Metabolism and Pharmacokinetics of Novel Selective Vascular Endothelial Growth Factor Receptor-2 Inhibitor Apatinib in Humans

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Non-standard abbreviations:

ABT, 1-aminobenzotriazole; AUC: Area under the concentration-time curve; CE, collision energy; CYP450, cytochrome P450; FMO, flavin-containing monooxygenase; HIMs, human intestinal microsomes; HLMs, human liver microsomes; HPMs, human pulmonary microsomes; HRMs, human renal microsomes; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

MDF, mass defect filter; MDR, multidrug resistance; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; PAI, pharmacological activity index; Q-TOF MS, quadrupole time-of-flight mass spectrometer; RTK, receptor tyrosine kinase; S.D., standard deviation; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra-performance liquid chromatography; VEGFR-2, vascular endothelial growth factor receptor-2.
Abstract

Apatinib is a new oral anti-angiogenic molecule that inhibits VEGFR-2. The present study aims to determine the metabolism, pharmacokinetics, and excretion of apatinib in humans and to identify the enzymes responsible for its metabolism. The primary routes of apatinib biotransformation included cis- and trans-cyclopentyl-3-hydroxylation, N-dealkylation, pyridyl-25-N-oxidation, 16-hydroxylation, dioxygenation, and O-glucuronidation after 3-hydroxylation. Nine major metabolites were confirmed by comparison with reference standards. The total recovery of the administered dose was 76.8% within 96 h post-dose, with 69.8% and 7.02% of the administered dose excreted in feces and urine, respectively. About 59.0% of the administered dose was excreted unchanged via feces. Unchanged apatinib was detected in negligible quantities in urine, indicating the systemically available apatinib was extensively metabolized. The major circulating metabolite was the pharmacologically inactive cis-3-hydroxy-apatinib-O-glucuronide (M9-2), the steady state exposure of which was 125% that of the apatinib. The steady state exposures of cis-3-hydroxy-apatinib (M1-1), trans-3-hydroxy-apatinib (M1-2), and apatinib-25-N-oxide (M1-6) were 56%, 22%, and 32% of parent drug exposure, respectively. Calculated as pharmacological activity index values, the contribution of M1-1 to the pharmacology of the drug was 5.42% to 19.3% that of the parent drug. The contribution of M1-2 and M1-6 to the pharmacology of the drug was less than 1%. Therefore, apatinib was a major contributor to the overall pharmacological activity in humans. Apatinib was primarily metabolized by CYP3A4/5 and, to a lesser extent, by CYP2D6, CYP2C9, and CYP2E1. UGT2B7 was the main enzyme responsible for M9-2 formation. Both UGT1A4 and UGT2B7 were responsible for trans-3-hydroxy-apatinib-O-glucuronide (M9-1) formation.
Introduction

New blood vessel formation (angiogenesis) is a critical step for tumor cell survival, proliferation, local invasion and metastasis (Carmeliet et al., 2000). Vascular endothelial growth factor receptors (VEGFRs) are receptor tyrosine kinases (RTKs) that function as key regulators of this process. The VEGFR-family proteins consist of three members: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4), among which VEGFR-2 is thought to be principally responsible for angiogenesis in malignancies (Glade-Bender et al., 2003). Small-molecule VEGFR inhibitors are widely used for the treatment of metastatic renal-cell carcinoma, gastrointestinal stromal tumors, hepatocellular carcinoma (Ivy et al., 2009), soft tissue sarcoma (Gennigens et al., 2012), and medullary thyroid cancer (Degrauwe et al., 2012), and in development of a number of other oncology indications, including colorectal cancer, non-small-cell lung cancer, pancreatic cancer, ovarian cancer, and breast cancer (Ivy et al., 2009).

Apatinib (N-[4-(1-cyano-cyclopentyl)phenyl]-2-(4-pyridylmethyl)amino-3-pyridine carboxamide, formerly known as YN968D1) is an orally administered small-molecule RTK inhibitor which selectively targets VEGFR-2 with an IC\textsubscript{50} of approximately 1 nM. Apatinib also less effectively inhibits the activities of PDGFR\textbeta, c-kit, and c-src which have all been implicated in the pathogenesis of human tumors (Song et al., 2005; Heinrich et al., 2002; Irby et al. 2000). Apatinib shows antitumor efficacy in mice when administered alone or in combination with chemotherapeutic drugs against a broad range of human tumor xenografts with good tolerance (Tian et al., 2011).

Apatinib reverses P-glycoprotein (ABCB1)- and ABCG2-mediated multidrug resistance (MDR) in drug-resistant solid tumor cells by inhibiting their transport function (Mi et al., 2010). It also targets to side population cells and
ABCB1-overexpressing leukemia cells to enhance the efficacy of chemotherapeutic drugs (Tong et al., 2012). These results suggest that apatinib may be used in combination with conventional ABCB1 and ABCG2 substrate chemotherapeutic drugs to overcome MDR in clinical cancer chemotherapy.

The results from a phase I clinical study conducted in patients with advanced solid tumors have shown that apatinib can be well tolerated and exhibits substantial antitumor activity across a broad range of malignancies at the recommended dose of 750 mg once daily (Li et al., 2010). The pharmacokinetic parameters of apatinib in plasma were also calculated using non-compartmental analysis. Apatinib is currently being evaluated in phase II/III clinical trials for the treatment of many advanced cancer types, including gastric carcinoma, hepatocellular carcinoma, breast cancer, and colorectal cancer.

Absorption, distribution, metabolism, and excretion studies have become an integral and critical part of drug development. The present study was undertaken to assess the metabolic profile, routes of excretion, and pharmacokinetics of apatinib in humans after single- or multiple-dose administration of 750 mg apatinib mesylate tablets (corresponding to 604 mg apatinib free base). Cytochrome P450 (CYP450) isozymes responsible for the oxidative metabolism of apatinib, as well as UDP-glucuronosyltransferase (UGT) isozymes involved in the O-glucuronidation of hydroxylated metabolites of apatinib, were also examined to help predict potential drug-drug interaction and variability in pharmacokinetics.
**Materials and Methods**

**Chemicals.** Apatinib mesylate tablets were manufactured by Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China) and used for clinical trials. Reference standards of apatinib mesylate, cis-3-hydroxy-apatinib (M1-1), trans-3-hydroxy-apatinib (M1-2), and vatalanib (internal standard for pharmacokinetic study) were also provided by Hengrui Medicine. The reference standard of apatinib-25-N-oxide (M1-6) was synthesized and purified in our laboratory. Reference standards of trans-3-hydroxy-16-hydroxy-N-dealkylated metabolite (M4-7), cis-3-hydroxy-16-hydroxy-N-dealkylated metabolite (M4-8), trans-3-hydroxy-25-N-oxide metabolite (M6-9), cis-3-hydroxy-25-N-oxide metabolite (M6-10), cis-3-hydroxy-O-glucuronide-N-dealkylated metabolite (M8-2), and cis-3-hydroxy-apatinib-O-glucuronide (M9-2) were isolated and purified from human urine in our laboratory. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): β-nicotinamide adenine dinucleotide 2’-phosphate reduced tetrasodium salt (NADPH), alamethicin, uridine 5’-diphosphoglucuronic acid (UDPGA), β-glucuronidase from *Helix pomatia* (type H-3), 1-aminobenzotriazole (ABT), sulfaphenazole, ticlopidine, quinidine, ketoconazole, chlormethiazole, racemic flurbiprofen, mefenamic acid, hecogenin, and all HPLC-grade solvents used for ultra-performance liquid chromatography-ultraviolet/quadrupole time-of-flight mass spectrometry (UPLC-UV/Q-TOF MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Other reagents used were of analytical grade (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China). Deionized water was purified by a Millipore Milli-Q Gradient Water Purification System (Molsheim, France). YMC*GEL® ODS-A-HG (12 nm S-50 μM, YMC Co. Ltd., Kyoto, Japan) was used to fractionate the urine samples before further purification.


Enzymes Sources. Pooled human liver microsomes (HLMs), recombinant human CYP450 isozymes (CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11), recombinant human flavin-containing monooxygenases (FMO1, FMO3, and FMO5), and recombinant human UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were purchased from BD Gentest (Woburn, MA, USA). Pooled human intestinal microsomes (HIMs), human pulmonary microsomes [HPMs(NS), non-smoker; HPMs(S), smoker], and human renal microsomes (HRMs) were purchased from Xenotech LLC (Lenexa, KS, USA).

Study Design, Dosing, and Sample Collection. Human studies were conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all of the subjects prior to enrollment.

Healthy Subject Study. This was a single-center, nonrandomized, open-label study. The study protocol and informed consent document were approved by the Ethics Committee of Teda International Cardiovascular Hospital (Tianjin, China). Twelve (six males and six females) healthy Chinese subjects with a mean age of 25.6 years (range 21 to 29 years) and a mean body mass index of 22.3 kg/m$^2$ (range 19.8 to 24.0 kg/m$^2$) participated in the study. The subjects were in good health as assessed by physical examination, without histories of drug or alcohol abuse, nonsmokers, and on no other medication at the time of the study. After at least a 10 h overnight fast, each subject received a single dose of 750 mg apatinib mesylate tablets (corresponding to 604 mg apatinib free base). Urine samples were collected at predose and between 0 to 4, 4 to 8, 8 to 12, 12 to 24, 24 to 36, 36 to 48, 48 to 72, and 72 to 96 h after dosing and
stored at –70 °C until analysis. Fecal samples were collected at pre-dose and after each bowel movement for up to 96 h post-dose and homogenized with 5 volumes of methanol. The fecal homogenates were stored at –70 °C until analysis.

