Evaluation of Metabolism and Disposition of GDC-0152 in Rats Using 14C Labeling Strategy at Two Different Positions: A Novel Formation of Hippuric Acid from 4-Phenyl-5-Amino-1,2,3-Thiadiazole

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Received May 31, 2012; accepted December 4, 2012

ABSTRACT

The compound (S)-1-((S)-2-cyclohexyl-2-((S)-2-[methylamino]propanamido)acetyl)-N-(4-phenyl-1,2,3-thiadiazol-5-yl)pyrrolidine-2-carboxamide (GDC-0152) is a peptidomimetic small molecule antagonist of inhibitor of apoptosis (IAP) proteins with antitumor activity. The mass balance, pharmacokinetics, tissue distribution and metabolism of GDC-0152 was investigated in rats following intravenous administration of 15 mg/kg of [14C]GDC-0152, labeled either at the terminal phenyl ring (A) or at the carbonyl of the 2-amino-2-cyclohexylacetyl moiety (B). In rats, 92.2%–95.1% of the radiolabeled GDC-0152 dose was recovered. Approximately 62.3% and 25.1% of A was excreted in urine and feces, respectively. By contrast, B was excreted almost equally in urine (27.2%), feces (32.2%), and expired air (27.5%). GDC-0152 underwent extensive metabolism, with less than 9% of the dose recovered as parent in excreta. Similarly, in plasma, GDC-0152 represented 16.7% and 7.5% of the area under the curve of the total radioactivity for A and B, respectively. The terminal half-life (t1/2) for total radioactivity was longer for B (21.2 hours) than for A (4.59 hours). GDC-0152 was highly metabolized via oxidation and amide hydrolysis, followed by subsequent sulfation and glucuronidation. The most abundant circulating metabolites were the amide hydrolyzed products, M26, M28, M30, M31, and M34, which ranged from 3.5% to 9.0% of total radioactivity. In quantitative whole-body autoradiography studies, the residence of radioactivity in tissues was longer for B than for A, which is consistent with the t1/2 of the total radioactivity in circulation. A novel 4-phenyl-5-amino-1,2,3-thiadiazole (M28) oxidative cleavage resulted in the formation of hippuric acid (M24). This biotransformation was also observed in rat hepatocyte incubations with para-substituted M28 analogs. In addition, the formation of M24 was inhibited by 1-aminobenzotriazole, which points to the involvement of P450 enzymes.

Introduction

Inhibitors of apoptosis (IAP) proteins are involved in regulating apoptosis or programmed cell death. IAP proteins act to suppress apoptosis, in part, through inhibition of activated cytosolic cysteine/aspartate-specific proteases (caspases) that are essential for apoptosis to occur (Flygare and Fairbrother, 2010; Varfolomeev and Vucic, 2011). The mammalian family of IAP proteins includes X-linked IAP (XIAP), cellular IAP 1 and 2 (cIAP1 and cIAP2), and melanoma IAP. IAP proteins are frequently overexpressed in cancer cells, where they serve as regulators of cancer cell survival and are often indicators of poor prognosis (Vucic and Fairbrother, 2007). By contrast, inhibition of IAP proteins sensitizes cancer cells to pro-apoptotic anticancer agents. Interest has, therefore, been shown in the development of IAP antagonists as potential anticancer agents.

The compound (S)-1-((S)-2-cyclohexyl-2-((S)-2-[methylamino]propanamido)acetyl)-N-(4-phenyl-1,2,3-thiadiazol-5-yl)pyrrolidine-2-carboxamide (GDC-0152) (Fig. 1) is a potent pan inhibitor of IAP proteins, with binding affinities to the third baculoviral IAP repeat domains of XIAP, cIAP1, cIAP2, and the baculoviral IAP repeat domain of melanoma IAP in the low nanomolar range (Ki < 50 nM) as determined by a fluorescence polarization-based competition assay (Flygare et al., 2012). GDC-0152 showed robust antitumor activity as a single agent in xenograft mice bearing MDA-MB-231 breast cancer tumors (Flygare et al., 2012). GDC-0152 is a peptidomimetic small molecule inhibitor that is anticipated to undergo amide hydrolysis in vivo, resulting in cleavage of the molecule. To thoroughly detect metabolites and better understand the disposition of GDC-0152 in rats, we conducted a detailed rat mass balance study with two different GDC-0152 molecules: one radio-labeled with 14C on the carbonyl of the 2-amino-2-cyclohexylacetyl moiety (B) (Fig. 1). The primary objectives of the present study were to characterize the disposition of GDC-0152, determine its routes of excretion, and

ABBREVIATIONS: ABT, 1-aminobenzotriazole; ACN, acetonitrile; amu, atomic mass unit; AUC, area under the plasma drug concentration-time curve; BDC, bile duct-cannulated; cIAP1, cellular inhibitor of apoptosis 1; cIAP2, cellular inhibitor of apoptosis 2; GDC-0152, (S)-1-((S)-2-cyclohexyl-2-((S)-2-[methylamino]propanamido)acetyl)-N-(4-phenyl-1,2,3-thiadiazol-5-yl)pyrrolidine-2-carboxamide; HPLC, high-performance liquid chromatography; IAP, inhibitor of apoptosis; LC, liquid chromatography; LE, Long-Evans; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z, mass to charge; SD, Sprague Dawley; t1/2, half-life, TMSC2H2N2, trimethylsilyldiazomethane; QWBA, quantitative whole-body autoradiography; XIAP, X-linked inhibitor of apoptosis.

