Absorption, Distribution, Metabolism and Excretion of [14C]GDC-0449 (Vismodegib), an Orally Active Hedgehog Pathway Inhibitor, in Rats and Dogs: A Unique Metabolic Pathway via Pyridine Ring Opening

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ABBREVIATIONS: GDC-0449 (Vismodegib), 2-chloro-N-(4-chloro-3-(pyridin-2-yl)-phenyl)-4-(methylsulfonyl)-benzamide; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; ADME, absorption, distribution, metabolism, excretion; AUC, area under the concentration-time curve; QWBA: quantitative whole-body autoradiography.
ABSTRACT:

GDC-0449 (also known as vismodegib) is a potent and selective first-in-class small molecule inhibitor of the Hedgehog signaling pathway and is currently in clinical development. In this study, we investigated the metabolic fate and disposition of GDC-0449 in rats and dogs following a single oral administration of [14C]GDC-0449. An average of 92.4% and 80.4% of the total administered radioactivity was recovered from urine and feces in rats and dogs, respectively. In both species, feces was the major route of excretion, representing 90.0% and 77.4% of the total dose in rats and dogs, respectively. At least 42.1% and 30.8% of the dose was absorbed in rats and dogs, respectively, based on the total excretion of radioactivity in bile and urine. GDC-0449 underwent extensive metabolism in rats and dogs with the major metabolic pathways being oxidation of the 4-chloro-3-(pyridin-2-yl)-phenyl moiety followed by phase II glucuronidation or sulfation. Three other metabolites resulting from an uncommon pyridine ring opening were found mainly in feces, representing 1.7-17.7% of the dose in total in rats and dogs. In plasma, the total radioactivity was absorbed quickly in both rats and dogs, and unchanged GDC-0449 was the predominant circulating radioactive species in both species (>95% of total circulating radioactivity). QWBA in rats showed that the radioactivity was well distributed in the body, except for in the central nervous system, and the majority of radioactivity was eliminated from most tissues by 144 h.
**Introduction**

GDC-0449 (Vismodegib Fig. 1) is a first-in-class small molecule inhibitor of the Hedgehog (Hh) signaling pathway being developed by Genentech, Inc. as an anti-cancer therapeutic. Hh pathway activation has been implicated in a number of cancers (Scales and de Sauvage, 2009; Von Hoff et al., 2009; Rudin, et al., 2009). Mutations in the Hh receptor components patched (PTCH1) or smoothened (SMO) result in constitutive pathway activation and have been identified in basal cell carcinoma (Hahn et al., 1996; Johnson et al., 1996) and medulloblastoma (Pietsch et al., 1997; Raffel et al., 1997; Vorechovsky et al., 1997). It has also been observed that aberrant Hh ligand production can drive the growth of other tumor types such as colorectal and pancreatic cancer (Yauch et al., 2008), prostate cancer (Fan et al., 2004), and B cell lymphoma (Dierks et al., 2007) through paracrine activation of the Hh pathway. Paracrine signaling typically involves ligand expressed on the cancer cells signaling adjacent stromal components in the case of solid tumors, or signaling from stromally produced Hh ligand to cancer cells in the case of hematopoietic cancers. GDC-0449 binds to and inhibits SMO, a seven-transmembrane protein (Robarge et al., 2009), effectively blocking the Hh signal. In a Phase I trial, GDC-0449 was well tolerated in patients with solid tumors and showed promising efficacy in advanced basal cell carcinoma (Von Hoff et al., 2009).

GDC-0449 has desirable preclinical ADME properties that have been previously described (Wong et al., 2009). GDC-0449 is relatively stable in hepatocyte and liver microsomal incubations in rat, dog, and human. In liver microsomes from rat, dog and human, two oxidative metabolites are formed. In rat and dog urine, these metabolites are shown to have gone through sequential metabolism to form glucuronides. In human liver
microsomes, these oxidative metabolites are mainly formed by CYP3A and CYP2C9. In rats and dogs, GDC-0449 exhibits low in vivo clearance and good oral bioavailability. Allometric scaling predicts clearance in humans to be low, which is consistent with the predicted human hepatic clearance using hepatocytes. More recently, the oral pharmacokinetics of GDC-0449 in humans was described (Von Hoff et al., 2009; Ding et al., 2010). The clinical pharmacokinetics of the drug are characterized by high plasma exposures and a long terminal plasma half life ($t_{1/2} = 286$ h). The primary objectives of the present study were to characterize the disposition of GDC-0449, determine its routes of excretion, and identify metabolites in plasma and excreta in rats and dogs after a single oral administration of radiolabeled [$^{14}$C]GDC-0449. In addition, the tissue distribution of radiolabeled [$^{14}$C]GDC-0449 in rats was characterized using QWBA.
Materials and Methods

**General chemicals.** HPLC grade acetonitrile (ACN), methanol, formic acid (FA), water, ammonium formate, ammonium hydroxide, ethyl acetate, and chloroform were purchased from either Mallinckrodt Baker, Inc (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Ammonium acetate (analytical grade, lot#089K1646), sulfatase from *Aerobacter aerogenes* (lot # 110K86101) type VI glycerol solution, and β-glucuronidase (from Helix pomatia, type H-1) were purchased from Sigma Chemical Co. (St. Louis, MO). Diazald, diethyl ether, and sodium methoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). The carbon-14 cocktail for the online β-RAM IN/US radioactive detection was purchased from AIM Research Co. (Newark, DE). The Ultima Gold XR scintillation cocktail and Carbosorb were obtained from PerkinElmer Life Science (Waltham, MA).

**Radiolabeled Drug and Reference Compounds.** [14C]GDC-0449 (also known as vismodegib) was synthesized by Selcia Limited (Essex, UK) as a free base with a chemical purity of >99% and a radiochemical purity of >99% as determined by NMR and radio-HPLC analysis. The radiolabel 14C was evenly distributed in the middle benzene ring of GDC-0449 (specific activity=149.73 μCi/mg; Fig. 1). Unlabeled GDC-0449 with a chemical purity of >99% was synthesized at Genentech, Inc. (South San Francisco, CA).

**Test animals.** Sprague Dawley (SD) and Long-Evans (LE) rats were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA). Beagle dogs were purchased from Covance Research Products, Inc. (Kalamazoo, MI). The animals were housed individually in suspended, stainless steel wire-mesh cages or metabolism cages and were acclimated for 4-7 days prior to dose administration. Animals were fasted overnight prior to administration of the dose and up to 4 h post-dose. Water was provided *ad libitum*. Each
animal was weighed and assigned a permanent identification number. All studies were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Rats.** SD rats were administered orally with a single dose of [14C]GDC-0449 at a target dose of 50 mg/kg (100 μCi/kg). [14C]GDC-0449 was formulated in 0.5% methylcellulose and 0.2% Tween 80 in reverse osmosis water (pH 3.0), and the specific activity of the dose was 1.96 μCi/mg. Four groups of rats were assigned in this study. The first group (n=3 per sex) was designated for mass balance determination. For group 1 animals, urine and feces were collected on dry ice at approximately 0-8 and 8-24 h post-dose and at 24-h intervals up to 240 h post-dose. The second group included bile duct-cannulated (BDC) rats (n=3 per sex) and was designated for absorption and biliary excretion determination. In BDC rats, a solution of taurocholic acid (2.3 mg/ml in 0.9% saline) was infused via the distal (duodenal) cannula at a rate of 0.9 ml/h during the sample collection. Urine and bile were collected in plastic containers on dry ice at 0-8, 8-24, 24-48, 48-72, 72-96, and 96-120 h post-dose. The third group of rats (n=3 per sex) was designated for pharmacokinetic analysis. Blood (approximately 0.25 ml) was collected from a jugular vein via a syringe and needle and transferred into tubes containing potassium EDTA at pre-dose and at 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, and 120 h. Blood was placed on wet ice or stored at approximately 5°C until centrifuged to obtain plasma for radioanalysis. The fourth group of rats (n=7 per sex) were dosed for identification of circulating metabolites. Animals were sacrificed via cardiac puncture at pre-dose and at 0.5, 4, 8, 24, 48, and 72 h post-dose, and blood (as much as possible) was collected into
tubes containing potassium EDTA. As with group 3, plasma was prepared from blood by centrifugation.

