M-1 is an important part of sarpogrelate disposition in humans. In experimental rats, the majority of circulating M-1 in the plasma after oral administration was determined to be glucuronide conjugates (Takada et al., 1997). In addition, metabolic analysis demonstrated that \sim 76% of M-1 was present as a glucuronide, whereas only 24% was present as free M-1 in bile from rats treated with sarpogrelate. Our results also suggest M-1 glucuronidation to be more efficient than that of sarpogrelate. Intrinsic clearance rates of M-1 *O*-glucuronides (SMG1 and SMG3) and *N*glucuronide (SMG2) were 10-fold higher than those of the sarpogrelate



Recombinant UGTs

Fig. 5. Formation of the metabolites SMG1 (A), SMG2 (B), and SMG3 (C) by recombinant human UGT isoforms. M-1 at two different concentrations of 15 (\Box) and 150 μ M (\blacksquare) was incubated with each recombinant UGT isoform (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) at a protein concentration of 0.25 mg/ml, and other conditions are the same with HLM. Each bar represents the mean \pm S.D. of triplicate determinations.

O-glucuronide (SG1) and *N*-glucuronide (SG2) in HLMs. Understanding the nature of M-1 glucuronidation is of interest because M-1 is known to be more pharmacologically active than its parent compound sarpogrelate.

In the present investigation, we demonstrated that sarpogrelate is metabolized to O-glucuronide (SG1) and N-glucuronide (SG2), whereas M-1 is transformed into two O-glucuronides (SMG1 and SMG3) and a N-glucuronide in the presence of UDPGA in HLMs (Fig. 1). The structures of M-1 glucuronides were characterized by mass and ¹H-NMR spectroscopy. No structural information could be obtained from product ion mass spectra of M-1 glucuronides because all were almost identical. NMR spectra showed that the ¹H chemical shifts of the anomeric proton of SMG1 and SMG3 were similar (4.45 and 4.64 ppm), whereas that of SMG2 was farther downfield, at 4.89 ppm. In general, the chemical shifts of anomeric protons in Oglucuronides were 4.4-4.6 ppm, whereas those in N-glucuronides were 5.1-5.5 ppm (Alonen et al., 2008; Zhu et al., 2008; Pallmann et al., 2010). Accordingly, SMG1 and SMG3 were tentatively assigned as O-glucuronides and SMG2 as a quaternary ammonium Nglucuronide of M-1. The structure of M-1 suggested that Oglucuronidation can occur only at the hydroxyl group located on the dimethylamino-2-hydroxy-propyl moiety. Considering the racemic mixture of sarpogrelate and M-1, SMG1 and SMG3 were postulated to be diastereomers of M-1 O-glucuronide. The stereochemistry of M-1 O-glucuronides (SMG1 and SMG3) was confirmed by the incubation of individual enantiomer of M-1 with HLMs. SMG1 was identified to be (R)-M-1 O-glucuronide and SMG3 was characterized as (S)-M-1

O-glucuronide. The structure of SMG2 was further supported by species differences in the formation of SMG2; SMG2 was generated only by HLMs and not by RLMs (Fig. 2). The formation of quaternary ammonium glucuronides from tertiary amines is a reaction largely restricted to humans and generally not observed in animal species, including rats, mice, dogs, and monkeys (Soars et al., 2001; Kaku et al., 2004; Kaivosaari et al., 2011). The structure of sarpogrelate glucuronides was confirmed by analysis of their hydrolysis products. Sarpogrelate can be hydrolyzed at the ester moiety in its chemical structure, leading to formation of M-1. The conversion of SG1 to M-1 in an aqueous solution indicated that SG1 was sarpogrelate Oglucuronide and the hydrolysis of the succinic glucuronide moiety from SG1 resulted in the formation of M-1. However, SG2 was hydrolyzed to SMG2 but not to M-1, suggesting that SG2 was a quaternary ammonium glucuronide of sarpogrelate and that a glucuronide moiety remained after the hydrolysis of succinic ester, leading to the formation of the N-glucuronide of M-1. The structure of SMG2 was further supported by the fact that SG2 formation occurred in HLMs but not in RMLs.

