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

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Abbreviations: ACN, acetonitrile; AUC, area under the curve; BDC, bile duct cannulated; CID, collision induced dissociation; Cys, cysteine; CRO, contract research organization; DMSO, dimethyl sulfoxide; ER, estrogen receptor; GSH, glutathione; HCD, higher-energy collisional dissociation; HLM, human liver microsomes; HLS9, human liver S9; HPLC, high-performance liquid chromatography; JVC, jugular vein cannulated; LC, liquid chromatography; LC-MS, liquid chromatography–tandem mass spectrometry; LSC, liquid scintillation counting; MCT, methylcellulose–and Tween 80; NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate; PEG 400, polyethylene glycol 400; S9 fraction, 9000g supernatant of a liver homogenate; SERD, selective estrogen receptor degrader; UDPGA, uridine 5’-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC-UV, ultra-performance liquid chromatography coupled with ultraviolet detection; UV, ultraviolet detection; XIC, extracted Ion Chromatogram.
ABSTRACT

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 \([^{14}C]GDC-0810\) synthetic M6 and M4 with liver microsomes, intestinal microsomes, and hepatocytes of different species as well as recombinant UGT enzymes to further understand the formation of M2. The results suggested that 1) M2 was more efficiently formed from M6 than from M4, 2) Acyl glucuronidation was mainly catalyzed by UGT1A8/7/1 that is highly expressed in the intestines while 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.
INTRODUCTION

Breast cancer is the most common form of cancer and the leading cause of cancer death in women worldwide, accounting for more than 1,300,000 new cases and nearly 460,000 cancer deaths annually (Jemal 2011). Approximately 75% of all breast cancers express and are dependent on the estrogen receptor (ER) for tumor growth and progression. Modulation of estrogen activity and/or synthesis is the mainstay of therapeutic approaches in women with ER+ breast cancer. However, despite the effectiveness of available hormonal therapies such as tamoxifen, aromatase inhibitors (e.g. anastrozole, letrozole and exemestane), and full ER antagonists/degraders (e.g. fulvestrant), many patients develop resistance to these agents and require further treatment. Therefore, there is a need for new ER-targeting therapies with increased anti-tumor activity to delay disease progression and/or overcome resistance to the currently available hormonal therapies and ultimately prolong survival in women with ER+ advanced breast cancer (Di 2010, Baselga 2012, Miller 2010 and Van 2012).

GDC-0810 is a small molecule therapeutic agent that competes with estrogens for binding to the ER with low nano-molar potency (Joseph 2016). Unlike fulvestrant, which is also an ER antagonist and degrader, GDC-0810 has a nonsteroidal chemical backbone and displays good oral bioavailability (Lai 2015).

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 2005 and Argikar 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 in vitro systems (liver microsomes, intestinal microsomes, hepatocytes and recombinant 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 (South San Francisco, CA). \[^{14}\text{C}\]GDC-0810 was synthesized by Selcia (Essex, UK) with a specific radioactivity of 8.8 kBq/mg. Reagents: 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 (DMSO), 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 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 and 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 microsome protein or 1 mg/mL of intestinal microsomal protein, 5 mM MgCl₂, 25 μg/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 h 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 × g for 20 min, 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 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 (rUGT) incubation mixtures contained GDC-0810, M4 or M6 (10 μM), 100 mM Tris buffer (pH 7.4), 0.25 mg/mL 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 h 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 × g for 20 min, 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 pre-warmed (37°C) InVitroGRO HT thawing medium in 50 mL centrifuge tubes. The tubes were centrifuged for 5 min at 60 × g and the supernatants were discarded. Cells were re-suspended with 50 mL of pre-warmed DMEM by gently inverting the tubes several times. The tubes were centrifuged for 5 min at 60 × 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 × 10⁶ cells/mL. The hepatocytes were incubated with a final [¹⁴C]GDC-0810 concentration of 10 μM, a final DMSO 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 h at 37°C. The hepatocyte incubation samples were protein precipitated with 10 volumes of ACN containing 0.1% formic acid and centrifuged at 2000 × g for 20 min. 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, CA).