**Dogs.** Four beagle dogs (n=2 per sex) and two BDC male beagle dogs were housed individually in stainless steel metabolism cages. [14C]GDC-0449 was formulated in the same ways as for rats with a specific activity of 3.51 μCi/mg in the final formulation. Each dog received [14C]GDC-0449 at a target oral dose of 5 mg/kg (20 μCi/kg) (actural dose at 4.8 mg/kg (17.8 μCi/kg)). Urine, feces, and bile (from BDC male dogs) were collected at pre-dose, 0-8, 8-24 h and then at 24 h intervals up to 336 h post-dose on dry ice (urine, bile, and feces (0-8 h)) and at room temperature (feces, intervals after 8 h)). Cage rinse and wipes were performed and collected at 24 h intervals up to 336 h post dose. Blood samples (~5 ml) were collected on wet ice at pre-dose, 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, 96, 120, 168, 240, 288, and 336 h post-dose, and plasma was obtained within 30 min.

**Determination of Radioactivity.** Radioactivity in plasma, urine, and bile was measured for at least 5 min or 100,000 counts using a Packard 2900TR liquid scintillation counter from PerkinElmer Life and Analytical Sciences (Waltham, MA). An Ultima Gold XR scintillation cocktail was used for sample analysis. All samples were analyzed in duplicate for rats and in triplicate for dogs. Scintillation counting data were automatically corrected for counting efficiency using the external standardization technique and an instrument-stored quench curve generated from a series of sealed quenched standards. The amount of radioactivity in plasma is expressed as μg-equivalents of GDC-0449 per gram plasma (μg-Eq/g) and was calculated using the specific activity of the administered dose. Pharmacokinetic parameters for radioactivity in plasma were calculated using
WinNonlin® Professional Edition, Version 4.1 from Pharsight Corporation. Inc. (Sunnyvale, CA) and are presented in Table 1.

For fecal samples, samples were homogenized with 80% ACN in water or with 100% water. Aliquots of fecal homogenates were air-dried before combustion using a Packard model A307 sampler oxidizer (Waltham, MA). Radioactivity in the combustion products was determined by trapping the liberated $^{14}$CO$_2$ in a mixture of Permafluor and CarboSorb prior to analysis by liquid scintillation counting.

**Extraction of Metabolites from Biological Samples.** Aliquots of urine, feces, and bile samples at various time points were pooled relative to the excreted volume or weight at each time point, so that each pooled sample represented >90% of the radioactivity excreted in that route. Rat urine, feces, and bile samples were pooled from 0-24, 0-48, and 0-24 h post-dose, respectively. Dog urine, feces, and bile samples were pooled from 0-192, 0-144, and 0-240 h post-dose, respectively. Pooled urine and bile samples were centrifuged and the supernatant was injected onto an HPLC column without further purification. The pooled fecal homogenates were treated with ACN, sonicated for 30 min, and centrifuged at 1400 x g for 10 min. The supernatants were separated, and the process was repeated until most radioactivity (>80%) was recovered. All the supernatants were combined and evaporated to dryness under nitrogen in a TurboVap® LV evaporator (Caliper life sciences, Inc., Hopkinton, MA) at 37°C. The residue was reconstituted with 20-50% ACN in water before injection onto an HPLC column.

Plasma samples collected at 0.5, 4, 8, 24, 48, and 72 h from group 4 rats and at 1, 3, 6, 12, and 48 h from dogs were extracted for metabolite profiling and identification. Rat plasma was not pooled, while dog plasma at each timepoint was pooled from two animals.
Pooled or individual plasma samples were subjected to protein precipitation by addition of three volumes of ACN followed by vortex mixing, sonication and centrifugation at 2095 x g for 10 min. The supernatant was separated and the process was repeated until most radioactivity (>80%) was recovered. All the supernatants were combined and evaporated to dryness under nitrogen in a TurboVap® LV evaporator at 37°C. The residue was reconstituted with 20-50% ACN in water before injection onto an HPLC column.

**Metabolite Profiling.** Metabolite profiling in rats was conducted using method-A on an HPLC system consisting of Shimadzu LC-10AD pumps and a degasser (Columbia, MD), an HTS PAL autosampler (Carrboro, NC), and a β-RAM radioactivity detector from AIM Research, Inc. (Newark, DE). The HPLC and β-RAM were externally controlled using a StopFlow™ LC-ARC system from AIM Research, Inc. (Newark, DE). Chromatography was performed on an Aqua C18 column (5 micron, 2.1 x 150 mm) from Phenomenex (Torrance, CA). The mobile phases consisted of 10 mM ammonium acetate (pH = 5, adjusted with 0.01% formic acid (FA)) in water (mobile phase A) and 10 mM ammonium acetate (pH = 5) in 90% ACN with 0.01% FA (mobile phase B). The flow rate was 0.2 ml/min. HPLC gradients were initiated with 10% B for 3 min, changed to 20% B over 7 min, followed by a shallow gradient to 35% B over 50 min, and then changed to 55% B over 10 min. The gradient was then changed to 98% B over 10 min and held there for 10 min. The gradient was then returned to the initial composition of 10% B within one minute. The system was allowed to equilibrate for 9 min before the next injection. The column effluent was split and approximately 70 µl/min was introduced into a 4000 QTRAP® linear ion trap mass spectrometer equipped with a TurboIonSpray source from AB Sciex, Inc. (Foster city, CA). The remaining effluent was directed to the flow cell
of the β-RAM. The quantitative assessment of metabolites was performed by integrating the areas of HPLC radioactive peaks using ARC software. Radioactive peaks were reported as a percentage of the total radioactivity collected during the entire HPLC run. The relative distribution of radioactive metabolites in urine, feces, and bile was calculated from the percentage of the dose excreted in the matrix multiplied by the percent distribution of metabolites in radiochromatograms of the matrix.

Metabolite profiling in dogs was conducted using method-B on a Waters 2695 HPLC system with a Waters 486 UV detector (Milford, MA) and an ISCO Foxy 200® fraction collector from ISCO, Inc. (Lincoln, NE). Chromatography was performed on a Synergi Hydro-RP C18 column (4 micron, 4.6 x 150 mm) from Phenomenex (Torrance, CA). The mobile phases consisted of 10 mM ammonium acetate (pH = 5) in water (mobile phase A) and 10 mM ammonium acetate (pH = 5) in 95% ACN (mobile phase B). The flow rate was 0.7 ml/min. HPLC gradients were the same as described above. Fractions of chromatography effluents were collected by time (15 sec/fraction) to Deepwell LumaPlate™-96 plates from PerkinElmer Life Science, Inc. (Meriden, CT). The plates were subsequently dried by a SpeedVac® concentrator from Savant Instrument, Inc. (Halbrook, NY) for up to 8 h. The radioactivity in each fraction was determined by Packard TopCount NXT™ Microplate Scintillation and Luminescence Counter technology (Meriden, CT). HPLC radiochromatograms were reconstructed using ARC® Convert and Evaluation software. Radioactivity peaks were integrated to determine the percent distribution of individual radioactivity peaks or regions in each sample. The relative distribution of radioactive metabolites in the matrix was then calculated as a percentage of the dose.
In method B, Synergi Hydro-RP C18 column replaced Aqua C18 column used in method A, due to improved separation of some metabolites, for example, M10 and M12. The pH of mobile phases and gradient in method A and B were the same. Rat urine and bile samples were also analyzed using method B to bridge metabolite identification in dogs.

**Metabolite Identification.** Mass spectrometry (MS) spectra were obtained on either a 4000 QTRAP® linear ion trap mass spectrometer equipped with a TurbolonSpray source from ABSciex, Inc. or an LTQ-Orbitrap® high resolution mass spectrometer equipped with a Max-ESI or Heated-ESI ionization source from Thermo, Inc. (San Jose, CA). In-line radioactivity detection coupled with LC-MS/MS was used to facilitate metabolite detection. The 4000 QTRAP® was operated in positive ion mode with the electrospray voltage set at 4500 V with nitrogen gas as a collision gas and the collision energy set at 75 ± 15 eV. Various scan modes were used for identifying metabolites, which included precursor information-dependent acquisition (IDA), neutral loss IDA, multiple-reaction monitoring IDA, multiple-ion monitoring IDA, and enhanced product ion scans. The LTQ-Orbitrap® mass spectrometer was used for accurate mass measurement and was connected with an Accela LC system from Thermo, Inc.. The same chromatographic condition was used as for metabolite profiling. The electrospray ion source voltage was 4.5-5.0 kV. The heated capillary temperature was 300-350°C. The scan-event cycle consisted of a full scan mass spectrum at a resolving power of 30,000 (at a mass-to-charge ratio (m/z) of 400), and the corresponding data-dependent tandem mass spectrometry (MS²) scans were acquired at a resolving power of 7500. Accurate mass measurements were performed using an external calibration.
NMR analysis. Isolated metabolites M1, M3, M13, M14, and M17 were prepared in methanol-D₄ or acetonitrile-D₃ from Cambridge Isotope, Inc. (Billerica, MA) containing 0.05% v/v trimethylsilane (TMS) as an internal chemical shift reference standard. NMR measurements for M3 and M17 were carried out on a Bruker Avance 3, 600 MHz spectrometer (Billerica, MA) equipped with a 5mm, TCI, Z-gradient CryoProbe. The others were measured on a Bruker Avance 500 MHz spectrometer equipped with a 5 mm, QNP CryoProbe. The sample temperature was maintained at 28°C for all data collections, and spectra were acquired and processed using version 2.1 and patch level 6 Bruker TopSpin software (Billerica, MA). 1D ¹H NMR spectra, 2D DQF ¹H/¹H-COSY, 2D ¹H/¹³C HSQC, 2D ¹H/¹³C and ¹H/¹⁵N HMBC, and selective 1D TOCSY were collected using the standard Bruker pulse sequences.

