In Vitro Metabolism of the Novel, Highly Selective Oral Angiogenesis Inhibitor Motesanib Diphosphate in Preclinical Species and in Humans

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Abbreviations:
- CID=collision-induced dissociation
- CYP=cytochrome P450
- DAD=diode array detector
- ESI=electrospray ionization
- HLM=human liver microsomes
- HMBC=heteronuclear multiple bond correlation
- HPLC=high-pressure liquid chromatography
- LC/MS=liquid chromatography/mass spectrometry
- LC/MS/MS=liquid chromatography/tandem mass spectrometry
- Kit=stem cell factor receptor
- M1=Conjugative metabolite of motesanib, indoline N-glucuronide
- M2=Oxidative metabolite of motesanib, structure unassigned
- M3=Oxidative metabolite of motesanib, pyridine N-oxide
- M4=Oxidative metabolite of motesanib, lactam (oxindole)
- M5=Oxidative metabolite of motesanib, carbaminolamine
- Ma=Conjugative metabolite of motesanib, indoline N-carbomyl glucuronide
- Mb=Conjugative metabolite of motesanib, indoline N-glucose
- Mc=Conjugative metabolite of motesanib, indoline N-N-acetylglucosamine
- Miv=Oxidative N-dealkylated metabolite of motesanib, lactam of Mv
- Mv=Oxidative N-dealkylated metabolite of motesanib, 2-aminonicotinamide
- Mx=Oxidative N-dealkylated metabolite of motesanib, isonicotinic acid
- NMR=nuclear magnetic resonance
- PDGF=platelet-derived growth factor
- Q-TOF=quadrupole time-of-flight
- UGT=UDP-glucuronosyltransferase
- UDP-GA=uridine diphosphate-glucuronic acid
- VEGF=vascular endothelial growth factor
Abstract

Motesanib diphosphate is a novel, investigational, highly selective oral inhibitor of the receptor tyrosine kinases vascular endothelial growth factor receptors 1, 2, and 3; platelet-derived growth factor receptor; and the stem cell factor receptor Kit. The in vitro metabolic profiles of $^{[14]}$C-motesanib were examined using microsomes and hepatocytes from preclinical species and humans. Several oxidative metabolites were observed and characterized by tandem mass spectrometry, nuclear magnetic resonance spectroscopy, and coinjection with authentic standards. Cytochrome P450 (CYP) 3A4 is the major isozyme involved in the oxidative biotransformation of motesanib, but the CYP2D6 and CYP1A isozymes also make minor contributions. In hepatocyte incubations, oxidative and conjugative pathways were observed for all species examined, and indoline N-glucuronidation was the dominant pathway. Three less common and novel phase II conjugates of the indoline nitrogen were detected in hepatocytes and in microsomes supplemented with specific cofactors, including N-carbamoyl glucuronide (Ma), N-glucose (Mb), and N-linked $\beta$-N-acetylglucosamine (Mc). An N-glucuronide metabolite (M1) was the most frequently observed phase II conjugate in liver microsomes of all species, whereas the N-acetylglucosamine conjugate was observed only in monkey liver microsomes. Incubations with recombinant human UDP-glucuronosyltransferases (UGTs) and inhibition by the UGT1A4 and UGT1A1 substrates/inhibitors imipramine and bilirubin suggested that UGT1A4 is the major UGT isozyme catalyzing the N-glucuronidation of motesanib, with a minor contribution from UGT1A1. The in vitro metabolic profiles were similar between the human and preclinical species examined. All metabolites found in humans were also detected in other species.
Introduction

Motesanib diphosphate (formerly known as AMG 706) (Figure 1) is a highly selective kinase inhibitor with both antiangiogenic and direct antitumor activity (Polverino et al., 2006; Rosen et al., 2007). Motesanib is an ATP-competitive inhibitor of vascular endothelial growth factor (VEGF) receptors 1, 2, and 3, platelet-derived growth factor (PDGF) receptor, and stem cell factor receptor (Kit), which have all been implicated in the pathogenesis of human tumors (Dvorak, 2002; Heinrich et al., 2002; Song et al., 2005). In preclinical models of human cancer, oral administration of motesanib potently inhibited VEGF-induced angiogenesis in the rat corneal model and induced regression of established A431 xenografts (Polverino et al., 2006). Furthermore, motesanib has shown antiangiogenic and antitumor activity in patients with advanced solid malignancies (Rosen et al., 2007). Additional studies of motesanib as monotherapy and in combination with various agents are currently ongoing.

Definitive characterization of metabolites and examination of species differences in metabolism are becoming increasingly important. An understanding of quantitative and qualitative differences in metabolic profiles, especially with respect to metabolites unique to humans, is essential to comparing exposure and safety of a drug in nonclinical species relative to humans during risk assessment (Baillie et al., 2002; Hastings et al., 2003). In addition, identification of enzymes involved in the metabolism of a drug can help predict potential drug-drug interactions and variability in pharmacokinetics. In this study, we report results from the first preclinical metabolic investigation of motesanib. Phase I oxidative and phase II conjugative metabolites of motesanib were detected and characterized using liver microsomes and hepatocytes from mouse, rat, dog, monkey, and human. The cytochrome P450 (CYP) isozymes responsible for oxidative
metabolism of motesanib, as well as UDP-glucuronosyltransferase (UGT) isozymes involved in N-glucuronidation of motesanib, were also examined.
Methods

Chemicals and Reagents

Motesanib, authentic metabolite standards M3 (N-oxide), M4 (lactam), M1 (indoline N-glucuronide), and 2-[(1H-pyrrolo[2,3-b]pyridin-4-ylmethyl)amino]-N-(1,2,3,4-tetrahydro-4,4-dimethyl-7-quinolinyl)-3-pyridinecarboxamide (internal standard used for CYP reaction phenotyping experiments) were synthesized at Amgen Inc. (Thousand Oaks, CA). [Methylene 14C]-motesanib (specific activity, 23 mCi/mmol) and [carbonyl 14C]-motesanib (specific activity, 54 mCi/mmol) (Figure 1) were synthesized by Moravek Biochemicals, Inc. (Brea, CA). A 1-mM (25 or 50 µCi/mL) stock solution of [methylene 14C]-motesanib dissolved in acetonitrile was used.

Hydroxy-diclofenac was purchased from Gentest (Woburn, MA). D9-OH-bufuralol was synthesized by PharmaCore (High Point, NC). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) and were of the highest quality available. The In-Flow 3 scintillant was purchased from IN/US Systems (Tampa, FL).

Hepatic microsomes from male CD-1 mice, male Sprague-Dawley rats, male beagle dogs, male cynomolgus monkeys, and humans (pooled sex mix) were purchased from Cedra (Austin, TX). Microsomes expressing human cDNA CYPs (Supersomes™); microsomes from engineered cell lines expressing individual UGTs; and monoclonal antibodies specific for CYP3A4, CYP2D6, and CYP1A were obtained from Gentest.

Cryopreserved monkey and human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD). Freshly isolated male beagle dog hepatocytes were purchased from Xenotech (Lenexa, KS). Hepatocytes were freshly isolated from 6-to-8-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and
CD-1 mice (Charles River Laboratories). Rat and mouse hepatocytes were isolated by in situ liver perfusion with blanch and collagenase media. The perfused liver was removed and cells were gently shaken into a calcium-free hepatocyte maintenance media. The nonhepatocyte cells, dead cells, and connective tissue remnants were removed from the viable hepatocytes by gently centrifuging and washing the cells 3 times with hepatocyte incubation media.

**Oxidative Microsomal Incubations**

[Methylene $^{14}$C]-motesanib (10 µM, 0.225 µCi/mL) was incubated with liver microsomes (1.0 mg/mL in 100 mM phosphate buffer, pH 7.4) from human, monkey, dog, mouse, and rat at 37°C for 30 minutes with or without NADPH (1 mM) in a total volume of 0.5 mL. Incubations were stopped by adding 250 µL ice-cold acetonitrile containing 0.1% formic acid followed by centrifugation at 14,000 rpm for 10 minutes. The supernatants were analyzed directly (without any further sample clean up) by high-performance liquid chromatography (HPLC) with a diode array detector (DAD) and a β-RAM radiometric detector. Selected samples were analyzed by HPLC with ion-trap mass spectrometry.

**Phase II Microsomal Incubations**

[Methylene $^{14}$C]-motesanib (10 µM, 0.225 µCi/mL) was incubated with liver microsomes (1.0 mg/mL in 100 mM phosphate buffer containing 10 mM magnesium chloride, pH 7.4) from human, monkey, dog, mouse and rat, respectively, at 37°C for 30 minutes, with or without phase II co-factors UDP-GA, UDP-glucose, UDP-galactose or UDP-N-acetylglucosamine (2 mM final concentration) in a total volume of 0.5 mL. Alamethicin (25 mg/mL in methanol) was added to the incubations at a final concentration of 100 µg/mg of protein. Incubations were stopped by adding 250 µL of ice-cold
acetonitrile followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant
was analyzed by HPLC with DAD, and radiometric detection. Selected samples were
analyzed by HPLC with ion trap mass spectrometry for metabolite identification. The
percentages of these phase II conjugates quantified by their UV peak areas were
identical to those quantified by radiometric detection. Subsequent measurements of N-
glucuronide (M1) in UGT phenotyping and kinetic experiments were therefore based on
UV peak areas, and no internal standard was used.