Drug Dosing, Sample Collection and Analysis

Eight female human subjects each received a single oral administration of 600 mg GDC-0810. The plasma samples pre-treated with citric acid (final concentration of 25 mM) were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8-h time points on day 1 (single dose) and at the corresponding time points on day 7 (the steady state). The samples were pre-treated with sodium citrate (final concentration of 25 mM). The plasma samples from four subjects were AUC pooled such that all collections on day 1 (from 0 to 8 h) were combined and all collections on day 7 were combined (Hop 1998). Plasma samples were treated with three volumes of ACN to precipitate the protein. After centrifugation, the supernatants were evaporated to about 150 μL using a SpeedVac® concentrator and ACN was added to a volume of about 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 h 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 session.

**Mass Spectrometric Methods**

The protonated molecular ions ([M+H]^+) in positive ion mode for analytes were determined from full scan mass spectrometry (MS) 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 7,500 and collision induced dissociation with a collision energy range of 15-35 ev.

**LC Separation Methods**

Two chromatographic methods were used. The first one, long gradient (60 min) and a high flow rate (1.0 mL/min), intended for experiments with radioactive material. A UPLC-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 x 150 mm, 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 min. It then increased to 50% B from 24 to 32 min and then to 95% B from 50 to 52 min. An organic wash for 3 min returned the column to its initial conditions before the next injection 5 min later.
Another short gradient method (25 min) with low flow rate (0.4 mL/min) used for non-radioactive material. A Synergi Polar-RP 100A column at 100 x 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 min. The gradient increased to 43% B from 11 to 19 min and then to 95% B within 2 min. An organic wash for 2 min returned the column to its initial conditions before the next injection 2 min 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 Supp. Fig. 1 (a, b). Their mass spectra as well as M1 spectrum were reported in Supp. Fig. 2 (c). Chromatogram comparisons of M6 and its migration in stock solution from a contract research organization (CRO), freshly prepared solution from powder and fresh human plasma (Day 1) were evaluated as shown in Supp. Fig. 3 (a).

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 Supp. Fig. 3 (b). From UV trace of Supp. Fig. 3 (a), 8%, 1% and <1% acyl-migration were observed for stock solution of M6 from CRO, freshly prepared stock solution from powder and fresh human plasma, respectively, which gives us a confidence that acyl-migration of M6 is not an issue in human plasma profiling if samples were pre-treated 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 Supp. Fig. 4 (a) and M6 was stable up to 5 days at room temperature except in 1% formic acid as shown in Supp. Fig. 4 (b). Acyl-migration of M6 was evaluated and was found stable under different conditions. M6 has much poorer ionization efficiency than M4 (up to 50-folds) in positive ion mode as shown in Supp. Fig. 1 (a), therefore, the estimation of M4 formation with peak area based on mass spectrometry response could be significant underestimation (by ~98%).
**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 as 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 Figure 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 Figure 2, as well as in Supp. Fig. 5 and Supp. 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 Supp. Table 1 and Supp. Fig. 7 and Supp. Fig. 8.

Metabolic Profiles of $[^{14}\text{C}]\text{GDC-0810}$ 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 (Supp. Table 2 and Figure 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 about 4-folds 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 at a much higher rate (>10x) 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 Figure 5(a). 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 thirteen 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 is shown in Figure 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 Supp. Fig. 9.

**In Vivo Metabolic Profiles of [14C]GDC-0810 in Rats**

The mass balance of [14C]GDC-0810 in rats following oral administration 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 (Supp. Table 3 and Supp. Fig. 10). All metabolites identified in humans and rats were 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, monoglucuronidation is common. For example, acyl-glucuronidation is a common metabolic pathway for a carboxylic acid containing drugs, such as gemfibrozil (Okerholm 1976), diclofenac (Kumar 2002), muraglitazar (Zhang 2011) and pelaglitazar (Zhang 2011 and Wang 2011). The acyl-glucuronide, M6, is a relatively stable metabolite compared to 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 2005 and Borlak 2006), amides, tertiary aliphatic amines (Kaku 2004) and aromatic N-heterocycles (Klieber 2008). The N-glucuronidation pathway is especially common in humans due to the activity of two “specialized” enzymes, UGT1A4 and 2B10.

