In Vitro Metabolism of BIIB021, an Inhibitor of Heat Shock Protein 90, in Liver Microsomes and Hepatocytes of Rats, Dogs, and Humans and Recombinant Human Cytochrome P450 Isoforms

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ABSTRACT:

Inhibition of heat shock protein 90 (HSP90) results in the degradation of oncoproteins that drive malignant progression and induce cell death, thus making HSP90 a potential target of cancer therapy. 6-Chloro-9-(4-methoxy-3,5-dimethyl-pyridin-2-yl)-purin-2-ylamine (BIIB021), a synthetic HSP90 inhibitor, exhibited promising antitumor activity in preclinical models. It is currently in phase II clinical trials for the oral treatment of breast cancer. The objective of this study was to obtain both quantitative and qualitative metabolic profiles of [14C]BIIB021 in rat, dog, and human liver microsomes and hepatocytes to provide support for in vivo safety and clinical studies. The metabolites of [14C]BIIB021 were identified using liquid chromatography-tandem mass spectrometry coupled with radiometric detection. BIIB021 was extensively metabolized in both liver microsomes and hepatocytes. The major oxidative metabolic pathways identified for all species were due to hydroxylation (M7) and O-demethylation (M2) of the methoxydimethylpyridine moiety. The majority of M7 in dog hepatocytes was further conjugated to form the glucuronide (M4). Oxidative dechlorination (M6), monooxygenation (M10), and oxidative N-dealkylation of the methoxy-dimethylpyridine moiety (M11 and M12) were observed as the minor metabolic pathways in hepatocytes of all three species. A glutathione conjugate (M18) was also identified in all species. Its formation was catalyzed, in part, by soluble glutathione transferase via direct displacement of the chlorine on the amino-chloropurine moiety. Subsequent minor secondary metabolites M13, M14, M15, and M17 were observed in human, dog, and rat hepatocytes. Results from incubations of BIIB021 with human recombinant cytochrome P450 P450 (P450) isoforms and a P450 antibody inhibition study in human liver microsomes suggested that the formation of M7 is mainly catalyzed by CYP2C19 and CYP3A4, whereas the formation of minor metabolite M2 in human liver microsomes probably could be attributed to CYP3A4.

INTRODUCTION

Heat shock protein (HSP) 90 is an abundant molecular chaperone that promotes the conformational maturation of “client” proteins and protects them from degradation (Pearl and Poddromou, 2006; Soo et al., 2008; Biamonte et al., 2010). Many of the known clients are protein kinase or transcription factors involved in multiple signal transduction pathways. HSP90 is also expressed in the activated form in cancer cells, whereas it was 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 subsequently degrade. Therefore, HSP90 inhibitors represent a promising approach to treating cancers driven by multiple molecular abnormalities.

6-Chloro-9-(4-methoxy-3,5-dimethylpyridin-2-yl)methyl-9H-purin-2-ylamine (BIIB021) is a synthetic HSP90 inhibitor and exhibited a strong antitumor effect as a single agent and increased 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 combination 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. BIIB021 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 a multidrug resistance expression cell line (Zhang et al., 2010). It is under development as an oral agent for the treatment of breast cancer.

Identification of metabolites of a new chemical entity in animals and humans is essential to pharmaceutical development and commercialization. The in vitro metabolism of BIIB021 was studied in liver microsomes and hepatocytes from rats, dogs, and humans to provide information to inform in vivo safety and clinical studies. In addition, the metabolism of BIIB021 in human liver microsomes and hepatocytes was compared with the metabolism in human, dog, and rat hepatocytes to identify species differences that may impact in vivo disposition.

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Identification of metabolites of a new chemical entity in animals and humans is essential to pharmaceutical development and commercialization. The in vitro metabolism of BIIB021 was studied in liver microsomes and hepatocytes from rats, dogs, and humans to provide information to inform in vivo safety and clinical studies. In addition, the metabolism of BIIB021 in human liver microsomes and hepatocytes was compared with the metabolism in human, dog, and rat hepatocytes to identify species differences that may impact in vivo disposition.
pound progression. The U.S. Food and Drug Administration (FDA) recommends that the metabolic profiles in humans of all new chemical entities should be characterized before initiation of large clinical trials. In vitro studies using preclinical species and human hepatocellular and subcellular fractions and/or recombinant human enzymes often provide valuable information on the metabolic pathways in humans in vivo (Baranczewski et al., 2006; Dalvie et al., 2009). The illustrated metabolic pathways in vitro in animals and humans also provide guidance to select the right animal species for long-term safety assessment studies and to ensure that the selected animal species are exposed to all major metabolic forms formed in humans (Baillie et al., 2002; FDA: Guidance for Industry: Safety Testing of Drug Metabolites, http://www.fda.gov/cder/guidance/6897fnl.pdf, 2008).