Identification of UGT isoforms responsible for the formation of M-1 glucuronides was demonstrated with several approaches. First, incubation with 12 recombinant human UGT isoforms showed that only UGT1A4 catalyzed *N*-glucuronidation of M-1. UGT1A4 is the major isoform involved in the glucuronidation of tertiary amines, including 1'-hydroxymidazolam, imipramine, and tamoxifen (Green and Tephly, 1996; King et al., 2000; Zhu et al., 2000). No UGT1A4 ortholog is present in rat tissues (King et al., 2000). Incubation of



Fig. 6. Effects of UGT isoform-selective inhibitors on the formation of the metabolites SMG1, SMG2, and SMG3 in HLMs. Niflumic acid (0.1 and 1 μ M, UGT1A9) (A), hecogenin (1 and 10 μ M, UGT1A4) (B), and fluconazole (1 and 3 mM, UGT2B4/2B7) (C) were used as inhibitors of each UGT isoform. Each bar represents the mean \pm S.D. of triplicate determinations.

sarpogrelate or M-1 with RMLs in the presence of UDPGA generated *O*-glucuronides (SG1, SMG1, and SMG3) but not *N*-glucuronides (SG2 and SMG2), consistent with previous reports that only the UGT1A4 isoform catalyzes *N*-glucuronidation of tertiary amines. SMG1 formation was catalyzed mainly by UGT1A9 with minor contributions by UGT 2B4 and 2B7, whereas SMG3 was generated by both UGT2B4 and 2B7, suggesting considerable stereo-selectivity in M-1 *O*-glucuronidation. UGT1A9 played a major role in glucuronidation of *R*-enantiomer whereas UGT2B4 and 2B7 were preferentially involved in glucuronidation of *S*-enantiomer of M-1.

Secondly, inhibition of M-1 glucuronide formation was evaluated using UGT-isoform-specific substrates and/or inhibitors. SMG1 formation was strongly inhibited by niflumic acid (1 μ M, ~80%), a selective inhibitor of UGT1A9 (Gaganis et al., 2007; Miners et al., 2010), whereas the formation of SMG3 was inhibited by fluconazole (3 mM, ~90%), a selective inhibitor of UGT2B7 (Uchaipichat et al., 2006b; Raungrut et al., 2010) (Fig. 7). SMG2 formation was markedly inhibited by hecogenin (10 μ M, ~60%), a potent UGT1A4 inhibitor (Uchaipichat et al., 2006a). Thirdly, correlations between M-1 glucuronidation and known UGT isoform activities were assessed using a panel of HLMs. Significant correlations were observed between the SMG1 formation and UGT1A9-mediated propofol glucuronidation rates (Court, 2005) (P = 0.0031), and between the SMG2 formation and UGT1A4-mediated trifluoperazine glucuronidation rates (Uchaipichat et al., 2006a) (P = 0.0015). Our results collectively suggested that UGT1A4 catalyzed only *N*-glucuronidation



Fig. 7. Correlations between formation of the SMG1 and propofol glucuronidation (A) and between formation of the SMG2 and trifluoperazine glucuronidation (B) in 20 different HLMs. P < 0.05 indicates statistical significance. Each data point represents the mean of duplicate determinations.



Fig. 8. Michaelis–Menten plots for formation of the SMG1, SMG2, and SMG3 in HLM (A), SMG1 by UGT1A9 and 2B4 (B), SMG2 by UGT1A4 (C), and SMG3 by UGT2B4 and 2B7 (D). HLMs or recombinant UGTs were incubated with 0–1000 μ M M-1 at 37°C for 20 minutes in the presence of UDPGA (5 mM). Each data point represents the mean \pm S.D. of triplicate determinations.

TABLE 1 Kinetic parameters of M1 glucuronidation by human liver microsomes and recombinant uridine 5'-diphospho-glucuronosyltransferases

		Kinetic Parameters		
		Km	$V_{\rm max}$	$CL_{\rm int} (V_{\rm max}/K_{\rm m})$
		μM	nmol/min per milligram protein	µl/min per milligram protein
O-Glucuronide I (SMG1)	HLM UGT1A9 UGT2B4	234.6 564.1 105.1	1.126 0.731 0.043	4.80 1.30 0.42
N-Glucuronide (SMG2)	HLM UGT1A4	534.6 476.2	0.621 0.298	1.16 0.63
O-Glucuronide II (SMG3)	HLM UGT2B4 UGT2B7	203.1 186.6 96.3	1.772 0.097 0.031	8.72 0.52 0.32

of M-1, whereas O-glucuronidation was mediated by UGT1A9, UGT2B4, and UGT2B7.

