Radiolabel uncovers nonintuitive metabolites of AMPAR potentiator BIIB104: Novel release of \[^{14}\text{C}]\text{cyanide from 2-cyanothiophene and subsequent formation of }[^{14}\text{C}]\text{thiocyanate}

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Abbreviations: ADME, absorption, distribution, metabolism, and excretion; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDC, bile duct-cannulated; CID, collision-induced dissociation; COSY, (homonuclear) correlation spectroscopy; DLM, dog liver microsomes; ESI, electrospray ionization; FDA, Food and Drug Administration; HLM, human liver microsomes; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; HRMS, high resolution mass spectrometry; HSQC, heteronuclear single quantum coherence; LC, liquid chromatography (which may refer to either HPLC or UPLC in this article); LSC, liquid scintillation counting; m/z, mass-to-charge ratio; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NOE, nuclear Overhauser effect; RLM, rat liver microsomes; ROI, region of interest; TMS, tetramethylsilane; TOCSY, (homonuclear) total correlation spectroscopy; TOF, time-of-flight; UPLC, ultraperformance liquid chromatography.
Abstract

BIIB104 (formerly PF-04958242), N-((3S,4S)-4-(4-(5-cyanothiophen-2-yl)phenoxy)tetrahydrofuran-3-yl)propane-2-sulfonamide, is an AMPAR potentiator investigated for the treatment of cognitive impairment associated with schizophrenia. Preliminary in vitro metabolism studies with non-radiolabeled BIIB104 in rat, dog, and human liver microsomes (RLM, DLM, and HLM) showed O-dealkylation in all 3 species, tetrahydrofuran hydroxylation dominating in DLM and HLM, and thiophene hydroxylation prevalent in RLM. However, a subsequent rat mass balance study with [nitrile-14C]BIIB104 showed incomplete recovery of administered radioactivity (~80%) from urine and feces over 7 days following an oral dose, and an exceptionally long plasma total radioactivity half-life. Radiochromatographic metabolite profiling and identification, including chemical derivation, revealed that [14C]cyanide was a major metabolite of [nitrile-14C]BIIB104 in RLM, but a minor and trace metabolite in DLM and HLM, respectively. Correspondingly in bile duct-cannulated rats, [14C]thiocyanate accounted for ~53% of total radioactivity excreted over 48 h postdose and it, as an endogenous substance, explained the exceptionally long plasma radioactivity half-life. The release of [14C]cyanide from the 2-cyanothiophene moiety is postulated to follow an epoxidation-initiated thiophene-opening based on the detection of non-radiolabeled counterpart metabolites in RLM. This unusual biotransformation serves as a lesson regarding placement of the radioactive label on an aryl nitrile when material will be used for evaluating the metabolism of a new drug candidate. Additionally, the potential cyanide metabolite of nitrile-containing drug molecules may be detected in liver microsomes with LC-MS following a chemical derivatization, so to be informed early during drug discovery.
Significance Statement.

Using [nitrile-14C]BIIB104, non-intuitive metabolites of BIIB104 were discovered involving a novel cyanide release from the 2-cyanothiophene motif via a postulated epoxidation-initiated thiophene-opening. This unusual biotransformation serves as a lesson regarding placement of the radioactive label on an aryl nitrile when material will be used for evaluating the metabolism of a new drug candidate.
Introduction

Drug metabolite identification is essential to small-molecule drug discovery and development to understand metabolic clearance mechanisms and their enzymology, guide drug design in discovery, and ensure the effectiveness and safety of candidate drugs (Schadt et al., 2018; Shanu-Wilson et al., 2020; Stepan et al., 2013). Advancements over recent decades in liquid chromatography – high-resolution mass spectrometry (LC-HRMS) and associated computer algorithms have made LC-HRMS-based metabolite identification highly efficient for most cases (Castro-Perez and Prakash, 2020; Ma and Chowdhury, 2011; Schadt et al., 2018). Consequently, radiolabeled drug-metabolism studies (with $^{14}$C or $^3$H) are now backloaded in drug development, with human radiolabeled absorption, distribution, metabolism and excretion (ADME) studies often occurring in parallel to Phase II or III studies (Schadt et al., 2018). However, radiolabeled studies are necessary to obtain comprehensive, reliable, and direct quantitative information on candidate drugs' ADME (Beaumont et al., 2014; Isin et al., 2012; Kingston et al., 2021). Often, radiolabeled studies reveal “new” metabolites that were not discovered previously with non-radiolabeled studies (Katyayan et al., 2020; Surapaneni et al., 2021; Takahashi et al., 2017). In a draft guidance for industry (FDA, 2022), the US Food and Drug Administration (FDA) recommended that human mass balance studies should be conducted at the latest before initiating any late-phase clinical trials. However, the FDA draft guidance does not cover animal mass balance studies.

BIIB104 (formerly PF-04958242) is an $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) potentiator that has been studied as a potential treatment for cognitive impairment associated with schizophrenia (Coppell et al., 2022; Ranganathan et al., 2017; Shaffer et al., 2015). To support BIIB104 clinical development, radiolabeled metabolite profiling and identification were conducted during its Phase II trial in rat, dog, and human liver microsomes (RLM, DLM and HLM) and in Wistar rat, a nonclinical toxicology species. Preliminary in vitro metabolism studies with non-radiolabeled BIIB104 in RLM, DLM and HLM did not suggest unusual metabolites, hence straightforward outcomes were anticipated for the radiolabeled mass-balance studies in rat. However, the total radioactivity recovery in urine and feces over 7 days following an oral dose of [nitrile-$^{14}$C]BIIB104 was incomplete in rats. Furthermore, exceptionally long blood and plasma total radioactivity half-lives were observed in rats (ca. 80 h), which starkly contrasted the BIIB104 plasma half-life of <1.7 h in rats determined previously in pharmacokinetic and toxicokinetic studies (unpublished internal Biogen data). To address the disconnect in pharmacokinetics between BIIB104-derived total radioactivity and BIIB104, urine and bile collected from bile duct-cannulated (BDC) rats, and plasma collected from intact rats, underwent radiochromatographic metabolite profiling. Additionally, metabolite profiling and identification of [nitrile-$^{14}$C]BIIB104 incubations in RLM, DLM, and HLM were performed to determine any species differences.
Metabolite analyses revealed that the nitrile carbon-placed radiolabel in $[^{14}\text{C}]$BIIB104 was metabolically lost as $[^{14}\text{C}]$cyanide, which was detoxified to $[^{14}\text{C}]$thiocyanate in rat in vivo.

