METABOLISM AND EXCRETION OF 6-CHLORO-9-(4-METHOXY-3,5-DIMETHYL-2-PYRIDINYL)-9H-PURIN-2-YLAMINE (BIIB021), AN HSP90 INHIBITOR, IN RATS AND DOGS, AND ASSESSMENT OF ITS METABOLIC PROFILE IN PLASMA OF HUMANS

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Running Title: Metabolism of a pyridinylmethyl-9H-purin-2-ylamine analog

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Abbreviations:; AO, aldehyde oxidase; ARC, accurate radioisotope counting; AUC, Area under curve; BDC, bile duct cannulated; CID, collision-induced dissociation; CYP450, cytochrome P450; FDA, food and drug administration; GSH, glutathione; HPLC, high performance liquid chromatography; HR: high resolution; HSP, heat shock protein; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; RAM, Radioactivity monitor; SD, Sprague Dawley
Abstract

6-Chloro-9-(4-methoxy-3, 5-dimethyl-pyridin-2-ylmethyl)-9H-purin-2-ylamine (BIIB021), a synthetic HSP90 inhibitor, exhibited promising antitumor activity in preclinical models and was in development for the treatment of breast cancer. The metabolism and excretion of BIIB021 was investigated in rats and dogs following oral administration of [^{14}\text{C}]BIIB021. The administered radioactive dose was quantitatively recovered in both species, and feces/bile was the major route of excretion. Metabolic profiling revealed that BIIB021 is extensively metabolized primarily via hydroxylation of the methyl group (M7), O-demethylation (M2) and to a lesser extent by glutathione conjugation (M8 & M9). M7 was further metabolized to form the carboxylic acid (M3) and glucuronide conjugate (M4). Human plasma obtained from the phase I study in cancer patients were also analyzed to assess the metabolism of BIIB021 in humans and to ensure that selected animal species were exposed to all human major metabolites. Results suggested that BIIB021 is metabolized via hydroxylation followed by carboxylation and glucuronidation in humans consistent with rat and dog; however, an additional dominant circulating metabolite, hydroxylation at the purine ring (M10), was identified in humans. Preliminary in vitro studies using liver cytosolic fractions indicated that M10 formation is primarily catalyzed by aldehyde oxidase (AO). AO catalytic activity for M10 formation was the highest in the monkey, followed by mouse, human, and rat. The apparent $K_m$ and $V_{\text{max}}$ values of M10 formation were $174 \pm 8 \, \mu\text{M}$ and $14.0 \pm 0.3 \, \text{pmol min}^{-1} \, \text{mg protein}^{-1}$ in human, and $132 \pm 9 \, \mu\text{M}$ and $131 \pm 4 \, \text{pmol min}^{-1} \, \text{mg protein}^{-1}$ in monkey, respectively.
Introduction

Heat shock protein 90 (HSP90) is an abundant molecular chaperone that promotes the conformational maturation of ‘client’ proteins and protects them from degradation (Biamonte et al., 2010; Pearl & Prodromou, 2006; Soo et al., 2008). Many of the known clients are protein kinases or transcription factors involved in multiple signal transduction pathways. HSP90 is also expressed in the activated form in cancer cells, whereas it is latent in normal somatic cells. Therefore, it has become an attractive target in oncology. Inhibition of HSP90 function causes many oncogenic client proteins to adopt aberrant conformations and subsequent degradation. Therefore, HSP90 inhibitors represent a promising approach to treat cancers driven by multiple molecular abnormalities.

BIIB021, 6-chloro-9-(4-methoxy-3,5-dimethylpyridin-2-yl)methyl-9H-purin-2-ylamine, is a synthetic HSP90 inhibitor and exhibits a strong antitumor effect as a single agent and increases the efficacy of radiation (Kasibhatla et al., 2007; Lundgren et al., 2009). Orally-administered BIIB021 demonstrated efficacy in the U87 glioblastoma, the N87 gastric carcinoma, and the BT474 breast carcinoma xenograft models in nude mice. The combinations of BIIB021 with paclitaxel or bortezomib demonstrated significant tumor growth inhibition compared with single-agent treatment or controls in ovarian tumor and myeloma xenograft models, respectively. It also synergizes with radiation, a commonly used therapy in the treatment of squamous cell carcinoma. BIIB021 is not a substrate of p-glycoprotein and showed comparable potency against multiple-drug-resistance expression cell line (Zhang et al., 2010). It was under development as an oral agent for the treatment of breast cancer. BIIB021 is rapidly absorbed in vivo and has a short half-life in mouse (Kasibhatla et al., 2007), rat, and dog. It shows moderate to high clearance in these animal species. Identification of
metabolites of a new molecular entity in animals and humans is essential to pharmaceutical
development and compound progression. The Food and Drug administration (FDA)
recommends that the metabolic profiles of all new molecular entities in humans should be
characterized before initiating large clinical trials. *In vitro* studies using hepatocellular and
subcellular fractions from preclinical species and humans and/or recombinant human enzymes
often provide valuable information on the expected metabolic pathways in humans *in vivo*
(Baranczewski et al., 2006; Dalvie et al., 2009). To improve patient safety and to avoid late-
stage clinical trial delays, the FDA and International Conference on Harmonization (ICH)
guidance recommend that the drug metabolites present in humans should also be present in at
least one of the animal species used for long term safety assessment (EMA, 2009; FDA, 2008).
Therefore, comparison of metabolic profiles between preclinical species and humans should be
assessed as early as possible during the drug-development process.

BIIB021 is extensively metabolized in both liver microsomes and hepatocytes (Xu et al.,
2012). The objectives of this study were to characterize the metabolism, pharmacokinetics, and
excretion of [14C]BIIB021 in Sprague Dawley (SD) rats and beagle dogs and to obtain both
quantitative and qualitative profiles of circulating metabolites in plasma obtained from a dose
escalation study in cancer patients. A single dose of [14C]BIIB021 was administered orally to
intact and bile duct cannulated (BDC) SD rats (3 mg/kg) and beagle dogs (6 mg/kg). Profiling
and identification of collected urine, bile, plasma and feces were achieved using LC-MS/MS in
combination with radioactivity and UV detection.
Materials and Methods

Reference Compounds and Chemicals  BIIB021, [14C]BIIB021, d3-BIIB021, and authentic standards, CF2246 (O-desmethyl BIIB021, 2-((2-amino-6-chloro-9H-purin-9-yl)methyl)-3,5-dimethylpyridin-4-ol, M2), 5-OH-1983 Gluc (5-hydroxymethyl glu, M4), and CF3785 (5-hydroxymethyl BIIB021, (6-((2-amino-6-chloro-9H-purin-9-yl)methyl)-4-methoxy-5-methylpyridin-3-yl)methanol, M7), and M10 (2-amino-6-chloro-9-((4-methoxy-3,5-dimethylpyridin-2-yl)methyl)-9H-purin-8-ol) were synthesized at Biogen Idec (Kasibhatla et al., 2005; Kasibhatla et al., 2007). M10 was also purified from the incubation mixture of the monkey liver cytosolic fraction with BIIB021.