**Patient Study.** This was a single-center, nonrandomized, open-label study. The study protocol was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (Shanghai, China). Patients with histologically confirmed advanced colorectal cancer for which no standard curative therapy was available were eligible for this study. Patient entry criteria included: age ≥ 18 years; an Eastern Cooperative Oncology Group performance status between 0 and 1; an estimated life expectancy ≥3 months; and adequate hematological, renal, and liver functions. Patients were excluded if they were pregnant or breast feeding, had a history of brain metastasis, uncontrolled hypertension, coronary disease or other significant cardiovascular disease, gastrointestinal disorder or other factors that could interfere with drug absorption, were on anti-coagulation therapy, had prior therapy with anti-VEGFR targeting agents, with a history of any other malignancy, or had recent prior chemotherapy, radiotherapy, or surgery.

Twenty (ten males and ten females) patients with a mean age of 55.2 years (range 36 to 68 years) and a mean body mass index of 24.3 kg/m\(^2\) (range 14.5 to 32.0 kg/m\(^2\)) participated in the study. These patients continuously received an oral dose of 750 mg apatinib mesylate tablets (corresponding to 604 mg apatinib free base) once daily for 28 d. Blood samples (2 ml to 3 ml) for metabolism and pharmacokinetic analyses were collected in heparinized tubes at 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h (before the next dose) on days 1 and 28, as well as 48 h after the last dosing. Samples were centrifuged at 1000 × g for 10 min to separate the plasma fractions. The plasma samples were stored at –20 °C until analysis.
Metabolite Profiling and Identification. Sample Preparation. Representative pooled human plasma, urine, and fecal homogenate samples were prepared for metabolite profiling. Equal volumes of plasma samples collected from all cancer patients at 0 h (pre-dose) and 4 and 8 h after the last dosing were pooled by time point. Excreta (urine and fecal homogenate) samples from all healthy subjects were pooled in proportion to the total sample weight over 24 h intervals. For every 200 μl aliquot of pooled plasma, urine, and fecal homogenate samples, 400 μl of acetonitrile was added. After vortex-mixing and centrifugation at 11000 × g for 5 min, the supernatants were evaporated to dryness under a stream of nitrogen at 40 °C. The residues were then reconstituted in 100 μl of acetonitrile-water (1:9). Reconstituted extract of pooled urine and fecal homogenate samples were diluted at ratios of 1:4 and 1:40, respectively, with acetonitrile-water (1:9) prior to analysis to achieve proper mass response. A 3 μl aliquot of the resulting solution was injected for metabolite identification.

UPLC-UV/Q-TOF MS Analysis. The LC separation of the metabolites was performed on an Acquity UPLC system (Waters Corporation, Milford, MA, USA) using an Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm particle size, Waters Corporation). The column temperature was set to 40 °C, and the flow rate was kept to 450 μl/min. The mobile phase consisted of solvent A (5 mM ammonium acetate solution with 0.05% formic acid) and solvent B (acetonitrile), and a gradient method was used. The mobile phase was initially composed of solvent A/solvent B (95:5) and held for 2 min, then linearly programmed to solvent A/solvent B (40:60) over 13 min and held for 1 min and to solvent B (100%) for 1 min. The mobile phase condition was returned to the starting solvent mixture and allowed to equilibrate for 3 min before the next injection. The effluent was monitored using a UV detector at 340
Mass spectrometry was performed using a Synapt Q-TOF high-resolution mass spectrometer (Waters Corporation) operating in positive ion electrospray mode. Nitrogen and argon were used as desolvation and collision gases, respectively. The capillary and cone voltages were set to 3.5 kV and 50 V, respectively. The desolvation gas was set to 700 l/h at a temperature of 350 °C. The source temperature was set to 100 °C. The scan range was set at $m/z$ 80−1000. All analyses were acquired using Lock Spray™ to ensure accuracy and reproducibility. Leucine-enkephalin ($m/z$ 556.2771) at a concentration of 400 ng/ml and a flow rate of 3 μl/min was used for the lock mass. Data were collected in centroid mode. Data acquisition was performed using an alternating low and elevated collision energy (CE) scan mode ($MS^E$), which allowed for the simultaneous acquisition of molecular ion and fragmentation ion data for all detected analytes within one single experiment. At low CE, the transfer and trap CEs were 2 and 4 eV, respectively. At high CE, the transfer CE was 15 eV and trap CE ramped from 5 eV to 25 eV.

Instrument control and post-acquisition analyses were performed using a MetaboLynx™ (v4.1) program (Waters Corporation), which utilizes mass defect filtering (MDF) and the Dealkylation™ tool to generate a series of extracted ion chromatograms (XICs). The XICs correspond to the ion currents that fall within a 40 mDa window around the $m/z$ values of the expected metabolites. The XICs of the control and samples were compared to eliminate the chromatographic peaks in the samples that also appear in the control (Zhang et al., 2008). Fragmentations were proposed using the Mass Frontier 7.0 software (Thermo Fisher Scientific Inc., San Jose, CA, USA).

**Enzyme Hydrolysis.** Pooled human plasma and urine samples were adjusted to pH
5 using citrate buffer and treated with 2000 units of β-glucuronidase. The mixture was incubated in a water bath at 37 °C for 16 h. Then the mixtures were treated as described above and analyzed. Incubated plasma and urine samples without the enzyme served as control.

*Reduction of N-oxide Metabolites with Titanium Trichloride (TiCl₃).* To identify N-oxides, the pooled human plasma and urine samples were treated with TiCl₃, which can selectively reduce N-oxides to their corresponding amines (Kulanthaivel et al., 2004). TiCl₃ solution (20 µl) was added to a 200 µl aliquot of plasma and urine. The mixtures were kept on ice for 2 h and neutralized with potassium hydroxide. Then the reaction mixtures were treated as described above and analyzed.

*Synthesis of Apatinib-25-N-oxide.* A solution of apatinib mesylate (100 mg in 1 ml methanol) was added to a solution of m-chloroperbenzoic acid (100 mg in 20 ml dichloromethane) and stirred at room temperature for 24 h (Chelucci et al. 2008). The mixture was evaporated and the residue was further purified using a Shimadzu LC-6AD semi-preparative HPLC apparatus equipped with a SPD-20A UV detector. Separation was achieved using a YMC-Pack ODS-A column (10 × 250 mm I.D., 5 µm; YMC Company Ltd., Kyoto, Japan). The column oven was set to room temperature. Elution was performed using methanol-water (70:30, v/v) at a flow rate of 3 ml/min. The detection wavelength was set to 340 nm. The eluate at 9.8 min was collected and evaporated to obtain the synthetic standard of M1-6 (70% yield).

*Preparation of the Major Metabolites from Human Urine.* Pooled bulk urine samples were precipitated with methanol and concentrated to dryness. The residue was dissolved in 50 ml of anhydrous methanol-water (1:9) and fractionated using YMC*GEL® ODS-A-HG column chromatography (10% to 100% anhydrous methanol), yielding seven fractions (A to G). Fraction D eluted by methanol-water
(4:6) and Fraction E eluted by methanol-water (5:5) were further purified on a Shimadzu LC-6AD semi-preparative HPLC apparatus equipped with an SPD-20A UV detector. Chromatographic separation was achieved using a YMC-Pack ODS-A column (10 × 250 mm I.D., 5 µm; YMC Company Ltd.). The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (methanol), and a gradient method was used. The detection wavelength was set to 340 nm. Fraction D afforded M8-2 (26 mg), and fraction E afforded M4-7 (3 mg), M6-9 (8 mg), M6-10 (15 mg), and M9-2 (28 mg).

**Quantification of Apatinib and Its Major Metabolites in Human Plasma.**
Apatinib, M1-1, M1-2, M1-6, and M9-2 were simultaneously determined utilizing a previously reported LC-MS/MS method (Ding et al., 2012). M9-1 was also semi-quantified using M9-2 as the calibration standard.

**Pharmacokinetic Analysis.** Standard noncompartmental methods were used to determine the pharmacokinetic parameters of apatinib and its metabolites using WinNonlin (Version 5.3, Pharsight, Mountain View, CA, USA). The peak plasma concentration ($C_{\text{max}}$) and time to reach $C_{\text{max}}$ ($T_{\text{max}}$) were taken directly from the pharmacokinetic concentration-time data. The elimination rate constant ($k_e$) value was estimated using least-squares regression of the terminal log-linear phase of the concentration-time curve, and the elimination half-life ($t_{1/2}$) was calculated as $\ln(2)/k_e$. The area under the plasma concentration–time curve (AUC$_{0-24\text{h}}$) was calculated using the linear-log trapezoidal method. The accumulation ratios (day 28/day 1) for AUC$_{0-24\text{h}}$ and $C_{\text{max}}$ were also calculated.

**Quantification of Apatinib and Its Major Metabolites in Human Urine and Feces.** The concentrations of apatinib and its metabolites M1-1, M1-2, M1-6, M4-7, M6-9, M6-10, M8-2, and M9-2 in urine samples, as well as apatinib and its
metabolites M1-1, M1-2, M1-6, and M6-9 (for the semi-quantification of total dioxygenated metabolites) in fecal samples, were determined using the LC-MS/MS method. This method was partially validated including selectivity, linearity, intra-day precision and accuracy, matrix effect, recovery, and stability.

