The Role of Aldehyde Oxidase and Xanthine Oxidase in the Biotransformation of a Novel Negative Allosteric Modulator of Metabotropic Glutamate Receptor Subtype 5^S

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ABSTRACT:

Negative allosteric modulation (NAM) of metabotropic glutamate receptor subtype 5 (mGlu₅) represents a therapeutic strategy for the treatment of childhood developmental disorders, such as fragile X syndrome and autism. VU0409106 emerged as a lead compound within a biaryl ether series, displaying potent and selective inhibition of mGlu₅. Despite its high clearance and short half-life, VU0409106 demonstrated efficacy in rodent models of anxiety after extravascular administration. However, lack of a consistent correlation in rat between in vitro hepatic clearance and in vivo plasma clearance for the biaryl ether series prompted an investigation into the biotransformation of VU0409106 using hepatic subcellular fractions. An in vitro appraisal in rat, monkey, and human liver S9 fractions indicated that the principal pathway was NADPH-independent oxidation to metabolite M1 (+16 Da). Both raloxifene (aldehyde oxidase

inhibitor) and allopurinol (xanthine oxidase inhibitor) attenuated the formation of M1, thus implicating the contribution of both molybdenum hydroxylases in the biotransformation of VU0409106. The use of ¹⁸O-labeled water in the S9 experiments confirmed the hydroxylase mechanism proposed, because ¹⁸O was incorporated into M1 (+18 Da) as well as in a secondary metabolite (M2; +36 Da), the formation of which was exclusively xanthine oxidase-mediated. This unusual dual and sequential hydroxylase metabolism was confirmed in liver S9 and hepatocytes of multiple species and correlated with in vivo data because M1 and M2 were the principal metabolites detected in rats administered VU0409106. An in vitro-in vivo correlation of predicted hepatic and plasma clearance was subsequently established for VU0409106 in rats and nonhuman primates.

Introduction

Defining the in vivo PK parameters and biotransformation pathways for a chemical series or new chemical entity (NCE) represents

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the first step in establishing the in vitro-in vivo correlation (IVIVC) of hepatic clearance and blood clearance in a nonclinical species. The benefits of establishing an IVIVC are 3-fold: 1) IVIVC assists confirmation that the species selected for PK screening will most closely mirror the hepatic extraction predicted for humans; 2) IVIVC provides the foundation for PK screens in discovery (e.g., in vivo cassette dosing and/or in vitro metabolic stability) for rank-ordering of compounds with respect to clearance and half-life; and 3) biotransformation data resulting from an IVIVC investigation may uncover species differences in metabolism or a human unique pathway, putting the development of an NCE at risk (Balani et al., 2005). Hence, selection of an appropriate subcellular fraction not only functions as a

ABBREVIATIONS: PK, pharmacokinetic(s); NCE, new chemical entity; IVIVC, in vitro-in vivo correlation; P450, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; AO, aldehyde oxidase; NAM, negative allosteric modulator; mGlu₅, metabotropic glutamate receptor subtype 5; 1-ABT, 1-aminobenzotriazole; rcf, relative centrifugal force; LC, liquid chromatography; MS/MS, tandem mass spectroscopy; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; MS, mass spectrometry; DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple bond correlation spectroscopy; COSY, correlation spectroscopy; HSQC, heteronuclear spin-quantum correlation spectroscopy; XO, xanthine oxidase; SGX523, 6-(6-(1-methyl-1H-pyrazol-4-yl)-[1,2,4]triazolo[4,3-b]pyridazin-3-ylthio)quinoline; BIBX 1382, N8-(3-chloro-4-fluorophenyl)-N2-(1-methyl-4-piperidinyl)-pyrimido[5,4-d]pyrimidine-2,8-diamine; VU0465585, 3-fluoro-5-((2-methylpyrimidin-5-yl)oxy)-N-(4-methylthiazol-2-yl)benzamide; VU0458442, 3-fluoro-5-((5-fluoropyridin-3-yl)oxy)-N-(4-methylthiazol-2-yl)benzamide.

critical link when an IVIVC of drug clearance is established but also informs the selection of an appropriate nonclinical species for safety assessment.

Facilitated by four decades of research into P450 function and interspecies expression and regulation (Guengerich, 2001; Ortiz de Montellano, 2005), disposition scientists have built confidence in scaling nonclinical in vitro and in vivo PK data to predicted human PK for compounds for which P450-mediated metabolism represents the primary route of clearance (Hosea et al., 2009; Hutzler et al., 2010). Similar traction has been realized in medicinal chemistry, in which chemists have succeeded in reducing P450-catalyzed clearance, either through the alteration of physicochemical properties or through hindering metabolism via structural modifications to the scaffold (Pryde et al., 2010). However, a major limitation of this approach to discovery DMPK screening, nonclinical PK scaling, and subsequent human PK prediction is the incidence of non-P450-mediated metabolism of NCEs and the significant species differences that accompany non-P450 metabolism and in vitro scaling (Obach et al., 1997). In particular, research and development organizations are experiencing an emergence of aldehyde oxidase (AO) in the metabolism of drug candidates (Dittrich et al., 2002; Dalvie et al., 2010; Diamond et al., 2010; Pryde et al., 2010; Akabane et al., 2011; Garattini and Terao, 2012). The escalation of efforts aimed to define interspecies AO expression and regulation (Garattini and Terao, 2012) and to establish improved in vitro screens for non-P450 substrates (Zientek et al., 2010; Deguchi et al., 2011; Hutzler et al., 2012) underscores the emerging role of AO in drug metabolism and the increased demand for approaches to adequately scale PK across species and predict human disposition.

VU0409106 was a lead compound that resided in a novel pyrimidine-containing biaryl ether class of negative allosteric modulators (NAMs) of the group I metabotropic glutamate receptor subtype 5 (mGlu₅) (Niswender and Conn, 2010; Emmitte, 2011). VU0409106 displayed inhibitory potency against the target receptor ($IC_{50} = 26$ nM) and selectivity against other group I, II and III mGlu receptor subtypes ($IC_{50} \geq 10 \mu M$) (Jones et al., 2011). Although continued interest was dampened due to solubility limited absorption and poor oral PK, VU0409106 proved to be a useful tool compound in that it produced concentration-dependent anxiolytic effects in multiple rat models (Jones et al., 2011). These observations underscore the potential therapeutic benefit of mGlu₅ antagonism in the treatment of childhood developmental disorders, such as fragile X syndrome and autism (Bear et al., 2004).

