

# Species-Specific Involvement of Aldehyde Oxidase and Xanthine Oxidase in the Metabolism of the Pyrimidine-Containing mGlu<sub>5</sub>-Negative Allosteric Modulator VU0424238 (Auglurant)<sup>§</sup>

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## ABSTRACT

Aldehyde oxidase (AO) and xanthine oxidase (XO) are molybdo-flavoenzymes that catalyze oxidation of aromatic azaheterocycles. Differences in AO activity have been reported among various species, including rats, humans, and monkeys. Herein we report a species difference in the enzymes responsible for the metabolism of the negative allosteric modulator of metabotropic glutamate receptor subtype 5 (mGlu<sub>5</sub> NAM) VU0424238 (VU238, auglurant). Hepatic S9 incubations with AO and XO specific inhibitors hydralazine and allopurinol indicated that rats and cynomolgus monkeys both oxidized VU238 to the 6-oxopyrimidine metabolite M1 via an AO-mediated pathway, whereas secondary oxidation to the 2,6-dioxypyrimidine metabolite M2 was mediated predominantly by AO in monkeys and XO in rats. Despite differences in enzymatic pathways, intrinsic clearance (CL<sub>int</sub>) of M1 was similar between species (cynomolgus and rat CL<sub>int</sub> = 2.00 ± 0.040 and 2.19 ± 0.201

μl/min per milligram of protein, respectively). Inhibitor studies in the S9 of multiple species indicated that oxidation of VU238 to M1 was mediated predominantly by AO in humans, cynomolgus and rhesus monkeys, rats, mice, guinea pigs, and minipigs. Oxidation of M1 to M2 was mediated predominantly by XO in rats and mice and by AO in monkeys and guinea pigs, whereas low turnover prevented enzyme phenotyping in humans and minipigs. Additionally, inhibitor experiments indicated that oxidation at the 2-position of the pyrimidine ring of the known AO substrate, BIBX1382, was mediated by AO in all species, although production of this metabolite was comparatively low in rats and mice. These data may suggest low reactivity of rat AO toward 2-oxidation of pyrimidine-containing compounds and highlight the importance of thoroughly characterizing AO-metabolized drug candidates in multiple preclinical species.

## Introduction

Aldehyde oxidase (AO) and xanthine oxidase (XO) belong to a family of molybdo-flavoenzymes that catalyze the oxidation of nitrogen-containing aromatic heterocycles. Consequently, metabolism mediated by these enzymes, particularly AO, is now frequently encountered among drug discovery and development programs, as the inclusion of azaheterocycles in small-molecule drug candidates has become a common tactic to evade cytochrome P450 metabolism or to engage particular targets such as kinases (Pryde et al., 2010). Species differences in the expression and activity of AO (Terao et al., 2016) present a challenge in predicting the metabolism and disposition of AO-metabolized compounds using traditional animal models; consequently, several AO-metabolized drug candidates have failed during clinical trials (Dittrich et al., 2002; Diamond et al., 2010; Akabane et al., 2011; Infante et al., 2013; Lolkema et al., 2015; Jensen et al., 2017). To usefully employ preclinical animal models for the prediction of drug metabolism and disposition of AO substrates, a better understanding of the species differences surrounding this enzyme is needed.

Both AO and XO are cytosolic enzymes that function as a homodimer requiring a molybdenum-containing cofactor and flavin adenine dinucleotide for catalytic activity (Pryde et al., 2010). Unlike AO, XO can interconvert between xanthine oxidase and xanthine dehydrogenase, whereas AO exists only as an oxidase (Pryde et al., 2010). Whereas AO and XO share similar substrate specificities (both oxidize aromatic azaheterocycles on an electrophilic carbon atom adjacent to a nitrogen atom), AO metabolizes a broader array of substrates relative to XO, which has a general preference for purine/pyrimidine analogs (Kitamura et al., 2006; Pryde et al., 2010). Inhibitor specificities for the two enzymes differ as well, resulting in the ability to differentiate between AO- and XO-mediated metabolism via the use of selective inhibitors such as hydralazine (Johnson et al., 1985; Strelevitz et al., 2012) and allopurinol (Massey et al., 1970; Zientek and Youdim, 2015), respectively.