The 14C-label was incorporated on the C-8 position of the purine ring (Fig. 1). It had a specific activity of 52.9 mCi/mmol and a radiochemical purity of >99%, as determined by HPLC using an in-line radioactivity detector. All other chemicals were of HPLC or analytical grade and were obtained from Fisher Scientific (Suwanee, GA), unless specified otherwise.

Pooled liver cytosols (10 mg/ml) of CD-1 mice (male), SD rats (male), beagle dogs (male), cynomolgus monkeys (male), and humans (100 male and 100 female) were purchased from XenoTech (Lenexa, Kansas).

Animals, Dosing, and Sample Collection

In-life portions of rat and dog studies were conducted at XenoBiotic Laboratories, Inc (Plainsboro, NJ). Intact and BDC SD rats (218-300 g) were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA). Beagle dogs (7.9-8.8 kg) were from an in-house colony of XenoBiotic laboratories Inc. and were originally obtained from Covance Laboratories (Madison, WI). Animals were quarantined for a minimum of 5 days before treatment and maintained on a 12-h light/dark cycle. The animals were housed individually in stainless steel metabolism cages.
The animals were provided water *ad libitum*. All studies were conducted in a research facility of XenoBiotic laboratories accredited by the American Association for the Accreditation of Laboratory Animal Care. All *in-vivo* studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

**Rat study.** A group of SD intact rats (n=3 /gender) and another group of bile-cannulated rats (n=3/gender) were administered a single 3 mg/kg (192 µCi/kg) oral dose of [¹⁴C]BIIB021 for the mass balance and biliary excretion experiments. The dose was formulated at 1 mg/mL in pH 2 HCl aqueous solution on the day prior to dose administration. Urine and feces were collected from intact animals for 7 days at 0-6, 6-12, 12-24, 24-48, 48-72, 72-96, 96-120, 120-144, and 144-168 h intervals after the dose. The first fecal sample was collected at 0 to 24 h after the dose. Bile and urine samples were collected from BDC animals at 0-6, 6-12, 12-24, and 24-48 h intervals after the dose. The volumes of urine and bile samples were recorded and all of the biological samples were stored at -20°C until analysis. For identification of circulating metabolites, a third group of intact SD rats (n=10/gender) was given an oral dose of 3 mg/kg (192 µCi/kg) [¹⁴C]BIIB021. Blood from two animals/gender was collected at 0.25, 0.5, 2, 6, and 12 h post dose into tubes containing anticoagulant K₂EDTA. The blood samples were centrifuged at 1000 g for 10 min to obtain the plasma. The obtained plasma was immediately transferred to clean tubes and stored at -20°C until analysis. The plasma samples at these five time points from the same gender were pooled by AUC method of Hamilton (Hamilton et al., 1981) prior to the LC-MS analysis.

**Dog study.** Three male and three female beagle dogs (7.9-8.8 kg) were administered a single oral 6 mg/kg (25 µCi/kg) dose of [¹⁴C]BIIB021. Urine and feces were quantitatively collected for at least 16 h before dosing, then at 0-6, 6-24, and over 24 h intervals through 168 h post dose.
Blood samples were collected by venipuncture of a cephalic vein at 0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h postdose. The blood samples were transferred into tubes containing K$_2$EDTA and immediately placed on wet ice. The plasma was obtained after the blood sample was centrifuged (1200 g) for 10 min at 4°C. The plasma samples (0-12 h) of each dog were pooled by AUC method of Hamilton prior to the LC-MS analysis.

**Human study.** Plasma samples were obtained from patients in phase I, open-labeled multiple dose escalation safety studies. Blood samples (~15 mL to provide approximately of 7.5 mL of plasma) were collected into tubes containing anticoagulant K$_2$ EDTA on Day 1 at 0 h (predose), and at 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 24 h postdose. Within 30 min of collection, the blood samples were centrifuged at approximately 1500x g for about 10 min at 4°C and the plasma was collected and stored at -70°C until analysis. Approximately 2.5 mL of each plasma sample was used for the identification of circulating metabolites. Plasma samples from five patients (A, B, C, D, and E) dosed at 200 mg BIIB021 and one patient (F) dosed at 300 mg were chosen for metabolite identification. The plasma samples of each subject at 0.5, 1, 1.5, 2, and 4 h postdose were pooled by AUC method of Hamilton prior to the LC-MS analysis. The brief information of six patients is listed as follows: patient A, female, age 63 with liomeyosarcoma; patient B, female, age 59 with colon cancer; patient C, male, age 80 with mesothelioma; patient D, female, age 57 with breast cancer; patient E, female, age 66 with breast cancer; patient F, male, age 49 with alveolar soft part sarcoma.

**Determination of Radioactivity**
The radioactivity in urine, bile, and plasma was determined by liquid scintillation counting. Triplicate aliquots of urine (0.1 g), bile (0.05 g), and plasma (100-250 µL) for each sampling time point, were mixed with 10-15 mL of scintillation fluid and analyzed directly by liquid scintillation counting using a LS-6500 liquid scintillation counter (Beckmann Instruments, Inc. Fullerton, CA). Fecal samples were homogenized with 3-7 x isopropyl alcohol:water (50:50) and triplicate aliquots of homogenates, equivalent to approximately 100 mg of fresh feces weight, were combusted on a Harvey biological sample oxidizer (Tappan, NY), followed by liquid scintillation counting. The samples obtained before dosing were also counted to obtain background count rate. Samples containing radioactivity (dpm) less than or equal to twice background for the system were assumed to contain 0 dpm in the calculation of the means. The amount of radioactivity in the dose was expressed as 100%, and the percentage of radioactivity in the urine and feces at each sampling time was expressed as the percentage of dose excreted in the respective matrices at the sampling time. The amount of radioactivity in plasma at each time point was calculated using the specific activity of the dose administered and was expressed as nanogram-equiv of parent drug per milliliter and percentage of total circulating radioactivity.

**Extraction of Metabolites from Biological Samples**

Pooling of plasma from all species was performed for each individual according to the AUC method of Hamilton such that each sample was representative of the total exposure (AUC) to metabolites relative to each other over the entire collection period. Pooled plasma samples (1 mL) were treated with two volumes of acetonitrile to precipitate plasma proteins, followed by vortexing for 2 min. After storage at 4°C for 30 min, the mixture was centrifuged at 10,000 g at 4°C for 10 min. The supernatant was then transferred to a 15 mL polypropylene conical tube.
The pellets were then suspended with water (1x, v/v) and extracted again with acetonitrile (2x, v/v) according to the procedure described above. The two acetonitrile extracts were combined and duplicate aliquots (0.2 mL) were analyzed by liquid scintillation counting. The mean recovery of radioactivity after extraction was above 90%. The combined extracts were evaporated to dryness under a nitrogen stream using a TurboVap® 96 evaporator (Biotage, Charlotte, NC). The residues were reconstituted with an appropriate volume of acetonitrile:water (1:1, v/v) for profiling of radiolabeled metabolites. Urine samples from rat and dog were pooled such that the pooled sample represented >90% of total urinary radioactivity. The pooled urine sample was centrifuged at 10,000 g at 4°C for 10 min to remove particulate materials and the supernatants were directly injected on the HPLC system. Bile samples from rat were pooled such that the pooled sample represented >90% of the total biliary radioactivity. The pooled sample was centrifuged and directly analyzed on the HPLC system. Fecal homogenates were combined such that at least 80% of the radioactivity excreted in feces was included. An aliquot (~1g) of each pooled fecal homogenate was diluted with 15 volumes of 0.4% formic acid solution. The mixture was vortexed for 2 min and five volumes of acetonitrile was added to the mixture and shaken for 15 min. The mixture was then centrifuged at 4°C for 10 min and the resulting supernatants were separated. The remaining solids were extracted two more times as described above. The three extracts were combined and aliquots were then taken and analyzed by LSC for the evaluation of extraction efficiency. The pooled extract was evaporated to dryness and reconstituted with a small volume (1.0 mL) of acetonitrile:water (1:1, v/v) for LC-RAM analysis.

