Identification of a novel N-carbamoyl glucuronide: In vitro, In vivo and mechanistic studies

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Abbreviations

UGTs, Uridine diphosphoglucuronosyl transferases; UDPGA, Uridine diphosphoglucuronic acid;

HLMs, Human Liver Microsomes; ESI, electrospray ionization; MeCN, acetonitrile; NaHCO₃,
sodium bicarbonate; CID, collision induced dissociation; DPP-4, dipeptidyl peptidase-4;
Abstract

1-[4-Aminomethyl-4-(3-chloro-phenyl)-cyclohexyl]-tetrahydro-pyrimidin-2-one, 1, was developed as an inhibitor of dipeptidyl peptidase-4 enzyme (DPP-4). Biotransformation studies with 1 revealed the presence of an N-carbamoyl glucuronide metabolite (M1) in rat bile and urine. N-carbamoyl glucuronides are rarely observed, and little is understood regarding the mechanism of N-carbamoyl glucuronidation. The objectives of the current investigation were to elucidate the structure of the novel N-carbamoyl glucuronide, to investigate the mechanism of N-carbamoyl glucuronide formation in vitro using stable labeled CO₂, UGT reaction phenotyping, and to assess whether M1 was formed to the same extent in vitro across species – mouse, rat, hamster, dog, monkey and human. Structure elucidation was carried out on a Thermo LTQ-Orbitrap® with accurate mass measurement and MSⁿ capabilities. ¹³C-labeled carbon dioxide (¹³CO₂) was used for identification of the mechanism of N-carbamoyl glucuronidation. Mechanistic studies with ¹³C-labeled CO₂ in rat liver microsomes revealed that CO₂ from the bicarbonate buffer (in equilibrium with exogenous CO₂) may be responsible for the formation of M1. M1 was formed in vitro in liver microsomes from multiple species – mainly rat and hamster, followed by similar formation in dog, monkey, mouse, human. M1 could be detected in UGT1A1, UGT1A3 and UGT2B7 Supersomes® in a CO₂ rich environment. In conclusion our study demonstrates that formation of M1 was observed in microsomal incubations across various species and strongly suggests the incorporation of CO₂ from the bicarbonate buffer, in equilibrium with exogenous CO₂ into the carbamoyl moiety of the formed N-carbamoyl glucuronide.
Introduction

Human UDP-glucuronosyltransferases catalyze the conjugation of glucuronic acid to a variety of xenobiotic and endobiotic molecules (Remmel et al., 2009). The UGTs are expressed in many tissues, including liver, kidney, intestine, colon, adrenal glands, spleen, lung, skin, testes, ovaries, olfactory glands, and brain (Tukey and Strassburg, 2000). Conjugation via glucuronidation is an important step in the elimination of many endogenous molecules from the body, including bilirubin, bile acids, steroid hormones, thyroid hormones, retinoic acids, etc (Burchell et al., 1998). The glucuronidation reaction proceeds by transfer of a glucuronic acid moiety from UDPGA to an acceptor molecule and occurs with a wide range of functional groups such as aromatic and aliphatic alcohols, carboxylic acids, thiols, primary, secondary, tertiary, and aromatic amino groups, and acidic carbon atoms (Remmel et al., 2007). One such conjugation reaction at a carbamic acid functionality results in the formation of an N-carbamoyl glucuronide. Carbamic acids are formed when carbon dioxide reacts with primary or secondary amines and subsequent glucuronidation of the carbamic acid results in N-carbamoyl glucuronide formation. Few reactions have been reported in the literature for N-carbamoyl glucuronidation. N-carbamoyl glucuronides of amino acids and certain xenobiotics have been reviewed by Schaefer (Schaefer, 2006). Other xenobiotics that are reported to form N-carbamoyl glucuronide are tocainide (Elvin et al., 1980), rimantidine (Brown et al., 1990), carvedilol (Schaefer, 1992), mofegiline (Dow et al., 1994), sertraline (Obach et al., 2005), sitagliptin (Vincent et al., 2007) and amosulalol (Suzuki and Kamimura, 2007). Little is known about the mechanism of this reaction and the human UGTs that catalyze it.
1 was developed as a small molecule inhibitor of DPP-4 (adenosine deaminase complexing protein-2) for the treatment of type II diabetes. In an effort to understand the mechanism of clearance of 1 in rats, metabolite identification studies were carried out in rat plasma, urine and bile samples. Biotransformation studies revealed that an N-carbamoyl glucuronide (M1) was present in rat bile and urine. The objectives of the current investigation were as follows – (i) to elucidate the structure of this novel N-carbamoyl glucuronide in rat bile and urine (ii) to study the mechanism of N-carbamoyl glucuronide formation in vitro in an appropriate model using $^{13}$C-labeled carbon dioxide (iii) to study if M1 could be biosynthesized in vitro in rat liver microsomes (iv) to compare the formation of M1 in liver microsomes across various species and, (v) to identify the UGT isozymes involved in the formation of M1 in vitro.
Materials and Methods