Metabolite Isolation and Derivatization. M1, M3, M4, M5, M13, M14, and M17 were isolated from either rat or dog bile or feces by solid phase extraction, liquid-liquid extraction, HPLC separation with fraction collection, and multiple TLC separations. M3 and M17 were extracted with ethyl acetate and purified with modified HPLC method A from rat bile. M3 was also isolated from dog feces. Dog feces was subjected to solid phase extraction with Oasis HLB extraction cartridge (35 cc, 6 g from Waters, Inc. (Milford, MA)) followed by fractionated HPLC separation. The fractions containing M1, M3 and M13 were concentrated and separated on preparatory normal phase TLC (silica gel 60 F₂₅₄ plates (5 x 20, and/or 20 x 20 cm; 250 μm) with chloroform, methanol and formic acid or ammonia hydroxide as developing systems. The TLC bands containing each metabolite were scraped off and further purified again with modified HPLC method B. M13 was also isolated from dog bile during the isolation of M14 under the same isolation
condition. A mixture of M4 and M5 was collected from dog bile with the HPLC fractionation using a modified method B.

**ß-Glucuronidase hydrolysis of M4 and M5.** The isolated M4 and M5 mixture was transferred into a standard vial and evaporated to dryness under a nitrogen stream. The residue was dissolved in a 0.1 M sodium phosphate buffer (pH 6.8), and 10 µl of ß-glucuronidase (53,000 units/ml) were added into the solution. After incubation at 37°C for 3 h, the reaction mixture was dried under a nitrogen stream, and the residue was reconstituted with ACN:H₂O (1:1) before analysis.

**M13 methylation.** An aliquot of isolated M13 was transferred into an HPLC vial. An aliquot of fresh prepared diazomethane solution (100 µl) was added (Glastrup, 1998), and the reaction mixture was vortexed. The reaction mixture was kept at room temperature for 1 h. At the end of the reaction, the reaction mixture was evaporated to dryness under nitrogen using an N-Evaporator from Organomation Associates, Inc. (Berlin, MA), and the dry residue was reconstituted in methanol before analysis.

**Sulfatase hydrolysis of M17.** An aliquot of the M17 fraction was dried and reconstituted in 0.1 M of phosphate buffer (pH=7.0), followed by addition of sulfatase solution (6.125 units/ml). The mixture was incubated for 16 h at 37°C and stopped with volumes of ACN. The resulting sample was centrifuged, and the supernatant was then transferred and concentrated before analysis.

**Hydrolysis of M18.** An aliquot of ethyl acetate extract of rat bile was dried and reconstituted in 0.5 N HCl solution. The mixture was incubated for 1 h at 50°C and extracted with ethyl acetate. The organic fraction was concentrated before analysis.
**QWBA.** Six adult male (217-238 g) and 6 adult female (142-161 g) pigmented LE rats received a single oral dose of [\(^{14}\text{C}\)]GDC-0449 in 0.5% w/v methycellulose (400 centipoise) and 0.2% v/v Tween-80 at a target dose of 2 mg/kg and approximately 50 μCi/rat. One male and 1 female pigmented rat per time point were euthanized at 1, 4, 8, 24, 72, and 144 h post-dose by deeply anesthetizing via isoflurane inhalation. Immediately following euthanasia, each rat was prepared for QWBA by immersion into a freezing chamber containing dry ice and hexanes for at least 15 min. Each carcass was embedded, cut into sagittal sections, and mounted for QWBA. Selected sections were exposed to phosphor image screens from Fuji Biomedical, Inc. (Stamford, CT), and tissue radioactivity concentrations were quantified from the whole-body autoradiograms using a validated image analysis system consisted of a Typhoon 9410™ image acquisition system from GE Healthcare/Molecular Dynamics, Inc. (Sunnyvale, CA) and version 7.0 MCID™ image analysis software from GE Healthcare/Imaging Research, Inc. (St. Catherines, Ontario, Canada). Concentrations of radioactivity were expressed as μCi/g and converted to microgram equivalents of GDC-0449 per gram of matrix (μg-Eq/g) using the specific activity (90.24 μCi/mg) of the administered formulated [\(^{14}\text{C}\)]GDC-0449. The lower limit of quantitation (LLOQ) was determined as 0.008 μg-Eq/g of tissue and the upper limit of quantitation (ULOQ) as 72.305 μg-Eq/g of tissue.
Results

Pharmacokinetics. The mean plasma concentration-time profiles and pharmacokinetic parameters of total radioactivity in rats and dogs following oral administration of \(^{14}\text{C}\)GDC-0449 are presented in Fig. 2 and Table 1, respectively. The results indicate that the plasma terminal elimination half-lives \((t_{1/2})\) of total radioactivity were comparable with mean estimates being 34.6-43.8 h after oral administration in rats and dogs, respectively. Despite high levels of radioactivity being achieved in both species, absorption appeared to be more rapid in rats based upon the lower \(t_{\text{max}}\) estimate. In rats, the mean AUC\(_{0-\infty}\) of the total radioactivity was 400 μg-Eq·h/g for male rats and 288 μg-Eq·h/g for female rats after a single oral dose of 50 mg/kg, while the mean AUC\(_{0-\infty}\) of the total radioactivity was 169 μg-Eq·h/g for male dogs and 179 μg-Eq·h/g for female dogs after a single oral dose of 5 mg/kg. On a per kilogram dose basis, higher exposures were achieved in dogs when compared to rats. The exposure of total radioactivity in males and females were comparable

Excretion and Mass Balance.

Rats. After a single oral dose of \(^{14}\text{C}\)GDC-0449 in SD rats, the radioactivity was excreted predominantly in feces. Table 2 shows the mean cumulative radioactivity recovery in the urine and feces over 240 h post-dose (detailed excretion data were presented in supplemental table 1). Excretion of radioactivity was similar in both males and females. At 240 h post-dose, the average cumulative excretion amounted to 2.4% in the urine and 90.0% in the feces with a majority of radioactivity being recovered within the first 48 h. In BDC rats following an oral dose of GDC-0449, an average of 38.9% of the administered radioactivity was excreted in the bile over 120 h post-dose (Table 2). Similar
to fecal excretion, the majority of radioactivity recovered in bile was excreted in the first 48 h. Based on the radioactivity excreted in bile and urine, approximately 42.1% of the dose was absorbed in rats.

**Dogs.** Similar to rats, the feces was the primary route of excretion in Beagle dogs after a single oral administration of 5 mg/kg of [14C]GDC-0449. The total radioactivities recovered over 336 h were 70.7% in male dogs (3.8% in urine and 66.9% in feces) and 90.2% in female dogs (6.1% in urine and 84.1% in feces) (Supplemental table 1). Over the first 48 h, radioactivity recovered in urine and feces was 49.1% of the dose on average in dogs (Table 2), which is less than observed in rats. In BDC male dogs, an average of 8.3%, 47.7% and 22.5% of the administered radioactivity was excreted in the urine, feces and bile over 336 h, respectively (female BDC dogs were not studied; Table 2). At least 30.8% of the dose was absorbed in dogs based on the radioactivity recovered in bile and urine.

**Metabolite Profiles of GDC-0449 in Rats and Dogs.** The metabolism of GDC-0449 in rats and dogs was assessed by profiling plasma, urine, feces, and bile. The samples from each matrix of rats and dogs were pooled to represent >95% of the total radioactivity in the corresponding excretion routes.

