Identification of Human UDP-Glucuronosyltransferases Involved in N-Carbamoyl Glucuronidation of Lorcaserin

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Running Title Page

Running Title:  Human UGT involved in lorcaserin N-carbamoyl glucuronidation

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Abbreviations:

HLM, human liver microsomes
UDGPA, uridine 5'-diphosphoglucuronic acid
UGTs, UDP-glucuronosyltransferases
LC/MS/MS, liquid chromatography/tandem mass spectrometry
Abstract

Lorcaserin, a selective serotonin 5-HT$_{2C}$ receptor agonist, is a weight management agent in clinical development. Lorcaserin N-carbamoyl glucuronidation governs the predominant excretory pathway of lorcaserin in humans. Human UDP-glucuronosyltransferases (UGTs) responsible for lorcaserin N-carbamoyl glucuronidation were identified herein. Lorcaserin N-carbamoyl glucuronide formation was characterized by the following approaches: metabolic screening using human tissues (liver, kidney, intestine, and lung) and recombinant enzymes, kinetic analyses, and inhibition studies. While microsomes from all human tissues studied herein were found to be catalytically active for lorcaserin N-carbamoyl glucuronidation, liver microsomes were the most efficient. With recombinant UGT enzymes, lorcaserin N-carbamoyl glucuronidation was predominantly catalyzed by three UGT2Bs (UGT2B7, UGT2B15, and UGT2B17), whereas two UGT1As (UGT1A6 and UGT1A9) played a minor role. UGT2B15 was most efficient, with an apparent $K_m$ of 51.6 ± 1.9 µM and $V_{max}$ value of 237 ± 2.8 pmol/mg protein/min. The rank order of catalytic efficiency of human UGT enzymes for lorcaserin N-carbamoyl glucuronidation was UGT2B15 > UGT2B7 > UGT2B17 > UGT1A9 > UGT1A6. Inhibition of lorcaserin N-carbamoyl glucuronidation activities of UGT2B7, UGT2B15, and UGT2B17 in human liver microsomes by mefenamic acid, bisphenol A, and eugenol further substantiated the involvement of these UGT2B isoforms. In conclusion, multiple human UGT enzymes catalyze N-carbamoyl glucuronidation of lorcaserin; therefore, it is unlikely that inhibition of any one of these UGT activities will lead to significant inhibition of the lorcaserin N-carbamoyl glucuronidation pathway. Thus, the potential for drug-drug interaction by concomitant administration of drug(s) that are metabolized by any of these UGTs is remote.
Introduction

UDP-glucuronosyltransferases (UGTs) compose a superfamily of endoplasmic reticulum membrane bound enzymes (Mackenzie et al., 1997; Meech and Mackenzie, 2010). These enzymes are expressed in liver and in extra-hepatic tissues such as kidney, intestine, brain, skin, breast, uterus, and prostate (Mackenzie et al., 1997; Tukey et al., 2000, 2001). UGTs glucuronidate many endobiotics and xenobiotics by transferring glucuronic acid from its co-factor uridine 5'-diphosphoglucuronic acid (UDPGA) to lipophilic substrates, thereby transforming them into hydrophilic glucuronides and facilitating their subsequent elimination via the biliary or renal routes. Consequently, glucuronidation is recognized as one of the major metabolic pathways for many drugs (Tukey et al., 2000; Bock, 2002). The majority of glucuronidation reactions in humans result in O- and N-glucuronides through conjugation of alcohols, carboxylic acids, and amines (Miniers and Mackenzie, 1991; Tukey et al., 2000; Bock, 2002). Although rare, S-glucuronidation (Ethell et al., 2003) and C-glucuronidation (Kerdpin et al., 2006) have also been reported in humans. Another class of glucuronidation reactions, the N-carbamoyl glucuronidation of primary and secondary amines, is being reported more frequently in the literature (Straub et al., 1988; Tremaine et al., 1989; Schaefer, 1992; 2006). In recent years, drugs such as sertraline (Obach et al., 2005), varenicline (Obach et al., 2006), and a novel dipeptidyl peptidase-4 (DPP-4) inhibitor (Gunduz et al., 2010) were shown to form N-carbamoyl glucuronide metabolites by UGT enzymes. Herein, N-carbamoyl glucuronidation of lorcaserin by human UGTs is reported.

