Identification of a Discrete Diglucuronide of GDC-0810 in Human Plasma after Oral Administration

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GDC-0810 is a small molecule therapeutic agent having potential to treat breast cancer. In plasma of the first-in-human study, metabolite M2, accounting for 20.7% of total drug-related materials, was identified as a discrete diglucuronide that was absent in rats. Acyl glucuronide M6 and N-glucuronide M4 were also identified as prominent metabolites in human plasma. Several in vitro studies were conducted in incubations of [14C]GDC-0810, synthetic M6 and M4 with liver microsomes, intestinal microsomes, and hepatocytes of different species as well as recombinant UDP-glucuronosyltransferase (UGT) enzymes to further understand the formation of M2. The results suggested that 1) M2 was more efficiently formed from M6 than from M4, and 2) acyl glucuronidation was mainly catalyzed by UGT1A8/7/1 that is highly expressed in the intestines whereas N-glucuronidation was mainly catalyzed by UGT1A4 that is expressed in the human liver. This complicated mechanism presented challenges in predicting M2 formation using human in vitro systems. The absence of M2 and M4 in rats can be explained by low to no expression of UGT1A4 in rodents. M2 could be the first discrete diglucuronide that was formed from both acyl- and N-glucuronidation on a molecule identified in human plasma.

SIGNIFICANCE STATEMENT
A discrete diglucuronidation metabolite of GDC-0810, a breast cancer drug candidate, was characterized as a unique circulating metabolite in humans that was not observed in rats or little formed in human in vitro system.
Metabolite profiling during the first-in-human study of GDC-0810 led to identification of a novel discrete diglucuronide metabolite (M2) as a primary circulating metabolite that had not been previously detected. Two different types of diglucuronides have been reported in literature. Type I is a result of an additional glucuronidation on the first glucuronide’s sugar moiety, called a “linked diglucuronide” (Murai et al., 2005; Argiak, 2012). Type II is a result of glucuronidation that occurs at two different functional groups on the same molecule. These compounds are called bis-glucuronides, or more accurately “discrete diglucuronides.” M2 is a type II discrete diglucuronide that was formed via acyl-glucuronidation and N-glucuronidation of GDC-0810.

The current study aims to understand the biotransformation of GDC-0810 for formation of M2 in vitro systems (liver microsomes, intestinal microsomes, hepatocytes, and recombinant UDP-glucuronosyltransferase [UGT] enzymes) and in vivo systems (rat mass balance and cold human plasma). The results suggest that M2 was mainly formed by sequential glucuronidation at the acyl moiety followed by N-glucuronidation at indazole of GDC-0810.

Materials and Methods

Chemicals and Reagents

GDC-0810 and its acyl-glucuronide (M6) and N-glucuronide (M4) metabolites as well as M8 were synthesized by Genentech (San Francisco, CA). [14C]GDC-0810 was synthesized by Selcia (Essex, UK) with a specific radioactivity of 8.8 kBq/mg. Acetonitrile (ACN) was purchased from EMD Chemicals (Gibbstown, NJ). Ultrapure HPLC water and formic acid were purchased from J.T. Baker (Center Valley, PA). Ammonium formate and ammonium hydroxide solution were purchased from Fluka (St. Louis, MO). Sodium citrate, dimethyl sulfoxide, alamethicin, uridine 5'-diphospho-glucuronic acid (UDPGA), the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), cysteine (Cys), glutathione (GSH), Trypan blue solution, and internal standard propranolol were purchased from Sigma-Aldrich (St. Louis, MO). 100 mM of Tris buffer and potassium phosphate buffer (pH 7.4), as well as magnesium chloride were provided from in-house media preparation group. Pico-Fluor 40 Carbon-14 cocktail for liquid scintillation counting (LSC) was purchased from PerkinElmer (Waltham, MA). InVitroGRO HT thawing medium was purchased from BioIVT (Westbury, NY), and Gibco Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Thermo Fisher Scientific (Waltham, MA).