The biaryl ether series exemplified by VU0409106 did not exhibit a consistent correlation between in vitro hepatic clearance in rat microsomes and in vivo plasma clearance in rat (i.e., IVIVC); in addition, the compound showed variability in the extent of hepatic extraction between rat and human microsomes. The inability to establish an IVIVC in rat, in addition to the variability between species, prompted an investigation into the biotransformation of the scaffold and a consequent move to hepatic S9 fractions in lieu of conventional hepatic microsomes for the primary metabolic stability screen. The in vitro hepatic S9 metabolism appraisal of VU0409106 indicated a significant species difference in the biotransformation of the compound across Sprague-Dawley rat, beagle dog, cynomolgus monkey, and human. With use of selective enzyme inhibitors, varied hepatic subcellular fractions, and isotopically labeled reagents (e.g., H₂¹⁸O), we elucidated a dual aldehyde oxidase and xanthine oxidase mode of metabolism of VU0409106 in humans. In addition, a closer examination of the in vitro experiments revealed the formation of a secondary metabolite that resulted from two sequential oxidations of VU0409106 by aldehyde oxidase and xanthine oxidase. This unusual sequential biotransformation

was confirmed in hepatocytes from multiple species and correlated with the metabolism and clearance observed in vivo.

Materials and Methods

Reagents. VU0409106, 3-fluoro-5-((2-methylpyrimidin-5-yl)oxy)-N-(4-methylthiazol-2-yl)benzamide (VU0465585), and 3-fluoro-5-((5-fluoropyridin-3-yl)oxy)-N-(4-methylthiazol-2-yl)benzamide (VU0458442) were prepared and characterized by the Department of Medicinal Chemistry within the Vanderbilt Center for Neuroscience Drug Discovery. Potassium phosphate, ammonium formate, formic acid, NADPH, MgCl₂, ¹⁸O-labeled water (H₂¹⁸O), raloxifene, hydralazine, 1-aminobenzotriazole (1-ABT), and allopurinol were purchased from Sigma-Aldrich (St. Louis, MO). Hepatic subcellular fractions were obtained from BD Biosciences (San Diego, CA). Cryopreserved hepatocytes were obtained from Celsis/In Vitro Technologies (Baltimore, MD). All solvents or reagents were of the highest purity commercially available.

Pharmacokinetic Studies. Rat PK. To ascertain the PK parameters of VU0409106 in vivo, male Sprague-Dawley rats ($n = 2$) weighing between 250 and 300 g were purchased from Harlan (Indianapolis, IN) with catheters surgically implanted in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately 1 week before dosing and provided food and water ad libitum. Parenteral administration of compound to rats was achieved via a jugular vein catheter at a dose of 1 mg/kg and a dose volume of 1 mL/kg (10% ethanol-70% polyethylene glycol 400–20% saline). Blood collections via the carotid artery were performed at 0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 h after administration. Samples were collected into chilled, EDTA-fortified tubes and centrifuged for 10 min (3000 rcf, 4°C), and the resulting plasma was stored at –80°C until analysis. For in vivo metabolite profiling of VU0409106, plasma from rats receiving a single intraperitoneal administration (56.6 mg/kg) of the test article was extracted by addition of 2 volumes of acetonitrile with subsequent centrifugation (3000 rcf, 10 min). The resulting supernatant was dried under a stream of nitrogen at ambient temperature, and the resulting residues were reconstituted in aqueous acetonitrile (15:85, v/v) in preparation for LC-MS/MS analysis. All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Monkey PK. The PK parameters for VU0409106 in vivo in male cynomolgus monkeys ($n = 2$) were determined from a study that was executed externally (Ricerca, Concord, OH). In brief, parenteral administration of VU0409106 to monkeys (≈ 3 kg) was achieved via the saphenous vein at a dose of 0.2 mg/kg and a dose volume of 1 mL/kg (10% ethanol-60% polyethylene glycol 400–30% isotonic saline). Blood collections via the femoral vein were performed at 0.033, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h after administration. Samples were collected into chilled, EDTA-fortified tubes and centrifuged for 10 min (3000 rcf, 4°C), and the resulting plasma was stored at –80°C until analysis. The procedures involving the care or use of animals in this study were approved by the Ricerca Institutional Animal Care and Use Committee before the initiation of the study.

Pharmacokinetic analysis. Pharmacokinetic parameters were obtained from noncompartmental analysis (WinNonlin, version 5.3; Pharsight, Mountain View, CA) of individual concentration-time profiles after the parenteral administration of a test article.

Liquid Chromatography-UV-Mass Spectrometry Analysis of Metabolites. An Agilent 1100 HPLC system was coupled to a Supelco Discovery C18 column (5 μm , 2.1 $\times$ 150 mm; Sigma-Aldrich). Solvent A was 10 mM (pH 4.1) ammonium formate, and solvent B was MeCN. The initial mobile phase was 85:15 A-B (v/v), and by linear gradient was transitioned to 20:80 A-B over 20 min. The flow rate was 0.400 mL/min. The HPLC eluent was first introduced into an Agilent 1100 diode array detector (single wavelength selected, 254 nm) followed by electrospray ionization-assisted introduction into a Finnigan LCQ Deca XP^{PLUS} ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) operated in either the positive or negative ionization mode. Ionization was assisted with sheath and auxiliary gas (ultra-pure nitrogen) set at 60 and 40 psi, respectively. The electrospray voltage was set at 5 kV with the heated ion transfer capillary set at 300°C and 30 V. Relative collision energies of 25 to 35% were used when the ion trap mass spectrometer was operated in the MS/MS or MSⁿ mode. The following are considerations with regard to high-resolution mass spectrometry of metabolites with use of a LTQ Orbitrap XL (Thermo Fisher Scientific): parent ion selection and subsequent tandem mass

spectrometry (MS/MS and MSⁿ) were performed manually. Molecular formulas from all fragments observed in the MS/MS and MS³ experiments were generated by XCalibur software (Thermo Fisher Scientific) using a formula generation algorithm. The formula generation algorithm used the following constraints: 10 ppm for maximum tolerance, nitrogen rule not used, and ring double bond equivalent values from -1.0 to 100.0.

Isolation of M1 and High-Field NMR Analysis of VU0409106 and M1. NMR experiments were acquired using a 14.0-T Bruker magnet equipped with a Bruker AV-III console operating at 600.13 MHz. All spectra were acquired in 3-mm NMR tubes using a Bruker 5-mm TCI cryogenically cooled NMR probe. Chemical shifts were referenced internally to DMSO-d₆ (2.5 ppm) or CD₃CN (1.98 ppm), which also served as the ²H lock solvents. For one-dimensional ¹H NMR, typical experimental conditions included multiple data points (32,000), 13-ppm sweep width, a recycle delay of 1.5 s and 32 to 256 scans depending on sample concentration. Heteronuclear multiple bond correlation (HMBC) data are available in Supplemental Fig. 3. The following ¹H signals were observed for VU0409106: 9.09 (1H, s), 8.77 (2H, s), 7.71 (1H, d, 9.27 Hz), 7.62 (1H, s), 7.43 (1H, d, 9.54 Hz), 6.83 (1H, s), and 2.28 (3H, s). The two-dimensional ¹H-¹H correlation spectroscopy (COSY) experimental conditions included a 2048 × 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 s, and 4 scans per increment. The data were processed using the squared sine-bell window function, symmetrized, and displayed in magnitude mode.