Lorcaserin is a novel and selective serotonin 5-HT$_{2C}$ receptor agonist in clinical development that has been shown to be an effective agent for weight management in phase 3 studies (Smith et al., 2010; Fidler et al., 2011). Lorcaserin, (R)-8-chloro-1-methyl-2,3,4,5-tetrahydro-1H-3-
benzazepine (Fig. 1A), is a secondary amine containing benzazepine. As with other benzazepines (Staub et al., 1988; Shaffer et al., 2009), we also observed that lorcaserin undergoes $N$-carbamoyl glucuronidation in vitro as well as in vivo. In humans, the $N$-carbamoyl glucuronidation was found to be the major clearance pathway of lorcaserin after oral administration (Morgan et al., 2008; Chen et al., 2008). In this study, we report the identity of the human liver UGTs responsible for the metabolism of lorcaserin into its $N$-carbamoyl glucuronide metabolite in human using in vitro systems. Microsomes from human liver, kidney, intestine, and lung were used to identify the most efficient organ for $N$-carbamoyl glucuronidation. Metabolic screening was performed with a battery of recombinant human UGTs to identify the specific UGT-isoforms involved in $N$-carbamoyl glucuronidation. Enzyme kinetic studies were performed with human liver as well as with those UGT enzymes that catalyze $N$-carbamoly glucuronidation efficiently. Experiments for inhibition of UGT-glucuronidation activity in human liver microsomes (HLM) were conducted using known high affinity UGT substrates to aid in the identification of specific UGT-isoforms involved in the lorcaserin $N$-carbamoyl glucuronidation. Two concentrations of lorcaserin were used for the inhibition experiments, 1 µM, which is clinically relevant (Smith et al., 2010), and 100 µM, which is in the proximity of $K_m$ values obtained with human liver microsomes for lorcaserin $N$-carbamoyl glucuronide formation.
Methods

Chemicals and enzyme sources. Lorcaserin hydrochloride hemihydrate was provided by Cilag AG (Schaffhausen, Switzerland). Lorcaserin N-carbamoyl glucuronide was supplied by SAFC Pharma (Manchester, UK). Lorcaserin sulfamate-\textsubscript{d6}, used as an internal standard, was synthesized at Arena Pharmaceuticals, Inc. (San Diego, CA). Alamethicin, magnesium chloride, dimethyl sulfoxide (DMSO), saccharolactone, uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA), mefenamic acid, bisphenol A, and eugenol were purchased from Sigma-Aldrich (St. Louis, MO). High-purity HPLC-grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). All reagents were of high purity grade. Microsomes from human liver, kidney, intestine, and lung (smoker and non-smoker) were purchased from Xenotech (Lenexa, KS). The following human recombinant UGTs expressed in insect cells were purchased from BD Biosciences (Woburn, MA): UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17. These recombinant UGTs were supplied as prepared microsomes.

Preparation of stock solutions: Lorcaserin, saccharolactone, and UDPGA were prepared in incubation buffer (sodium bicarbonate buffer, pH 7.4). Eugenol and bisphenol A were dissolved in acetonitrile and water mixture (1:1, v/v). Mefenamic acid was dissolved in acetonitrile and DMSO mixture (1:1, v/v). Alamethicin was dissolved in methanol.

Metabolic assays. All metabolic assays were performed using modifications of a previously reported method (Schaefer, 1992). In our assay, we did not observe enhancement of lorcaserin N-carbamoyl glucuronide production by adding carbon dioxide gas to the sodium bicarbonate buffer, and 100 mM sodium bicarbonate buffer alone was found to be adequate as a source of carbon dioxide for the reaction. Therefore, all metabolic reactions were conducted in 100 mM
sodium bicarbonate buffer along with other necessary reagents (Shaefer, 1992; Kirsch et al., 2000). All metabolic assays including enzyme kinetics assays were performed in triplicate.

**Assays with microsomes from liver, kidney, intestine, and recombinant UGTs.** Incubations for initial screening were carried out to determine the extent of lorcaserin N-carbamoyl glucuronide formation in various human tissue microsomes (liver, kidney, intestine, and lung) as well as in recombinant human UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17). Incubation mixtures contained human tissue microsomes (0.25 mg microsomal protein/mL) or recombinant UGT (0.125 mg protein/mL), saccharolactone (2 mM), magnesium chloride (10 mM), alamethicin (25 μg/mL), and 100 mM sodium bicarbonate buffer (pH 7.4). The incubation mixtures were kept on ice for 15 min followed by the addition of lorcaserin (100 μM). All incubations were pre-warmed at 37°C for 5 min and then initiated by the addition of UDPGA (3 mM), bringing the total reaction volume to 500 μL. After 20 min incubation at 37°C, the reaction was terminated by adding 500 μL of ice-cold acetonitrile containing the internal standard, lorcaserin sulfamate-d₆ (25 ng/mL).