BIIB021 is rapidly absorbed in vivo and has a short half-life in mouse (Kasibhatla et al., 2007), rat, and dog. It shows moderate or high clearance in these animal species. The elimination of BIIB021 is probably through metabolism (Xu et al., 2010). The objectives of this study were to obtain both quantitative and qualitative metabolite profiles of [14C]BIIB021 after incubations in rat, dog, and human liver microsomes and hepatocytes. The metabolites were characterized by high-resolution LC-MS/MS and by comparisons of their retention times on HPLC and MS spectra with those of the synthetic standards.

In addition, incubations using cDNA-expressed human P450s were performed to determine the P450s responsible for the formation of major oxidative metabolites (M2 and M7). The role of cytosolic enzymes such as aldehyde oxidase (AO) and glutathione transferase (GST) on metabolism of BIIB021 was also investigated.

Materials and Methods

Reference Compounds and Chemicals. BIIB021, [14C]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-hydroxymethyl-BIIB021 glucuronide (5-hydroxymethylglucuronide, M4), CF3785 [5-(hydroxymethyl) BIIB021, (6-((2-amino-6-chloro-9H-purin-9-yl)methyl)-4-methoxy-5-methylpyridin-3-ylmethanol, M7], and CF2483 [2-amino-6-chloro-9-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]-9H-purin-8-ol, M10] were synthesized at Biogen Idec (Kasibhatla et al., 2005, 2007).

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 radioactive purity of >99%, as determined by HPLC using an in-line radioactivity detector. 7-Chloro-1H-[1,2,3]triazolo[4,5-d]pyrimidin-5-amine was purchased from TCI America (Portland, OR). Allupurinol, ethacrynic acid, isovanillin, menadione, and raloxifene were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were HPLC or analytical grade and were obtained from Thermo Fisher Scientific (Waltham, MA), unless specified otherwise.

Pooled liver microsomes (20 mg/ml) of Sprague-Dawley rats, beagle dogs, and humans were purchased from XenoTech, LLC (Lenexa, KS). The pooled human liver microsomes (lot 910251) consisted of liver samples from 50 donors of both genders. Ten donors (five male and five female) pooled human cryopreserved hepatocytes were purchased from CellzDirect (Durham, NC).

For the rat, male and female cryopreserved hepatocytes were ordered from CellzDirect and pooled first before the incubation with BIIB021.

Microsomes from baculovirus-infected S9 insect cells expressing P450 reductase and each individual human P450 were purchased from BD Biosciences (San Jose, CA). The monoclonal antibodies against CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were also purchased from BD Biosciences.

Microsomal, Cytosolic, and Recombinant Human P450 Incubations. The metabolism of BIIB021 was studied in duplicate in rat, dog, and human liver microsomes (LM) and cytosolic subcellular fractions and recombinant human P450s. For liver microsomes, the incubation mixture (300 μl in the total volume) consisted of the following components: 1 mg/ml microsomal protein, 10 μM [14C]BIIB021, 0.5 μCi/ml (diluted from 100 μM, 5 μCi/ml test solution), 1 mM NADPH, 3.3 mM MgCl2, and 100 mM potassium phosphate, pH 7.4. Reactions were started by addition of NADPH (final concentration at 1 mM) and were incubated at 37°C in a shaking incubator block (450 rpm). After 90 min, reactions were terminated with 300 μl of ice-cold acetonitrile containing 0.1% formic acid. Control incubations were performed without NADPH. Samples were then centrifuged, and supernatants were directly analyzed by LC-RAM-MS.

For the liver cytosolic incubations, the mixtures contained 1 mg/ml 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 a 30-min incubation. The quenched reaction mixture was vortexed and centrifuged for 10 min at 4°C. The supernatant was directly analyzed by LC-MS analysis. Incubations were conducted in duplicate, and expressed human P450s include CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

In the recombinant human P450 incubations, the mixtures (300 μl) contained microsomes (50 pmol/ml) from baculovirus-infected S9 insect cells and 10 μM (0.5 μCi/ml) [14C]BIIB021 in a 100 mM phosphate buffer (pH 7.4). The incubations were initiated by addition of NADPH with a final concentration at 1 mM. The mixture was incubated with gentle shaking for 30 min at 37°C and terminated by addition of 300 μl of ice-cold acetonitrile containing 0.1% formic acid. Samples were processed the same as above before LC-MS analysis. Incubations were conducted in duplicate, and expressed human P450s include CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

In the glutathione-fortified incubations, 300-μl mixtures contained 10 μM [14C]BIIB021 (0.5 μCi/ml) and 1 mg/ml enzymes (either human liver microsomes or cytosols) in 100 mM potassium phosphate buffer (pH 7.4). Reactions were started by addition of GSH (final concentration at 10 mM) or water in controls. The GST inhibitor ethacrynic acid (100 μM) was pretreated with the incubation mixture for 5 min before addition of BIIB021. After 90-min incubations, reactions were terminated with 300 μl of ice-cold acetonitrile. Samples were processed the same as described above before LC-MS analysis.

