Metabolic Chiral Inversion of Brivanib and Its Relevance to Safety and Pharmacology

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Abbreviations: VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; Brivanib (BMS-540215), (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yloxy)propan-2-ol; LC/MS, liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; ESI, electron-spray ionization; HLM, human liver microsomes; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; NADPH, β-Nicotinamide adenine dinucleotide phosphate-reduced form; NADH, nicotinamide adenine dinucleotide-reduced form; NAD⁺, nicotinamide adenine dinucleotide.
Abstract

Brivanib alaninate is an orally administered alanine prodrug of brivanib, a dual inhibitor of the VEGF and FGF signaling pathways. It is currently in clinical trials for the treatment of hepatocellular carcinoma and colorectal cancer. Brivanib has a single asymmetric center derived from a secondary alcohol. The potential for chiral inversion was investigated in incubations with liver subcellular fractions, and in animals and humans following oral doses of brivanib alaninate. Incubations of [14C]brivanib alaninate with liver microsomes and cytosols from rats, monkeys and humans followed by chiral chromatography resulted in two radioactive peaks, corresponding to brivanib and its enantiomer. The percent of the enantiomeric metabolite relative to brivanib in microsomal and cytosolic incubations of different species in the presence of NADPH ranged from 11.6-15.8% and 0.8-3.1%, respectively. The proposed mechanism of inversion involves the oxidation of brivanib to a ketone metabolite which is subsequently reduced to brivanib and its enantiomer. Following oral doses of brivanib alaninate to rats and monkeys, the enantiomeric metabolite was a prominent drug-related component in plasma, with the percent of AUC 94.7 and 39.7%, respectively, relative to brivanib. In humans, the enantiomeric metabolite was a minor circulating component, with the AUC <3% of brivanib. Pharmacological studies indicated that brivanib and its enantiomer had similar potency toward the inhibition of VEGFR-2 and FGFR-1 kinases. Because of low plasma concentration in humans, the enantiomeric metabolite was not expected to contribute significantly to target-related pharmacology of brivanib. Moreover, adequate exposure in the toxicology species suggested no specific safety concerns with respect to exposure to the enantiomeric metabolite.
Introduction

Brivanib (BMS-540215, (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yloxy)propan-2-ol) is a novel dual inhibitor of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) signaling pathways (Borzilleri et al., 2005; Bhide et al., 2006; Dempke and Zippel, 2010). VEGF and FGF play a critical role in physiology and pathology of angiogenesis (Ferrara et al., 2003; Klebl and Muller, 2005; Tassi and Wellstein, 2006) and are important targets in anti-cancer therapy. Brivanib alaninate, an alanine prodrug of brivanib, is currently under clinical development for the treatment of hepatocellular carcinoma and colorectal cancer (Diaz-Padilla and Siu, 2011; Park et al., 2011). Brivanib possesses a single asymmetric center derived from a secondary alcohol with an R-configuration. Asymmetric secondary alcohols have been shown to undergo chiral inversion to their enantiomeric metabolites through an alcohol-ketone inter-conversion (Zhang et al., 1994; Skálová et al., 1999; Skálová et al., 2003; Itoh et al., 2006; Mannens et al., 2007). The mechanism typically involves an oxidation of the chiral secondary alcohol to the corresponding achiral ketone which is subsequently reduced back to a mixture of enantiomers.

Chiral inversion of drugs varies in extent and significance, and the mechanisms of inversion can be enzymatic or non-enzymatic (Reist et al., 2000; Wsol et al., 2004). As the resulting stereoisomer and the parent drug differ in their three-dimensional structures, their interactions with naturally asymmetric protein targets, metabolic enzymes and transporters could be different, leading to alteration of pharmacokinetic and pharmacodynamic behaviors (Brocks et al., 2004; Hao et al., 2005). Moreover, chiral inversion of a drug candidate following dose administration to humans may raise safety concerns in clinical practice (Brocks et al., 2004; Wsol et al., 2004).