The preparation procedures for the urine samples were similar to those for the plasma, except that the resulting solution was diluted 10-fold with mobile phase before analysis. To a 10 µl aliquot of fecal homogenous, 960 µl of IS solution (500 ng/mL vatalanib) was added, and the mixture was thoroughly vortex-mixed. A 20 µl aliquot of the resulting solution was mixed with 180 µl of mobile phase before analysis. The LC-MS/MS analytical conditions for urine and fecal sample analysis were similar to those for plasma, except that more reaction monitoring transitions were selected: m/z 339 to 137 for M4-7, m/z 430 to (212+108) for M6-9 and M6-10, and m/z 499 to 323 for M8-2 were added for urine analysis; m/z 430 to 430 for M6-9 was added for fecal sample analysis. For urine sample analysis, the method was linear over the concentration range of 30.0–10000 ng/ml for each analyte. For fecal sample analysis, the method was linear over the concentration ranges of 2.50–2000, 0.500–400, 0.250–200, 0.250–200, and 2.50–200 µg/g for apatinib, M1-1, M1-2, M1-6, and M6-9, respectively. The amount of apatinib and its major metabolites excreted in urine and feces during each collection interval was calculated by multiplying the concentration of each analyte by the volume or weight of the matrix collected over that interval. The total urinary recovery or fecal recovery was calculated as the cumulative amount excreted over all the collection periods and expressed as a percentage of the administered dose.

The Binding of Apatinib and Its Major Metabolites to Human Plasma Proteins.

The binding of apatinib, M1-1, M1-2, and M1-6 to human plasma proteins was
measured by ultracentrifugation with similar methods as described previously (Verbeke et al., 1979). Triplicate samples of apatinib, M1-1, M1-2, and M1-6 at concentrations in the range of therapeutic plasma levels (500 ng/ml for apatinib; 50 ng/ml for M1-1, M1-2, and M1-6) were tested, and data are presented as mean ± standard deviation.

**In Vitro Pharmacological Activity of Major Metabolites.** Metabolites M1-1, M1-2, M1-6, and M9-2 were screened for their activity toward the inhibition of VEGFR-2, PDGFRβ, and c-kit RTKs utilizing a previously reported assay format (Hunt et al., 2004). For comparison, the pharmacological activity of apatinib was also evaluated under the same conditions. A pharmacological activity index (PAI) was calculated by dividing the unbound concentration/in vitro potency ratio of each major metabolite by the corresponding ratio of the parent drug, to quantify the contribution of each major metabolite to the overall effect of the drug (Leclercq et al., 2009). As described previously, a PAI value > 25% means the metabolite may make a significant contribution to the effect of the drug activity (Smith et al., 2005).

**Oxidative Metabolism of Apatinib in Microsomes and Recombinant CYP450 and FMO Isoforms.** Recombinant human CYP enzymes (CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11) or FMO enzymes (FMO1, FMO2, and FMO3) or microsomes (HLMs, HIMs, HPMs, and HRMs) were incubated with apatinib. The incubation mixtures (200 µl) contained phosphate buffer (100 mM, pH 7.4), P450 (50 pmol P450/ml) or FMO (0.25 mg protein/ml) or microsomes (1 mg protein/ml), apatinib (2 µM), and NADPH (2 mM). After 1-h incubation at 37 °C, ice-cold acetonitrile (200 µl) was added to each incubation to stop the reaction. Incubations were performed in duplicate and analyzed by LC-MS/MS and UPLC-UV/Q-TOF MS.
HLM Incubations in the Presence of Selective CYP450 Inhibitors. The duration of incubation and the protein concentrations used above were useful for initial experiments aimed at identification of metabolites in microsomal incubations, but they may not represent linear conditions. To determine conditions that are linear for time of incubation and protein concentration, 2 µM apatinib was incubated in HLMs (0–1.0 mg protein/ml) or P450 enzymes (0–50 pmol P450/ml, CYP3A4, CYP3A5, or CYP2D6) with 2 mM NADPH at 37 °C across a range of incubation times (0–90 min). Based on the results obtained, a 10-min incubation and a final HLM concentration of 0.2 mg protein/ml and P450 enzyme concentration of 10 pmol P450/ml represented conditions that are linear and were used in the subsequent experiments.

The incubation mixtures (200 µl in duplicate) contained phosphate buffer (100 mM, pH 7.4), HLM (0.2 mg protein/ml), apatinib (2 µM), NADPH (1 mM), and a single selective P450 inhibitor. The chemical inhibitors used were α-naphthoflavone (1 µM) for CYP1A1/2, sulfaphenazole (6 µM) for CYP2C9, ticlopidine (10 µM) for CYP2B6 and CYP2C19, quinidine (2 µM) for CYP2D6, ketoconazole (1 µM) for CYP3A4/5, chlormethiazole (24 µM) for CYP2E1, and ABT (1 mM) for all the P450s. Metabolism-dependent inhibitor ticlopidine was preincubated with HLM in the presence of NADPH for 10 min before the substrate was added. After substrate addition, the samples were then incubated at 37 °C for 10 min. Reactions were terminated by the addition of equal volume of ice-cold acetonitrile. The formations of M1-1, M1-2, M1-3, M1-5, M1-6, M2, and M3-1 in the presence of inhibitors were compared to metabolites formed in their absence.

To provide additional evidence for the contribution of FMOs to the formation of M1-6 (pridine N-oxide), HLMs were heat-treated at 45°C for 5 min before incubation with apatinib. These treatment conditions would be expected to inactivate the
heat-sensitive FMOs but do not appreciably affect P450 enzyme activities (Tugnait et al., 1997). Incubation conditions and sample preparation were similar as described above.

**The Formation of M1-1, M1-2, and M1-6 by Recombinant CYP450 and FMO Isoforms.** To further probe the specific isoforms involved in the formation of major oxidative metabolites (M1-1, M1-2, and M1-6), 2 $\mu$M apatinib was incubated with recombinant human CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11 (10 pmol P450/ml) or FMO1, FMO2, and FMO3 (0.2 mg protein/ml) at 37°C for 10 min (final incubation volume 200 $\mu$l). The incubations were initiated by addition of 1 mM NADPH and terminated by addition of 200 $\mu$l of ice-cold acetonitrile. Incubations were conducted in duplicate and analyzed by LC-MS/MS.

**In vitro enzyme kinetic study.** Enzyme kinetic parameters for the formation of M1-1, M1-2, and M1-6 were evaluated in HLM (0.2 mg protein/ml) and recombinant CYP3A4, CYP3A5, and CYP2D6 enzymes (10 pmol P450/ml). Incubations were performed at 37 °C for 10 min. The substrate concentration ranged from 0.1 to 15 $\mu$M. Data are presented as averages of triplicate experiments. Kinetic constants were obtained by fitting Michaelis–Menten equation ($V = V_{\text{max}} \times S/(K_m + S)$) to experimental data using nonlinear regression (Prism 5.0, GraphPad Software Inc., San Diego, CA). $V$ is the reaction velocity, $V_{\text{max}}$ is the maximum velocity, $K_m$ is the Michaelis constant (substrate concentration at 0.5 $V_{\text{max}}$), and $S$ is the substrate concentration. *In vitro* intrinsic clearance (CL$_{int}$) was calculated as $V_{\text{max}}/K_m$. The fitness of the model was investigated based on visual inspection of the Eadie-Hofstee plots (corresponding to $V$ against $V/S$) and calculated correlation coefficient ($R^2$).

**Phase II Microsomal Incubations.** The protein content and time linearity were proven in the preceding analysis. M1-1 and M1-2 (20 $\mu$M) were incubated with HLMs,
HIMs, HPMs, and HRMs (0.5 mg protein/ml in 100 mM Tris-HCl buffer containing 8 mM MgCl₂ and 25 μg/ml alamethicin, pH 7.5) at 37 °C for 1 h with or without 2 mM UDPGA in a total volume of 200 μl. After preincubation of the reaction mixtures for 5 min at 37°C, the reactions were initiated by the addition of UDPGA and terminated by the addition of 200 μl ice-cold acetonitrile. Incubations were performed in duplicate and analyzed by UPLC-UV/Q-TOF MS.

**O-Glucuronidation of M1-1 and M1-2 by Recombinant UGT Isoforms.** The enzymes involved in the O-glucuronidation of M1-1 and M1-2 were determined using a panel of recombinant human UGT enzymes, including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17. The concentration of the UGT enzymes was 0.5 mg protein/ml. Incubations were performed in duplicate and analyzed by LC-MS/MS.

**Inhibition of O-Glucuronidation of M1-1 and M1-2 by Selective UGT Inhibitors.** Mefenamic acid (Mano et al., 2007a) and flurbiprofen (Mano et al., 2007b), two potent inhibitors of UGT2B7, as well as hecogenin (Uchaipichat et al., 2006), a potent inhibitor of UGT1A4, were tested for their inhibitory effect on the O-glucuronidation of M1-1 and M1-2 in HLMs. To determine the IC₅₀ values of these inhibitors, M1-1 and M1-2 (20 μM) were incubated in the absence and presence of mefenamic acid (0.2–20 μM), racemic flurbiprofen (1–200 μM), or hecogenin (1–100 μM) in HLMs. Incubations were performed in duplicate and analyzed by LC-MS/MS.
Results

**Metabolite Profiling and Identification.** Metabolites of apatinib in human plasma, urine, and fecal samples were identified based on the generated elemental composition of ions obtained from accurate mass measurements, specific MS fragmentation pattern and co-chromatography (when standard available). The chromatographic and MS fragmentation behaviors of apatinib were first investigated to aid in the structural assignment of the metabolites. Apatinib had a retention time of 11.4 min on the HPLC system and showed a protonated molecule ion at m/z 398.189. The high CE mass spectrum (Fig. 1A) showed a major fragment ion at m/z 212.082, which was formed through cleavage of the nicotinamide amide bond and charge retention on the pyridyl moiety. The fragment ion at m/z 371.190 was formed through the cleavage of the cyano group. The fragment ion at m/z 184.087 represented the picolyl-aminopyridine moiety. The fragment ion at m/z 92.049 represented the picolyl group. Based on the MS fragment pattern, the structure of apatinib was divided into parts A, B, and C (Fig. 1B). The structures of the metabolites were then tentatively characterized by determining the changes in the m/z values of these three segments. The proposed MS fragmentation pattern of apatinib is shown in Fig. 1B.

