Identification of the Oxidative and Conjugative Enzymes Involved in the Biotransformation of Brivanib

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ABSTRACT:

Brivanib alaninate, the L-alanine ester prodrug of brivanib, is currently being developed as an anticancer agent. In humans, brivanib alaninate is rapidly hydrolyzed to brivanib. Prominent biotransformation pathways of brivanib included oxidation and direct sulfate conjugation. A series of in vitro studies were conducted to identify the human esterases involved in the prodrug hydrolysis and to identify the primary human cytochrome P450 and sulfotransferase (SULT) enzymes involved in the metabolism of brivanib. Brivanib alaninate was efficiently converted to brivanib in the presence of either human carboxylesterase 1 or carboxylesterase 2. Because esterases are ubiquitous, it is likely that multiple esterases are involved in the hydrolysis. Oxidation of brivanib in human liver microsomes (HLM) primarily formed a hydroxylated metabolite (M7). Incubation of brivanib with human cDNA-expressed P450 enzymes and with HLM in the presence of selective chemical inhibitors and monoclonal P450 antibodies demonstrated that CYP1A2 and CYP3A4 were the major contributors for the formation of M7. Direct sulfation of brivanib was catalyzed by multiple SULT enzymes, including SULT1A1, SULT1B1, SULT2A1, SULT1A3, and SULT1E1. Because the primary in vitro oxidative metabolite (M7) was not detected in humans after oral doses of brivanib alaninate, further metabolism studies of M7 in HLM and human liver cytosol were performed. The data demonstrated that M7 was metabolized to the prominent metabolites observed in humans. Overall, multiple enzymes are involved in the metabolism of brivanib, suggesting a low potential for drug-drug interactions either through polymorphism or through inhibition of a particular drug-metabolizing enzyme.

Introduction

Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) signaling pathways play critical roles in angiogenesis, a process associated with vascular hyperproliferative disorders, particularly in cancer (Ellis and Fidler, 1996; Carmeliet and Jain, 2000; Gerwinski et al., 2000; Cross and Claesson-Welsh, 2001). In efforts to develop novel chemotherapeutic agents, both VEGF and FGF have become compelling targets for small molecule kinase inhibitors (Herbst, 2006; Tassi and Wellstein, 2006; Kiseliov et al., 2007). It is now well established that inhibitors of VEGF and/or FGF signaling pathways prevent tumor growth and metastasis through the inhibition of tumor-induced angiogenesis (Manetti and Botta, 2003; Underliner et al., 2004; Baka et al., 2006). Brivanib (Fig. 1), (R)-1-(4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-5-methylpyrrolo[1,2,4]triazin-6-yl)propan-2-ol, is a dual inhibitor of both VEGF and FGF signaling pathways (Borzilleri et al., 2005; Bhide et al., 2006; Ayers et al., 2007; Cai et al., 2008; Marathe et al., 2009). Its L-alanine ester prodrug, brivanib alaninate (Fig. 1), is currently being investigated in phase II/III clinical studies for the treatment of cancer, either as a single oral agent or in combination with other anticancer drugs (Diaz-Padilla and Siu, 2011). Brivanib has displayed promising anticancer activity in both preclinical and clinical studies and was well tolerated in humans when administered orally (Dempke and Zippel, 2010; Mekhail et al., 2010; Park et al., 2011).

Studies have shown that brivanib alaninate was rapidly converted to brivanib in liver and intestinal fractions from animals and humans with a half-life of less than 10 min (Marathe et al., 2009). In addition, after an oral dose of brivanib alaninate to rats, monkeys, and humans, the exposure to the prodrug was minimal to none, suggesting rapid presystemic hydrolysis. Brivanib was the prominent circulating species in plasma and was extensively metabolized before excretion (Gong et al., 2011). The primary biotransformation pathways of brivanib in humans were methyl oxidation and direct sulfation (Gong et al., 2011).