Biotransformation-mediated cyanide release from small-molecule drugs has been previously reported for: a 2-cyanopyridine moiety (Takahashi et al., 2017), a 2-cyanopyrazin-6-one moiety (Lin et al., 2005), aliphatic nitriles with a hydroxylation-prone adjacent aliphatic carbon (Le et al., 2016; Zheng et al., 2018), and, notably, sodium nitroprusside (Hall and Guest, 1992). Cyanide occurs naturally as cyanogenic glycosides in many plants (Buonvino et al., 2022; Gleadow and Moller, 2014). The primary route for detoxification of cyanide in mammals is biotransformation to thiocyanate mediated by the mitochondrial enzyme rhodanese (also called thiosulfate sulfurtransferase) utilizing endogenous sulfur donors such as thiosulfate ($\text{S}_2\text{O}_3^{2-}$) (Buonvino et al., 2022; Isom et al., 2016). The level of rhodanese is higher in animals which digest a greater quantity of dietary cyanide, thus herbivores have higher levels of rhodanese than carnivores (Buonvino et al., 2022). In this article, we will share our thought processes in identifying the initially unexpected metabolites of BIIB104, discuss caveats of the approaches taken or adopted for the identification of cyanide and thiocyanate, and consider takeaway messages regarding drug-metabolism studies on nitrile-containing compounds exemplified by this case.
Materials and Methods

Materials

Non-radiolabeled BIIB104 was made by Pfizer Inc. (Groton, CT). The $^1$H and $^{13}$C NMR data of BIIB104 are available in Supplemental Table 1. [nitrile-$^{14}$C]BIIB104 (specific activity 58.6 mCi/mmol, radiochemical purity >99%, chemical purity by UV >96%) was supplied as a solution of 0.69 mCi/ml in acetonitrile by ViTrax (Placentia, CA). Radiolabeled reference standards K$^{14}$CN (100 µCi/ml in methanol, specific activity 56.0 mCi/mmol, >95% purity) and KS$^{14}$CN (100 µCi/ml in MeOH, specific activity 58.0 mCi/mmol, >98% purity) were also acquired from ViTrax. Bromobimane, Ellman's reagent, KCN, NADPH, 2,3-naphthalenedicarbaldehyde, and taurine were obtained from Sigma-Aldrich Inc. (St. Louis, MO). KSCN was obtained from Strem Chemicals (Newburyport, MA) and KS$^{13}$C$^{15}$N was obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). All solvents were LC-MS grade.

Liver microsomes for metabolite profiling presented in this article were bought from Sekisui XenoTech LLC (Kansas City, KS), including RLM (male Wistar, product number R3000), DLM (male beagle, product number D1000) and mixed gender HLM (product number H2610). The HLM (product number HL-Mix-102) for biosynthesis of metabolites M1 was obtained from Gentest (now part of Discovery Life Sciences, Huntsville, AL). RLMs for biosynthesis of metabolites M2 twice were obtained from BioIVT (Westbury, NY) and from Sekisui XenoTech, respectively.

Generating In Vitro Metabolites in Liver Microsomes

RLM, DLM, and HLM Incubations of [nitrile-$^{14}$C]BIIB104. The incubation reaction mixtures in a volume of 2 ml contained a final concentration of 10 µM [nitrile-$^{14}$C]BIIB104 (specific activity 58.6 mCi/mmol), 0.1 M potassium phosphate buffer pH 7.4, 3.3 mM MgCl$_2$, 1 mg protein/ml liver microsomes, 2 mM NADPH. The incubation reaction was performed at 37 °C. At 0, 30 and 60 min, respectively, an aliquot (500 µl) the reaction mixture was quenched with an equal volume of ice-cold acetonitrile, vortex-mixed and then centrifuged (17,000x g for 10 min) under 4 °C to precipitate microsomal proteins. An aliquot (15 µl) of clear supernatant was injected from the autosampler chilled at 8 °C for radiochromatography or LC-HRMS and MS/MS analysis.

Biosynthesis of metabolites M1 and M2 from non-radiolabeled BIIB104. To prepare M1, BIIB104 (0.01 mM), pooled HLM (1 mg/mL), MgCl$_2$ (3.3 mM), and NADPH (1.3 mM) in 50 ml 0.1 M potassium phosphate buffer pH 7.4 was incubated for 1 h at 37 °C. HCl (1 M, 5 mL) was added and the resulting mixture (at pH~3) was
extracted with methyl t-butyl ether (2 × 15 mL), and the organic fraction was evaporated in vacuo. The residue was reconstituted in acetonitrile (2.5 mL), sonicated, and 47.5 mL water containing 0.1% formic acid was added. The material was spun in a centrifuge at 40,000× g and the supernatant was applied to a Varian Polaris C18 column (4.6 x 250 mm; 5 µ) at 0.8 mL/min using a Jasco pump. The column was transferred to an HPLC-UV-MS system and the gradient described later in LC Method 1. The eluent was collected into 20 s fractions. Fractions containing M1 were pooled, the liquid removed in vacuo and the residue subjected to NMR analysis. To prepare metabolite M2, an identical procedure was followed, except that 0.6 mg/mL Wistar rat liver microsomes were used instead of human liver microsomes. Also, the supernatant obtained after the 40,000× g spin was further clarified by passing the material through a 0.45 µm filter prior to loading onto the HPLC column.

To generate an additional sample of M2 with enough material for two-dimensional HMBC NMR, BIIB104 (20 µM) was incubated in 6 x 10 mL 100 mM potassium phosphate buffer pH 7.4 containing 5 mM MgCl, 2 mM NAPDPH, and rat liver microsomes (final concentration 1 mg/ml protein) for 1.5 hours at 37°C. Proteins were precipitated by an addition of 10 mL cold acetonitrile followed by centrifugation. The supernatant was concentrated to approximately 6 mL using a vacuum evaporator and a preliminary cleanup was performed using solid phase extraction. A Sep-Pak C18 Plus Light Cartridge (Waters, Milford, MA) was conditioned/equilibrated with acetonitrile followed by water, then the entire sample was loaded, washed with 1 mL water, and eluted with 1 mL 40% acetonitrile. The eluate was diluted with 0.5 mL water prior to semipreparative LC. Purification was performed on an Agilent 1260 semipreparative LC (Santa Clara, CA) with Waters XBridge BEH OBD C18 column @ 25°C (10 x 150 mm, 5 µm) and a faction collector. Chromatographic parameters include an injection volume of 800 µL, mobile phases of 0.1% formic acid water (A) and acetonitrile (B), from 35 to 55% B over 10 min at a flow rate of 5 mL/min, followed by a column wash of 95% B. The fraction collector was triggered by a UV threshold of 10 mAU @ 320 nm for the M2 peak at 6.1 min. The fractions from multiple batches were pooled and vacuum-evaporated overnight to obtain approximately 130 µg of M2 (as estimated by LC-UV signal against the parent drug standard).