After being processed using the MDF and dealkylation techniques, a total of 23, 35, and 14 metabolites of apatinib were detected in human plasma, urine, and feces, respectively (Figs. 2A, 2C and 2E). The corresponding UV chromatograms are shown in Figs. 2B, 2D, and 2F, respectively. Based on the UV chromatograms, M9-2 was the major circulating drug-related component at steady state, followed by M0, M1-1, M1-6, and M1-2. The major components in urine included M1-1, M1-2, M6-9, M6-10, M8-2, and M9-2. M0 was the predominant component in feces, followed by M1-1, M6-7, M6-5, and M1-2. Table 1 lists the detailed information of the possible
metabolites of apatinib, including protonated molecule ion, proposed elemental composition, retention time of each chromatographic peak, relative MS peak area, and the characteristic fragment ions. The proposed metabolic pathways of apatinib in humans are shown in Fig. 3. A description of the information used for the assignment of each metabolite is detailed in the supplemental data. Nine major metabolites (M1-1, M1-2, M1-6, M4-7, M4-8, M6-9, M6-10, M8-2, and M9-2) were confirmed by comparison with reference standards. The $^1$H nuclear magnetic resonance (NMR) data of reference standards are listed in Table 2.

**Pharmacokinetic Evaluation in Patients with Advanced Colorectal Cancer.** The plasma concentration-time profiles of apatinib and its metabolites M1-1, M1-2, M1-6, M9-1, and M9-2 after oral administration of 750 mg apatinib mesylate once daily for 28 d to patients with advanced colorectal cancer are shown in Fig. 4. A summary of their main pharmacokinetic parameters is presented in Table 3.

After the first dose, the $C_{\text{max}}$ of apatinib, M1-1, M1-2, M1-6, M9-1, and M9-2 were achieved approximately 2.9 to 10.2 h post-dose, with mean values of 3819, 849, 339, 375, 36.5, and 1543 nmol/l, respectively. During the first 24 h after dosing, the systemic exposures of M1-1, M1-2, M1-6, M9-1, and M9-2 (based on molar concentrations) were approximately 40%, 16%, 21%, 2%, and 88% that of the parent drug, respectively.

After the last dose, the $C_{\text{max}}$ of apatinib, M1-1, M1-2, M1-6, M9-1, and M9-2 were achieved approximately 4.7 to 11.5 h post-dose, with mean values of 3935, 1470, 571, 786, 94.3, and 2843 nmol/l, respectively. During the first 24 h after dosing, the systemic exposures of M1-1, M1-2, M1-6, M9-1, and M9-2 (based on molar concentrations) were approximately 56%, 22%, 32%, 5%, and 125% that of the parent drug, respectively. Therefore, M9-2 was the major circulating drug-related component.
at steady state, followed by parent drug, M1-1, M1-6, M1-2, and M9-1. The $t_{1/2}$ of apatinib, M1-1, M1-2, M1-6, M9-1, and M9-2 averaged 18.6, 24.7, 23.2, 21.2, 26.3, and 23.8 h, respectively, indicating that systemic clearance for metabolites was slightly slower than that for the parent compound. After repeated administration, the AUC$_{0-24\text{h}}$ accumulation ratios of apatinib and its metabolites were 1.77 to 3.69. The $C_{\text{max}}$ accumulation ratios of apatinib and its metabolites were 1.16 to 3.01, respectively. Gender difference in the pharmacokinetics of apatinib in humans was not observed (data not shown).

**Excretion of Apatinib in Humans.** The cumulative recovery of total drug-related compounds in urine, feces, and the sum of urine and feces after a single oral administration of 750 mg apatinib mesylate to 12 healthy subjects is shown in Table 4. The total drug-related compounds were excreted predominantly in feces (69.8% ± 16.1% of dose) and only a minor proportion was excreted renally (7.02% ± 1.77% of dose). The average cumulative excretion was 76.8% ± 14.9%. As shown in Fig. 5, 59.0% of the administered dose was excreted as unchanged drug in feces. The metabolites M1-1, M1-2, M1-6, and total dioxygenated metabolites excreted in feces accounted for 2.76%, 1.20%, 0.02%, and 6.80% of the administered dose, respectively. Unchanged apatinib was detected only in negligible quantities in urine. The metabolites M1-1, M1-2, M1-6, M4-7, M6-9, M6-10, M8-2, and M9-2 excreted in urine accounted for 0.567%, 0.386%, 0.982%, 0.101%, 0.472%, 1.10%, 1.87%, and 1.53% of the dose, respectively. Gender difference in the excretion of apatinib was not observed.

**The Binding of Apatinib and Its Major Metabolites to Human Plasma Proteins.** The percentage protein binding of apatinib, M1-1, M1-2, and M1-6 at concentrations in the range of therapeutic plasma levels are listed in Table 5.
In Vitro Pharmacological Activity of Major Metabolites. In vitro pharmacological study results of major metabolites are listed in Table 5. M1-1 potently inhibited VEGFR-2, PDGFRβ, and c-kit with IC₅₀ of 19, 47, and 125 nM, respectively. M1-2 moderately inhibited VEGFR-2, PDGFRβ, and c-kit with IC₅₀ of 179, 1352, and 2511 nM, respectively. M1-6 moderately inhibited VEGFR-2, PDGFRβ, and c-kit with IC₅₀ of 265, 369, and 981 nM, respectively. M9-2 did not inhibit the kinase activity of either enzyme to any significant extent. The PAI values of M1-1 toward VEGFR-2, PDGFRβ, and c-kit were 5.42%, 19.3%, and 5.66% compared with parent drug, respectively. The PAI values of M1-2 and M1-6 toward VEGFR-2, PDGFRβ, and c-kit were all < 1% compared with the parent drug.

Oxidative Metabolism of Apatinib in Microsomal and CYP Enzyme Incubations. As shown in Fig. 6, after 1-h microsomal or CYP enzyme incubations, approximately 50% of the parent compound was consumed in the HLM incubations, whereas only 13% was utilized in the HIM incubations. Apatinib was not metabolized by HRMs and HPMs. Therefore, the liver was suspected to be the major oxidative biotransformation site for apatinib. More than 95% of the parent compound was consumed in CYP2D6, CYP3A4, and CYP3A5 incubations. Within 30% to 50% of the parent compound was consumed in CYP2C9 and CYP2E1 incubations. Within 5% to 15% of the parent compound was consumed in CYP1B1, CYP2C8, and CYP2C19 incubations. Less than 5% of the parent compound was consumed in CYP1A2, CYP2A6, CYP2B6, CYP4A11, FMO1, FMO3, and FMO5 incubations, indicating that these CYP and FMO enzymes contributed minimally to the metabolism of apatinib.

In an NADPH-dependent manner, apatinib was metabolized by HLMs into seven metabolites: M1-1, M1-2, M1-3, M1-5, M1-6, M2, and M3-1. M1-2 was the main in
vitro metabolite based on the peak areas obtained. The absence of NADPH in the incubations implies the absence of any metabolites.

**HLM Incubations in the Presence of Selective CYP450 Inhibitors.** The inhibition results generated from selective chemical inhibitors on the formation of M1-1, M1-2, M1-3, M1-5, M1-6, M2, and M3-1 in HLM incubations are shown in Fig. 7. Compared with the control samples without inhibitors, incubation with α-naphthoflavone (CYP1A1/2 inhibitor) and chlormethiazole (CYP2E1 inhibitor) had no substantial effect on the formation of all the metabolites. Sulphaphenazole (CYP2C9 inhibitor) inhibited the formation of M1-1, M1-2, M1-3, M1-6, and M2 by 18% to 39%, and did not inhibit the formation of M1-5 and M3-1. Ticlopidine (inhibitor of CYP2B6 and CYP2C19) inhibited the formation of M1-2 and M1-3 by 17% and 29%, respectively, and did not inhibit the formation of other metabolites. Quinidine (CYP2D6 inhibitor) inhibited the formation of M1-1, M1-2, M1-3, and M1-6 by 18% to 36%, and did not inhibit the formation of M1-5, M2, and M3-1. Ketoconazole (CYP3A4/5 inhibitor) inhibited the formation of M1-1, M1-2, M1-3, M1-6, M2, and M3 by 44%, 82%, 91%, 66%, 75%, and 96%, respectively, but did not inhibit the formation of M1-5. ABT (inhibitor of all the CYP enzymes) inhibited the formation of M1-1, M1-2, M1-3, M1-5, M1-6, M2, and M3 by 78%, 95%, 100%, 46%, 97%, 82%, and 100%, respectively, suggesting the involvement of CYP450s in all metabolites yield. Heat treatment of HLM did not affect M1-6 (pridine-N-oxide) formation, thus excluding involvement of FMOs in M1-6 formation.

**The Formation of Major Oxidative Metabolites by Recombinant CYP450 Isoforms.** As shown in Fig. 8, FMOs were not capable of apatinib metabolism. All tested CYP isoforms were capable of M1-1 and M1-2 formation. M1-1 was produced to the greatest extent by CYP2D6 (0.226 pmol/min/pmol protein), and to a lesser
extent by CYP3A4 (0.037 pmol/min/pmol protein) and CYP3A5 (0.014 pmol/min/pmol protein). Other CYPs also catalyzed the production of M1-1 but with much less extent. M1-2 was produced to the greatest extent by CYP3A4 (0.748 pmol/min/pmol protein) and CYP3A5 (0.710 pmol/min/pmol protein). M1-6 was predominantly formed by CYP2D6 (1.28 pmol/min/pmol protein), and to a lesser extent by CYP3A4 (0.210 pmol/min/pmol protein) and CYP3A5 (0.148 pmol/min/pmol protein).