**Assay with human liver microsomes and recombinant UGTs for kinetic analyses.** The kinetic studies were performed using human liver microsomes and recombinant UGTs (UGT1A9, UGT2B7, UGT2B15, and UGT2B17). Incubation mixtures were prepared by combining human liver microsomes (0.25 mg microsomal protein/mL) or recombinant UGT (0.125 mg protein/mL), saccharolactone (2 mM), magnesium chloride (10 mM), alamethicin (25 μg/mL), and 100 mM sodium bicarbonate buffer (pH 7.4). Samples were kept on ice for 15 min followed by the addition of varying concentration of lorcaserin, ranging from 0.78 to 2000 μM depending on the enzyme system. All incubations were pre-warmed at 37°C for 5 min and then reactions
were initiated by the addition of UDPGA (3 mM), bringing the total reaction volume to 500 µL. After 20 min of incubation at 37°C, the reactions were quenched by adding 500 µL of ice-cold acetonitrile containing the internal standard, lorcaner sulfamate-d₆ (25 ng/mL).

**Effect of chemical inhibitors on human liver UGT activity.** High-affinity UGT substrates such as mefenamic acid (Gaganis et al., 2007; Mano et al., 2007a, 2007b), bisphenol A (Hanioka et al., 2008), and eugenol (Turgeon et al., 2003) were utilized as inhibitors to block UGT2B7-, UGT2B15-, and UGT2B17-catalyzed lorcaner N-carbamoyl glucuronidation activity in human liver microsomes (HLM), respectively. Two concentrations of lorcaner, 1 µM (clinically relevant) and 100 µM (in the proximity of the $K_m$ value of 128 µM in HLM), were used during inhibition studies. Two concentrations of mefenamic acid (5 and 25 µM), bisphenol A (5 and 25 µM), and eugenol (20 and 100 µM) were used to inhibit UGT2B7-, UGT2B15-, and UGT2B17-mediated lorcaner N-carbamoyl glucuronidation in HLM, respectively. The concentrations of mefenamic acid, bisphenol A, and eugenol, selected to inhibit the reaction, were approximately equal to (low) or five times greater than (high) their respective $K_m$ values, so that the maximum inhibitory effect of these compounds might be observed while maintaining UGT selectivity. Additionally, two mixtures of mefenamic acid, bisphenol A, and eugenol together, one at 30 µM (mixture of three low concentrations of three inhibitors) and another at 150 µM (mixture of three high concentrations of three inhibitors), were used to inhibit lorcaner N-carbamoyl glucuronidation in HLM.

Typical incubation mixtures for UGT inhibition assays contained HLM (0.25 mg microsomal protein/mL), saccharolactone (2 mM), magnesium chloride (10 mM), alamethicin (25 µg/mL), and 100 mM sodium bicarbonate buffer (pH 7.4). Samples were kept on ice for 15 min followed...
by the addition of lorcaserin (1 or 100 μM) and UGT inhibitors (mefenamic acid, bisphenol A or eugenol as mentioned above). For the control activity, no inhibitors but appropriate volume of solvents were included in the incubation mixtures. All incubations were pre-warmed at 37°C for 5 min and reactions were then initiated by adding UDPGA (3.0 mM), bringing the total reaction volume to 500 μL. After 20 min of incubation at 37°C, the reactions were quenched with the addition of 500 μL of ice-cold acetonitrile containing the internal standard, lorcaserin sulfamate-d₆ (25.0 ng/mL).

**Analyses for lorcaserin N-carbamoyl glucuronide.** The quenched samples were centrifuged for 15 min at 2572g (3700 RPM, Allegra 25-R Centrifuge, Beckman Coulter) and then 150 μL of supernatant was transferred to another 96-well plate. Lorcaserin N-carbamoyl glucuronide was analyzed by LC/MS/MS using lorcaserin sulfamate-d₆ as an internal standard. A 10 μL sample was introduced for chromatographic separation using a Mac Mod HALO C18 (2.7 μm, 3 × 30 mm) column (MAC-MOD Analytical, Inc., Chadds Ford, PA) at a flow rate of 0.60 mL/min. A binary gradient was applied using water containing 0.1% formic acid (mobile phase A) and methanol (mobile phase B). Initially 50% of mobile phase B was applied for 30 seconds and then switched to 95% B over 60 seconds. The column was then washed with 95% B for 15 seconds followed by a gradient change from 95% to 50% B over 5 seconds. Finally, the column was re-equilibrated with 50% B for 70 seconds. The total analysis time was approximately 3 min. Mass spectrometric detection was achieved with an MDS Sciex API-4000 (Applied Biosystems/MDS Sciex, Foster City, CA) operated in negative ion mode and using multiple reaction monitoring. Lorcaserin N-carbamoyl glucuronide (analyte) and lorcaserin sulfamate-d₆ (internal standard) were monitored with Q1/Q3 transitions of m/z 414.1/193.8 and 280.0/79.8,
respectively. Quantification was performed with regression analysis generated from calibration standards.