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 2005 and Murai 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 1957 and 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 (Trdana 2011), morphine (YEH 1977), quercetin and isorhamnetin (Nakamura 2018); genistein (Hosoda 2010); hesperetin and naringenin (Stevens 2019). Posaconazole was reported to produce a discrete diglucuronide, but the positions of the conjugation could not be identified (Krieter 2004). Discrete diglucuronides on two different types of functional groups were rare (Burkon 2008 and Springer 2019) (King 1991). Two of resveratrol-2C, 4O-diglucuronides were only reported by Alexander Burkon and Veronika Somoza 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 2013). Discrete diglucuronide of diflunisal through a subsequent glucuronidation of diflunisal acyl glucuronide. Diflunisal acyl-glucuronide, not the phenolic glucuronide, was reported to undergo subsequent glucuronidation at the phenolic group to form diflunisal 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 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 thirteen 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 human hepatocytes and human liver microsomes. In incubations with M4 or M6, M2 formation from M6 was much more (>10x) from M4 in human liver microsomes. In human liver S9
incubation, M2 was also formed from M6, not 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 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, 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 M2 formation from M6. UGT1A4 is extensively expressed in human liver and plays a crucial role in N-glucuronidation, which is lack in rodents (Fujiwara 2018). Moreover, the N-glucuronidation rates in humans are unique or much higher than in animals (Kaivosaari 2011 and Fujiwara 2018). In general, UGT enzymes are located predominantly in the endoplasmic reticulum (ER) of the liver. However, UGT1A1, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 are also found in the intestine (Yan 2004) and in other extra-hepatic systems (Rowland, 2013). UGT1A8, and UGT1A7 have been only found in jejunum, ileum and colon but not in liver (Cheng 1998, Mojarrabi 1998 and Wu 2011). Furthermore, UGT1A1 is expressed a much higher abundance in human intestine than in human liver (Rouleau 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 was 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 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 2003). However, acyl-glucuronide M6 was stable and degradation was insignificant. Although acyl-glucuronidation activated the α,β-unsaturated carboxyl acid, there was no adverse effects known to associate with this bioactivation (Mulder 2020).
The circulation of metabolites generally depends on physicochemical properties (logP, pKa, TPSA), permeability, protein binding, role of transporters, and fractional formation from parent (fm) (Smith & Dalvie, 2012). Glucuronide conjugation leads to substantial changes to the physiochemical 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 (Kosoglou 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 mechanism presented challenges in predicting the metabolism of GDC-0810 using human in vitro systems. The lack of N-glucuronidation in rats led to no formation of M2 and M4 in rats.

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Authorship Contributions

Participated in research design: Zhang C, Liu, Zhang D
Conducted experiments: Zhang D, Zhang C, Bobba, Jorski, Quynh
Performed data analysis: Zhang D, Zhang C, Su, Quynh, Wang, Kenny, Khojasteh
Wrote or contributed to the writing of the manuscript: Zhang D, Zhang C, Su, Khojasteh, Choo, Kenny
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LIST OF FIGURES

Figure 1  Proposed Metabolic Pathways of GDC-0810 in plasma of Humans Following Oral Administration

Figure 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

Figure 3  Radioactive Chromatograms Following Incubations of $^{[14C]}$GDC-0810 in Hepatocytes of Human, Monkey, Dog, Rat, Mouse and Rabbit

Figure 4  Comparison of Metabolites Formed in Incubations of GDC-0810, M4 and M6 in Human Liver Microsomes and Human Liver S9 Fractions in the Presence of UDPGA

Figure 5  Comparison of Metabolites Formed in Incubations of GDC-0810, M4 and M6 with 13 Recombinant UGT Enzymes in the Presence of UDPGA
### Table 1. Characterization of GDC-0810 and its Metabolites