**Rats.** In plasma, GDC-0449 was the only major radioactive peak, accounting for >95% of plasma radioactivity at 0.5, 4, 8, and 24 h. No radioactive peaks were detected at 48 and 72 h. Minor mono-oxidative metabolites (M1, M3, M10, M11 and M14) and subsequent glucuronides (M4 and M5) were detected in plasma by LC-MS/MS, but these metabolites were below the detection limit of online flow scintillation detection.
Representative HPLC radiochromatograms of the extracted urinary (pooled from 0-24 h), fecal (pooled from 0-48 h), and bile (pooled from 0-24 h) samples from rats are shown in Fig. 3, and the tabulated mean percentages in these matrices are presented in Table 3. In urine, each of the observed radiochromatographic peaks (Fig. 3A) was <1% of the total dose with the exception of M9 (a pyridine ring opening metabolite), which accounted for an average of 1.1% of the dose in female rats and 0.3% in male rats. The radio-peak that eluted at 20 min was not identified since no molecular ion was detected and it was below 1% of the dose. In feces, GDC-0449 was the most abundant drug-related component, which accounted for on average of 44.7% and 40.2% of the dose in male and female rats, respectively. The most abundant metabolite was M3 (oxidative metabolite), representing 15.9% and 10.2% of the dose in males and female rats, respectively. In females, M9 radioactivity accounted for 5.7% of the dose, but in males, M9 accounted for 1.7% of the dose. No glucuronide metabolites were detected in feces. The four major metabolites found in rat bile were M3 (an oxidative metabolite), M4 and M5 (glucuronide metabolites), and M17 (a sulfate metabolite), with each one accounting for 0.5-12.7% of the dose. The parent drug represented <1% of the dose in bile. Many minor metabolites were also detected, but they represented <1% of the dose.

_Dog._ Similar to what was observed in rats, GDC-0449 accounted for >95% of the total circulating radioactivity in dog plasma. M5 was the only metabolite detected by LC-MS/MS in MRM mode.

Representative HPLC radiochromatograms of the extracted urinary (pooled from 0-192 h), fecal (pooled from 0-144 h), and bile (pooled from 0-240 h) samples from dogs are shown in Fig. 4, and the tabulated mean percentages in these matrices are presented in
Table 3. In urine, 10 discernible peaks, including unchanged GDC-0449, were observed in the HPLC radiochromatogram. Unchanged GDC-0449 represented <1% of the dose excreted in urine. Each metabolite identified in urine was ≤1.2% of the dose, and all together they accounted for 2.6-4.1% of the dose. In feces, 6 major peaks were observed in the HPLC radiochromatogram. As in rats, GDC-0449 was the most abundant drug-related component in feces, which accounted for an average of 23.0% and 13.2% of the dose in male and female dogs, respectively. The most abundant metabolite was M3 (a mono-oxidative metabolite), representing 18.9% and 28.0% of the dose in male and female dogs, respectively. The pyridine ring opening metabolites, M13 and M18, were the second most abundant in feces, accounting for 10.4% and 17.7% of the dose in total in male and female dogs, respectively. Two other metabolites, M1 and M15 (oxidative metabolites) were identified, each accounting for 3.1-3.2% of the dose in male and 4.3-5.6% in female dog feces. In dog bile, GDC-0449 and M5 were the most abundant radioactive peaks, each accounting for 3.8% of the dose. M3, M14, M15 (oxidative metabolites), M4 (a glucuronide), M13 and M18 and M16 were identified and accounted for 0.7-3.1% of the dose.

**Metabolite Identification.** Overall, 17 metabolites were identified in rats and dogs (Table 4) in the current \(^{14}\)C labeled study. Metabolites M1, M3, M4, M5, and M6 had been identified from previous *in vitro* and *in vivo* studies (Wong *et al.*, 2009). M2 reported in the previous study was not identified in the current study. This might be due to the higher doses used (500 mg/kg and 1200 mg/kg PO) in previous studies. Metabolite structures were elucidated using various LC-MS/MS techniques, including enhanced product ion scans and/or acquisition of their accurate mass data. Metabolites M1, M3, M13, M14, and
M17 were isolated, and their NMR spectra were obtained for further characterization. Chemical or enzymatic reactions were performed to confirm the existence of certain functional groups, such as glucuronides, sulfates, and carboxylic acids.

GDC-0449 eluted at 71.4 min (method-A) and had a protonated molecular ion \([\text{M+H}]^+\) signal at \(m/z\) 421. It had an isotope pattern at \(m/z\) 421, 423 and 425 with intensity ratios of 9:6:1, which is characteristic for the presence of two chlorine atoms. The product ion spectrum obtained by collision induced dissociation (CID) at \(m/z\) 421 contained major fragment ions at \(m/z\) 342, 313, 306, 217, 204, 188, 155, and 139 (Table 4 and Fig. 5). The fragmentation interpretation of GDC-0449 is presented in Fig. 5. The fragment ion at \(m/z\) 342 resulted from loss of the methyl sulfonyl moiety. The subsequent loss of HCl from the ion at \(m/z\) 342 led to the formation of the ion at \(m/z\) 306. The cleavage of the amide bond resulted in the fragment ion at \(m/z\) 204 and the characteristic fragment ion at \(m/z\) 217, corresponding to the substructure of the 2-chloro-4-methylsulfonyl benzamide moiety. From the ion at \(m/z\) 217, loss of the methyl sulfonyl moiety led to formation of the ion at \(m/z\) 139. The accurate mass of the fragment ion at \(m/z\) 154.9907 suggests that oxygen migration occurred while the methyl sulfonyl moiety cleaved from the ion at \(m/z\) 217. This type of rearrangement was previous reported (Baarschers and Krupay, 1971). This fragmentation pattern plus the chlorine isotope pattern were used to facilitate GDC-0449 metabolite identification and structure elucidation. The precursor ion scans of \(m/z\) 342, 204, and 217 were further used to identify metabolites that had modifications on the methyl sulfonyl, 4-chloro-3-(pyridin-2-yl)-phenyl, and 2-chloro-4-methylsulfonyl benzamide moieties, respectively.
The $^1$H-NMR data of GDC-0449 are summarized in Table 5. The $^1$H-NMR spectrum of GDC-0449 showed ten aromatic protons at $\delta$ 7.40-8.69 (H$_3$, H$_5$, H$_6$, H$_2$, H$_5'$, H$_6'$, H$_3''$, H$_4''$, H$_5''$, and H$_6''$) and one methyl group at $\delta$ 3.14 (CH$_3$). The assignment of the proton chemical shifts was confirmed by $^1$H-$^1$H COSY analysis (supplemental figure 1). Typical substituted aromatic proton coupling patterns were observed and used to determine the location of modifications in the metabolites.

Metabolites M1, M10, M11, M12, and M14 have a [M+H]$^+$ signal at $m/z$ 437, which is 16 amu higher than the GDC-0449 molecular ion and is indicative of mono-oxidation. Even though the base fragment peak across the product ion spectra of these oxidative metabolites is different, they have the same fragments as the parent compound at $m/z$ 217, 155, and 139, indicating that the 2-chloro-4-methylsulfonyl benzamide moiety remained unchanged and modification occurred on the 4-chloro-3-(pyridin-2-yl)-phenyl moiety (Table 4). M1 and M14 were isolated from dog feces and bile, and their $^1$H-NMR data are presented in Table 5. Both M1 and M14 had one less aromatic proton compared to GDC-0449, which was consistent with hydroxyl substitution. In M1, the signal of H$_6'$ at $\delta$ 7.06 ppm changed to a broad singlet from a double doublet at $\delta$ 7.76 ppm (d, J=7 Hz) in GDC-0449 and indicated that hydroxylation occurred at H$_5'$. In M14, the coupling constant of H$_6'$ on the chloroaniline ring changed from double doublets to a doublet, indicating that hydroxylation occurred at H$_2'$. The signal of H$_5'$, which shifted from $\delta$ 7.52 to $\delta$ 7.18 ppm, also indicated that hydroxylation occurred at H$_2'$.