Metabolite **M1** was obtained from rat and/or cynomolgus monkey S9 incubations of VU0409106. The supernatants from S9 reactions were subjected to chromatographic purification, and **M1** was subsequently purified from the in vitro milieu. The sample containing **M1** was reconstituted in DMSO-d₆. The following ¹H signals were observed for **M1**: 8.42 (1H, s), 7.86 (1H, s), 7.53 (1H, d, 9.19 Hz), 7.36 (1H, s), 6.99 (1H, d, 9.19 Hz), 6.74 (1H, s), and 2.26 (3H, s). The two-dimensional ¹H-¹H COSY experimental conditions included a 2048 × 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 s, and 4 scans per increment. The data were processed using the squared sine-bell window function, symmetrized, and displayed in magnitude mode. Multiplicity-edited heteronuclear spin-quantum correlation spectroscopy (HSQC) experiments were acquired using a 1024 × 256 data matrix, a J_{C-H} value of 145 Hz, which resulted in a multiplicity selection delay of 34 ms, a recycle delay of 1.5 s, and 16 scans per increment along with GARP decoupling on ¹³C during the acquisition time (150 ms). The data were processed using a $\pi/2$ shifted squared sine window function and displayed with CH/CH₃ signals phased positive and CH₂ signals phased negative. ¹J_{C-H} filtered HMBC experiments were acquired using a 2048 × 256 data matrix, a J_{C-H} value of 9 Hz for detection of long-range couplings resulting in an evolution delay of 55ms, ¹J_{C-H} filter delay of 145 Hz (34 ms) for the suppression of one-bond couplings, a recycle delay of 1.5 s, and 128 scans per increment. The HMBC data were processed using a $\pi/2$ shifted squared sine window function and displayed in magnitude mode.

In Vitro Biotransformation of VU0409106 in Hepatic S9 Fractions and Hepatocytes. *Hepatic S9 fractions.* The in vitro metabolism of VU0409106 was investigated using hepatic S9 fractions from Sprague-Dawley rats (62 males, pooled), beagle dogs (5 males, pooled), cynomolgus monkeys (3 males, pooled), and humans (150-donor UltraPool, BD Biosciences). A potassium phosphate-buffered reaction (0.1 M, pH 7.4) of VU0409106 (25 μ M), hepatic S9 fractions (5 mg/ml), and MgCl₂ (3 mM) was incubated at 37°C in borosilicate glass test tubes under ambient oxygenation for 1 h with select experiments being fortified with NADPH (2 mM). All S9 reactions were initiated by the addition of VU0409106 (or test article) to the in vitro milieu. When appropriate, incubations were fortified (50–100 μ M) with the molybdenum hydroxylase inhibitors allopurinol (XO), raloxifene (AO), hydralazine (AO), and 1-ABT (time-dependent P450 inhibitor) before the addition of the test article, VU0409106. When required for mechanistic studies, rat hepatic S9 fractions were suspended in potassium phosphate buffer (0.1 M) that had been reconstituted in ¹⁸O-labeled water (75% v/v). Protein was precipitated by the addition of 2 volumes of MeCN with subsequent centrifugation (3000 rcf, 10 min). The supernatant was dried under a stream of nitrogen and reconstituted in 85:15 (v/v) ammonium formate (10 mM, pH 4.1)-MeCN in preparation for LC-MS analysis.

Hepatocytes. Cryopreserved Sprague-Dawley rat (male, 14 donor pool), beagle dog (male, 3 donor pool), cynomolgus monkey (male, 4 donor pool), rhesus monkey (male, 3 donor pool), and human (mixed gender, 20 donor pool) hepa-

tocytes (2 × 10⁶ cells/ml) suspended in Krebs-Henseleit buffer were incubated with VU0409106 (25 μ M) at 37°C for 4 h. Cells were subsequently precipitated by the addition of 2 volumes of MeCN with subsequent centrifugation (3000 rcf, 10 min, 4°C). The resulting supernatant was dried under a stream of nitrogen and reconstituted in 15% MeCN (aqueous) in preparation for LC-MS analysis.

Hepatic S9, Cytosol, and Hepatocyte Metabolic Stability Assessment of VU0409106. The metabolic stability of VU0409106 was investigated in rat, cynomolgus monkey, and human (150 donor UltraPool) hepatic S9 fractions or the corresponding cytosolic fraction (rat and human only) using substrate depletion methodology (percentage of parent compound remaining). In 96-well plates a potassium phosphate-buffered (0.1 M, pH 7.4) solution of VU0409106 (1 μ M), S9 (2.5 mg/ml; cytosol, 1 mg/ml), $\pm$ NADPH (1 mM) was incubated at 37°C under ambient oxygenation; reactions were initiated by the addition of VU0409106 (or test article). At the designated times (t = 0, 3, 7, 15, 25, and 45 min), an aliquot of the incubation mixture was removed and precipitated by the addition of 2 volumes of ice-cold MeCN containing carbamazepine as an internal standard (50 ng/ml). Hepatocyte metabolic stability assessment of VU0409106 was performed in a similar manner and under ambient oxygenation, using 2 × 10⁶ cells/ml per incubation (20 donors pooled, mixed gender). At the designated times (t = 0, 15, 30, 60, and 120 min), an aliquot was removed and precipitated by the addition of 2 volumes of ice-cold MeCN containing carbamazepine as an internal standard (50 ng/ml). The plates were centrifuged at 3000 rcf (4°C) for 10 min. The resulting supernatants were diluted 1:1 (supernatant-water) into new 96-well plates in preparation for LC-MS/MS analysis. All samples were analyzed via electrospray ionization on an AB Sciex API-5500 QTrap (Applied Biosystems, Foster City, CA) instrument that was coupled with LC-20AD pumps (Shimadzu, Columbia, MD) and a CTC PAL autosampler (Leap Technologies, Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 column (2.1 × 50 mm, 3 μ m; Fortis Technologies Ltd., Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted); mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 30% B after a 0.2-min hold and was linearly increased to 90% B over 0.8 min, held at 90% B for 0.5 min, and returned to 30% B in 0.1 min followed by a reequilibration (0.9 min). The total run time was 2.5 min, and the HPLC flow rate was 0.5 ml/min. The source temperature was set at 500°C, and mass spectral analyses were performed using multiple reaction monitoring, with transitions and voltages specific for each compound using a TurboIonSpray source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.5.1 software. Each compound was assayed in triplicate within the same 96-well plate. The substrate depletion methodology (Obach and Reed-Hagen, 2002) was used to estimate the in vitro intrinsic clearance (CL_{int}, ml · min⁻¹ · kg⁻¹, eq. 1) of VU0409106 in S9 fractions (120.7 mg of protein), cytosol (80.7 mg of protein), and hepatocytes (Houston and Galetin, 2008):