**Liver cytosolic incubation of BIIB021**
Metabolism of BIIB021 was studied in duplicate in mouse, rat, dog, monkey, and human liver cytosolic subcellular fractions. The incubation mixtures contained 1 mg/mL mouse, rat, dog, monkey, or human cytosolic protein and 10 µM [14C]BIIB021 (0.5 µCi/mL) in a final volume of 500 µL of 100 mM phosphate buffer (pH 7.4). The reaction was quenched with ice-cold acetonitrile (500 µL) after 50-min incubation. The quenched reaction mixture was vortexed and centrifuged for five min at 4°C. The supernatant was directly analyzed by LC-RAM-MS. In the kinetic study, the incubation conditions for M10 formation in liver cytosol (monkey and human) were optimized with respect to protein concentration and incubation time. The reaction rate was linear with protein concentration and incubation time up to 1 mg of protein/ml incubation mixture and 60 min, respectively. The substrate BIIB021 concentration was chosen as 0.5, 2, 5, 20, 50, 200, and 500 µM. The incubation time was 15 and 8 mins for human and monkey, respectively. For the quantitative analysis of M10 by LC-MS, deuterium labeled BIIB021 was used as an internal standard.

**Instrumentation.** The LC-RAM-MS system consisted of an Agilent 1290 Infinity UHPLC system (Santa Clara, CA), a v.ARC radiometric detector (Hockessin, Delaware), and a Thermo LTQ Orbitrap™ high resolution mass spectrometer (San Jose, CA). The separation of metabolites was performed on a Luna® analytical C18 (2) column (4.6 x 150 mm, 3 µm particle size, Phenomenex, Torrance, CA) with on-line radioactivity monitoring using a mobile phase containing a mixture of 0.4% formic acid in water (pH 3.72; solvent A) and acetonitrile (solvent B). The column was held at 35°C and the samples were eluted at a flow rate of 0.70 mL/min. The mobile phase was initially composed of solvent A/solvent B (100:0) for 3 min. It was then programmed with five subsequent linear gradients (5 min from 0% to 6% B; 5 min from 6% to 10% B; 20 min from 10% to 16% B; 14 min from 16% to 30% B; 8 min from 30% to 100% B),
and finally with 5 min at 100% B. It was returned to the starting solvent mixture over 15 min. The system was allowed to equilibrate for approximately 15 min before the next injection. The HPLC effluent was directly infused into the radiometric detector or mass spectrometer. StopFlow AD™ cocktail (AIM, Hockessin, Delaware) was used as the scintillation liquid. Radiolabeled metabolites and [\(^{14}\text{C}\)]BIIB021 were quantitatively assessed by the integration of each radio-chromatographic peak.

The LTQ Orbitrap™ mass spectrometer was operated in the positive electrospray ionization mode. The heated capillary temperature was maintained at 250 °C; the sheath gas and auxiliary gas flow rates were set to 60 and 40 units, respectively. The ion spray voltage, capillary voltage, and tube lens offset voltage were adjusted to 3 kV, 40 V and 85 V, respectively. The normalized collision energy was 30% during MS/MS acquisition and helium was used as the collision gas. High resolution mass measurement was performed in Orbitrap™ mode with a resolution of 15K. The instrument was calibrated with a mixture of solution (Thermo, San Jose, CA) containing caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621. High resolution data was expressed as four decimal atomic mass units and mass difference (ΔM) between measured mass and the calculated mass was expressed in parts per million (ppm) units. Elemental compositions were calculated based on high resolution mass data and McLafferty's nitrogen rule.

**Quantitative Assessment of Metabolites in Humans**

The following LC-MS/MS bioanalytical assays were used for the quantitation of BIIB021 and metabolites M3 and M7 in human plasma. The internal standard was stable isotopically labeled d3-BIIB021 and spiked into each plasma sample before the process. Plasma samples (0.10 mL) were treated with two volumes of acetonitrile to precipitate plasma proteins, followed by
vortexing for 2 min. After storage at 4°C for 30 min, the mixture was centrifuged at 10,000 g at 4°C for 10 min. The supernatant (0.01 mL) was then injected onto the LC-MS/MS system for analysis. BIIB061, M3, M7, and the internal standard were eluted from a Waters XBridge C18 (2.5 μm, 2.1x30 mm) column using a gradient of water/acetonitrile with 0.4% formic acid as mobile phases. BIIB061, M3, M7, and the internal standard were detected in positive ESI mode by MRM transitions of m/z 319>150, 349>150, 335>138, and 322>153, respectively. The concentrations of BIIB021, M3 and M7 in the study samples were calculated using the peak area ratios of unknown against a standard curve relating the peak area ratios to the analyte concentrations in a linear, 1/x2 regression.

To estimate the relative concentration of circulating metabolites in humans, a reference correction factor was established in plasma (Yi & Luffer-Atlas, 2010; Yu et al., 2007). Rat bile or plasma samples, containing [14C]BIIB021 and metabolites [14C]M3, [14C]M4, [14C]M7, [14C]M10 were spiked into extracted control human plasma at a concentration of 10% by volume. The spiked samples were analyzed by LC-MS and LC with radiometric detection using a Beckman Coulter LSC (Beckman Coulter, Fullerton, CA) with peak integration via AIM software (AIM, Hockessin, Del). The relative radioactive response of metabolites to the parent, as compared with the relative MS response of each metabolite to the parent was used to generate correction factors as follows:

\[
\text{Correction factor} = \frac{\text{ARC}_{\text{metabolite}} / \text{ARC}_{\text{parent}}}{\text{MS}_{\text{metabolite}} / \text{MS}_{\text{parent}}}
\]

Where \(\text{ARC}_{\text{metabolite}}\) and \(\text{ARC}_{\text{parent}}\) are the peak areas of each metabolite and BIIB021, respectively, as determined by radiometric detection, and \(\text{MS}_{\text{metabolite}}\) and \(\text{MS}_{\text{parent}}\) are the peak areas of each metabolite and the parent compound as determined by MS detection. The
correction factors were applied to the % peak area calculations for each metabolite by multiplying the MS peak area response of the metabolite by the respective correction factor.
Results

\(^{14}\text{C} \text{Excretion}\)

\textbf{Rats.} The recovery of the radiolabeled material after oral administration of a single 3 mg/kg dose of \[^{14}\text{C}]\text{BIIB021} to SD rats was essentially complete within a period of 168 h postdose. Approximately 68.8\% of the dose was excreted in the feces, whereas 26.2\% of the dose was excreted in the urine and 5\% in the cage wash after 168 h, resulting in an overall recovery of 100\% (Table 1). More than 95\% of the excreted radioactivity recovery occurred in the first 48 h. Mean total recoveries of dosed radioactivity from BDC rats were 26.2\% in urine, 46.2\% in bile, and 22.0\% in the feces (Table 1). No significant gender differences in the excretion pattern of rats were observed.

