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


Received April 10, 2012; accepted June 18, 2012

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 (+18 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 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 tool for defining the in vitro metabolism of a target compound, but also as a method to identify and validate pathways that mirror the hepatic extraction predicted for humans.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health National Institute of Mental Health [Grant 5-R01-MH62646-13]; the National Institutes of Health National Institute of Neurological Disorders and Stroke [2-R01-NS31373-16]; and Seaside Therapeutics, Inc. (Cambridge, MA).

Article, publication date, and citation information can be found at http://dx.doi.org/10.1124/dmd.112.046136. The online version of this article (available at http://dmd.aspetjournals.org) contains supplemental material.

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 spectrometry; 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; X0, 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 DMFK 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; Emmite, 2011). VU0409106 displayed inhibitory potency against the target receptor (IC50 = 26 nM) and selectivity against other group I, II and III mGlu receptor subtypes (IC50 ≥10 μM) (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 isotypically labeled reagents (e.g., H18O), 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 characterized and procured 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, hydroxylamine, 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 Celsa/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 recf, 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 recf, 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 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 recf, 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-UVMass Spectrometry Analysis of Metabolites. An Agilent 1100 HPLC system was coupled to a Supelco Discovery C18 column (5 μm, 2.1 × 50 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, 215 nm) followed by electrospray ionization-assisted introduction into a Finnigan LCQ Deca XP+™ 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 MS3 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 MS³) 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 equivalence 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 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, 9.54 Hz), 6.74 (1H, s), and 2.26 (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. Multiplicity-edited heteronuclear spin–quantum correlation spectroscopy (HSQC) experiments were acquired using a 1024 × 64 data matrix, 12 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. 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 (50-donor UltraPool, BD Biosciences). A potassium phosphate-buffered (0.1 M, pH 7.4) solution of VU0409106 (1 μM), S9 (2.5 mg/ml; cytosol, 1 mg/ml), ± 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 analysis. All samples were analyzed via electrospray ionization on an AB Sciei 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 μm; 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 TurbolonSpray source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciei 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 (CLᵢᵣᵣ, 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):

\[
\text{CLᵢᵣᵣ} = \frac{\ln 2}{t \frac{1}{2} (\text{min})} \times \frac{1 \text{ ml}}{2.5 \text{ mg protein}_{\text{M}} \times \frac{120.7 \text{ mg protein}_{\text{M}}}{1 \text{ g liver weight}} \times \frac{(A)\text{g liver weight}}{kg \text{ body weight}}
\]

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 CLᵢᵣᵣ calculation for hepatocyte metabolic stability assessment. A predicted hepatic clearance (CLᵢᵣᵣᵣ, milliliters per minute per kilogram) was subsequently calculated using the well stirred model, uncorrected for the fraction unbound in plasma (eq. 2):

\[
\text{CLᵢᵣᵣᵣ} = \frac{\text{Q}_{\text{HI}} \times \text{CLᵢᵣᵣ}}{\text{Q}_{\text{HI}} + \text{CLᵢᵣᵣ}}
\]

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,
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

---

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>VU0409106</td>
<td><img src="image1" alt="Structure" /></td>
<td>14.5</td>
<td>331</td>
<td>313, 304, 217, 116</td>
</tr>
<tr>
<td>M1</td>
<td><img src="image2" alt="Structure" /></td>
<td>12.55</td>
<td>347</td>
<td>329, 291, 251, 233, 205</td>
</tr>
<tr>
<td>M2</td>
<td><img src="image3" alt="Structure" /></td>
<td>12.3</td>
<td>363</td>
<td>345, 320, 267, 249, 224, 221</td>
</tr>
<tr>
<td>M3</td>
<td><img src="image4" alt="Structure" /></td>
<td>13.4</td>
<td>361</td>
<td>343, 265, 247, 219</td>
</tr>
<tr>
<td>M4</td>
<td><img src="image5" alt="Structure" /></td>
<td>11.8</td>
<td>347</td>
<td>319, 276, 234, 217</td>
</tr>
<tr>
<td>M5</td>
<td><img src="image6" alt="Structure" /></td>
<td>12.9</td>
<td>347</td>
<td>329, 251, 233</td>
</tr>
</tbody>
</table>

---
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 cryopreserved hepatocytes; M1 was also observed in the plasma of rats (male) receiving intraperitoneal administrations of the test article. In addition to water loss (Δ m/z 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 (Δ-56 Da) indicated an oxidative biotransformation of the pyrimidine moiety.
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, [M + 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, [M + 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 a 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, [M + 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 MS³ (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, [M + 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 × 10⁸ 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-
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 mM) 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 mM) addition and quantitatively after the addition of hydralazine (100 mM) to the in vitro incubations. The biotransformation of VU0409106 in rat appeared to be predominantly catalyzed by AO, because the selective XO inhibitor allopurinol (100 mM) 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 mM) 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 → M2), we

![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.