Reaction phenotyping studies, where enzymes involved in the metabolism of a drug candidate are determined, have become an integral part of drug development and are extremely valuable in predicting the potential for drug-drug interactions (Rodrigues, 1999; Lu et al., 2003; Zhang et al., 2007). The enzymes most commonly involved in the drug metabolism are cytochrome P450 (P450) enzymes, which catalyze a variety of biotransformation reactions such as hydroxylation, epoxidation, and dealkylation (Guengerich, 2001, 2004). Another
superfamily of metabolic enzymes includes the cytosolic sulfotransferases (SULTs), which catalyze the sulfate conjugation of xenobiotics and therapeutic drugs by transferring a sulfonate group from 3’-phosphoadenosine 5’-phosphosulfate (PAPS) to an acceptor group such as –OH or –NH2 of a substrate (Glatt et al., 2000, 2001; Gamage et al., 2006). Less noticed but important drug metabolism enzymes are esterases in the blood, intestine, and liver that are often involved in the activation of prodrugs and detoxification of xenobiotics (Satoh et al., 2002). In particular, carboxylesterases (CEs) expressed in intestine and liver cytosol play central roles in the bioactivation of many drugs, including irinotecan and capetebitine (Xu et al., 2002; Tabata et al., 2004a,b; Fujiyama et al., 2010). The objective of this study was to characterize the primary human P450 and SULT enzymes involved in the metabolism of brivanib, and the human CEs involved in the hydrolysis of brivanib alinate. In addition, further metabolism studies of the primary oxidative metabolite (M7) were conducted in liver subcellular fractions to confirm the link between M7 and the secondary metabolites of brivanib that were observed in vivo (Gong et al., 2011).

Materials and Methods

Chemicals and Reagents. Pooled HLM (20 donors male/female, 20 mg protein/ml) and human cDNA-expressed P450 enzymes (1 nmol/ml) were purchased from BD Gentest (Woburn, MA). Human recombinant SULTs (10 mg protein/ml) were purchased from XenoTech, LLC (Lenexa, KS). Human CE1 (50 mg protein/ml) and CE2 (50 mg protein/ml) were purchased from Cyrex Ltd. (Dundee, UK). Inhibitory monoclonal anti-human P450 antibodies for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 were obtained from the National Institutes of Health (Bethesda, MD). Brivanib was supplied by the Department of Chemical Synthesis, Bristol-Myers Squibb Research and Development (Stamford, CT). [14C]Brivanib alinate ([1R], 25)-2-aminopropionic acid 2-[4-(4-fluoro-2-methyl-1H-indol-5-yl)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yl]oxy)-1-methylethyl ester, 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. [14C]M7 (a methyl hydroxy metabolite of brivanib), [14C]M25 (a direct sulfate conjugate of brivanib), and [14C]M26 (a carboxylic acid metabolite of brivanib) were obtained according to the procedures described previously (Gong et al., 2011). Datasatin (BMS-354825), used as internal standard (IS) for the liquid chromatography/mass spectrometry (LC/MS) quantitation of M7 and M25, was supplied by the Department of Chemical Synthesis, Bristol-Myers Squibb Research and Development. It was selected because it shared structural similarities with these metabolites. All other chemicals, namely, furafylline, tranylcypromine, orphenadrine, montelukast, sulfaphenazole, benzylviranol, quinidine, diethyldithiocarbamate, ketocnazole, 1-aminoenzatriazole (1-ABT), PAPS, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate, ammonium acetate, and formic acid were obtained from EM Scientific (Gibbstown, NJ). Ecolite liquid scintillation cocktail was purchased from MP Biomedicals (Solon, OH). Type 1 reagent grade water was prepared with a Milli-Q Plus ultra pure water system (Millipore Corporation, Billerica, MA). Acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI).

For incubations with radiolabeled compounds, [14C]brivanib alinate was used as substrate because [14C]brivanib was not available. During incubation, [14C]brivanib was generated in situ through the rapid hydrolysis of [14C]brivanib alinate (Marathe et al., 2009). In incubations with recombinant enzymes and with HLM in the presence of selective P450 inhibitors and monoclonal P450 antibody, brivanib was used as substrate.

Ester Hydrolysis of Brivanib Alinate. [14C]Brivanib alinate, at concentrations of 2 or 20 μM, was incubated with human CE1 or CE2 at 37°C in a shaking water bath. The incubation mixtures consisted of enzyme (1 mg protein/ml), [14C]brivanib alinate (2 or 20 μM), and phosphate buffer (100 mM, pH 7.4), at a final volume of 0.5 ml. The mixtures were allowed to react for 30 min and were then quenched with ice-cold acetonitrile (0.5 ml). Negative control incubations were performed in the same manner but lacked enzymes. The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was injected into LC/MS for biotransformation profiling and mass-spectral analysis.

Incubation with HLM and Human Liver Cytosol. HLM-mediated oxidation of [14C]brivanib ([14C]brivanib alinate was added to the incubation mixture), [14C]M7, and [14C]M25 were carried out at 37°C in a shaking water bath. Individual test compound (2 μM, 11.6 × 10^−3 μCi/ml) was incubated with HLM (1.0 mg protein/ml), NADPH (1.0 mM), and MgCl2 (0.5 mM) in potassium phosphate buffer (100 mM, pH 7.4) at a final volume of 0.5 ml. The mixture was allowed to react for 60 min before being quenched with ice-cold acetonitrile (0.5 ml). The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the 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 lacked either NADPH or HLM.