Cryopreserved hepatocytes were purchased from BioIVT (Westbury, NY) and prepared from both male and female human donors (n = 10), male monkeys, dogs, and rats, and female mice and rabbits (n = 10). Liver microsomes and liver S9 fractions (20 mg/ml of protein) were obtained from BD Biosciences (Billerica, MA) and prepared from female human donors (n = 10), male monkeys, dogs, and female rats, mice, and rabbits. Intestinal microsomes were purchased from XenoTech (Kansas City, KS) and prepared from female human donors, male monkeys and dogs, and female rats and mice. Recombinant UGT enzymes (rUGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17) were purchased from Corning (Corning, NY).

Methods

In Vitro Incubations.

Incubation mixtures contained [14C]GDC-0810 (10 μM) in 100 mM potassium phosphate (pH 7.4), 1 mg/ml of liver microsomal protein or 1 mg/ml of intestinal microsomal protein, 5 mM MgCl2, 25 mg/ml of alamethicin, and 2 mM UDPGA with or without 2 mM NADPH in a final volume of 0.5 ml. The incubations were performed on a shaker at 50 rpm for 2 hours in a 37°C water bath. The reactions were quenched by protein precipitation with 10 volumes of ACN containing 0.1% formic acid. Following centrifugation at 2000 x g for 20 minutes, the resulting supernatant was concentrated to near dryness under nitrogen evaporation and reconstituted with 150 μl of water: ACN (2:1, v/v). The radioactivity in the supernatant was quantitatively recovered. The samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS) with fraction collection.

GDC-0810, M4, or M6 were also incubated in human liver microsomes each at 10 μM, as well as in human liver S9 fractions at 20 μM under similar conditions, and analyzed by LC-MS according to the short gradient method (see below). The remaining molecules were incubated as described in the previous paragraph.

Human recombinant UGT incubation mixtures contained GDC-0810, M4, or M6 (10 μM), 100 mM Tris buffer (pH 7.4), 0.25 mg/ml of protein of recombinant enzymes UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, or 2B17, 5 mM MgCl2, and 5 mM UDPGA in a final volume of 200 μl. The incubation was performed on the Jitterbug microplate incubator shaker from Boekel Scientific (Feasterville, PA) for 1 hour in 37°C. The reactions were stopped by adding 50 μl ACN containing the internal standard, propranolol (0.05 μM in final). Following centrifugation at 2000 x g for 20 minutes, the resulting supernatants were directly injected onto the LC-MS according to the short gradient method.

[14C]GDC-0810 was incubated with cryopreserved hepatocytes, which were thawed using prewarmed (37°C) InVitroGRO HT thawing medium in 50-ml centrifuge tubes. The tubes were centrifuged for 5 minutes at 60 x g and the supernatants were discarded. Cells were resuspended with 50 ml of pre-warmed DMEM by gently inverting the tubes several times. The tubes were centrifuged for 5 minutes at 60 x g and the supernatants were discarded. The total cell count and the number of viable cells were determined by the trypan blue exclusion method. Incubations were carried out in scintillation vials containing 1.0 ml of hepatocyte suspension (65%–85% viable). Cells were suspended in pre-warmed DMEM incubation medium to approximately 2 x 10⁶ cells/ml. The hepatocytes were incubated with a final [14C]GDC-0810 concentration of 10 μM, a final dimethyl sulfoxide concentration of 0.1%, and less than 0.5% of ACN. The scintillation vials were placed on an orbital shaker rotating at 50 rpm in an incubator for 4 hours at 37°C. The hepatocyte incubation samples were protein precipitated with 10 volumes of ACN containing 0.1% formic acid and centrifuged at 2000 x g for 20 minutes. The radioactivity in the supernatant was quantitatively recovered. The resulting supernatant was concentrated to a near-dry residue under nitrogen evaporation and reconstituted with 150 μl of water:ACN (2:1, v/v). The samples were analyzed by LC-MS with fractions collected at 15-second intervals and analyzed by TopCount solid scintillation counting (PerkinElmer, Shelton, CT).