**NMR Spectroscopy for Structural Characterization of Biosynthesized M1 And M2**

M1 sample for all NMR data and the M2 sample for initial 1H NMR data were dissolved in 0.15 mL of DMSO-d6 “100%” (Cambridge Isotope Laboratories, Andover, MA) and placed in a 3 mm NMR tube under a dry argon atmosphere. NMR spectra were recorded on a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA) controlled by Topspin V2.1 and equipped with a 5 mm TCI Cryo probe. One-dimensional 1H spectra were typically recorded using a sweep width of 7500 Hz and a total recycle time of 7 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to
noise. Two-dimensional NMR data of COSY, TOCSY, multiplicity edited HSQC and HMBC were recorded using the standard pulse sequences provided by Bruker. A 1K × 128 (COSY, multiplicity edited HSQC and HMBC) or a 1K × 256 (TOCSY) data matrix was acquired using a minimum of 16 scans and 16 dummy scans with a spectral width of 9500 Hz in the f2 dimension. The data was zero-filled to a size of 1K × 1K. One-dimensional NOE experiments were performed with a cycle time of 7.5 s and a mixing time of 0.6 s. A mixing time of 80 ms was used in the TOCSY experiments. All spectra were referenced using residual DMSO (δ=2.50 ppm relative to TMS δ=0.00).

Resynthesized M2 sample of a larger amount for $^{1}\text{H}-^{13}\text{C}$ two-dimensional NMR was dissolved in 0.2 mL of DMSO-d6 and placed in a 3 mm NMR tube. The NMR data including HSQC and HMBC were acquired on a Bruker AVNACE NEO 600MHz NMR spectrometer equipped with a 5 mm QCI Cryoprobe at 298K. The spectrometer is operating at 600.18 MHz and is installed with Topspin 4.1.1 (Bruker Biospin Corporation). Water suppression was achieved with excitation sculpting included in the Bruker supplied pulse sequence zgesgp.

**Rat In Vivo Sampling and Sample Preparation**

Rat Mass balance study was conducted at Charles River Laboratories (Ashland LLC., Ashland, OH). A single oral dose of [nitrile-$^{14}\text{C}]$BIIB104 (3 mg/kg, approximately 200 µCi/kg) was given to three groups of male and female Wistar rats, including a group of intact rats for plasma exposure of drug-derived radioactivity up to 48 h postdose, a group of intact rats for excreted radioactivity in urine and feces up to 168 h postdose, and a group of BDC rats for excreted radioactivity in bile, urine, and feces over 48 h postdose. Aliquots of known size of each plasma, bile, urine, cage rinse, and cage wash samples were mixed with appropriate volume of scintillation cocktail and analyzed by liquid scintillation counting (LSC). Aliquots of whole blood, fecal homogenate (homogenized with deionized water), and carcass (as necessary) were processed and oxidized. The $^{14}\text{CO}_2$ liberated during combustion was trapped in a suitable medium and analyzed by LSC.

**Urine and bile sample preparation.** Rat urine or bile collected from the group of BDC animals in the rat mass balance study were pooled based on sex and proportionally to original sample collection weights of individual time intervals, resulting in one representative pooled urine sample or pooled bile sample for the males and females, respectively. Aliquots of 1 ml pooled urine were mixed with 0.2 ml of cold acetonitrile and then centrifuged at 3220x g for 10 min. Aliquots of 1 ml pooled bile were mixed with cold acetonitrile of equal volume to precipitate proteins and then centrifuged at 3220x g for 10 min. The clear supernatant of the urine and the bile samples were transferred to autosampler vials, respectively. An aliquot of 40 µl was injected from the autosampler chilled at 8 °C for radiochromatography or an aliquot of 10 µl was injected for LC- HRMS and MS/MS analysis.
Plasma sample preparation. Rat plasma samples collected from the group of rats for the plasma exposure of administered radioactivity in the rat mass balance study were pooled based on sex using Hamilton method (Hamilton et al., 1981; Hop et al., 1998), resulting in one pooled sample each for male and female rats respectively, representing the average concentration of area under the curve from 0 to 48 h postdose.

Proteins in 475 µl pooled plasma samples were precipitated by mixing with cold acetonitrile of 3 times of the plasma volume. The plasma sample mixtures were vortexed and then centrifuged at 3220 g for 10 min. The supernatant was dried down under nitrogen gas until an approximate volume of 150 µl and then approximately 15 µl of acetonitrile was added. The samples were sonicated, and the clear solution was transferred to autosampler vials for the analysis by radiochromatography and LC-HRMS and MS/MS.

Chemical Derivatizations

Cyanide in RLM. The reaction was adapted from the literature (Bhandari et al., 2014a; Lacroix et al., 2011). Stock solutions of 5 mM taurine and 4 mM 2,3-naphthalenedicarboxaldehyde were respectively prepared in 0.1 M potassium phosphate buffer (pH 8.0). Then, 50 µl of taurine and 50 µl of 2,3-naphthalenedicarboxaldehyde were added to 250 µl of the clear supernatant of RLM incubation sample. After reaction under darkness for 10 min at 4 °C, an aliquot of 15 µl clear reaction mixture was injected from the autosampler chilled at 8 °C for LC-HRMS and MS/MS analysis.

Thiocyanate in rat urine. The reaction was adapted from the literature (Bhandari et al., 2014a). A solution of 10 mM Ellman’s reagent was prepared in 10 mM potassium phosphate buffer (pH 7.0) and a solution of 4 mM bromobimane was prepared in 0.1 M potassium phosphate buffer (pH 8.0). An aliquot of 100 µL of 10 mM Ellman’s reagent was added into 200 µl of the prepared supernatant of pooled rat urine sample and the mixture was vortex-mixed for 1 min. Then 100 µL of 4 mM bromobimane was added into the above solution and the mixture was heated on a block heater at 70 °C for 15 min. The reaction solution was centrifuged for 10 min at 17000x g and the supernatant was transferred into autosampler vials. An aliquot of 50 µl supernatant was injected from the autosampler chilled at 8 °C for LC-HRMS and MS/MS analysis.

Metabolite Profiling and Identification Using Radiochromatography and LC-UV-HRMS and MS/MS.

The analytical system consists of an Acquity UPLC system equipped with a photodiode array UV detector (Waters), a β-RAMS radio flow detector (Lablogic systems, Inc., Tampa, FL) or a MFx fraction collector (Trajan...
Scientific and Medical, Austin TX) followed by off-line radioactivity counting, and a TripleTOF 6600 mass spectrometer (AB Sciex LLC, Framingham, MA). A few LC methods were used in this study.

LC Method 1: A Varian Polaris C18, 5 µm, 4.6 mm x 250 mm (Agilent) was used at room temperature. Mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at the flow rate of 0.8 ml/min. Linear gradients started at 5% B and held for the first 5 min, then to 25% B at 10 min, to 70% B at 60 min, to 95% B at 61 min and held until 64 min, back to 5% B at 65 min for equilibration.

LC Method 2: An Acquity UPLC HSS T3, 1.8 µm, 2.1 mm x 50 mm (Waters) was used at 50 °C, with the same mobile phase as Method 1 at the flow rate of 0.45 ml/min. Linear gradients started at 5% B for 0.2 min, then to 50% B at 9 min, to 95% B at 10 min and held until 11 min, then back to 5% B for equilibration.