Human LM Incubations in the Presence of P450 Monoclonal Antibody. For liver microsomes, the incubation mixture (300 μl in the total volume) consisted of the following components: 1 mg/ml microsomal protein, 10 μM [14C]BIIB021, 0.5 μCi/ml (diluted from 100 μM, 5 μCi/ml test solution), 1 mM NADPH, 3.3 mM MgCl2, and 100 mM potassium phosphate buffer (pH 7.4). Reactions were started by addition of NADPH (final concentration at 1 mM) and incubated at 37°C in a shaking incubator block (450 rpm). After 90 min, reactions were terminated with 300 μl of ice-cold acetonitrile containing 0.1% formic acid. Control incubations were performed without NADPH. Samples were then centrifuged, and supernatants were directly analyzed by LC-RCAM-MS.

The incubations were conducted in duplicate at a final volume of 300 μl. Each reaction mixture consisted of human liver microsomal proteins (1 mg/ml), 10 μM [14C]BIIB021 (0.5 μCi/ml), 1 mM NADPH, 3.3 mM MgCl2, and 15 μl anti-P450 antibodies in phosphate buffer (100 mM, pH 7.4). Incubations without antibody were conducted as the positive controls. All components except BIIB021 and NADPH were mixed and preincubated for 15 min on ice and then 5 min at 37°C. BIIB021 and NADPH were added, and the mixture was incubated for an additional 60 min at 37°C. The mixture was quenched with 300 μl of ice-cold acetonitrile containing 0.1% formic acid. The samples were then centrifuged, and supernatants were directly analyzed by LC-RCAM-MS.
determine the concentration of M7. To achieve maximum inhibition of M7 formation, different volumes of anti-CYP2C19 and anti-CYP3A4 were used in the human liver microsomal incubations.

**Hepatocyte Incubations.** Metabolism of [14C]BIIB021 was evaluated in rat, dog, and human hepatocytes. Incubations were performed in duplicate in Krebs-Henseleit buffer. The final substrate concentration in the cell suspension was 10 μM (0.5 μCi/ml) in a volume of 0.5 ml at a cell density of 1 × 10^6 cells/ml. Incubations proceeded for 1.5 h at 37°C in the 24-well incubation plate under 95% O2/5% CO2. 7-Ethoxycoumarin was incubated as a positive control for the hepatocytes. To ensure maximum recovery of compound, a 10-μl aliquot of each incubation mixture was added to 5 ml of scintillation fluid before the reaction was quenched. The samples were then quenched with 50 μl of ice-cold trichloroacetic acid and were put on ice. The samples were sonicated for 20 min, vortexed, and centrifuged for 20 min. The radioactivity recovery was measured by comparing recovery before and after the reaction was terminated. The supernatant was then analyzed by LC-RAM-MS.

**Instrumentation.** The LC-RAM-MS system consisted of an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA), a v.ARC radiometric detector (AIM Research, Hockessin, DE), and a Thermo LTQ Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific). The separation of metabolites was performed on a Luna analytical C18(2) column (4.6 × 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 four 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; and 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 Research) was used as the scintillation liquid. 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 Fisher Scientific) containing caffeine, 1-methionyl-arginy1-phenylalanine, and Ultramark 1621. High-resolution data were expressed as four decimal places.

**Results**

**Metabolite Profiles of [14C]BIIB021 in LM and Hepatocytes.** Recovery of radioactivity from all in vitro incubations of [14C]BIIB021 in liver microsomes and hepatocytes was >85%. The supernatant from each incubation mixture was used for radioactivity profiling of metabolites.

The representative HPLC radiochromatograms of metabolites in rat, dog, and human LM incubations are shown in Fig. 2. The relative percentage of individual radioactivity peaks is summarized in Table 1. BIIB021 was moderately stable in liver microsomes at the clinical percentage of individual radioactivity peaks is summarized in Table 1. BIIB021 was relatively stable in rat LM, 9.5 and 36.2% in dog, and 3.6 and 42.2% in human, respectively. Oxidative N-dealkylation of the 4-methoxy-3,5-dimethylpyridine moiety M11 and M12 was found as an additional metabolic pathway in rat LM, and the resulting metabolites; M11 and M12 represented ~6.9% of the radioactivity. A minor metabolite M10 (monohydroxylation, ~1.2%) was also identified in rat, dog, and human LM at 1.3, 0.3, and 0.5% of the radioactivity, respectively.