**In vitro enzyme kinetic study.** The formation of M1-1, M1-2, and M1-6 by HLMs, CYP3A4, CYP3A5, and CYP2D6 exhibited Michaelis-Menten kinetics (Fig. 9). Table 6 presents the enzyme kinetic variables for the formation of M1-1, M1-2, and M1-6 in HLMs, CYP3A4, CYP3A5, and CYP2D6. $V_{\text{max}}$ and $K_m$ values for the formation of M1-1, M1-2, and M1-6 in CYP3A4 were all higher than that observed in CYP3A5, resulting the comparable in vitro intrinsic clearance ($V_{\text{max}}/K_m$) in CYP3A4 and CYP3A5. The high $V_{\text{max}}$ and $CL_{\text{int}}$ values for the formation of M1-1 and M1-6 in CYP2D6 suggest that CYP2D6 has a high capability for the formation of M1-1 and M1-6. Enzyme kinetics of M1-2 formation in CYP2D6 was not determined because CYP2D6 catalyzed the formation of negligible M1-2.

**Microsomal Conjugation of M1-1 and M1-2.** M9-2 and M9-1 are cis and trans isomers. M9-2 was the major circulating drug-related component at steady state, while M9-1 was present only in negligible quantities in human plasma. To investigate the selectivity, M1-1 and M1-2 were individually incubated with HLMs, HIMs, HPMs, and HRMs. O-glucuronidation of M1-1 to form M9-2 in the HLMs and HRMs was similar, while negligible amounts of M9-2 were formed in HIMs and HPMs (data not shown). The highest M9-1 formation was observed in HLMs. The formation of M9-1 in HRMs was approximately 50% that in HLMs. Negligible amounts of M9-1 were
formed in HIMs and HPMs (data not shown). In HLMs, the formation of M9-2 was eight times higher than that of M9-1. In HRMs, the formation of M9-2 was sixteen times higher than that of M9-1.

**UGT Enzymes Responsible for the Glucuronidation of M1-1 and M1-2.** M1-1 and M1-2 were incubated with 12 individual UGT isoforms to identify the UGT enzymes responsible for the formation of M9-2 and M9-1 (Fig. 10). UGT1A1, UGT1A3, UGT1A4, UGT1A8, UGT1A9, UGT2B4, UGT2B7, and UGT2B17 all catalyzed the formation of M9-2, with UGT2B7 having the highest activity. UGT1A1, UGT1A3, UGT1A4, UGT1A8, UGT2B4, and UGT2B7 all catalyzed the formation of M9-1, with UGT1A4 having the highest activity, followed by UGT2B7. UGT2B7 is abundantly expressed in the liver, small intestine, colon, and kidney, while UGT1A4 is only expressed at relatively low levels in the liver (Ohno et al., 2009). Thus, UGT1A4 and UGT2B7 may both play important roles in the formation of M9-1.

The inhibitory effect of mefenamic acid (UGT2B7 inhibitor), racemic flubiprofen (UGT2B7 inhibitor), and hecogenin (UGT1A4 inhibitor) on the formation of M9-2 and M9-1 in HLMs was evaluated. As shown in Fig. 11, mefenamic acid inhibited M9-2 and M9-1 formation in HLMs with IC50 of 2.64 µM (2.17 to 3.23 µM) and 8.35 µM (6.74 to 10.7 µM), respectively. Racemic flubiprofen inhibited M9-2 and M9-1 formation in HLMs with IC50 of 13.6 µM (10.6 to 17.0 µM) and 44.4 µM (36.8 to 54.2 µM), respectively. Hecogenin showed little inhibitory effect on M9-2 formation. The IC50 of hecogenin was 45.7 µM (24.1 to 138 µM) for M9-1 formation. These results strongly suggested that UGT2B7 was the main enzyme responsible for M9-2 formation, and both UGT1A4 and UGT2B7 were responsible for M9-1 formation.
The studies summarized in this article focus on the metabolism, pharmacokinetics, and excretion of apatinib after oral administration of 750 mg apatinib mesylate to humans. Plasma samples for metabolism and pharmacokinetic analysis were collected from patients with advanced colorectal cancer. Urine and fecal samples for metabolism and excretion studies were collected from healthy subjects.

A total of 23, 35, and 14 metabolites were detected in human plasma, urine, and feces, respectively, by UPLC/Q-TOF MS. The proposed structures of the nine major metabolites M1-1, M1-2, M1-6, M4-7, M4-8, M6-9, M6-10, M8-2, and M9-2 were confirmed by comparison with reference standards after HPLC co-chromatography, mass spectrometry, and NMR. The metabolic scheme of apatinib in humans is shown in Fig. 3. Systemically available apatinib was extensively metabolized in humans. The primary routes of apatinib biotransformation involved cis- and trans-cyclopentyl-3-hydroxylation, N-dealkylation, pyridyl-25-N-oxidation, 16-hydroxylation, dioxygenation, and O-glucuronidation after cyclopentyl-3-hydroxylation. Apatinib shared some common metabolic pathways with its analog motesanib (Li et al., 2009), such as pyridyl-N-oxidation and N-dealkylation. However, the major metabolic reactions of apatinib occurred on the cyclopentyl group, including hydroxylation and further O-glucuronidation.

Total recovery of drug-related compounds accounted for 76.8% of the dose. The majority of the recovered dose was eliminated via feces (69.8% of the dose). Only a minor proportion was excreted through urine (7.02% of the dose). About 59.0% of the total intake dose of apatinib was excreted unchanged within 0-96 h via feces. The substantial fecal recovery of apatinib may be related to 1) non-absorbed drug, 2) biliary excretion of apatinib, 3) unstable metabolites that convert back apatinib by
intestinal bacterial enzymes after biliary transport into the intestine, such as the $N$-oxide (el-Mekkawy et al., 1993) and/or 4) direct intestinal excretion after absorption. Apatinib exhibited poor oral bioavailability after a single oral administration of apatinib mesylate to dogs (9.24% in male dogs, 15.4% in female dogs, unpublished data). Thus, the apatinib recovered in feces was suspected to represent mainly the non-absorbed drug. In addition, the pyridyl-25-$N$-oxide metabolite (M1-6), which was found in plasma and urine, was detected only in negligible quantities in feces. These observations were in agreement with the reductive potential of human intestinal bacteria toward $N$-oxides. The excretion of M1-1, M1-2, M1-6, and total dioxygenated metabolites in feces accounted for 2.76%, 1.20%, 0.02%, and 6.80% of the administered dose, respectively. Unchanged apatinib was detected only in negligible quantities in urine, indicating that the systemically available apatinib was extensively metabolized. The major metabolites M1-1, M1-2, M1-6, M4-7, M6-9, M6-10, M8-2, and M9-2 excreted in urine each accounted for less than 2% of the dose. Gender difference in the excretion of apatinib was not observed.

The major circulating metabolites may contribute to the overall efficacy and toxicity of the drug. Thus, determination of the pharmacokinetics of the major metabolites of a new drug is essential. The pharmacokinetics of unchanged apatinib in this study was comparable to data previously reported in cancer patients (Li et al., 2010). $C_{\text{max}}$ of apatinib was achieved 2.9 to 4.7 h post-dose. Significant inter-patient variability with apatinib indicated that dose modification may be needed to meet individual needs. The major circulating metabolite was the pharmacologically inactive metabolite M9-2, the steady state exposure of which was 125% that of the parent drug at steady state. The systemic exposures of M1-1, M1-2, and M1-6 were 56%, 22%, and 32% that of the parent drug, respectively. Calculated as pharmacological activity
index (PAI) values, the contribution of M1-1 to the pharmacology of the drug was 5.42% to 19.3% that of the parent drug, and the contribution of M1-2 and M1-6 to the pharmacology of the drug was less than 1% that of the parent drug. Therefore, among the prominent systemically available drug-related components, the parent drug was considered to have major contributions to the overall pharmacological activity in humans; active monooxygenated metabolites M1-1, M1-2, and M1-6 were considered to have only minor contributions. The systemic clearance for all of the metabolites quantified was slightly slower than that for the parent drug. The accumulation degrees of apatinib and its metabolites were observed to be moderate, with mean AUC₀⁻²₄ h accumulation ratios ranging from 1.77 to 3.69.

The oxidative metabolites of apatinib were mainly formed in the liver in an NADPH-dependent manner. In vitro metabolism studies with recombinant human isozymes and inhibition studies with selective chemical inhibitors of human P450 enzymes demonstrated that the oxidative metabolism of apatinib was mediated by CYP3A4/5 and, to a lesser extent, by CYP2D6, CYP2C9, and CYP2E1. All tested CYP isoforms were capable of M1-1 and M1-2 formation. M1-1 was produced to the greatest extent by CYP2D6, and to a lesser extent by CYP3A4 and CYP3A5. M1-2 was produced to the greatest extent by CYP3A4 and CYP3A5. M1-6 was predominantly formed by CYP2D6, and to a lesser extent by CYP3A4 and CYP3A5. N-dealkylation of apatinib was predominantly mediated by CYP3A4/5. Hydroxylation and dealkylation are most common metabolic pathways observed in CYP2D6 substrates, while N-oxidation is far less common in CYP2D6 substrate. However, there are a few prior examples of CYP2D6 mediated formation of N-oxide. Procainamide (Lessard et al., 1999), Sparteine (Zhou et al., 2009), citalopram (Olesen et al., 1999), AZD0328 (Zhou et al., 2011) and another AZD0328 structure similar α7
NNR agonist (Shaffer et al., 2007) probably are the only limited number of compounds reported previously to have N-oxidation catalyzed by CYP2D6.