The biotransformation and disposition of brivanib in animals and humans have been characterized previously (Mekhail et al., 2010; Gong et al., 2011). Following oral doses of [14C]brivanib alaninate to rats, monkeys and humans, the radioactive dose was mainly cleared by metabolism and the majority of
drug-derived radioactivity was excreted in feces (Gong et al., 2011). Sample analysis, conducted with achiral LC/MS methods, suggested that brivanib underwent both oxidative metabolism and sulfate conjugation. A ketone metabolite, that can be potentially reduced to brivanib and its enantiomer, was observed in incubations of brivanib with human liver microsomes (HLM) (Gong et al., 2012), and in rats, monkeys and humans following oral doses of brivanib alaninate (Gong et al., 2011). This study describes the characterization of brivanib chiral inversion in incubations with liver subcellular fractions, and its exposure in animals and humans following oral doses of brivanib alaninate. The safety and pharmacology related to the enantiomeric metabolite in humans are also discussed.
Materials and Methods

Chemicals and Reagents. Pooled liver microsomes (20 mg protein/mL) and liver cytosols (10 mg protein/mL) from rats, monkeys and humans were purchased from BD Bioscience (Woburn, MA). Brivanib ((R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yloxy)propan-2-ol), brivanib alaninate ((1R, 2S)-2-aminopropionic acid 2-[4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy]-1-methylethyl ester), stable labeled $^{13}$C$_3$,$^{15}$N$_2$-brivanib and the enantiomeric metabolite (the S-isomer of brivanib) (Figure 1) were supplied by the Department of Chemical Synthesis, Bristol-Myers Squibb Research & Development (Princeton, NJ). $[^{14}$C$]$Brivanib alaninate (13.1 µCi/mg, radiochemical purity 98.5%) was supplied by the radiochemistry group of the Department of Chemical Synthesis, Bristol-Myers Squibb Research & Development (Princeton, NJ). 3′-phosphoadenosine 5′-phosphosulfate (PAPS), β-Nicotinamide adenine dinucleotide phosphate-reduced form (NADPH), nicotinamide adenine dinucleotide-reduced form (NADH), and nicotinamide adenine dinucleotide (NAD$^+$) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Potassium phosphate and formic acid were obtained from EM Science (Gibbstown, NJ). Ecolite™ liquid scintillation cocktail was purchased from MP Biomedicals (Irvine, CA). Type I reagent grade water was prepared with a MilliQplus ultrapure water system (Millipore Corp, Bedford, MA). Acetonitrile and methanol were purchased from Burdick & Jackson Inc (Muskegon, MI). All organic solvents and water were of HPLC grade.

Chiral Inversion of Brivanib in Microsomal and Cytosolic Incubations. $[^{14}$C$]$Brivanib alaninate was used as the substrate in incubations because $[^{14}$C$]$brivanib was not available. During the incubation, $[^{14}$C$]$brivanib was generated in situ through the rapid hydrolysis of $[^{14}$C$]$brivanib alaninate (Marathe et al., 2009). The incubation mixtures consisted of $[^{14}$C$]$brivanib alaninate (2 µM, 11.6×10$^{-3}$ µCi/mL), enzymes (liver microsomes or cytosols from rat, monkey or human, 1.0 mg protein/mL), cofactor (NADPH, NADH or NAD$^+$, 1.0 mM), MgCl$_2$ (0.5 mM) and potassium phosphate buffer (100 mM, pH
7.4) with a final volume of 0.5 mL. Incubations were carried out at 37 °C in a shaking water bath. The mixture was allowed to react for 60 min before being quenched with cold acetonitrile (0.5 mL). The samples were vortex mixed and centrifuged for 10 min at 14000 rpm. A portion of supernatant (50-80 µL) was injected into LC/MS for biotransformation profiling and mass-spectral analysis. Negative control incubations were performed in the same manner, but either lacked cofactor or enzymes.

**Incubation of the Enantiomeric Metabolite with Liver Microsomes.** Incubations were carried out at 37 °C in a shaking water bath. The enantiomeric metabolite (10 µM) was incubated with rat, monkey or human liver microsomes (1.0 mg protein/mL), NADPH (1.0 mM) and MgCl₂ (0.5 mM) in potassium phosphate buffer (100 mM, pH 7.4) with a final volume of 0.5 mL. The mixture was allowed to react for 60 min before being quenched with cold acetonitrile (0.5 mL) containing 2 µM stable labeled ¹³C₃,¹⁵N₂-brivanib as internal standard. The samples were vortex mixed and centrifuged for 10 min at 14000 rpm. The supernatant was diluted 10 times with water, and a portion of diluted solution (50-80 µL) was injected into LC/MS for the quantitative analysis of brivanib and its enantiomer. Negative control incubations were performed in the same manner, but either lacked NADPH or enzymes.