Drug Dosing, Sample Collection, and Analysis. Eight female human subjects each received a single oral administration of 600 mg GDC-0810. The plasma samples pretreated with citric acid (final concentration of 25 mM) were collected at 0-, 0.5-, 1-, 1.5-, 2-, 3-, 4-, 6-, and 8-hour time points on day 1 (single dose) and at the corresponding time points on day 7 (the steady state). The samples were pretreated with sodium citrate (final concentration of 25 mM). The plasma samples from four subjects were area under the curve (AUC)-poled such that all the collections on day 1 (from 0 to 8 hours) were combined and all collections on day 7 were combined (Hop et al., 1998). Plasma samples were treated with three volumes of ACN to precipitate the protein. After centrifugation, the supernatants were evaporated to approximately 150 μl using a SpeedVac concentrator, and ACN was added to a volume of approximately 300 μl. After a second centrifugation, supernatants (10 μl) were injected into the LC-MS.

The human study was performed in accordance with the principles of the Declaration of Helsinki and its amendments, and the study protocol was approved by the institutional review board and radiation safety committee at the investigational site. All subjects were in good health and gave written, informed consent to participate in the study. All of other details were mentioned in the supplemental.

All animal housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Animal rooms were maintained on a 12-hour light/dark cycle. The rat mass balance study, study information, sample pooling and extractions, radio-analysis and pharmacokinetic calculations, assessment of extractions and column recoveries, metabolite profiling, and offline radio-detection are all summarized in the supplemental section.

Mass Spectrometric Methods. The protonated molecular ions ([M+H]+) in positive ion mode for analytes were determined from full scan mass spectrometry data acquired using an LTQ-Orbitrap and Fusion LUMOS high-resolution mass spectrometers equipped with an electrospray ionization source (Thermo Scientific, San Jose, CA). The electrospray voltage was set at 4.0 kV, and the
capillary temperature was 270°C. Full-scan mass spectra were obtained at a resolving power of 30,000 with accurate mass measurements using external calibration. The corresponding data dependent tandem mass spectrometry (MS/MS) scans were acquired at a resolving power of 7500 and collision induced dissociation with a collision energy range of 15–35 ev.

**Liquid Chromatography Separation Methods.** Two chromatographic methods were used. The first one, long gradient (60 minutes) and a high flow rate (1.0 ml/min), was intended for experiments with radioactive material. An ultra-performance liquid chromatography coupled with UV detection (UV) system was equipped with an Accela solvent delivery system (built-in degasser) and an Accela Photodiode Array Detector from Thermo Scientific (San Jose, CA), hybridized with an HTS PAL autosampler and a Pal HTS-XT fraction collector from Leap Technologies (Carrboro, NC). A Polaris C18-A column with 4.6 × 150 nm, 3 μm from Agilent Technologies (Santa Clara, CA) was used. The autosampler temperature and column temperature were maintained at 10°C and 30°C, respectively. Samples were injected in 10:1 post-column split mode. Mobile phase A was 10 mM ammonium formate in water with 0.1% formic acid, and mobile phase B was ACN. The gradient started with 5% B and increased to 37% from 1 to 7 minutes. It then increased to 50% B from 24 to 32 minutes and then to 95% B from 50 to 52 minutes. An organic wash for 3 minutes returned the column to its initial conditions before the next injection 5 minutes later.

Another short gradient method (25 minutes) with low flow rate (0.4 ml/min) used for nonradioactive material. A Synergi Polar-RP 100A column at 100 × 2 mm, 2.5 μm from Phenomenex (Torrance, CA) was used. There was no post-column split. The gradient started with 5% B and increased to 39% from 1 to 3 minutes. The gradient increased to 43% B from 11 to 19 minutes and then to 95% B within 2 minutes. An organic wash for 2 minutes returned the column to its initial conditions before the next injection 2 minutes later.