$$CL_{int} = \frac{\ln 2}{t_{1/2} \text{ (min)}} \times \frac{1 \text{ ml}}{2.5 \text{ mg protein}_{S9}} \times \frac{120.7 \text{ mg protein}_{S9}}{1 \text{ g liver weight}} \times \frac{(A) \text{ g liver weight}}{\text{kg body weight}} \quad (1)$$

where $t_{1/2}$ is the substrate depletion half-life (minutes) and A = 20 (human), 45 (rat), or 30 (monkey). The value of 135 × 10⁶ hepatocyte/g liver was used in the CL_{int} calculation for hepatocyte metabolic stability assessment. A predicted hepatic clearance (CL_{HEP}, milliliters per minute per kilogram) was subsequently calculated using the well stirred model, uncorrected for the fraction unbound in plasma (eq. 2):

$$CL_{HEP} = \frac{Q_H \times CL_{int}}{Q_H + CL_{int}} \quad (2)$$

Results

Characterization of VU0409106 Metabolites by LC-MS/MS and NMR. LC-MS/MS and high-field NMR were used to characterize the metabolites of VU0409106 produced in vitro from rat, dog, monkey, and human hepatic S9 fractions and hepatocytes (Table 1). Circulating metabolites of VU0409106 were also identified in rats receiving a single,

TABLE 1
LC-MS/MS fragmentations of VU0409106 and observed metabolites

Metabolite	Structure	Retention Time <i>min</i>	[M + H] ⁺	Mass Fragments
VU0409106		14.5	331	313, 304, 217, 116
M1		12.55	347	329, 291, 251, 233, 205
M2		12.3	363	345, 320, 267, 249, 224, 221
M3		13.4	361	343, 265, 247, 219
M4		11.8	347	319, 276, 234, 217
M5		12.9	347	329, 251, 233

intraperitoneal administration of VU0409106 (56.6. mg/kg). The metabolites of VU0409106 are depicted in Scheme 1. For the metabolites in which there were insufficient levels detected in biological media (e.g., plasma or in vitro milieu), the regiochemistry was tentatively assigned. HRMS data provided additional evidence for the proposed structures of metabolites; observed data are compared with the corresponding calculated values based on a metabolite's empirical formula.

VU0409106. The protonated molecular ion, [M + H]⁺, for the pyrimidine-containing biaryl ether VU0409106 was observed at *m/z* 331 (HRMS, observed 331.0666 versus calculated 331.0660). Eluting at 14.5 min, the MS/MS fragmentation of VU0409106 produced a fragment ion at *m/z* 313 (Table 1), corresponding to the loss of water from the parent molecule (−H₂O; 18 Da). The fragment ion at *m/z* 304 corresponded to the loss of HCN (−27 Da) from the pyrimidine

SCHEME 1. Proposed metabolism of VU0409106 in vitro in rat (r), monkey (m), and human (h) and in vivo in rat plasma (R).

moiety (HRMS, observed 304.0555 versus calculated 304.0551). A key ion corresponded to the fragmentation of the amide bond and subsequent formation of an acylium ion at m/z 217 (HRMS, observed 217.0409 versus calculated 217.0408). The formation of m/z 217 proved to be a useful fragmentation, subsequently eliminating the biotransformation of the thiazole moiety in the formation of the primary oxidative metabolites.

Metabolite M1. Oxidation of the pyrimidine moiety resulted in the formation of a principal oxidative metabolite (**M1**) that eluted at 12.5 min and produced a protonated molecular ion, $[M + H]^+$, at m/z 347 (HRMS, observed 347.0613 versus calculated 347.0609). The formation of **M1** was observed from incubations of VU0409106 in rat (male), monkey (male), and human hepatic S9 fractions and cryopre-

served hepatocytes; **M1** was also observed in the plasma of rats (male) receiving intraperitoneal administrations of the test article. In addition to water loss (-18 ; m/z 329), a primary fragmentation observed for **M1** (Table 1) was the gas-phase, water-assisted fragmentation of the amide bond that resulted in the formation of an ion at m/z 251 (100% relative; HRMS, observed 251.0463 versus calculated 251.0463). At lower abundance (60% relative) the fragment ion at m/z 233 corresponded to a 16-Da increase over the acylium ion fragment (m/z at 217) (HRMS, observed 233.0356 versus calculated 233.0357) observed in the parent VU0409106, indicating that the thiazole moiety was unchanged. The observation of a fragment ion at m/z 291 (-56 Da) indicated an oxidative biotransformation of the pyrimidine moiety.

FIG. 1. Comparison of ^1H NMR of **M1** (A) and VU0409106 (B).

Metabolite **M1** was subsequently isolated from rat S9 incubations and characterized by high-field NMR. The structure for **M1** was partially determined on the basis of integrated spectral intensity for all peaks, which was the expected 1:1 ratio except for methyl at 2.26 that gave a 3:1 ratio compared with the aromatic protons (Fig. 1). HSQC data clearly showed six unique C–H groups for the peaks between 8.42 and 6.74 ppm with carbon chemical shifts ranging from 107.1 to 164.9 ppm (Fig. 2A). HMBC data were used to establish correlations between the methyl protons (2.26) and the C–H at 107.8 (6.73) for the methylthiazole moiety (Supplemental Fig. 3). The central aryl group protons at 7.53, 7.36, and 6.99 were all assigned on the basis of correlations from both ^1H - ^1H COSY (Fig. 2B) and HMBC experiments. With the assignment of four of the six detected C–H groups, two unique protons were assigned to the pyrimidinone ring, indicating the absence of one of the three C–H groups that were observed in VU0409106.

Metabolite M2. A secondary hydroxylation of the pyrimidine moiety was proposed to result in the formation of a metabolite (**M2**), eluting at a retention time of 12.3 min and producing a protonated molecular ion, $[\text{M} + \text{H}]^+$, for this principal metabolite at m/z 363 (HRMS, observed 363.0555 versus calculated 363.0558). The formation of **M2** was observed from incubations of VU0409106 in rat (male), monkey (male), and human hepatic S9 fractions and cryopreserved hepatocytes; **M2** was also observed in the plasma of rats (male) receiving intraperitoneal administrations of the test article. The formation of **M2** was observed at trace levels in human in vitro incubations.

Similar to metabolite **M1**, the fragmentation of the secondary metabolite **M2** produced two key fragmentations (Table 1) resulting from the gas-phase cleavage and rearrangement of the amide, yielding ions at m/z 249 (HRMS, observed 249.0307 versus calculated 249.0306) and 267 (HRMS, observed 267.0409 versus calculated 267.0412). Each of these ions indicated dual oxidation of the biaryl ether portion of the parent compound VU0409106 (i.e., +32 Da over 217 of VU0409106 and +16 Da over 251 of **M1**, respectively). The ion at m/z 320 (−43 Da) corresponds to the fragmentation of the oxidized pyrimidine moiety (Table 1).