*Enzyme kinetic analysis.* Kinetic parameters were estimated using Sigma Plot software (Systat Software, Inc., Richmond, CA) designed for a nonlinear least-square fit to the standard Michaelis-Menten equation: \( v = \frac{V_{\text{max}} [S]}{K_m + [S]} \); where \( v \) is the rate of reaction, \( V_{\text{max}} \) is the maximum reaction velocity, \( K_m \) is Michaelis constant (substrate concentration at 0.5 of \( V_{\text{max}} \)), and \([S]\) is the substrate concentration (Segel; 1976). Goodness of fit for Michaelis-Menten kinetic model was assessed from the standard error (SE), 95% confidence interval and \( r^2 \) values. The kinetic constants reported as the mean ±SE and the \( r^2 \) values were shown in Table 2.
Results

**Lorcaserin N-carbamoyl glucuronide formation in human tissue microsomes.** Incubation of lorcaserin (100 µM) with liver, kidney, intestine, and lung microsomes generated the N-carbamoyl glucuronide metabolite of lorcaserin (Table 1). HLM generated substantially greater amounts of lorcaserin N-carbamoyl glucuronide than microsomes from other tissues. The liver microsomes produced approximately 6- and 8-fold more lorcaserin N-carbamoyl glucuronide than the kidney and intestine microsomes, respectively. Lorcaserin N-carbamoyl glucuronide production in liver microsomes was approximately 900- and 2000-fold higher than lung microsomes of smokers and non-smokers, respectively. The rank order of the lorcaserin N-carbamoyl glucuronide formation rate in these tissue microsomes was liver (1028.0 ± 21.6 pmol/mg protein/min) > kidney (185.9 ± 5.2 pmol/mg protein/min) > intestine (133.1 ± 0.3 pmol/mg protein/min) > lung (1.116 ± 0.060 pmol/mg protein/min for smokers and 0.493 ± 0.070 pmol/mg protein/min for non-smokers). Because liver tissue was the most efficient in the formation of the lorcaserin N-carbamoyl glucuronide, further kinetic and inhibition studies were performed with microsomes from human liver only.

**Lorcaserin N-carbamoyl glucuronide formation in recombinant UGTs.** Twelve recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in insect cells were used to determine their relative ability to form lorcaserin N-carbamoyl glucuronide (Fig. 2). UGT1A6, UGT1A9, UGT2B7, UGT2B15, and UGT2B17 catalyzed lorcaserin N-carbamoyl glucuronidation (Fig. 1). The rank order of the catalytic activity was UGT2B15 > UGT2B7> UGT2B17> UGT1A9> UGT1A6. UGT1A6 was excluded from further kinetic studies due to its negligible catalytic activity.
Kinetics studies with HLM. Kinetic analyses for the formation of lorcaserin N-carbamoyl glucuronide were performed with human liver microsomes using a wide range of concentrations (0.78 - 1000 μM) of lorcaserin. The reactions were saturated at ~ 400 μM, and no substrate inhibition was observed with increased lorcaserin concentration up to 1000 μM in the kinetic reactions (Fig. 3). The $K_m$ and $V_{max}$ values calculated using a nonlinear least-square fit to the standard Michaelis-Menten equation were $128.1 \pm 5.5 \, \mu M$ and $2379.2 \pm 28.5 \, \text{pmol/mg protein/min}$, respectively. The intrinsic clearance ($CL_{int} = V_{max}/K_m$) in human liver microsomes was $18.57 \, \mu \text{L/mg protein/min}$ (Table 2).

Kinetics studies with recombinant human UGTs. Kinetic analysis for the determination of $K_m$ and $V_{max}$ values were performed with UGT1A9, UGT2B7, UGT2B15, and UGT2B17 using a wide range (0.78 – 2000 μM) of lorcaserin concentrations depending on the enzyme system. Like human liver microsomes, no substrate inhibition was observed for these UGT isoforms (Fig. 4A-D). The $K_m$ values for UGT1A9, UGT2B7, UGT2B15, and UGT2B17 were $518.0 \pm 63.8$, $93.8 \pm 8.6$, $51.6 \pm 1.9$, and $254.1 \pm 13.3 \, \mu M$, respectively, and the $V_{max}$ values were $103.3 \pm 5.1$, $186.4 \pm 4.4$, $237.4 \pm 2.8$, and $155.3 \pm 2.8 \, \text{pmol/mg protein/min}$, respectively (Table 2).