**In Vivo Chiral Inversion of Brivanib in Rats, Monkeys and Humans.** Rat and monkey studies were conducted under the standards recommended by the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. All human subjects provided written consent before participation in the study and the protocol for human study was approved by the institutional review board. Blood samples were collected into tubes containing K₂EDTA. All blood samples were placed on ice immediately and were centrifuged within 30 minutes after collection for 15 minutes at approximately 1000 g to obtain plasma. All plasma samples were stored at -80 °C prior to analysis.
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**Rat.** Male Sprague-Dawley rats (n = 6, weighing approximately 250-300 g) received single oral doses of brivanib alaninate administered by gavage at a target dose level of 60 mg/kg. The brivanib alaninate dosing solution (10 mg/mL) was prepared in 50 mM sodium citrate buffer (pH 3.5). Animals were fasted overnight before dosing. Approximately 4 hours after dosing, animals were fed Certified Rodent Diet 5002 (PMI Nutrition International, Inc.) and were given unlimited access to water. Serial blood samples were collected from animals via tail veins before dosing and at 1, 2, 4, 6, 8, and 24 h after dosing.

**Monkey.** Male cynomolgus monkeys (n = 3, weighing approximately 4.5-5.8 kg) received single oral doses of brivanib alaninate administered by gavage at a target dose level of 10 mg/kg. The brivanib alaninate dosing solution (10 mg/mL) was prepared in 50 mM sodium citrate buffer (pH 3.5). Animals were fasted overnight before dosing. Approximately 4 hours after dosing, animals were fed Certified Primate Diet 5048 (PMI Nutrition International, Inc.) and were given unlimited access to water. Serial blood samples were collected from animals via a vascular access port before dosing and at 1, 2, 4, 6, 8, and 24 h after dosing.

**Human.** A total of 16 healthy volunteers (male and female, 18-55 years of age) participated in the study. Each subject was administered a single oral dose of brivanib alaninate (film-coated tablets, each containing 200 mg of drug substance) at a dose level of 400 mg. Serial blood samples were collected before dosing and at 1, 2, 3, 4, 6, 8 and 12 h after dosing.

**Concentration-Dependent Sulfation of Brivanib and Its Enantiomer.** Incubations of brivanib and its enantiomer with cytosols were conducted following the procedures previously described (Gong et al., 2012). The kinetics were determined at 9 substrate concentrations (1, 2, 5, 10, 20, 30, 60, 80 and 120 µM). The formation of the sulfate metabolites was linear up to 30 min of incubation time in cytosols from rat, monkey and human with a protein concentration of 0.2 mg/mL. The incubation mixtures consisted of brivanib or its enantiomer (1-120 µM), PAPS (0.5 mM), MgCl$_2$ (0.5 mM) and cytosols from rat, monkey and human (0.2 mg protein/mL) in phosphate buffer (100 mM, pH 7.4) with a final
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volume of 0.5 mL. After incubation, the concentrations of the sulfation metabolites were determined with a LC/MS/MS method reported previously (Gong et al., 2012).

**In Vitro Pharmacological Activities of Brivanib and Its Enantiomer.** In vitro pharmacology of brivanib and its enantiomeric isomer was evaluated in both a kinase inhibition assay and a cell proliferation inhibition assay. For the kinase inhibition assay, recombinant proteins containing the cytoplasmic domains of the kinases were used. Stock solutions of the test compounds were prepared in DMSO, and the final concentrations of the test compounds were in the range of 0-45 µM. Assay conditions and data analysis were described in published papers (Borzilleri et al., 2005 and Bhide et al., 2006). Inhibition of cell proliferation was assessed using 5-bromo-2-deoxy Uridine (BrdU) incorporation as a direct measure of DNA synthesis and propidium iodide (PI) stained cells as an indirect measure of cell number. The effect of various concentrations of test compounds (0-45 µM) was evaluated for cells stimulated either by VEGF, FGF or both together. Detailed assay conditions have been described previously (Borzilleri et al., 2005).