LC Method 3: A xBridge® C18, 3.5 µM, 3 mm x 150 mm (Waters) was used at 50 °C. Mobile phase consisted of 10 mM ammonium formate pH 9 (A) and acetonitrile (B) at the flow rate of 0.80 ml/min. Linear gradients started at 5% B and held for the first 5 min, then to 30% B at 30 min, to 95% B at 34 min and held until 39 min, back to 5% B at 40 min for equilibration.

The β-RAM5 operation conditions include a flow cell 250 µl, the FlowLogic U (Lablogic Systems, Inc.) scintillant flow rate of 1.6 ml/min, and “active counting mode”. Laura 4 (Lablogic Systems, Inc.) was used to process radiochromatographic data that were acquired with β-RAM5 radio flow detector. The LC fractions (7 s each) of rat plasma samples were collected into LumaPlate-96 “DeepWell” (holding up to 300 µl) white opaque 96-well microplates with solid scintillant coated on the bottom (PerkinElmer, Waltham, MA), using the MFx collector with a “dynamic flow reservoir” of 250 µl. The LumaPlate-96 microplates with collected fractions were completely dried down in fume hood overnight. The radioactivity in the LumaPlate-96 microplates was counted using a MicroBeta2 Microplate Counter 2450 equipped with 12 detectors (PerkinElmer). The counting time was 10 min. Microsoft Excel (Microsoft, Redmond, WA) was used to construct radiochromatograms of plasma samples from the off-line countered radioactivity data.

Negative electrospray ionization (ESI) was adopted in all LC-MS and MS/MS experiments in this study. The mass spectrometer was operated in “information dependent acquisition” mode scanning from m/z 50 – 1000 for MS scans, and m/z 50-1000 for MS/MS scans with a maximum of 4 precursors chosen per MS scan. On one occasion, the MS was scanned from as low as m/z 20, so to test whether CN⁻ can be detected in negative ion LC-MS. The mass spectrometer operation parameters included an ion spray voltage of -4500 V, the heater gas temperature of 500 °C, declustering potential of 80 V, collision energy of 10 eV for survey scan and 40 eV for
“information-dependent acquisition”. The collision gas was nitrogen. PeakView version 2.1 and MetabolitePilot 2.0 (AB Sciex) were used to process LC-MS and MS/MS data.

Radioactivity recovery was determined for acetonitrile extracts of liver microsomal incubation samples and pooled rat plasma samples. Supernatant aliquots were mixed with an appropriate volume of scintillation cocktail and analyzed by LSC. The radioactivity recovery was calculated by comparing with aliquots of respective samples without adding acetonitrile.
Results

In Vitro Metabolism of [Nitrile-\textsuperscript{14}C]BIIB104 in RLM, DLM, and HLM

The sample preparation of protein precipitation using acetonitrile was efficient to recover [nitrile-\textsuperscript{14}C]BIIB104-derived radioactivity from liver microsomal incubation mixtures to respective supernatant for metabolite profiling and identification. Extraction recoveries for all samples were 97% or better.

Figure 1. shows radiochromatographic metabolite profiles of [nitrile-\textsuperscript{14}C]BIIB104 in RLM, DLM and HLM, respectively (panels A, B and C). Also provided are LC-HRMS extracted ion chromatograms of BIIB104 and the metabolites (panels D, E, F of Fig. 1), including non-radiolabeled metabolites M8a/b and M9 identified in RLM. A schematic overview of cross-species biotransformation of [nitrile-\textsuperscript{14}C]BIIB104 in liver microsomes is proposed in Scheme 1. Tetrahydrofuran hydroxylation (M1) was the major metabolism observed in DLM and HLM.

Thiophene hydroxylation (M2) was a major metabolite in RLM. Phenoxy O-dealkylation (M3) occurred in all 3 species as a minor metabolic pathway. Collision-induced dissociation (CID) product ion spectra of M1 and M2 obtained in LC-MS/MS experiment cannot discern the exact site of hydroxylation, e.g., whether hydroxylation of M2 is on the thiophene or phenyl moiety (Supplemental Fig. 1). Metabolites M1 and M2 were biosynthesized from non-radiolabeled BIIB104 using HLM and RLM, respectively, then isolated and subjected to NMR analysis.

The \textsuperscript{1}H spectrum of M1 indicates the thiophene, phenoxy and isopropyl resonances are present and only slightly changed in chemical shift (Fig. 2A). Additionally, the \textsuperscript{1}H spectrum indicates the M1 isolate is a mixture of two closely related compounds. The resonances of H2'/H6' (inset of Fig. 2A) clearly demonstrate two resonances with the same coupling pattern (doublets) and constants (\textit{J}=8.5 Hz) at approximately a ratio of 1:0.7, which is consistent with the diastereomer epimerization. The existence of a pair of diastereomers is also supported by the data from the COSY and TOCSY experiments (data not shown). The multiplicity edited HSQC spectrum (not shown) contains a single methylene resonance (one less than BIIB104) and four methine resonances (one more than BIIB104) between 5.5 and 3.0 ppm. The methine \textsuperscript{1}H resonance at 5.2 ppm is correlated with a \textsuperscript{13}C resonance at 99.0 ppm which is indicative of an aliphatic methine with two attached oxygen atoms (HSQC spectrum not shown). All these data indicate either C2” or C5” of BIIB014 has been hydroxylated. One-dimensional NOE experiment was performed by excitation of the 5.2 ppm resonance, which resulted in the enhancement of the resonances at 7.07 and 4.51 ppm (Fig. 2B). The enhancement of the 7.07 ppm resonance strongly suggests the hydroxylation is at C5”.

The \textsuperscript{1}H spectrum of M2 indicates the amino tetrahydrofuran, isopropyl and phenoxy of BIIB104 are present and unmodified (Supplemental Fig. 2). Furthermore, the thiophene two spin system (\textit{\delta} 7.57 and 7.95 ppm, \textit{J}=3.9
Hz) of doublets observed in the $^1$H spectrum of BIIB104 (Supplemental Table 1) is absent in the spectrum of M2. The $^1$H spectrum of M2 also contains a new singlet at $\delta$ 6.29 ppm, which integrates to a single proton. All these data are consistent with the hydroxylation of either C3 or C4 of the thiophene.