\textbf{Dogs.} Following a single oral dose of \[^{14}\text{C}]\text{BIIB021} to beagle dogs, an overall mean of 91.3\% of the total dose was recovered in the urine, feces, and cage wash through 168 h postdose (Table 1). The mean cumulative doses recovered in the feces and urine were 51.7 and 34.0\%, respectively. Approximately 89\% of the excreted radioactivity was recovered in the first 48 h. No gender related differences were observed in the excretion pattern of radioactivity in dogs.

\textbf{Metabolite Profiles}

\textbf{Rat urine.} A representative HPLC-radiochromatogram of urinary metabolites from an intact rat is shown in Figure 2A. The metabolites were quantified by integration of the radiochromatographic peaks. The mean percentages of metabolites detected in the urine of rats (male and female pooled), expressed as percentage of administered doses, are shown in Table 2. Unchanged parent was not detected and a total of seven major metabolites were detected in the urine. Those metabolites were M2, M3, M4, M5, M6, and M7 and accounted for 1.38, 1.27,
1.04, 0.87, 1.20, and 14.2% of the dose, respectively. Metabolite M9 was detected as a minor metabolite in the radiochromatogram and accounted for 0.28% of the dose.

**Rat feces.** A representative HPLC-radiochromatogram of fecal metabolites from an intact rat is shown in Figure 2B. The mean percentages of detected fecal metabolites, expressed as percentage of administered dose, are shown in Table 2. A total of six metabolites were detected in the feces. These metabolites were M1, M2, M3, M5, M6, and M7 and accounted for 6.06, 7.51, 3.07, 2.81, 3.18, and 15.6% of the dose, respectively. Parent BIIB021 accounted for only 0.92% of the dose.

**Rat bile.** A representative HPLC-radiochromatogram of biliary metabolites from one BDC rat is shown Figure 2C. The major metabolites detected in bile were M2, M3, M4, M5, M6, M7, M8, and M9, representing 1.32, 4.65, 10.8, 1.26, 1.86, 10.2, 2.47, and 0.66% of the total administrated dose in BDC rats, respectively. The remaining metabolites including BIIB021 each represented at <2% of the total administered dose.

**Rat plasma.** A representative HPLC radiochromatogram of circulating metabolites from one rat is shown in Figure 2D. Mean percentages of total circulating radioactivity are shown in Table 3. A total of six metabolites were detected. The majority of circulating radioactivity was attributed to unchanged parent (49.3%) followed by M7 (18.5%) and M4 (6.40%). The remaining metabolites (M2, M3, M6 and M10) each represented less than <5% of total circulating radioactivity.

**Dog urine.** An average of 32% of the dose was recovered in the urine of dogs during the 0-24 h time periods. A representative HPLC-radiochromatogram of metabolites from one dog urine sample (0-24 h period) is shown in Figure 3A. The metabolites were quantified by integration of the radiochromatographic peaks. The mean percentages of urinary metabolites detected in the
urine of dogs, expressed as percentages of administered dose, are shown in Table 2. A total of six metabolites were detected. M4 represented the major metabolite at 15.0% of the dose. Unchanged parent accounted for only 0.10% of the dose and the remaining metabolites (M2, M3, M5, M6 and M7) each represented <3% of the dose.

**Dog feces.** An average of 53% of the dose was recovered in the feces of dogs during the 0-48 h time period. The 0-48 h pooled samples were extracted and a representative HPLC-radiochromatogram of metabolites from one extracted fecal sample is shown in Figure 3B. The mean percentages of metabolites detected in the fecal extracts of dogs, expressed as percentage of administered dose, are shown in Table 2. A total of four metabolites were detected in the feces of male and female dogs. Unchanged drug accounted for 1.08% of the total administered dose, while major metabolites M2 and M7 represented 10.8 and 11.6% of the dose, respectively. The other two metabolites M3 and M6 represented 1.08 and 1.50% of the dose. Many minor radioactive signals (Figure 3B) in the HPLC-radiochromatogram, whose components could not be determined, were integrated and accounted for remaining 27% of the dose. The origin of so many radioactive peaks just above the baseline was unclear and the percentage of identified major metabolites may be underestimated.

**Dog plasma.** A representative HPLC radiochromatogram of circulating metabolites from one dog is shown in Figure 3C. Mean percentages of total circulating radioactivity are shown in Table 3. A total of six metabolites were detected. The majority of circulating radioactivity was attributed to M4 (61.5%) followed by unchanged parent (4.75%) and M2 (4.35%). The remaining metabolites (M3, M6, M7 and M10) each represented less than 3% of the total circulating radioactivity.
**Human plasma.** Metabolites in human plasma were identified via their UV and MS spectra. BIIB021 and its metabolites exhibited a UV maximum absorbance at 310 nm. A representative HPLC-UV (310 nm) chromatogram of circulating metabolites from one pooled human sample is shown in Figure 4. Compared to the chromatogram from the corresponding predose sample (Figure 4), major metabolites (M3, M4, M7, and M10) and BIIB021 were clearly identified. To quantitatively estimate each metabolite concentration, a reference correction factor of each metabolite between radioactivity response and mass spectrometry response was established. Since BIIB021 and metabolites M3 and M7 were quantified by LC-MS/MS assay, the estimated concentrations of M3 and M7 in pooled plasma using the correction factor approach were compared with measured concentrations (Table 4). For M3, five out of six patients had estimated concentrations falling within 72 to 109% of measured concentration. For M7, the estimated concentration of all patients was relatively low by comparison and fell within 58.8 to 78.6% of measured concentration. Using the same approach, the concentrations of M4 and M10, which were not quantified in the initial bioanalysis, were estimated (Table 5). Based on these results, the mean percentages of total identified circulating drug related components are calculated and shown in Table 3. A total of four metabolites were detected. M3, M4, M7, and M10 represented 18.1, 12.9, 24.2, and 18.6% of the total drug related material, respectively. Unchanged parent represented 26.2% of total components.