**Preparation of Plasma Samples for Quantitative Analysis.** Sample preparation was conducted using a liquid-liquid extraction method. In brief, an aliquot of plasma sample (0.2 mL) was mixed with 0.05 mL of stable labeled $^{13}$C$_3$,$^{15}$N$_2$-brivanib internal standard (2500 ng/mL), and 0.025 mL ammonium acetate buffer (1 M) containing 4% acetic acid in a 96-well plate. To the mixture, 0.6 mL methyl tert-butyl ether was added with a Tomtec Quadra 3 SPE (Tomtec, Hamden, Connecticut). The plate was vortex mixed for 15 min, and centrifuged at 4000 rpm for 5 min. The supernatant (0.40 mL) was transferred to a clean 96-well plate with a Tomtec Quadra 3 SPE and evaporated under nitrogen for 15 min. The residue was reconstituted with 0.1 mL isopropyl alcohol by vortex mixing for 5 min, and then centrifuged for 5 min at 4000 rpm. An aliquot of the final solution (10 µL) was injected into LC/MS for quantitative analysis.
HPLC Profiling and Metabolite Identification. Biotransformation profiling was performed on an Agilent 1100 HPLC system interfaced with a Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). Achiral chromatographic separation was achieved with a Zorbax SB C18 HPLC column (4.6 × 250 mm, 5 µm, Agilent Technologies, Palo Alto, CA). A gradient of two-solvent system, A and B, was used for HPLC profiling. Solvent A consisted of 0.1% formic acid in water and solvent B was acetonitrile. The HPLC flow rate was 1.0 mL/min. The gradient consisted of an initial condition of 10% B, increased to 55% B over the course of 60 min, followed by a column wash with 90% B for 5 min, and returned to the initial condition of 10% B over the course of 4 min. Chiral chromatographic separation was achieved with a Chirapak IC HPLC column (150 mm x 4.6 mm, 5 µm particles, from Chiral Technology, West Chester, PA). Samples were eluted with an isocratic elution consisting of 14% isopropyl alcohol and 86% n-heptanes at a flow rate of 0.2 mL/min.

For radio-profiling, the HPLC eluate was split via a flow splitter (Dionex Co., Sunnyvale, CA) where 75% of eluate was collected into Deepwell LumaPlate 96-well plates at 0.25 min intervals. The run time was 70 min. The remaining 25% of the eluate was directed into a Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) for metabolite identification. The plates were dried in a Speed-Vac (Savant Instruments Inc., Holbrook, NY) and the radioactivity was counted for 5 min per well with a Packard TopCount radioactivity detector (PerkinElmer Biosciences, Downers Grove, IL).

Mass spectrometer analysis was performed on a Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). The ESI source was set in the positive ion mode using the following parameters: voltage, 4 kV; current, 10 µA; and capillary temperature, 275 °C. MS data were collected for a mass range of 100-1000 amu, and MS/MS data were acquired with the following parameters: isolation width, 1.5 amu; collision energy, 35 eV; and activation time, 30 ms. The nitrogen flow rate, spray current and voltages were adjusted to give maximum sensitivity. MS and MS/MS analysis were performed to identify the drug related compounds.
Chiral Bioanalytical Method for the Quantification of Brivanib and Its Enantiomer.

Quantitative analysis was carried out with a Shimadzu Class VP HPLC system interfaced with an API 4000 Q-trap mass spectrometer (Sciex, Toronto, Canada). The HPLC system was equipped with two pumps (model LC-10AT), an HTC PAL auto sampler (Leap Technologies, Cary, NC), and a diode array detector (SPC-M10A). A Chirapak IC HPLC column (150 mm x 4.6 mm, 5 μm particles, from Chiral Technology, West Chester, PA) was used for chromatographic separation with an isocratic elution consisting of 14% isopropyl alcohol and 86% n-heptane at a flow rate of 0.2 mL/min. The Q-trap mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source, was operated in the positive ionization mode. The selected reaction monitoring (SRM) transition was $m/z$ 371.2 → $m/z$ 313.2 for brivanib and its enantiomer and $m/z$ 376.2 → $m/z$ 318.2 for the internal standard. The limit of quantitation for brivanib and its enantiomer was 20 ng/mL.
Results

Brivanib Chiral Inversion in Incubations with Liver Subcellular Fractions. To evaluate in vitro chiral inversion of brivanib, \([^{14}C]\)brivanib alaninate was incubated with liver microsomes from rat, monkey and human in the presence or absence of NADPH. The incubation mixtures were analyzed with both achiral and chiral LC/MS methods. Metabolite profiles (Figure 2), generated with an achiral LC/MS method, were qualitatively similar across species. The radioprofile of the incubation mixture with HLM has been described previously (Gong et al., 2012) and is also included here as a comparison. In addition to the hydroxy metabolite (M7) and the carboxylic acid metabolite (M26), a ketone metabolite (M31) was observed as a prominent metabolite in microsomal incubations in the presence of NADPH. Brivanib alaninate was not observed after incubation due to rapid hydrolysis. When the incubation mixtures were profiled with a chiral LC/MS method, two major radioactive peaks were displayed on radiochromatographs, corresponding to brivanib (24.6 min) and its enantiomeric isomer (22.5 min) (Figure 3). The percent of brivanib converted to the enantiomeric metabolite after incubation ranged from 11.6 to 15.8% across species (Table 1). In the absence of NADPH or microsomes, the formation of M31 and the enantiomeric metabolite was negligible.