**Hepatocyte Incubations**

Hepatocytes were suspended in Krebs-Henseleit buffer at a cell concentration of $1 \times 10^6$
cells/mL. In a 24-well plate, [methylene $^{14}$C]- or [carbonyl $^{14}$C]-motesanib was incubated
at 10 µM (0.25 or 0.5 µCi/mL) in 0.5 mL of cell suspension with rat, mouse, monkey,
dog, and human hepatocytes for 0, 2, and 4 hours. The positive control was 7-ethoxy
coumarin at a concentration of 50 µM. Incubations were conducted at 37°C in the
presence of 5% CO$_2$ with saturating humidity and with gentle shaking every 10 to 15
minutes. Each treatment was performed in duplicate, and the incubations were
terminated using 250 µL acetonitrile. The total volume was transferred to a 1.5-mL
conical centrifuge tube and vortexed, centrifuged, and the supernatant was frozen at
−80°C until direct analysis by HPLC with radiometric detection and by mass
spectrometry.

**Metabolite Biosynthesis and Isolation for Nuclear Magnetic Resonance**

**Spectroscopy Characterization**

To isolate sufficient quantities (50–100 µg) of metabolites (M3, M4, and M5) for
structural characterization by nuclear magnetic resonance (NMR) spectroscopy,
incubations of nonradioactive motesanib with mouse and human liver microsomes in the
presence of NADPH were scaled up. Incubation conditions were first optimized for metabolite production in terms of motesanib concentration, microsomal protein concentration, and incubation time. Motesanib (50 µM) was then incubated with human or mouse liver microsomes (2 mg/mL) and 1 mM NADPH for 1 h in a total volume of 100 mL. To isolate enough M1 (~150 µg) for NMR confirmation, incubation of cold motesanib (25 µM) with human liver microsomes (1 mg/mL) in the presence of uridine diphosphate-glucuronic acid (UDP-GA, 2 mM) was also scaled up in a total volume of 20 mL and incubated for 1 h. Metabolite Ma was biosynthesized by incubating motesanib (0.25 mg/mL) with monkey liver microsomes (1 mg/mL) in potassium carbonate buffer (100 mM and saturated with dry ice). UDP-GA (2 mM), alamethicin (60 µg/mg microsomes), and D-saccharolactone (5 mM) were added, and the mixture was incubated in a closed-system CO2 atmosphere at 37°C for 20 hours.

Reactions were stopped by adding an equal volume of acetonitrile. Supernatants were diluted and extracted by solid-phase extraction (Oasis HLB, 6 cc/500 mg, conditioned with 2 × 6 mL of methanol followed by 2 × 6 mL H2O). Metabolites were isolated by semipreparative HPLC using a YMC ODS-AQ column (10 mm × 100 mm) at a flow rate of 5 mL/min. Separation was carried out using a linear gradient of 10% B to 90% B for 20 minutes (A = 90/10 H2O/CH3CN, 5 mM NH4OAC; B = 90/10 CH3CN/H2O, 5 mM NH4OAC). Isolated metabolites were concentrated and desalted using an Oasis HLB cartridge (1 cc/30 mg), eluted with methanol, and dried under nitrogen. The purities of metabolites were verified by HPLC and characterized by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Samples were stored at −20°C before dissolving in methanol-d4 for NMR analysis.
Oxidative Metabolism of Motesanib by Recombinant Cytochrome P450 Isoforms

Incubations were performed with microsomes from engineered cell lines expressing individual CYPs. Recombinant (Supersomes™) CYP1A1+OR, 1A2+OR, 1B1+OR, 2A6+OR+b5, 2B6+OR+b5, 2C8+OR+b5, 2C9*1+OR+b5, 2C9*2+OR, 2C18+OR, 2C19+OR+b5, 2D6*1+OR, 2E1+OR+b5, 3A4+OR+b5, 3A5+OR, 3A7+OR+b5, and 4A11+OR were tested. Incubations were conducted with [methylene 14C]-motesanib (10 µM) and CYP isozyme (50 pmol/mL), with or without 1 mM NADPH, in a total volume of 300 µL at 37°C for 30 minutes. The incubations were quenched with 150 µL ice-cold acetonitrile, vortexed, and centrifuged at 14,000 rpm for 10 minutes. Supernatants were analyzed by HPLC with UV and radiometric detection.

Inhibition of Motesanib Oxidative Metabolism by Selective Cytochrome P450 Inhibitors

For CYP-mediated reactions, experimental conditions were optimized and linear conditions were established with respect to protein, time of incubation, and substrate concentration. Based on these results and the turnover of metabolites, a 10-µM motesanib concentration was selected to allow for formation of metabolites and to provide adequate signal and sensitivity for detection of significant inhibition (>90%) when incubated with inhibitors or antibodies. In addition, the concentrations used were projected to be within 2 times the maximal concentrations expected in the clinic at the time of the study. Inhibition studies with CYP isoform-selective chemical inhibitors were carried out using motesanib (10 µM) with human liver microsomes (1 mg/mL) in phosphate buffer (100 mM, pH 7.4) and NADPH (1 mM) at 37°C for 30 minutes. Experiments were conducted using 2 different inhibitor concentrations in a total incubation volume of 200 µL (see Results for concentrations of each inhibitor). The reactions were stopped using 100 µL ice-cold acetonitrile containing 0.6 µM internal
standard (a proprietary structural analog of motesanib). The supernatants obtained after centrifuging for 10 minutes at 14,000 rpm were diluted using equal volumes of HPLC-grade water and analyzed by selected ion monitoring of metabolites using positive ion electrospray ionization (ESI) on an ion-trap mass spectrometer (LCQ classic, Thermo Electron, San Jose, CA).

Control experiments were performed using known selective CYP substrates and inhibitors. The control probe reactions used included the following: phenacetin O-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6, diclofenac 4’ hydroxylation for CYP2C9, S-mephénytoin 4-hydroxylation for CYP2C19, bufuralol 1’ hydroxylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1, and midazolam 1’-hydroxylation for CYP3A4. Mechanism-based inhibitors, such as 8-methoxypsoralen and diethylidithiocarbamate, were preincubated with microsomes and NADPH for 5 minutes before the addition of the respective substrates. Analysis of samples was performed using an API3000 triple quadrupole mass spectrometer (MDS Sciex, Concord, ON, Canada) using specific multiple reaction monitoring transitions for the marker metabolites.

**Inhibition of Motesanib Oxidative Metabolism by Specific Anti–Cytochrome P450 Antibodies**

Human liver microsomes (2 mg/mL in phosphate buffer) were added to monoclonal antibodies (10 mg/mL) specific for CYP3A4, CYP2D6, and CYP1A at 2 different antibody concentrations (2 and 10 µg of IgG per 0.1 mg of microsomes) and incubated on ice for 15 minutes. Monoclonal antibodies specific for CYP1A were preincubated at room temperature for 30 minutes. Motesanib reaction mixtures (20 µM motesanib and 2 mM NADPH in 100 mM phosphate buffer, pH 7.4) were preincubated at 37°C for 5 minutes
and added to equal volumes of each antibody mixture. Samples were incubated at 37°C for 30 minutes. Final concentrations of motesanib, NADPH, and monoclonal antibody were 10 µM, 1 mM, and 2 µg/0.1 mg microsomal protein or 10 µg/0.1 mg microsomal protein. Incubations were terminated by the addition of one half volume of ice-cold acetonitrile containing 0.6 µM internal standard. Samples were vortexed and centrifuged for 10 minutes at 14,000 rpm, and then analyzed by ion-trap mass spectrometry using selected ion monitoring of metabolites, as in the case for selective chemical inhibitors.

Positive control experiments using probe substrates of CYP3A4 (midazolam), CYP2D6 (bufuralol), and CYP1A (phenacetin) were carried out using similar incubation conditions.