To differentiate C3 and C4, M2 was biosynthesized again to obtain a large amount of sample for the acquisition of HMBC spectrum. HSQC spectrum of M2 shows only three types of aromatic carbons having proton attached (Fig. 3 and Supplemental Fig. 3, red signals), consistent with the disappearance of a thiophene proton from BIIB104. Not all anticipated multiple bond $^1$H-$^13$C correlations were detected in HMBC, due to still the limited amount of material and perhaps interference with water in the sample. For example, none of HMBC correlations from H3’ and H4” of the tetrahydrofuran moiety were detected (Fig. 3). Fortunately, diagnostic correlations to C5 from phenyl H3’/5’ and thiophene H4 were observed in HMBC (Fig. 3). H4-C4 of M2 resulted from 3-hydroxy was not assigned by HSQC alone, because observed carbon shift (119.9 ppm) of the remaining thiophene C-H cannot discern 3-hydroxy and 4-hydroxy (Table 1, C4 of 3-hydroxy and C3 of 4-hydroxy, respectively). Rather, 3-hydroxy was determined by the observed chemical shift of C5 (144.4 ppm) which is in good agreement with predicted chemical shift of C5 for 3-hydroxy at thiophene by two types of commonly used NMR predictor software (Table 1): ACD/Spectrus Processor 2018 (version S80541, build 103775, ACD/Labs, Toronto, ON, Canada) and MestReNova (v14.3.0-30573, Mestrelab Research S. L., Escondido, CA). If M2 had been 4-hydroxy at the thiophene, predicted chemical shift of C5 would be lower by as much as 20 ppm (Table 1). It has been previously reported that comparing observed carbon chemical shifts with predicted carbon chemical shifts of putative structures can be an effective approach for structural elucidation when $^1$H NMR information is limited at the chemical structure of biotransformation (Gu et al., 2015).

M13 of [nitrile-$^{14}$C]BIIB104 was detected by radiochromatography, but not by MS (even when scanning from m/z 20 in negative ion mode). M13 was identified and verified by two experiments. First, M13 was identified using a cyanide-derivatization method (Bhandari et al., 2014a; Lacroix et al., 2011; Lin et al., 2005; Sano et al., 1992) that converts cyanide to an organic anion compatible to LC-MS analysis. After the derivatization reaction with clear supernatant of a RLM-incubated $[^{14}$C]BIIB104 sample, a new radiochromatographic peak arose and the M13 peak was diminished (Supplemental Fig. 4); the M13-derived product had the same retention time and fragmentation pattern as the KCN-derived product (i.e. positive control, Fig. 4, panel A vs. B). Second, the identity of M13 was further confirmed by the identical radiochromatographic retention time with commercially obtained K$^{14}$CN (Supplemental Fig. 5). The chromatography of $[^{14}$C]cyanide was not optimal, as peak splitting was observed for both K$^{14}$CN and M13 (Supplemental, Fig. 5 and Fig. 1A).
Non-radiolabeled metabolites M8a/b and M9 were structurally characterized by LC-MS/MS CID spectra showing the accurate mass of fragment ions (Fig. 5). Neutral losses of CO₂ in the spectra indicated a carboxylic acid group in both M8a and M9 (Fig 4). All major fragment ions of M8a and M9 can be readily interpreted with proposed structures. M8b resembled that of M8a, although differing in ratios of fragment ions (Supplemental Table 2). M8a and M8b are proposed to be cis- and trans-carboxyacryloyl isomers (i.e. Z and E; indicated by cross double bond in Scheme 1). The same type of rearranged fragmentation of [M-H]⁻ ions was observed between M8a/M9 and BIIB104, producing rearranged fragment ions m/z 216 and 172 for M8a, and m/z 174 for M9 (Fig. 5 and Supplemental Fig. 6). Chromatographic peak splitting observed for M8a/b and M9 (Fig. 1D) was likely caused by ionized carboxylic acids in the injected supernatant sample with high organic solvent (50% acetonitrile) at neutral pH, versus the LC mobile phase starting with low organic solvent at a low pH (5% acetonitrile with 0.1% formic acid).

The [¹⁴C]BIIB104 material used for this study was highly pure. Moreover, non-radiolabeled metabolites M8 and M9 were absent from both [¹⁴C]BIIB104 and nonradiolabeled BIIB104. Thereby, all 9 metabolites shown in Figure 1 and Scheme 1 were formed by LMs.

**Rat In Vivo Metabolism of [Nitrile-¹⁴C]BIIB104**

Mass balance study with [nitrile-¹⁴C]BIIB104 (single oral dose of 3 mg/kg, approximately 200 µCi/kg) was performed in male and female Wistar rats. In intact rats, a similar excretion pattern was observed in both sexes (Supplemental Fig. 7A). Using sex-combined data, 36.7% (urine) and 39.1% (feces) of administered radioactivity was recovered over 168 hours postdose. In BDC rats, a similar excretion pattern was also observed between genders (Supplemental Fig 7B). Using sex-combined data, 67.1% of administered radioactivity was recovered over 48 hours postdose in urine (10.2%), bile (51.1%), and feces (5.8%). The majority (61.3%) of the administered dose was eliminated in bile and urine within 48 h postdose vs. the feces (5.8%), suggesting good oral absorption of [nitrile-¹⁴C]BIIB104.

The terminal half-lives of blood and plasma radioactivity were too long to be determined in the mass balance study with blood and plasma sampling from the Wistar rats only up to 48 h. However, the elimination of total radioactivity from blood and plasma for Long Evans male rats was determined in a separate study for quantitative whole-body autoradiography which lasted for a total of 28 days, with an estimated terminal half-life of 81.4 and 76.6 h in the blood and plasma, respectively (Supplemental Fig. 8).
Figure 6 displays radiochromatographic metabolite profiles in accumulative-pooled 0 to 48 h urine and bile samples of male BDC rats, as well as AUC-pooled 0 to 48 h plasma sample of male intact rats, receiving an oral dose of [nitrile-14C]BIIB104. Table 2 lists excreted metabolites via bile and urine of both male and female BDC rats over 48 h postdose, as percentages of administered radioactivity, respectively. BIIB104 was extensively metabolized in rat, with <0.1% of unchanged [nitrile-14C]BIIB104 in the pooled bile or urine. Conjugate metabolites M15, M16, M17, and M18 (Scheme 2) were identified by LC-HRMS and MS/MS (Supplemental Table 2). The thiophene hydroxylation (M2) was a major metabolite in RLM but was only a minor metabolite in rat bile and urine. Metabolites M15, M17 and M18 are likely downstream metabolites of M2, including the major glucuronidation metabolite M15 in rat bile (Scheme 2). The O-dealkylation (M3) was a minor metabolite in rat liver microsomes but was not detected in rat in vivo samples, as M3 has presumably undergone further metabolisms, e.g., sulfation to M16 (Scheme 2).

M14 eluting at the solvent front in LC Method 2 (Fig. 6) was retainable with an amino column under normal-phase LC conditions (Supplemental Fig. 9). An LC-HRMS extracted ion chromatogram of the S14CN- exact mass suggested that M14 could be thiocyanate (Supplemental Fig. 9B). However, normal-phase LC is not suitable for the analysis of thiocyanate, as the injection carryover and LC peak distortion were confirmed with KSCN and KS13C15N standards in the normal-phase LC with high organic content mobile phase (data not shown, worse in acetonitrile than in methanol). Endogenous thiocyanate at a much higher level than [14C]BIIB104-derived thiocyanate also interfered with the M14 identification (Supplemental Fig. 9, panels C vs. B). Furthermore, thiocyanate shows only weak signal in LC-MS due to poor ionization efficiency in the electrospray. Hence, a reported chemical derivatization method for thiocyanate (Bhandari et al., 2014a) was adopted in this study.