Some compounds can be substrates for both AO and XO, such as 6-deoxyacyclovir and 5-fluorouracil (Kitamura et al., 2006). In the case of 5-fluorouracil, oxidation by AO and XO occurs at the same site to produce the metabolite, 5-fluoro-6-oxouracil. Alternatively, 6-deoxyacyclovir is predominantly oxidized at the 6-position by XO and the 8-position by AO (Kitamura et al., 2006; Pryde et al., 2010). We previously reported on the role of AO and XO in the sequential metabolism of VU0409106, a

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**ABBREVIATIONS:** AO, aldehyde oxidase; AOX1–4, aldehyde oxidase 1–4; CL<sub>int</sub>, intrinsic clearance; CL<sub>p</sub>, plasma clearance; DCM, dichloromethane; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; mGlu<sub>5</sub>, metabotropic glutamate receptor subtype 5; NAM, negative allosteric modulator; P450, cytochrome P450; 6-TX, 6-thioxanthine; XO, xanthine oxidase.

negative allosteric modulator of the metabotropic glutamate receptor subtype 5 (mGlu<sub>5</sub> NAM) (Felts et al., 2013), in which case in vitro oxidation of the pyrimidine ring to the principle 6-oxopyrimidine metabolite was mediated by AO in humans, monkeys, and rats (Morrison et al., 2012). In vitro and in vivo experiments in Sprague-Dawley rat using the XO inhibitor allopurinol indicated that a secondary oxidation, resulting in the formation of a 2,6-dioxypyrimidine metabolite, was mediated by XO (Morrison et al., 2012; Crouch et al., 2016). These metabolites were also produced in the cynomolgus monkey; however, experiments with allopurinol were not conducted.

We recently reported on the discovery of the novel mGlu<sub>5</sub> NAM VU0424238 (VU238, augurant) as a potential clinical candidate for the treatment of a variety of psychiatric and neurodegenerative disorders (Felts et al., 2017). Similar to VU0409106, VU238 contains a pyrimidine head group that could be susceptible to oxidation by AO and/or XO. Accordingly, we found that VU238, like VU0409106, is also metabolized in an NADPH-independent manner to 6-oxopyrimidine (M1) and 2,6-dioxypyrimidine (M2) metabolites. During studies using pharmacologic inhibitors to evaluate the involvement of AO and XO in the formation of these metabolites, we discovered an apparent species difference in the primary enzyme responsible for the formation of the 2,6-dioxypyrimidine metabolite, M2, between rats and cynomolgus monkeys. A multispecies evaluation was conducted to further investigate species differences in the contributions of these two enzymes to the metabolism of VU238.

## Materials and Methods

### Materials

*N*-(5-fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (VU0424238, VU238), *N*-(5-fluoropyridin-2-yl)-6-methyl-4-((pyrimidin-5-yl-2-d)oxy)picolinamide (VU6010180, VU180), and *N*-(5-fluoropyridin-2-yl)-6-methyl-4-((6-oxo-1,6-dihydropyrimidin-5-yl)oxy)picolinamide (VU0652922, VU922) were prepared and characterized by the Division of Medicinal Chemistry within the Vanderbilt Center for Neuroscience Drug Discovery. VU0424238 was prepared and characterized as previously described (Felts et al., 2017). Synthetic procedures for the preparation of VU0652922 and VU6010180 are detailed as follows. Potassium phosphate, ammonium formate, formic acid, magnesium chloride, carbamazepine, hydralazine hydrochloride, and allopurinol were purchased from Sigma-Aldrich (St. Louis, MO). BIBX1382 dihydrochloride was purchased from Tocris Bioscience (R&D Systems, Minneapolis, MN); 6-thioxanthine was purchased from Carbosynth (San Diego, CA), and 6-thiouric acid was purchased from Ark Pharm (Arlington Heights, IL). Pooled human hepatic S9 (150 donors, 50% male, 50% female, lot 38289), pooled male Sprague-Dawley rat ( $n = 36$ , lot 4029004), pooled male cynomolgus monkey ( $n = 2$ , lot 5035001MUF), pooled male rhesus monkey ( $n = 6$ , lot 1), and pooled male CD-1 mouse ( $n = 170$ , lot 5023003) hepatic S9 fractions were obtained from Corning Inc. (Tewksbury, MA). Pooled male Hartley guinea pig ( $n = 50$ , lot 0510020) hepatic S9 was purchased from XenoTech (Lenexa, KS). Pooled male Gottingen minipig ( $n = 7$ , lot KQQ) hepatic S9 and pooled female Sprague-Dawley rat ( $n = 6$ , lot BKM), and pooled female cynomolgus monkey hepatic S9 fractions ( $n = 3$ , lot HWH) were purchased from BioreclamationIVT (Baltimore, MD). All solvents used for bioanalysis were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA) and were of high-performance liquid chromatography (HPLC) grade.