**Motesanib N-glucuronidation by Recombinant UDP-glucuronosyltransferase Isoforms**

Microsomes (Supersomes) from engineered cell lines expressing individual UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT2B7, and UGT2B15) were tested for glucuronidation activity. UGT isozymes (0.5 mg/mL in 50 mM Tris-HCl buffer, 1 mM MgCl₂; pH 7.5) were preincubated with alamethicin (100 µg/mg microsomal protein) for 15 minutes on ice, and the motesanib substrate (10 µM) was added and preincubated at 37°C for 5 minutes. Reactions were initiated by adding UDP-GA (5 mM) and incubated at 37°C for 30 minutes. Incubations were quenched with equal volumes of ice-cold acetonitrile, vortexed, and centrifuged. The supernatants were analyzed by HPLC with UV detection.
Inhibition of Motesanib N-glucuronidation by Selective UDP-glucuronosyltransferase Substrates/Inhibitors

The rate of formation of N-glucuronide metabolite M1 in human liver microsomes was linear for 10 minutes of incubation at a final protein concentration of 0.25 mg/mL in 100 mM phosphate, pH 7.4, containing 10 mM MgCl₂. Therefore, these reaction conditions were used for inhibition studies. The microsomal protein was preincubated with alamethicin on ice for 15 minutes (70 µg alamethicin/mg microsomal protein; alamethicin was prepared as a 25-mg/mL stock solution in methanol). Immediately before adding motesanib (final concentration, 10 µM), imipramine (1 or 5 mM) or bilirubin (25 or 50 µM) was added as a competitive inhibitor of UGT1A4 and UGT1A1. Reactions were initiated by adding 2 mM UDP-GA and then incubated at 37°C for 10 minutes. Incubations were quenched with one half volume of ice-cold acetonitrile and then vortexed and centrifuged. The resulting supernatants were diluted using equal volumes of HPLC-grade water and then analyzed by HPLC with UV detection.

Enzyme Kinetics of Motesanib N-glucuronidation in Humans, Rats, Dogs, and Monkeys

Incubation conditions for motesanib N-glucuronidation in human, rat, dog, and monkey liver microsomes were optimized for microsomal protein concentration and incubation time, and the incubations were conducted under initial-rate conditions. All incubations were conducted in phosphate buffer (100 mM) containing 10 mM MgCl₂ buffer (pH 7.4) and preincubated with 70 µg alamethicin/mg microsomal protein for 15 minutes before reaction initiation by UDP-GA (2 mM). To investigate the enzyme kinetics of N-glucuronidation in human liver microsomes, motesanib (1–400 µM) was incubated with microsomal protein (0.25 mg/mL) at 37°C for 10 minutes for motesanib concentrations up to 25 µM and for 30 minutes for motesanib concentrations higher than...
25 µM. Using rat liver microsomes, motesanib (1–200 µM) was incubated with microsomal protein (0.5 mg/mL) at 37°C for 20 minutes. Using dog and monkey liver microsomes, motesanib (1–600 µM) was incubated with microsomal protein (1 mg/mL) at 37°C for 30 minutes. Incubations were stopped by adding one half volume of ice-cold acetonitrile followed by centrifugation at 14,000 rpm for 10 minutes. The supernatants were diluted 1:1 with H₂O and analyzed by HPLC with a DAD (λ=338 nm). Motesanib N-glucuronide (M1) quantification was based on the UV peak area. Kinetic data (Kₘ and Vₘₐₓ) were obtained using the Michaelis-Menten equation, curve fitting with simple weighting, and the least square minimization procedure (regression analysis) using SigmaPlot™ version 8.0 (Systat Software Inc., San Jose, CA).

Liquid Chromatography with Radiometric Detection and Liquid Chromatography/Mass Spectrometry Analysis

Samples from microsomal and hepatocyte metabolic-profiling experiments were analyzed by reverse-phase HPLC (Agilent 1100, Palo Alto, CA) with diode array UV (λ=338 nm) and a Flo-One (Packard Instrument Company, Downers Grove, IL) or a β-RAM (IN/US Systems) radiochemical flow scintillation analyzer. Selected samples were also analyzed by HPLC with ion-trap mass spectrometry (LCQ classic, Thermo Electron) using ESI in positive-ion mode. Separation was performed using a Phenomenex Luna C18 column (3 µm, 4.6 x 150 mm) and a mobile phase consisting of 90/10 H₂O/CH₃CN containing 5 mM NH₄OAc (A) and 90/10 CH₃CN/H₂O containing 5 mM NH₄OAc (B). Because some of the phase II metabolites were acid labile, acid was avoided in chromatographic analysis. A step gradient (0–2 min, 0% B, linear to 90% in 32 min, then to 100% at 32.1 min, held at 100% B until 40 min) at a flow of 1 mL/min was used. The LC flow after UV detection was split via a zero-volume tee with 80% of the flow to the β-RAM and 20% of the flow to the mass spectrometer. The β-RAM was
equipped with a liquid flow cell of 500 µL, and the LC flow was mixed with liquid scintillant at a ratio of 1:3. Column recovery of injected sample radioactivity was evaluated by comparing the amount of radioactivity injected (determined using liquid scintillation counting) and the amount of total radioactivity detected by β-RAM. No loss of radioactivity on the column was found for either microsomal or hepatocyte samples.

Ion-trap mass spectrometer parameters were optimized using a motesanib standard solution (1 µM). ESI source parameters were set at sheath gas, 80 (arbitrary units); auxiliary gas, 5; capillary, 275°C; collision energy, 35% (arbitrary units); mass range, 100 to 750 amu.

In the CYP phenotyping experiments using chemical inhibitors and CYP-specific antibodies, a shorter LC/MS method with a linear gradient of 15% B to 95% B (same mobile phase as above) in 10 minutes was used. The separation of motesanib and its metabolites was carried out using a YMC ODS-AQ column (3 µm, 2.0 × 50 mm) at a flow rate of 0.25 mL/min. Metabolites M3, M4, and M5 and the internal standard, were detected by selected ion monitoring of 390.2, 388.2, 372.2, and 427.1 ions, respectively. The ratio of each metabolite’s peak area to the internal standard was used to calculate the percentage of inhibition by chemical inhibitors or CYP antibodies.

**Accurate Mass Spectrometry Analysis**

High resolution mass spectrometric measurements of Mx were performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer (API-US; Waters Corporation, Milford, MA) equipped with a dual orthogonal Z SPRAY ESI source. Separation was performed by reverse-phase HPLC (Agilent 1100), using acidic mobile phases (A = 0.1% formic acid in H₂O, B = 0.1% formic acid in acetonitrile) for better retention of Mx on the
HPLC column. Concentrated samples from rat hepatocyte incubation of motesanib were injected into a YMC ODS-AQ C18 column (5 µm, 4.6 × 250 mm) at a flow rate of 1.0 mL/min with a 40-minute gradient (0–5 min, 0% B; 5–35 min, linear to 60% B, 36 min, 95% B, held until 40 min). Q-TOF was operated under V-Mode and calibrated with polyethyleneglycol; reserpine (1 µg/mL) was used as lock spray at a flow rate of 3 µL/min.

**Nuclear Magnetic Resonance Spectroscopy Analysis**

The structures of motesanib metabolites M1, Ma, M3, M4, and M5 were solved by NMR analysis and by acquiring a combination of 1D proton, 2D $^1$H-$^1$H homonuclear correlation spectroscopy (COSY), 2D $^1$H-$^1$H nuclear Overhauser effect spectroscopy (NOESY), 2D $^1$H-$^{13}$C heteronuclear single-bond correlation (HSQC), and 2D $^1$H-$^{13}$C heteronuclear multiple-bond correlation (HMBC) spectra using 600 MHz NMR spectrometers (Bruker Biospin Inc., Billerica, MA) equipped with either a 5-mm broadband inverse probe (Nalorac/Varian Inc., Palo Alto, CA) or 5-mm TCI cryoprobe (Bruker Biospin Inc.). Purified metabolites (M1, M3, M4, and M5; 20–120 µg) were dissolved in 300 µL methanol-d4 and transferred to 5-mm susceptibility-matched Shigemi tubes. Isolated metabolite Ma was dissolved in 160 µL of MeOD-d4 and transferred to a 3-mm tube. All spectra were recorded with standard experiments using a Bruker pulse program library. Spectral resolution for $^1$H was 0.3 Hz/pt and 3 Hz/pt in 1D and 2D experiments, respectively.
Results

Liver Microsomal Oxidative Metabolism of Motesanib

Several oxidative metabolites were observed in incubations of both human and animal liver microsomes. The microsomal oxidative profiles of motesanib and the percentages of motesanib and its oxidative metabolites relative to the total radioactivity are shown in Figure 2 and Table 1. Among the species investigated, mouse liver microsomes exhibited the highest extent of in vitro oxidative metabolism of motesanib. All metabolites formed in human liver microsomes were also detected in other species.