Human liver cytosol-mediated oxidation of [14C]M7 was carried out at 37°C in a shaking water bath. The incubation mixture consisted of [14C]M7 (2 μM, 11.6 × 10^−3 μCi/ml), cytosol (1.0 mg protein/ml), NADPH (1.0 mM), and MgCl2 (0.5 mM) in potassium phosphate buffer (100 mM, pH 7.4) at a final volume of 0.5 ml. Incubations in the absence of NADPH were conducted in a similar manner. The mixture was allowed to react for 60 min before being quenched with ice-cold acetonitrile (0.5 ml). The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was injected into LC/MS for biotransformation profiling and mass-spectral analysis.

Incubations with Human cDNA-Expressed P450 Enzymes. Brivanib was incubated in triplicate with human cDNA-expressed P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) at 37°C in a shaking water bath. The reaction mixtures consisted of individual P450 enzyme (100 pmol/ml), brivanib (2 or 20 μM), NADPH (1.0 mM), MgCl2 (0.5 mM), and phosphate buffer (0.1 M, pH 7.4) at a final volume of 0.5 ml. The mixtures were allowed to react for 20 min before being quenched with ice-cold acetonitrile (0.5 ml) containing dasatinib as IS (100 mM). The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was analyzed by LC/MS to determine the concentration of M7.

HLM Incubation in the Presence of Chemical Inhibitors of P450. All experiments were conducted in triplicate at a final volume of 0.5 ml. The incubation mixtures consisted of HLM (250 μg protein/ml), brivanib (2 or 20 μM), NADPH (1.0 mM), MgCl2 (0.5 mM), phosphate buffer (0.1 M, pH 7.4), and individual inhibitors. Chemical inhibitors were furafylline (10 μM) for CYP1A2, tranylcypromine (2 μM) for CYP2A6, orphenadrine (50 μM) for CYP2B6, montelukast (3 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, quinidine (1 μM) for CYP2D6, diethylthiocarbamate (50 μM) for CYP2E1, ketocnazole (1 μM) for CYP3A4/5, and 1-ABT (1 mM) for all P450s. Incubations without inhibitors were also conducted as the positive controls. For incubations with direct chemical inhibitors (tranylcypromine, montelukast, sulfaphenazole, quinidine, and ketocnazole), all ingredients were mixed together except NADPH. The mixtures were warmed to 37°C and NADPH was then added to the mixtures to initiate the reactions. The mixtures were allowed to react for 30 min before being quenched with ice-cold acetonitrile (0.5 ml). For incubations with time-dependent inhibitors (furafylline, orphenadrine, diethylthiocarbamate, and 1-ABT), the inhibitors were preincubated with HLM in the presence of NADPH at 37°C for 15 min before the substrate was added. The mixtures were incubated for an additional 20 min at
37°C before being quenched with ice-cold acetonitrile (0.5 ml) containing dasatinib as IS (100 nM). The quenched reaction mixtures were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was analyzed by LC/MS to determine the concentration of M7.

**HLM Incubations in the Presence of P450 Antibody Inhibitors.** Experiments were conducted in triplicate at a final volume of 0.25 ml. Each reaction mixture consisted of HLM (250 μg protein/ml), brivanib (2 or 20 μM), NADPH (1 mM), MgCl2 (0.5 mM), and 5 μl of anti-P450 antibodies (anti-CYP1A2, anti-CYP2B6, anti-CYP2C9, anti-CYP2C19, anti-CYP2D6, or anti-CYP3A5) in phosphate buffer (100 mM, pH 7.4) (Gelboin and Krausz, 2006). Incubations without antibody were conducted as the positive controls. For each experiment, all ingredients were added to the Eppendorf tube except brivanib and NADPH. The mixture was preincubated for 20 min on ice, and then 10 min at 37°C. Brivanib and NADPH were added and the mixture was incubated for an additional 30 min at 37°C. After that time, the mixtures were quenched with ice-cold acetonitrile (0.25 ml) containing IS (100 nM). Samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was injected into LC/MS to determine the concentration of M7.

**Brivanib Concentration-Dependent Metabolite Formation.** The kinetics of M7 formation in HLM, cDNA-expressed CYP3A4, and cDNA-expressed CYP1A2 were determined at brivanib concentrations ranging from 1 to 80 μM. Because of the solubility limit of brivanib in aqueous solution, a further increase in concentration could not be achieved in the incubation mixtures. The formation of M7 was linear when brivanib was incubated with HLM (0.1 mg protein/ml), CYP3A4 (25 pmol/ml), or CYP1A2 (10 pmol/ml) up to 30 min at 10 μM concentration of brivanib. The formation of M7 was evaluated in triplicate at eight substrate concentrations (1, 2, 5, 10, 20, 40, 60, and 80 μM). The incubation mixtures consisted of brivanib (1–80 μM), NADPH (1 mM), MgCl2 (0.5 mM), and protein (HLM, 0.1 mg protein/ml; CYP3A4, 25 pmol/ml; or CYP1A2, 10 pmol/ml) in phosphate buffer (100 mM, pH 7.4). The mixtures were allowed to react for 20 min before being quenched with ice-cold acetonitrile (0.5 ml) containing IS (100 nM). The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was analyzed by LC/MS to determine the concentration of M7.