### Synthesis of *N*-(5-Fluoropyridin-2-yl)-6-Methyl-4-((6-oxo-1,6-Dihydropyrimidin-5-yl)oxy)Picolinamide (M1, VU0652922)

**4-(Benzyloxy)-5-Bromopyrimidine (1).** Benzyl alcohol (1.02 mL, 9.82 mmol, 2.00 eq) was dissolved in 12 mL of dimethylformamide (DMF) (12 mL), and sodium hydride (248 mg, 9.82 mmol, 2.00 eq) was added. After 5 minutes 5-bromo-4-chloro-pyrimidine (950 mg, 4.91 mmol, 1.00 eq) was added as a solution in DMF (4 mL). The reaction was stirred overnight and then quenched with a saturated solution of ammonium chloride. The mixture was diluted with ethyl acetate and washed with water (2×). The aqueous washes were back-

extracted with ethyl acetate, and the combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification using 0%–20% hexanes/ethyl acetate afforded 1.04 g (80%) of the title compound as a clear oil: ES-MS [ $M + 1$ ]<sup>+</sup>: 265.0.

**4-(4-(Benzyloxy)pyrimidin-5-yl)Oxy)-6-Methylpicolinonitrile (2).** Compound 1 (797 mg, 3.01 mmol, 1.00 eq), potassium hydroxide (506 mg, 9.02 mmol, 3.00 eq), tris(dibenzylideneacetone)-dipalladium(0) (165 mg, 0.180 mmol, 0.0600 eq), and 2-di-*tert*-butylphosphino-2',4',6'-triisopropylbiphenyl (153 mg, 0.361 mmol, 0.120 eq) were suspended in dioxane (7.5 mL) and water (7.5 mL) in a microwave vial and heated in a microwave reactor at 150°C for 15 minutes. The reaction was neutralized to pH 4 or pH 5 with 2N HCl and then extracted with ethyl acetate (1×) and 3:1 (CHCl<sub>3</sub>/IPA) (2×). The combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification using 0%–5% dichloromethane (DCM)/MeOH afforded 368 mg (64%) of the title compound as an off-white solid: ES-MS [ $M + 1$ ]<sup>+</sup>: 203.4.

**4-(4-(Benzyloxy)pyrimidin-5-yl)Oxy)-6-Methylpicolinonitrile (3).** Compound 2 (388 mg, 1.92 mmol, 1.00 eq), 4-chloro-6-methyl-pyridine-2-carbonitrile (293 mg, 1.92 mmol, 1.00 eq), and potassium carbonate (530 mg, 3.84 mmol, 2.00 eq) were suspended in DMF (5.8 mL) and heated overnight at 100°C. (Note: Some amount of benzyl transfer is observed. Nuclear overhauser effect spectroscopy (NOESY) nuclear magnetic resonance (NMR) confirmed the less polar product by LC-MS to be the desired product). After cooling to room temperature, the reaction was filtered and washed with ethyl acetate. The mixture was washed with water (2×), and the aqueous washes were back-extracted with ethyl acetate. The combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification using 0%–40% hexanes/ethyl acetate afforded 247 mg (40%) of the title compound as a yellow solid: ES-MS [ $M + 1$ ]<sup>+</sup>: 319.3.