Metabolite M3. The oxidative metabolite **M3** was detected in hepatocytes from multiple species and in plasma from rats receiving an intraperitoneal administration of VU0409106. Eluting with a retention time of 13.4 min, **M3** produced a protonated molecular ion, $[\text{M} + \text{H}]^+$, at m/z 361. Proposed as an oxidatively defluorinated **M2**, metabolite **M3** produced an MS/MS-induced loss of water, yielding fragment 343 as well as the key fragment ions at m/z 247 and 265 that were consistent with the formation of an acylium ion and a gas-phase, water-assisted fragmentation of the amide bond, respectively (Table 1). Consistent with an NADPH-dependent defluorination, each of these key fragment ions represented a net 2-Da reduction in mass compared with its fluorinated version, **M2**.

Metabolite M4. The oxidative metabolite **M4** was detected in rat plasma, eluted with a retention time of 11.8 min, and produced a protonated molecular ion, $[\text{M} + \text{H}]^+$, at m/z 347. Proposed as an NADPH-dependent oxidation of the thiazole moiety, **M4** produced an MS/MS-induced loss of carbon monoxide (−28 Da; −CO), yielding a fragment ion at m/z 319, as well as key fragment ions at m/z 217 (acylium ion) and 234 (unsubstituted amide), which were consistent with a metabolite structure that was unchanged on the biaryl ether portion of the scaffold (Table 1). In addition, consistent with this proposed structure for **M4**, was the MS^3 (347 → 319)-mediated production of the fragment ion at m/z 276, corresponding to cleavage of the thiazole ring system.

Metabolite M5. The formation of metabolite **M5** was discovered at trace levels in rat S9 and plasma, eluting at 12.9 min. This NADPH-dependent metabolite produced a protonated molecular ion, $[\text{M} + \text{H}]^+$, at m/z 347 and an MS/MS fragmentation spectrum (Table 1) similar to that of the NADPH-independent and principal metabolite, **M1**. That is, an MS/MS-induced fragmentation of **M5** catalyzed the gas-phase loss of water from the amide bond (m/z 329) as well as the direct cleavage (m/z 233; acylium ion) and water-assisted cleavage (m/z 251) of the amide bond. Without an isolated metabolite of **M5** for additional regiochemical assignment, a preliminary structure was proposed that corresponded to the oxidation of the pyrimidine of VU0409106.

Species Differences in the Non-P450-Mediated Biotransformation of VU0409106 in Vitro. LC-UV-MS analysis of VU0409106 (25 μM) metabolism in hepatic S9 fractions (5 mg/ml) indicated that the principal metabolite observed in rat, monkey, and human was **M1**, a metabolite that represented a single oxidation (+16 Da) of the pyrimidine moiety (Fig. 3, A, B, D, and E, respectively); **M1** was also the principal metabolite observed in rat, monkey, and human hepatocytes (1×10^6 cells/ml) (Supplemental Fig. 1). Subsequent high-field NMR analysis of **M1**, isolated from rat S9 incubations, indicated that the oxidation was at C-6 of the pyrimidine ring system (Scheme 1). Of interest, the S9 metabolism of VU0409106 to the principle metabolite, **M1**, was discovered to be NADPH-independent, thus removing cytochrome P450 enzymes from consideration in this biotransformation. Low-level formation of an NADPH-dependent metabolite, **M3**, was observed at trace levels in monkey and human S9 and at trace levels in rat, monkey, and human hepatocytes (Supplemental Fig. 2); me-

FIG. 2. ^1H - ^{13}C HSQC (A) and ^1H - ^1H COSY (B) of **M1** in $\text{DMSO}-d_6$ support oxidation at C-6 of the pyrimidine moiety.

FIG. 3. Representative LC/MS total ion current (TIC) (A) of extracts from a rat hepatic S9 incubation of VU0409106 and LC-UV chromatograms (overlay) of extracts from rat (B), dog (C), monkey (D), and human (E) hepatic S9 incubations of VU0409106 showing principal metabolites and the species differences in metabolism. An LC-UV trace of rat plasma (F) shows additional metabolites not detected in vitro.

metabolite **M3** is proposed to originate from oxidative defluorination of **M2** (Scheme 1). Particularly noteworthy was the observation that the dog was incapable of metabolizing VU0409106 to **M1** in hepatic S9 (Fig. 3C) or hepatocytes (Supplemental Fig. 1). In view of the NADPH-independent formation of **M1** and the absence of this biotransformation in the dog, a species known to lack a specific aldehyde oxidase activity common to rodents, nonhuman primates, and humans (Garattini and Terao, 2012), we considered this molybdenum-containing hydroxylase to be a likely contributor to the metabolism of VU0409106. These data were also consistent with data from hepatic cytosol incubations of VU0409106 indicating the formation of **M1** in rat, monkey, and human (vide infra). In addition to **M1**, a secondary metabolite (**M2**) was also observed at appreciable levels in vitro in rat and monkey and trace levels in human hepatic S9 (Scheme 1) and hepatocyte incubations of VU0409106, corresponding to a net addition of 32 Da over VU0409106. Incidentally, the formation of **M2** was also NADPH-independent and was absent in incubations of VU0409106 in dog hepatic subcellular fractions (Fig. 3C). In addition to the LC-MS/MS-assisted structure assignment for **M2** and consistent with the proposed structure for this metabolite, hepatic rat S9-mediated metabolism of the methylated analog VU0465585 in the absence of NADPH was limited to a *single oxidation* of the methylpyrimidine moiety (Scheme 2A) and indicated that the methyl group had effectively blocked the secondary oxidation (net +32 Da over VU0465585) of the pyrimidine moiety at the C-2 position (data not shown).

Biotransformation of VU0409106 Is Mediated by Aldehyde Oxidase and Xanthine Oxidase. To probe the involvement of AO, VU0409106 (1 μ M) and rat, monkey, and human hepatic S9 fractions (2.5 mg/ml protein) were incubated in the presence of selective inhibitors of both AO and XO (Johnson et al., 1985; Obach, 2004; Obach et al., 2004). The presence of the selective AO inhibitors in the rat S9 incubations of VU0409106 (Fig. 4A) reduced the formation of **M1** by greater than 50% in the case of raloxifene (100 μ M) addition and quantitatively after the addition of hydralazine (100 μ M) to the in vitro incubations. The biotransformation of VU0409106 in rat ap-

peared to be predominantly catalyzed by AO, because the selective XO inhibitor allopurinol (100 μ M) had little effect on the metabolism of VU0409106 in vitro (Fig. 4A). Likewise, the addition of raloxifene and hydralazine to human hepatic S9 fractions resulted in quantitative inhibition of VU0409106 metabolism to **M1** (Fig. 4B). However, unlike the observations in rat S9, addition of allopurinol (100 μ M) to human S9 incubations (Fig. 4B) resulted in >50% of VU0409106 metabolism in vitro, thus indicating a contribution from XO in the metabolism of this novel compound and highlighting a species difference between rat and human in the primary biotransformation of VU0409106.