**Metabolite Identification and Characterization.** The structures of metabolites were elucidated by electrospray LC/MS/MS using a combination of full scan MS and CID product ion (MS²/MS³) spectra. A total of 10 metabolites were identified in animals and humans. BIIB021 contains one chlorine atom that has two major isotopes, ³⁵Cl and ³⁷Cl, in a natural abundance.
ratio of ca. 3 to 1. Retention time of radioactive peaks in chromatograms and the characteristic $^{35/37}$Cl ion clusters observed in LC/MS data were used to find and confirm the molecular ions of BIIB021 and its metabolites that retained the chlorine atom. The structures of each metabolite were elucidated by interpreting the mass change of the molecular ion and fragmentation patterns, compared to those of BIIB021. The exact location of modification in major metabolites was determined by comparing the retention time, elemental composition, and product ion spectra of available synthetic standards. Metabolites M2, M4, M6, and M7 were fully characterized from in vitro metabolism studies (Xu et al., 2012) and they were identified in vivo samples based on their LC retention times and characteristic product ion spectra. M2 and M7 were identified as $O$-desmethyl BIIB021 (CF2246) and 5-hydroxymethyl-BIIB021 (CF3785), respectively, by comparison with the reference standard CF2246 and CF3785. M4 was identified as the glucuronide conjugate of 5-hydroxymethyl-BIIB021 by the characteristic glucuronic acid neutral loss (-176 Da) in the MS/MS spectra. M6 was identified as a dechlorinated hydroxylated metabolite of BIIB021 suggested by the loss of isotopic ratio (3:1) of the chlorine atom. The structural determinations of M1, M3, M5, M8, M9, and M10 are described below.

**Parent (BIIB021)** BIIB021 eluted at ~47.9 min by HPLC and produced protonated molecular ions at $m/z$ 319/321 (Xu et al., 2012). The product ion (MS/MS) spectrum of BIIB021 with proposed characteristic fragmentations is displayed in Figure 5. Loss of an HCl molecule resulted in the product ion at $m/z$ 283, which gave a product ion at $m/z$ 268 via further loss of an amino group. The characteristic ion at $m/z$ 150 is formed by cleavage of the C-N bond between the purine moiety and the pyridine ring with charge retention at the pyridine moiety. The counterpart 2-amino-4-chlorine-purine moiety produced the ion at $m/z$ 170. The subsequent loss of the formaldehyde from ion at $m/z$ 150 produced a fragment at $m/z$ 120. The characteristic ions
at m/z 150 and 120 proved to be very useful for finding and confirming the structural changes of BIIB021 and its metabolites that retained the intact pyridine ring. ¹H NMR Data (DMSO-d6/CF₃COOD, δ, 600 MHz): 8.44 (s, 1H, H-13), 8.18 (s, 1H, H-8), 5.57 (s, 2H, H-10), 3.98 (s, 3H, H-19), 2.38 (s, 3H, H-20), 2.34 (s, 3H, H-17).

**Metabolite M1**  Metabolite M1 was detected only in the rat feces and eluted at ca. 17.6 min by HPLC. It produced the protonated molecular ions at m/z 321/323 (Supplemental Figure 1), which were 2 Da higher than BIIB021 and 16 Da higher than M2 (the desmethylated metabolite). The product ion at m/z 303 was formed from loss of a H₂O molecule. MS³ spectrum of the product ion m/z 303 provided several characteristic ions at m/z 267, 170, and 134. The product ion at m/z 267 was formed from loss of an HCl molecule (-36 Da). The ion at m/z 170 corresponded to the unchanged 2-amino-4-chlorine-purine moiety. The ion at m/z 134 was 16 Da less than the fragment ion m/z 150 from BIIB021, suggesting a demethylated pyridine moiety. M1 was tentatively proposed as the mono-hydroxylated and demethylated BIIB021. The exact location of hydroxylation in M1 is unknown.

**Metabolite M3.** Metabolite M3 was detected in rat and dog urine, and rat bile and feces. It eluted at ca. 22.3 min by HPLC and had protonated molecular ions at m/z 349/351, which is 30 Da higher than BIIB021. The product ion spectrum of M3 is depicted in Figure 5. The product ions at m/z 313 and 269 were formed from loss of an HCl molecule and subsequent loss of a carboxyl group. Further loss of the amino group from ions at m/z 313 and 269 produced the ions at m/z 298 and 254, respectively. The product ions at m/z 180 and 150 were 30 Da higher than the fragment ions at m/z 150 and 120 from BIIB021, suggesting that 30 Da mass unit, such as a carboxyl group, is added to the pyridine moiety. M3 also has the same HPLC retention time and product ion spectra to those of the reference standard, 6-((2-amino-6-chloro-9H-purin-9-
yl)methyl)-4-methoxy-5-methylnicotinic acid (CF4176). Therefore, the carboxyl group was assigned at 5’ position of pyridine moiety and M3 was identified as CF4176.

**Metabolite M5.** Metabolite M5 was detected in rat and dog urine, and rat bile and feces. It eluted at ca. 28.0 min by HPLC and had protonated molecular ions at \( m/z \) 351/353 (Supplemental Figure 2), which is 32 Da higher than BIIB021. The product ions at \( m/z \) 333 and 297 were formed from loss of H₂O and subsequent loss of HCl molecule. The product ion at \( m/z \) 186, 16 Da higher than the fragment ion at \( m/z \) 170 from BIIB021, suggested that the hydroxylation had occurred at the 2-amino-4-chlorine-purine moiety. The product ion at \( m/z \) 166, 16 Da higher than the fragment ion \( m/z \) 150 from BIIB021, suggested that another hydroxylation had occurred at the pyridine ring moiety. M5 was tentatively proposed as the di-hydroxylated BIIB021. The exact location of hydroxylation in the metabolite is unknown based on current data.

**Metabolite M8.** Metabolite M8 was detected in rat bile. It eluted at ca. 41.5 min by HPLC and had the protonated molecular ion at \( m/z \) 461, which is 142 Da higher than BIIB021. HR mass analysis of M8 produced a [M+H]^+ at \( m/z \) 461.1698, consistent with the formula of C₁₉H₂₅O₄N₈S (-3.51 ppm), which is in agreement with a cysteinylglycine conjugate of dechlorinated BIIB021.

The product ion spectrum of M8 is depicted in Supplemental Figure 3. The product ions at \( m/z \) 386 and 358 were formed from loss of a glycine moiety (-75 Da) and subsequent loss of a CO moiety (-28 Da). The MS³ of ion 386 provided fragment ions at \( m/z \) 316, 237, 150, and 120. The ion at \( m/z \) 316 was formed by loss of the cysteinylglycine moiety and the ion at \( m/z \) 237 was formed by loss of a pyridine ring and a glycine moiety. Unchanged product ions at \( m/z \) 150 and 120 suggested that the pyridine ring moiety was intact. M8 was tentatively identified as a cysteinylglycine conjugate of BIIB021.
**Metabolite M9.** Metabolite M9 was detected in rat urine and bile. It eluted at ca. 38.2 min by HPLC and had a protonated molecular ion at $m/z$ 446, which is 127 Da higher than BIIB021. The product ion spectral data of M9 are depicted in Figure 6. The product ions at $m/z$ 317 and 404 (Figure 6A) were formed from loss of the acetylcysteine (-129 Da) and acetyl moiety, respectively. Further fragmentation of the production ion at $m/z$ 317 (Figure 6B) yielded informative ions at $m/z$ 284, 168, 150, and 120. The product ions at $m/z$ 284 were formed from loss of the acetylcysteine moiety and a sulfur atom. Similar to what was observed in M8, the product ion at $m/z$ 168 corresponded to a 2-amino-4-thiol-purine moiety. The product ions at $m/z$ 150 and 120 suggested that the pyridine moiety was unchanged. M9 was thus tentatively identified as an N-acetylcysteine conjugate of BIIB021.