Results

**Chromatographic Separation and Evaluation of Metabolite Stability.** GDC-0810 and its isomer M8, as well as glucuronide metabolites M2, M4, and M6 were separated well chromatographically with both long and short gradient methods as shown in Supplemental Fig. 1, A and B. Their mass spectra as well as M1 spectrum were reported in Supplemental Fig. 2C. Chromatogram comparisons of M6 and its migration in stock solution from a contract research organization, freshly prepared stock solution from powder, and fresh human plasma (day 1) were evaluated as shown in Supplemental Fig. 3A.

M1/M2 was tentatively identified as an acyl-migration isomer (Table 2). Significant acyl-migration of M2 was found to form M1 only in a year-old plasma sample (Supplemental Fig. 3B). From UV trace of Supplemental Fig. 3A, 8%, 1%, and <1% acyl-migration were observed for stock solution of M6 from a contract research organization, freshly prepared stock solution from powder, and fresh human plasma, respectively, which gives us confidence that acyl-migration of M6 is not an issue in human plasma profiling if samples were pretreated with citric acid. Chemical stabilities of M4 and M6 were evaluated in different pH in 1% and 0.1% formic acid, 7.4 and 8.5. M4 was stable up to 3 days at room temperature except in 1% formic acid as indicated in Supplemental Fig. 4A, and M6 was stable up to 5 days at room temperature except in 1% formic acid as shown in Supplemental Fig. 4B. Acyl-migration of M6 was evaluated and was found stable under different conditions. M6 has much poorer ionization efficiency than M4 (up to 50-fold) in positive ion mode as shown in Supplemental Fig. 1A, therefore, the estimation of M4 formation with peak area based on mass spectrometry response could be significant underestimation (by ~98%).

![Fig. 1. Proposed metabolic pathways of GDC-0810 in plasma of humans following oral administration.](image-url)
Metabolite Identification. GDC-0810 metabolites were identified in samples from the first-in-human study, various in vitro studies, and in vivo rat mass balance studies. The metabolites were mainly formed as a result of phase I oxidation and phase II glucuronidation (Table 1). The following metabolites were identified based on high resolution full scans followed by fragmentation patterns and comparisons with synthetic standards: M1 and M2 (discrete diglucuronidation), M3 (oxidative diglucuronidation), M4 (N-glucuronidation), M5/M5a/M5b/M5c (acyl-glucuronidation plus mono-oxidation at various sites), M6 (acyl-glucuronidation), M7/M7a/M7b/M13 (mono-oxidation at various sites), and M8 (a minor isomer of GDC-0810).

M2 is the Major Circulating Metabolite in Human Plasma. GDC-0810 is the major component (57%–92% of total UV absorbance) in AUC-pooled plasmas from human subjects (Table 2). In particular, GDC-0810, M2, M4, and M6 represent 57%–73%, 13%–29%, 0%–2%, and 6%–12% of total UV absorbance, respectively, in human plasmas at day 7 (multiple doses at steady state). Therefore, M2, the discrete diglucuronide, is a major circulating metabolite in human. The results also suggest significant individual variability in metabolite formation. A scheme of the proposed metabolic pathways of GDC-0810 human plasma is presented in Fig. 1.
Metabolic Profiles of [14C]GDC-0810 in Liver and Intestinal Microsomes of Humans and Animal Species. The results of liver microsomal incubations with [14C]GDC-0810 in the presence of UDPGA and/or NADPH are shown in Table 3 and Fig. 2, as well as in Supplemental Fig. 5 and Supplemental Fig. 6. The discrete diglucuronide M2 was detected only in human liver microsome incubations but at a trace level. In contrast to the in vivo observations, the acyl-glucuronide M6 was the major human metabolite in these incubations. M6 was also the major metabolite across all species tested in liver microsomes. The N-glucuronide M4 was a major metabolite in human but not in animal species, even not detected in rat and mouse.