Initial inhibition experiments measuring the formation of the primary metabolite **M1** indicated that allopurinol significantly inhibited the formation of **M2** in rat S9 (data not shown). Suspecting the role of XO in the sequential metabolism of VU0409106 (i.e., **M1** $\rightarrow$ **M2**), we

SCHEME 2. Metabolism of the methylated analog VU0465585 (A) and the fluoropyridine analog VU0458442 (B) in rat S9 indicates the sensitivity of AO and XO metabolism to pyrimidine substitution.

FIG. 4. Raloxifene (100 μ M, AO inhibitor), hydralazine (100 μ M, AO inhibitor), and allopurinol (100 μ M, XO inhibitor) inhibit the metabolism of VU0409106 metabolism in rat (A) and human (B) hepatic S9 (–NADPH), effectively reducing the formation of **M1** (percentage of control).

subjected an aliquot of isolated **M1** to rat hepatic S9 incubations (–NADPH) in the presence and absence of AO and XO inhibitors (100 μ M) to determine the role of these molybdenum hydroxylases in the secondary biotransformation of **M1**. Whereas allopurinol had a minimal effect on the formation of **M1** in rat S9, it nearly quantitatively prevented the formation of the secondary metabolite **M2** (Fig. 5) and thus implicated XO in the conversion of **M1** to **M2**. The present example of a sequential primary-to-secondary metabolism of a small molecule by mixed contributions from AO and XO is unusual and underscores the need for continued investigation of the interspecies differences in AO and XO multispecies expression, function, and regulation.

Incorporation of ^{18}O -Labeled Water into **M1 and **M2** Confirms the Hydroxylase-Mediated Metabolism of VU0409106 In Vitro.** Both AO and XO use water (H_2O) in their catalytic mechanisms of oxidation (Garattini and Terao, 2012) rather than molecular oxygen (O_2), as is the case with the cytochrome P450 oxidases (Guengerich, 2001). The aforementioned data provided strong evidence for the involvement of AO and XO in the sequential biotransformation of VU0409106; thus, we performed a mechanistic experiment using H_2^{18}O (Dalvie et al., 2010; Diamond et al., 2010; Hutzler et al., 2012) to confirm the contribution of these molybdenum-containing hydroxy-

lases in the biotransformation of VU0409106. After rat and human S9 (5 mg/ml) incubations of VU0409106 (25 μ M) in the presence of H_2^{18}O , we observed the incorporation of ^{18}O into **M1**, because an $[\text{M} + \text{H}]^+$ molecular ion was detected at m/z 349 (i.e., $\text{M} + 2$ Da) (Fig. 6A) and at a relative abundance (69%) consistent with the percentage of H_2^{18}O in the reaction (75% v/v). A subsequent LC-MS/MS experiment also produced a major fragment ion at m/z 253 (Fig. 6A, inset), representing a gas-phase, water-assisted loss of the aminothiazole moiety. The detection of the 235 and 253 fragment ions represented an 18-Da increase over the same fragment ions produced for the parent VU0409106 (m/z 217 and 235, respectively) and thus confirmed the incorporation of ^{18}O into **M1**. Together with the AO inhibitor results (mixed AO/XO in human), this mechanistic experiment confirmed a hydroxylase-mediated biotransformation of VU0409106. Likewise, the xanthine oxidase-mediated oxidation of **M1** resulted in the formation of **M2** and a corresponding second addition of H_2^{18}O into the metabolite. The LC/MS spectrum of **M2** indicated that a second ^{18}O atom (i.e., $\text{M} + 4$ Da) had been incorporated, producing an $[\text{M} + \text{H}]^+$ molecular ion at m/z 367 (53%) (Fig. 6B), the relative abundance of which was consistent with the percentage of H_2^{18}O in the reaction (75% v/v). Consistent with the characteristic MS/MS pattern observed for **M1**, the detection of the 253 and 271 fragment ions (Fig. 6B, inset) represented a 36-Da increase over the same fragment ions produced for parent VU0409106 (m/z 217 and 235, respectively). Moreover, the fragment ion detected at m/z 322 (2 Da increase over the m/z 320 fragment ion for **M2** in Table 1) indicated a remnant ^{18}O atom in the molecule and was again consistent with the proposed structure for **M1**. These LC-MS/MS data indicated an incorporation of two ^{18}O atoms into **M2** and confirmed a sequential hydroxylase mechanism of VU0409106 oxidation in vitro (i.e., AO $\rightarrow$ XO oxidation).

M1 Formation Represents the Principal Biotransformation of VU0409106 In Vivo in Rat. The in vivo metabolism of VU0409106 was examined in vivo in male Sprague-Dawley rats that had received a single intraperitoneal dose of VU0409106 (56.6 mg/kg). Consistent with the in vitro hepatic metabolism of VU0409106, the principal metabolites detected by LC-MS/MS analysis were **M1** and **M2** (Fig. 3F), the formation of which indicated an in vivo role for AO and XO in the metabolism of VU0409106 in Sprague-Dawley rats. In addition to **M1** and **M2**, we observed three metabolites at low levels, including the defluorinated metabolite **M3**, a hydroxylated thiazole metabolite (**M4**), and a hydroxylated pyrimidine metabolite (**M5**), each observed in the circulation of rats receiving a single intraperitoneal dose of VU0409106 (Fig. 3F; Scheme 1). The formation of metabolites **M3**,

FIG. 5. The selective XO inhibitor allopurinol (100 μ M), but not the AO inhibitors (raloxifene or hydralazine, 100 μ M), prevent the biotransformation of **M1** to **M2** in rat hepatic S9 (**M2** formation as percentage of control).

FIG. 6. Mass spectra of **M1** (A) and **M2** (B) from rat hepatic S9 incubations performed in $H_2^{18}O$ indicate the incorporation of ^{18}O into the respective metabolites. The insets to A and B are MS/MS spectra of ^{18}O -labeled metabolites and provide evidence for regiochemical insertion of ^{18}O into **M1** and **M2**.

M4, and **M5** was attenuated with the addition of the pan-P450 inactivator, 1-ABT, in rat and human hepatocytes and thus indicated that the biotransformation of VU0409106 probably involved a minor contribution from P450 enzymes (data not shown).