Hepatocyte Metabolism of Motesanib

Motesanib was extensively metabolized in hepatocyte incubations, especially in human hepatocytes, which had approximately 15% motesanib remaining after 4 hours of incubation (Figure 3, Table 2). Phase II conjugative pathways (M1, Ma, Mb, and Mc) accounted for 53%, 47%, 31%, 81%, and 93% of total metabolism in rat, mouse, dog, monkey, and human hepatocytes, respectively. The major human metabolite was M1 (indoline N-glucuronide), which accounted for >90% of total metabolism and was also formed in all other species examined. The phase II conjugates Ma (N-carbamoyl glucuronide) and Mb (N-glucose) were detected in rat, monkey, mouse, and dog hepatocyte incubations, but not in human hepatocytes. Metabolite Mc (N-acetylg glucosamine conjugate) was detected only in monkey hepatocyte incubations. In addition to the phase II conjugates, oxidative metabolites such as M2, M3, M4, and M5 were also detected in some species (Figure 3, Table 2). Metabolites resulting from N-dealkylation of methyl pyridyl, Mx, Miv and Mv, were also detected.
Liver Microsomal Phase II Metabolism of Motesanib

The species differences observed in the formation of the phase II conjugates M1, Mb, and Mc were further examined in liver microsomes using different UDP-sugar cofactors. The N-glucuronide metabolite M1 was formed in all species examined (Figure 4, Table 3). The rate and extent of M1 formation was much greater in human liver microsomes than in other species. The N-glucose adduct Mb was formed in the presence of UDP-glucose in all species except human, and the highest rate of its formation was in monkey liver microsomes (Table 3). No adduct was detected in any of the species examined using UDP-galactose as a cofactor. When motesanib was incubated with liver microsomes using UDP-N-acetylglucosamine as a cofactor, only monkey liver microsomes generated the N-acetylglucosamine conjugate Mc (Table 3). Overall, the results obtained using liver microsomes agreed with results obtained using hepatocyte incubations.

Identification of Metabolites

Preliminary identification of metabolites was based on LC/MS, LC/MS/MS, and NMR. Using full-scan positive ion ESI, motesanib and most of its metabolites were found to occur predominantly as protonated molecules [M+H]+. Therefore, the molecular weight of each metabolite was readily obtained. Furthermore, multistage mass spectra obtained from ion-trap mass spectrometry of motesanib and its metabolites were generated using positive- or negative-ion ESI to identify the potential sites of metabolism. A summary of molecular ions and fragment ions of motesanib and its metabolites is shown in Table 4.
Motesanib

The molecular ion of motesanib [M+H]+ was observed at m/z 374, and the peak at 2 mass units higher (m/z 376) corresponds with [14C]-motesanib (Figure 5). The fragmentation of the [M+H]+ ion at m/z 374 generated by collision induced dissociation (CID) was interpreted and used to aid in the structural assignment of the metabolites. The tandem mass spectrum of the [M+H]+ ion at 35% collision energy (arbitrary unit) displayed 2 major fragment ions at m/z 212 and 189. Fragment ion at m/z 212 corresponded to the cleavage of nicotinamide amide bond and charge retention on 1 pyridyl moiety. Fragment ion at m/z 189 corresponded to the indoline formamide moiety. The proton NMR chemical shifts and coupling constants of motesanib are included in Table 5.

Metabolite M3

M3 exhibited molecular ion [M+H]+ at m/z 390 in the ESI positive-ion full-scan mass spectrum, implying a mono-oxygenated metabolite (Figure 6). The tandem mass spectrum of protonated M3 showed a major fragment ion at m/z 372, corresponding to the loss of H2O, and 2 other minor fragment ions at m/z 282 and 252, likely resulting from cleavage of the methyl pyridyl group. The results of the negative-ion tandem mass spectra of M3 were in agreement with the positive ion tandem mass spectra, suggesting that oxidation likely occurred on the methyl pyridyl moiety. This oxidation induced a fragmentation in M3 that is quite different from that of motesanib. The structural identity of M3 was confirmed to be pyridyl N-oxide by NMR analysis of the isolated M3 from microsomal incubations. The position of the N-oxide moiety was determined by the characteristic changes in chemical shifts and coupling pattern of protons H1 and H2 - relative to motesanib, as shown in Table 5. The structure was further confirmed by
comparison with a synthetic N-oxide standard, which showed chromatographic, mass spectral, and NMR characteristics identical to isolated metabolite M3.

**Metabolite M4**

Motesanib metabolite M4 was generated in vitro in both microsomal incubations and hepatocyte incubations of all species investigated. ESI positive-ion full-scan MS of M4 showed predominantly a protonated molecule at m/z 388, 14 amu higher than that of protonated motesanib. The MS/MS of protonated M4 produced 1 major fragment ion at m/z 212 (Figure 7), so the 14-amu increment must have occurred on the indoline moiety. The structure of M4 was confirmed by NMR analysis of the isolated M4 from microsomal incubations, and its proton NMR characteristic is shown in Table 5. The key observation in this experiment was the long-range methyl protons to carbonyl carbon (at 185 ppm) coupling from an HMBC experiment. The structure of M4 was also confirmed with cochromatography with authentic standards.

**Metabolite M5**

M5 was one of the major microsomal metabolites of motesanib detected in all species investigated (Figure 2, Table 1). The positive-ion full-scan ESI spectrum of M5 clearly showed a predominant ion at m/z 372 (Figure 8), 2 amu less than that of protonated motesanib (Figure 5). As shown in Figure 8, the tandem mass spectrum of M5 was similar to that of protonated motesanib except for the fragment at m/z 189 for motesanib was shifted 2 amu less to m/z 187. NMR analysis of M5 (Table 5) indicated that it was a carbinolamine. The carbinolamine metabolite dehydrated readily during the ESI process, generating dehydrated ions in both positive- and negative-ion modes. The key observations in the HMBC experiment were long-range proton-carbon couplings 10(10')-
C_{11}(C_{11}'), where \( \delta_{C_{11}} = 100.3 \text{ ppm}, \quad ^1J_{C_{11}-H_{11}} = 161 \text{ Hz}, \quad \delta_{C_{11}'} = 182.4 \text{ ppm}, \quad \text{and} \quad ^1J_{C_{11}-H} = 182 \text{ Hz.} \) The NMR data were essential to determining the structure of M5.

**Metabolite M2**

Metabolite M2 was a minor metabolite detected in mouse and dog microsomal incubations with motesanib (Figure 2, Table 1). The ESI positive-ion full-scan mass spectrum of M2 exhibited predominantly an ion at m/z 388, a 14-amu increment comparable with that of protonated motesanib (data not shown). The M2 tandem mass spectrum was different from that of M4, and showed several diagnostic fragment ions at m/z 370, 212, and 159, suggesting that oxidation occurred on the indoline moiety. Therefore, M2 is possibly a sequential metabolite from M5. However, complete structure assignment of M2 was not possible based on MS data alone.

**Metabolite M1**

Motesanib metabolite M1 was the major metabolite formed in human hepatocytes (Figure 3, Table 2). M1 was also formed in hepatocyte incubations of other species examined, but to a lesser extent than in the human sample. When motesanib was incubated with liver microsomes in the presence of UDP-GA, M1 was detected in all species. However, human liver microsomes exhibited the highest percentage of M1 formation (Figure 4, Table 3).

The ESI positive-ion full-scan mass spectrum of M1 showed predominantly the [M+H]^+ ion at m/z 550, which is 176 amu higher than that of protonated motesanib and suggests direct glucuronidation of motesanib. The tandem mass spectrum of M1 displayed an abundant fragment ion at m/z 374 corresponding to the loss of a glucuronide moiety from motesanib (Figure 9).
M1 was isolated from in vitro experiments and fully characterized by NMR. Identifiable proton and carbon chemical shifts are listed in Table 5. The 1D proton spectrum displayed chemical shifts from both motesanib and glucuronide moieties. The glucuronide anemic proton appeared as a doublet at $\delta_H 4.82/\delta_C 85.3$ ppm, and its large H12-H13 scalar coupling (8.9 Hz) indicates a $\beta$-configuration at the anomeric carbon. HMBC correlations were observed between the methylene carbon in the indoline ring (C11, 59.9 ppm) and the anemic proton (H12) of the glucuronide, and a 1D slice from the 2D $^{13}C-^1H$ HMBC spectrum at the chemical shift of C11 is displayed in Figure 10. The 3-bond correlations to the anemic proton and both methyl protons confirm that glucuronic acid is attached to the indoline nitrogen. In addition, the indoline N-glucuronide authentic standard was synthesized and showed identical chromatographic, mass spectral, and NMR characteristics compared with the isolated M1.

Metabolite Ma

Motesanib metabolite Ma was detected in hepatocyte incubations of several species (Figure 3, Table 2). The ESI positive-ion full-scan mass spectrum of Ma identified predominantly the [M+H]$^+$ ion at m/z 594. The protonated Ma fragmented exclusively to m/z 418 upon collision-induced dissociation, and the neutral loss of 176 amu again indicated glucuronide conjugation. MS$^3$ further revealed a neutral loss of 44 amu (CO$_2$) to produce protonated motesanib at m/z 374 as the predominant ion. Therefore, the structure of Ma was proposed to be N-carbamoyl glucuronide.