Metabolites M3 and M17 eluted at 67.5 and 47.2 min (method-A), respectively. The [M+H]$^+$ signal of M3 is 16 amu higher than that of GDC-0449, which is indicative of mono-oxidation. In the product ion spectrum of M3, the presence of the same fragments
as the parent compound at \( m/z \) 217, 155, and 139 indicated that the 2-chloro-4-methylsulfonyl benzamide moiety remained unchanged. The ion at \( m/z \) 188 was not present and an ion at 16 amu higher (\( m/z \) 204) was observed instead, suggesting that mono-oxygenation occurred on the 4-chloro-3-(pyridin-2-yl)-phenyl moiety (Table 4). The \(^1\)H-NMR spectrum of M3 is similar to that of GDC-0449 except for the aromatic proton signals on the pyridine ring (Table 5). In M3, the missing H$_{5''}$ resonance signal indicated that the hydroxyl was substituted on C$_{5''}$ on the pyridine ring (the meta position relative to nitrogen). Because of the hydroxyl substitution on C$_{5''}$, the signals of H$_{4''}$ and H$_{6''}$ in the ortho and para positions relative to nitrogen are strongly shifted to the high-field compared to those of GDC-0449 (Table 5). The [M+H]$^+$ signal of M17 at \( m/z \) 517 is 80 amu higher than that of M3 at \( m/z \) 437, indicating that M17 is a sulfate of M3. This was confirmed by the formation of M3 when isolated M17 was incubated with sulfatase. The \(^1\)H-NMR signals of M17 were largely unchanged compared to M3 except for H$_{4''}$ and H$_{6''}$, which show a down-field shift of approximately 0.5 and 0.2 ppm, respectively, due to substitution with a strong electro-withdrawing group (Table 5). Thus, the \(^1\)H-NMR data supports the structure elucidation of M17 as a sulfate of M3. The chemical shift assignments of each proton in M3 and M17 are based on the results of 2D DQF \(^1\)H/\(^1\)H-COSY, 2D \(^1\)H/\(^13\)C HSQC, 2D \(^1\)H/\(^13\)C and \(^1\)H/\(^15\)N HMBC, and selective 1D TOCSY.

Metabolites M4, M5 and M6 eluted at 30.4, 32.5 and 33.4 min (method-A), respectively, and had a [M+H]$^+$ signal at \( m/z \) 613, which is 176 amu higher than that of the mono-oxidative metabolite of GDC-0449 at \( m/z \) 437 and is indicative of glucuronides of the oxidative metabolites of GDC-0449 (Table 4). The product ion spectra of the aglycones of M4 and M5 were very similar to those of M1 and M3, suggesting that M4 and
M5 were likely glucuronides of M1 and M3, respectively. In bile, M5 was the most abundant glucuronide, and it was not detected in feces. On the other hand, M3 was the predominant oxidative metabolite in feces (Table 3). This data further suggests that M3 is probably the aglycone of M5. This was confirmed by the incubation of isolated M4 and M5 with β-glucuronidase.

Metabolites M7 and M8 were minor metabolites that was present only in rat bile, representing <1% of the dose. These two metabolites eluted at 31.6 and 45.2 min (method-A), respectively, and had a [M+H]+ signal at m/z 726, which is 305 amu higher than that of GDC-0449, indicating that these metabolites were glutathione (GSH) conjugates of the parent drug. The product ion spectra of m/z 726 gave major fragment ions at m/z 597, 494, 453, 374, 217, 155, and 139 (Table 4). The fragment ion at m/z 597, formed by a typical neutral loss of 129 amu from the [M+H]+ signal at m/z 726, suggests a loss of glutamic acid from the GSH moiety. The most abundant fragment ion at m/z 453 was the result of loss of glutamyl-dehydroalanyl-glycine by fragmentation of the S-C bond on the cysteinyl-β-carbon sulfur bond. The ion at m/z 374 corresponded to loss of a methyl sulfonyl moiety from m/z 453. The same fragments as the parent compound at m/z 217, 155, and 139 indicated that the 2-chloro-4-methylsulfonyl benzamide moiety remained unchanged. These data suggest that GSH was conjugated to the 4-chloro-3-(pyridin-2-yl)-phenyl moiety.

Metabolite M9 eluted at 49.9 min (method-A) and had a [M+H]+ signal at m/z 443.0224, indicated that its empirical formula is C_{18}H_{17}O_{5}N_{2}Cl_{2}S (-1.23 ppm). The high resolution MS/MS spectrum of m/z 443 and MS³ spectrum of m/z 443→426 gave fragments at m/z 425.9978, 398.0032, 379.9922, 216.9729, and 154.9907 (Fig. 6). The
same fragments as the parent compound at \( m/z \) 217 and 155 suggest that the 2-chloro-4-methylsulfonyl benzene moiety remained unchanged and that the modification occurred on the pyridine-chloro-aniline moiety. The loss of formamide \( \text{CONH}_3 \) from \( m/z \) 443 to 398 indicated that M9 had an amide moiety, suggesting that this modification likely involved pyridine ring opening. The \( \text{MS}^n \) spectrum of M9 was very similar to that of M13 except that its \([\text{M+H}]^+\) is 1 amu lower than M13. M13 was confirmed to be a carboxylic acid via pyridine ring opening by \(^1\text{H}-\text{NMR}\) data (see below). Therefore, M9 is proposed to be an amide metabolite.

Metabolite M13 eluted at 40.5 min (method-B) in dog feces, and slightly earlier at 37.5 min, in dog urine and bile. The different observed retention time of M13 in urine, bile and feces was perhaps due to matrix effects, but its spectra in different matrices were identical. M13 was isolated and purified from dog feces. High resolution mass analysis produced a \([\text{M+H}]^+\) signal at \( m/z \) 444.0050, which is consistent with a molecular formula of \( \text{C}_{18}\text{H}_{16}\text{O}_6\text{NCl}_2\text{S} \) (-4.54 ppm). The intensity of the \([\text{M+H}]^+\) signal is much lower than that of the \([\text{M+NH}_4]^+\) signal at \( m/z \) 461/463 or that of the ion at \( m/z \) 426/428, which results from loss of water in the ionization source. This suggests a structural modification that resulted in formation of a metabolite with reduced proton affinity. The product ion mass spectrum of \( m/z \) 444 contains the fragment ions at \( m/z \) 426, 398, 217 and 155, which are the same as those of M9, in which the 2-chloro-4-methylsulfonyl benzamide moiety remained unchanged. The fragment at \( m/z \) 398 resulted from the neutral loss of formic acid from \( m/z \) 444 and indicated that M13 had a carboxylic acid moiety.

The \(^1\text{H}-\text{NMR}\) spectrum of M13 showed six aromatic protons at \( \delta \) 7.46-8.11 ppm, four aliphatic protons at \( \delta \) 2.63 ppm and \( \delta \) 3.13 ppm, and one methyl group at \( \delta \) 3.15 ppm.
The six aromatic protons displayed coupling patterns consistent with the typical 1, 2, 4 or 1, 3, 4 aromatic substitutes and were assigned to the proton signals of H₃, H₅, H₆, H₂', H₅', and H₆'. In comparison to GDC-0449, the aromatic proton signals of the pyridine ring were missing in M13. Instead, four aliphatic protons at δ 2.63 ppm and δ 3.13 ppm appeared as two triplets and were coupled to each other (Fig. 7 and supplemental figure 2), indicating the existence of a CH₂CH₂ moiety. Combined with accurate masses of the [M+H]⁺ and fragment ions, the structure of M13 is proposed to be a carboxylic acid formed via pyridine ring opening. To further confirm the structure of M13, isolated M13 was derivatized with diazomethane, and methylated M13 was detected by LC-MS/MS with a fragmentation pattern that supported the proposed structure.

Metabolite 15 eluted at 51.0 min (method-B) and the high resolution mass analysis produced a [M+H]⁺ signal at m/z 453.0055, which is consistent with a molecular formula of C₁₉H₁₅O₅N₂Cl₂S (-4.04 ppm). The molecular weight is 32 amu higher than that of GDC-0449, which is indicative of a di-oxygenated metabolite. The product ions at m/z 435 and 417, formed by loss of water and HCl from the [M+H]⁺, respectively, are also 32 amu higher than the corresponding ions of GDC-0449 (Table 4). The product ion at m/z 356 (-97 amu) resulted from the loss of methyl sulfonyle and water and further fragmented into the ions at m/z 338 (-H₂O) and 328 (-CO). The product ions at m/z 217 and 155, similar to those of GDC-0449, indicated that the 2-chloro-4-methylsulfonyl benzamide remained unchanged and that oxygenation most likely occurred on the pyridine-2-chloro-5-aminobenzene moiety.

Metabolite M16 eluted at 40.0 min (method-B), and the high resolution mass analysis produced a [M+H]⁺ signal at m/z 599.06802, which is consistent with a molecular
formula of C_{25}H_{25}O_{9}N_{2}Cl_{2}S (4.62 ppm). The molecular weight is 162 amu higher than the mono-oxygenated metabolite at m/z 437, which is indicative of glucosidation. Product ions at m/z 437 and the chlorine satellite at m/z 439 were detected, both of which are formed as a result of loss of sugar (-162 amu). These ions further fragmented to ions at m/z 419/421 (loss of water), m/z 401 (loss of HCl), and m/z 340/342 (loss of the 2-chloro-4-methylsulfonyl benzamide moiety). Product ions at m/z 217 and 155, similar to those of GDC-0449, indicated that the 2-chloro-4-methylsulfonyl benzamide remained unchanged and suggests that the oxidation and glucosidation most likely occurred on the pyridine-2-chloro-5-aminobenzene moiety.