Chemicals and Reagents. 1 was synthesized at Novartis Institutes for Biomedical Research (Basel, Switzerland). Liver microsomes (sprague dawley rat, beagle dog, cynomolgus monkey, syrian hamster and CD1 mouse; 20 mg/mL) were purchased from XenoTech, LLC (Lenexa, Kansas) and human liver microsomes (20 mg/mL, pooled lot # 20) were purchased from Cellzdirect (Austin, TX). β-glucuronidase (isolated from *Helix Pomatia*) and UDPGA were purchased from Sigma-Aldrich (St.Louis, MO). UGT Supersomes® at 5 mg/mL protein concentration (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17) were obtained from BD Biosciences (Franklin Lakes, NJ). Solvents and reagents were MS grade and were purchased from J.T. Baker (Phillipsburg, NJ). NaH$^{13}$CO$_3$ (99% pure) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA).

In vivo Studies in SD Rats. The in-life portion of the study was conducted at Novartis Institutes for Biomedical Research (Cambridge, MA) in accordance with institutional animal care and use committee (IACUC) protocols. Three male sprague dawley rats with cannulated bile ducts were administered an intravenous 1mg/kg dose of 1, formulated in 100 mM phosphate buffer, pH 7.4. Pre-dose (0 hour) and post-dose bile and urine were collected up to 30 hours, (at intervals of 0 to 4 hours, 4 to 7 hours, 7 to 30 hours). Plasma was obtained from the blood samples collected at pre-dose (0 hour) and post-dose (1 and 6 hours) for metabolic profiling. Rat bile, urine and plasma were stored at -80°C until analysis.
LC-MS<sup>n</sup> method for metabolite identification. Urine and bile samples were pooled from each time interval (equal volumes) and centrifuged at 6440 x g for 5 minutes prior to analysis. An aliquot (0.2 mL) of each plasma sample was transferred to a 2 mL eppendorf tube containing MeCN (0.2 mL) and the samples were vortexed and centrifuged (3200 x g for 5 minutes) to precipitate the proteins. The respective supernatants were removed and transferred to 1 mL conical glass inserts prior to analysis. Pre-dose samples (0 hours) were utilized as a control samples (background ion spectra) for LC-MS<sup>n</sup> analyses. Samples were analyzed for presence of metabolites with the help of a Thermo LTQ Orbi-Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) capable of MS<sup>n</sup> scanning and accurate mass measurement interfaced with a 3X Ti HPLC pump and CTC PAL autosampler (Leap Technologies, Carrboro, NC). The analytes (injected as 20 μL aliquots) were separated on a Waters Symmetry C18 analytical column (5μm, 2.1 x 150 mm, Waters, Milford, MA) with a 35 minute gradient elution method. Mobile phase A consisted of 10 mM ammonium formate in MS grade water with 0.1% formic acid. Mobile phase B consisted of MS grade acetonitrile with 0.1% formic acid. The sample aliquots were eluted with a flow rate of 0.25 mL/min with 5% B over 5 minutes. Thereafter, the percentage of mobile phase B was gradually increased to 40 %B over 20 minutes and to 95% over 5 minutes. Following the elution of 1 and its metabolite, the column was returned over 1 minute to 5% B, where it was held for 3 minutes before the next injection. ESI in positive mode was carried out at a capillary temperature of 375°C, a sheath gas flow rate of 35 mL/min, source voltage of 4.20 kV, source current of 100 μA, capillary voltage of 35 V and tube lens voltage equal to 100 V. Multipole RF amplifier was set at 400 Vp-p, along with multipole ‘0’ offsets at -1.25 V and -4.75 V, lens ‘0’ voltage at -6.50 V, lens ‘1’ voltage at -13 V, gate lens offset at -62 V, multipole ‘1’ offset at -7 V and front lens at -5 V. The instrument calibration was set at a
multiple RF frequency of 2824 Hz, main RF frequency of 1187.5 Hz and pulsed Q dissociation collision energy factor of 10. Fourier Transformation Mass Spectrometry (FTMS) enabled accurate mass measurement was carried out at a mass resolution of 60,000. Ion Trap Mass Spectrometry (ITMS) based data dependent scan after collision induced dissociation was carried out at a normalized collision energy of 35. Activation Q was set at 0.25 and activation time was 30 milliseconds.