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Received May 31, 2012; accepted December 4, 2012

http://dx.doi.org/10.1124/dmd.112.047019

Drug Metab Dispos 41:508–517, February 2013

Supplemental material to this article can be found at: http://dx.doi.org/10.1124/dmd.112.047019

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http://dx.doi.org/10.1124/dmd.112.047019

Drug Metabolism & Disposition
identify metabolites in plasma and excreta in rats following a single i.v. administration of these two [14C]GDC-0152 molecules. The tissue distribution of the two [14C]GDC-0152 molecules was also characterized in rats in a quantitative whole-body autoradiography (QWBA) study.

Materials and Methods

General Chemicals

High-performance liquid chromatography (HPLC)—grade acetonitrile (ACN), methanol, formic acid, water, ammonium formate, ammonium hydroxide, ethyl acetate, and diethyl ether were purchased from either Mallinckrodt Baker, Inc. (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Ammonium acetate (analytical grade), trimethylsilyldiazomethane (TMSCH2N2), trifluoroacetic acid, l-aminobenzyltriazole (ABT), sulfatase from Aerobacter aerogenes type VI glycerol solution and β-glucuronidase (from Helix pomatia, type H-1) were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Phenyl-5-amino-1,2,3-thiadiazole and its analogs [4-(4-chlorophenyl)-1,2,3 thiadiazole-5-amine (G1); 4-(4-isonicotinamidophenyl)-1,2,3 thiadiazol-5-amine (G2); and 4-(4-methoxyphenyl)-1,2,3-thiadiazol-5-amine (G3)] were purchased from Oakwood Products, Inc. (West Columbia, SC). Cryopreserved rat hepatocytes were obtained from Celsis In Vitro Technology (Baltimore, MD). The 14C cocktail for the online β-RAM IN/US Systems, Inc. radioactive detection was purchased from AIM Research Co. (Newark, DE). The Ultima Gold XR scintillation cocktail and Carbo-Sorb II were obtained from PerkinElmer Life Science (Waltham, MA).

Radionuclide and Reference Compounds

[14C]GDC-0152 was synthesized by Selcia Limited (Essex, UK) as a free base with a chemical and radiochemical purity of >98% as determined by nuclear magnetic resonance spectroscopy and radio-HPLC analysis. Compound A was radiolabeled with 14C evenly distributed in the benzene ring with a specific activity of 66.9 mCi/mmol; compound B was 14C radiolabeled on the carbonyl of the 2-amino-2-cyclohexylacetyl moiety with a specific activity of 66.9 mCi/mmol (Fig. 1). Unlabeled GDC-0152 with a chemical purity of >99% was synthesized at Genentech, Inc. (South San Francisco, CA).

Animals, Dosing and Sample Collection

Femoral or jugular vein and bile duct–cannulated (BDC) Sprague Dawley (SD) and Long-Evans (LE) rats were purchased from Hilltop Laboratory Animals, Inc. (Scottdale, PA). The animals were housed individually in suspended, stainless steel wire-mesh cages and were acclimated for at least 2 days prior to dose administration. Food (Certified Rodent Diet #5002, PMI Nutrition International, Brentwood, MO) and water were provided ad libitum. Animals were fasted overnight (at least 12 hours) prior to administration of the dose and up to 4 hours post dose. During the test period, the animals were placed in plastic metabolism cages. 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.

SD rats were administered intravenously with a single dose of compound A or B at a target dose of 15 mg/kg (100 μCi/kg). GDC-0152 with and without 14C labeling was formulated in 15% hydroxypropyl-β-cyclodextrin and 20 mM succinic acid in water. The formulations were stirred continuously on a magnetic stir plate to achieve and maintain homogeneity throughout the dose administration procedure. The specific activity of the dosing formulation was 6.42 and 6.23 μCi/mg for compounds A and B, respectively. The stability of compounds A and B in the dosing formulation was qualitatively assessed by analyzing predose and postdose aliquots of each formulation with the established radio-HPLC method.

Four groups of rats were assigned and dosed separately with either compound A or B. The first group of SD rats (n = 3 per sex) was designated for mass balance determination. Urine and feces were collected on dry ice at approximately 0–8 and 8–24 hours post dose and at 24-hour intervals up to 240 hours post dose.

The second group of BDC SD rats (n = 3 per sex) was designated for biliary excretion determination. In BDC rats, a solution of taurocholic acid (2.3 mg/mL in 0.9% saline) was infused via a 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 hours post dose. The samples were stored at −20°C until sample analysis.

The third group of SD rats (n = 7 per sex) were designated for pharmacokinetic analysis and identification of circulating metabolites. Animals were sacrificed via cardiac puncture after anesthetization at predose and at 0.25, 1, 3, 6, 24, and 48 hours post dose. The terminal blood sample was collected from each animal into tubes containing potassium EDTA. Plasma was prepared from blood by centrifugation and stored at −70°C until sample analysis.

The fourth group of rats (n = 6 per sex) consisted of pigmented LE rats and was designated for tissue distribution QWBA. One male and one female were euthanized per timepoint at 0.083, 1, 6, 12, 24, and 120 hours post dose after deep anesthetization via isoflurane inhalation. Immediately following euthanasia, each rat was immersed into a freezing chamber containing dry ice and hexanes. The frozen carcass was stored at −20°C for QWBA.