**Human Liver Cytosol-Mediated Sulfation.** Human liver cytosol-mediated sulfation of [14C]brivanib ([14C]brivanib alaninate was added to the incubation mixture), [13C]M7, and [13C]M26 was conducted at 37°C in a shaking water bath. Individual test compound (2 μl, 11.6 × 10−3 μCi/ml) was incubated with cytosol (1.0 mg protein/ml), PAPS (0.5 mM), and MgCl2 (0.5 mM) in potassium phosphate buffer (100 mM, pH 7.4) at a final volume of 0.5 ml. The mixture was allowed to react for 2 h before being quenched with ice-cold acetonitrile (0.5 ml). Negative control incubations were performed in the same manner but lacked either PAPS or cytosol. The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was injected into LC/MS for biotransformation profiling and mass-spectral analysis.

**Incubations with cDNA-Expressed SULTs.** Brivanib was incubated in triplicate with human cDNA-expressed SULTs (SULT1A1, SULT1A3, SULT1B1, SULT2A1, and SULT1E1) at 37°C at a final volume of 0.5 ml. The reaction mixtures consisted of protein (20 μg protein/ml), brivanib (2 or 20 μM), PAPS (0.5 mM), MgCl2 (0.5 mM), and phosphate buffer (50 mM, pH 7.4). The mixtures were allowed to react for 30 min before being quenched with ice-cold acetonitrile (0.5 ml) containing IS (100 nM). The samples were mixed by vortexing and were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 μl) was analyzed by LC/MS to determine the concentration of M25.

**High-Performance Liquid Chromatography Profiling and Metabolite Identification.** Biotransformation profiling was performed on a Shimadzu Class-VP high-performance liquid chromatography (HPLC) system equipped with two pumps (model LC-10AT), an autoinjector (SIL-10AD), and a diode array detector (SPC-M10A) (Shimadzu Europe). Chromatographic separation was achieved with a reverse-phase HPLC column (Zorbax SB C18, 2.1 × 150 mm, 5 μm; Agilent Technologies) at room temperature. A gradient with two solvents, 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 program started at an initial condition of 10% B, which was 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. For radio profiling, the HPLC eluate was split via a flow splitter (Dionex Co., Sunnyvale, CA) where 75% of eluate was collected into 96-well Deepwell LumaPlate plates at 0.25-min intervals (PerkinElmer Life and Analytical Sciences, Waltham, MA). The run time was 70 min. The remaining 25% of the eluate was directed into a Finnigan LTQ ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) for metabolite identification. The plates were dried in a Speed-Vac (Thermo Fisher Scientific), and the radioactivity was counted for 5 min per well with a Packard TopCount (PerkinElmer Life and Analytical Sciences).

Mass spectrometer analysis was performed on a Finnigan LTQ ion-trap mass spectrometer (Thermo Fisher Scientific). The electrospray ionization source was operated in the positive ion mode with the following parameters: voltage, 4 kV; current, 10 μA; and capillary temperature, 275°C. MS data were collected for a mass range of 100 to 1000 amu, and tandem mass spectrometry (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 maximal sensitivity. MS and MS/MS analyses were performed to identify the drug-related compounds in the samples.

**LC/MS/MS Quantification of M7 and M25.** Quantitative analysis of M7 and M25 was performed with a Shimadzu Class-VP (Shimadzu Europe) HPLC system interfaced with an API 4000 Q-trap mass spectrometer (MDS Sciex, Concord, ON, Canada) that was equipped with a Turboionspray source. The HPLC system was equipped with two pumps (model LC-10AT), an HTC PAL autosampler (LEAP Technologies, Carrboro, NC), and a diode array detector (SPC-M10A) (Shimadzu Europe). Chromatographic separation was achieved with a reverse-phase HPLC column (Zorbax SB C18, 2.1 × 150 mm, 5 μm; Agilent Technologies) at room temperature. A gradient with two solvents, A and B, was used for HPLC profiling. Solvent A consisted of 0.1% formic acid in water and solvent B was acetonitrile. The gradient program started at an initial condition of 10% B for 1 min, which was increased linearly to 90% over 14 min. The gradient was held at 90% B for 2 min and then was returned to the initial condition over 1 min. The HPLC flow rate was 0.3 ml/min. The Q-trap mass spectrometer was operated in positive electrospray ionization mode. Nitrogen was used as the nebulizer and auxiliary gas. The desolvation temperature was 300°C, and the source temperature was 150°C. Detection of M7, M25, and IS was achieved through multiple reaction monitoring. The individual selected reaction monitoring transitions were m/z 387.2 → m/z 329.2 for M7, m/z 451.4 → m/z 371.2 for M25, and m/z 488.2 → m/z 401.2 for IS. The standard curve ranged from 0.05 to 3.0 μg/ml and was fitted to a 1/x^2 weighted quadratic regression model.