The representative HPLC radiochromatograms of metabolites in rat, dog, and human hepatocytes and in buffer are shown in Fig. 3. The relative percentage of individual radioactivity peaks is summarized in Table 1. A total of 12 (11 radioactive and 1 nonradioactive) metabolites were identified from these incubations. BIIB021 was extensively metabolized in rat and dog hepatocytes, and less than 5 and 0.8% of [14C]BIIB021 remained unchanged in rat and dog, respectively, after 90-min incubations. BIIB021 was relatively stable in human hepatocytes, and ~38% remained unchanged after incubation. In rat, dog, and human hepatocytes, M7 was identified as a major metabolite, representing ~37.5, 3, and 49% of the radioactivity, respectively. The majority of M7 in dog was further biotransformed to a glucuronide conjugate (M4), representing ~44.8% of radioactivity. M4 represented 10.2% of radioactivity in the rat but was not detected in the human hepatocytes. O-Demethylation (M2) was also determined to be one of the major metabolic pathways, and it represented ~8.7, 25.5, and 0.5% of the radioactivity in rat, dog, and human hepatocytes. Consistent with rat LM, oxidative N-dealkylation was found only in rat hepatocytes, and the resulting metabolites, M11 and M12, both represented ~11.1% of the radioactivity. Several GSH conjugates were also identified in the incubations. M18, representing 13.1 and 9% of radioactivity in rat and dog hepatocytes, was formed by direct displacement of the chlorine atom by GSH. M14 was a GSH-substituted dechlorinated M2, representing ~7% of radioactivity in dog hepatocytes. None of these metabolites was identified in human hepatocytes. M17 was identified as a GSH-substituted dechlorinated monohydroxylated BIIB021, and it presented 9.1, 3, and 1.8% of radioactivity in rat, dog, and human hepatocytes. M6 and M15, which were formed by oxidative dechlorination of BIIB021 and M7, were found to be dominant in human hepatocytes, representing 4.4 and 3.4% of radioactivity, respectively. Again, M6 was formed in control Krebs’ buffer, suggesting that it is, in part, formed nonenzymatically. A minor metabolite, M10 (monohydroxylation), represented only less than 2% of radioactivity across all species. Other metabolites, M13 and M16, were formed by combination of these primary pathways. M13 was detected at 1% of radioactivity in dog hepatocytes. M16 (purine oxidation and O-demethylation), accounting for 1.9% of the radioactivity, was found only in human hepatocytes.

**Metabolic Identification and Characterization.** A total of 13 metabolites were identified in liver microsomes and hepatocytes incubations by high-resolution LC-MS/MS. BIIB021 contains one chlorine atom that has two major isotopes, 35Cl and 37Cl, in a natural abundance ratio of approximately 3:1. Retention times of radioactive peaks in chromatograms and the characteristic 35/37Cl 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. [14C]BIIB021 has a high percentage in the incubation solution with a ratio of [14C]BIIB021 to [14C]BIIB021 close to 1:3. Therefore, the 12C/13C ion clusters along with the chlorine pattern observed in the LC-MS data were also used for detecting metabolites and interpreting their fragmentation patterns in MS/MS spectra. The structures of each metabolite were elucidated by interpreting the mass change of the molecular ion and similarity/change of fragmentation patterns, compared with those of BIIB021. The exact location of modification in
Fig. 2. HPLC radiochromatograms of [14C]BIIB021 metabolites with human LM control without NADPH (A), human LM fortified with NADPH (B); dog LM fortified with NADPH (C), and rat LM fortified with NADPH (D).
major metabolites was determined by comparing the retention time, elemental composition, and product ion spectra of available synthetic standards.

BIIB021 eluted at ~47.98 min on HPLC and produced protonated molecular ions at m/z 319/321/323 at the ratio of 1:3:3:1. The product ion (MS/MS) spectrum of BIIB021 with proposed characteristic fragmentations is displayed in Fig. 4. 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 a methyl group. The product ions at m/z 302/304 were formed from the loss of an NH3 (17 Da). The other characteristic ion at m/z 150 is formed by cleavage of the C=N bond between the pyridine moiety and the pyridine ring with charge retention at the pyridine moiety. The subsequent loss of the formaldehyde from the 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 in BIIB021 and its metabolites that retained the intact pyridine ring.

Metabolite M2 was detected in all three species. It eluted at approximately 23.74 min on HPLC and produced protonated molecular ions at m/z 305/307. The product ions at m/z 269/271 were formed from loss of an HCl molecule (36 Da). The product ions at m/z 170/172 and 136 corresponded to the 2-amino-4-chlorine-purine moiety and the pyridine ring moiety, respectively (Supplemental Fig. 1). The 14-Da loss from the pyridine ring moiety suggested that M2 was an O-desmethyl metabolite of BIIB021. M2 also has the same HPLC retention time and product ion spectra as those of the reference standard, 2-(2-amino-6-hydroxy-9-Y-purin-9-yl)methyl-3,5-dimethylpyridin-4-ol (CF2246). Therefore, M2 was identified as O-desmethyl BIIB021.

Metabolite M4 was detected in rat and dog hepatocyte incubations. It eluted at approximately 27.23 min and produced protonated molecular ions at m/z 511/513. The product ions at m/z 335/337 (Supplemental Fig. 2) corresponded to loss of a glucaric acid moiety (~176 Da). This aglycone had a molecular ion that was 16 Da higher than that of BIIB021, suggesting that it is a hydroxylated metabolite. The product ion at m/z 166 obtained from the aglycone (m/z 335) was formed from the pyridine ring moiety, suggesting that the hydroxylation had occurred on this ring. M4 has the same HPLC retention time as that of a reference standard of 5-hydroxymethyl-BIIB021 glucuronide (5-OH 1983 Gluc). All product ions of M4 were consistent with those of 5-OH 1983 Gluc. Therefore, M4 was identified as the glucuronide conjugate of 5-hydroxymethyl-BIIB021.