The kinetic analysis indicated higher $V_{\text{max}}$ but lower binding affinity for the formation of M1-1, M1-2, and M1-6 in CYP3A4 than that observed in CYP3A5, indicating the comparable in vitro intrinsic clearance ($V_{\text{max}}/K_m$) in CYP3A4 and CYP3A5. Although CYP2D6 has a higher capability for the formation of M1-1 and M1-6 than CYP3A4 and CYP3A5, results of the in vitro inhibition studies with selective chemical inhibitors indicated that CYP2D6 are not expected to play a significant role in the hepatic clearance of apatinib in humans.

The concomitance of known CYP3A4/5 inducers, inhibitors, or substrate may influence drug levels of apatinib. Further in vivo studies will be conducted to evaluate the clinical drug-drug interaction. Hypertension is observed as an adverse effect of all the oral VEGF tyrosine kinase inhibitors (Izzedine et al., 2007), which have occurred because inhibition of VEGFR in arterial endothelial cells decreases the release of nitric oxide (Tang et al., 2004). In phase I clinical studies of apatinib, 68% of the patients who took the drug experienced hypertension. Many antihypertensive drugs, such as amlodipine, diltiazem, felodipine, nicardipine, nifedipine, and verapamil were the substrates or inhibitors of CYP3A. Thus, the concomitant use of these antihypertensive medications with apatinib in patients may induce drug-drug interaction. The potential for drug interactions with M1-1 and M1-6 (metabolite present at $> 25\%$ of parent drug AUC) should be considered in the following studies according to the FDA guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf).

M9-2 ($O$-glucuronide of M1-1) and M9-1 ($O$-glucuronide of M1-2) were cis and
trans isomers. Measured as AUC₀⁻²⁴ h ratios at steady state, the plasma exposure of M1-1 was 1.6 folds higher than that of M1-2, while the plasma exposure of M9-2 was 25.6 folds higher than that of M9-1. These observations suggested that the formation of M9-2 is preferred over M9-1. To test this, M1-1 and M1-2 were individually incubated with HLMs, HIMs, HPMs, and HRMs. Liver and kidney were found to be both important biotransformation sites for the formation of M9-2 and M9-1. In HLMs, the formation of M9-2 was 8 times higher than that of M9-1. In HRMs, the formation of M9-2 was 16 times higher than that of M9-1. These observations were in agreement with the preference in vivo. Incubations with recombinant human UGTs and inhibition studies with selective chemical inhibitors suggested that UGT2B7 was the major UGT isozyme catalyzing the formation of M9-2, while UGT1A4 and UGT2B7 were both important UGT isozymes catalyzing the formation of M9-1.

M1-1 (cis-3-hydroxy-apatinib) and M1-2 (trans-3-hydroxy-apatinib) were cis and trans isomers. Measured as AUC₀⁻²⁴ h ratios at steady state, M1-1 was 1.6 times higher than M1-2, and the sum of M1-1 and its O-glucuronide conjugate (M9-2) was 5.87 times higher than the sum of M1-2 and its O-glucuronide conjugate (M9-1). M1-1 in urine and feces was also higher than M1-2. Therefore, a preference was identified in humans for the formation of M1-1 to its trans isomer M1-2. However, the in vitro microsomal incubation studies and the chemical oxidative reactions both showed a preference for the formation of the trans isomer M1-2. Further investigation is needed to reveal the exact causes.
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Authorship contributions.

Participated in research design: Ding, Zhong, Chen, Jiang, and Zhang

Conducted experiments: Ding, Li, Gao, Dai, and Xie.

Contributed new reagents or analytic tools: Ding, Zhong, and Chen.

Performed data analysis: Ding, Zhong, Chen, Gao, and Li.

Contributed to the writing of the manuscript: Ding and Zhong.
References


Legends for figures

Fig. 1. Mass spectrum of apatinib at high collision energy (A) and the tentative structures of the most informative fragment ions for apatinib (B). The structure of apatinib could be divided into three parts (A, B, and C) based on the fragmentation pattern.

Fig. 2. Metabolic profiles of apatinib after oral administration of 750 mg apatinib mesylate to humans. (A) MDF metabolic profile of pooled plasma samples collected 8 h post-dose at steady state; (B) UPLC-UV chromatogram of pooled plasma samples collected 8 h post-dose at steady state; (C) MDF metabolic profile of 0–24 h pooled urine samples; and (D) UPLC-UV chromatogram of pooled 0–24 h urine samples; (E) MDF metabolic profile of 24–48 h pooled fecal samples; and (F) UPLC-UV chromatogram of pooled 24–48 h fecal samples.

Fig. 3. Proposed metabolic pathways of apatinib in humans.

Fig. 4. Mean plasma concentration–time profiles of apatinib, M1-1, M1-2, M1-6, M9-1, and M9-2 following oral administration of 750 mg apatinib mesylate once daily for 28 d to 20 patients with advance colorectal cancer on days 1 (A) and 28 (B).

Fig. 5. Mean cumulative urine excretion (A) and fecal excretion (B) of apatinib and its major metabolites following a single oral administration of 750 mg apatinib mesylate to 12 healthy Chinese volunteers.

Fig. 6. Incubation of apatinib (2 μM) in recombinant human P450 and FMO enzymes
and HLMs in the presence of 2 mM NADPH at 37°C for 1 h. Each bar represents the mean of duplicate determinations.

**Fig. 7.** Effect of P450 selective chemical inhibitors on the formation of major metabolites in HLMs. A 10-min incubation and a final HLM concentration of 0.2 mg protein/ml represented conditions that are linear and were used in this experiments. Each column represents the mean percentage activity relative to vehicle control from duplicate measurements.

**Fig. 8.** Formation of M1-1, M1-2, and M1-6 from apatinib (2 μM) incubation in individual human recombinant P450 enzyme. Each bar represents the mean activity from duplicate measurements.

**Fig. 9.** Kinetic analysis for the formation of M1-1, M1-2, and M1-6 in HLMs as well as human recombinant CYP3A4, CYP3A5, and CYP2D6. Apatinib was incubated at concentrations in the range of 0.1 ~ 15 μM in triplicate.

**Fig. 10.** O-Glucuronidation of M1-1 to form M9-2 (■) and M1-2 to form M9-1 (□) at substrate concentration of 20 μM by 12 human recombinant UGTs. Each column represents the mean of duplicate determinations.

**Fig. 11.** Inhibitory effects of mefenamic acid, flurbiprofen, and hecogenin on the O-glucuronidation of M1-1 to form M9-2 (A) and M1-2 to form M9-1 (B) at substrate concentration of 20 μM in HLMs. Data represent the mean of duplicate determination.
Tables

Table 1. Identification of apatinib metabolites in human plasma, urine, and feces after oral administration of 750 mg apatinib mesylate tablets using UPLC-UV/Q-TOF mass spectrometry.