Metabolite M18 eluted at 26 min (method-A) and the accurate mass of the [M+H]^+ was observed at m/z 471.0168 and 473.0135 with the distinct two chlorine pattern. The empirical formula of m/z 471.0168 is proposed as C_{19}H_{17}O_{6}N_{2}Cl_{2}S (-3.18 ppm), which is the addition of CO compared to the formula of M9. The high resolution MS/MS spectrum of m/z 471.0168 generated fragments at m/z 453.0072, 443.0237, 425.9978, 425.0130, and 397.0179 (Fig. 8). The direct loss of carbonyl from the [M+H]^+ suggests the existence of an aldehyde moiety in M18. The fragment ion at m/z 425.9978 has the exact same accurate mass as one of the fragments of M9, but the corresponding structures are different, as illustrated in Fig. 6 and Fig. 8, because of the lack of a further loss of the carbonyl moiety to form the fragment ion at m/z 398.0032 in M18. The fragment ion at m/z 425.0130 is formed by the loss of formic acid from the parent ion, indicating the existence of a carboxylic acid in M18, therefore, the structure of M18 was tentatively proposed as illustrated in Fig. 8. In addition, M18 converted to M13 after it was incubated
with 0.5 N HCl for 1 h, suggesting that M18 is likely an intermediate for the formation of M13.

**QWBA.** The tissue distribution of GDC-0449 was investigated using QWBA. A representative whole-body autoradiogram (female) showing patterns of radioactivity distribution in tissues is illustrated in Fig. 10, and the concentrations of drug-derived radioactivity in the tissues of rats are summarized in supplemental table 2.

In male rats, drug-derived radioactivity was absorbed and widely distributed to tissues, with concentrations present in almost all tissues at 1-24 h post-dose. $C_{max}$ was observed at 1 h post-dose for most tissues (35 of 39 tissues evaluated at each time point). Overall, distribution of drug-derived radioactivity was similar in female rats. In both male and female rats, the highest radioactivity concentration in blood was observed at 1 h post-dose (1.677 and 2.592 μg-Eq/g, respectively), and concentrations were detectable through 24 h post-dose in males (0.044 μg-Eq/g) and 144 h post-dose in females (0.011 μg-Eq/g). Concentrations of radioactivity in the excretory tissue system were generally higher than blood concentrations at all time points, while other organ system tissues showed concentrations similar to those in blood.

Concentrations of radioactivity in all tissues in male rats declined over the course of the study and most (32 of 39) tissues were below the quantifiable limit (BQL) by 144 h post-dose. However, elimination was not complete as radioactivity remained in the uvea of the eye (0.088 μg-Eq/g), the aorta (0.085 μg-Eq/g), the liver (0.063 μg-Eq/g), the Harderian gland (0.051 μg-Eq/g), the kidney cortex (0.025 μg-Eq/g), the adrenal gland (0.025 μg-Eq/g), the lung (0.009 μg-Eq/g), and the esophagus (0.009 μg-Eq/g). Similarly, in female rats concentrations of radioactivity in all tissues declined over the course of the
study, and most (31 of 38) tissues were BQL by 144 h post-dose. Elimination was not complete in females as radioactivity remained in the uvea of the eye (0.184 μg-Eq/g), the aorta (0.081 μg-Eq/g), the adrenal gland (0.063 μg-Eq/g), the liver (0.055 μg-Eq/g), the kidney cortex (0.024 μg-Eq/g), the pigmented skin (0.018 μg-Eq/g), and the blood (0.011 μg-Eq/g). The association of [14C]GDC-0449-derived radioactivity with melanin-containing tissues appeared to be reversible and would be expected to be eliminated if the study were conducted for a longer time, because radioactivity in pigmented skin and the uveal tract decreased steadily. Furthermore, radioactivity in the meninges and choroid plexus of the brain, which is known to contain melanin, was not observed in the autoradiographs of male and female rats. This data supports the notion that the association between [14C]GDC-0449-derived radioactivity with melanin is reversible and that elimination of such radioactivity from ocular melanin is predicted.
Discussion

Here we report on the metabolic fate and disposition of GDC-0449 in rats and dogs, the species used for safety assessment. These studies ultimately provide insight in our understanding of the route of excretion and the contribution of metabolism to the overall clearance in these two preclinical species. These studies will also aid in our understanding of metabolic information generated in human mass balance studies.

GDC-0449 was $^{14}$C labeled in the central ring to avoid possible loss of the radiolabel via metabolic modification. GDC-0449 was dosed orally at pharmacologically and toxicologically relevant doses of 5 mg/kg in dogs and 50 mg/kg in rats. In male and female rats, 92.4% of the dose was recovered, with the majority excreted in feces in the first 48 h post-dose. In dogs, the recoveries in males were lower, at an average of 71%, compared to females, at an average of 90%. In male and female BDC rats, an average of 38.9% of the administered radioactivity was excreted in bile and 3.2% in urine, indicating that at least 42.1% of the dose was absorbed. This data is similar to a previously reported rat oral bioavailability of 52.9% when the animals were dosed 5 mg/kg in a suspension in 0.5% methylcellulose with 0.2% Tween 80 (Wong et al., 2009). In male BDC dogs, the radioactivity recovered in bile and urine revealed that at least 30.8% of dosed GDC-0449 was absorbed, consistent with the 32.9% oral bioavailability in dogs reported previously (Wong et al., 2009).

An interesting aspect of the radioactivity recovery in dog feces was that significant amounts of radioactivity were recovered far beyond the mean GI transit time of ~13 h (Davies and Morris, 1993). Previous work performed in dogs using an oral
physiologically-based pharmacokinetic (PBPK) model suggested that GDC-0449 exhibited non-sink permeation characteristics due to its low solubility, high permeable and extremely low metabolic clearance (Wong et al., 2010). Based on this proposed oral PBPK model, GDC-0449 could undergo exsorption moving from the systemic circulation, across the intestinal membrane, and back into the lumen of the intestinal tract. The detection of radioactivity in feces at time intervals far exceeding GI transit time is consistent with our previously proposed model, since the concentration gradient to drive the exsorption process would be sufficient only following the transit of unabsorbed GDC-0449 into the feces.

There was no substantial sex difference in the total circulating radioactivity following oral administration to male and female rats and dogs. In both rats and dogs, the predominant component of the radioactivity present in plasma was parent GDC-0449 (>95%). The observed plasma $t_{1/2}$ of radioactivity in dogs (~35 h) was similar to the previously reported estimate of 42 h. In contrast, the $t_{1/2}$ of radioactivity in rats (~44 h) was longer than the previously reported estimate of 1.3 h (Wong et al., 2009). A discrepancy in the $t_{1/2}$ estimate when using radiolabeled compounds is often related to the presence of metabolites in plasma. However, since the primary component in plasma was unchanged GDC-0449, the difference in $t_{1/2}$ is more likely related to differences in dose of GDC-0449 given to rats in the previous study (1 mg/kg IV) and the current experiment (50 mg/kg PO). The higher dose administered may have led to metabolic saturation of the clearance pathway resulting in a longer estimate of terminal half-life.

In the QWBA study, drug-derived radioactivity was widely distributed to tissues of pigmented male and female rats through 24 h post-dose. High levels of drug-related
radioactivity in the contents of the alimentary canal are consistent with a portion of the dose not being absorbed in the rat mass balance study, as describe above. Elimination was not complete at the end of the QWBA study (144 h) with notable concentrations of radioactivity observed in the eye uvea (Fig. 10), suggesting that an association exists between GDC-0449 and melanin. However, this association appears reversible, and radioactivity in pigmented skin and the eye uveal decreased steadily over the study period. In addition, radioactivity in the meninges and choroid plexus of the brain, both of which are known to contain melanin, was not observed in the autoradiographs of male and female rats. On the basis of these observations, the complete elimination of [14C]GDC-0449-derived radioactivity from ocular melanin would be predicted if the study duration were to be extended.