Based on the intrinsic clearance ($CL_{int} = V_{max}/K_m$) values in Table 2, the rank order for the formation of lorcaserin N-carbamoyl glucuronide was UGT2B15 > UGT2B7 > UGT2B17 > UGT1A9.

Inhibition of UGT activity in HLM. As shown in Fig. 5A, a low concentration of mefenamic acid (5 μM), bisphenol A (5 μM), or eugenol (20 μM), added either separately or combined (total inhibitor concentration = 30 μM, designated as Combined-Low) to the HLM incubation containing 1 μM lorcaserin (substrate), inhibited ≤ 5% of lorcaserin N-carbamoyl glucuronidation. However, increased concentrations of mefenamic acid (25 μM), bisphenol A
(25 μM), and eugenol (100 μM) inhibited 23%, 37%, and 18% of lorcaserin N-carbamoyl glucuronidation, respectively (Fig. 5A). The addition of a mixture containing the increased concentrations of these inhibitors to the incubation (total inhibitor concentration = 150 μM designated as Combined-High) resulted in 66% inhibition of lorcaserin N-carbamoyl glucuronidation in liver microsomes (Fig. 5A), which is similar to the summed effect (23% + 37% + 18% = 78%) exerted by the individual inhibitors.

When the lorcaserin (substrate) concentration was increased to 100 μM, mefenamic acid (25.0 μM), bisphenol A (25.0 μM), and eugenol (100 μM) inhibited 15%, 30%, and 11% of lorcaserin N-carbamoyl glucuronidation, respectively, under identical conditions (Fig. 5B). Addition of a mixture of these three inhibitors (total inhibitor concentration = 150 μM) caused 53% inhibition of lorcaserin N-carbamoyl glucuronidation in liver microsomal incubation (Fig. 5B), which is close to the combined effect (15% + 30% + 11% = 56%) exerted by individual inhibitors under identical substrate conditions (100 μM lorcaserin). No inhibitory effect was observed with low inhibitor concentrations (5 and 20 μM) incubated with 100 μM of lorcaserin concentration in HLM (Fig. 5B).
Discussion

While N-carbamoyl glucuronidation is considered to be rare, it represents another important metabolic pathway which has increasingly drawn attention from drug metabolism researchers in recent years. Although a number of publications investigated its possible mechanism (Schaefer, 1992, 2006), few studies report the identification of UGT enzymes that catalyze the N-carbamoyl glucuronidation pathway (Obach et al., 2005, 2006; Gunduz et al., 2010). In this study, we identified the human UGT enzymes involved in N-carbamoyl glucuronidation of lorcaserin.

First, we examined the formation of lorcaserin N-carbamoyl glucuronide in microsomes prepared from various human tissues which include liver, kidney, intestine, and lung. Among these organs, human liver was by a substantial margin the most efficient for lorcaserin N-carbamoyl glucuronidation. We also identified that UGT2B7, UGT2B15, and UGT2B17 are the primary enzymes involved in lorcaserin N-carbamoyl glucuronidation. These UGT enzymes are abundantly expressed in liver compared to other tissues (Turgeon et al., 2003; Ohno and Nakaji, 2009). For example, quantitative estimation showed that UGT2B7 is 2-fold and UGT2B15 is 25-fold higher in liver compared to the small intestine (Ohno and Nakaji, 2009). UGT2B15 has the highest intrinsic clearance followed by UGT2B7 for lorcaserin N-carbamoyl glucuronidation. Thus, human liver tissues have a high pool of catalytic UGT2B enzymes involved in lorcaserin metabolism. Therefore, the high intrinsic clearance for lorcaserin N-carbamoyl glucuronidation by liver microsomes appears to be associated with higher expression of these enzymes in the liver which is clearly in accordance with these literature reports (Ohno and Nakaji, 2009; Turgeon et al., 2003).