Due to the recovery of only 59% of radioactivity in excreta from group 1 following administration of compound B, we subsequently dosed compound B to three additional male SD rats housed in sealed glass metabolism cages that were designed for expired air collection in addition to urine and feces sample collection. The system was sealed, and air was entered through the inlet of the system with airflow drawn by a vacuum pump through a series of two traps. The two traps (designated scrubbers A and B) were arranged in series. Any expired [14C]CO2 gas was trapped when bubbled through glass cylinders containing a solution of Carbo-Sorb II and methoxyethanol (1:1). The [14C] CO2 scrubber liquids were exchanged at 24-hour intervals up to 168 hours post dose. The total weight of each [14C]CO2 scrubber liquid specimen was measured. The samples were stored at −20°C until sample analysis.

Determination of Radioactivity

Radioactivity in plasma, urine, and bile was measured for at least 5 minutes or 100,000 counts using a Packard 2800TR liquid scintillation counter from PerkinElmer Life and Analytical Sciences (Waltham, MA). The Ultima Gold scintillation cocktail was used for sample analysis. All samples were analyzed in duplicate. 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 microgram equivalents of GDC-0152 per mL plasma (μg-Eq/mL) and was calculated using the specific activity of the administered dose.

Fecal samples were homogenized with 3 volumes of water. Triplicate-weighed 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 [14C]CO2 in a mixture of Pemafluor and Carbo-Sorb II prior to analysis by liquid scintillation counting.

QWBA

The frozen carcasses were embedded in a 2% carboxymethylcellulose matrix and mounted on a microtome stage (Leica CM3600 Cryomacrot, Nussloch, Germany, or Vibratome 9800, St. Louis, MO). Three quality control standards,
which were plasma fortified with [14C]glucose at one concentration (approximately 0.05 μCi/g; lot JAR090313) were placed into each frozen block and were used for section thickness quality control. Sections approximately 40 μm thick were taken in the sagittal plane and captured on adhesive tape (3M Ltd., St. Paul, MN). Selected sections along with calibration standards of [14C]glucose mixed with blood at 10 different concentrations (0.00075540 to 6.52477612 μCi/g) were exposed to phosphor image screens from Fuji Biomedical, Inc. (Stamford, CT). The tissue radioactivity concentrations were quantified using a validated image analysis system consisted of a Typhoon 9410 image acquisition system with MCID image analysis software (version 7.0) from GE Healthcare/Molecular Dynamics (Sunnyvale, CA) and Imaging Research, Inc. (St. Catherines, Ontario, Canada), respectively. Concentrations of radioactivity were expressed as microgram equivalents of GDC-0152 per gram of matrix (μg-Eq/g) using the specific activity of the administered formulated compounds A and B at 6.42 μCi/μg and 6.23 μCi/mg, respectively. For compound A, the lower limit of quantitation was determined to be 0.118 μg-Eq/g of tissue, and the upper limit of quantitation was 110.1 μg-Eq/g of tissue. For compound B, the lower limit was determined to be 0.121 μg-Eq/g of tissue, and the upper limit was 1046.98 μg-Eq/g of tissue.

**Pharmacokinetic Analysis**

Pharmacokinetic parameters for total radioactivity in plasma, GDC-0152, and major circulatory metabolites were calculated by noncompartmental methods using WinNonlin Professional Edition, version 5.2 from Pharsight Corporation (Mountain View, CA). Area under the drug plasma concentration-time curve (AUC) was determined from time 0 to the last measurable concentration.

**Extraction of Metabolites from Biologic Samples**

Aliquots of urine and feces and bile samples obtained at various timepoints were pooled relative to the excreted volume or weight at each timepoint, 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 hours post dose, respectively, except that rat urine following compound B administration was pooled from 0 to 72 hours. Pooled urine and bile samples were centrifuged with or without dilution with 1 volume of ACN, and then the supernatant was injected onto an HPLC column without further purification. The pooled fecal homogenates (approximately 2 g) were extracted with 4 mL of ACN for compound A or with methanol for compound B by sonication and vortexing. This mixture was centrifuged, and the supernatant was removed and saved. The residue was extracted an additional time with 4 mL of 90% ACN or methanol in water as previously described. Both supernatant fractions were combined and evaporated under steam of nitrogen gas in a TurboVap LV evaporator (Caliper Life Sciences, Inc., Hopkinton, MA) at 37°C. The residues were reconstituted to 0.4 mL with 50% ACN or methanol in water and centrifuged before injection onto an HPLC column (30 μL). Aliquots of 20 μL of this solution were counted in a liquid scintillation counter for radioactivity to determine the overall recovery of the extraction process.

Plasma samples collected at 0.5, 1, 3, 6, 24, and 48 hours from group 3 rats were extracted for metabolite profiling and identification. The individual plasma samples were subjected to protein precipitation by addition of three volumes of ACN followed by vortex mixing, sonication, and centrifugation at 2095g for 10 minutes. The supernatant was evaporated under nitrogen gas in an EVX-192 Apricot Evaporex evaporator (Apricot Designs, Inc., Covina, CA). The residues were reconstituted to 100% of sample volume with 50% ACN or methanol in water and centrifuged before injection onto an HPLC column (30 μL). Aliquots of 20 μL of this solution were counted in an liquid scintillation counter for radioactivity to determine the recovery of the extraction process.