Formation of M2, M4, and M6 were investigated in intestinal microsomes in the presence of UDPGA and NADPH. M2 and M4 were not observed in these incubations. M6 was as a minor metabolite in human (0.4%) and as a major metabolite in monkey (26.4%) and dog (17.4%). The results are presented in Supplemental Table 1 and Supplemental Fig. 7 and Supplemental Fig. 8.

**TABLE 2**
Estimated relative abundance of GDC-0810 and its metabolites in AUC pooled (0–8 h) human plasma samples at day 1 (single dose) following daily oral doses of 600 mg

<table>
<thead>
<tr>
<th>Analyte</th>
<th>m/z</th>
<th>Biotransformation</th>
<th>RT (min)</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Ave (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0810</td>
<td>447.1256</td>
<td>Parent</td>
<td>17.3</td>
<td>92.0</td>
<td>80.0</td>
<td>90.6</td>
<td>67.6</td>
<td>82.5</td>
</tr>
<tr>
<td>M1</td>
<td>799.1924</td>
<td>Discrete diglucuronidation</td>
<td>4.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2</td>
<td>799.1922</td>
<td>Discrete diglucuronidation</td>
<td>4.9</td>
<td>3.1</td>
<td>9.7</td>
<td>3.6</td>
<td>19.8</td>
<td>8.9</td>
</tr>
<tr>
<td>M3</td>
<td>815.1877</td>
<td>Oxidative diglucuronidation</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M4</td>
<td>623.1580</td>
<td>N-glucuronidation</td>
<td>7.0</td>
<td>1.0</td>
<td>2.2</td>
<td>1.2</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>M5</td>
<td>639.1535</td>
<td>Oxidative acyl-glucuronidation</td>
<td>7.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M6</td>
<td>623.1585</td>
<td>Acyl-glucuronidation</td>
<td>10.0</td>
<td>3.1</td>
<td>6.6</td>
<td>4.6</td>
<td>4.6</td>
<td>7.1</td>
</tr>
<tr>
<td>M7</td>
<td>463.1212</td>
<td>Mono-oxidation</td>
<td>13.4</td>
<td>0.8</td>
<td>1.4</td>
<td>0.6</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>M8</td>
<td>447.1263</td>
<td>Isomer of parent</td>
<td>15.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M9</td>
<td>472.1256</td>
<td>Parent</td>
<td>17.3</td>
<td>72.7</td>
<td>65.2</td>
<td>57.1</td>
<td>62.9</td>
<td>64.5</td>
</tr>
</tbody>
</table>

**TABLE 3**
Percent radioactivity of [14C]GDC-0810 and its metabolites in incubations with liver microsomes of human, monkey, dog, rat, mouse, and rabbit in the presence of UDPGA and NADPH

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Distribution of Radioactivity of [14C]GDC-0810 from Incubations in Liver Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>CLM</td>
</tr>
<tr>
<td>GDC-0810</td>
<td>51.0</td>
</tr>
<tr>
<td>M1/M2</td>
<td>D</td>
</tr>
<tr>
<td>M4</td>
<td>18.3</td>
</tr>
<tr>
<td>M5</td>
<td>1.2</td>
</tr>
<tr>
<td>M6</td>
<td>20.0</td>
</tr>
<tr>
<td>M7</td>
<td>D</td>
</tr>
<tr>
<td>M7a</td>
<td>ND</td>
</tr>
<tr>
<td>M7b</td>
<td>0.9</td>
</tr>
<tr>
<td>M13</td>
<td>ND</td>
</tr>
<tr>
<td>M14</td>
<td>1.8</td>
</tr>
<tr>
<td>M15</td>
<td>1.0</td>
</tr>
</tbody>
</table>

CLM, cynomolgus monkey liver microsomes; D, detected by LC-MS only; DLM, dog liver microsomes; HLM, human liver microsomes; MLM, mouse liver microsomes; RLM, rat liver microsomes; RabLM, rabbit liver microsomes; ND, not detected.