Among extra hepatic tissues, kidney was most efficient at producing the N-carbamoyl glucuronide followed by intestine and lung. These findings suggest that both hepatic and
extrahepatic tissues are involved in lorcaserin \textit{N}-carbamoyl glucuronidation in humans. Human liver microsomes also showed a much higher turnover rate than any individual recombinant UGT enzyme examined. This finding is similar to the observation previously reported for oxazepam glucuronidation in HLM compared to recombinant UGTs, where HLM produced more oxazepam glucuronide than individual recombinant enzymes (Court et al., 2002). Formation of lorcaserin \textit{N}-carbamoyl glucuronide in the microsomes of lung was the least efficient compared to microsomes from other organs, consistent with observations for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone glucuronidation (Ren et al., 2000). Ullrich et al (1997) reported that lung microsomes from smokers have about 2-fold higher glucuronidation activity compared to non-smokers suggesting a functional increase of UGT activity in the lung of smokers. UGT2B7 (Ren et al., 2000) and UGT2B17 (Beaulieu et al., 1996; Ohno and Nakaji, 2009) are constitutively expressed in human lung. Several studies have also suggested the induction of UGT2B7 and UGT2B17 in tobacco smokers’ lung (Ren et al., 2000; Carmella et al., 2002; Gallagher et al., 2007). In line with these literature reports, a likely explanation for the 2-fold increased formation of lorcaserin \textit{N}-carbamoyl glucuronidation in lung microsomes from smokers is the induction of one or more of these UGTs (UGT2B7 and UGT2B17), which are involved in this metabolic pathway of lorcaserin along with UGT2B15.

Further, lorcaserin is rapidly and highly absorbed after oral administration in humans. It is metabolized mainly in the liver. Based on total radioactivity >90\% of lorcaserin dose is excreted in the urine and ~33\% of dose is accounted for \textit{N}-carbamoyl glucuronidation (Morgan et al., 2008; Chen et al., 2008). It is conceivable that the sum of activity of each UGT isoforms expressed in human liver microsomes involved with lorcaserin \textit{N}-carbamoyl glucuronidation is equal to the UGT activity of human liver microsomes. Therefore, the contribution of UGT1A9,
UGT2B7, UGT2B15 and UGT2B17 to the observed activity in human liver microsomes ($V_{\text{max,UGT}}/V_{\text{max,HLM}}$) may be estimated as 4.3, 7.8, 10.0 and 6.5%, respectively. Interestingly, the sum of these estimated fractions (~29%) is close to the fraction accounted for $N$-carbamoyl glucuronidation in human urine (~33%). Thus, multiple UGTs are involved in this pathway and the contribution of each individual UGT appeared not to be >10%.

Second, it was shown that the UGT2B family, namely UGT2B7, UGT2B15, and UGT2B17, predominantly catalyzed lorcaserin $N$-carbamoyl glucuronidation and that UGT2B15 was the most efficient UGT followed by UGT2B7 and UGT2B17. Interestingly, UGT2B15 and UGT2B17 share high amino acid sequence identity (>92%), tissue distribution, and specificity for substrates such as steroids (Hum et al., 1999; Tukey et al., 2000; Turgeon et al., 2001). In contrast, UGT2B7 shares less amino acid sequence identity (>78%) with UGT2B15 and UGT2B17 and has a much broader substrate specificity, as it is involved in the metabolism of drugs from all major classes, including opioids, cancer drugs and steroids (Tukey et al., 2000; Innocenti et al., 2001; Turgeon et al., 2003). Despite their differences in sequence similarity, specificity and catalytic rate, UGTs show a great deal of substrate redundancy (Miniers and Mackenzie, 1991; Tukey et al., 2000). For example, UGT2B7, UGT2B15, and UGT2B17 all catalyze glucuronidation of testosterone, dihydrotestosterone, and androstane-3α-17β-diol (Turgeon et al., 2001). We observed that lorcaserin $N$-carbamoyl glucuronidation is also catalyzed by these three steroid metabolizing enzymes, with comparable rates. Among these three UGTs, UGT2B7 was shown to be the only UGT involved in $N$-carbamoyl glucuronidation of varenicline (Obach, 2006), and was the major UGT for sertraline (Obach, 2005); both of these drugs are secondary amines. In contrast, UGT2B7 plays a very minor role in $N$-carbamoyl glucuronidation of a primary amine DPP-4 inhibitor (Gunduz et al., 2010). We observed, for the
first time, that UGT2B15 and UGT2B17, in addition to UGT2B7, are involved in \( N \)-carbamoyl glucuronidation of a secondary amine, lorcaserin. These examples suggest that carbamic acids of secondary amines may be a preferred or at least a good substrate for UGT2B7 mediated \( N \)-carbamoyl glucuronidation.