**Data Analysis.** Radiochromatographic profiles were prepared by plotting the net counts-per-minute values obtained from the TopCount versus time after injection using Microsoft Excel (version 2007; Microsoft, Redmond, WA). For each radioprofile, the average counts-per-minute values of the first eight fractions were subtracted from the counts-per-minute values of each subsequent fraction. The K_{m} and V_{max} values of each enzyme were estimated by fitting the data to the Michaelis-Menten equation, V = V_{max} · S/(K_{m} + S), using a nonlinear regression analysis (GraphPad Prism, version 5.04; GraphPad Software Inc., San Diego, CA).

**Results**

**Hydrolysis of Brivanib Alaninate.** The involvement of human enzymes in the hydrolysis of brivanib alaninate was examined with two commercially available human CE enzymes, CE1 and CE2. Incubation of [14C]brivanib alaninate with CEs resulted in the formation of a new radioactive peak corresponding to brivanib (Supplemental Fig. S1). At 2 μM concentration of [14C]brivanib alaninate, approximately 81.7 and 79.3% of the prodrug, respectively, was converted to its active moiety in the presence of human CE1 and CE2. In a control experiment in which [14C]brivanib alaninate was incubated under similar conditions with no enzymes, brivanib was not observed in the reaction mixtures.
Metabolism of Brivanib in HLM. As an initial step toward the identification of the primary metabolic enzymes, incubations were conducted in HLM with [14C]brivanib alaninate as the substrate; the results are illustrated in Fig. 2A. The prodruk was converted completely via hydrolysis to brivanib after incubation in the presence and absence of NADPH. Approximately 8% of brivanib was further metabolized in HLM in the presence of NADPH. LC/MS analysis indicated that brivanib was metabolized primarily to a hydroxy metabolite with a protonated molecular ion at m/z 387. This metabolite had the same HPLC retention time, as well as MS and MS/MS fragmentation patterns, as M7 that was characterized in a previous study (Gong et al., 2011). In addition, a carboxylic acid derivative (M26) and a ketone metabolite (M31), formed at much lower levels than M7, were also observed in the incubation mixture. The structures of the metabolites, as well as the in vitro biotransformation pathways of brivanib, are illustrated in Fig. 3. Only a negligible amount of metabolites were formed in the absence of NADPH, and no metabolites were observed in the absence of enzymes.

Metabolism of Brivanib by cDNA-Expressed Human P450 Enzymes. To evaluate the activities of P450 enzymes for the formation of the primary metabolite M7, brivanib was incubated with individual human cDNA-expressed enzyme in the presence of NADPH. Concentrations of M7 were quantified with a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method and are summarized in Supplemental Table S1. Conversion of brivanib to M7 was catalyzed by CYP1A1, CYP1A2, CYP1B1, and CYP3A4, and to a lesser extent by CYP2D6, CYP3A5, CYP2C19, CYP2C8, CYP2A6, CYP2B6, and CYP2C9. To better estimate the relative contribution of each P450 enzyme to the overall clearance of brivanib in humans, the enzyme activities were normalized to the average content of each enzyme in HLM (Rodrigues, 1999). Results indicated that CYP1A2 and CYP3A4 would be predicted to be the major contributors for the formation of M7 (Fig. 4).

Chemical and Antibody Inhibition. To further confirm the primary P450 enzymes involved in brivanib metabolism, selective inhibitors of the P450 enzymes were evaluated for their inhibitory effects on the formation of M7. In the presence of 1-ABT, a general P450 inhibitor, formation of M7 was inhibited by ~95% relative to the positive control, suggesting that M7 was formed primarily by P450 enzymes (Fig. 5). When brivanib was incubated with HLM in the presence of furafylline, a selective inhibitor of CYP1A2, or ketoconazole, a selective inhibitor of CYP3A4/5, the formation of M7 was inhibited by ~60 and ~50%, respectively. Chemical inhibitors for other P450 enzymes showed little or no inhibitory effects on the formation of M7. Likewise, the formation of M7 was inhibited by approximately 55 and 50%, respectively, when incubated with monoclonal antibodies (Gelboin and Krausz, 2006) of CYP1A2 and CYP3A4/5 (Fig. 5).