Metabolic Profiles of [14C]GDC-0810 in Hepatocytes. Consistent with the liver microsomal results, M2 was only detected in human hepatocytes at a low level by mass spectrometry, not by radioactive detector (Supplemental Table 2; Fig. 3). M4 was detected as a unique and major metabolite in human hepatocytes and was not formed in the hepatocytes of all animal species. M6 was formed at approximately 4-fold less than M4 in human hepatocytes, but a major metabolite in monkey and dog hepatocytes. These results suggest that N-glucuronidation (M2/M4) could be the major metabolic pathway in human hepatocytes, and acyl-glucuronidation (M6) could be the major metabolic pathway in animal hepatocytes.

Metabolic Profiles of GDC-0810, M4, or M6 in Human Liver Microsomes and S9. The synthetic M4 and M6 were incubated in human liver microsomes and S9 fractions, separately. Figure 4 shows a metabolic comparison between incubations of GDC-0810, M4, or M6 in human liver microsomes and S9. Incubations of M4 or M6 in human liver microsomes and S9 in the presence of NADPH and UDPGA showed that formation of M2 from M6 was 4-fold higher in hepatocytes than in liver microsomes.
Fig. 2. Radioactive chromatograms following incubations of [14C]GDC-0810 in liver microsomes of human, monkey, dog, rat, mouse, and rabbit in the presence of UDPGA and NADPH.
at a much higher rate (>10×) than from M4. M6 was also hydrolyzed to the parent, which was then converted to M4 via N-glucuronidation.

**Reaction Phenotyping of GDC-0810, M4, and M6.** Thirteen human recombinant UGT enzymes were tested in incubations with GDC-0810 in the presence of UDPGA. The experiments showed that the acyl glucuronide M6 was mainly formed by UGT1A1, 1A8, 1A7, and 1A3 as shown in Fig. 5A. UGT1A4 is the major and only enzyme responsible for M4 formation from GDC-0810. No M2 was detected in any incubations of GDC-0810 with 13 human recombinant UGT enzymes when incubated separately.

Reaction phenotyping experiments were also conducted with authentic M4 or M6 to investigate the formation of the discrete diglucuronide, M2. The data are shown in Fig. 5, B and C, and indicated that M2 was mainly formed from M6 by UGT1A4 (~65% of total) and much less formed from M4 by UGT1A8, 1A1, 1A3, and 1A9 (<5% of total), where UGT1A8 is the major responsible enzyme to convert M4 to M2. Details on the potential UGT enzymes responsible for formation of the major GDC-0810 metabolites in vitro are depicted in Supplemental Fig. 9.

**In Vivo Metabolic Profiles of [14C]GDC-0810 in Rats.** The mass balance of [14C]GDC-0810 in rats following oral administration

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**Fig. 3.** Radioactive chromatograms following incubations of [14C]GDC-0810 in hepatocytes of human, monkey, dog, rat, mouse, and rabbit.
was investigated and metabolites in plasma, urine, bile, and feces were identified by LC-MS/MS and radioactivity profiles. M6 was the major metabolite of [14C]GDC-0810 in rat plasma, feces, and bile. No M2 and M4 were detected in rats. The full set of results is provided in the supplemental section (Supplemental Table 3; Supplemental Fig. 10). All metabolites identified in humans and rats are listed in Table 4.