Metabolite profile analysis indicated that GDC-0449 underwent extensive oxidative and conjugative metabolic reactions in rats and dogs. The structures of metabolites and the metabolic pathways are illustrated in Fig. 9. In rats, the majority of radioactivity was found in feces of which unabsorbed GDC-0449 (42% of the dose) was the primary drug related component. The other major drug-related components (>1%) in rat feces were M3 (13%) and M9 (pyridine ring opening at an average of 3.7%). GDC-0449 was detected in rat bile at <1% of the dose, and the majority of the metabolites (>1%) in this matrix consisted of the oxidative sulfate (M17 at 9.9%), mono-oxidative metabolite (M3 at 6.1%), and glucuronides of oxidative GDC-0449 (M4 at 1.5% and M5 at 7.3%). The conjugative metabolites excreted in bile were not detected in feces, most likely due to hydrolysis by gut microflora. Two minor GSH conjugates (M7 and M8) were also detected in the bile, each of which accounts for <0.5% of the dose. These GSH metabolites were not detected
in rat feces, perhaps due to a minimal relative presence compared to other radioactive peaks. These GSH conjugates were also not detected in dogs (bile or feces).

Similar to rats, feces was the predominant route of GDC-0449 elimination in dogs. The amount of GDC-0449 recovered in feces was on average approximately 18% of the dose. Considering ~30% of the dose was estimated to be absorbed in the dog, unchanged GDC-0449 expected to be recovered in the feces was ~70% of the dose. To investigate this discrepancy, selected female dog feces was profiled by time intervals and revealed that GDC-0449 was the most abundant radioactive component (~35% of the radioactivity) over the first 48 h. In subsequent days, M3 became the most abundant radioactive component. Due to only unchanged GDC-0449 detected in plasma, this time dependent change in abundance of GDC-0449 and M3 in feces suggested that GDC-0449 might be metabolized in the gut resulting in the formation of M3 and subsequently M13 and M18. In dogs, the BDC study was conducted only in males, and samples were collected for 336 h. Unlike rat bile, no oxidative sulfate metabolite (M17) was detected in dogs. More unchanged GDC-0449 was eliminated in bile in dogs than in rats (3.8% vs. 0.4%). The major metabolites (>1% of the dose) in dog bile were mono-oxidative metabolites (M3 and M14), glucuronides (M4 and M5), pyridine ring-opened products (M13 and M18), a dioxidative metabolite (M15) and a glucose conjugate (M16).

A total of three uncommon pyridine ring-opened metabolites were detected (M9, M13 and M18) in rats and dogs. M9, the major ring-opened product in rats, was found in feces, urine, and to a lesser degree in bile. In dogs, M9 was not detected, and the major ring-opened product found was M13, a hydrolyzed product of M18 and possibly of M9. However, these metabolites were not detected when GDC-0449 was incubated in liver
microsomes or hepatocytes (Wong et al., 2009). The absence of pyridine ring-opened metabolites in vitro in hepatic incubations may be a consequence of either very slow hepatic turnover of GDC-0449 observed in microsomes and hepatocytes or the involvement of extra-hepatic enzymes in their formations.

Enzyme catalyzed ring opening of heteroaromatic compounds have been reported in the literature including furans, thiophenes, thiazoles, and thiazolidinediones (Kalgutkar, et al., 2005). In the case of ring opening of pyridines, they are rare but have been observed in microbial metabolism reported by Kaiser et al., (1996), Chaiyen et al., (1997), and McCulloch et al., (2009). For example, 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase was identified involved in the degradation of vitamin B6 in bacteria and catalyzed oxidative ring opening of 2-methyl-3-hydroxypyridine-5-carboxylic acid to form E-2-acetaminomethylene succinate (Chaiyen et al., 1997, and McCulloch et al., 2009). In addition to oxidative ring opening of hydroxylated pyridines, pyridine ring opening has also been proposed via reduction followed by ring fission under anaerobic conditions by Kaiser et al., (1996). The mechanism leading to the pyridine ring opening to from M18, M9 and M13 is not clear, but M18 may likely be an intermediate which could further be hydrolyzed to form M9 or M13. Investigations are underway to study the formation mechanism of M9, M13 and M18 and to determine the enzymes responsible for the observed biotransformation.

Absorbed GDC-0449 underwent extensive metabolism in rats and dogs. The metabolites identified in this study, compared to metabolites (M1 and M3) reported in human liver microsomal incubation (Wong et al., 2009), suggests that the toxicology species provides adequate coverage of the reported human metabolites. The final
In conclusion, GDC-0449 is reasonably well absorbed in rats and dogs and is widely distributed to tissues. This molecule undergoes extensive metabolism and is primarily eliminated via bile to feces. The metabolism of GDC-0449 occurs largely via oxidation on the 4-chloro-3-(pyridin-2-yl)-phenyl moiety followed by sequential glucuronidation and sulfation and pyridine ring-opening.
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Contributed new reagents or analytical tools: Reynolds, Deese

Performed data analysis: Yue, Mulder, Wong, Deese, Solon, Hop, Khojasteh

Wrote or contributed to the writing of the manuscript: Yue, Wong, Hop, Khojasteh
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410.
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Legends for figures

FIG. 1. The chemical structure of $[^{14}\text{C}]\text{GDC-0449}$. The star (*) denotes the location of the uniformly distributed $^{14}\text{C}$-radiolabel in the phenyl ring of the 4-chlorine-aniline moiety.

FIG. 2. Mean plasma concentration-time profiles of total radioactivity in rats and dogs after oral administration of $[^{14}\text{C}]\text{GDC-0449}$. A: Rats; B: Dogs

FIG. 3. Representative radiochromatograms of GDC-0449 and its metabolites in urine (A), feces (B), and bile (C) after a single 50 mg/kg oral dose of $[^{14}\text{C}]\text{GDC-0449}$ in rats.

FIG. 4. Representative radiochromatograms of GDC-0449 and its metabolites in urine (A), feces (B), and bile (C) after a single 5 mg/kg oral dose of $[^{14}\text{C}]\text{GDC-0449}$ in dogs

FIG. 5. The product ion spectrum of GDC-0449 at $m/z$ 421 and its fragmentation interpretation.

FIG. 6. The MS, MS/MS, and MS$^3$ spectra of M9 by LTQ-Orbitrap in a positive ion mode

FIG. 7. The $^1\text{H}-^1\text{H}$ COSY NMR spectra of M13 in acetonitrile-$d_3$. The insert is the expended aliphatic region $\delta$ 2.5~3.3 ppm (see Table 5)

FIG. 8. The MS, MS/MS, and MS$^3$ spectra of M18 by LTQ-Orbitrap in a positive ion mode

FIG. 9. The proposed metabolic pathways for biotransformation of $[^{14}\text{C}]\text{GDC-0449}$ in rats and dogs. Bold line represents major metabolic pathway in either rats or dogs.

GS=glutathione; Gluc=glucuronic acid; O=oxygen; Glc=glucose

FIG. 10. A whole-body autoradiogram of the radioactivity distribution in female Long-Evans rats at 1 and 144 h post-dose following a single PO administration of $[^{14}\text{C}]-\text{GDC-0449}$ (2 mg/kg).
TABLE 1
Pharmacokinetic parameters of total radioactivity in rats and dogs following oral administration of $[^{14}C]$GDC-0449

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Dose (mg/kg)</th>
<th>$C_{\text{max}}$ (µg-Eq/g)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>$t_{1/2}$ (h)</th>
<th>AUC$_{0-\infty}$ (µg-Eq·h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Male (n=3)</td>
<td>50</td>
<td>40.0</td>
<td>1</td>
<td>45.2</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Female (n=3)</td>
<td>50</td>
<td>29.0</td>
<td>1</td>
<td>42.4</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td>34.5</td>
<td>1</td>
<td>43.8</td>
<td>344</td>
</tr>
<tr>
<td>Dog</td>
<td>Male (n=2)</td>
<td>5</td>
<td>3.44</td>
<td>3</td>
<td>36.8</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Female (n=2)</td>
<td>5</td>
<td>2.85</td>
<td>15</td>
<td>32.5</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td>3.15</td>
<td>9</td>
<td>34.6</td>
<td>174</td>
</tr>
</tbody>
</table>

$AUC_{0-\infty} =$ area under the plasma concentration-time curve; $C_{\text{max}} =$ highest observed mean concentration; $t_{1/2} =$ half life; $t_{\text{max}} =$ time at which $C_{\text{max}}$ is observed.
TABLE 2
Mean Percentage of Radioactive Dose Excreted in Urine, Feces and Bile from Rats and Dogs after a Single Oral Administration of [14C]GDC-0449a