Third, due to the substrate overlap among UGTs and lack of specific chemical inhibitors of UGTs, known high-affinity UGT substrates are often used to inhibit UGT activity towards other substrates, assuming the competitive nature of substrate specificity and binding (Williams et al., 2004). A mixture of three UGT substrates, bisphenol A (UGT2B15), mefenamic acid (UGT2B7), and eugenol (UGT2B17), decreased \( N \)-carbamoyl glucuronidation activity by 66% and 53% with 1 \( \mu \)M and 100 \( \mu \)M of lorcaserin (substrate), respectively, suggesting that at either concentration of substrate, lorcaserin \( N \)-carbamoyl glucuronidation activity was inhibited, presumably through competition for UGT2B7, UGT2B15, and UGT2B17 activity in HLM. These findings are consistent with the results obtained from recombinant UGT isoforms that UGT2B7, UGT2B15 and UGT2B17 are the major isoforms catalyze lorcaserin \( N \)-carbamoyl glucuronidation. Some UGT substrates exhibit a high \( K_m \) value, thus requiring higher concentration for inhibition when used as a substrate (Williams et al., 2004). For example, up to 500 \( \mu \)M of fenamates, including mefenamic acid, were utilized to inhibit UGT2B7 catalyzed 4-methylumbelliferone glucuronidation in human kidney cortical microsomes. However, in some instances, a lower concentration of mefenamic acid was sufficient, for example, to inhibit gemfibrozil glucuronidation in human liver microsomes (Mano et al., 2007b). In the case of lorcaserin, a combined mixture of three inhibitors (150 \( \mu \)M) was in the proximity of the \( K_m \) value of 128 \( \mu \)M for its \( N \)-carbamoyl glucuronide formation in HLM. When the three inhibitors were tested individually, the sum of inhibitory effect was 56% (100 \( \mu \)M lorcaserin), which is
essentially the same as the 53% inhibition of N-carbamoyl glucuronidation when they were applied combined (150 µM). This observation, taken together with the $K_m$ values of UGT2B7, UGT2B15, and UGT2B17 for lorcaserin N-carbamoyl glucuronidation, suggests either minimal or absence of non-specific inhibitory effects (Mano et al., 2007b). A much higher combined concentration of these inhibitors (1200 µM) abolished the lorcaserin N-carbamoyl glucuronidation activity in HLM (data not shown). However, such a high inhibitor concentration has no clinical relevance and might have an increased non-specific effect (Williams et al., 2004). It is important to note that the inhibitory effect of individual inhibitors used on lorcaserin N-carbamoyl glucuronidation activity in liver microsomes was in the range of 11-37% with either concentration (1 or 100 µM) of lorcaserin suggesting that a single inhibitor did not sufficiently inhibit lorcaserin N-carbamoyl glucuronidation. This may be due to the wide range of $K_m$ values (Table 2) of the UGTs involved in lorcaserin N-carbamoyl glucuronidation. Considering the differences in kinetic parameters of multiple UGTs involved in lorcaserin N-carbamoyl glucuronidation pathway, it is unlikely that one substrate (inhibitor) will inhibit all of these UGT enzymes altogether. In a separate study, we also demonstrated that multiple cytochrome P450s and flavin-containing monooxygenase (phase I) mediated pathways are involved in lorcaserin clearance (our accompanied manuscript). Thus, inhibition of one pathway may not greatly influence the overall clearance of lorcaserin.

In conclusion, this in vitro study demonstrated that multiple human UGT2B isoforms such as UGT2B7, UGT2B15, and UGT2B17, predominantly catalyze lorcaserin N-carbamoyl glucuronidation. UGT1A isoforms such as UGT1A6 and UGT1A9 exhibit a minor contribution to this pathway. Considering the fraction of lorcaserin cleared as N-carbamoyl glucuronide, the involvement of multiple UGTs and contribution from other phase I enzymes in lorcaserin
metabolism, it is unlikely that inhibition of any of the UGTs will cause a significant impact on lorcaserin clearance in humans. Thus, drug-drug interaction through inhibition of the \(N\)-carbamoyl glucuronidation pathway is unlikely for lorcaserin when taken with concomitant medication.
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Conducted experiments: Usmani and Palamar.

Performed data analysis: Usmani, Palamar and Sadeque.

Wrote or contributed to the writing of the manuscript: Sadeque.
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Footnotes

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b) Part of this work was presented at the 15th ISSX North American Regional Meeting, October 12-16, 2008, San Diego, California, USA.
Legends for figures

**Figure 1.** (A) Lorcaserin, and (B) Lorcaserin N-carbamoyl glucuronide catalyzed by human UDP-glucuronosyltransferase, UGT1A6, UGT1A9, UGT2B7, UGT2B15, and UGT2B17. The contributions of these UGTs in this pathway were identified using recombinant UGT enzymes and inhibition of lorcaserin N-carbamoyl glucuronidation activity in human liver microsomes by chemical inhibitors.

