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

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ABSTRACT:

Brivanib alaminate is an orally administered alanine prodrug of brivanib, a dual inhibitor of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (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 after oral doses of brivanib alaminate. Incubations of [14C]brivanib alaminate 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 percentage of the enantiomeric metabolite relative to brivanib in microsomal and cytosolic incubations of different species in the presence of NADPH ranged from 11.6 to 15.8 and 0.8 to 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. After oral doses of brivanib alaminate to rats and monkeys, the enantiomeric metabolite was a prominent drug-related component in plasma, with the percentages of area under the curve (AUC) at 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 VEGF receptor-2 and FGF receptor-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)-5-methylpyrrolo[1,2,4]triazin-6-yl)oxy)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yl)oxy)-5-methylpyrrolo[1,2,4]triazin-6-yl)oxy)propan-2-ol; M7, (R)-1-([4-(4-fluoro-2-hydroxymethyl)-1H-indol-5-yloxy]-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-ol; M26, (R)-4-fluoro-5-[(6-(2-hydroxypropoxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-4-yloxy)-1H-indole-2-carboxylic acid; M31, 1-[(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-1one; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; AUC, area under the curve; NAD+ nicotinamide adenine dinucleotide; HUVECs, human umbilical vein endothelial cells.

ABBREVIATIONS: BMS-540215, brivanib; (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yloxy)propan-2-ol; M7, (R)-1-([4-(4-fluoro-2-hydroxymethyl)-1H-indol-5-yloxy]-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-ol; M26, (R)-4-fluoro-5-[(6-(2-hydroxypropoxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-4-yloxy)-1H-indole-2-carboxylic acid; M31, 1-[(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-one; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; AUC, area under the curve; NAD+, nicotinamide adenine dinucleotide; HUVECs, human umbilical vein endothelial cells.

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Brivanib alaninate was used as the substrate in incubations because (Muskegon, MI). All organic solvents and water were of HPLC grade. Acetonitrile and methanol were purchased from Burdick & Jackson Laboratories and a Milli-Q Plus ultrapure water system (Millipore Corporation, Bedford, MA). Type I reagent grade water was prepared with a scientific (Gibbstown, NJ). Ecolite liquid scintillation cocktail was purchased from BD Bioscience (Woburn, MA). Brivanib, brivanib alaninate [(1R, 2S)-2-(4-(5-fluoro-2-methyl-1H-indol-5-yl)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yl)-1-methylethyl] ester, stable labeled 13C3,15N2-aminopropionic acid 2-[4-(4-fluoro-2-methyl-1H-indol-5-yl)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yl]-1-methylethyl ester, stable labeled 13C3,15N2-brivanib, and the enantiomeric metabolite (the S-isomer of brivanib) (Fig. 1) were supplied by the Department of Chemical Synthesis, Bristol-Myers Squibb Research and Development (Princeton, NJ). [14C]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 and Development. 3’-Phosphoadenosine 5’-phosphosulfate, NADPH, NADH, and nicotinamide adenine dinucleotide (NAD+) were purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate and formic acid were obtained from EM Science (Gibbstown, NJ). Potassium phosphate and formic acid were obtained from MP Biomedicals (Irvine, CA). All organic solvents and water were of HPLC grade. Brivanib alaninate (20 mg/ml protein) and liver cytosols (10 mg/ml protein) from rats, monkeys, and humans were purchased from BD Bioscience (Woburn, MA). Brivanib, brivanib alaninate [(1R, 2S)-2-(4-(5-fluoro-2-methyl-1H-indol-5-yl)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yl)-1-methylethyl ester, stable labeled 13C3,15N2-brivanib, and the enantiomeric metabolite (the S-isomer of brivanib) (Fig. 1) were supplied by the Department of Chemical Synthesis, Bristol-Myers Squibb Research and Development (Princeton, NJ). [14C]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 and Development. 3’-Phosphoadenosine 5’-phosphosulfate, NADPH, NADH, and nicotinamide adenine dinucleotide (NAD+) were purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate and formic acid were obtained from EM Science (Gibbstown, NJ). Potassium phosphate and formic acid were obtained from MP Biomedicals (Irvine, CA). Type I reagent grade water was prepared with a Milli-Q Plus ultrapure water system (Millipore Corporation, Bedford, MA). Acetonitrile and methanol were purchased from Burdick & Jackson Laboratories (Muskegon, MI). All organic solvents and water were of HPLC grade.