Brivanib Concentration-Dependent Metabolite Formation. Kinetic studies were performed with HLM and cDNA-expressed CYP3A4 and CYP1A2 for the formation of the primary oxidative metabolite M7. The kinetic parameters were estimated using a nonlinear regression fitting of the data to the Michaelis-Menten equation and are summarized in Table 1. HLM, CYP3A4, and CYP1A2 showed K_m values of 29.1, 32.7, and 45.0 μM for the formation of M7, respectively. The V_max values were 89.1 (pmol/mg protein/min), 0.53 (pmol/pmol enzyme/min), and 1.39 (pmol/pmol enzyme/min), respectively, for HLM, CYP3A4, and CYP1A2.

Sulfation of Brivanib in Human Liver Cytosol. Figure 6A shows the biotransformation profiles of [14C]brivanib alaninate in human liver cytosol in the presence of PAPS, a cofactor of SULTs. Similar to HLM-mediated metabolism, the prodruk was not observed after incubation due to rapid hydrolysis. Metabolism of brivanib in cytosol in the presence of PAPS yielded a sulfate conjugate with HPLC retention time, as well as MS and MS/MS fragmentation patterns identical to M25, a direct sulfate conjugate of brivanib characterized previously (Gong et al., 2011).

Metabolism of Brivanib by Human SULTs. To identify the enzymes involved in the formation of M25, a panel of cDNA-expressed recombinant SULT enzymes (SULT1A1, SULT1A3, SULT2A1, SULT1B1, and SULT1E1) were screened for their activities. Results, summarized in Fig. 7, indicated that multiple SULT enzymes were involved in the formation of M25. Direct sulfation of brivanib was catalyzed by SULT1B1, SULT1E1, and SULT1A1 and, to a lesser extent, by SULT1A3 and SULT2A1.

Metabolism of [14C]M7 in HLM and Human Liver Cytosol. [14C]M7 was incubated with HLM or human liver cytosol to evaluate whether M26 was formed through the further oxidation of M7. Results, illustrated in Fig. 2B, indicated approximately 45% conversion of M7 to M26 in HLM in the presence of NADPH, with no other metabolites observed. No reaction occurred in HLM in the absence of NADPH. In addition, the formation of M26 was observed when [14C]M7 was incubated with human liver cytosol in the presence or absence of NADPH, with similar extent of conversion as in HLM.

Sulfation of [14C]M7 and [14C]M26 in Human Liver Cytosol. To investigate whether oxidative metabolites M7 and M26 can form sulfate conjugates, incubations of [14C]M7 and [14C]M26 were conducted in human liver cytosol in the presence of PAPS. The radiochromatographic profile of the reaction mixture of [14C]M26 displayed a major peak eluting at 33.2 min, with a protonated
molecular ion at \( m/z \ 481 \) (Fig. 6C) and major fragments at \( m/z \ 401 \) and \( m/z \ 343 \). Both the HPLC retention time and MS fragments were identical to those of M33 (Fig. 3) previously identified in humans after oral doses of \( [14C] \) brivanib alaninate (Gong et al., 2011). Likewise, the radioprofile (Fig. 6B) of the incubation mixture of M7 displayed a peak eluting at 31.3 min, with a protonated molecular ion at \( m/z \ 467 \) and MS fragment at \( m/z \ 387 \), consistent with the sulfate metabolite M19 (Fig. 3) (Gong et al., 2011). Metabolite M33 was also observed in the incubation mixture of M7. This metabolite was presumably formed through the oxidation of M7 to M26 and further sulfation of M26. In the absence of PAPS, no sulfate conjugates were observed.

**Oxidation of M25 by Human P450 Enzymes.** In theory, M19 and M33 can also be generated from M25 through P450-mediated oxidation. To test the possibility of this alternative metabolic pathway, the sulfate conjugate of brivanib, \( [14C]M25 \), was incubated with HLM in the presence of NADPH. The resulting radiochromatograms displayed two new radioactive peaks eluting at 31.4 and 33.2 min, with MS and MS/MS fragmentation identical to those of M19 and M33, respectively (Fig. 2C). In the absence of NADPH, the metabolism of M25 was negligible.