In Vitro and In Vivo Pharmacokinetic Analysis Confirms the Relevance of AO/XO in the Clearance of VU0409106 in Nonclinical Species. *In vitro pharmacokinetics.* Using substrate depletion methodology for the determination of half-life ($t_{1/2}$), CL_{int} , and subsequent CL_{HEP} (Obach and Reed-Hagen, 2002; Zientek et al., 2010), we observed low to moderate CL_{HEP} for VU0409106 (1 μM) in hepatic S9 (2.5 mg/ml), cytosol (1.0 mg/ml), and hepatocytes from Sprague-Dawley rats ($CL_{HEP} = 30, 13$, and 40 ml/min/kg, respectively) (Table 2) as well as a moderate CL_{HEP} in cynomolgus monkey

hepatic S9 and hepatocytes ($CL_{HEP} = 20$ and 36 ml $\cdot$ min $^{-1}$ $\cdot$ kg $^{-1}$, respectively). Hepatocyte data obtained from rhesus monkey also revealed a similar fate for VU0409106, because the primary biotransformation pathway was M1 formation at a rate similar to that observed in cynomolgus monkey (Supplemental Fig. 1). Of interest, VU0409106 (1 μM) was stable in human S9 fractions without NADPH ($CL_{int} = \leq 1$ ml $\cdot$ min $^{-1}$ $\cdot$ kg $^{-1}$) and moderately stable in the corresponding human cytosol ($CL_{int} = 3\text{--}4$ ml $\cdot$ min $^{-1}$ $\cdot$ kg $^{-1}$), whereas a marked clearance of the compound was observed in S9 fractions that had been fortified with NADPH (Table 2). The latter finding, indicating a role for P450 under more kinetic appropriate conditions, represented a disconnect from what was observed in the human S9 (5 mg/ml + NADPH) biotransformation of VU0409106

TABLE 2

In vitro clearance of VU0409106 (CL_{int} and CL_{HEP}) in multiple species hepatic S9, cytosol, and hepatocytes as well as CL_p in Sprague-Dawley rat and cynomolgus monkey

In vitro clearance was calculated as described under *Materials and Methods*.

	Rat			Monkey			Human	
	CL_{int}^a	CL_{HEP}^b	CL_p^c	CL_{int}	CL_{HEP}	CL_p	CL_{int}	CL_{HEP}
				$ml \cdot min^{-1} \cdot kg^{-1}$				
S9 minus NADPH	25	18		34	19		1.0	1.0
S9 + NADPH	54	30		37	20		15	8.6
Cytosol	16	13		N.A.	N.A.		3.7	3.2
Hepatocytes	96	40		194	36		6.9	5.1
In vivo			43			24		

CL_{int} , intrinsic clearance; CL_{HEP} , predicted hepatic clearance; CL_p , plasma clearance; N.A., not available.

shown previously in Fig. 3. The fact that human hepatocytes metabolized VU0409106 primarily to **M1** at rates ($CL_{int} = 6.9 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$; $CL_{HEP} = 5.1$) similar to those observed in the cytosol experiment (Table 2) provided evidence for AO and XO in the *in vitro* metabolism of the compound. The exact percentage of AO and XO contributions in humans have not been rigorously defined *in vitro* and require a more thorough kinetic analysis, the subject of which is currently under investigation in our laboratory.

In vivo pharmacokinetics. Considering that AO was determined to be the primary route of metabolism for this series, we investigated whether this non-P450 pathway discovered *in vitro* was predictive of the *in vivo* scenario. Therefore, male Sprague-Dawley rats were administered a single intravenous dose (1 mg/kg) of VU0409106 to determine its PK profile *in vivo*. Consistent with the *in vitro* PK data, noncompartmental analysis (Gabrielsson and Weiner, 2000) of the resulting time-concentration data indicated that VU0409106 was a moderate to high clearance compound (Table 2), displaying a plasma clearance (CL_p) of less than hepatic blood flow for the species ($CL_p = 43 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ versus $CL_{HEP} = 30\text{--}40 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ *in vitro*). Coupled with a corresponding volume of distribution predicted at steady state (V_{ss}) of 0.8 l/kg, VU0409106 displayed an effective $t_{1/2}$ of <15 min (data not shown). An IVIVC with respect to clearance was also established in cynomolgus monkey because the clearance generated from *in vitro* experiments in liver S9 and hepatocytes ($CL_{HEP} = 20$ and $33 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, respectively) was predictive of the plasma clearance ($CL_p = 24 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) in animals receiving a 1 mg/kg i.v. administration of VU0409106 (Table 2). The metabolism data indicating AO-mediated clearance *in vitro* in rat and monkey are consistent with the *in vivo* data obtained for VU0409106.

Discussion

We have described the biotransformation of the novel biaryl ether compound, VU0409106, in hepatic S9 and hepatocytes of multiple species. The discovery that rat, monkey, and human metabolized VU0409106 in an NADPH-independent manner through a primary pathway *in vitro* in hepatic S9 indicated a likely contribution of a molybdenum hydroxylase in the metabolism of this novel mGlu₅ NAM. Indeed, the collective use of cofactor manipulation (S9 incubations) and selective inhibitors implicated AO and XO in the biotransformation of VU0409106 to the oxidized metabolite **M1** in rat and human (Scheme 1). Furthermore, the absence of **M1** formation in dog hepatic S9 fractions implicated the involvement of AOX1, a protein absent in dogs (Garattini and Terao, 2012). Of importance, the use of ¹⁸O-labeled water in S9 experiments provided additional evidence for the role of AO and/or XO in the biotransformation of VU0409106 because MS/MS data indicated the incorporation of an ¹⁸O atom on the pyrimidine moiety of **M1** as well as with **M2**.

Although the contemporary DMPK literature is replete with examples of AO-mediated drug metabolism (Lake et al., 2002; Beedham et al., 2003; Klecker et al., 2006; Diamond et al., 2010; Pryde et al., 2010; Akabane et al., 2011), we observed two findings regarding the biotransformation of VU0409106 that were particularly noteworthy. First, results of chemical inhibitor experiments (e.g., hydralazine/AO and allopurinol/XO) indicated a divergence in the primary biotransformation pathway for VU0409106 in rat and human (S9 and hepatocytes); the principal biotransformation of VU0409106 to **M1** was catalyzed predominantly by AO in rats, whereas the formation of **M1** in human S9 was discovered to be catalyzed by both AO and XO. Second, rat S9 incubations of isolated **M1** resulted in the NADPH-independent formation of **M2** (Scheme 1); the secondary metabolite was also observed at trace levels *in vitro* in monkey and human. Surprisingly, the biotransformation of **M1** to the secondary metabolite **M2** was discovered to be exclusively XO-mediated. Consistent with the structural assignment for **M2**, we observed no XO-mediated oxidation of the C-2 methyl-substituted analog, VU0465585 (Scheme 2A). Although the present biotransformation involving a dual role for AO and XO is unusual, Beedham and coworkers (Rashidi et al., 1997) had reported such sequential metabolism for the purine analog antiviral drug, famciclovir. However, unlike the instance with famciclovir and opposite to our initial proposal of **M1** structure, the primary oxidation site mediated by AO and XO was at the C-6 position of the pyrimidine moiety rather than the C-2 position that one would expect given purine catabolism and purine drug metabolism data (Rashidi et al., 1997; Pryde et al., 2010). Of importance, the present metabolism structure-activity relationship has been extended to other analogs within the biaryl ether pyrimidine series (data not shown).