**Chiral Inversion of Brivanib in Microsomal and Cytosolic Incubations.** [14C]Brivanib alaninate was used as the substrate in incubations because [14C]brivanib was not available. During the incubation, [14C]brivanib was generated in situ through the rapid hydrolysis of [14C]brivanib alaninate (Marathe et al., 2009). The incubation mixtures consisted of [14C]brivanib alaninate (2 µM, 11.6 × 10−3 µCi/ml), enzymes (liver microsomes or cytosols from rat, monkey or human, 1.0 mg/ml protein), cofactor (NADPH, NADH, or NAD+), 1.0 mM), MgCl2 (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 ice-cold acetone (0.5 ml). The samples were vortex mixed and centrifuged for 10 min at 14,000 rpm. A portion of supernatant (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 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/ml protein). 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 ice-cold acetone (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 14,000 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 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 centrifuged within 30 min after collection for 15 min at −1000g to obtain plasma. All plasma samples were stored at −80°C before analysis.

**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 h after dosing, animals were fed certified rodent diet 5002 (PMI Nutrition International, Inc., St. Louis, MO) and 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 h after dosing, animals were fed Certified Primate Diet 5048 (PMI Nutrition International, Inc.) and 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.

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 described previously (Gong et al., 2012). The kinetics were determined at nine 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), 3′,5′-phosphoadenosine 5′-phosphosulfate (0.5 mM), MgCl₂ (0.5 mM), and cytosols from rat, monkey, and human (0.2 mg/ml protein) in phosphate buffer (100 mM, pH 7.4) with a final 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 each species catalyzed the chiral conversion of brivanib, with the percent-
ages 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 cofactor. 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. After incubations with liver microsomes from rat, monkey, and human in the presence of NADPH, approximately 3.5, 2.2, and 2.5% of the enantiomeric isomer, respectively, were 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 after single oral doses of brivanib alinate. The concentration-time profiles of brivanib and its enantiomer in plasma from humans and animals are shown in Fig. 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 (Tₘₐₓ) at 2 and 4 h, respectively, in

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>NADPH</th>
<th>NADH</th>
<th>NAD⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver microsomes</td>
<td>13.8</td>
<td>3.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Monkey liver microsomes</td>
<td>15.8</td>
<td>2.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>11.6</td>
<td>7.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rat liver cytosol</td>
<td>0.8</td>
<td>N.D.</td>
<td>N.D.⁺</td>
</tr>
<tr>
<td>Monkey liver cytosol</td>
<td>2.5</td>
<td>N.D.</td>
<td>N.D.⁺</td>
</tr>
<tr>
<td>Human liver cytosol</td>
<td>3.1</td>
<td>N.D.</td>
<td>N.D.⁺</td>
</tr>
</tbody>
</table>

N.D., not detected.  
⁺The ketone metabolite (M31) was observed after incubation.

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**Fig. 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 because of low abundance. CPM, counts per minute.

**Fig. 2.** Metabolite profiles of incubations of [¹⁴C]brivanib alaninate with rat liver microsomes (A), monkey liver microsomes (B), and human liver microsomes (C). 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 because the samples were analyzed with an achiral HPLC column. CPM, counts per minute.

**Fig. 4.** A representative metabolite profile, generated with a chiral HPLC method, of an incubation mixture of [¹⁴C]brivanib alaninate with monkey liver microsomes.
rats (C\text{max} = 2.6 \, \mu g/ml) and monkeys (C\text{max} = 0.5 \, \mu g/ml) after an oral dose (Table 2). Area under curve (AUC) values of the enantiomeric metabolite as a percentage of brivanib were 94.7 and 39.7% in rats and monkeys, respectively. After oral doses of brivanib alaninate in healthy volunteers, the exposures of the enantiomeric metabolite were low compared with 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 toward 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 Supplementary Table S1. The K\text{m} values for both the isomers in cytosols from animals and humans ranged from 0.7 to 4.1 \mu M 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\text{50} values ranging from 20 to 28 nM. Furthermore, both compounds displayed comparable activities toward the proliferation inhibition of HUVECs stimulated with either VEGF, FGF, or both. The IC\text{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\text{50} values of brivanib and its enantiomer were 695 and 1011 nM, respectively. When both growth factors were present, the IC\text{50} values were 841 and 807 nM for brivanib and the enantiomeric metabolite, respectively.