Metabolite M6 was detected in all samples. It eluted at approximately 33.1 min and produced protonated molecular ions at m/z 301/303. The molecular mass (18 Da lower than that of BIIB021) and the ratio of isotopic ions 301/303 (1:3) suggest the loss of a chlorine atom and the addition of a hydroxyl group to BIIB021. The product ions at m/z 152/154, 18 Da lower than the ions at m/z 170/172 from BIIB021 (Supplemental Fig. 3), indicated that dechlorination and hydroxylation had occurred at the 2-amino-4-chlorine-purine moiety. The product ions at m/z 150 and 120 were the same as those observed for BIIB021. M6 was tentatively identified as a dechlorinated-hydroxylated metabolite of BIIB021.

Metabolite M7 was detected in all species. It eluted at approximately 35.98 min on HPLC and produced protonated molecular ions at m/z 335/337. With a 16 Da higher molecular ion than that of BIIB021, it was proposed as a hydroxylated metabolite. The product ion at m/z 166, 16 Da higher than the fragment ion at m/z 150 from the parent, suggested that the hydroxylation had occurred at the pyridine ring (Fig. 5A). M7 has the same HPLC retention time as that of a reference standard, 5-hydroxymethyl-BIIB021 (CF3785). All product ions of M7 were consistent with those of CF3785. Therefore, M7 was identified as 5-hydroxymethyl-BIIB021.

Metabolite M10 was detected in all species. It eluted at approximately 40.56 min on HPLC and produced protonated molecular ions at m/z 335/337, 16 Da higher than those of BIIB021. The product ion at m/z 284, 16 Da higher than the fragment ion at m/z 268 from the parent, suggested the addition of one oxygen atom to the purine moiety (Fig. 5B). The product ion at m/z 186, 16 Da higher than 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. M10 has the same HPLC retention time as that of a reference standard, 2-amino-6-chloro-9-(4-methoxy-3,5-dimethylpyridin-2-yl)methyl-9H-purin-8-ol (CF2483). All product ions of M10 were consistent with those of CF2483. Therefore, M10 was identified as a hydroxylated metabolite, the isomer of M7.

Metabolites M11 and M12 were detected only in the rat hepatocytes. M11 eluted at approximately 13.8 min on HPLC and produced protonated molecular ions at m/z 170/172, consistent with the ion of the 2-amino-4-chlorine-purine moiety. All product ions had isotopic ratios that were consistent with commercial 7-chloro-1H-[1,2,3]triazolo[4,5-d]pyrimidin-5-amine (Supplemental Fig. 4). Therefore, M11 was identified as 7-chloro-1H-[1,2,3]triazolo[4,5-d]pyrimidin-5-amine.

M12 eluted at approximately 14.3 min on HPLC and produced a protonated molecular ion at m/z 168 as a nonradiolabeled metabolite. The product ions at m/z 150 and m/z 120 were formed from consecutive losses of a water molecule and a methoxyl group (Supplemental Fig. 5). Therefore, M12 was tentatively assigned as (4-methoxy-3,5-dimethylpyridin-2-yl)methanol.

Metabolite M13 was detected in rat and dog hepatocyte incubations. It eluted at approximately 17.89 min on HPLC and had protonated molecular ions at m/z 493/495. The HRMS of molecular [M + H] + ion 493.1675 suggests a molecular formula of C20H23N5O4. The ratio of ions at m/z 493/495 was 1:3, indicating loss of the chlorine atom. The product ions at m/z 317/319 and 299/301 corresponded to the sequential loss of a glucaric acid moiety (~176 Da) and a water molecule (Supplemental Fig. 6). The product ion at m/z 166, 16 Da higher than the fragment ion at m/z 150 from the parent, suggested the addition of an oxygen atom to the pyridine moiety. M13 was tentatively assigned as the 2-((6-((2-amino-6-hydroxy-9H-purin-9-yl)methyl)-4-methoxy-5-methylpyridin-3-yl)methyl)-4-hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol formed via oxidative dechlorination of the 2-amino-4-aminochloropurine moiety and hydroxylation followed by glucuronidation on the pyridine moiety.

Metabolite M14 was detected only in dog hepatocytes. It eluted at approximately 18.23 min and produced protonated molecular ions at m/z 576/578. The ratio of isotopic ions 580 (M + 4) versus 576 was reduced to 0.15:1, suggesting loss of a chlorine atom. The product ions at m/z 447/449 and 303/305 were formed from loss of the anhydroglutamic acid (~129 Da) and glutamine-alanine-glycine.
FIG. 3. HPLC radiochromatograms of [14C]BIIB021 and metabolites with Krebs’ buffer as control (A), human hepatocytes (B), dog hepatocytes (C), and rat hepatocytes (D).
(−273 Da), suggesting that it was a glutathione conjugate (Supplemental Fig. 7). MS³ spectra of ions at m/z 303/305 provided fragments at m/z 270/272 and 136 via loss of an SH and the purine moiety, respectively. The fragment ion at m/z 136, 14 Da lower than the fragment ion at m/z 150 from the parent, suggested demethylation at the pyridine ring. HRMS of [M + H⁺]⁺ ion at m/z 576.1985 suggests a molecular formula of C₂₃H₂₉N₉O₇S. Loss of the GSH (C₁₀H₁₇N₃O₆S) from the molecular formula gave an ion at m/z 269, which is a typical product ion of M2 by loss of an HCl molecule. M14 was therefore tentatively assigned as a GSH conjugate of deschloro M2.