The potential of chiral inversion of brivanib was also tested in liver cytosols from animals and humans. Liver cytosolic fractions catalyzed the conversion of brivanib to its enantiomer in a NADPH-dependent manner. Approximately 0.8-3.1% of brivanib underwent chiral inversion after cytosol-mediated incubations (Table 1).

In addition to NADPH, other cofactors including NADH and NAD\(^+\) were tested for their influence on the catalytic activities of microsomes and cytosols for the brivanib chiral inversion. In the presence of NADH, but not NAD\(^+\), liver microsomes from all 3 species catalyzed the chiral conversion of brivanib, with the percent of chiral inversion lower than those with NADPH (Table 1). In cytosolic fractions, the formation of the enantiomeric metabolite was not observed when either NADH or NAD\(^+\) was present as
co-factor. A small portion of brivanib (<1%) was oxidized to M31 in liver cytosols when NAD$^+$ was used as the cofactor.

**Chiral Inversion of the Enantiomeric Metabolite.** To test whether brivanib chiral inversion is bidirectional, the conversion of the enantiomeric metabolite to brivanib was investigated. Following incubations with liver microsomes from rat, monkey and human in the presence of NADPH, approximately 3.5, 2.2, 2.5% of the enantiomeric isomer, respectively, was converted to brivanib. No reaction occurred in microsomes in the absence of NADPH.

**In Vivo Chiral Inversion of Brivanib.** In vivo chiral inversion of brivanib was assessed in male Sprague-Dawley rats, male cynomolgus monkeys and healthy volunteers, following single oral doses of brivanib alaninate. The concentration-time profiles of brivanib and its enantiomer in plasma from humans and animals are shown in Figure 4, and the mean pharmacokinetic parameters are summarized in Table 2. In both rats and monkeys, the enantiomeric metabolite was a prominent circulating component. The plasma concentration of the enantiomeric metabolite reached a maximum ($T_{max}$) at 2 h and 4 h, respectively, in rats ($C_{max} = 2.6 \mu g/mL$) and monkeys ($C_{max} = 0.5 \mu g/mL$) after an oral dose (Table 2). Area under curve (AUC) values of the enantiomeric metabolite as a percent of brivanib were 94.7 and 39.7% in rats and monkeys, respectively. Following oral doses of brivanib alaninate in healthy volunteers, the exposures of the enantiomeric metabolite were low compared to those in rats and monkeys. Mean concentrations of the enantiomeric metabolite were consistently below 3% of brivanib concentration. In humans, the AUC value of the enantiomeric metabolite was 1.6% relative to brivanib.

**Concentration-Dependent Sulfation of brivanib and Its Enantiomer.** Kinetic studies towards sulfation of brivanib and its enantiomer were performed with liver cytosols from rat, monkey and human for the formation of sulfate metabolites. The study was conducted to compare the rate of clearance of brivanib and its enantiomer through sulfate conjugation, a primary clearance pathway of brivanib in humans. The kinetic parameters were estimated with a nonlinear regression by fitting the data to the
Michaelis-Menten equation, and are summarized in Table S1 (Supplemental Information). The $K_m$ values for both the isomers in cytosols from animals and humans ranged from 0.7-4.1 µM for the formation of sulfate metabolite. The intrinsic clearance of two isomers were comparable in cytosols from the same species.

**Pharmacological Activities of Brivanib and Its Enantiomer.** In vitro pharmacology of brivanib and its enantiomer was evaluated for their activities toward the inhibition of VEGFR-2 and FGFR-1 kinases, and their ability to inhibit cellular proliferation of growth-factor-stimulated human umbilical vein endothelial cells (HUVECs). Results, summarized in Table 3, revealed that both isomers had similar activities toward the inhibition of VEGFR-2 and FGFR-1 kinases with IC$_{50}$ values ranging from 20-28 nM. Additionally, both compounds displayed comparable activities toward the proliferation inhibition of HUVECs stimulated with either VEGF, FGF or both. The IC$_{50}$ values of brivanib and its enantiomer were 618 and 687 nM, respectively, against HUVECs stimulated with VEGF. For cells stimulated with FGF, the IC$_{50}$ values of brivanib and its enantiomer were 695 and 1011 nM, respectively. When both growth factors are present, the IC$_{50}$ values were 841 and 807 nM for brivanib and the enantiomeric metabolite, respectively.
Discussions