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bile duct intact animals</th>
<th>Bile duct cannulated animalsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
</tr>
<tr>
<td>Ratsc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8</td>
<td>0.8</td>
<td>2.0</td>
</tr>
<tr>
<td>0-24</td>
<td>2.3</td>
<td>84.1</td>
</tr>
<tr>
<td>0-48</td>
<td>2.4</td>
<td>89.4</td>
</tr>
<tr>
<td>0-72</td>
<td>2.4</td>
<td>89.7</td>
</tr>
<tr>
<td>0-96</td>
<td>2.4</td>
<td>89.8</td>
</tr>
<tr>
<td>0-120</td>
<td>2.4</td>
<td>89.9</td>
</tr>
<tr>
<td>0-240</td>
<td>2.4</td>
<td>90.0</td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8</td>
<td>0.0</td>
<td>4.3</td>
</tr>
<tr>
<td>0-24</td>
<td>1.0</td>
<td>24.1</td>
</tr>
<tr>
<td>0-48</td>
<td>1.9</td>
<td>47.1</td>
</tr>
<tr>
<td>0-72</td>
<td>2.6</td>
<td>57.2</td>
</tr>
<tr>
<td>0-96</td>
<td>3.2</td>
<td>64.4</td>
</tr>
<tr>
<td>0-120</td>
<td>3.5</td>
<td>68.6</td>
</tr>
<tr>
<td>0-336</td>
<td>4.9</td>
<td>75.5</td>
</tr>
</tbody>
</table>

a mean of male and female; b male only for dogs; c samples were collected and analyzed at 24 h intervals; "-"=no samples
### TABLE 3
Relative distribution of metabolites in pooled urine, feces, and bile following oral administration of [14C]GDC-0449 to rats and dogs

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percentage of dose in rats</th>
<th>Percentage of dose in dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine (0-24 h)</td>
<td>Feces (0-48 h)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>GDC-0449</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>M1</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>M3</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>M4</td>
<td>0.2</td>
<td>D</td>
</tr>
<tr>
<td>M5</td>
<td>D</td>
<td>0.3</td>
</tr>
<tr>
<td>M6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M9</td>
<td>0.3</td>
<td>1.1</td>
</tr>
<tr>
<td>M10</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>M11</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>M12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M13</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>M14</td>
<td>0.5</td>
<td>D</td>
</tr>
<tr>
<td>M15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M17</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>M18</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

---

M=male; F=female; D=detected by LC-MS/MS and radiochromatographic peak was not discernible; "-"=not detected.

"The percentage of the dose was calculated as the % of the sample multiplied by the percent of excretion from n=2 for dogs and n= 3 for rats. "This metabolite co-eluted with M9. "These figures represent the total percentage of the dose of the listed metabolites; the remainder of the radioactivity was distributed in various minor peaks (each representing <5% of the dose) throughout the radiochromatogram. These minimal peaks were not characterized. "This data represents the total percentage of the dose excreted in each route over the time period of sample pooled.
# TABLE 4

Major collision-induced product ions of GDC-0449 and its metabolites in rats and dogs

<table>
<thead>
<tr>
<th>Analyte</th>
<th>[M+H]$^+$ (m/z)</th>
<th>Major fragment ions$^a$ (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0449</td>
<td>421</td>
<td>342 (100), 313, 306, 217, 188, 155, 139</td>
</tr>
<tr>
<td>M1</td>
<td>437</td>
<td>419 (100), 340/342, 217, 139</td>
</tr>
<tr>
<td>M3</td>
<td>437</td>
<td>419, 401, 358 (100), 322, 247, 217, 204, 155, 139</td>
</tr>
<tr>
<td>M4</td>
<td>613</td>
<td>437 (100), 419, 340/342, 217</td>
</tr>
<tr>
<td>M5</td>
<td>613</td>
<td>437 (100), 419, 401, 358/360, 322, 204, 155</td>
</tr>
<tr>
<td>M6</td>
<td>613</td>
<td>595, 437 (100), 421, 401, 358</td>
</tr>
<tr>
<td>M7</td>
<td>726</td>
<td>709, 663, 597, 580, 494, 453 (100), 374, 217, 155, 139</td>
</tr>
<tr>
<td>M8</td>
<td>726</td>
<td>709, 663, 597, 580, 494, 453 (100), 217, 199, 155, 139</td>
</tr>
<tr>
<td>M9</td>
<td>443</td>
<td>426 (100), 398, 380, 217, 155</td>
</tr>
<tr>
<td>M10</td>
<td>437</td>
<td>401, 409, 383 (100), 323, 217, 139</td>
</tr>
<tr>
<td>M11</td>
<td>437</td>
<td>419, 409 (100), 383, 374, 339, 304, 229, 217, 204, 168, 155</td>
</tr>
<tr>
<td>M12</td>
<td>437</td>
<td>419, 401, 383 (100), 358, 323, 217, 155</td>
</tr>
<tr>
<td>M13</td>
<td>444</td>
<td>426 (100), 398, 380, 362, 301, 217, 155</td>
</tr>
<tr>
<td>M14</td>
<td>437</td>
<td>419, 401, 383 (100), 391, 217, 155</td>
</tr>
<tr>
<td>M15</td>
<td>453</td>
<td>435 (100), 417, 356, 338, 328, 217, 155</td>
</tr>
<tr>
<td>M16</td>
<td>599</td>
<td>437 (100), 419, 401, 340, 217, 155</td>
</tr>
<tr>
<td>M17</td>
<td>517</td>
<td>437, 419, 358 (100), 322, 217, 204, 155, 139</td>
</tr>
<tr>
<td>M18</td>
<td>471</td>
<td>453, 443, 426, 425, 397</td>
</tr>
</tbody>
</table>

$^a$Base peak is labeled as 100 in parentheses.
# TABLE 5

1H NMR spectroscopy data of GDC-0449, M1, M3, M13, M14, and M17

<table>
<thead>
<tr>
<th>Proton</th>
<th>GDC-0449</th>
<th>M1</th>
<th>M3</th>
<th>M13</th>
<th>M14</th>
<th>M17</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH</td>
<td>1H, 9.14 s</td>
<td>1H, 8.94 s</td>
<td>1H, 8.97 s</td>
<td>1H, 8.07 d, 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1H, 8.05 d (1.6)</td>
<td>1H, 8.03, br s</td>
<td>1H, 8.08 d (1.6)</td>
<td>1H, 8.11 d (2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1H, 7.95 dd (1.7, 7.8)</td>
<td>1H, 7.93, d (8.0)</td>
<td>1H, 7.98 dd (1.7, 8.0)</td>
<td>1H, 8.0 dd (8.0, 2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1H, 7.81 d (8.0)</td>
<td>1H, 7.84, d (8.0)</td>
<td>1H, 7.84 d (8.1)</td>
<td>1H, 7.83 d (8.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>1H, 7.91 d (2.6)</td>
<td>1H, 8.16, br s</td>
<td>1H, 7.91 d (2.7)</td>
<td>1H, 7.73 d (2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>1H, 7.54 d (8.7)</td>
<td>---</td>
<td>1H, 7.52 d (8.7)</td>
<td>1H, 7.46 d (8.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6'</td>
<td>1H, 7.76 dd (2.7, 8.7)</td>
<td>1H, 7.06, br s</td>
<td>1H, 7.70 dd (2.6, 8.6)</td>
<td>1H, 7.97 dd (8.5, 2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3''</td>
<td>1H, 7.71 m, (1.0, 7.8)</td>
<td>1H, 7.67, d (8.0)</td>
<td>1H, 7.61 dd (0.5, 8.5)</td>
<td>2H, 3.13 t (7.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4''</td>
<td>1H, 7.88 m (1.8, 7.8)</td>
<td>1H, 7.81, m</td>
<td>1H, 7.32 dd (2.9, 8.5)</td>
<td>2H, 2.63 t (7.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5''</td>
<td>1H, 7.40 m (1.0, 4.9, 7.9)</td>
<td>1H, 7.31, m</td>
<td>-</td>
<td>1H, 7.73 m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6''</td>
<td>1H, 8.69 m (1.0, 4.9)</td>
<td>1H, 8.66, d (4.6)</td>
<td>1H, 8.33 d (2.4)</td>
<td>1H, 8.71 d (4.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH₃</td>
<td>3H, 3.14 s</td>
<td>3H, 3.15, s</td>
<td>3H, 3.15 s</td>
<td>3H, 3.13 s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

s=singlet; d=doublet; dd=doublet of doublets; m=multiplet.
FIG 2

A: Rat

B: Dog

Plasma Concentrations (ug-Eq/ml)

Time (hrs)

Male
Female
FIG 3

A: Urine (0-24 h)

B: Feces (0-48 h)

C: Bile (0-24 h)

Retention Time (min)

CPM

Unknown
M5
M14
M10
M3

GDC-0449

M9
M3
M9
M3
M17
M5
M4
M8
M3
FIG 10