Ma was also formed in the in vitro incubations of motesanib with liver microsomes from several species in a CO$_2$-rich atmosphere in carbonate buffer and in presence of UDP-GA. The reaction in monkey liver microsomes was scaled up, and under the experimental conditions, 30% of Ma and 10% of M1 were formed. Ma was isolated,
purified, and fully characterized by NMR spectra. Its identifiable chemical shifts are listed in Table 5. The 1D proton spectrum of the metabolite showed peaks from both motesanib and glucuronide. Comparison of chemical shifts from motesanib and Ma indicated that changes were localized to the indoline moiety. The anomic proton appeared at 5.57 (d, 7.1 Hz, β-anomer) with a carbon chemical shift of 95.2 ppm, indicating an O-glucuronide. HMBC correlations were also observed from H11 to the carbonyl carbon (C17) at 151.5 ppm. The combined evidence strongly supports the presence of an N-carbomyl glucuronide and the site of conjugation being on the indoline nitrogen.

Metabolite Mb

Motesanib metabolite Mb was detected in hepatocyte incubations of several species other than humans (Figure 3, Table 2). Mb exhibited molecular ion [M+H]+ at m/z 536. MS/MS of protonated Mb produced abundant fragment ions at m/z 374 and 518, which correspond to the intact protonated motesanib and loss of H2O from Mb, respectively. Incubation of motesanib with liver microsomes of several species in the presence of UDP-glucose also yielded Mb (Table 3), but similar incubations in the presence of UDP-galactose did not. The structure of Mb was therefore identified as an N-glucose conjugate of motesanib, and the site of conjugation was likely on the indoline nitrogen, which is the site of conjugation for glucuronidation (M1 and Ma). Mb was not detectable in human liver microsomal incubations, which was consistent with the results from human hepatocyte incubation.

Metabolite Mc

Motesanib metabolite Mc was detected in monkey hepatocyte incubations, but not in other species (Figure 3, Table 2). Mc exhibited molecular ion [M+H]+ at m/z 577 in the
positive-ion ESI mass spectrum. MS/MS of protonated Mc produced predominantly a fragment ion at m/z 374 corresponding to the intact protonated motesanib. Further fragmentation of the 374 ion produced ions at m/z 212 and 189 in the MS³ spectrum. Mass spectrometry data indicated direct conjugation and a mass increment of 203 amu, and the conjugate moiety contained an odd number of nitrogen atoms. As a result, Mc was proposed to be an indoline N-linked β-N-acetylglucosamine adduct of motesanib. Because in vitro experiments using motesanib incubated with monkey liver microsomes in the presence of UDP-N-acetylglucosamine also produced the same metabolite (Table 3), the structure of Mc was confirmed to be the β-N-acetylglucosamine adduct of motesanib, and the site of conjugation is likely on the indoline nitrogen based on unequivocally assigned M1 and Ma metabolites. Both microsomal incubations in the presence of UDP-N-acetylglucosamine and hepatocyte incubations indicated that Mc was a unique metabolite formed in monkeys.

Metabolites Miv and Mv
Motesanib metabolites Miv and Mv were not detected by radiometry when [methylene ¹⁴C]-motesanib was used as substrate, but they were detected by UV and MS. Miv and Mv did not retain the radiolabel after N-dealkylation of the methyl pyridyl moiety. When [carbonyl ¹⁴C]-motesanib was incubated with rat hepatocytes, Miv and Mv, but not Mx, were detected by radiometry (Figure 11), which confirmed that Miv and Mv were metabolites resulting from the loss of methyl pyridine and that Mx contained the methyl pyridyl moiety.

The ESI full-scan mass spectrum of Mv showed an abundant ion at m/z 283, and its tandem mass spectrum exhibited 2 unique fragment ions at m/z 189 and 121. Consequently, the identity of Mv has been proposed to be an amino nicotinamide
metabolite. Metabolite Miv eluted earlier than Mv, and it exhibited a molecular ion at m/z 297, 14 amu higher than that of Miv. The tandem mass spectrum of protonated Miv revealed a predominant ion at m/z 121, suggesting that the amino pyridine moiety remained unchanged. Thus, the structure of Miv was proposed to be the lactam of Mv.

Metabolite Mx

Motesanib metabolite Mx was an extremely polar metabolite that eluted in the solvent front under analysis conditions. In comparison, Mx was better retained on a YMC ODS-AQ column using acid mobile phases. Using positive-ion ESI on the Q-TOF mass spectrometer, exact mass measurement confirmed the identity of Mx to be isonicotinic acid. The exact mass of [M+H]+ of Mx was detected at m/z 124.0395 (−3.2 ppm from the theoretical mass of protonated isonicotinic acid, C₆H₅NO₂ 124.0399). The synthetic standard of isonicotinic acid showed a similar retention time and mass spectrum, thus confirming that Mx and isonicotinic acid are the same.

Oxidative Metabolism of Motesanib by Recombinant Cytochrome P450 Isoforms

In an NADPH-dependent manner, motesanib was metabolized by human liver microsomes into 3 major metabolites: M3 (N-oxide), M4 (lactam), and M5 (carbinolamine). Identification of the CYP isozymes responsible for oxidative metabolism of motesanib was performed using a combination of approaches, including metabolism by recombinant CYP isozymes and the use of CYP inhibitors and specific anti-CYP antibodies.

Motesanib (10 µM) was incubated with recombinant human CYPs. Of the recombinantly expressed isoforms tested, CYP1A1, CYP2B6, CYP2D6, CYP3A4, CYP3A5, and CYP3A7 were capable of metabolizing motesanib. CYP2D6 was capable of forming M3
(N-oxide), M4 (lactam), and M5 (carbinolamine) in vitro, whereas CYP1A1 and CYP3A only formed lactam (M4) and carbinolamine M5 metabolite. CYP2B6 formed only the N-oxide M3 metabolite. The rates of formation of these oxidative metabolites by recombinant human CYPs are summarized in Figure 12.

Inhibition of Motesanib Oxidative Metabolism by Selective Cytochrome P450 Inhibitors

The results of experiments using specific CYP inhibitors to prevent the formation of motesanib metabolites are summarized in Figure 13. Incubation with ketoconazole, quinidine, 8-methoxypsoralen, sulphaphenazole, and furafylline resulted in less than approximately 20% inhibition of M3 formation. The formation of M4 (lactam) and M5 (carbinolamine) metabolites of motesanib were both markedly inhibited in the presence of the CYP3A4 inhibitor, ketoconazole. Ketoconazole resulted in approximately 50% metabolic inhibition at a 1-µM concentration and approximately 70% inhibition at a 5-µM concentration. The formation of M4 was only moderately inhibited (<20%) by quinidine (CYP2D6), sulphaphenazole (CYP2C9), lansoprazole (CYP2C19), and 8-methoxypsoralen (CYP2A6). The formation of M5 was moderately inhibited (<20%) by quinidine (CYP2D6) and sulphaphenazole (CYP2C9). Results from positive control experiments confirmed that the incubations were performed under optimum conditions (Ono et al., 1996).

Inhibition of Motesanib Oxidative Metabolism by Specific Anti–Cytochrome P450 Antibodies

The results of anti–CYP antibody inhibition of motesanib oxidative metabolism are shown in Figure 14. At the concentration of 10 µg/0.1 mg HLM, anti–CYP3A4 markedly inhibited formation of metabolites M4 and M5, similar to results from experiments that
used specific CYP inhibitors. The formation of metabolite M3 was inhibited by approximately 50% compared with the control using the anti–CYP1A antibody. The anti–CYP2D6 antibody displayed a modest ability to prevent the formation of motesanib metabolites. Inhibition studies with human liver microsomes in presence of CYP3A4-, CYP2D6-, and CYP1A-specific antibodies showed that CYP3A4 was the major CYP isoform responsible for the formation of the M4 and M5 metabolites; CYP2D6 and CYP1A may play a minor role in metabolism.

**Motesanib N-Glucuronidation by Recombinant UDP-glucuronosyltransferase Isoforms**

Motesanib (10 µM) was incubated with microsomes expressing human UGTs. UGT1A4 and UGT1A1 were capable of catalyzing the N-glucuronidation of motesanib, with UGT1A4 exhibiting greater catalytic activity. The rates of N-glucuronide metabolite formation by UGT1A4 and UGT1A1 were 88.1 and 8.9 pmol/mg protein per minute, respectively. No other recombinantly expressed isoforms tested formed N-glucuronide metabolite (M1).

**Inhibition of Motesanib N-glucuronidation by Selective UDP-glucuronosyltransferase Substrates/Inhibitors**

The effect of the UGT1A1 and UGT1A4 substrates bilirubin and imipramine on N-glucuronidation of motesanib was tested in human liver microsomes. The results indicate that imipramine inhibited M1 formation by 64% at 1 mM and by 100% at 5 mM. Bilirubin, a specific substrate of UGT1A1, exhibited 49% inhibition at 25 µM but caused only 58% inhibition at 50 µM. However, bilirubin at higher concentrations is also known to inhibit UGT1A4 activity (Ghosal et al., 2004). Together, these inhibition data are in
agreement with UGT isoform activity and demonstrate that UGT1A4 plays a major role in
the formation of M1, whereas UGT1A1 is less important for M1 formation.