**Metabolite M10** Metabolite M10 was detected in the plasma samples of rat, dog, and human. It eluted at ca. 40.56 min by HPLC and had a protonated molecular ion at $m/z$ 335, which is 16 Da higher than BIIB021 (Figure 5 in Xu et al., 2012). The product ion at $m/z$ 284, 16 Da higher than that of the fragment ion at $m/z$ 268 from the parent, suggested the addition of one oxygen atom to the purine moiety (Table 6). The product ion at $m/z$ 186, 16 Da higher than that of the fragment ion at $m/z$ 170, also indicated that the hydroxylation had occurred at the 2-amino-4-chlorine-purine moiety. The fragment ions of $m/z$ 120 and 150 were unchanged. Therefore, M10 was identified as a hydroxylated metabolite, an isomer of M7. It was also identified as a metabolite in liver cytosolic fraction (Xu et al., 2012) and had the same retention time in LC as the synthetic standard (M10). To further confirm the exact location of hydroxylation, M10 was isolated from monkey liver cytosolic incubation and its $^1$H NMR spectra was compared with that of BIIB021 (Figure 7). In $^1$H NMR spectra of BIIB021, it contained two aromatic $^1$H signals at 8.18 (H8) and 8.44 (H13) ppm, respectively. However, in the $^1$H NMR spectra of M10 only one aromatic
1H signal at 8.52 ppm (H13) remained and one additional exchangeable proton (probably NH of urea) appeared at 11.50 ppm. The numbers of all aliphatic 1H signals remained the same in the two compounds. Therefore, the oxidation of M10 was assigned at the C8 position of 2-amino-4-chlorine-purine moiety. 1H NMR Data (DMSO-d6/CF3COOD, δ, 600 MHz): 11.50 (s, 1H, NH-7), 8.52 (s, 1H, H-13), 5.18 (s, 2H, H-10), 4.02 (s, 3H, H-19), 2.34 (s, 3H, H-20), 2.27 (s, 3H, H-17).

Kinetic Analysis of M10 Formation

The formation of metabolite M10 was primarily catalyzed by AO (Xu et al., 2012). To determine and compare the kinetic activity of M10 formation in different species, BIIB021 was incubated in the liver cytosolic fractions of rat, dog, monkey, mouse, and human. At 10 µM BIIB021, the monkey cytosolic fraction had the highest catalytic activity and approximately 23% BIIB021 was converted into M10 (Figure 8). In mouse, human, and rat, 7.3, 2.6, and 1.4% of BIIB021 were converted into M10, respectively. M10 was not formed in the dog liver cytosolic fraction due to the lack of AO.

The kinetics of M10 formation was further determined in human and monkey liver cytosolic fractions and Michaelis-Menten kinetics were observed at the concentrations of 0.5 to 500 µM BIIB021 (Figure 9). The apparent $K_m$ and $V_{max}$ values of M10 formation in human were $174 \pm 8 \mu M$ and $14.0 \pm 0.3$ pmol min$^{-1}$ mg protein$^{-1}$. The apparent $K_m$ and $V_{max}$ values of M10 formation in monkey were $132 \pm 9 \mu M$ and $131 \pm 4$ pmol min$^{-1}$ mg protein$^{-1}$.
Discussion

We report the metabolism and excretion of BIIB021 in rats and dogs, the preclinical species used for long-term safety assessment, and assessment of metabolic profiles of human plasma obtained from a dose escalation study in cancer patients. [14C]BIIB021 was administered orally to SD rats and beagle dogs. Excretion of radioactivity was nearly complete within 48 h after dosing and the majority of dose was recovered from feces in both species. In a separate BDC study in rats, 46.2% and 26.2% of the radioactivity were recovered in the bile and urine, suggesting that BIIB021 was well absorbed (>72%) and both biliary and urinary excretion were the route of elimination of BIIB021-derived radioactivity. The urine and fecal HPLC-radiochromatograms of rats and dogs indicated that BIIB021 is extensively metabolized in vivo. Unchanged BIIB021 was detected as a trace amount in urine and fecal samples. A total of ten metabolites in rats and seven metabolites in dogs were identified. Three primary metabolic pathways of BIIB021 included hydroxylation of the 3-methyl group, O-demethylation of the 4-methoxy pyridine, and direct displacement of the chlorine with GSH. Various combinations of these primary pathways and conjugations yielded other metabolites (Figure 1). The desmethyl metabolite (M2), monohydroxylated BIIB021 (M7), and its glucuronide conjugate (M4) were the major metabolites in rat and dog excreta. The dechlorinated metabolite, M6, also as a degradant of BIIB021 (Xu et al., 2012), was detected in most of the excreta of two species. The cysteinylglycine conjugate M8 and mercapturic acid M9 were identified as the minor metabolites in rat bile, suggesting that this glutathione conjugation was a minor biotransformation pathway. The identification of M8 and M9 suggested that BIIB021 was conjugated with glutathione via chlorine substitution. This observation was consistent with in vitro metabolism study results that showed BIIB021 was conjugated with glutathione (Xu et al., 2012).
The HPLC-radiochromatogram of circulating metabolites in rats and dogs were significantly different. In rats, unchanged BIIB021 was a major circulating radioactive component, accounting for approximately 49.3% of the total plasma radioactivity. M4 and M7 were the major circulating metabolites. In contrast, the majority of circulating radioactivity in dogs was comprised of metabolites. M4 was the dominant circulating metabolite. Other metabolites M2, M3 (carboxy-), M6, and M10 each accounted for less than 5% of radioactivity in dog plasma. Interestingly, monohydroxylated metabolite M10, whose formation is mainly catalyzed by AO (Xu et al., 2012), was observed as a minor metabolite in dog plasma. Since dog is known to be devoid of AO, the existence of M10 may be attributed to minimal aldehyde oxidase or other oxidase catalyzed oxidation.

BIIB021 is a clastogenic compound and radiolabeled human mass balance studies could not be conducted on healthy volunteers but rather must be conducted in cancer patients. It is difficult for cancer patients to stay at the clinical site for a week. Therefore, the metabolic profiles were obtained in human plasma collected from a dose escalation study in cancer patients. To identify and estimate the quantity of circulating metabolites of BIIB021 in humans, two approaches were applied. The first approach was to quantify oxidative metabolites which were predicted based on knowledge of major circulating metabolites in rat and dog and metabolites identified in vitro incubations. Therefore, M3 and M7 were chosen. As shown in Table 5, four out of six patients had concentrations of M3 and M7 higher or equivalent to those of parent BIIB021. The second approach was to conduct metabolite scouting of the same samples using LC-UV and LC-MS. The identified metabolites were semi-quantified via the correction factor of each metabolite between radioactivity response and mass spectrometry response using parent drug BIIB021 as a pseudo internal standard. This approach has
successfully been used in previous studies (Yi & Luffer-Atlas, 2010; Yu et al., 2007). The concentrations of M3 and M7 were also estimated and compared with the concentrations obtained from a validated bioanalytical assay. The estimated concentrations in five out of six patients were 70% of the measured concentrations. The estimated M7 concentration in all patients was systematically lower than the measured concentration and the reason is not clear. In addition to M3 and M7, two other major metabolites M4 and M10 were identified and semi-quantified. The glucuronide conjugate M4 had relatively variable concentrations across subjects. Surprisingly, M10 had almost an equivalent concentration to parent BIIB021 and this outcome was not predicted from previous studies. M10 was identified as a metabolite which is present at high levels in humans than in the animal species. It was a pharmacologically active metabolite of BIIB021, whose formation was catalyzed by AO (Xu et al., 2012).