Metabolite M15 was detected only in human hepatocytes. It eluted at 21.82 min and produced the protonated molecular ions at m/z 317/319. The 1:3 ratio of isotopic ions at m/z 317/319 suggested loss of a chlorine atom. The product ions at m/z 152/154, 18 Da lower than those of the 2-amino-4-chlorine-purine moiety (Supplemental Fig. 8), indicated that oxidative dechlorination had occurred at this moiety. The product ion at m/z 166, 16 Da higher than the fragment ion at m/z 150 from the parent, suggested that hydroxylation had occurred at the pyridine ring. M15 was tentatively assigned as the monohydroxylated M6. The hydroxylation site could not be fully determined by MS data.

Metabolite M16 was detected only in human hepatocytes. It eluted at 22.83 min on HPLC and had protonated molecular ions at m/z 321/323. The product ions at m/z 186/188, 16 Da higher than the fragment at m/z 170/172 from the parent (Supplemental Fig. 9), suggested that the hydroxylation had occurred at the 2-amino-4-chlorine-purine moiety. The product ions at m/z 136 and 108 were

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<th>Metabolite</th>
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<th>Dog</th>
<th>Rat</th>
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FIG. 4. Collision-induced dissociation (CID) product ion spectra of monoisotopic molecular ions 319 (A, 35Cl), 321 (B, 37Cl), and 323 (C, 35Cl, 14C) of [14C]BIIB021.
formed from the pyridine ring moiety by subsequent losses of the 2-amino-4-chlorine-purine and CO moieties (−28 Da). The ion at m/z 136, 14 Da lower than the fragment ion at m/z 150 from the parent molecule, suggested the loss of a methyl group from the pyridine ring. M16 was tentatively assigned as a demethylated M10 or monohydroxylated M2.

Metabolite M17 was detected in hepatocytes of all species. It eluted at 25.06 min and had protonated molecular ions at m/z 606/608. The ratio of isotopic ions 610 (M + 4) versus 606 was reduced to 0.15:1, suggesting loss of the chlorine. The product ions at m/z 477/479 corresponded to loss of the anhydroglutamic acid (−129 Da). The product ions at m/z 333/335 were due to cleavage of the S–CH₂ bond, leading to a neutral loss of 273 Da (Supplemental Fig. 10). HRMS of [M + H]⁺ ion at m/z 606.2085 suggests a molecular formula of C₂₄H₃₁N₉O₈S. Loss of GSH (C₁₀H₁₇N₃O₆S) from the molecular ion gave the same ion at m/z 299, which is a typical product ion of M7 by loss of an HCl molecule. M17 was therefore tentatively assigned as the GSH conjugate of deschlorinated M7. The location of hydroxylation could not be determined by MS data.

Metabolite M18 was detected in rat and dog hepatocytes. It eluted at approximately 34.6 min and had protonated molecular ions at m/z 590/592/594. The ratio of isotopic ions 594 (M + 4) versus 590 was reduced to 0.20:1, suggesting loss of a chlorine atom. The product ion at m/z 461/463 corresponded to the loss of the anhydroglutamic acid (−129 Da) (Fig. 6A). The product ions at m/z 317/319 were due to cleavage of the S–CH₂ bond, leading to a neutral loss of 273 Da. The product ions at m/z 150 and 120 in the MS² spectrum (Fig. 6B) were the same as those observed in BIIB021. HRMS of [M + H]⁺ ion 590.2130 suggests a molecular formula of C₂₄H₃₁N₉O₇S. Loss of GSH (C₁₀H₁₇N₃O₆S) from the molecular formula gave the same ion at m/z 283, which is a typical product ion of BIIB021 by loss of an HCl molecule. M18 was tentatively identified as the GSH conjugate of deschloro-BIIB021.

Metabolism by cDNA-Expressed P450s and Monoclonal Antibody Inhibition. Human recombinant CYP2C19, CYP2C9, and CYP3A4 isozymes showed catalytic activity of BIIB021 metabolite formation (Fig. 7). [¹⁴C]BIIB021 was completely metabolized by CYP2C19. Average 5 and 89% radioactivity presented as M2 and M7, respectively. With human recombinant CYP3A4, 70% radioactivity remained as the parent. Approximately 5 and 21% radioactivity was detected as metabolite M7. The rest of the radioactivity remained as the parent. It appeared that CYP2C19 and CYP3A4 were the main liver microsomal enzymes responsible for the formation of hydroxyl (M7) and desmethyl (M2) metabolites.