The objective of the present study was to investigate the potential for chiral inversion of brivanib in incubations with liver subcellular fractions as well as in animals and humans following oral doses of brivanib alaninate. These studies were necessary to completely establish the safety and pharmacology profiles of brivanib in humans and to ensure appropriate coverage of the metabolite in toxicological species. The chirality of brivanib is derived from an asymmetric secondary alcohol, a common structural motif that can undergo metabolic chiral inversion (Wsol et al., 2004). In addition, a ketone metabolite (M31), an intermediate in the inter-conversion, was observed previously in HLM-mediated incubation and in vivo in animals and humans following oral doses of [$^{14}$C]brivanib alaninate (Gong et al., 2011). These data suggested the potential of brivanib to undergo chiral inversion, and hence the need for a detailed characterization.

Biotransformation of brivanib in HLM has been described previously. Since the samples were analyzed by achiral LC/MS methods, no information regarding chiral inversion was obtained from those studies (Gong et al., 2012). Therefore, additional studies in liver microsomes from rat and monkey were conducted to understand species differences in the metabolism and chiral inversion of brivanib. Metabolite profiles of incubation mixtures of [$^{14}$C]brivanib alaninate in liver microsomes from rat and monkey, generated with an achiral LC method, were qualitatively similar to that with HLM, in which M31 was a prominent drug-related component. Subsequent chiral analysis revealed that indeed a portion of brivanib was converted to its enantiomer after incubation, with the extent of chiral inversion comparable across species (Table 1 and Figure 3). No reaction occurred in the absence of liver microsomes suggesting that the asymmetric center was stable under physiological condition without enzymes. Additionally, liver cytosols fractions from animals and humans displayed activity for the chiral inversion of brivanib, indicating that both microsomal and cytosolic enzymes were likely involved in this reaction (Table 1).
A two-step biotransformation pathway for the formation of the enantiomeric metabolite is proposed based on the in vitro biotransformation data (Figure 5). The mechanism involves both oxidation and reduction, and chiral inversion is the net result of these processes. The individual enzyme involved in each reaction was not identified. Brivanib underwent chiral inversion when incubated with either microsomal or cytosolic fractions, with NADPH favored over NADH as the cofactor in both systems. Multiple enzymes including P450s, alcohol dehydrogenase and many oxidoreductases, can catalyze the alcohol oxidation and ketone reduction (Barski et al., 2008; Plapp, 2010). P450s are membrane-bound enzymes mainly present in microsomes whereas alcohol dehydrogenase is a cytosolic enzyme. Oxidoreductases can be found in both microsomal and cytosolic fractions. The majority of oxidoreductases prefer NADPH over NADH as cofactor, similar to P450s (Barski et al., 2008). The NADPH-dependence in microsomes indicates that both P450 enzymes and oxidoreductases could play a role in the chiral inversion. The cytosolic activity for brivanib chiral inversion is presumably contributed from NADPH-dependent cytosolic oxidoreductases such as aldo-keto reductases and ketone reductase. Furthermore, the formation of M31, but not the enantiomeric metabolite, in cytosol in the presence of NAD\(^+\) suggests that NAD\(^+\)-dependent alcohol dehydrogenase might catalyze the oxidation of brivanib (Plapp, 2010). These data seems to suggest that multiple enzymes are involved in the chiral inversion of brivanib.

To date, most of the literature examples of chiral inversion are bidirectional in nature with the enantiomeric metabolite and the parent drug being metabolically inter-convertible (Wsol et al., 2004; Yi et al., 2007). There are also examples of unidirectional chiral inversion in which the enantiomeric metabolite is not converted to the parent drug (Hao et al., 2005; Xin et al., 2010). The basis for bidirectional or unidirectional inversion via alcohol/ketone inter-conversion is attributed to the stereoselectivity of the oxidative enzymes and to the stereospecificity of the reducing enzymes (Brocks et al., 2004; Babú et al., 2008). To investigate whether brivanib chiral inversion was unidirectional or
bidirectional, the enantiomeric metabolite was incubated with liver microsomes from animals and humans. Subsequent chiral analysis confirmed the presence of brivanib in the incubation mixtures, demonstrating that the chiral inversion was bidirectional. However, the extent of the reverse conversion (<3%) was less compared to brivanib conversion under similar conditions, presumably due to slow oxidation of the enantiomeric metabolite relative to the oxidation of brivanib.