![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.](https://example.com/scheme2.png)
subjected an aliquot of isolated M1 to rat hepatic S9 incubations (−NADPH) in the presence and absence of AO and XO inhibitors (100 μM) 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 18O-Labeled Water into M1 and M2 Confirms the Hydroxylase-Mediated Metabolism of VU0409106 In Vitro. Both AO and XO use water (H2O) in their catalytic mechanisms of oxidation (Garattini and Terao, 2012) rather than molecular oxygen (O2), 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 H218O (Dalvie et al., 2010; Diamond et al., 2010; Hutzler et al., 2012) to confirm the contribution of these molybdenum-containing hydroxylases in the biotransformation of VU0409106. After rat and human S9 (5 mg/ml) incubations of VU0409106 (25 μM) in the presence of H218O, we observed the incorporation of 18O into M1, because an [M + H]+ molecular ion was detected at m/z 349 (i.e., M + 2 Da) (Fig. 6A) and at a relative abundance (69%) consistent with the percentage of H218O 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 18O 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 H218O into the metabolite. The LC/MS spectrum of M2 indicated that a second 18O atom (i.e., M + 4 Da) had been incorporated, producing an [M + H]+ molecular ion at m/z 367 (53%) (Fig. 6B), the relative abundance of which was consistent with the percentage of H218O 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 18O atom in the molecule and was again consistent with the proposed structure for M1. These LC-MS/MS data indicated an incorporation of two 18O atoms into M2 and confirmed a sequential hydroxylase mechanism of VU0409106 oxidation in vitro (i.e., AO → 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,
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 · min$^{-1}$ · 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$_{int}$ = 1 ml · min$^{-1}$ · kg$^{-1}$) and moderately stable in the corresponding human cytosol (CL$_{int}$ = 3–4 ml · min$^{-1}$ · 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.
shown previously in Fig. 3. The fact that human hepatocytes metabolized VU0409106 primarily to M1 at rates (CLint = 6.9 ml·min⁻¹·kg⁻¹; CLHEP = 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 has 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 (CLp) of less than hepatic blood flow for the species (CLp = 43 ml·min⁻¹·kg⁻¹ versus CLHEP = 30–40 ml·min⁻¹·kg⁻¹ in vitro). Coupled with a corresponding volume of distribution predicted at steady state (Vss) of 0.8 l/kg, VU0409106 displayed an effective t1/2 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 (CLHEP = 20 and 33 ml·min⁻¹·kg⁻¹, respectively) was predictive of the plasma clearance (CLp = 24 ml·min⁻¹·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 and 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 a 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 antimarial drug, falcicloril. However, unlike the instance with falcicloril 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 CLHEP = 30 versus CLp = 43 ml·min⁻¹·kg⁻¹). Hepatocytes also produced a predicted clearance for VU0409106 in rats that correlated well with the observed in vivo clearance (hepaticocyte CLHEP = 30 ml·min⁻¹·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 (CLHEP = 36 ml·min⁻¹·kg⁻¹) also correlated with the in vivo clearance (CLp = 24 ml·min⁻¹·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,

<table>
<thead>
<tr>
<th>Species</th>
<th>CLint a</th>
<th>CLHEP b</th>
<th>CLp</th>
<th>CLint a</th>
<th>CLHEP b</th>
<th>CLp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>25</td>
<td>18</td>
<td>34</td>
<td>19</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>S9 + NADPH</td>
<td>54</td>
<td>30</td>
<td>37</td>
<td>20</td>
<td>15.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Cytosol</td>
<td>16</td>
<td>13</td>
<td>N.A.</td>
<td>N.A.</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>96</td>
<td>40</td>
<td>194</td>
<td>36</td>
<td>6.9</td>
<td>5.1</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CLint, intrinsic clearance; CLHEP, predicted hepatic clearance; CLp, plasma clearance; N.A., not available.

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 CLHEP = 30 versus CLp = 43 ml·min⁻¹·kg⁻¹). Hepatocytes also produced a predicted clearance for VU0409106 in rats that correlated well with the observed in vivo clearance (hepaticocyte CLHEP = 30 ml·min⁻¹·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 (CLHEP = 36 ml·min⁻¹·kg⁻¹) also correlated with the in vivo clearance (CLp = 24 ml·min⁻¹·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-[(1-methyl-1H-pyrazol-4-yl)-[1,2,4]triazolo[4,3-b]pyridazin-3-yl]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)-pyrimidinol[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 (Ditrich 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, Kaligutkar 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 hydroxylase (AO and XO) drug metabolism and pharmacokinetics. This was accomplished in part through the successful prediction of human 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.

Acknowledgments
We thank Dr. J. Matthew Hutzler (Boehringer-Ingelheim, Ridgefield, CT) for helpful discussions regarding the present investigation.

Authorship Contributions
Participated in research design: Morrison, Blobaum, Sanchez-Ponce, Rush, Niswender, Ermittie, and Daniels.

Conducted experiments: Morrison, Blobaum, Byers, Santamango, Bridges, Stec, Brewer, Sanchez-Ponce, Corlew, Venable, Rodriguez, and Jones.

Contributed new reagents or analytic tools: Felts, Manka, Bates, Lindsley, and Ermittie.

Performed data analysis: Morrison, Blobaum, Stec, Sanchez-Ponce, Rodriguez, Jones, and Daniels.

Wrote or contributed to the writing of the manuscript: Morrison, Stec, Sanchez-Ponce, Rush, Conn, and Daniels.

References