**Enzyme Kinetics of Motesanib N-glucuronidation in Microsomes**

The enzyme kinetics of motesanib N-glucuronidation in human, rat, dog, and monkey
liver microsomes are shown in Table 6. Human liver microsomes showed the highest
efficiency for motesanib N-glucuronidation, with an intrinsic clearance \((V_{\text{max}}/K_m)\) of
49.4 µL/min/mg protein. Rat, dog, and monkey liver microsomes showed a much lower
intrinsic clearance of 7.8, 5.0, and 4.2 µL/min/mg protein, respectively.
Discussion

The in vitro metabolism of motesanib—a novel, highly selective oral inhibitor of VEGF receptors 1, 2, and 3; PDGF receptor, and Kit—was investigated using liver microsomes and hepatocytes from human and preclinical species. Motesanib undergoes NADPH-dependent oxidation and oxidative metabolism in human liver microsomes similar to nonhuman species. The major oxidative metabolites of motesanib were identified as N-oxide (M3), indoline lactam (M4), indoline carbinolamine (M5), and N-dealkylated metabolite isonicotinic acid (Mx).

Identification of motesanib metabolites was first based on full-scan and LC/MS/MS data. The high sensitivity, selectivity, and speed of the LC/MS technique have made it an extremely useful tool for the identification of metabolites both in vitro and in vivo (Clarke et al., 2001; Nassar and Talaat, 2004; Ma et al., 2006). With “soft” atmospheric pressure ionization techniques (either ESI or atmospheric pressure chemical ionization) most xenobiotics and their metabolites produce protonated [M+H]+ and deprotonated [M-H]− molecules in positive and negative ionization modes, respectively. Molecular weight and type of biotransformation can be readily identified. With LC/MS/MS data, CID fragment patterns of the metabolites can be compared with that of the parent drug, and sites and types of biotransformation can often be pinpointed to specific substructures (Levensen et al., 2005). However, there are limitations of LC/MS data for metabolite structure assignments. For example, in the present report, the carbinolamine metabolite M5 was quite labile, and full-scan ESI produced a dehydrated ion rather than the intact protonated molecule. NMR data provided definitive structural confirmation of metabolite M5, and these data were also essential for the full structural characterization of the N-oxide (M3) and lactam (M4) metabolites.
Carbinolamines of aliphatic amines are generally unstable and rapidly undergo nonenzymatic dissociation resulting in N-dealkylation (Rose and Castagnoli, 1983). The carbinolamines of the N-heterocyclic class of compounds, however, are more stable, and their detection and isolation have been reported (Murphy, 1973; Streeper et al., 1997; Vickers and Polsky, 2000; Cui et al., 2004). The carbinolamine M5 metabolite of motesanib was sufficiently stable to allow isolation, purification, and characterization by NMR. M5 was a major oxidative metabolite of motesanib in microsomes and was detected in the human and preclinical species tested. From reaction phenotyping experiments using recombinant human CYPs, selective chemical inhibitors, and specific CYP inhibitory antibodies, CYP3A4 was found to be the major CYP isozyme responsible for M5 formation, with possible minor contributions from CYP2D6, CYP2C9, and CYP1A1. Approximately 70% of M5 formation was inhibited by ketoconazole and the anti-CYP3A4 monoclonal antibody.

Further oxidation of carbinolamine M5 can lead to formation of the M4 lactam metabolite. In human liver microsomes, both the CYP3A4 inhibitor ketoconazole and the CYP3A4-specific antibody inhibited M4 formation by 70%, a trend similar to their inhibition of M5 formation. CYP1A1, CYP2D6, CYP2C9, CYP2C19, and CYP2A6 appear to play minor roles in regulating the formation of M4. Cytochrome P450 phenotyping experiments indicated the involvement of CYP2D6 and CYP1A in the formation of the pyridyl N-oxide metabolite M3. The involvement of flavin-containing mono-oxygenases in M3 formation was not examined in this study and cannot be ruled out.

In hepatocyte incubations, motesanib was extensively metabolized and species differences in metabolism were observed. A summary of the in vitro biotransformation of motesanib in preclinical species and in humans is shown in Figure 15.
metabolites appeared to dominate in hepatocytes, especially in humans, with the indoline N-glucuronide M1 as the major metabolite (accounting for >90% of total metabolism). M1 was also detected in rat, mouse, dog, and monkey hepatocytes. Using liver microsomes and UDP-GA as a cofactor, M1 was produced in all species, but its formation was most efficient in humans. This observation is consistent with reports that species differences for N-glucuronidation appeared to be predominantly quantitative in nature for primary and secondary amine forming nonquaternary N-glucuronides (Chiu and Huskey, 1998). M1 was isolated and purified from scaled-up incubations of motesanib with human liver microsomes and UDP-GA, and its structure was fully characterized by LC/MS and NMR. M1 was very labile and susceptible to cleavage back to motesanib and glucuronic acid under very mild acidic conditions, which is consistent with reports for other unstable and acid labile primary and secondary amine N-glucuronides (Babu et al., 1992; Green and Tephly, 1998). In general, glucuronidation is considered a key pathway for detoxifying and eliminating drugs; thus, it is important to more fully understand the mechanism of N-glucuronidation, the enzymes involved, and their regulation. Our results indicate that in humans, motesanib indoline N-glucuronidation is catalyzed mainly by human UGT1A4 and to a lesser extent by UGT1A1.

In addition to indoline N-glucuronide metabolite M1, 3 other unusual conjugates at the alicyclic nitrogen were observed in the hepatocyte incubations of motesanib. The first, Ma, an N-carbamoyl glucuronide metabolite found in all species except humans, was presumably formed by reaction of the amine with dissolved CO$_2$ to form a transient carbamic acid intermediate, followed by subsequent conjugation with glucuronic acid. N-carbamoyl glucuronidation is an uncommon metabolic reaction but has been reported for several primary and secondary amines, including tocainide (Elvin et al., 1980), carvedilol...
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(Schaefer, 1992), a dipeptidyl peptidase IV inhibitor ILT-threo (Beconi et al., 2003), and sertraline (Obach et al., 2005). In vitro formation of N-carbamoyl glucuronide metabolites have also been reported in liver microsomes incubated in a CO₂-rich atmosphere and in carbonate buffer in the presence of UDP-GA. Obach and coworkers (Obach et al., 2005) have also reported that sertraline N-carbamoyl glucuronidation was primarily formed by UGT2B7.

Metabolite Mb, an N-glucoside conjugate and second unusual conjugate at the acyclic nitrogen, was detected in hepatocyte incubations of motesanib in all preclinical species but not in human hepatocytes. The UGTs catalyze the conjugation of a variety of substrates with a sugar using UDP-sugar (UDP-GA, UDP-galactose, UDP-glucose, or UDP-xylose) as the sugar donor. N-glucuronidation is a quite common and important phase II reaction for amine-containing compounds. However, N-glucosidation is known to be a metabolic reaction only for a limited number of compounds, including barbiturates (Tang, 1990; Toide et al., 2004) and varenicline (Obach et al., 2006). The formation of N-glucoside metabolite Mb in microsomal incubations derived from rat, mouse, dog, and monkey with UDP-glucose as a cofactor is consistent with results obtained using hepatocytes. The structural requirements for glucoside formation are unknown; however, they appear to share a requirement for glucuronides, and glucosidation is typically a minor pathway where glucuronidation is possible (Tang, 1990).

Metabolite Mc (N-acetylglucosamine conjugate), a third unusual conjugate at the acyclic nitrogen, was detected only in monkey hepatocyte incubations. Although N-acetylglucosaminidation has been reported as a selective conjugation reaction for ursodeoxycholic acid (Marschall et al., 1994) via O-linkage, formation of N-linked β-N-
Acetylglucosamine is extremely rare. In 1 report, an N-linked β-N-acetylglucosamine metabolite for the HIV-1 reverse-transcriptase inhibitor delavirdine was identified in monkey urine (Chang et al., 1997). Using UDP-N-acetylglucosamine as a cofactor, we observed that the metabolite Mc was produced exclusively in monkey liver microsomes.