Discussion

Discrete diglucuronide M2 was first detected in the first-in-human study of GDC-0810 as an abundant circulating metabolite. However, M2 was basically absent in rats following administration of [14C]GDC-0810 or in incubations in liver fractions of humans and animal species. Here, we report that M2 was formed through sequential acyl- and N-glucuronidation of GDC-0810. Although the sequence of diglucuronidation has not been previously reported in the literature, mono-glucuronidation is common. For example, acyl-glucuronidation is a common metabolic pathway for a carboxylic acid containing drugs, such as gemfibrozil (Okerholm et al., 1976), diclofenac (Kumar et al., 2002), mura-glutazar (Zhang et al., 2011), and peliglitzar (Wang et al., 2011; Zhang et al., 2011). The acyl-glucuronide, M6, is a relatively stable metabolite compared with many other acyl-glucuronides. N-Glucuronidation is also a common metabolic pathway for a wide variety of nitrogen-containing compounds, including primary and secondary amines (Kaji and Kunne, 2005; Borlak et al., 2006), amides, tertiary aliphatic amines (Kaku et al., 2004), and aromatic N-heterocycles (Klieber et al., 2008). The N-glucuronidation pathway is especially common in humans due to the activity of two “specialized” enzymes, UGT1A4 and UGT1A10.

Many unusual glucuronides were reported (Argikar, 2012) such as type I (linked) diglucuronides in human, including testosterone-17-O-diglucuronide and estradiol-17-O-diglucuronide (Murai et al., 2005, 2006). Although type II discrete diglucuronides are not very common, the naturally occurring human discrete diglucuronide, bilirubin acyl-diglucuronide, was the first reported diglucuronide on two different carboxylic acids (Billing et al., 1957; Schmid, 1957). A limited number of drug-related discrete diglucuronides occurring on two different but the same types of functional groups were reported, such as from two phenolic hydroxyl groups of raloxifene (Trdan et al., 2011), morphine (Yeh et al., 1977), quercetin and isorhamnetin (Nakamura et al., 2018), genistein (Hosoda et al., 2010), and hesperetin and naringenin (Stevens et al., 2019). Posaconazole was reported to produce a discrete diglucuronide, but the positions of the conjugation could not be identified (Krieter et al., 2004). Discrete diglucuronides on two different types of functional groups were rare (King and Dickinson, 1991; Burkon and Somoza, 2008; Springer and Moko, 2019). Two of resveratrol-2C, 4O-diglucuronides were only reported by Burkon and Somoza (2008) as novel resveratrol metabolites in human plasma and human urine. Gut microbiota is one of main reasons to cause a strong inter-individual responding difference of resveratrol in humans (Bode et al., 2013). Discrete diglucuronide of diffunisal through a subsequent glucuronidation of diffunisal acyl glucuronide. Diffunisal acyl-glucuronide, not the phenolic glucuronide, was reported to undergo subsequent glucuronidation at the phenolic group to form diffunisal discrete diglucuronide. Acyl-glucuronide M6 undergoes subsequent N-glucuronidation at NH of indazole more efficiently than acyl glucuronidation of N-glucuronide M4 to form M2. Interestingly, only two selected regio-isomers (the 2-O-linked and 3-O-linked positional isomers, not the 1-O-linked and 4-O-
linked ones) of migrated diflunisal acyl-glucuronide formed the corresponding discrete diglucuronide (King and Dickinson, 1991). Different from diflunisal acyl-glucuronide, acyl-migration of M6 before converting to M2 was not observed.

To better understand the mechanism of M2 formation, we conducted incubations of [14C]GDC-0810 in across species of liver microsomes, intestinal microsomes, and hepatocytes. Additional incubations were conducted with UDPGA and GDC-0810, M4, or M6 in human liver microsomes and S9, as well as in the presence of 13 recombinant UGT enzymes. The mono-glucuronide, M4, was identified as the prominent metabolite in human hepatocytes and in human liver microsomes but M6 was formed in all species. In incubation with [14C]GDC-0810 or GDC-0810, M2 was a very minor (only detected by mass spectrometry) only in human hepatocytes and human liver microsomes. In incubations with M4 or M6, M2 formation from M6 was much more (>10×) than from M4 in human liver microsomes. In human liver S9 incubation, M2 was also formed from M6, neither from GDC-0810 nor M4. Interestingly, M2 and M4 were not formed by [14C]GDC-0810 directly in various in vitro systems of any animal species. M2 was observed minor metabolite in monkeys (Mulder et al., 2020) where it was called M1. Following a single oral dose of [14C]GDC-0810 in rats, M6 was the major metabolite observed in rat plasma (4.9%) and rat bile (10.2%). The complete metabolite profiles indicate that neither M2 nor M4 was formed in rats.