**Metabolite Profiling in Rats**

Metabolite profiling was conducted on a HPLC system consisting of Shimadzu LC-10AD pumps and a degasser (Columbia, MD), an HTS PAL autosampler (Carborro, NC), and a β-RAM radioactivity detector from AIM Research, Inc. The HPLC and β-RAM were externally controlled using a StopFlow LC-ARC system from AIM Research, Inc. Chromatography was performed on a Proteo C18 column (5 μm, 4.6 × 250 mm) from Phenomenex, Inc. (Torrance, CA). The mobile phases consisted of 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1% trifluoroacetic acid in methanol (mobile phase B). The flow rate was 0.7 mL/min. HPLC gradients were initiated with 5% B for 11 minutes, changed to 66% B over 98 minutes, and followed by an increase to 88% B over 22 minutes. The gradient was then changed to 98% B over 3 minutes and held there for 9 minutes. The gradient was then returned to the initial composition of 5% B within 1 minute. The system was allowed to equilibrate for 21 minutes before the next injection. Plasma samples were run using a slightly different HPLC gradient from the other matrices, and a liquid chromatography (LC) factor of 40 controlled by ARC software was used to improve sensitivity. The LC factor of 40 indicates that the mobile phase flow was reduced to 40% when a peak was detected by β-RAM detector. The column effluent was split, and approximately 250 μL/min was introduced into a 4000 QTRAP 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 the radiochromatograms of the matrix.

**Metabolite Identification**

Mass spectrometry (MS) spectra were obtained on either a 4000 QTRAP or an LTQ-Orbitrap mass spectrometer equipped with a Max-ESI source from Thermo, Inc. (San Jose, CA). Online radioactivity detection coupled with liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used to facilitate metabolite detection. The 4000 QTRAP was operated in positive ion mode with the electrospray voltage set at 3500 V and nitrogen gas as a collision gas with the collision energy set at 40 ± 15 eV. Various scan modes were used for identifying metabolites, including 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 chromatographic condition for metabolite identification used was the same as for metabolite profiling. The electrospray ion source voltage was 4.5 kV. The heated capillary temperature was 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. The two most intense ions detected during full scan MS triggered data-dependent MS/MS scanning with the combined use of a parent ion list and dynamic exclusion. The collision energy was normalized at 20% for MS/MS scans and at 35% for MS³ scans. Accurate mass measurements were performed using an external calibration.

**Metabolite Derivatization**

**β-Glucuronidase Hydrolysis.** Male urine was adjusted to pH 5 with HCl, and β-glucuronidase (68,000 unit/mL) was added to the solution for 15-hour incubation at 37°C (Jackson et al., 1991). The reaction was stopped with 2 volumes of ACN and centrifuged, and the supernatant was concentrated before analysis.

**M24 and Hippuric Acid Methylation.** Aliquots of plasma from the 3-hour timepoint and purchased hippuric acid were dried under nitrogen and reconstituted in 1:1 methanol:diethyl ether. Solutions were cooled to 0°C in an ice bath before adding 2.0 M TMSCH,N2 in ether dropwise until a yellow color persisted for 30 minutes. The reaction mixtures were centrifuged at 2095g for 10 minutes and concentrated to remove solvents prior to analysis.

**Hydrolysis by sulfatase.** An aliquot of a urine sample was adjusted to pH 7 using Tris buffer, followed by addition of sulfatase solution (5 units/mL). The mixture was incubated for 16 hours at 37°C, and the reaction was stopped with the addition of two volumes of ACN. The resulting sample was centrifuged at 2095g for 10 minutes, and the supernatant was then transferred and concentrated before analysis.
**In Vitro Hepatocyte Incubation**

$[^{14}C]GDC-0152$, M28, or its three analogs were incubated at a concentration of 5 or 20 $\mu M$ in rat hepatocytes ($1 \times 10^3$ cells/mL) for 3 hours at 37°C with or without a 15-minute preincubination with 1 mM ABT. The reactions were quenched with 3 volumes of acetonitrile, the samples were centrifuged at 14,000 $g$ for 5 minutes, and the supernatant was concentrated under a stream of nitrogen gas. A total of 20 $\mu L$ of supernatant were injected into the LC-MS/MS system for analysis after reconstitution with 300 $\mu L$ of 33% methanol in water. For $[^{14}C]GDC-0152$ incubation samples, the LC-MS/MS method described in the metabolite profiling section was used. The mass spectrometer was operated in a positive scan mode. For samples of M28 and its analogs, the LC method was referenced from Penner et al. (2010) and performed on a Thermo Hypersil Gold C18 column (1.9 $\mu M$, 2.1 $\times$ 150 mm). The mobile phases consisted of 20 mM ammonium carbonate in water (pH 7.5, mobile phase A) and methanol (mobile phase B). The flow rate was 0.4 mL/min. HPLC gradients were initiated with 5% B for 1 minute, changed to 50% B over 19 minutes, followed by an increase to 98% B over 5 minutes, and held for 3 minutes. The gradient was then returned to the initial composition of 5% B within 1 minute. The mass spectrometric analysis was performed on an LTQ Orbitrap Velos (Thermo, Inc.) operated in a negative ion scan mode.

**Results**

**Excretion and Mass Balance.**

Table 1 presents the radioactivity recovered in excreta, including urine, feces, and expired air, following a single i.v. administration of compound A or B in rats. In rats given compound A, 62.3% of the radioactivity was found in urine whereas 25.1% was found in feces; a majority of the radioactivity (>93.5%, not reported in Table 1) was excreted within the first 24 hours. In rats given Compound B, the total recovery was much lower, with 26.2% of the total radioactivity found in urine and 29.4% in feces, which accounts for only 58.7% of the total. As a result of the incomplete mass balance, additional male rats were intravenously administered compound B, and the expired $[^{14}C]$CO$_2$ was trapped, which accounted for 27.5% of the total radioactivity. When either compound A or B were given to BDC rats, the radioactivity recovered in bile over 120 hours was similar, ranging from 33.0% to 37.0% of the dose. Most of the bile radioactivity was recovered within the first 8 hours. Excretion of radioactivity was similar in both male and female rats.