To further confirm the primary P450 enzymes involved in BIIB021 metabolism, several monoclonal antibodies of P450 enzymes were added to human liver microsomal incubations (Fig. 8A). With anti-CYP2C19 or anti-CYP3A4, maximum inhibition of M7 formation was achieved at 75.6 or 34%, respectively (Fig. 8B). Other monoclonal antibodies such as CYP1A2, CYP2A6, CYP2B6, CYP2D6, and CYP2E1 did not show significant inhibition of M7 formation (Fig. 8A). The inhibitory effects of monoclonal antibodies on M2 formation were not observed significantly in this experiment.

Metabolism by Cytosolic Enzymes. Metabolite M10 was detected as a non-NADPH/NAD-dependent metabolite in human cytosolic incubation. To determine the major enzymes involved in the formation of M10, specific inhibitors of xanthine oxidase and AO were added to cytosolic incubation. As shown in Fig. 9, M10 formation was not affected by addition of the xanthine oxidase inhibitor, allopurinol (Clarke et al., 1995; Rashidi et al., 2007). In contrast, M10 formation was significantly inhibited by AO inhibitors, isovanillin, menadione, and raloxifene (Obach, 2004; Rashidi et al., 2007). The results suggested that the formation of M10 is probably catalyzed by AO.

Glutathione conjugate metabolite M18 was formed both chemically and enzymatically catalyzed by GST. After [¹⁴C]BIIB021 was incubated in GSH-fortified phosphate buffer and human liver microsomes, approximately 20% total radioactivity was identified as M18 in both
incubations (Fig. 10), suggesting a nonenzymatic GSH substitution reaction. When incubated with GSH-fortified human liver cytosol, \( \sim 55\% \) [\(^{14}\text{C}\)]BIIB021 was converted to M18. After the GST inhibitor ethacrynic acid was added to the cytosolic incubation, the amount of M18 was reduced to 20%, which was equivalent to the extent of M18 formation observed in the buffer. This result suggested that M18 formation was predominantly catalyzed by cytosolic GST in the liver. Similar chlorine displacement by water (M6) was also observed, but the percentage of M6 remained 1 to 3% across all incubations (data not shown).

**Discussion**

The in vitro metabolism of BIIB021 in liver microsomes and hepatocytes of rats, dogs, and humans has been elucidated to support preclinical safety and clinical studies. Rats and dogs were chosen in this study because they are the species used for preclinical safety assessments. BIIB021 is extensively metabolized in both liver microsomes and hepatocytes, and a total of 13 metabolites were identified by LC-MS/MS. The major metabolites were similar in all species (Table 3; Fig. 11). However, several additional metabolites were identified in rat hepatocytes. The primary major metabolic pathways were due to hydroxylation of the methyl group, O-demethylation, N-dealkylation of the methoxy-dimethylpyridine moiety, oxygenation of the purine, and direct displacement of the chlorine with GSH or water (Fig. 11). The other metabolites were due to combinations of these primary pathways. Most of these metabolites do not exhibit pharmacological activity except for M7 and M10, which were 10- and 5-fold less potent than the parent BIIB021, respectively.

After incubation of [\(^{14}\text{C}\)]BIIB021 with liver microsomes fortified with NADPH, monohydroxylation and O-demethylation at the methoxy-dimethylpyridine moiety were identified as the major metabolic pathways in three species and yielded metabolites M7 and M2, respectively. Consistent with LM incubations, monohydroxylation at the methoxy-dimethylpyridine moiety was also observed as a major metabolic pathway in hepatocytes. The majority of M7 in dog hepatocytes was further metabolized to form a glucuronide conjugate M4. O-Demethylation (M2) was also a major metabolic pathway in rat and dog. Qualitative identification using cDNA-expressed P450s suggested that monohydroxylation and demethylation are mainly catalyzed by CYP2C19 and CYP3A4. Although CYP2C19 showed higher catalytic activity of monohydroxylation, the amount of CYP2C19 in human liver is less than 1% of the total P450s (Inoue et al., 1997). The contribution to monohydroxylation of BIIB021 could be balanced by...
both CYP2C19 and the dominant P450 enzyme CYP3A4. In the P450 antibody inhibition study in human liver microsomes, it appeared that CYP2C19 and CYP3A4 were responsible for 65 to 75% and 25 to 35% formation of M7. Likewise, oxidative O-demethylation is expected to be mainly catalyzed by human CYP3A4 when approximately equal amounts of M2 formation were observed by using equal molarity of human recombinant CYP2C19 and CYP3A4.

The oxidative N-dealkylation of the 4-methoxy-3,5-dimethylpyridine moiety (M11 and M12) was a unique metabolic pathway in the rat. It was not observed either in human or in dog. The nonenzymatic oxidative dechlorination of BIIB021 (M6) and its metabolites (M13 and M15) observed during the incubation was indicative of the instability of the BIIB021 chlorine moiety in aqueous solution. M13 and M15 were probably formed from nonenzymatic oxidative dechlorination of M4 and M7, respectively.