Brivanib has manageable safety profiles in long term toxicology and clinical studies (Mekhail et al., 2010; Park et al., 2011). The toxicology profile of the enantiomeric metabolite was not directly studied in preclinical species. In addition, it was not clear whether the enantiomeric metabolite contributed to the target-related pharmacological activity. These concerns prompted us to conduct in vivo studies in toxicological species and in humans to determine the systemic exposures of brivanib and the enantiomeric isomer. Following oral administration of brivanib alaninate to rats and monkeys at well-tolerated dose levels, the enantiomeric metabolite was a prominent drug-related component in plasma in both species suggesting that indeed chiral inversion occurred in vivo. Considering the dose-proportional pharmacokinetics of brivanib (Marathe et al., 2009), the total exposure of brivanib and its enantiomer in humans and animals were comparable to the previously reported results (Gong et al., 2011). The low plasma concentrations of the enantiomeric metabolite in humans compared to those in toxicological species demonstrate adequate toxicological coverage for this metabolite. In addition, the results suggested that an achiral bioanalytical method that measured total concentration of brivanib and the enantiomeric metabolite was sufficient to provide an accurate representation of the pharmacokinetics of brivanib in the clinical studies.

The low exposure of the enantiomeric metabolite in human was surprising given the fact that the extent of brivanib chiral inversion was similar across species after incubation with liver sub-fractions. The reason behind this remains unclear. Many factors could contribute to the limited exposure of the enantiomeric metabolite in human. For example, the enantiomeric metabolite could be subjected to
higher systemic clearance in humans mediated by either metabolism or active transport. Previous studies demonstrated that sulfate conjugation of brivanib was a primary clearance pathway in humans. To address whether this pathway could address the lower exposure to the enantiomer in humans, the kinetic parameters for the formation of sulfate conjugate were determined for both isomers in liver cytosols from rat, monkey and human. The results suggest both isomers had comparable rate of intrinsic clearance through sulfation pathway (Table S1, Supplemental Information). Although this was a preliminary study with limited substrate concentrations, it was obvious that there were no dramatic differences between the isomers through a major metabolic pathway. More studies on the role of the active transporters in the disposition of brivanib and the enantiomeric metabolite may reveal the reason for the exposure difference between humans and animals.

Brivanib and its enantiomer displayed similar potency when tested for their activities toward the inhibition of VEGFR-2 and FGFR-1 kinases, and for their ability to inhibit proliferation of growth factor-stimulated HUVEC. This was consistent with the previous studies around the pharmacology activity of structural analogues of brivanib, including brivanib metabolites, which suggested that modification on the alkyl side chain had little influence on the inhibition potency against VEGFR-2 and FGFR-1 (Bhide et al., 2006; Cai et al., 2008). However, given the low exposure of the enantiomer in humans, it is unlikely that it contribute significantly to the overall pharmacology of brivanib.

In summary, brivanib underwent chiral inversion in incubations with liver subcellular fractions from rat, monkey and human. Following oral doses of brivanib alaninate, the exposure of the enantiomeric metabolite was significant in rats and monkeys and low in humans, indicating that the contribution to the pharmacology activity from the enantiomeric metabolite in humans was insignificant relative to brivanib. Furthermore, adequate exposure to the enantiomeric metabolite in toxicological species suggested no safety concerns in humans.
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Authorship Contributions

Participated in research design: Gong, Gan, Masson, Syed, Williams, Humphreys, and Iyer.

Conducted experiments: Gong, Xia, Masson, Syed, Williams, Jemal.

Contributed new reagents or analytic tools: Pursley.

Performed data analysis: Gong, Gan, Syed, Williams, Masson, and Iyer.

Wrote or contributed to the writing of the manuscript: Gong, Masson, Syed, Williams, Humphreys, Pursley, and Iyer.
References


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Footnotes

Current affiliation for Yuan-Qing Xia: AB Sciex (YX), Framingham, MA 01701, USA
Legends for Figures

Figure 1. Structures of brivanib, the enantiomeric metabolite of brivanib, [14C]brivanib alaninate and stable labeled brivanib internal standard. The site labeled with C-14 is indicated with an asterisk.

Figure 2. Metabolite profiles of incubations of [14C]brivanib alaninate with (A) rat liver microsomes; (B) monkey liver microsomes; and (C) human liver microsomes. Samples were profiled with an achiral HPLC method. The profiles are background-subtracted reconstructed radiochromatograms of 15-s fractions collected from an HPLC run. Structures of M7 and M26 have been reported previously. The peak labeled brivanib is a mixture of brivanib and its enantiomer since the samples were analyzed with an achiral HPLC column.