**Pharmacokinetics.**

Table 2 presents the pharmacokinetic parameters of the total radioactivity, GDC-0152, and the abundant circulating metabolites formed following i.v. administration of compound A or B in rats. The mean $C_{max}$ values of the total radioactivity following i.v. administration of compounds A and B were similar at 7.5 and 7.8 $\mu g$-Eq/mL, respectively. However, the mean plasma AUC of the total radioactivity for B (114 $\mu g$-Eq/mL) was greater than 2-fold more than that for compound A (43.8 $\mu g$-Eq/mL). Accordingly, a longer plasma terminal elimination $t_{1/2}$ was observed for the total radioactivity for B (21.2 hours) than for compound A (4.6 hours). The estimated AUCs of GDC-0152 for compounds A and B were similar at 7.3 and 8.5 $\mu g$-Eq/mL, respectively, when administered intravenously in rats. The AUCs of GDC-0152 were substantially less than those of the total radioactivity, suggesting that the circulating metabolites accounted for more of the observed radioactivity than the parent drug. Since the AUCs of GDC-0152 labeled at the A or B positions were similar, the differences between the AUC and $t_{1/2}$ of the total radioactivity for A and B previously noted suggest that the metabolites derived from compound B were present much longer in plasma than those from A. No sex difference was observed in AUCs of the total radioactivity in rats.

**QWBA.**

The tissue distribution of GDC-0152 was investigated using QWBA. The concentrations of drug-derived radioactivity in the tissues of rats are summarized in Supplemental Tables 1 and 2. As illustrated in Fig. 2, drug-derived radioactivity was widely distributed to tissues after a single slow bolus i.v. injection of compound A or B to pigmented LE rats. The concentrations in all tissues decreased steadily, but more tissues had measurable concentrations at the last timepoint (120 hours post dose) for compound B than for A, which suggests that the radioactive metabolites of B were present in the body longer than those of A. These observations are consistent with the plasma pharmacokinetic data that showed AUC and $t_{1/2}$ values for total radioactivity for compound B at approximately 2- to 4-fold higher than those determined for compound A. Excretory, endocrine, secretory, and alimentary canal tissues of male and female rats showed the highest concentrations of radioactivity, whereas tissues of the central nervous system, bone, white adipose, and the lens of the eye had the lowest concentrations. For compound A, maximum blood concentrations were observed at 1 hour post dose for males and females at 7.329 and 5.750 $\mu g$-Eq/g, respectively. Similarly, for B, maximum blood concentrations were observed at 0.083 hour post-dose for males at 7.929 $\mu g$-Eq/g and at 1 hour post-dose for females at 6.292 $\mu g$-Eq/g. There were no obvious sex-related differences between the tissue distributions of A and B.
Mass Spectral Fragments of GDC-0152 and Identification of Major Metabolites.

GDC-0152 had a protonated molecular ion at m/z 499 with a retention time of 104.6 minutes. The product ion spectrum of m/z 499 generated by collision-induced dissociation gave a few pairs of fragment ions resulting from amide bond cleavage at m/z 178/322, m/z 275/225, and m/z 414/86 (Fig. 3). The fragment ions m/z 414 and 275 were further cleaved by the loss of N2 to form the fragment ions m/z 386 and 247, respectively. The loss of CO from the fragment m/z 225 led to the formation of the second-most abundant fragment m/z 197. The fragment ions m/z 112 and 70 represent cyclohexyl methylaminium and pyrrolidinium, respectively. All the fragment ions were confirmed by accurate mass measurement. On the basis of observations of mass shifts of −14, +16, +32, or +176 atomic mass units (amu) on one or more of the aforementioned fragments, the demethylation, monooxidation, dioxidation and glucuronidation of GDC-0152 were readily identified.

Amide hydrolysis led to the formation of M14, M28, and M34 (Table 3), and their molecular formulas were elucidated with accurate mass measurement. M28 is a major amide hydrolysis product of GDC-0152, with a chromatographic retention time of 68.9 minutes and a protonated molecular ion at m/z 178.0428 in its full-scan spectrum. The measured accurate mass suggests that the elemental composition of M28 is C8H8N3S (3.12 ppm), which corresponds to 4-phenyl-5-amino-1,2,3-thiadiazole. The fragment ion spectrum of m/z 178 contained the fragment ions m/z 160, 150, 133, 123, 117, 106, 90, and 77. The most abundant fragment ion, m/z 105, corresponds to a benzylideneoxonium. The fragment ion m/z 134 was formed by the loss of 46 amu from m/z 180.1652, which corresponds to loss of a H2CO2, indicative of the presence of a carboxylic acid in M24. The mass difference between the fragments m/z 134 and m/z 105 is 29 amu, suggesting that a benzylideneoxonium fragment was formed by the loss of a methanimine (NHCH2) from the fragment ion m/z 134. Elucidation of the collision-induced disassociation product ion spectrum of M24 suggested that M24 is hippuric acid. To further confirm the structure of M24, the radioactive fraction of M24 was collected and methylated with TMSCH2N2. The methylated M24 had the same retention time as the methylated hippuric acid standard, and their product ion spectra were identical.