This is the first systematic investigation of the in vitro metabolism of motesanib, a novel, highly selective oral kinase inhibitor for the treatment of solid tumors. In human hepatocytes, motesanib undergoes extensive indoline N-glucuronidation (>90% of total metabolism) and to a much lesser extent, oxidative metabolism (<10%). Motesanib metabolites detected from human samples in vitro were also present in samples from rats, dogs, and monkeys, and no unique human metabolites were found. These in vitro metabolism studies and the examination of species differences in metabolism are critical for selecting the appropriate species for safety studies and for identifying the metabolites that may potentially be active or toxic, especially in light of recent FDA guidance on safety testing of metabolites. Predicting human in vivo metabolites and ensuring sufficient coverage of metabolites in appropriate preclinical safety studies are important for successful drug development. Follow-up absorption, distribution, metabolism, and excretion studies with radiolabeled motesanib in rats and dogs indicated very good correlation between in vitro and in vivo metabolic profiles (Amgen Inc., data on file) and will be the subject of a separate publication. The in vitro data accurately predicted the in vivo metabolic profile of motesanib and indicate that the metabolism of motesanib occurs via multiple oxidative and conjugative pathways. Furthermore, multiple CYP isozymes (CYP3A4, CYP2D6, and CYP1A1) and UGT isozymes (UGT1A4 and UGT1A1) are involved in the biotransformation of motesanib, thereby reducing the likelihood of potential drug interactions from other coadministered xenobiotics.
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References


Footnotes

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Legends for Figures

Figure 1. Chemical structures of $^{14}$C-labeled motesanib.

Figure 2. In vitro oxidative metabolism profiles of motesanib in liver microsomes from human and preclinical species.

Figure 3. In vitro metabolism profiles of motesanib in hepatocytes from human and preclinical species.

Figure 4. Formation of motesanib N-glucuronide (M1) in liver microsomes across species with UDP-GA co-factor.

Figure 5. Positive-ion ESI full-scan and tandem mass spectra of motesanib.

Figure 6. Positive-ion ESI full-scan and tandem mass spectra of motesanib metabolite M3.

Figure 7. Positive-ion ESI full-scan and tandem mass spectra of motesanib metabolite M4.

Figure 8. Positive-ion ESI full-scan and tandem mass spectra of motesanib metabolite M5.

Figure 9. Positive-ion ESI full-scan and tandem mass spectra of motesanib metabolite M1.

Figure 10. 1D proton trace extracted from 2D $^{13}$C-$^1$H correlation spectrum of metabolite M1 at carbon chemical shift of C11.
Figure 11. In vitro metabolism profiles of motesanib in rat hepatocytes and comparison between 2 different $^{14}$C labels of motesanib (denoted by * in the structures).

Figure 12. Formation of oxidative metabolites of motesanib by recombinant human cytochrome P450.

Figure 13. Effect of isoform-selective cytochrome P450 inhibitors on the oxidative metabolism of motesanib by human liver microsomes. In positive control experiments using specific CYP inhibitors against known substrate reactions, ketoconazole (1 and 5 µM) inhibited 1'-OH midazolam formation from midazolam (5 µM, with 0.1 mg/mL HLM, 5 min) by 93.1% and 98.1%; quinidine (1 and 5 µM) inhibited 1'-OH bufuralol formation from bufuralol (15 µM, 0.25 mg/mL HLM, 10 min) by 77.0% and 85.3%; sulphaphenazole (1 and 10 µM) inhibited 4'-OH diclofenac formation from diclofenac (5 µM, with 0.1 mg/mL HLM, 10 min) by 70.9% and 95.5%; lansoprazole (10 µM) inhibited 4'-OH mephenytoin formation from S-mephenytoin (50 µM, with 0.5 mg/mL HLM, 30 min) by 86.5%; Furafllyline (10 µM) inhibited acetaminophen formation from phenacetin (50 µM with 0.5 mg/mL HLM, 20 min) by 86.9%; Diethyldithiocarbamate (30 µM) inhibited 6-OH chlorzoxazone formation from chlorzoxazone (30 µM with 0.5 mg/mL HLM, 20 min) by 73.3%; and 8-methoxypsoralen (0.2 µM) inhibited 7-OH coumarin formation from coumarin (2 µM with 0.2 mg/mL HLM, 10 min) by 94.1%.
Figure 14. Effect of isoform-selective cytochrome P450 antibodies (10 µg/0.1 mg human liver microsomes) on the oxidative metabolism of motesanib by human liver microsomes. In positive control experiments using anti-CYP antibodies (10 µg/0.1 mg of HLM) against known substrate reactions, CYP3A4 antibody inhibited 1'-OH midazolam formation from midazolam (5 µM) by 41.3%; CYP2D6 antibody inhibited 1'-OH bufuralol formation from bufuralol (15 µM) by 76.2%; and CYP1A antibody inhibited acetaminophen formation from phenacetin (50 µM) by 90.5%.

Figure 15. Summary of in vitro metabolic pathways of motesanib.
TABLE 1

The composition of oxidative metabolites of motesanib in microsomes across species

<table>
<thead>
<tr>
<th>Species</th>
<th>Mx</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>Parent</th>
<th>Others&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>4.2</td>
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<td>18.3</td>
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<td>3.0</td>
<td>8.1</td>
<td>74.9</td>
<td>1.5</td>
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<tr>
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<td>6.6</td>
<td>10.2</td>
<td>75.1</td>
<td>ND</td>
</tr>
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</table>

ND=not detected

<sup>a</sup>Results obtained by HPLC with a β-RAM radiometric detector. Numbers indicate mean percentages, which represent the compositions of microsomal incubations (n=2 for each species) using motesanib (10 µM) and microsomal protein (1 mg/mL) incubated in the presence of NADPH (1 mM) at 37°C for 30 min.

<sup>b</sup>Peaks not identified by LC/MS.
TABLE 2

The composition of oxidative and conjugative metabolites of motesanib in hepatocytes across species

<table>
<thead>
<tr>
<th>Species</th>
<th>Mx</th>
<th>M1</th>
<th>Ma</th>
<th>Mb</th>
<th>Mc</th>
<th>M2</th>
<th>M3</th>
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<td>3.2</td>
<td>20.2</td>
<td>ND</td>
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</tr>
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<td>5.3</td>
<td>11.7</td>
<td>3.0</td>
<td>10.8</td>
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<td>ND</td>
<td>ND</td>
<td>16.6</td>
<td>6.8</td>
<td>45.9</td>
<td>ND</td>
</tr>
<tr>
<td>Dog</td>
<td>3.4</td>
<td>7.2</td>
<td>ND</td>
<td>3.8</td>
<td>ND</td>
<td>ND</td>
<td>4.5</td>
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<td>6.4</td>
<td>64.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Monkey</td>
<td>4.9</td>
<td>33.0</td>
<td>7.1</td>
<td>7.2</td>
<td>11.1</td>
<td>ND</td>
<td>ND</td>
<td>8.9</td>
<td>ND</td>
<td>27.9</td>
<td>ND</td>
</tr>
<tr>
<td>Human</td>
<td>ND</td>
<td>79.3</td>
<td>Trace</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.4</td>
<td>4.7</td>
<td>ND</td>
<td>14.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND=not detected

*Results obtained by HPLC with a β-RAM radiometric detector. Numbers indicate mean percentages, which represent the compositions of hepatocyte incubations (n=2 or 3) using motesanib (10 µM) and hepatocytes (1 × 10⁶ cells/mL) at 37°C for 4 h.
### TABLE 3

*The composition of conjugative metabolites of motesanib in microsomes across species*

| Cofactor                  | Metabolite | % of Metabolite Formation⁣
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>UDP-GA</td>
<td>M1</td>
<td>20.5</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>Mb</td>
<td>5.1</td>
</tr>
<tr>
<td>UDP-N-acetylglucosamine</td>
<td>Mc</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND=not detected

⁣Results obtained by HPLC with a β-RAM radiometric detector. Percentages represent mean relative amount of conjugative metabolites M1, Mb, and Mc formed in microsomal incubations (n=2 for each species) using motesanib (10 µM) and microsomal protein (1 mg/mL) incubated in the presence of cofactor (2 mM) at 37°C for 30 min.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M+H]+, m/z</th>
<th>Major LC/MS/MS Fragments, m/z</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent drug</td>
<td>374</td>
<td>212, 189</td>
<td>Motesanib</td>
</tr>
<tr>
<td>Mx</td>
<td>124</td>
<td>–</td>
<td>Isonicotinic acid</td>
</tr>
<tr>
<td>M2</td>
<td>388</td>
<td>370, 212, 159</td>
<td>N-oxide</td>
</tr>
<tr>
<td>M3</td>
<td>390</td>
<td>372, 282, 252</td>
<td>Lactam (oxindole)</td>
</tr>
<tr>
<td>M4</td>
<td>388</td>
<td>212</td>
<td>Carbinolamine</td>
</tr>
<tr>
<td>M5</td>
<td>372</td>
<td>212, 187</td>
<td>Indoline N-glucuronide</td>
</tr>
<tr>
<td>M1</td>
<td>550</td>
<td>532, 374, 212, 189</td>
<td>Indoline N-carbomyl glucuronide</td>
</tr>
<tr>
<td>Ma</td>
<td>594</td>
<td>418, 374</td>
<td>Indoline N-glucose</td>
</tr>
<tr>
<td>Mb</td>
<td>536</td>
<td>518, 374</td>
<td>Indoline N-glucose</td>
</tr>
<tr>
<td>Mc</td>
<td>577</td>
<td>374, 212, 189</td>
<td>Indoline N-N-acetylglucosamine</td>
</tr>
<tr>
<td>Mv</td>
<td>283</td>
<td>189, 121</td>
<td>2-Amino nicotinamide metabolite</td>
</tr>
<tr>
<td>Miv</td>
<td>297</td>
<td>121</td>
<td>Lactam of Mv</td>
</tr>
</tbody>
</table>