In summary, our results demonstrated that the sarpogrelate active metabolite M-1 was transformed to SMG1, SMG2, and SMG3 in HLMs. Additionally, UGT1A9, UGT1A4 and UGT2B4 are the major isoforms responsible for SMG1, SMG2, and SMG3 formation, respectively. Identification of the UGT1A9, UGT1A4, and UGT2B4 isoforms as responsible for M-1 glucuronidation will prove valuable for determination of the impact of these isoform polymorphisms on

both the variability in M-1 pharmacokinetics and the magnitude of drug-drug interactions.

Acknowledgments

The authors thank Dr. Yohan Park (College of Pharmacy, Inje University) for helpful comments on the interpretation of ¹H-NMR spectra.

Authorship Contributions

Participated in research design: Lee, J.-G. Shin, D.-H. Kim.

- Conducted experiments: H.-J. Kim, Jeong, Seo.
- Contributed new reagents or analytic tools: K.J. Shin, Choi.

Performed data analysis: H.-J. Kim, Jeong, Seo, K.J. Shin, Ghim, Sohn, J.-G. Shin, D.-H. Kim.

Wrote or contributed to the writing of the manuscript: H.-J. Kim, D.-H. Kim.

References

Alonen A, Jansson J, Kallonen S, Kiriazis A, Aitio O, Finel M, and Kostiainen R (2008) Enzymeassisted synthesis and structure characterization of glucuronic acid conjugates of losartan, candesartan, and zolarsartan. *Bioorg Chem* 36:148–155.

Court MH (2005) Isoform-selective probe substrates for *in vitro* studies of human UDP-glucuronosyltransferases. *Methods Enzymol* 400:104–116.

Furukawa K, Tanabe T, Hoshino S, Mishima Y, Shionoya S, Yoshizaki S, Katsumura T, and Nakashima M (1991) Therapeutic effects of sarpogrelate hydrochloride (MCI-9042) on chronic arterial occlusive disease: a double-blind comparison with ticlopidine hydrochloride. Jpn J Clin Pharmacol Ther 7:1747–1770.

Gaganis P, Miners JO, and Knights KM (2007) Glucuronidation of fenamates: kinetic studies using human kidney cortical microsomes and recombinant UDP-glucuronosyltransferase (UGT) 1A9 and 2B7. *Biochem Pharmacol* 73:1683–1691.

Green MD and Tephly TR (1996) Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. Drug Metab Dispos 24:356–363.

Cullemeter C (2003) Pharmacogenomics of human UDP-glucuronosyltransferase enzymes. Pharmacogenomics J 3:136–158.