When compound B was dosed to animals, metabolites M33 and M35 were detected and identified as the hydroxylation and acetylation products, respectively, of M34, (S)-2-amino-2-cyclohexylacetic acid, which was formed via the hydrolysis of two amide bonds of GDC-0152. Additional minor metabolites derived from M28 and M34, such as m/z 258, 194 and 370, were also present in rat urine, bile, and feces, but no structures were able to be assigned for these metabolites because of their minimal MS responses.

Metabolite Profiles of GDC-0152 in Rats.

The metabolite profile of GDC-0152 in rats was analyzed by profiling plasma, urine, feces, and bile following a single i.v. administration of
compound A or B. The plasma samples were analyzed individually, and the samples from excreta of rats were pooled to represent >95% of the total radioactivity in the corresponding excretion routes. The extraction recovery from all the matrices was greater than 89%.

In plasma, the four abundant metabolites derived from compound A, as summarized in Table 2 and shown in Fig. 4, were the amide hydrolyzed product, 4-phenyl-5-amino-1,2,3-thiadiazole (M28), and the methylated, hydroxylated and glucuronidated products of M28 (M26, M30, and M31), which in total accounted for 24.1% of the total radioactivity. There were many other circulating metabolites that each accounted for less than 3% of the total radioactivity. When compound B was dosed, the major circulating metabolite was (S)-2-amino-2-cyclohexylacetic acid (M34), representing 9.0% of the total radioactivity in rat plasma. The $C_{\text{max}}$ of M34 was 2-fold greater than other circulating metabolites. The $t_{\text{max}}$ of metabolites formed from compound A or B were all in the range of 1–2 hours with the exception of M28, which had a $t_{\text{max}}$ of 0.63 hour.

**Fig. 3.** The product ion spectrum of GDC-0152 at $m/z$ 499 and its fragmentation interpretation.

**TABLE 3**

Fragment ions generated by collision-induced dissociation of GDC-0152 and its major metabolites

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$[\text{MH}]^+\ (m/z)$</th>
<th>RT (min)</th>
<th>Fragment Ions ($m/z$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDC-0152</td>
<td>499</td>
<td>104.6</td>
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<td>182, 154, 125, 97, 76</td>
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RT, retention time.
Table 4 presents the relative abundance of metabolites in rat excreta following a single i.v. administration of compound A or B. GDC-0152 was a minor component of the total radioactivity in feces (<2%) and bile (<1%) but more abundant in urine (3.7%–8.0%). These low values are indicative of extensive metabolism of GDC-0152. The major metabolic pathways are proposed in Fig. 4. Multiple monohydroxylated metabolites (M1–M4) on the cyclohexyl moiety of GDC-0152 were observed from rat excreta after administration of either compound A or B. M1 was the most abundant metabolite detected in bile, representing 7.8%–8.4% of the dose. This metabolite was also excreted in feces at 2.5%–2.6% of the dose. The hydroxylated 2-amino-2-cyclohexylacetic acid metabolite, M33, derived from compound B, was the most abundant drug-related radioactive component in feces, representing 6.4% of the dose.

In rat urine, the majority of GDC-0152 metabolites were derived from compound A via amide hydrolysis, including 4-phenyl-5-amino-1,2,3-thiadiazole (M28), and through the hydroxylation, methylation, glucuronidation and sulfation (M22–M31) of M28. These metabolites corresponded to 43.3% of the total dose in rats. The most abundant urinary metabolite was M26, which was a hydroxylated and methylated product of M28 and represented 16.9% of the total dose. Glucuronidation and/or methylation of M28 represented 0.4%–9.0% of the dose (M27, M30, and M31). M24, the hippuric acid derivative of M28, corresponded to 5.3% of the dose in rats. Additionally, three unknown metabolites derived from B represented 3.0%, 4.2%, and 2.7% of the dose in rat urine.

**Formation of M24 and M28 in Rat Hepatocyte Incubations.**

The pathway for formation of M28 and M24 (hippuric acid) from GDC-0152 was investigated in rat hepatocytes. Less than 30% of GDC-0152 remained after a 3-hour incubation in rat hepatocytes, and M28, M24, various glucuronides, and the oxidative metabolites M1–M4 were detected. Benzoic acid, a proposed precursor of hippuric acid, however, was not identified. The absence of benzoic acid in GDC-0152 incubations may be due to the acid’s rapid conjugation with glycine, resulting in formation of hippuric acid. When GDC-0152 was preincubated with ABT, formation of M24 was reduced.

**Fig. 4.** The proposed major metabolic pathways of [14C]GDC-0152 in rats. The symbols (*) and (#) denote the location of 14C for A and B, respectively. Gluc, glucuronic acid conjugate. Metabolites that are underlined represent >5% of the dose in one of the matrices.

**TABLE 4**

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<tr>
<th>Metabolites</th>
<th>AB</th>
<th>A</th>
<th>B</th>
<th>A</th>
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<th>A</th>
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<td>1.3</td>
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<td>—</td>
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<td>28.7</td>
<td>32.2</td>
<td>27.5</td>
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*< 0.1% = below the detection limit; NA = not applicable; [14C]CO₂ was trapped in a solution of Carbo-Sorb II and methoxyethanol (1:1).

> total percentage of dose of listed metabolites. The remainder of the radioactivity was distributed in various peaks (each representing less than 5% of dose) throughout the radiochromatogram; these peaks were not characterized.