**Discussion**

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 after 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 was derived from an asymmetric secondary alcohol, a common structural motif that can undergo metabolic chiral inversion (Wsdol et al., 2004). In addition, a ketone metabolite (M31), an intermediate in the interconversion, was observed previously in studies with liver microsomes (Wsoł et al., 2004). In the present study, no new enantiomeric metabolite was observed. This was not unexpected since the compound was manufactured to be optically pure.

**Table 2**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose</th>
<th>Analyte*</th>
<th>C\text{max} (\mu g/ml)</th>
<th>T\text{max} (h)</th>
<th>AUC\text{0-24} (\mu g \cdot 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>
</tr>
<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>0.05</td>
<td>5</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enantiomeric metabolite</td>
<td>0.05</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The concentration of brivanib and the enantiomeric metabolite were determined with a chiral LC/MS method.

**Table 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kinase Inhibitiona</th>
<th>Proliferation Inhibition of HUVECs Stimulated with Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\text{50} (nM)</td>
<td>VEGF</td>
</tr>
<tr>
<td>Brivanib</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>Enantiomeric metabolite</td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

a IC\text{50} values of brivanib were similar to those reported previously (Bhide et al., 2006).
HLM-mediated incubation and in vivo in animals and humans after oral doses of [14C]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. Because 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 [14C]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; Fig. 3). No reaction occurred in the absence of liver microsomes, suggesting that the asymmetric center was stable under physiological condition without enzymes. Furthermore, liver cytosols fractions from animals and humans displayed activity for the chiral inversion of brivanib, indicating that both microsomal and cytosolic enzymes were probably 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 (Fig. 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 seem 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 interconvertible (Wsöl 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 interconversion is attributed to the stereoselectivity of the oxidative enzymes and the stereospecificity of the reducing enzymes (Babi 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 with brivanib conversion under similar conditions, presumably because of 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 humans to determine the systemic exposures of brivanib and the enantiomeric isomer. After 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 with 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 subfractions. 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 that both isomers had comparable rate of intrinsic clearance through the sulfation pathway (Supplementary Table S1). 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-driven cell lines. The low exposure of the enantiomeric metabolite in human was significant relative to the overall pharmacology of brivanib. In summary, brivanib underwent chiral inversion in incubations with liver subcellular fractions from rat, monkey, and human. After oral dosing of brivanib metabolite, the exposure of the enantiomeric metabolite was significant in both species and humans, indicating that the contribution to the pharmacology activity from the enantiomeric metabolite in humans was significant 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, Masson, Syed, Xia, Williams, and Jemal.

Contributed new reagents or analytic tools: Pursley.

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

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

References


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Table S1. Enzyme kinetic parameters for the sulfation of brivanib and its enantiomer in liver cytosols from rat, monkey and human. Incubations were conducted at 37°C with brivanib or the enantiomer of brivanib (1, 2, 5, 10, 20, 30, 60, and 120 µM) and cytosols (0.1 mg protein/ml) in the presence of NADPH. Enzyme kinetic parameters were estimated by fitting the data to the Michaelis-Menten equation using a nonlinear regression analysis with GraphPad Prism (version 5.04; GraphPad Software Inc.).

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Rat Liver Cytosols</th>
<th>Monkey Liver Cytosols</th>
<th>Human Liver Cytosols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brivanib</td>
<td>Enantiomeric Metabolite</td>
<td>Brivanib</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>439</td>
<td>405</td>
<td>1901</td>
</tr>
<tr>
<td>$K_{\text{m}}$ (µM)</td>
<td>2.4</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>$C_{\text{Lint}}$ ((V_{\text{max}} /K_{\text{m}}))</td>
<td>181</td>
<td>421</td>
<td>715</td>
</tr>
</tbody>
</table>

The unit for $V_{\text{max}}$ was picomole per milligram of protein per minute.
The unit for $C_{\text{Lint}}$ was microliter per milligram of protein per minute.