- Hara H, Osakabe M, Kitajima A, Tamao Y, and Kikumoto R (1991) MCI-9042, a new antiplatelet agent is a selective S2-serotonergic receptor antagonist. *Thromb Haemost* 65:415–420. Kaivosaari S, Finel M, and Koskinen M (2011) N-glucuronidation of drugs and other xenobiotics
- by human and animal UDP-glucuronosyltransferases. *Xenobiotica* **41**:652–669.
- Kaku T, Ogura K, Nishiyama T, Ohnuma T, Muro K, and Hiratsuka A (2004) Quaternary ammonium-linked glucuronidation of tamoxifen by human liver microsomes and UDPglucuronosyltransferase 1A4. *Biochem Pharmacol* 67:2093–2102.
- Kamatsu T, Kitazawa K, Izawa O, Inoue S, Ohshima M, and Iida S (1992) Study of the metabolic fate of (±)-2-(dimethyl-amino)-1-[[o-(m-methoxyphenethyl)-phenoxy]methyl]ethyl hydrogen succinate hydrochloride (MCI-9042). (I). Metabolism of MCI-9042 in rats, dogs, monkeys and human. Yakubutsu Dotai 6:353–375.
- King CD, Rios GR, Green MD, and Tephly TR (2000) UDP-glucuronosyltransferases. Curr Drug Metab 1:143–161.
- Liston HL, Markowitz JS, and DeVane CL (2001) Drug glucuronidation in clinical psychopharmacology. J Clin Psychopharmacol 21:500–515.
- Miners JO, Mackenzie PI, and Knights KM (2010) The prediction of drug-glucuronidation parameters in humans: UDP-glucuronosyltransferase enzyme-selective substrate and inhibitor probes for reaction phenotyping and *in vitro-in vivo* extrapolation of drug clearance and drugdrug interaction potential. *Drug Metab Rev* 42:196–208.
- Nishihira K, Yamashita A, Tanaka N, Kawamoto R, Imamura T, Yamamoto R, Eto T, and Asada Y (2006) Inhibition of 5-hydroxytryptamine receptor prevents occlusive thrombus formation on neointima of the rabbit femoral artery. J Thromb Haemost 4:247–255.
- Pallmann T, Jonas U, Wagner M, Thevis M, Kaeferstein H, Rothschild MA, and Bender K (2010) Enzyme-assisted synthesis and structural characterization of pure benzodiazepine glucuronide epimers. Eur J Pharm Sci 39:233–240.
 Rashid M, Manivet P, Nishio H, Pratuangdejkul J, Rajab M, Ishiguro M, Launay JM,
- Rashid M, Manivet P, Nishio H, Pratuangdejkul J, Rajab M, Ishiguro M, Launay JM, and Nagatomo T (2003) Identification of the binding sites and selectivity of sarpogrelate, a novel 5-HT2 antagonist, to human 5-HT2A, 5-HT2B and 5-HT2C receptor subtypes by molecular modeling. *Life Sci* **73**:193–207.
- Raungrut P, Uchaipichat V, Elliot DJ, Janchawee B, Somogyi AA, and Miners JO (2010) In vitroin vivo extrapolation predicts drug-drug interactions arising from inhibition of codeine glucuronidation by dextropropoxyphene, fluconazole, ketoconazole, and methadone in humans. J Pharmacol Exp Ther 334:609–618.
- Saini HK, Takeda N, Goyal RK, Kumamoto H, Arneja AS, and Dhalla NS (2004) Therapeutic potentials of sarpogrelate in cardiovascular disease. *Cardiovasc Drug Rev* 22:27–54.

- Shimizu T, Yamada Y, Tashita A, Yamamoto K, Kotaki H, Sawada Y, and Iga T (1999) [Pharmacokinetic analysis of antiplatelet effect of sarpogrelate hydrochloride and its application to drug dosage regimen—modeling based on reversible inhibition of 5-HT2 serotonergic receptor in the platelet membrane by sarpogrelate hydrochloride and its metabolite]. Yakugaku Zasshi 119:850–860.
- Shin JG, Soukhova N, and Flockhart DA (1999) Effect of antipsychotic drugs on human liver cytochrome P-450 (CYP) isoforms in vitro: preferential inhibition of CYP2D6. *Drug Metab Dispos* 27:1078–1084.
- Shinohara Y, Nishimaru K, Sawada T, Terashi A, Handa S, Hirai S, Hayashi K, Tohgi H, Fukuuchi Y, and Uchiyama S, et al.; S-ACCESS Study Group (2008) Sarpogrelate-Aspirin Comparative Clinical Study for Efficacy and Safety in Secondary Prevention of Cerebral Infarction (S-ACCESS): A randomized, double-blind, aspirin-controlled trial. Stroke 39: 1827–1833.
- Soars MG, Riley RJ, and Burchell B (2001) Evaluation of the marmoset as a model species for drug glucuronidation. *Xenobiotica* 31:849–860.
- Takada Y, Takada A, and Urano T (1997) MCI-9042, the new selective antagonist of serotonergic (5-HT_{2A}) receptors. *Cardiovasc Drug Rev* 15:101–121.
- Uchaipichat V, Mackenzie PI, Elliot DJ, and Miners JO (2006a) Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone) "probes" for human udp-glucuronosyltransferases. *Drug Metab Dispos* 34:449–456.
- Uchaipichat V, Winner LK, Mackenzie PI, Elliot DJ, Williams JA, and Miners JO (2006b) Quantitative prediction of *in vivo* inhibitory interactions involving glucuronidated drugs from *in vitro* data: the effect of fluconazole on zidovudine glucuronidation. *Br J Clin Pharmacol* 61: 427–439.
- Zhu B, Bush D, Doss GA, Vincent S, Franklin RB, and Xu S (2008) Characterization of 1'hydroxymidazolam glucuronidation in human liver microsomes. *Drug Metab Dispos* 36: 331–338.

Address correspondence to: Dr. Dong-Hyun Kim, Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, #633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, Korea. E-mail: dhkim@inje.ac.kr