<table>
<thead>
<tr>
<th>Metabolite Characterization</th>
<th>RT (^a) (min)</th>
<th>Observed [M + H](^+) (m/z)</th>
<th>Theoretical [M + H](^+) (m/z)</th>
<th>Mass Accuracy (ppm)</th>
<th>Chemical Formula</th>
<th>Detecting Species</th>
<th>Major Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0810 Parent</td>
<td>17.3</td>
<td>447.1275</td>
<td>447.1270</td>
<td>-1.1</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_2)(^+)</td>
<td>All</td>
<td>429.1172, 311.0641, 299.0755, 284.0523, 293.0219, 273.1021, 169.0762</td>
</tr>
<tr>
<td>M1(^*) Discrete diglucuronide</td>
<td>4.6</td>
<td>799.1921</td>
<td>799.1912</td>
<td>-1.1</td>
<td>C(<em>{38})H(</em>{37})ClFN(<em>2)O(</em>{14})(^+)</td>
<td>H</td>
<td>623.1580, 447.1257</td>
</tr>
<tr>
<td>M2 Discrete diglucuronide</td>
<td>4.9</td>
<td>799.1921</td>
<td>799.1912</td>
<td>-1.3</td>
<td>C(<em>{38})H(</em>{37})ClFN(<em>2)O(</em>{14})(^+)</td>
<td>H</td>
<td>623.1580, 447.1257</td>
</tr>
<tr>
<td>M3 Oxidative diglucuronide</td>
<td>5.2</td>
<td>815.1877</td>
<td>815.1873</td>
<td>-0.5</td>
<td>C(<em>{38})H(</em>{37})ClFN(<em>2)O(</em>{15})(^+)</td>
<td>H, Rab</td>
<td>not available</td>
</tr>
<tr>
<td>M4 N-glucuronide</td>
<td>7.0</td>
<td>623.1594</td>
<td>623.1591</td>
<td>-0.5</td>
<td>C(<em>{32})H(</em>{29})ClFN(_2)O(_6)(^+)</td>
<td>H</td>
<td>605.1491, 447.1278, 299.0724</td>
</tr>
<tr>
<td>M5a Oxidative glucuronide</td>
<td>14.5(^#)</td>
<td>639.1541</td>
<td>639.1558</td>
<td>1.6</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_3)(^+)</td>
<td>R, Rab</td>
<td>463.1167, 445.1080, 315.0644</td>
</tr>
<tr>
<td>M5b Oxidative glucuronide</td>
<td>18.5(^#)</td>
<td>639.1542</td>
<td>639.1552</td>
<td>1.2</td>
<td>C(<em>{32})H(</em>{26})ClFN(_2)O(_9)(^+)</td>
<td>All</td>
<td>463.1249, 445.1058, 315.0673</td>
</tr>
<tr>
<td>M5c Oxidative glucuronide</td>
<td>19.2(^#)</td>
<td>639.1542</td>
<td>639.1552</td>
<td>3.2</td>
<td>C(<em>{32})H(</em>{26})ClFN(_2)O(_9)(^+)</td>
<td>R, M, Rab</td>
<td>621.1401, 463.1187, 445.1085, 315.0664</td>
</tr>
<tr>
<td>M6 Acyl-glucuronide</td>
<td>10.0</td>
<td>623.158</td>
<td>623.1591</td>
<td>1.1</td>
<td>C(<em>{32})H(</em>{28})ClFN(_2)O(_8)(^+)</td>
<td>All</td>
<td>447.1274, 311.0645</td>
</tr>
<tr>
<td>M7 Mono-oxidative</td>
<td>40.1(^#)</td>
<td>463.1226</td>
<td>463.1219</td>
<td>-0.7</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_3)(^+)</td>
<td>H, C, Rab</td>
<td>446.1216, 315.0700, 311.0648, 185.0713</td>
</tr>
<tr>
<td>M7a Mono-oxidative</td>
<td>34.4(^#)</td>
<td>463.1215</td>
<td>463.1219</td>
<td>4.0</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_3)(^+)</td>
<td>R, M</td>
<td>446.1203, 411.1513</td>
</tr>
<tr>
<td>M7b Mono-oxidative</td>
<td>31.0(^#)</td>
<td>463.12</td>
<td>463.1219</td>
<td>1.9</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_3)(^+)</td>
<td>All</td>
<td>445.1116, 315.0728, 311.0639, 272.0641, 161.0203</td>
</tr>
<tr>
<td>M13 Mono-oxidative</td>
<td>18.5(^#)</td>
<td>463.1248</td>
<td>463.1219</td>
<td>-2.1</td>
<td>C(<em>{26})H(</em>{21})ClFN(_2)O(_3)(^+)</td>
<td>All</td>
<td>446.1180, 414.0915, 315.0768, 224.0574, 208.0779</td>
</tr>
<tr>
<td>M14 GSH Conjugate</td>