**In vitro Generation of N-Carbamoyl Glucuronide M1.** Glucuronidation experiments were conducted by modification of previously reported protocols (Shaffer et al., 2005; Argikar and Remmel, 2009b; Argikar and Remmel, 2009a). Incubations (0.5 mL) were conducted in 2 mL DW-96 well plates (Analytical Sales and Products, Inc., Pompton Plains, NJ) under CO2-rich atmosphere, at 37°C in a shaking water bath. A constant environment of CO2 was maintained by slow, but continuous purging with CO2 gas. The incubations contained liver microsomes (either rat, human, monkey, hamster, mouse or dog) at a protein concentration of 1 mg/mL or human UGT isoforms (at a protein concentration of 0.2 mg/mL in 0.1 M NaHCO3 buffer (pH 7.5), substrate, 1 (20 μM), alamethicin (50 μg), MgCl2 (5mM), D-saccharolactone (1 mg/mL). Each reaction was started by the addition of UDPGA (3.2 mg/mL). After 2 hours of incubation, the reaction was quenched with 0.5 mL of MeCN and centrifuged for 5 minutes at 4630 x g. The resulting supernatants were transferred to a clean DW-96 well plates with glass inserts (1 mL) for LC-MSn analysis.

**In vitro Generation of N-Carbamoyl Glucuronide M1 using 13CO2.** Incubations (1 mL) were conducted in round bottom borosilicate glass tubes under 13CO2-rich atmosphere at 37°C in a
shaking water bath. $^{13}$CO$_2$ gas was generated by addition of HCl to a saturated solution of NaH$^{13}$CO$_3$. A constant environment of CO$_2$ was maintained by slow, but continuous purging with CO$_2$ gas. The incubations contained rat liver microsomes at a protein concentration of 1 mg/mL in 0.1 M NaHCO$_3$ buffer (pH 7.5), substrate, i.e., 1 (20 $\mu$M), alamethicin (50 $\mu$g), MgCl$_2$ (5mM), D-saccharolactone (1 mg/mL). The reaction was started by the addition of UDPGA (3.2 mg/mL). After 2 hours of incubation, each reaction was quenched with 1 mL of MeCN and centrifuged for 5 minutes at 4630 x g. The resulting supernatants were transferred to a clean DW-96 well plate with glass inserts (1 mL) for LC-MS$n$ analysis.

**In vitro Generation of N-Carbamoyl Glucuronide M1 using KH$_2$PO$_4$ buffer.** Incubations (0.5mL) were conducted in a DW-96 well plate (2 mL) under CO$_2$-rich atmosphere at 37$^\circ$C in a shaking water bath. Each incubation contained rat liver microsomes (protein concentration of 1 mg/mL ) in 0.1 M KH$_2$PO$_4$ buffer (pH 7.5), substrate, 1 (20 $\mu$M), alamethicin (50 $\mu$g/mg of protein), MgCl$_2$ (5 mM), D-saccharolactone (1 mg/mL) and UDPGA (3.2 mg/mL). After 2 hours of incubation, the reactions were quenched with 0.5 mL of MeCN and centrifuged for 5 minutes at 4630 x g. The resulting supernatants were transferred to a clean DW-96 well plate with glass inserts (1 mL) for LC-MS$n$ analysis.