The current kinetic results showed BIIB021 had similar affinity to human and monkey AO but Vmax in monkeys was almost 10-fold higher than the value of in humans. The catalytic activity in rats was lower than that in humans and no catalytic activity in dog was measurable in vitro. The kinetic results were consistent with the observation in vivo where the extent of M10, as the percentage of total circulating drug components, was lower in rats and dogs than that of humans. The data also suggested that the monkey could be a better species for safety assessment of BIIB021 and M10 than the dog. Understanding the metabolic pathways and intrinsic clearance of major metabolites across species is important in choosing the right preclinical species for metabolite safety coverage and avoiding potential human disproportionate metabolites. In addition, M10 was not detected as a major metabolite in vitro in human liver microsomal, cytosolic, and hepatocyte incubations (Xu et al., 2012). The average intrinsic clearance (Vmax/Km) of M10 formation was $8.0 \times 10^{-5}$ ml/min/mg protein in human liver cytosol and it
was far lower than the intrinsic clearance of AO catalyzed vanillic acid formation at 1.74 ml/min/mg protein (Sahi et al., 2008). However, M10 was found as a major circulating metabolite in humans and represented more than 15% of the total BIIB021 related components. Although the reason is not fully understood, underestimation of the portion of M10 in circulation may be explained as follows. AO is an ubiquitous enzyme expressed in multiple tissues (Kitamura et al., 2006) and, although the intrinsic clearance is low, the combination of all AO catalytic formation of M10 from different tissues, could exceed the presumably dominant hepatic M10 formation. Contribution of M10 formation in liver subcellular fractions or hepatocytes may underestimate the total AO catalytic activity \textit{in vivo}. Another explanation could be that the AO catalytic activity on M10 formation \textit{in vitro} preparations is much lower than the AO catalytic activity \textit{in vivo}. The high concentration of M10 was not expected based on \textit{in vitro} human analyses and \textit{in vivo} studies in preclinical species. Therefore, as recommended in the guidance on the safety testing of drug metabolites issued from FDA (FDA, 2008) and ICH (EMA, 2009), the early assessment of metabolite abundance of drug candidates in human plasma from Phase 1 clinical trials is very important. The metabolite scouting method using LC-MS in combination with radioactivity and MS response factors appeared feasible and efficient to estimate the metabolite abundance in human plasma samples from phase I clinical studies. The limitation of this approach is that it could not provide the information about the overall clearance pathways of BIIB021 in human, which is normally obtained from the radiolabeled mass balance study.

In summary, the results of this study provide the first analysis of formation and excretion of metabolites of BIIB021 in rats, dogs, and humans. BIIB021 was extensively metabolized and eliminated mainly by metabolism. Both parent and oxidative metabolites were dominant components in circulation in all species. The CYP catalyzed hydroxylation of the methyl group
followed by subsequent glucuronidation and carboxylation are dominant in all species. The AO catalyzed purine oxygenation was dominant in humans compared to rats and dogs. O-Demethylation and dechlorination facilitate the elimination of BIIB021. The glutathione conjugation and subsequent mercapturic acid formation represents a minor metabolic pathway. Combined with previous in vitro metabolism study (Xu et al., 2012), existence of multiple metabolic pathways of BIIB021 in humans mitigates the potential of drug-drug interactions in the proposed combination therapy against cancer.
Acknowledgments

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Authorship Contributions

- Participated in research design: Lin Xu, Chandra Prakash.
- Conducted experiments: Caroline Woodward, Jing Dai
- Contributed new reagents or analytic tools: Caroline Woodward, Jing Dai
- Performed data analysis: Lin Xu, Caroline Woodward, Chandra Prakash
- Wrote or contributed to the writing of the manuscript: Lin Xu, Chandra Prakash
References


Lundgren, K., Zhang, H., Brekken, J., Huser, N., Powell, R. E., Timple, N., Busch, D. J., Neely, L., Sensintaffar, J. L., Yang, Y. C., McKenzie, A., Friedman, J., Scannevin, R., Kamal,


Footnotes

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email:lin.xu@biogenidec.com
Figures

FIG 1. Proposed biotransformation pathways of BIIB021

FIG 2. Representative radiochromatograms of rat urine (panel A), feces (panel B), bile (panel C), and plasma (panel D).

FIG 3. Representative radiochromatograms of dog urine (panel A), feces (panel B), and plasma (panel C).

FIG 4. Representative UV (310 nm) chromatograms of predose (A) and AUC pooled (0-4 h, B) human plasma samples.

FIG 5. CID product ion spectra of BIIB021 (A) and M3 (B)

FIG 6. CID product spectrum of M9 (A) and MS3 of product ion 317 (B)

FIG 7. 1H NMR spectra of BIIB021 and metabolite M10

FIG 8. M10 formation rate in mouse, rat, dog, monkey, and human

FIG 9. Kinetic of M10 formation in human (A) and monkey (B) cytosols
Table 1. Material balance (mean ± SD) and route excretion of [\(^{14}\text{C}\)]BIIB021 drug-related material in rats and dogs

<table>
<thead>
<tr>
<th>Species</th>
<th>% of Administered Dose</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Bile</td>
<td>Total</td>
</tr>
<tr>
<td>Rat</td>
<td>26.2 ± 3.5</td>
<td>68.8 ± 12.0</td>
<td>NA</td>
<td>100 ± 12</td>
<td></td>
</tr>
<tr>
<td>Rat (BDC)</td>
<td>26.2 ± 10.2</td>
<td>22.0 ± 2.4</td>
<td>46.2 ± 7.6</td>
<td>95.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>34.0 ± 6.3</td>
<td>51.7 ± 8.6</td>
<td>NA</td>
<td>91.3 ± 3.3</td>
<td></td>
</tr>
</tbody>
</table>

*aPercentage of dose also includes cage washes*
Table 2. Relative abundance of urinary, biliary, and fecal metabolites of BIIB021 in rats and dogs following oral administration of [\(^{14}\text{C}\)]BIIB021 (% of total administrated dose)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces (Bile)</td>
</tr>
<tr>
<td>BIIB021</td>
<td>ND</td>
<td>0.92 (0.29)</td>
</tr>
<tr>
<td>M1</td>
<td>ND</td>
<td>6.06</td>
</tr>
<tr>
<td>M2</td>
<td>1.38</td>
<td>7.51 (1.32)</td>
</tr>
<tr>
<td>M3</td>
<td>1.27</td>
<td>3.07 (4.65)</td>
</tr>
<tr>
<td>M4</td>
<td>1.04</td>
<td>(10.8)</td>
</tr>
<tr>
<td>M5</td>
<td>0.87</td>
<td>2.81 (1.26)</td>
</tr>
<tr>
<td>M6</td>
<td>1.20</td>
<td>3.18 (1.86)</td>
</tr>
<tr>
<td>M7</td>
<td>14.2</td>
<td>15.6 (10.2)</td>
</tr>
<tr>
<td>M8</td>
<td></td>
<td>(2.47)</td>
</tr>
<tr>
<td>M9</td>
<td>0.28</td>
<td>(0.66)</td>
</tr>
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</table>
Table 3. Relative abundance of circulating metabolites of BIIB021 in rats, dogs, and human following oral administration of BIIB021