The relevance of the AO-mediated metabolism of VU0409106 in rats (mixed AO/XO in human) was evident from the *in vitro-in vivo* correlation we observed for VU0409106 and the analogs of the series with respect to metabolism-mediated clearance (hepatic S9 $CL_{HEP} = 30$ versus $CL_p = 43 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). Hepatocytes also produced a predicted clearance for VU0409106 in rats that correlated well with the observed *in vivo* clearance (hepatocyte $CL_{HEP} = 30 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). Of importance, LC-MS/MS analysis of plasma from rats receiving extravascular administration of VU0409106 indicated that **M1** was the principal metabolite *in vivo*, thus establishing AO metabolism as a relevant primary clearance mechanism *in vivo*. Likewise, the AO-mediated clearance observed in cynomolgus monkey hepatocytes ($CL_{HEP} = 36 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) also correlated with the *in vivo* clearance ($CL_p = 24 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) observed in nonhuman primates. Recent hepatocyte data obtained from rhesus monkey, a species proposed to closely mirror human AO metabolism (for references, see Garattini and Terao, 2012), indicated a similar fate for VU0409106,

because the primary biotransformation pathway observed was M1 formation at rates similar to those observed in cynomolgus monkey.

Regardless of the apparent IVIVC established in rats and monkeys for a particular AO substrate, the ability to predict human clearance and half-life was in question, given the precedent variability with respect to AO catalytic activity, expression, and enzyme regulation among mammalian species (e.g., rat, guinea pig, monkey, and human) (Sahi et al., 2008; Garattini and Terao, 2012). Located in the cytosol (Rashidi et al., 1997), AO is ubiquitously expressed throughout the tissues in multiple species, and the elevated expression in the hepatic tissue can contribute to extensive first-pass metabolism with oral dosing. Furthermore, the incidence of potential human AO variability has been reported from in vitro investigations (Hutzler et al., 2012), and, coupled with an inability to confidently predict human PK from scaling rodent and nonrodent clearance and half-life (Zientek et al., 2010), the DMPK community continues to debate the development of small-molecule therapeutic agents that are exclusively cleared by AO-mediated (or XO) metabolism.

Examples of AO- and XO-mediated drug metabolism have garnered attention from the drug safety and disposition community as well. Diamond et al. (2010) published an account of obstructive nephropathy in cynomolgus monkeys that was proposed to be induced by an abundant metabolite formed through AO metabolism of the investigational drug, 6-(6-(1-methyl-1*H*-pyrazol-4-yl)-[1,2,4]triazolo[4,3-*b*]pyridazin-3-ylthio)quinoline (SGX523). Their report of the AO-specific formation of a problematic metabolite underscored the importance of appropriate selection of safety species during preclinical development. Boehringer-Ingelheim had to discontinue a clinical trial of an investigational drug [N8-(3-chloro-4-fluorophenyl)-N2-(1-methyl-4-piperidinyl)-pyrimido[5,4-*d*]pyrimidine-2,8-diamine (BIBX 1382)], in part because of a “preclinically unknown metabolite” that had originated from AO metabolism of the parent compound in human subjects (Dittrich et al., 2002). The particular revelation of extensive AO-mediated metabolism of Boehringer-Ingelheim’s epidermal growth factor receptor inhibitor was consistent with low bioavailability in the clinic and raised potential questions with respect to appropriate species selection (Balani et al., 2005; Smith and Obach, 2009) during the nonclinical safety assessment. Finally, Kalgutkar and coworkers (Sharma et al., 2011) recently reported on an XO-catalyzed primary biotransformation of a quinoxaline-containing G protein-coupled receptor agonist in plasma (in vitro and ex vivo) from rodents (mouse, rat, and guinea pig). This previously undefined plasma-specific biotransformation prevented a definitive PK assessment for the quinoxaline analog and has presented questions with regard to the impact of XO in the systemic clearance of nitrogen-containing heterocycles in vivo.

Considering the inability to confidently assess predicted human PK with compounds primarily cleared by AO and in view of the cited accounts of research and development hurdles associated with AO and XO metabolism, we attempted to shift metabolism of the pyrimidine-containing mGlu₅ NAM series away from molybdenum hydroxylases and toward P450-mediated metabolism using various medicinal chemistry strategies. For example, outright replacement of the pyrimidine with a fluoropyridine moiety (Scheme 2B) represented a broad change to the biaryl ether scaffold that prevented AO metabolism and shifted the principal biotransformation pathway to P450. However, such broad alteration of the scaffold was accompanied with P450 inhibition liabilities and undesirable physicochemical properties. Although successful in blocking AO metabolism, the installation of various substitutions at the C-6 center of the pyrimidine moiety proved ineffective, the net effect being loss of potency on the receptor, decreased GPCR selectivity, or both. Likewise, we also considered the installation of electron-donating groups on the pyrimidine moiety as a

means to reduce the rate of AO-mediated metabolism, given that the mechanism of AO-mediated metabolism involves nucleophilic (i.e., H₂O) attack of a developing electrophilic intermediate. Again, consistent with the aforementioned strategy, we observed intolerance for substitution on the pyrimidine moiety as evidenced by a loss of potency or selectivity at the receptor. More recently we have observed a reduction of AO-mediated metabolism, albeit not at 100%, by simply installing P450-labile alkyl groups (for example) distal to the pyrimidine moiety. The rational design of a compound displaying a balanced P450:AO metabolism phenotype (e.g., 50:50) has been observed with new analogs in humans but not in rats in vitro. Perhaps this observation was not surprising, given the presently referenced accounts of the unpredictable variability between rat and human AO in their metabolism of xenobiotics.

Historically, academicians and industrial DMPK scientists have been successful in the prediction of human PK from nonclinical species, in which P450-mediated metabolism was defined as the primary route of clearance for small-molecule drug candidates (Hosea et al., 2009). Likewise, contemporary DMPK approaches have been extended to the successful prediction of human clearance and half-life for experimental therapeutic agents for which glucuronidation (Deguchi et al., 2011) and esterase hydrolysis (Liu et al., 2011) represent the primary mechanisms of clearance. However, because of the species differences noted for AO catalysis, expression, and enzyme regulation (Rashidi et al., 1997), the current approach in the prediction of human PK of AO substrates seems to be limited to the assessment of clearance and half-life in human hepatocytes (Zientek et al., 2010; Hutzler et al., 2012). Given the emergence of AO substrates from drug discovery organizations, continued basic science development in molybdenum hydroxylase (AO and XO) drug metabolism and pharmacokinetics is warranted to implement drug candidate optimization programs, assess the drug safety of drug candidates and metabolites, and ultimately predict the drug metabolism and disposition in humans when non-P450 metabolism surfaces.

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Authorship Contributions

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