**Treatment of M1 with $\beta$-Glucuronidase.** The bile sample collected from 0 - 4 hours (0.5 mL) containing M1 was treated with $\beta$-glucuronidase (0.5 mL) at an enzyme concentration of 5000 unit/mL. Control bile sample (0.5 mL) was treated with KH$_2$PO$_4$ buffer (0.5 mL, pH 7.4). Each solution was incubated in a shaking water bath at 37$^\circ$C for two hours. Aliquots were obtained
from the incubation at 0 and 2 hours. Each time point was quenched with MeCN (0.2 mL),
vortexed and centrifuged at 6440 x g for 5 minutes before removing the supernatant for analysis
by LC-MSn.
Results

Structural rationalization of 1. Upon ESI in positive ion mode at a collision energy of 35, 1 showed a protonated molecular ion [M+H] of m/z 322. The LC retention time was approximately 15.5 minutes (Figure 1). The CID product ion spectrum (Figure 2) afforded fragment ions with m/z values of 305, 222, 205 and 101. Fragment ion m/z 305 was formed by the loss of ammonium moiety. Fragment ion m/z 222 was formed by the loss of tetrahydropyrimidin-2(1H)-one moiety. Subsequent loss of ammonia from fragment ion m/z 222 resulted in an ion of m/z value 205. Fragment ion m/z 101 was confirmed as tetrahydropyrimidin-2(1H)-one moiety of 1 by accurate mass measurement.

Structural rationalization of M1, N-carbamoyl glucuronide of 1. ESI of M1 in positive ion mode at a collision energy of 35 resulted in a protonated molecular ion of m/z value 542, higher than 1 by 220 amu (Figure 3). This was detected at retention time of approximately 19.1 minutes (Figure 1). The CID product ion spectrum of M1 afforded (1 + 44 + 176) suggesting an addition of CO2 and glucuronic acid. A peak at m/z 542 was also observed as a single peak in a neutral loss of 176 scan. The fragment ion m/z 366 resulted from the loss of a glucuronide moiety as a neutral fragment and fragment ion m/z 322 corresponding to 1, resulted from the loss of glucuronide and CO2 moieties. These indicative fragments revealed that M1 was an N-carbamoyl glucuronide of 1.

Results from β-glucuronidase treatment. As described in the methods section, the rat bile containing M1 was treated with β-glucuronidase for two hours. The enzyme treated bile sample showed almost complete loss of M1 and a marked generation of the parent, 1 in comparison to
the control sample containing buffer instead of the enzyme β-glucuronidase. No parent was observed in the pretreatment control (0 - 4 hours bile sample, with no treatment with β-glucuronidase). The corresponding carbamic acid of 1 is presumably unstable and was not observed after β-glucuronidase mediated hydrolysis. This data supports the fact that m/z 542 was indeed a glucuronide that reverted back to the parent, as illustrated in Figure 4.

**Results from experiments with $^{13}$C-labeled CO$_2$.** In experiments carried out under an environment of labeled $^{13}$CO$_2$, m/z corresponding to 543 was observed (Figure 5). This matched with the expected stable labeled N-carbamoyl glucuronide of 1 containing $^{13}$CO$_2$. Furthermore, MS/MS of ion 543 revealed m/z 367 along with other fragments that accurately matched the fragment ions from MS/MS of m/z 542 (the N-carbamoyl glucuronide containing CO$_2$). The fragment m/z 367 corresponded to a stable labeled carbamic acid of 1 (Figure 5). Due to the incorporation of labeled $^{13}$CO$_2$, not only was m/z 367 one amu higher than the previously identified m/z 366; but also was in disagreement with the Nitrogen Rule, as expected. This shows that incorporation of $^{13}$CO$_2$ to yield $^{13}$C-labeled M1 was successful in an environment of $^{13}$CO$_2$.

**Species selectivity and phenotyping.** To compare the formation of M1 across species, incubations were carried out with 1 in liver microsomes from multiple species. Among the species tested, M1 was formed readily in rat and hamster, followed by comparable formation in dog, monkey, mouse and human (Figure 6). Due to lack of a synthetic standard of M1, formation of M1 relative to 1 was based on MS response and was considered a measure of the extent of M1 formation in incubations with liver microsomes and cloned expressed UGTs. UGT phenotyping
experiments in UGT supersomes® under an environment of CO₂ revealed that formation of M1 was catalyzed by UGT1A3, UGT1A1 and UGT2B7, as shown in (Figure 7).
Discussion

N-carbamoyl glucuronidation is a rarely observed disposition process. To date N-carbamoyl glucuronidation has been reported for a small number of drugs containing primary and secondary amino functionalities, for example, sitagliptin (Vincent et al., 2007) and sertraline (Obach et al., 2005). Few of the earlier reports for N-carbamoyl glucuronidation were indeed for tocainide (Elvin et al., 1980; Ronfeld et al., 1982). Since then, such a metabolic pathway has been reported for rimantidine (Brown et al., 1990), carvedilol (Schaefer, 1992), mofegiline (Dow et al., 1994) and most recently for amosulalol (Suzuki and Kamimura, 2007), in addition to sitagliptin and sertraline.