> total percentage of excretion from each route in the time period of the sample pooled.
To further assess hippuric acid formation, incubations were conducted in rat hepatocytes with either an M28 standard or an M28 analog with a para-substituent (Table 5). The analogs were substituted with either an electron-withdrawing group, Cl (G1) or CF₃ (G2), or an electron-donating group, OCH₃ (G3). The substituted benzoic acid or hippuric acid product could, thus, be distinguished from any naturally occurring compounds in the hepatocyte incubation medium. This design also aided in understanding the influence of these groups on 1,2,3-thiadiazole cleavage and the formation of benzoic acid. These incubations were conducted with or without ABT preincubation to determine P450 involvement in the formation of M24. For analysis of these experiments, the LC mobile phase was adjusted to pH 7.5, and the MS detection was operated in a negative scan mode for increased sensitivity.

As shown in Table 5, in the absence of ABT, hippuric acid and its analogs were formed in incubations with M28, G1, G2, and G3. Notably, trifluoromethyl benzoic acid was detected only in the G2 incubation. The absence of benzoic acids formed from the other analogs might be a result of rapid conjugation with glycine. In the presence of ABT, the formation of substituted hippuric acid was inhibited 3- to 5-fold for G1, G2, and G3. Likewise, the formation of trifluoromethyl benzoic acid from G2 was inhibited 2-fold by ABT. On the other hand, formation of hippuric acid from M28 was not appreciably affected by the addition of ABT. This was likely due to endogenous levels of hippuric acid present in the incubations.

**Discussion**

This study describes the disposition and metabolism of the peptidomimetic compound GDC-0152 in rats by utilizing ¹⁴C labels, either evenly distributed on the phenyl ring (A) or on the carbonyl of the 2-amino-2-cyclohexylacetyl moiety (B). As reported in the literature, the multiple-position radiolabeling strategy has been used to assess the metabolic fate of molecules that potentially cleave into much smaller parts (Penner et al., 2012). In the current study, the use of ¹⁴C labels at two different positions within different molecules allowed the metabolites formed through amide hydrolysis of GDC-0152 to be traced. This approach enabled us to identify two unique metabolic pathways involving decarboxylation to liberate CO₂ from (S)-2-amino-2-cyclohexylacetic acid (M34) or hippuric acid formation from 4-phenyl-5-amino-1,2,3-thiadiazole (M28).

More than 92.2% of the administered radioactive dose of compound A in rats was recovered from urine and feces, whereas only 58.7% of the radioactive dose of B was recovered. QWBA studies showed no significant radioactivity remaining in any tissue at the end of study. To investigate whether the remaining radioactivity of compound B was eliminated via expired air, an additional group of male SD rats was dosed with compound B and housed in sealed glass metabolism cages to collect expired air along with urine and feces samples. In total, 95.1% of the dosed radioactivity was recovered from this study, with 27.2% of the radioactive dose recovered in the expired air. This radioactivity could be the result of ¹³C₂O₂ release via decarboxylation from metabolite M34, which was identified as a cyclohexanyl substituted glycine with ¹³C on the carbonyl moiety. The decarboxylation metabolic pathway is commonly observed for amino acids via decarboxylases (EC 4.1.1) (Chang A and Schomburg D, 2001).

GDC-0152-related radioactivity was rapidly eliminated, and elimination was nearly complete within 48 hours after dosing of either compound A or B in rats. Although a majority of the radioactivity from compound A (62.3%) was excreted in urine, the radioactivity from compound B was almost evenly recovered in urine, feces, and expired air. There were no discernible sex differences in the excretion pattern of radioactivity, metabolite profiles, and the pharmacokinetics of the total radioactivity and unchanged GDC-0152 in rats after i.v. administration of compound A or B. Unchanged GDC-0152 displayed similar pharmacokinetic properties regardless of which compound was dosed to rats. However, when compound B was dosed, the total radioactivity in plasma circulation had a 2- to 3-fold higher exposure (AUC), and the t₁/₂ was nearly 5-fold longer than when compound A was dosed. On the basis of AUC estimates, the parent compound accounted for only 16.7% and 7.5% of the total radioactivity in rats for A and B, respectively, thus the majority of the circulating radioactivity was attributed to metabolites. This indicated that metabolites derived from B have greater plasma AUCs than those derived from A. The major circulating GDC-0152 metabolites identified were M26, M28, M30, and M31 from compound A and M34 from compound B, each accounting for 3.54%-9.0% of the total circulating radioactivity (Table 2). The greater AUC and longer t₁/₂ of the total radioactivity than those of GDC-0152 indicated that circulating metabolites cleared more slowly than GDC-0152. Decreased volumes of distribution of metabolites relative to GDC-0152 would provide an alternate explanation for the high AUC of radioactivity, but this would also

**TABLE 5**

<table>
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<th>R</th>
<th>[M+H]⁺ m/z</th>
<th>Major MS² Fragment Ion m/z</th>
<th>ABT-/ABT⁺</th>
<th>[M+H]⁺ m/z</th>
<th>Major MS² Fragment Ion m/z</th>
<th>ABT-/ABT⁺</th>
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<td>168.0222</td>
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<td>145.0271</td>
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</table>

¹¹: not detected under experimental conditions; ABT, 1-aminobenzotriazole; MS, mass spectroscopy.

Ratio of metabolic formation without ABT pretreatment to metabolic formation with ABT pretreatment.
result in a decreased metabolite t1/2, which is not consistent with observed increase in t1/2 of total radioactivity.

GDC-0152 was metabolized and subsequently excreted in urine, bile, feces, and as CO2 in expired air. The metabolites with molecular weights less than 400 amu were excreted in urine, as expected (Klaassen, 2007), and resulted from amide hydrolysis and subsequent oxidation, methylation, and/or conjugation. The larger metabolites (>400 amu) were excreted mostly in bile and were derived from direct oxidation and subsequent glucuronidation (Table 4).