**Discussion**

A human absorption, distribution, metabolism, and excretion study with \( [14C] \) brivanib alaninate indicated that brivanib was extensively metabolized in humans before excretion (Gong et al., 2011). The primary metabolic pathways responsible for the elimination of brivanib in humans included both oxidation and sulfate conjugation. A number of metabolites, including M19, M25, M26, and M33, were identified in humans. Metabolites M25 and M26 resulted from direct sulfation and oxidation of brivanib, respectively, whereas M19 and M33 were generated through both oxidative metabolism and sulfation. Based on the human absorption, distribution, metabolism, and excretion results, it was estimated that at least 19% of the dose underwent oxidative metabolism, and 26% of the dose underwent direct sulfate conjugation. Approximately 35% of the dose underwent both oxidation and conjugative metabolism (M19 and M33). The current studies described here were conducted to characterize P450s and SULTs involved in the oxidation and sulfation of brivanib, respectively, with the aim to better predict the potential for drug-drug interactions either through polymorphism or through inhibition of a particular drug-metabolizing enzyme.

Brivanib alaninate was rapidly hydrolyzed when incubated with HLM and human liver cytosol, as well as in serum from animals and...
humans (Marathe et al., 2009). The reaction was presumably catalyzed by esterases because the prodrug hydrolysis in human plasma can be prevented through the inhibition of esterase activity (Fung et al., 2010; Gong et al., 2011). The presystemic hydrolysis of brivanib alaninate after oral administration to humans and animals (Marathe et al., 2009; Gong et al., 2011) suggested that the reaction was primarily catalyzed by the esterases localized in the intestine and/or liver. In the present study, we examined the activities of two human CEs for their activities. CE1 is primarily expressed in human liver, and CE2 is primarily localized in the intestine and liver (Satoh et al., 2002). Both enzymes were highly active in the hydrolysis of brivanib alaninate to brivanib, indicating possible roles of human CEs in the reaction. However, the current data do not rule out the possibility of the involvement of other intestinal and liver esterases in the hydrolysis. Furthermore, given that the brivanib alaninate is readily hydrolyzed in serum from humans and animals, it is expected that esterases expressed in other tissues are capable of hydrolyzing the prodrug. Taken together, these data suggest that multiple esterases are likely to be involved in the hydrolysis of brivanib alaninate.

P450 enzymes play a major role in drug metabolism and are responsible for the clearance of many drugs (Lamb et al., 2007). Alteration of the expression levels or the activities of these enzymes is the major cause of metabolic drug-drug interactions. Initial in vitro studies with HLM demonstrated that brivanib predominantly underwent hydroxylation, leading to the formation of M7. However, this primary in vitro oxidative metabolite was not observed in humans after administration of brivanib alaninate (Gong et al., 2011), suggesting further metabolism to form secondary metabolites (Fig. 3). To identify the P450 enzymes involved in the formation of M7, we screened the activities of a panel of 14 human cDNA-expressed P450 enzymes. Multiple enzymes were found to be involved in the hy-

### TABLE 1

Enzyme kinetic parameters for the formation of M7 in incubations of brivanib with HLM, cDNA-expressed CYP3A4, and cDNA-expressed CYP1A2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ ($\mu$M/min)</th>
<th>$CL_{int}$ ($\mu$L/min)</th>
<th>$V_{max}/K_m$</th>
<th>$CL_{int}/V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>29.1 ± 7.9</td>
<td>89.1 ± 8.8</td>
<td>3.1</td>
<td>0.016</td>
<td>0.031</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>32.7 ± 10.9</td>
<td>0.53 ± 0.11</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>45.0 ± 16.2</td>
<td>1.39 ± 0.25</td>
<td>0.031</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The unit for $V_{max}$ was picomole per milligram of protein per minute for HLM and picomole per micromole of enzyme per minute for expressed enzymes.

* The unit for $CL_{int}$ was microliter per milligram of protein per minute for HLM and microliter per micromole of enzyme per minute for expressed enzymes.
droxylation of brivanib, including CYP1A2, CYP3A4, and extrahepatic enzymes (CYP1A1 and CYP1B1). However, after taking into account the average enzyme content in HLM (Rodrigues, 1999), CYP1A2 and CYP3A4 were identified to be the major metabolic enzymes involved in the formation of M7 (Fig. 4). This conclusion was further supported by the inhibitory studies where a number of well documented chemical inhibitors and monoclonal antibodies of P450s were used to assess their inhibitory potential on brivanib biotransformation. HLM activity was inhibited significantly by chemical inhibitors or antibodies of CYP1A2 or CYP3A4, whereas inhibitors and antibodies of the other P450 enzymes had little or no inhibitory effects (Fig. 5). The contribution of CYP3A4 and CYP1A2 in the oxidation of brivanib is in agreement with the kinetic results which suggest that the $K_m$ for the formation of M7 in HLM is close to that observed in expressed enzymes. Even though CYP1A2 had a slightly higher rate of clearance than CYP3A4, based on the protein expression levels in HLM (CYP3A4 > CYP1A2) (Rodrigues, 1999), we expect both enzymes to have similar contribution to the formation of M7.