<table>
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<th>Name</th>
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<th>(m/z)</th>
<th>Formula</th>
<th>Error (PPM)</th>
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<td>3.0 8.6</td>
<td>0.13 n.d. n.d. n.d.</td>
<td>212.082</td>
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<tr>
<td>M1-5 mono-oxygenation</td>
<td>414.195</td>
<td>C₁₈H₂₁N₅O₂</td>
<td>5.4 10.1</td>
<td>0.10 n.d. n.d. 0.87</td>
<td>228.079, 92.050</td>
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<tr>
<td>M1-6* 25-N-oxidation</td>
<td>414.192</td>
<td>C₁₈H₂₁N₅O₂</td>
<td>-1.6 10.9</td>
<td>1.70 2.28 2.35 0.12</td>
<td>397.191, 228.078, 108.044</td>
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<tr>
<td>M2 N-dealkylation</td>
<td>307.157</td>
<td>C₁₈H₁₈N₄O</td>
<td>3.1 10.0</td>
<td>2.67 3.55 n.d. 1.05</td>
<td>121.039</td>
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<tr>
<td>M3-1 trans 3-hydroxylation and N-dealkylation</td>
<td>323.157</td>
<td>C₁₈H₁₈N₄O</td>
<td>20.6 6.2</td>
<td>1.51 2.85 2.26 n.d.</td>
<td>121.040</td>
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<tr>
<td>M3-2 cis 3-hydroxylation and N-dealkylation</td>
<td>323.148</td>
<td>C₁₈H₁₈N₄O</td>
<td>-9.9 6.6</td>
<td>1.65 1.73 0.52 n.d.</td>
<td>121.038</td>
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<td>M3-3 mono-oxygenation and N-dealkylation</td>
<td>323.157</td>
<td>C₁₈H₁₈N₄O</td>
<td>19.0 7.1</td>
<td>0.40 0.74 0.17 n.d.</td>
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<td>M3-4 mono-oxygenation and N-dealkylation</td>
<td>323.157</td>
<td>C₁₈H₁₈N₄O</td>
<td>19.9 9.6</td>
<td>0.19 0.42 0.10 n.d.</td>
<td>137.035</td>
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<tr>
<td>M4-1 di-oxygenation and N-dealkylation</td>
<td>339.144</td>
<td>C₁₈H₁₈N₄O</td>
<td>-5.0 4.5</td>
<td>n.d. n.d. 0.19 n.d.</td>
<td>121.038</td>
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<td>M4-2 di-oxygenation and N-dealkylation</td>
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<td>M4-8* cis 3-hydroxylation, 16-hydroxylation and N-dealkylation</td>
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<td>M4-9 di-oxygenation and N-dealkylation</td>
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<td>M5-1 mono-oxygenation and dehydrogenation</td>
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<td>M5-2 mono-oxygenation and dehydrogenation</td>
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<td>C₁₉H₂₂N₂O</td>
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<td>M5-3 mono-oxygenation and dehydrogenation</td>
<td>412.181</td>
<td>C₁₉H₂₂N₂O</td>
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<td>0.15 n.d. 0.19 n.d.</td>
<td>108.043</td>
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M5-4  mono-oxygenation and dehydrogenation 412.177 C₉H₁₀N₂O₂ 0.2 8.6 0.08 n.d. n.d. n.d. 212.085
M5-1  di-oxygenation 430.195 C₉H₁₀N₂O₂ 16.1 5.7 n.d. n.d. 0.28 n.d. 212.088
M6-2  di-oxygenation 430.197 C₉H₁₀N₂O₂ 20.5 6.2 n.d. n.d. 1.36 0.31 212.085
M6-3  di-oxygenation 430.185 C₉H₁₀N₂O₂ -7.2 6.5 0.17 0.18 2.97 0.32 212.036
M6-4  di-oxygenation 430.178 C₉H₁₀N₂O₂ -22.3 6.7 0.18 0.20 2.71 0.99 212.038
M6-5  di-oxygenation 430.195 C₉H₁₀N₂O₂ 15.4 6.8 n.d. n.d. 3.64 228.088, 92.053
M6-6  di-oxygenation 430.189 C₉H₁₀N₂O₂ 3.5 7.1 n.d. n.d. 0.91 0.31 212.083
M6-7  di-oxygenation 430.195 C₉H₁₀N₂O₂ 16.3 7.2 n.d. n.d. 4.42 228.089, 92.056
M6-8  di-oxygenation 430.202 C₉H₁₀N₂O₂ 33.1 7.6 n.d. n.d. 0.26 228.091, 92.058
M6-9ª  trans-3-hydroxylation and 25-N-oxidation 430.188 C₈H₉N₂O₂ -0.2 7.8 0.17 0.22 2.97 n.d. 212.036, 108.020
M6-10ª cis-3-hydroxylation and 25-N-oxidation 430.180 C₈H₉N₂O₂ -18.2 8.1 0.20 0.39 5.21 n.d. 212.027, 108.037
M6-11 di-oxygenation 430.185 C₈H₉N₂O₂ -6.5 8.5 n.d. n.d. 0.67 n.d. 212.082, 108.043
M7-1  mono-oxygenation and sulfation 494.151 C₉H₁₀N₂OS 2.2 7.0 n.d. n.d. 0.21 0.15 212.081
M7-2  mono-oxygenation and sulfation 494.150 C₉H₁₀N₂OS 1.0 7.3 0.08 n.d. 0.43 0.33 212.086
M8-1  trans-3-hydroxylation, N-dealkylation and O-glucuronidation 499.174 C₁₀H₁₀N₂O₅S -17.8 5.1 n.d. 0.16 3.01 n.d. 323.151, 121.038
M8-2ª cis-3-hydroxylation, N-dealkylation and O-glucuronidation 499.181 C₁₀H₁₀N₂O₅S -3.7 5.5 0.52 0.82 10.3 n.d. 323.151, 121.038
M9-1  trans-3-hydroxylation and O-glucuronidation 590.220 C₁₃H₁₄N₂O₆ 9.1 6.5 0.69 0.53 1.07 n.d. 414.195, 212.082
M9-2ª cis-3-hydroxylation and O-glucuronidation 590.213 C₁₃H₁₄N₂O₆ -16.0 6.9 0.20 0.44 1.30 n.d. 414.183, 212.079, 92.047
M10 di-oxygenation and glucuronidation 606.210 C₁₃H₁₄N₂O₆ -16.0 6.9 0.20 0.44 1.30 n.d. 430.183, 108.042

ª metabolites which were confirmed by comparison with reference standards after HPLC co-chromatography, mass spectrometry, and NMR.

ª not detected.
Table 2. \(^1\)H NMR data of apatinib (M0), cis-3-hydroxy-apatinib (M1-1), trans-3-hydroxy-apatinib (M1-2), apatinib-25-N-oxide (M1-6), trans-3-hydroxy-16-hydroxy-N-dealkylated metabolite (M4-7), cis-3-hydroxy-16-hydroxy-N-dealkylated metabolite (M4-8), trans-3-hydroxy-25-N-oxide metabolite (M6-9), cis-3-hydroxy-25-N-oxide metabolite (M6-10), cis-3-hydroxy-O-glucuronide-N-dealkylated metabolite (M8-2), and cis-3-hydroxy-apatinib-O-glucuronide (M9-2).

<table>
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<tr>
<th>Position</th>
<th>δH(1H)</th>
<th>Apatinib</th>
<th>M1-1</th>
<th>M1-2</th>
<th>M1-6</th>
<th>M4-7</th>
<th>M4-8</th>
<th>M6-9</th>
<th>M6-10</th>
<th>M8-2</th>
<th>M9-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2,4,5</td>
<td>2.40(m,2H)</td>
<td>2.73(dd,J=13.5,6.0,1H)</td>
<td>2.53(m,1H)</td>
<td>2.42(m,2H)</td>
<td>2.62(m,1H)</td>
<td>2.82(dd,J=14.0,6.0,1H)</td>
<td>2.64(m,1H)</td>
<td>2.82(dd,J=14.0,6.4,1H)</td>
<td>2.75(dd,J=14.8,6.4,1H)</td>
<td>2.87(dd,J=14.8,6.4,1H)</td>
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<tr>
<td>H-3</td>
<td>1.88(s,3H)</td>
<td>2.36(m,1H)</td>
<td>2.09(m,2H)</td>
<td>1.99(m,4H)</td>
<td>2.21(m,2H)</td>
<td>2.36(m,3H)</td>
<td>2.22(m,2H)</td>
<td>2.37(m,2H)</td>
<td>2.20(m,2H)</td>
<td>2.19(m,2H)</td>
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<td>H-7,11</td>
<td>7.49(d,J=8.7,2H)</td>
<td>7.49(d,J=8.7,2H)</td>
<td>7.46(d,J=8.5,2H)</td>
<td>7.46(d,J=8.6,2H)</td>
<td>7.43(d,J=8.7,2H)</td>
<td>7.51(d,J=8.2,2H)</td>
<td>7.47(d,J=8.4,2H)</td>
<td>7.52(m,overlapped,2H)</td>
<td>7.56(d,J=8.6,2H)</td>
<td>7.55(d,J=8.4,2H)</td>
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<tr>
<td>H-8,10</td>
<td>7.78(d,J=8.7,2H)</td>
<td>7.78(d,J=8.7,2H)</td>
<td>7.75(d,J=8.2,2H)</td>
<td>7.73(d,J=8.2,2H)</td>
<td>7.76(d,J=8.7,2H)</td>
<td>7.63(d,J=8.2,2H)</td>
<td>7.73(d,J=8.4,2H)</td>
<td>7.73(d,J=8.4,2H)</td>
<td>7.70(d,J=8.2,2H)</td>
<td>7.72(d,J=8.4,2H)</td>
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<tr>
<td>H-15</td>
<td>8.15(dd,J=7.1,1.4,1H)</td>
<td>8.09(dd,J=7.5,2.0,1H)</td>
<td>8.08(dd,J=7.5,2.0,1H)</td>
<td>8.05(dd,J=7.1,1.5,1H)</td>
<td>5.72(d,J=9.5,1H)</td>
<td>5.73(d,J=9.6,1H)</td>
<td>8.05(dd,J=7.1,1.4,1H)</td>
<td>8.06(dd,J=7.1,2.1,1H)</td>
<td>8.11(m,1H)</td>
<td>8.09(m,1H)</td>
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<td>H-16</td>
<td>6.74(dd,J=7.5,0.1,1H)</td>
<td>6.68(dd,J=7.5,0.1,1H)</td>
<td>6.68(dd,J=7.5,0.1,1H)</td>
<td>6.69(dd,J=7.8,4.9,1H)</td>
<td>6.70(dd,J=7.6,4.8,1H)</td>
<td>6.70(dd,J=7.6,4.8,1H)</td>
<td>6.75(dd,J=7.6,5.2,1H)</td>
<td>6.70(dd,J=7.6,5.2,1H)</td>
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<td>H-17</td>
<td>8.10(dd,J=5.0,1.4,1H)</td>
<td>8.14(dd,J=5.0,2.0,1H)</td>
<td>8.14(dd,J=5.0,2.0,1H)</td>
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<td>7.98(d,J=9.6,1H)</td>
<td>8.12(dd,J=8.1,6,1H)</td>
<td>8.12(dd,J=8.1,2,1H)</td>
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<td>8.07(m,1H)</td>
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<td>H-21</td>
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<td>4.67(d,J=5.9,2H)</td>
<td>4.60(d,J=5.0,2H)</td>
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<td>4.78(c,2H)</td>
<td>4.78(c,2H)</td>
<td>4.80(c,2H)</td>
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<td>H-23,27</td>
<td>7.90(d,J=6.0,2H)</td>
<td>7.27(d,J=6.0,2H)</td>
<td>7.28(d,J=6.0,2H)</td>
<td>7.50(d,J=7.0,2H)</td>
<td>7.52(d,J=6.4,2H)</td>
<td>7.54(m,overlapped,2H)</td>
<td>7.54(m,overlapped,2H)</td>
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<td>H-24,26</td>
<td>8.78(d,J=6.0,2H)</td>
<td>8.45(d,J=6.0,2H)</td>
<td>8.44(d,J=6.0,2H)</td>
<td>8.24(d,J=6,0,2H)</td>
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\(^a\) DMSO-\(d_6\), 500MHz.