The metabolite profiles from feces and bile were qualitatively similar; however, a few metabolites were quantitatively different between the two matrices (Table 4). As expected, the glucuronides (M27 and M30) were detected in bile and, to a much smaller extent, in feces, indicating that these metabolites were likely hydrolyzed by intestinal β-glucuronidase and excreted as the corresponding aglycones (M28 and M31) in feces (Rozman and Klaassen, 2001). This hypothesis is consistent with our observation that the percentage of dose of aglycones in feces was similar to the total percentage of glucuronides and aglycones in bile. The oxidative metabolite M1 was greater than 3-fold more abundant in bile than in feces, suggesting that M1 might undergo further hydrolysis or other chemical and enzymatic degradation in the intestine. Metabolites M33 and M34 were relatively abundant metabolites detected in feces (6.4% and 1.6%) and, to a lesser extent, in urine, but they were not detectable in bile, suggesting that M33 and M34 might be directly transferred from blood into the intestines via passive diffusion (Rozman, 1986) or via active intestinal secretion (Lauterbach, 1977).

In addition, an unusual metabolic pathway of formation of hippuric acid (M24) from GDC-0152 was observed. Hippuric acid is formed as a product of glycine conjugation to benzoic acid (Hutt and Caldwell, 1990). Glycine conjugation is recognized as an important mechanism for the excretion and detoxification of aromatic carboxylic acids (Kasuya et al., 1996). Hippuric acid is generally present at concentrations of g/L in the urine of humans and rats (Angerer and Horsch, 1992). The presence of hippuric acid in the pre-dose urine of rats, therefore, resulted in a great challenge for identification of M24. Many steps were taken to confirm the identity of M24 as hippuric acid. When hippuric acid was observed to co-elute with the radioactive peak of M24, the first step was to attempt a separation between the two compounds. Hippuric acid was suspected to cause ion suppression of the mass spectrometric response of M24, resulting in masking of the true molecular ion of M24. However, regardless of the purification technique, including hydrophilic interaction chromatography, liquid–liquid extraction and solid phase extraction under acidic or basic conditions, hippuric acid persistently co-eluted with M24. The second attempt in identifying M24 was to make a chemical derivative. This was done by methylation of the LC fraction corresponding to this metabolite and of a hippuric acid standard with TMSCH2N2. The isolated methylated radiolabeled M24 had the exact retention time and high-resolution MS/MS fragmentation pattern as those of methylated hippuric acid. On the basis of these observations, M24 was assigned to be hippuric acid, a metabolite of GDC-0152 through ring cleavage of 4-phenyl-5-amino-1,2,3-thiadiazole. This metabolite was formed in rat hepatocyte incubations and inhibited by ABT, a broad P450 inactivator, implicating P450 enzymes in the involvement of this biotransformation.

To gain mechanistic insight into the formation of hippuric acid, in vitro studies were conducted by incubating synthetic 4-phenyl-5-amino-1,2,3-thiadiazole (M28) or a para-substituted chloro, trifluoromethyl, or methoxyl analog (G1, G2, and G3, respectively) in rat hepatocytes in the presence or absence of ABT. Hippuric acid or the corresponding substituted hippuric acid metabolite was formed in the incubations. As was observed for GDC-0152, formation of these metabolites was inhibited by ABT. Importantly, trifluoromethyl benzoic acid was detected in the G2 (CF3 substituted) incubation (Table 5), indicating that benzoic acid was an intermediate in the formation of hippuric acid from 4-phenyl-5-amino-1,2,3-thiadiazole.

On the basis of these findings, we propose two metabolic pathways catalyzed by P450 enzymes to generate reactive intermediates I and II (Fig. 5). Intermediate I is formed by epoxidation of the thiadiazole,

![Fig. 5. The proposed mechanism of hippuric acid formation from 4-phenyl-5-amino-1,2,3, thiadiazole.](image_url)
leading to a loss of \( N_2 \) from the ring and ultimately to the loss of \( H_2S \). This results in the formation of benzyol cyanide, which is hydrolyzed to form benzoic acid. Intermediate II is formed by \( S \)-oxidation that interrupts the aromaticity of the thiadiazole. The ring becomes electrophilic and reacts with a water molecule at the benzylic position. Upon water attack, the ring falls apart to release \( N_2 \) and forms 2-oxo-2-thiophene. This proposed intermediate forms 2-oxo-2-thiophene bioactivation (Dansette et al., 2005).

In summary, peptidomimetic GDC-0152 underwent extensive metabolism in rats. Utilizing two \([14C]\)GDC-0152 molecules labeled at two different positions greatly facilitated metabolite identification and led to the discovery of \( CO_2 \) liberation and to a unique metabolic pathway leading from a 4-phenyl-5-amino-1,2,3-thiadiazole to hippuric acid.

Acknowledgments

All the authors were at Genentech when this work was done except for Eric Solon, who is at QPS, Inc. The authors thank Ronitte Libedinsky for her editorial contribution.

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Participated in research design: Yue, Wong, Rudewicz, Hop, Khojasteh, Ware, Budha.

Conducted experiment: Mulder, Yue, Solon.

Performed data analysis: Yue, Mulder, Wong, Solon, Lyssikatos.

Wrote or contributed to the writing of the manuscript: Yue, Wong, Mulder, Hop, Khojasteh.

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