Carbamic acids are known to be intrinsically unstable and are likely forming without enzymatic catalysis under physiological conditions. However, there has been some evidence for characterization of carbamic acids of xenobitics in solution phase by NMR analysis. Carbamic acid derivatives of piperazine (Kirsch et al., 2000) and des-methyl org1377 (Delbressine et al., 1990) were identified in this manner, generated after reacting NaHCO3 with respective parent molecules. However, stable carbamic acids have also been reported by Shaffer and coworkers (Shaffer et al., 2005) and Hayakawa and colleagues (Hayakawa et al., 2003). A transesterification type reaction has been employed as a method of trapping an unstable carbamic acid. Straub and coworkers were successful in isolation of ethyl ester of benzazepine after ethanolysis of benzazepine N-carbamoyl glucuronide in the presence of sodium metal and anhydrous ethanol (Straub et al., 1988). Liu and co-workers employed a similar ethanolysis reaction to form an ethyl ester of a carbamate of “compound I” (Liu et al., 2001).
N-carbamoyl glucuronides of unstable carbamic acids can convert back to parent moieties upon hydrolysis (Tremaine et al., 1989), or even during sample preparation. Rimantidine-N-carbamoyl glucuronide identified by post-trimethylsilyl or pentafluorobenzyl derivatization GC-MS, was documented to revert back to the parent after treatment with gluculase (Brown et al., 1990). For N-carbamoyl glucuronides of unstable carbamic acids, complete hydrolysis back to the parent compound has been reported after enzymatic hydrolysis. In the present study, we were unable to isolate and identify the corresponding carbamic acid of 1 upon treatment with β-glucuronidase (Figure 4). However, we were able to observe complete conversion of the glucuronide to the parent, as compared to the control. Furthermore, the fragment ion m/z 322 corresponding to 1 was formed by loss of both CO₂ and glucuronide moieties. This is diagnostic fragmentation pattern for N-carbamoyl glucuronides (Shaffer et al., 2005), coupled with the accurate mass enabled LC-MSⁿ characterization of the intact N-carbamoyl glucuronide provides firm evidence for the novel N-carbamoyl glucuronide formation.

The mechanism of N-carbamoyl glucuronidation has not been experimentally proven until now. Spontaneous formation of carbamic acids has been documented for amino acids in the literature (Morrow et al., 1974). Formation of carbamic acids is proposed to be pH dependent. As amines may be protonated at lower pH values and the concentration of dissolved carbon dioxide decreases with increase in pH, formation of carbamate is optimal in the pH range of 6 to 9 (Schaefer, 2006). Additionally, due to their nature of being relatively unionized at physiological pH of 7.4, amino groups with lower pKa may form corresponding carbamic acids more readily. We attempted to identify the source of carbon dioxide that results in formation of an N-carbamoyl glucuronide. In vitro incubations with 1 were carried out in RLM under a saturated
labeled $^{13}$CO$_2$ environment at atmospheric pressure, in NaHCO$_3$ buffer. Control incubations were carried out under a saturated environment of CO$_2$ at atmospheric pressure, with NaHCO$_3$ buffer.

MS/MS analysis revealed that $N$-(13C)carbamoyl glucuronide was formed in the earlier incubations in proportions equal to $N$-(12C)-carbamoyl glucuronide (Figure 5). $N$-(12C)-carbamoyl glucuronide alone was formed in control incubations. This finding of incorporation of $^{13}$CO$_2$ in the product in equal proportions to CO$_2$ strongly indicates the prevalence of an equilibrium between CO$_2$(gaseous) and dissolved CO$_2$. At the incubation pH of 7.4, the equilibrium extends further between the dissolved CO$_2$ and the various anionic forms of bicarbonate. Whether the formation of the corresponding glucuronide occurs as a sequential reaction after formation of carbamic acid of 1 or if the reaction proceeds through a concerted mechanism with a transition state involving the substrate, CO$_2$ and UDPGA in the active site of UGTs cannot be deciphered at this time.