Figure 7A shows that after derivatization reaction of bromobimane with a rat urine sample, a new radiochromatographic peak for the expected product occurred (~26% region of interest, ROI) and the M14 peak was diminished (~57% ROI; reduced from ~80% ROI before derivatization). LC-MS spectra of derivation products indicated that [nitrile-14C]BIIB104-derived thiocyanate was indeed at a much lower level than endogenous thiocyanate in rat urine (Fig 7B vs 7C). The resolution of TOF mass spectrometry instrument used for this study was approximately 12,000 in the mass range around m/z 250, as measured by peak width at half height. The resolution is not high enough to separate the S14CN derivative (exact mass = m/z 250.0532) from the M+2 isotope peak of the SCN derivative (exact mass = m/z 250.0479), i.e. the composite isotope peak of naturally occurring isotopes (including one 34S, two 13C, one 18O, or 13C + 15N, etc.) as calculated at the resolution of 12,000 by isotopic distribution calculator within PeakView software (AB Sciex). If a mass spectrometer with very high
resolution of 500,000 were to be used, the $^{14}$CN derivative of bromobimane would be almost baseline-separated from M+2 natural isotopes of the SCN derivative in mass spectra.

Nevertheless, slightly higher relative abundance of m/z 250 in the [nitrile-$^{14}$C]BIIB104-dosed rat urine than the vehicle control observed in Fig. 7B vs 7C suggested that $^{14}$CN$^-$ was at approximately 3% level of all SCN$^-$ at m/z 248, while relative abundances of M+1 isotopic ion (m/z 249) were identical between the [nitrile-$^{14}$C]BIIB104-dosed and vehicle control rat urine (Fig. 7B vs 7C). Since the ratio of [nitrile-$^{14}$C]BIIB104 : unlabeled BIIB104 in the dosing formulation to rats was close to 1:1, the $^{14}$CBIIB104-derived thiocyanate metabolite (M14) is estimated to account for approximately 6% of total thiocyanate present in the rat urine of 0 to 48 h postdose. Finally, the identity of M14 in rat urine and bile was further verified by matching its retention time with commercially obtained KS$^{14}$CN standard (LC Method 1; Supplemental Fig. 10). It is noteworthy that bromobimane ionizes well in positive ESI. During this study, negative ESI was chosen as default ionization mode for being suitable to analyze the parent drug and metabolites. Nevertheless, it is interesting that chemical structure of bromobimane and the SCN derivative allows ionization in negative ESI adopted from the literature (Bhandari et al., 2014a), i.e., by deprotonation from a carbon, of which the negative charge is presumably stabilized by resonant structures.

A similar metabolite profile was observed between male and female BDC rats in pooled 0 to 48 h bile or urine (Table 2). Combining data between sexes listed in Table 2, $^{14}$Cthiocyanate accounted for approximately 53% of the total radioactivity excreted in bile and urine over 48 h and comprised approximately 31% of the administered radioactive dose (Table 2).

In pooled 0 to 48 h plasma of intact rats, $^{14}$Cthiocyanate accounted for virtually all plasma radioactivity in male rats (Fig. 6C) and female rats (not shown). A sample recovery test indicated that the plasma radioactivity was completely recovered into supernatant after protein precipitation by acetonitrile. The recovery of radioactivity from rat plasma to the supernatant was determined to be 100% and the residual radioactivity in the protein pellets of rat plasma were only 0.3%.
Discussion

The $^{14}$C-radiolabel of a candidate drug for ADME studies needs to be on a carbon atom that is stable from drug metabolism and relatively easy to synthesize. [Nitrile-$^{14}$C]BIIB104 was made by following a previously proven easy synthetic route. Unexpectedly, the nitrile radiolabel uncovered nonintuitive metabolites of BIIB104 arising from loss of cyanide from the thiophene substituent.

The highly polar radiochromatographic nature of M14 (Fig. 6) hinted that it could be $^{14}$C-thiocyanate upon detoxification of $^{14}$C-cyanide that was somehow released from [nitrile-$^{14}$C]BIIB104. However, the path of identifying $^{14}$C-thiocyanate in vivo and $^{14}$C-cyanide in vitro was not straightforward. Cyanide is not detectable by LC-MS, and thiocyanate has a very weak signal in LC-MS. A thiocyanate metabolite without radiolabel in a non-radiolabeled study would be difficult to discern relative to endogenous thiocyanate, especially when the drug dose is low as it is for BIIB104 (Takahashi et al., 2017; Zheng et al., 2018). Bhandari et al. have previously reported simultaneous analysis of cyanide and thiocyanate in swine plasma samples by chemical ionization gas chromatography-mass spectrometry (GC-MS) following derivation with pentafluorobenzyl bromide (Bhandari et al., 2014b; Bhandari et al., 2012). But nowadays, GC-MS has become rare in biopharmaceutical companies and the limited GC-MS resource may not be accessible for the analysis of radiolabeled samples, since LC-MS has dominated mass spectrometric analysis across the industry.

$^{14}$C-cyanide (M13) was hypothesized to be released from [nitrile-$^{14}$C]BIIB104 upon oxidative ring-opening of 2-cyanothiophene and subsequent hydrolysis of the acyl-cyanide intermediate (Scheme 3), which was supported by the identification of non-radiolabeled counterpart metabolites M8a/b and M9 in RLM. The release of $^{14}$C-cyanide from 2-cyanothiophene was not a one-step substitution as for a previously reported case of a 2-cyanopyridine moiety (Takahashi et al., 2017). The mechanistic difference between our present case of 2-cyanothiophene and the literature case of 2-cyanopyridine reported by Takahashi et al.(2017) is justifiable, as thiophene is relatively electron rich whereas pyridine is relatively electron deficient. Unsurprisingly, our initial search for counterpart non-radiolabeled metabolites having a 2-hydroxythiophene moiety was futile. To consider a likely oxidative thiophene-opening mechanism, data mining was conducted to search for thiophene-opened non-radiolabeled metabolites of $^{14}$C]BIIB104 in RLM by looking for UV-chromatographic peaks at wavelengths lower than the maximum absorption wavelength of BIIB104 from the raw data acquired with a photodiode array detector. In BIIB104, the 2-cyanothiophene and phenoxy moieties are in the same plane according to X-ray crystal structure (Shaffer et al., 2015). Thus, π-conjugation between the two aromatic systems is probably a major factor contributing to the maximum absorption around 310 nm. Oxidative thiophene-opening metabolism would result in the loss of the 2-cyanothiophene chromophore and its π-conjugation to the
phenoxy chromophore (Scheme 3). From data mining, UV-chromatographic peaks M8a/b and M9 were found in the RLM sample (Supplemental Fig. 11). M9 is not visible in the chromatogram at maximum absorption wavelengths of BIIB104, whereas M8a is observed (Supplemental Fig. 11A). Likely π-conjugation of carboxyacryloyl with phenoxy in M8a may explain its UV absorption at a higher wavelength than M9.