CID=collision-induced dissociation; LC/MS/MS=tandem mass spectrometry
TABLE 5

Nuclear magnetic resonance characteristics of motesanib and its metabolites M3, M4, M5, M1, and Ma

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Motesanib</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M1</th>
<th>Ma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Position</strong></td>
<td><strong>'H (ppm) (mult, J in Hz)</strong></td>
<td><strong>'C (ppm)</strong></td>
<td><strong>'H (ppm) (mult, J in Hz)</strong></td>
<td><strong>'C (ppm)</strong></td>
<td><strong>'H (ppm) (mult, J in Hz)</strong></td>
<td><strong>'C (ppm)</strong></td>
</tr>
<tr>
<td>1</td>
<td>8.45 (d, 6)</td>
<td>146.5</td>
<td>8.28 (d, 6.7)</td>
<td>138.0</td>
<td>8.46 (d, 6)</td>
<td>148.1</td>
</tr>
<tr>
<td>2</td>
<td>7.43 (d, 6)</td>
<td>123.0</td>
<td>7.56 (d, 6.7)</td>
<td>124.8</td>
<td>7.44 (d, 6)</td>
<td>122.1</td>
</tr>
<tr>
<td>3</td>
<td>4.78 (s)</td>
<td>43.1</td>
<td>4.78 (s)</td>
<td>42.2</td>
<td>4.8 (s)</td>
<td>42.7</td>
</tr>
<tr>
<td>4</td>
<td>8.12 (dd, 4.7, 1.7)</td>
<td>150.4</td>
<td>8.14 (dd, 4.7, 1.7)</td>
<td>150.3</td>
<td>8.16 (dd, 4.7, 1.7)</td>
<td>150.4</td>
</tr>
<tr>
<td>5</td>
<td>6.7 (dd, 7.7, 4.7)</td>
<td>111.5</td>
<td>6.72 (dd, 7.7, 4.7)</td>
<td>111.4</td>
<td>6.73 (dd, 7.7, 4.7)</td>
<td>111.3</td>
</tr>
<tr>
<td>6</td>
<td>8.04 (dd, 7.7, 1.7)</td>
<td>136.7</td>
<td>8.04 (dd, 7.7, 1.7)</td>
<td>136.5</td>
<td>8.08 (dd, 7.7, 1.7)</td>
<td>136.7</td>
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<tr>
<td>7</td>
<td>7.08 (d, 1.7)</td>
<td>104.8</td>
<td>7.08 (d, 1.7)</td>
<td>103.5</td>
<td>7.51 (m)</td>
<td>6.89 (d, 1.7)</td>
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<tr>
<td>8</td>
<td>6.94 (dd, 8.1, 1.7)</td>
<td>113.4</td>
<td>6.94 (dd, 8.1, 1.7)</td>
<td>112.0</td>
<td>7.25 (m)</td>
<td>6.9 (dd, 8.1, 1.7)</td>
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<tr>
<td>9</td>
<td>7.02 (d, 8.1)</td>
<td>121.6</td>
<td>7.03 (d, 8.1)</td>
<td>121.3</td>
<td>7.25 (m)</td>
<td>7 (d, 8.1)</td>
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<tr>
<td>10</td>
<td>1.31 (s)</td>
<td>26.4</td>
<td>1.32 (s)</td>
<td>26.4</td>
<td>1.38 (s)</td>
<td>1.38 (s)</td>
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<tr>
<td>10'</td>
<td>1.29 (s)</td>
<td>27.8</td>
<td>1.29 (s)</td>
<td>27.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3.29 (s)</td>
<td>60.8</td>
<td>3.3 (s)</td>
<td>61.2</td>
<td>NA</td>
<td>4.52 (s)</td>
</tr>
</tbody>
</table>

Ma
<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td>4.82</td>
<td>(d, 8.9)</td>
<td>85.3</td>
<td>5.57</td>
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<tr>
<td>13</td>
<td></td>
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<td></td>
<td>3.6</td>
<td>(t, 8.5)</td>
<td>70.6</td>
<td>3.52</td>
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<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>3.53</td>
<td>(t, 8.5)</td>
<td>77.9</td>
<td>3.52</td>
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<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td>3.49</td>
<td>(t, 8.5)</td>
<td>72.3</td>
<td>3.48</td>
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<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>3.73</td>
<td>(t, 8.5)</td>
<td>77.0</td>
<td>3.82</td>
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<td>17</td>
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<td></td>
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</tbody>
</table>

Notations: Br=broad peak; d=doublet; dd=doublet of a doublet; Hz=Hertz; J=scalar coupling; m=multiplet; mult= multiplicity; NA=not available; ppm=parts per million; s=singlet.

\(^a\)All spectra were acquired in MeOD-\(d_4\) and internally referenced to solvent (CD2HOD) resonance at \(\delta_H\ 3.32 / \delta_C \ 47.8\) ppm. All signal integrals in 1D 1H NMR spectra were consistent with the spectral assignments.

\(^b\)Pseudo doublet, strongly coupled AA’BB’

\(^c\)Strongly coupled ABX spin system

\(^d\)The 2 methyls are diastereotopic.

\(^e\)AB multiplet
TABLE 6

Enzyme kinetics of motesanib N-glucuronidation in human, rat, dog, and monkey liver microsomes

<table>
<thead>
<tr>
<th>Species</th>
<th>$V_{\text{max}}$, pmol/min/mg protein</th>
<th>$K_m$, µM</th>
<th>$\text{CL}_{\text{int}}$, µL/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>1463</td>
<td>29.6</td>
<td>49.4</td>
</tr>
<tr>
<td>Monkey</td>
<td>281</td>
<td>67.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Dog</td>
<td>795</td>
<td>159.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Rat</td>
<td>346</td>
<td>44.6</td>
<td>7.8</td>
</tr>
</tbody>
</table>

$\text{CL}_{\text{int}}=$intrinsic clearance
Figure 1.

$^{14}$C-motesanib ($^{14}$C on methylene)  $^{14}$C-motesanib ($^{14}$C on carbonyl)
Figure 2.

Radioactive Intensity, Arbitrary Units

Time, min

Rat Liver Microsomes

Mouse Liver Microsomes

Dog Liver Microsomes

Monkey Liver Microsomes

Human Liver Microsomes

Motesanib

M5

M3

M4

M2

M5

M5

M3

M4

M5

M3

M4

M5
Figure 4.

Radioactive Intensity, Arbitrary Units

Time, min

Rat Microsomes
M1 (21%)
Motesanib

Mouse Microsomes
M1 (6%)
Motesanib

Dog Microsomes
M1 (14%)
Motesanib

Monkey Microsomes
M1 (29%)
Motesanib

Human Microsomes
M1 (68%)
Motesanib
Figure 6.
Figure 7.
Figure 8.
Figure 11.
Figure 12.

Metabolite Formation, pmol/nmol CYP/min

Recombinant Cytochrome P450

- M3 (N-oxide)
- M4 (Lactam)
- M5 (Carbinolamine)
Figure 13.

Formation of Metabolite Relative to Control Incubations (Without Inhibitor)

- M3 (N-oxide)
- M4 (Lactam)
- M5 (Carbinolamine)

Agents:
- Ketoconazole (1 nM, CYP3A4)
- Ketoconazole (5 nM, CYP3A4)
- Quinidine (1 nM, CYP2D6)
- Quinidine (5 nM, CYP2D6)
- Sulphaphenazole (1 nM, CYP2C9)
- Sulphaphenazole (10 nM, CYP2C9)
- Lansoprazole (10 nM, CYP2C19)
- Furafylline (10 nM, CYP1A2)
- Diethylidithiocarbamate (30 nM, CYP2E1)
- 8-Methoxypsoralen (0.2 nM, CYP2A6)
Figure 14.

The figure illustrates the formation of metabolites relative to control incubations (without antibody) for different antibodies against cytochrome P450 enzymes. The x-axis represents the cytochrome P450 antibodies: Anti-CYP3A4, Anti-CYP2D6, and Anti-CYP1A. The y-axis represents the formation of metabolites, with values ranging from 0 to 100.

- **M3 (N-oxide)**
- **M4 (Lactam)**
- **M5 (Carbinolamine)**

Each antibody group shows bars for M3, M4, and M5 metabolites, with error bars indicating the standard deviation. The bars for M4 (Lactam) and M5 (Carbinolamine) are consistently higher than those for M3 (N-oxide) in all antibody groups.
Figure 15.