In the process of studying $N$-carbamoyl glucuronidation of carvedilol in dog and rat liver microsomes, Schaefer calculated the dissolved CO$_2$ in the in vitro incubation medium (Schaefer, 1992). Bicarbonate buffer was reported to have 200 fold higher dissolved CO$_2$ than phosphate buffer at atmospheric pressure. Furthermore, incubations containing bicarbonate buffer under a saturated CO$_2$ environment resulted in ten fold higher CO$_2$ concentrations than those containing bicarbonate buffer devoid of a CO$_2$ environment. We wanted to further elucidate the role of bicarbonate buffer in formation of 1-$N$-carbamoyl glucuronide. Therefore, the formation of M1 was evaluated in incubations containing NaHCO$_3$ buffer at pH 7.4 and potassium phosphate buffer at pH 7.4. As measured by LC-MS/MS, the formation of M1 was approximately 16 fold higher in the incubation containing NaHCO$_3$ buffer as compared to the corresponding incubation
in phosphate buffer, under an environment of CO₂. This result supplements the equilibrium hypothesis and suggests that bicarbonate buffer may directly facilitate the carbamic acid formation by this equilibrium mechanism and hence, N-carbamoyl glucuronide formation due to higher amounts of dissolved CO₂.

Preceding information reveals lack of species specificity for formation of N-carbamoyl glucuronides. In vitro, N-carbamoyl glucuronides have been reported in a wide range of species, for example, for carvedilol in rat and dog liver microsomes (Schaefer, 1992), BVT.2938 in rat, monkey and human liver microsomal incubations (Edlund and Baranczewski, 2004) for ILT-threo and ILT-allo (Beconi et al., 2003). N-carbamoyl glucuronides have not only been reported in \textit{in vitro} incubations, but also in \textit{in vivo} tissues or body fluids. For example, N-carbamoyl glucuronide conjugate of mofegiline was identified in dog urine (Dow et al., 1994), amosulol in mouse bile (Suzuki and Kamimura, 2007), varenicline in rat, monkey and human plasma and urine (Obach et al., 2006), ropinrole in monkey and human urine (Ramji et al., 1999) and that of tocainide was identified in urines of guinea pig, dog, cat, rabbit and monkey (Gipple et al., 1982). We attempted to examine if M1 was formed across multiple species and to measure the ratio of relative intensities of the N-carbamoyl glucuronide metabolite (M1/parent) across a variety of species. Among the species tested, M1/parent ratio was maximal in liver microsomal incubations from rat and hamster, followed by comparable ratios in dog, human, monkey and mouse (Figure 6). The extent of M1 formation in vivo, in the species mentioned earlier was beyond the scope of this investigation and remains unknown.
Literature reports on human UGT reaction phenotyping studies for N-carbamoylation reaction are even fewer. Varenicline-N-carbamoyl metabolite was reported to be formed by UGT2B7 only (Obach et al., 2006). Obach and co-workers reported that sertraline-N-carbamoyl glucuronidation was carried out principally by UGT2B7 in vitro. Measurable activities were also observed with UGT1A3, UGT1A6 and UGT2B4 (Obach et al., 2005). In the current investigation, we carried out in vitro incubations with cloned expressed UGT Supersomes® to identify the isozymes responsible for N-carbamoyl glucuronidation of 1. We identified that UGT1A1, UGT1A3 and UGT2B7 were responsible for N-carbamoyl glucuronidation of 1 in vitro. Based on the metabolite to parent mass spectrometric responses (M1/parent) in incubations carried out with recombinant UGT enzymes, UGT1A3 showed the maximal contribution in vitro (Figure 7). It must be noted however, that these results are based on the relative activity of each UGT isozymes in the Supersomes® and may not represent the relative contribution of each UGT isozymes in vivo.

N-carbamoyl glucuronides, being rare are not as well studied from a bio-analytical perspective. Liu and Pereira reported an identification of an N-carbamoyl glucuronide of a thiazolidine containing ‘compound I’, that was initially believed to be a mass spectroscopic interference (Liu and Pereira, 2002). Additionally, in-source fragmentation may lead to a dramatic decrease in signal or even lack of detection of N-carbamoyl glucuronides, especially if these glucuronides co-elute with the parent. It is worth mentioning that more carbamoyl glucuronides have been identified in the past decade than before. Whether this can be attributed to newer mass spectrometers, advanced soft ionization techniques such as electrospray ionization and advanced data acquisition techniques like data dependent scanning methodology, or if this is due to an
increasing number of biotransformation studies on compounds containing primary and secondary amino groups remains to be ascertained.