The finding of UV-chromatographic peaks (Supplemental Fig. 11) guided the identification of M8a/b and M9 by LC-HRMS and MS/MS. The occurrence of rearranged fragment ions for M8 and M9 (Fig. 5), as well as BIIB104 (Supplemental Fig. 1), can be interpreted by an intramolecular substitution of the phenoxy oxygen by the nitrogen anion formed from their respective [M-H]⁻ ions (Supplemental Fig. 6). This hypothesis was verified by the formation of the m/z 225 ion through further fragmentation of the m/z 285 ion in a MS³ experiment (i.e. MS/MS/MS) of non-radiolabeled BIIB104 using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The similarity in the rearranged fragmentation provided further structural evidence for that non-radiolabeled M8a/b and M9 in RLM were derived from [¹⁴C]BIIB104. Also note that if the cleavage of the sulfonamide bond is not a major fragmentation for [M-H]⁻ ions of a metabolite, this type of arranged fragment ions would not be seen (e.g., M1 and M2 in Supplemental Fig. 1B and 1C, respectively).

After the identification of counterpart non-radiolabeled metabolites M8a/b and M9 in RLM, experiments with M13 and M14 were more confirmatory, including matching LC retention times with commercial standards of K¹⁴CN and K⁵¹⁴CN, respectively, as well as derivatizations converting cyanide (M13) and thiocyanate (M14) to expected products of respective derivatives compatible with LC-MS/MS analysis. Caveats with the derivation methods have been noticed during our experiments. When testing with authentic standard in different blank sample matrices, we have found that the derivatization of cyanide (Bhandari et al., 2014b; Takahashi et al., 2017) is incomplete in liver microsomes, showed even less conversion in plasma, and did not work in urine and bile. This is not surprising, because cyanide derivatization (Supplemental Fig. 12) may be hindered by nucleophiles in biological matrices besides taurine and cyanide during the reaction step with 2,3-naphthalenedicarboxaldehyde and during the nucleophilic addition to the iminium intermediate, respectively. When the literature method for derivatizing cyanide was applied to plasma samples, stable isotope-labeled cyanide (K¹³C¹⁵N or Na¹³C¹⁵N) was used as an internal standard to ensure accurate quantitative analysis (Bhandari et al., 2014a; Lacroix et al., 2011).

The epoxidation proposed for the formation of [¹⁴C]cyanide in Scheme 3 is presumably mediated by cytochrome P450 enzymes. An inhibition experiment using a broad P450 inhibitor 1-aminobenzotriazole (1 mM) together with P450 2C9 selective inhibitor tienilic acid (3 μM) (Linder et al., 2009) has resulted in an approximately 2/3 decrease in the formation of [¹⁴C]cyanide from [nitrile-¹⁴C]BIIB104 in RLM (Supplemental Fig.
The P450 dependence does not contradict to the proposed epoxidation mechanism, although P450 enzymes also mediate other types of oxidations. Furthermore, M2 can be derived from the same 2,3-thiophene-epoxide intermediate (Scheme 3), supporting the epoxidation mechanism. Our proposed mechanism for 2,3-thiophene epoxide leading to M2, as well as to the intermediate for thiophene-opening, somehow resembles that Rademacher et al. previously reported for a hydroxythiophene metabolite via arene oxide formation and subsequent rearrangement (Rademacher et al., 2012).

Metabolic release of [14C]cyanide from 2-cyanothiophene moiety of BIIB104 is a dominant pathway in rat, however, is not a rat only phenomenon. Following a single oral-dose of [nitrile-14C]BIIB104 (0.1 mg/kg, 14.8 μCi/kg) to BDC beagle dogs, [14C]thiocyanate was excreted in dog bile and a mixture of [14C]cyanide and [14C]thiocyanate was excreted in dog urine (unpublished internal Biogen data). The sum of [14C]thiocyanate and [14C]cyanide accounted for approximately 8% of the total radioactivity excreted in bile and urine over 48 h postdose and comprised 2.8% of the administered dose in BDC dogs. The combined percentages of [14C]thiocyanate and [14C]cyanide in dog were lower than those corresponding data of [14C]thiocyanate in rat, indicating that in vivo release of [14C]cyanide from [nitrile-14C]BIIB104 in dog was minor compared to rat. This qualitatively correlates with in vitro difference in [14C]cyanide metabolite between DLM and RLM. However, [14C]thiocyanate in systemic circulation still resulted in exceptionally long half-lives of plasma radioactivity in bile duct-intact dogs (ca. 150 h, unpublished internal Biogen data).

Liver microsomal metabolite profiling indicated that in vitro release of cyanide from [nitrile-14C]BIIB104 was trace in human (Fig. 1). Also, given the very low clinical dose of BIIB104 (≤1 mg, PO daily), the metabolic release of cyanide from BIIB104 is not considered a risk for humans. It is noteworthy that sufficient safety margins over BIIB104 clinical exposures have been previously established by nonclinical toxicology studies in rats and dogs. Nevertheless, the metabolic loss of radiolabel from [nitrile-14C]BIIB104 may hinder a radiolabeled human ADME study, since formed [14C]thiocyanate could still result in an exceedingly long terminal half-life of radioactivity as thiocyanate is endogenous. This may result in an incomplete mass balance or a prolonged time for necessary mass balance recovery leading to extended in-clinic patient stays. Therefore, an alternate radiolabel at a metabolically stable site of BIIB104 is needed to facilitate the human ADME study. This has led to the synthesis of [phenyl-14C]BIIB104, of which the dosed radioactivity to rats was fully excreted over 48 h postdose (unpublished internal Biogen data). Considering this and other examples (Takahashi et al., 2017), it is inadvisable to use nitrile as the site of carbon-14 incorporation when the intended use of the reagent is for in vivo ADME studies, despite the factor that radiosynthesis may be easier for such reagents relative to placing the radionuclide at an alternate position.
This report with a 2-cyanothiophene and the previously reported case with a 2-cyanopyridine (Takahashi et al., 2017) are both examples for the metabolism-mediated release of cyanide from aromatic-nitriles which was once considered unlikely when compared to aliphatic-nitriles in drug design (Fleming et al., 2010). A question may be raised regarding how to spot the release of cyanide from nitrile-containing drug molecules earlier in drug discovery. While the detection of an in vivo thiocyanate metabolite without radiolabel is practically impossible due to relatively high levels of endogenous cyanate, the potential cyanide metabolite of nitrile-containing small molecules may be detected in LC-MS following the derivatization with 2,3-naphthalenedicarboxaldehyde and taurine in liver microsomes.
Acknowledgments

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper including the Supplemental Data.
Authorship Contribution

Participated in research design: Gu, Obach, Shaffer

Conducted experiments: Huang, Muste, Zhong, Walker, Obach

Contributed new reagents or analytic tools: N/A

Performed data analysis: Gu, Huang, Muste, Zhong, Walker

Wrote or contributed to the writing of the manuscript: Gu, Huang, Muste, Zhong, Walker, Obach, Shaffer
References


Footnotes

This work received no external funding.