M2 formation required at least two UGT enzymes, UGT1A8 or UGT1A7, as well as UGT1A1/3 for acyl-glucuronidation and UGT1A4 for N-glucuronidation. UGT1A8 and UGT1A1 showed higher acyl glucuronidation of GDC-0810 for form M6 and of M4 to form M2. Only UGT1A4 is involved for N-glucuronidation as observed in formation of M4 from GDC-0810 as well as of M2 from M6. UGT1A4 is extensively expressed in human liver and plays a crucial role in N-glucuronidation, which is lacking in rodents (Fujitwara et al., 2018). Moreover, the N-glucuronidation rates in humans are unique or much higher than in animals (Kaivosaari et al., 2011; Fujitwara et al., 2018). In general, UGT enzymes are located predominantly in the ER of the liver. However, UGT1A1, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, and 2B15 are also found in the intestine (Yan and Caldwell, 2004) and in other extra-hepatic systems (Rowland et al., 2013). UGT1A8 and UGT1A7 have been only found in jejunum, ileum, and colon but not in liver (Cheng et al., 1998, Mojarrab and Mackenzie, 1998; Wu et al., 2011). Furthermore, UGT1A1 is expressed a much higher abundance in human intestine than in human liver (Rouleau et al., 2017). Collectively, M2 is formed in the liver by UGT1A4-catalyzed N-glucuronidation of M6 that was largely formed by UGT1A8/7/1 catalysis of GDC-0810 in human intestines. Intestinal glucuronidation of GDC-0810 further confirmed that...
M6 was only formed in human intestinal microsomal incubations of [14C]GDC-0810. Interestingly, extensive glucuronidation of raloxifene in the intestines limited its bioactivation in the liver of humans (Dalvie et al., 2008). Acyl-glucuronides due to acyl-migration could react with nucleophilic moieties of serum proteins, cellular proteins, or DNA to form adducts and have been implicated in adverse effects (Shipkova et al., 2003).

However, acyl-glucuronide M6 was stable and degradation was insignificant. Although acyl-glucuronidation activated the α,β-unsaturated carboxylic acid, there was no adverse effects known to associate with this bioactivation (Mulder et al., 2020).

The circulation of metabolites generally depends on physicochemical properties (logP, pKa, topological polar surface area), permeability, protein binding, role of transporters, and fractional formation (fm) from parent (Smith and Dalvie, 2012). Glucuronide conjugation leads to substantial changes to the physicochemical properties of a compound. The resulting metabolites are more soluble and less permeable than the parent compound, leading to a lower likelihood that these discrete diglucuronides will be found in circulation. Given the generally high solubility and low permeability of discrete diglucuronides, the high circulating concentration of M2 in human plasma may be due to an extensive formation, unknown transporter effect, and a low volume of distribution (>99% plasma protein binding, data not shown). Drug transporter-mediated enterohepatic recycling to extend the residence time was also reported (Kosoglu et al., 2005).

In summary, formation of diglucuronide M2 required two sequential reactions of acyl-glucuronidation of GDC-0810 (forming M6) in the intestines followed by N-glucuronidation in the liver. This complicated metabolic pathway presented challenges in predicting the metabolism of GDC-0810 using in vitro systems. The lack of N-glucuronidation in rats led to no formation of M2 and M4 in rats.

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