**Figure 2.** Lorcaserin N-carbamoyl glucuronidation activity of various recombinant human UGT enzymes. The enzymatic assay contained 0.125 mg/mL recombinant protein and 100 µM of lorcaserin, UDPGA, saccharolactone, and alamethicin in sodium bicarbonate buffer (pH 7.4). Detailed incubations were described in methods section.

**Figure 3.** Michaelis-Menten kinetic plot for lorcaserin N-carbamoyl glucuronide formation catalyzed by human liver microsomes (HLM). The values are average of triplicate (±SE) incubations.

**Figure 4.** Michaelis-Menten kinetic plots for lorcaserin N-carbamoyl glucuronide formation catalyzed by human recombinant UGTs (A) UGT1A9, (B) UGT2B7, (C) UGT2B15, and (D) UGT2B17. The enzymatic assays were conducted with 0.125 mg/mL recombinant protein and a wide range of lorcaserin concentration (up to 2000 µM depending on the system), UDPGA, saccharolactone and alamethacine in sodium bicarbonate buffer (pH 7.4). The values are average of triplicate (±SE) incubations. Details incubations were described in the methods section.

**Figure 5.** Inhibition of lorcaserin N-carbamoyl glucuronidation activity in human liver microsomes by mefenamic acid (5 and 25 µM), bisphenol A (5 and 25 µM), eugenol (20 and 100
μM), and mixtures of these inhibitors. Combined Low: mixture of mefenamic acid (5 μM), bisphenol A (5 μM), and eugenol (20 μM) (total of 30 μM of three inhibitors). Combined High: mixture of mefenamic acid (25.0 μM), bisphenol A (25.0 μM), and eugenol (100 μM) (total of 150 μM of three inhibitors). Two concentrations of lorcaserin (substrate), 1 μM, and 100 μM, were used in this experiment.
Tables

**Table 1.** Formation of Lorcaserin N-Carbamoyl Glucuronide in Human Liver, Kidney, Intestine, and Lung (Smoker & Non-Smoker) Microsomes in the Presence of 100 µM Lorcaserin (substrate)

<table>
<thead>
<tr>
<th>Experimental Tissue</th>
<th>Lorcaserin N-Carbamoyl Glucuronide (pmol/mg protein/min) ±SE</th>
<th>Fold-less Compared to Liver Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1028.0 ± 21.6</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>185.9 ± 5.2</td>
<td>6-fold</td>
</tr>
<tr>
<td>Intestine</td>
<td>133.1 ± 0.3</td>
<td>8-fold</td>
</tr>
<tr>
<td>Lung (smoker)</td>
<td>1.116 ± 0.060</td>
<td>921-fold</td>
</tr>
<tr>
<td>Lung (non-smoker)</td>
<td>0.493 ± 0.070</td>
<td>2084-fold</td>
</tr>
</tbody>
</table>
Table 2. Kinetic Parameters, $K_m$ and $V_{max}$, for Loracserin N-Carbamoyl Glucuronide Formation by Human Liver Microsomes and Recombinant UGT Enzymes

<table>
<thead>
<tr>
<th>UGT Enzymes source</th>
<th>Kinetic Parameters</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM) ±SE</td>
<td>$V_{max}$ (pmol/mg protein/min) ±SE</td>
<td>CL$_{int}$ (µL/mg protein/min)</td>
<td>$r^2$</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>128.1 ± 5.6</td>
<td>2379.2 ± 28.5</td>
<td>18.57</td>
<td>1.0</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>518.0 ± 63.8</td>
<td>103.3 ± 5.1</td>
<td>0.199</td>
<td>0.97</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>93.8 ± 8.6</td>
<td>186.4 ± 4.4</td>
<td>1.987</td>
<td>0.98</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>51.6 ± 1.9</td>
<td>237.4 ± 2.8</td>
<td>4.601</td>
<td>1.0</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>254.1 ± 13.3</td>
<td>155.3 ± 2.8</td>
<td>0.611</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4A.
Figure 4C.

UGT2B15

Lorcaserin N-carbamoyl glucuronide (pmol/mg protein/min)

[0, 50, 100, 150, 200, 250] vs [0, 100, 200, 300, 400, 500]
Figure 4D.

![Graph showing the relationship between Lorcaserin N-carbamoyl glucuronide and [Lorcaserin] (µM)](image-url)
Figure 5A.

% Remaining (N-carbamoyl glucuronidation activity)

Inhibitor Concentration

- 5 μM - MFA
- 25 μM - MFA
- 5 μM - BSP
- 25 μM - BSP
- 20 μM - EUG
- 100 μM - EUG
- Combined - Low
- Combined - High

(A): 1 μM Lorcaserin
Figure 5B.