<td>7.5(^#)</td>
<td>754.2100</td>
<td>754.2102</td>
<td>0.3</td>
<td>C(<em>{36})H(</em>{38})ClFN(_2)O(_6)(^+)</td>
<td>In Vitro</td>
<td>447.1272, 405.1167, 340.0946, 299.0743, 203.0624, 162.0217, 130.0501, 84.0457</td>
</tr>
<tr>
<td>M15 Oxidation methylation</td>
<td>41.0(^#)</td>
<td>477.1352</td>
<td>477.1381</td>
<td>2.9</td>
<td>C(<em>{27})H(</em>{23})ClFN(_2)O(_3)(^+)</td>
<td>In Vitro</td>
<td>459.1288</td>
</tr>
</tbody>
</table>
C=monkey, D=dog, GSH=glutathione, H=human, M mouse, m/z=mass-to-charge ratio, R=rat, Rab=rabbit, RT=retention time (based on the short gradient)
*: M1/M2 were acyl-migration isomers.
#: Retention time (RT) referred to the long gradient.
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) and Day 7 (Multiple Doses at Steady State) 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>Estimated Relative Abundance Based on UV @ Day 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td><strong>GDC-0810</strong></td>
<td>447.1256</td>
<td>Parent</td>
<td>17.3</td>
<td>92.0</td>
</tr>
<tr>
<td>M1*</td>
<td>799.1924</td>
<td>Discrete diglucuronidation</td>
<td>4.6</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>
</tr>
<tr>
<td>M3*</td>
<td>815.1877</td>
<td>Oxidative diglucuronidation</td>
<td>5.2</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>
</tr>
<tr>
<td>M5*</td>
<td>639.1535</td>
<td>Oxidative acyl-glucuronidation</td>
<td>7.6</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>
</tr>
<tr>
<td>M7</td>
<td>463.1212</td>
<td>Mono-oxidation</td>
<td>13.4</td>
<td>0.8</td>
</tr>
<tr>
<td>M8*</td>
<td>447.1263</td>
<td>Isomer of parent</td>
<td>15.2</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>m/z</th>
<th>Biotransformation</th>
<th>RT (min)</th>
<th>Estimated Relative Abundance Based on UV @ Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td><strong>GDC-0810</strong></td>
<td>447.1256</td>
<td>Parent</td>
<td>17.3</td>
<td>72.7</td>
</tr>
<tr>
<td>M1</td>
<td>799.1924</td>
<td>Discrete diglucuronidation</td>
<td>4.6</td>
<td>0.4</td>
</tr>
<tr>
<td>M2</td>
<td>799.1922</td>
<td>Discrete diglucuronidation</td>
<td>4.9</td>
<td>13.4</td>
</tr>
<tr>
<td>M3*</td>
<td>815.1877</td>
<td>Oxidative diglucuronidation</td>
<td>5.2</td>
<td>0</td>
</tr>
<tr>
<td>M4</td>
<td>623.1580</td>
<td>N-glucuronidation</td>
<td>7.0</td>
<td>1.8</td>
</tr>
<tr>
<td>M5</td>
<td>639.1535</td>
<td>Oxidative acyl-glucuronidation</td>
<td>7.6</td>
<td>0</td>
</tr>
<tr>
<td>M6</td>
<td>623.1585</td>
<td>Acyl-glucuronidation</td>
<td>10.0</td>
<td>7.7</td>
</tr>
<tr>
<td>M7</td>
<td>463.1212</td>
<td>Mono-oxidation</td>
<td>13.4</td>
<td>4.0</td>
</tr>
<tr>
<td>M8*</td>
<td>447.1263</td>
<td>Isomer of parent</td>
<td>15.2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Detected only by mass spectrometry; UV: 220~350 nm; RT = retention time; m/z = mass-to-charge ratio; Oral dose: 600 mg; Time points of day 1 & 7: 0, 0.5, 1.5, 2, 3, 4, 6 and 8 h.
Table 3. Percent Radioactivity of $[^{14}\text{C}]$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 $[^{14}\text{C}]$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>0.9</td>
</tr>
<tr>
<td>M15</td>
<td>1.8</td>
</tr>
</tbody>
</table>