Mechanistic studies with $^{13}$C-labeled CO$_2$ suggest that formation of M1 was driven by exogenous CO$_2$ (in equilibrium with CO$_2$ dissolved in the buffer) in rat liver microsomes. This to our knowledge is the first report documenting the formation of N-carbamoyl glucuronidation by employment of $^{13}$CO$_2$ gas. Treatment of M1 with β–glucuronidase resulted in the formation of 1 and not the corresponding carbamate. It cannot be ascertained at this point if 1 forms a carbamate before glucuronidation or whether the formation of M1 is a concerted process. Our data suggests that the N-carbamoyl glucuronide metabolite (M1) of 1 was observed in vitro in liver microsomes, across all the tested species, namely – rat, mouse, hamster, dog, monkey and human. Based on the ratio of relative ion intensities (M1/parent), the N-carbamoyl glucuronide was formed in rat and hamster liver microsomes to a greater extent as compared to other species. UGT1A1, UGT1A3 and UGT2B7 (Supersomes®) were observed to catalyze the N-carbamoyl glucuronidation of 1. It is noteworthy that human UGTs other than human UGT2B7 may be involved in formation of carbamoyl glucuronides, as demonstrated by the novel N-carbamoyl glucuronide of 1. In conclusion, our study demonstrates the MS$^n$ based characterization, identification of mechanism of formation and cross-species comparison of a novel N-carbamoyl glucuronide metabolite (M1), in addition to UGT phenotyping.
Acknowledgements

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Figure Legend

Figure 1

Base Peak MS Chromatogram of 1 and its N-carbamoyl glucuronide Metabolite (M1) in vivo (rat bile), are represented. With the gradient and column conditions as mentioned in the methods section, 1 eluted at 15.5 minutes and M1 eluted at 19.1 minutes.

Figure 2

MS² spectrum of 1 obtained in positive ion mode electrospray ionization is shown in the figure. Collision induced dissociation was carried out at a collision energy of 35. The parent, 1 is seen as m/z 322, at approximately 10% signal intensity as compared to the base peak m/z 101. Other diagnostic fragments include ions obtained from piperimidinone and cyclohexyl moieties of the parent m/z 322.

Figure 3

MS² spectrum of M1, the putative N-carbamoyl glucuronide of 1, as obtained in positive ion mode electrospray ionization is shown in the figure. Collision induced dissociation was carried out at a collision energy of 35. M1, m/z 542 completely fragments to result in ions - m/z 366, equivalent to the corresponding carbamate and m/z 322, matching the parent.

Figure 4

Illustration of β-glucuronidase mediated hydrolysis of M1 in rat bile samples collected from 0-4 hours post-dose. Upon treatment with β-glucuronidase, M1 converted back to the parent compound, 1. Corresponding carbamic acid of 1 could not be detected.
**Figure 5**

MS² spectra of N-carbamoyl glucuronide metabolite of 1 (M1) generated *in vitro* by carrying out the incubation of 1 in RLM, in a CO₂ environment (top) and ¹³CO₂ environment (bottom). Each chromatogram is represented in terms of relative intensity of M1 peak. The retention times and absolute intensities of M1 in incubations under CO₂ environment and ¹³CO₂ environment were similar; 19.1 minutes each, and 7.2 x 10⁴ and 7.4 x 10⁴ respectively.

**Figure 6**

Cross species comparison. M1 formation, in liver microsomal incubations from multiple species, was carried out in NaHCO₃ buffer under an environment of CO₂. The relative formation of M1 is depicted as a ratio of ion intensities of M1/1, as measured in duplicate by LC-MSⁿ.

**Figure 7**

UGT Phenotyping. Evaluation of formation of M1 in human UGT supersomes®. *In vitro* incubations with UGT1A1, UGT1A3 and UGT2B7 resulted in formation of M1, when carried out under a, environment of CO₂ in NaHCO₃ buffer. The relative formation of M1 is depicted as a ratio of ion intensities of M1/1, as measured in duplicate by LC-MSⁿ.
Figure 4

Cl

\[ \begin{align*}
\text{Cl} & \quad \text{NH}_2 \\
\text{Cl} & \quad \text{NH} \\
\text{Cl} & \quad \text{OH} \\
\text{Cl} & \quad \text{CO}_2 \\
\end{align*} \]

N-Cabamoyl glucuronide (M1)

β-glucuronidase

Carbamate
Figure 5

322

366

322

367

M1
Figure 6

Relative Formation of M1

- Human
- Dog
- Monkey
- Rat
- Mouse
- Hamster