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rat</th>
<th>Dog</th>
<th>Human&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIIB021</td>
<td>49.3</td>
<td>4.75</td>
<td>26.2</td>
</tr>
<tr>
<td>M2</td>
<td>1.59</td>
<td>4.35</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>3.40</td>
<td>1.10</td>
<td>18.1</td>
</tr>
<tr>
<td>M4</td>
<td>6.40</td>
<td>61.5</td>
<td>12.9</td>
</tr>
<tr>
<td>M6</td>
<td>1.34</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>18.5</td>
<td>2.35</td>
<td>24.2</td>
</tr>
<tr>
<td>M10</td>
<td>3.06</td>
<td>2.00</td>
<td>18.6</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>83.6</strong></td>
<td><strong>78.0</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>Based on MS response using a conversion factor
Table 4. The comparison of measured concentration vs estimated concentration of metabolites M3 and M7 in the AUC pooled (0-4 h) plasma samples of patients following oral administration of BIIB021

<table>
<thead>
<tr>
<th>Patient</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured Concentration (ng/mL)</td>
<td>322</td>
<td>76.9</td>
<td>328</td>
<td>478</td>
<td>74.5</td>
<td>549</td>
</tr>
<tr>
<td>M3</td>
<td>Estimated Concentration (ng/mL)</td>
<td>295</td>
<td>43.6</td>
<td>246</td>
<td>428</td>
<td>78.2</td>
</tr>
<tr>
<td></td>
<td>Accuracy (%)</td>
<td>91.6</td>
<td>56.7</td>
<td>75.0</td>
<td>109</td>
<td>105</td>
</tr>
<tr>
<td>Measured Concentration (ng/mL)</td>
<td>493</td>
<td>92.1</td>
<td>344</td>
<td>451</td>
<td>285</td>
<td>652</td>
</tr>
<tr>
<td>M7</td>
<td>Estimated Concentration (ng/mL)</td>
<td>367</td>
<td>64.1</td>
<td>244</td>
<td>265</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Accuracy (%)</td>
<td>74.4</td>
<td>69.6</td>
<td>70.9</td>
<td>58.8</td>
<td>78.6</td>
</tr>
</tbody>
</table>
Table 5. The measured and estimated concentration of BIIB021 and its metabolites in the AUC pooled (0-4 h) plasma samples of patients following oral administration of BIIB021

<table>
<thead>
<tr>
<th>Patient</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIIB021 (ng/mL)</td>
<td>284</td>
<td>592</td>
<td>215</td>
<td>179</td>
<td>478</td>
<td>619</td>
</tr>
<tr>
<td>Measured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3 (ng/mL)</td>
<td>322</td>
<td>76.9</td>
<td>328</td>
<td>478</td>
<td>74.5</td>
<td>549</td>
</tr>
<tr>
<td>M7 (ng/mL)</td>
<td>493</td>
<td>92.1</td>
<td>344</td>
<td>451</td>
<td>285</td>
<td>652</td>
</tr>
<tr>
<td>Estimated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4 (ng/mL)</td>
<td>212</td>
<td>50</td>
<td>206</td>
<td>691</td>
<td>87</td>
<td>156</td>
</tr>
<tr>
<td>M10 (ng/mL)</td>
<td>218</td>
<td>293</td>
<td>237</td>
<td>225</td>
<td>322</td>
<td>346</td>
</tr>
<tr>
<td>Assignment</td>
<td>$^{12}$C-Mass [M+H] m/z</td>
<td>Proposed Structure$^a$</td>
<td>Biotransformation</td>
<td>Major fragments</td>
<td>Rt (min)</td>
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<tr>
<td>------------</td>
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<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>BIIB021</td>
<td>319.1070</td>
<td><img src="image" alt="BIIB021 structure" /></td>
<td></td>
<td>283, 268, 170, 150, 120</td>
<td>47.89</td>
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<tr>
<td>M1</td>
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<td><img src="image" alt="M1 structure" /></td>
<td>demethylation and oxidation</td>
<td>267, 170, 134</td>
<td>17.6</td>
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<tr>
<td>M2</td>
<td>305.0914</td>
<td><img src="image" alt="M2 structure" /></td>
<td>demethylation</td>
<td>269, 170, 136, 108</td>
<td>23.74</td>
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<td>M3</td>
<td>349.0825</td>
<td><img src="image" alt="M3 structure" /></td>
<td>oxidation</td>
<td>313, 298, 269, 254, 180, 150</td>
<td>17.6</td>
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<td>M4</td>
<td>511.1338</td>
<td><img src="image" alt="M4 structure" /></td>
<td>Hydroxylation and glucuronidation</td>
<td>335, 317, 299, 281, 166</td>
<td>27.23</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. (continued) Assignment and Proposed Structures of Metabolites of [14C]BIIB021

<table>
<thead>
<tr>
<th>Assignment</th>
<th>12C-Mass [M+H] m/z HRMS</th>
<th>Proposed Structure</th>
<th>Biotransformation</th>
<th>Major fragments</th>
<th>Rt (min)</th>
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<tbody>
<tr>
<td>M5</td>
<td>351.0971</td>
<td><img src="image" alt="Structure M5" /></td>
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<td>333, 297, 186, 166</td>
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<td>M6</td>
<td>301.1405</td>
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<td>Oxidative dechlorination</td>
<td>152, 150, 120</td>
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<td>M7</td>
<td>335.1016</td>
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<td>Hydroxylation</td>
<td>299, 281, 166</td>
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<td>M8</td>
<td>461.1698</td>
<td><img src="image" alt="Structure M8" /></td>
<td>Glycycysteine conjugation</td>
<td>386, 358, 316, 237, 150, 120</td>
<td>41.5</td>
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</table>
Table 6. (continued) Assignment and Proposed Structures of Metabolites of [\textsuperscript{14}C\textsubscript{]}BIIB021

<table>
<thead>
<tr>
<th>Assignment</th>
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<th>Proposed Structure</th>
<th>Biotransformation</th>
<th>Major fragments</th>
<th>Rt (min)</th>
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<td>M9</td>
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<td>Acetyl-cysteine conjugation</td>
<td>404, 317, 284, 168, 150, 120</td>
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<td>M10</td>
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<td>hydroxylation</td>
<td>284, 186, 150, 120</td>
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Figure 3
Figure 5

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6

A

B
Figure 8

Percentage of M10 Formation

- Human
- Monkey
- Dog
- Rat
- Mouse