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

$[^{14}\text{C}]$GDC-0810: 10 µM; LM: 1 mg/mL of protein; UDPGA: 2 mM; NADPH: 2 mM; Incubation time: 2 hr.
Table 4. In Vivo and In Vitro Comparisons of GDC-0810 and its Identified Metabolites in Human and Rat

<table>
<thead>
<tr>
<th>Analyte</th>
<th>GDC-0810</th>
<th>[\textsuperscript{14}C]GDC-0810</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Vivo</td>
<td>In Vitro</td>
</tr>
<tr>
<td></td>
<td>(Human)</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8h</td>
<td>HLM</td>
<td>HLM</td>
</tr>
<tr>
<td></td>
<td>64.5</td>
<td>51.0</td>
</tr>
<tr>
<td>M1</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>M2</td>
<td>20.7</td>
<td>D</td>
</tr>
<tr>
<td>M4</td>
<td>1.3</td>
<td>18.3</td>
</tr>
<tr>
<td>M5</td>
<td>2.9</td>
<td>ND</td>
</tr>
<tr>
<td>M5a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M5b</td>
<td>ND</td>
<td>1.17</td>
</tr>
<tr>
<td>M5c</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M6</td>
<td>9.2</td>
<td>20.0</td>
</tr>
<tr>
<td>M7</td>
<td>1.1</td>
<td>D</td>
</tr>
<tr>
<td>M7b</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>M13</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M13a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M13b</td>
<td>ND</td>
<td>0.2</td>
</tr>
</tbody>
</table>

D = detected by mass spectrometry only, ND = not determined, HLM = human liver microsomes (1 mg/mL), HIM = human intestinal microsomes (1 mg/mL), Hhep = human hepatocyte (cell density: \(2 \times 10^6\) cells/mL), NADPH = the reduced form of nicotinamide adenine dinucleotide phosphate (2 mM), UDPGA = uridine 5'-diphosphoglucuronic acid (2 mM), Incubation time: 1 or 4 h.

Human oral dose: 600 mg; Rat oral dose: 75 mg/kg (400 µCi/kg)
Figure 1.
Figure 2

Cy_LM = cynomolgus monkey liver microsomes, D_LM = dog liver microsomes, H_LM = human liver microsomes, M_LM = mouse liver microsomes, R_LM = rat liver microsomes, Rab_LM = rabbit liver microsomes, UDPGA = uridine 5’-diphosphoglucuronic acid.

[^14C]GDC-0810: 10 µM; LM: 1 mg/mL of protein; UDPGA: 2 mM; NADPH: 2 mM; Incubation time: 2 hr.
CyH = cymologous monkey hepatocytes, DH = dog hepatocytes, HH = human hepatocytes,
P = parent, RH = rat hepatocytes, MH = mouse hepatocytes, RabH = rabbit hepatocytes.

$[^14]C\text{GDC-0810: 10 \text{ M}}$; Hepatocyte cell density: $2 \times 10^6 \text{ cells/mL}$; Incubation time: 4 hr.
(a) UV comparisons (250~300 nm)

(b) Formation of M2

HLM = human liver microsomes, HLS9 = human liver S9 fractions, I = interference peak.
GDC-0810: 10 or 20 µM; HLM and HLS9: 1 mg/mL of protein; NADPH: 2 mM; Incubation time: 1 hr.

Figure 4
**Figure 5. Comparison of Metabolites Formed in Incubations of GDC-0810, M4 and M6 with 13 Recombinant UGT Enzymes**

(a) 60 min Incubation of GDC-0810 in 13 Recombinant UGT Enzymes

(b) 60 min Incubation of M4 in 13 Recombinant UGT Enzymes

(c) 60 min Incubation of M6 in 13 Recombinant UGT Enzymes

GDC-0810, M4 and M6: 10 μM; UDPGA: 5 mM; Incubation time: 1 hr.