In this study, several GSH adducts formed via the displacement of the chlorine on the amino-chloropurine moiety were observed in hepatocyte incubations. M18 was formed via direct GSH substitution of chlorine on the purine moiety. The substitution was predominantly catalyzed by cytosolic GST. The GSH substitution on 2-chloropyridines has been reported, but it is catalyzed by microsomal GST (Inoue et al., 2009). M14 and M17 were probably formed from GSH substitution of M2 and M7, respectively. The evidence of GSH substitution could suggest potential covalent binding of BIIB021 or its metabolites to endogenous proteins (Nakayama et al., 2011). After incubation of [14C]BIIB021 with hepatocytes, the recovery of radioactivity was greater than 85%, indicating that the covalent protein binding potential may be lower than 15% and GSH may play an important role in protecting against undesired covalent binding of endogenous proteins.

Because this substitution of chlorine with GSH occurred both chemically and enzymatically catalyzed by cytosolic GST, any undesired toxicity could be assessed in rat and dog toxicological studies. The GSH substitution pathway was also confirmed during metabolic profiling studies in vivo, and mercapturic acid of substituted BIIB021 was detected in urine (Xu et al., 2010).

Monohydroxylation on the purine moiety (M10) was identified as a minor metabolic pathway in liver microsomes and hepatocytes. Of interest, M10 formation was found to be NADPH- and NADH-independent, and the hydroxylation occurred on the hetero-aromatic purine moiety. These observations suggested that M10 formation was probably catalyzed by a cytosolic enzyme such as AO, xanthine dehydrogenase, or xanthine oxidase. The NADH independence of the reaction excluded the possibility of a xanthine dehydrogenase. The further inhibition studies demonstrated that the formation of M10 was completely inhibited by several AO inhibitors but not by a xanthine oxidase inhibitor, allopurinol. Therefore, AO appeared to catalyze the formation of M10. It has been reported that AO expression levels vary markedly across species with high activity in monkey and human, relatively lower activity in rat and mouse, and the lowest activity in dog and bird (Sahi et al., 2008). The trace amount of M10 formation in the LM incubation might be attributed to incomplete isolated cytosolic AO. The trace amount of M10 was also observed in dog liver cytosol with GSH, the recovery of radioactivity was greater than 85%, indicating that the covalent protein binding potential may be lower than 15% and GSH may play an important role in protecting against undesired covalent binding of endogenous proteins.
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<th>Assignment</th>
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<th>Major Fragments</th>
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M10 formation. In addition to M10, the formation of M16, which was identified only in human hepatocyte incubation, was also catalyzed by AO. It was formed by the combination of purine hydroxylation and O-demethylation via M10 or M2. The combined radioactivity of M10 and M16 in hepatocyte incubation was 3.6% in human, 0.4% in dog, and 0.4% in rat. Higher turnover of AO-catalyzed biotransformation of BIIB021 in human than in dog or rat was consistent with reported species variability of the AO expressing level. Meanwhile, AO is not only expressed in liver, it is also predominantly expressed in the lung, kidney, and other tissues (Kitamura et al., 2006). The interindividual variability of AO activity was also observed to be high in the liver (Sugihara et al., 1997; Lake et al., 2002). Hence, the contribution of AO to BIIB021 metabolism in hepatocytes only may underestimate its actual effect in clinic pharmacokinetic and metabolism. In vivo and in vitro metabolism of BIIB021 in dogs and rats might also underestimate the AO contribution in humans.

**FIG. 11.** Proposed metabolic pathways of BIIB021 in vitro.

**TABLE 3—Continued**

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HRMS, high-resolution mass spectrometry; Rt, retention time.
The FDA (Guidance for Industry: Safety Testing of Drug Metabolites, http://www.fda.gov/cder/guidance/6897final.pdf, 2008) and International Conference on Harmonisation (Guidance on NonClinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf, 2009) have recently issued a formal guidance on the safety testing of drug metabolites. On the basis of the current 14C-labeled in vitro metabolism study, the majority of human metabolites identified will be covered by preclinical species. Monohydroxylated metabolite M7 and O-demethylated metabolite M2 were also predominant in animal species. However, formation of metabolites such as M10 and M16 via the AO pathway is relatively low or negligible in rat and dog. To have safety coverage of M10 in preclinical species, higher dose treatment in toxicology studies and a large safety window of BIIB021 are required.

In summary, the results of this study provide the first analysis of characterization of metabolites of BIIB021 in liver microsomes and hepatocytes from rat, dog, and human. On the basis of the structures of metabolites, four major metabolic pathways involving two hydroxylation, nonenzymatic/enzymatic water/GSH substitution, and O-demethylation were observed in all species. The oxidative N-dealkylation pathway was unique in the rat. On the basis of these data, it is expected that BIIB021 will be biotransformed in humans by multiple pathways. Two monohydroxylation pathways were catalyzed by two polymorphic enzymes, CYP2C19 (Shon et al., 2002) and AO, which could contribute to the interindividual metabolite concentration of M7 or M10. The results of this study will aid in the identification of metabolites of BIIB021 in humans in vivo and the evaluation of potential drug-drug interactions in the clinic.

Acknowledgments
We thank Dr. Ryan Van de Water for synthesizing [14C]BIIB021 and Dr. Marco Biomonte, Kevin Hong, and Jiandong Shi for providing synthetic standards of metabolites.

References