Figure 3. A representative metabolite profile, generated with a chiral HPLC method, of an incubation mixture of [14C]brivanib alaninate with monkey liver microsomes. The profiles are background-subtracted reconstructed radiochromatograms of 15-s fractions collected from an HPLC run. The ketone metabolite was not identified due to low abundance.

Figure 4. Mean plasma concentration-time profiles of brivanib and the enantiomeric metabolite on a logarithmic scale in (A) rats, (B) monkeys and (C) humans after oral doses of brivanib alaninate. Plasma samples were analyzed with a chiral LC/MS method.

Figure 5. Proposed biotransformation pathways for the formation of the enantiomeric metabolite of brivanib.

Table 1. Percent of chiral inversion of brivanib in incubations with liver subcellular fractions from rat, monkey and humans, in the presence of NADPH, NADH or NAD+. Incubations were conducted for 60 min with protein concentration of 1.0 mg/mL.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Percent of conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>13.8</td>
</tr>
<tr>
<td>Tissue Type</td>
<td>Value 1</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Monkey liver microsomes</td>
<td>15.8</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>11.6</td>
</tr>
<tr>
<td>Rat liver cytosol</td>
<td>0.8</td>
</tr>
<tr>
<td>Monkey liver cytosol</td>
<td>2.5</td>
</tr>
<tr>
<td>Human liver cytosol</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ketone metabolite (M31) was observed after incubation.

ND: not detected;
Table 2. Mean pharmacokinetic parameters of brivanib and its enantiomer in rats, monkeys and humans after oral doses of brivanib alaninate.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose</th>
<th>Analyte</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µg·h/mL)</th>
<th>AUC % of the enantiomeric metabolite relative to brivanib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>60 mg/kg</td>
<td>Brivanib</td>
<td>3.5</td>
<td>1</td>
<td>7.6</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enantiomeric metabolite</td>
<td>2.6</td>
<td>2</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>10 mg/kg</td>
<td>Brivanib</td>
<td>1.4</td>
<td>1</td>
<td>6.8</td>
<td>39.7</td>
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<tr>
<td></td>
<td></td>
<td>Enantiomeric metabolite</td>
<td>0.5</td>
<td>4</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>400 mg</td>
<td>Brivanib</td>
<td>3.5</td>
<td>2</td>
<td>21.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enantiomeric metabolite</td>
<td>0.05</td>
<td>5</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

a. The concentration of brivanib and the enantiomeric metabolite were determined with a chiral LC/MS method.
Table 3. IC₅₀ values of brivanib and its enantiomer for inhibition of VEGFR-2 and FGFR-1 kinases and for proliferation inhibition of HUVECs stimulated with growth factors

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ Values (nM)</th>
<th>Kinase inhibition¹</th>
<th>Proliferation inhibition of HUVECs stimulated with growth factors</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VEGFR-2</td>
<td>FGFR-1</td>
</tr>
<tr>
<td>Brivanib</td>
<td></td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>Enantiomeric metabolite</td>
<td></td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

¹ IC₅₀ values of brivanib were similar to those reported previously (Bhide et al., 2006).
Figure 1

Brivanib

S-isomer

Brivanib alaninate
* C-14 label

Stable isotope-labelled brivanib
Figure 2

A

Brivanib
M7
M26
Ketone (M31)

Time (min)

CPM

M7
M26
Ketone (M31)

50 55 60 65 70

0 5 10 15 20 25 30 35 40

50 55 60 65 70

B

Brivanib

M7
M26
Ketone (M31)

Time (min)

CPM

M7
M26
Ketone (M31)

50 55 60 65 70

0 5 10 15 20 25 30 35 40

50 55 60 65 70

C

Brivanib

M7
M26
Ketone (M31)

Time (min)

CPM

M7
M26
Ketone (M31)

50 55 60 65 70

0 5 10 15 20 25 30 35 40

50 55 60 65 70

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Figure 3

The figure shows a chromatogram with two peaks. The first peak is labeled as "Brivanib." The second peak is labeled as "Enantiomeric metabolite." The x-axis represents time in minutes (0 to 40), and the y-axis represents CPM (counts per minute).
Figure 4

A  
- Brivanib
- Enantiomeric Metabolite

B  
- Brivanib
- Enantiomeric Metabolite

C  
- Brivanib
- Enantiomeric Metabolite
Figure 5

Brivanib

Enantiomeric Metabolite

Ketone Metabolite (M31)

Oxidation
Reduction
Oxidation
Reduction