\(^b\) CD\(_3\)OD, 400MHz.
Table 3. Pharmacokinetic parameters (mean ± S.D.) of apatinib and its major metabolites in human plasma following oral administration of 750 mg apatinib mesylate once daily for 28 d to 20 patients with advance colorectal cancer.

<table>
<thead>
<tr>
<th>Time</th>
<th>Parameter</th>
<th>Apatinib</th>
<th>M1-1</th>
<th>M1-2</th>
<th>M1-6</th>
<th>M9-1</th>
<th>M9-2</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>$T_{\text{max}}$ (h)</td>
<td>2.9 ± 1.4</td>
<td>5.2 ± 4.8</td>
<td>5.0 ± 4.8</td>
<td>7.7 ± 6.3</td>
<td>7.1 ± 4.5</td>
<td>10.2 ± 3.7</td>
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<tr>
<td></td>
<td>$C_{\text{max}}$ (nmol/l)</td>
<td>3819 ± 2204</td>
<td>849 ± 268</td>
<td>339 ± 131</td>
<td>375 ± 172</td>
<td>36.5 ± 22.0</td>
<td>1543 ± 819</td>
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<tr>
<td></td>
<td>$\text{AUC}_{0-24 \text{h}}$ (nmol·h/l)</td>
<td>30941 ± 18794</td>
<td>12458 ± 4380</td>
<td>4796 ± 2049</td>
<td>6605 ± 2806</td>
<td>555 ± 322</td>
<td>27276 ± 14147</td>
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<tr>
<td>Day 28</td>
<td>$T_{\text{max}}$ (h)</td>
<td>4.7 ± 2.2</td>
<td>7.2 ± 6.2</td>
<td>7.0 ± 6.1</td>
<td>5.5 ± 2.4</td>
<td>11.5 ± 8.0</td>
<td>11.3 ± 6.9</td>
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<tr>
<td></td>
<td>$C_{\text{max}}$ (nmol/l)</td>
<td>3935 ± 2211</td>
<td>1470 ± 418</td>
<td>571 ± 162</td>
<td>786 ± 210</td>
<td>94.3 ± 55.5</td>
<td>2843 ± 1226</td>
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<td>$\text{AUC}_{0-24 \text{h}}$ (nmol·h/l)</td>
<td>46285 ± 17883</td>
<td>26014 ± 8895</td>
<td>10001 ± 4107</td>
<td>14596 ± 4090</td>
<td>2168 ± 1184</td>
<td>57631 ± 24888</td>
</tr>
<tr>
<td>Day 28/Day 1</td>
<td>$t_{1/2}$ (h)</td>
<td>18.6 ± 9.2</td>
<td>24.7 ± 12.9</td>
<td>23.2 ± 12.3</td>
<td>21.2 ± 12.7</td>
<td>26.3 ± 15.9</td>
<td>23.8 ± 9.7</td>
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<tr>
<td></td>
<td>$C_{\text{max}}$ ratio</td>
<td>1.16 ± 0.82</td>
<td>1.78 ± 0.79</td>
<td>1.76 ± 0.97</td>
<td>2.41 ± 1.75</td>
<td>3.01 ± 2.15</td>
<td>1.84 ± 1.13</td>
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<tr>
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<td>$\text{AUC}_{0-24 \text{h}}$ ratio</td>
<td>1.77 ± 1.17</td>
<td>2.18 ± 1.13</td>
<td>2.20 ± 1.38</td>
<td>2.51 ± 1.79</td>
<td>3.69 ± 2.95</td>
<td>2.17 ± 1.35</td>
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</table>
Table 4. Excretion of total drug-related compounds in urine and feces after a single oral administration of 750 mg apatinib mesylate tablets to 12 healthy subjects.

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<th>Time period</th>
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<th>%Dose recovered by subject</th>
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<th>Mean ± S.D.</th>
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<td>4</td>
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<td>6</td>
<td>1</td>
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<td>3</td>
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<td>5</td>
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<td>6</td>
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<tr>
<td>Urine</td>
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<td>Mean ± S.D.</td>
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<td></td>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
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<tr>
<td>0-4 h</td>
<td>0.924</td>
<td>0.453</td>
<td>0.757</td>
<td>0.560</td>
<td>0.780</td>
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<td>79.1</td>
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<td>12.1</td>
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</table>

N.A., not applicable.
Table 5. Binding of apatinib, M1-1, M1-2, and M1-6 to human plasma proteins and the \textit{in vitro} pharmacological study results of M1-1, M1-2, M1-6, and M9-2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Analyte</th>
<th>Percent protein binding (%)$^a$</th>
<th>IC$_{50}$ (nM)</th>
<th>PAI (%)$^b$</th>
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<tr>
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<td></td>
<td>VEGFR-2</td>
<td>PDGFR$^\beta$</td>
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<tr>
<td>1</td>
<td>Apatinib</td>
<td>92.4 ± 3.2</td>
<td>1.90</td>
<td>16.5</td>
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<tr>
<td></td>
<td>M1-1</td>
<td>90.1 ± 2.2</td>
<td>19.2</td>
<td>46.9</td>
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<tr>
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<td>M1-2</td>
<td>88.0 ± 3.2</td>
<td>179</td>
<td>1352</td>
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<tr>
<td>2</td>
<td>Apatinib</td>
<td>92.4 ± 3.2</td>
<td>3.30</td>
<td>11.4</td>
</tr>
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<td></td>
<td>M1-6</td>
<td>75.8 ± 3.7</td>
<td>265</td>
<td>369</td>
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<tr>
<td></td>
<td>M9-2</td>
<td>not measured</td>
<td>Inactive</td>
<td>Inactive</td>
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</table>

$^a$Percent protein binding was measured by ultracentrifugation.

$^b$PAI was calculated as follows: PAI = (metabolite AUC at steady state × percent protein binding of metabolite × IC$_{50}$ of parent)/(parent AUC at steady state × parent percent protein binding × IC$_{50}$ of metabolite) × 100.
Table 6. Kinetic parameters (mean ± S.D.) for the formation of M1-1, M1-2, and M1-6 in HLMs as well as in human recombinant CYP3A4, CYP3A5, and CYP2D6.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Kinetic parameters</th>
<th>HLM</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>CYP2D6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1-1</strong></td>
<td>$K_m$ (μM)</td>
<td>1.93 ± 0.33</td>
<td>0.57 ± 0.13</td>
<td>0.28 ± 0.05</td>
<td>0.69 ± 0.15</td>
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<tr>
<td></td>
<td>$V_{max}$</td>
<td>3.28 ± 0.19</td>
<td>0.05 ± 0.003</td>
<td>0.02 ± 0.0007</td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>CL$_{int}$</td>
<td>1.70 ± 0.21</td>
<td>0.09 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>1.56 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.9726</td>
<td>0.9125</td>
<td>0.9318</td>
<td>0.9455</td>
</tr>
</tbody>
</table>

| M1-2       | $K_m$ (μM)         | 2.18 ± 0.29 | 0.90 ± 0.15 | 0.26 ± 0.03 | N.A. |
|            | $V_{max}$          | 39.1 ± 1.79 | 1.29 ± 0.06 | 0.88 ± 0.02 | N.A. |
|            | CL$_{int}$         | 17.9 ± 1.74 | 1.44 ± 0.20 | 3.38 ± 0.35 | N.A. |
|            | $R^2$              | 0.9849 | 0.9644 | 0.9753 | N.A. |

| M1-6       | $K_m$ (μM)         | 1.41 ± 0.22 | 1.02 ± 0.17 | 0.30 ± 0.03 | 0.53 ± 0.12 |
|            | $V_{max}$          | 9.82 ± 0.46 | 0.31 ± 0.01 | 0.16 ± 0.004 | 4.60 ± 0.24 |
|            | CL$_{int}$         | 7.01 ± 0.83 | 0.30 ± 0.04 | 0.52 ± 0.05 | 8.68 ± 1.61 |
|            | $R^2$              | 0.9765 | 0.9687 | 0.9792 | 0.9388 |

$a$ The unit for $V_{max}$ was pmol/min/mg protein for HLM and pmol/min/pmol P450 for CYP3A4, CYP3A5, and CYP2D6.

$b$ The unit for CL$_{int}$ was μl/min/mg protein for HLM and μl/min/pmol P450 for CYP3A4, CYP3A5, and CYP2D6.

N.A., not applicable.
Figure 4

A

B

Concentration (nM)

Time (h)

Concentration (nM)

Time (h)
Figure 5

A

B

Cumulative urine excretion (%)

Cumulative fecal excretion (%)

Time (h)

Apatinib
M1-1
M1-2
M1-6
M4-7
M6-9
M6-10
M8-2
M9-2
Total

Dioxygenated metabolites

Total

Time (h)
Figure 8

Metabolite formation (pmol/min/pmol protein)

CYP1A2  CYP1B1  CYP2A6  CYP2B6  CYP2C8  CYP2C9  CYP2C19  CYP2D6  CYP2E1  CYP3A4  CYP3A5  CYP4A11  FMO1  FMO3  FMO5

M1-1  M1-2  M1-6
Figure 11

A

- ■ Mefenamic acid
- ● Flubiprofen
- ▲ Hecogenin

```
Inhibitors (μM)

% of control
```

B

- ■ Mefenamic acid
- ● Flubiprofen
- ▲ Hecogenin

```
Inhibitors (μM)

% of control
```