SULT-mediated sulfation is an important conjugative metabolism for many therapeutic drugs (Gamage et al., 2006; Li et al., 2008). The human absorption, distribution, metabolism, and excretion study showed that sulfate conjugation played an important role in the elimination of brivanib (Gong et al., 2011). Reaction phenotyping analysis with recombinant human SULTs indicated that SULT1B1, SULT1A1, and SULT1E1 were the most active enzymes toward the conjugation of brivanib. Other SULTs also catalyzed the reaction, albeit less efficiently (Fig. 7). The relative abundance of each SULT in human liver is critical in estimating the major enzyme involved in the formation of M25. Several studies have reported the quantitation of SULT enzyme expressions in human liver (Fujita et al., 1999; Homma et al., 2002; Nishimura and Naito, 2006; Teubner et al., 2007). In these studies, SULT1A1 was found to be the most abundant enzyme, whereas the expressions of SULT1B1, SULT1E1, and SULT1A3 were either low or not detected. It is thus anticipated that SULT1A1 would be one of the major contributors for the formation of M25. However, because of lack of specific inhibitors for various SULTs, the contribution of other SULTs to the formation of M25 cannot be ruled out completely.

Metabolite M7 was not detected as a significant metabolite in vivo, even though it was a primary metabolite in vitro. Therefore, experiments were conducted to understand the further metabolism of M7 to its secondary metabolites. Definitive structure characterization demonstrated that both M7 and M26 resulted from the oxidation of the methyl group on the indol of brivanib (Gong et al., 2011), and it was thus hypothesized that M7 was the precursor for the formation of M26. In vitro studies (Figs. 2 and 6B) suggested that indeed M7 could be further metabolized to M26 in HLM in an NADPH-dependent manner or in human liver cytosol in an NADPH-independent manner. This clearly indicates that M26 was formed in a two-step process: methyl oxidation to a hydroxy methyl, followed by further oxidation to give a carboxylic acid metabolite. Additional studies revealed that M7 could also undergo conjugative metabolism to M19 and M33 in human liver cytosol in the presence of PAPS (Fig. 6B).

There are two possible pathways leading to the formation of M19 and M33: 1) oxidation of brivanib to M7 and M26, followed by sulfate conjugation; and 2) sulfation of brivanib to M25, followed by P450-mediated oxidation (Fig. 3). The relative contribution of each pathway in vivo is important in determining the role of metabolic enzymes in the elimination of brivanib. If both M19 and M33 were formed exclusively through brivanib oxidation followed by sulfate conjugation, P450 enzymes would be responsible for the elimination of 54% of the dose in humans. On the other hand, if sulfate conjugation was
the first metabolic reaction, SULT enzymes would be the major enzymes responsible for the elimination of 67% of the dose in humans. To understand this, a series of in vitro studies were conducted in both HLM and human liver cytosol. Incubation of brivanib in HLM generated M7 and M26, which were converted to their corresponding sulfate conjugates when incubated individually with human liver cytosol in the presence of PAPS (Fig. 6, B and C). Likewise, brivanib underwent sulfate conjugation in cytosol to yield M25, which was further oxidized to M19 and M33 in HLM (Fig. 2). The results suggest that both pathways are feasible in vitro, and it is likely that in vivo, multiple pathways contributed to the overall clearance of brivanib.

The oxidation of brivanib to a ketone metabolite M31 (Fig. 3) was a minor metabolic pathway both in HLM-mediated incubations and in humans after administration of brivanib alaninate (Gong et al., 2011). However, this pathway may be underestimated because of the potential role of M31 back to both brivanib and possibly the enantiomer of brivanib. This is a potential mechanism of metabolite chiral inversion of brivanib to its enantiomeric metabolite. The relative concentration of the enantiomeric metabolite was not determined in the current study (detailed information on the brivanib chiral inversion will be described in a separate manuscript).

In summary, several approaches were adopted in the current study to identify primary enzymes involved in the metabolism of brivanib. Hydrolysis of brivanib alaninate to brivanib was efficiently catalyzed by human CEs localized in intestine and liver. CYP1A2 and CYP3A4 were the primary contributors for the oxidation of brivanib to M7, and multiple SULT enzymes were involved in the sulfation of brivanib. The results suggested that the overall clearance of brivanib does not depend on a single enzyme, and coadministration of brivanib with any specific enzyme inhibitor should not have a dramatic impact on brivanib exposures.

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Authorship Contributions

Participated in research design: Gong, Gan, and Iyer.

Conducted experiments: Gong.

Contributed new reagents or analytic tools: Gong.

Performed data analysis: Gong and Iyer.

Wrote or contributed to the writing of the manuscript: Gong, Gan, and Iyer.

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