CG, JH, CM, JZ, and CLS are employee of Biogen and may hold Biogen stock. GSW and RSO are employee of Pfizer and may hold Pfizer stock.
Scheme Legends

**Scheme 1.** Proposed cross-species liver microsomal biotransformation of [nitrile-\(^{14}\)C]BIIB104. The cross double bond of M8a/b indicates a mixture of cis & trans (Z & E) configurations.

**Scheme 2.** Observed metabolites of [nitrile-\(^{14}\)C]BIIB104 in rats

**Scheme 3.** Postulated mechanism for the formation of \(^{14}\)Ccyanide and \(^{14}\)Cthiocyanate from [nitrile-\(^{14}\)C]BIIB104. Additionally, M2 can be derived from the same epoxide intermediate as proposed for metabolic release of \(^{14}\)Ccyanide from 2-cyanothiophene.
Figure Legends

**Fig. 1.** Radiochromatographic metabolite profiles of [nitrile-\(^{14}\text{C}\)] BIIB104 (obtained using LC Method 1) following 0.5 h incubation of in RLM (A), DLM (B) and HLM (C), respectively. LC-HRMS extracted ion chromatograms of the exact mass of [M-H]\(^{-}\) ions of BIIB104 and its metabolites identified in RLM (D), DLM (E), and HLM (F). Both \(^{14}\text{C}\)-labeled and non-radiolabeled [M-H]\(^{-}\) monoisotopic ions are extracted for BIIB104 and the metabolites, except for non-radiolabeled metabolites M8a/b and M9.

**Fig. 2.** \(^1\text{H}\) NMR spectrum of M1 biosynthesized using human liver microsomes (A) and one-dimensional NOE NMR by excitation of the 5.2 ppm resonance (B).

**Fig. 3.** Portion of HSQC and HMBC two-dimensional NMR spectra of M2 biosynthesized using rat liver microsomes, showing diagnostic \(^1\text{H}\)-\(^{13}\text{C}\) correlations from both H3'/5' and H4 to C5 in HMBC. Full spectra are available in Supplemental Data.

**Fig. 4.** Chemical derivatization to convert cyanide to an organic anion. LC-HRMS extracted ion chromatograms (obtained using LC Method 2) and high-resolution MS/MS CID product ion spectra showing the expected reaction product for \(^{14}\text{C}\)-cyanide metabolite M13 in RLM (A) and KCN standard in phosphate buffer (B).

**Fig. 5.** LC - high resolution MS/MS CID spectra of [M-H]\(^{-}\) ions of M8a (A) and M9 (B). Ionizing deprotonation could occur at either the carboxylic acid or the sulfonamide, as marked in red. Any hydrogen transfer across a cleaved bond is not shown in the spectral interpretation.

**Fig. 6.** Radiochromatographic metabolite profiling of [nitrile-\(^{14}\text{C}\)] BIIB104 (obtained using LC Method 3) in pooled 0 to 48 h rat urine (A) and bile (B) collected from male BDC rats, and plasma (C) collected from male intact rats receiving an oral administration of [nitrile-\(^{14}\text{C}\)] BIIB104.

**Fig. 7.** Radiochromatogram (obtained using LC Method 3) after a thiocyanate derivatization reaction for pooled 0 to 48 h urine collected from male rats receiving an oral dose of [nitrile-\(^{14}\text{C}\)] BIIB104 (A). LC-HRMS spectra showing molecular ion region of the derivatization product in pooled 0 to 48 h urine collected from rats receiving an oral dose of [nitrile-\(^{14}\text{C}\)] BIIB104 (B) vs. a rat receiving blank formulation vehicle (C).
TABLE 1. NMR chemical shifts of aromatic carbons in BIIB104 and M2 comparing with predicted shifts of BIIB104, M2 and a hypothetical 4-hydroxy at the thiophene. The diagnostic carbon chemical shift of M2 and pertinent predicted shifts are marked in *italic*.

![BIIB104](image)

<table>
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<tr>
<th>Carbon</th>
<th>BIIB104</th>
<th>M2 (3-hydroxy)</th>
<th>Hypothetical (4-hydroxy)</th>
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<tr>
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<td>129.1</td>
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δObs., Observed chemical shift; δPred., Predicted chemical shift; –, Not detected in HMBC of metabolite sample; ACD/Labs, ACD/Spectrus Processor 2018 Build 103775; MNova, MestReNova v14.3.0-30573.
### Tables 2. Metabolites of [nitrile-\(^{14}\text{C}\)]BIIB104 excreted in rat bile and urine over 48 h post dose

<table>
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<tr>
<th>Component</th>
<th>Metabolism</th>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>M2</td>
<td>hydroxylation at thiophene</td>
<td>–</td>
</tr>
<tr>
<td>M14</td>
<td>thiocyanate</td>
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<td>glucuronidation following hydroxylation</td>
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<td>M16</td>
<td>sulfation following O-dealkylation</td>
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</tr>
<tr>
<td>M17</td>
<td>glucuronidation following dihydroxylation</td>
<td>–</td>
</tr>
<tr>
<td>M18</td>
<td>sulfation following O-dealkylation and hydroxylation</td>
<td>0.2</td>
</tr>
<tr>
<td>BIIB104</td>
<td>parent drug</td>
<td>–</td>
</tr>
<tr>
<td>sum</td>
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\(\sim\), Not detected
Scheme 1

RLM, DLM and HLM denote rat, dog and human liver microsomes respectively.
Scheme 2

- M16
  - Identified in rat liver microsomes
  - Identified in rat liver microsomes

- M14
  - N=C
  - N=C–S

- M17
  - BIIIB104

- M2
  - Gluc

- M15
  - Gluc

* indicates the site of $^{14}$C radiolabel
Figure 1
Figure 2
Figure 3

Note: $^1$H NMR was acquired with water suppression.
Figure 4
Figure 5
Figure 6
* Matrix background ions, not isotopic peak of the SCN or $S^{14}$CN derivative

Bromobimane + SCN$^-$ Thiocyanate $\rightarrow$ [M-H]$^-$, m/z 248.0499

Bromobimane + $S^{14}$CN$^-$ $[^{14}$C] Thiocyanate $\rightarrow$ $^{14}$C [M-H]$^-$, m/z 250.0532