**4-(4-(Benzyloxy)pyrimidin-5-yl)Oxy)-6-Methylpicolinamide (4).** Compound 3 (247 mg, 0.776 mmol, 1.00 eq) was dissolved in dioxane (3.9 mL) and 2 N NaOH (1.55 mL, 3.10 mmol, 4.00 eq) was added. The reaction was heated at 60°C for 6 hours, at which point it was cooled and brought to pH 4 to pH 5 with 2N HCl. The mixture was concentrated to dryness, dissolved in 5% MeOH/DCM, and filtered. The organics were concentrated to afford 233 mg (89%) of the title compound as a brown solid that was used without further purification: ES-MS [ $M + 1$ ]<sup>+</sup>: 337.2.

**4-(4-(Benzyloxy)Pyrimidin-5-yl)oxy)-N-(5-Fluoropyridin-2-yl)-6-Methylpicolinamide (5).** Compound 4 (233 mg, 0.693 mmol, 1.00 eq), 2-bromo-5-fluoropyridine (183 mg, 1.04 mmol, 1.50 eq), palladium (II) acetate (7.78 mg, 0.0346 mmol, 0.0500 eq), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (40.1 mg, 0.0693 mmol, 0.100 eq), and sodium *tert*-butoxide (93.2 mg, 0.970 mmol, 1.40 eq) were dissolved in toluene (3.5 mL) and purged with nitrogen. The reaction was heated at 100°C overnight, cooled, and filtered over a Celite pad. The pad was washed with 5% methanol/DCM, and the combined organics were concentrated in vacuo. Purification using 0%–40% hexanes/ethyl acetate afforded 156 mg (52%) of the title compound as a white solid: ES-MS [ $M + 1$ ]<sup>+</sup>: 432.3.

***N*-(5-Fluoropyridin-2-yl)-6-Methyl-4-((6-Oxo-1,6-Dihydropyrimidin-5-yl)Oxy)Picolinamide (M1-VU0652922).** Compound 5 (156 mg, 0.362 mmol, 1.00 eq), was dissolved in chloroform (1.8 mL), and methanesulfonic acid (234  $\mu$ L, 3.62 mmol, 10.0 eq) was added. The reaction was stirred for 30 minutes and then neutralized with a saturated solution of sodium bicarbonate. The layers were separated, and the aqueous phase was extracted with 3:1 CHCl<sub>3</sub>/IPA (2×). The combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification by reverse-phase chromatography afforded 92 mg (75%) of the title compound as a white solid: <sup>1</sup>H nuclear magnetic resonance (NMR) (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.47 (s, 1H), 8.39 (d,  $J = 2.5$  Hz, 1H), 8.29–8.24 (m, 2H), 8.14 (s, 1H), 7.86 (td,  $J = 2.6, 8.6$  Hz, 1H), 7.43 (s, 1H), 7.19 (s, 1H), 2.56 (s, 3H); ES-MS [ $M + 1$ ]<sup>+</sup>: 342.2.

### Synthesis of *N*-(5-Fluoropyridin-2-yl)-6-Methyl-4-((Pyrimidin-5-yl-2-d)Oxy)Picolinamide (VU6010180)

**2-Bromo-5-((2-Methoxyethoxy)Methoxy)Pyrimidine (6).** 2-Bromo-5-hydroxypyrimidine (500 mg, 2.86 mmol, 1.00 eq), and *N,N*-diisopropylethylamine (1.49 mL, 8.57 mmol, 3.00 eq) were dissolved in DCM (7.2 mL) and cooled to 0°C. 2-Methoxyethoxymethyl chloride (653  $\mu$ L, 5.71 mmol, 2.00 eq) was added as a solution in DCM (3.6 mL), and the reaction was allowed to warm to room temperature. After determination of completion by LC-MS, a saturated solution of

sodium bicarbonate was added, and the layers were separated. The aqueous phase was extracted with an additional portion of DCM, and the combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification using 0%–15% hexanes/ethyl acetate afforded 484 mg (64%) of the title compound as a clear oil: ES-MS [*M* + 1]<sup>+</sup>: 263.0.

**5-(2-Methoxyethoxy)MethoxyPyrimidine-2-d (7).** Compound 6 (384 mg, 1.46 mmol, 1.00 eq) was dissolved in tetrahydrofuran (3.6 ml) and diethyl ether (3.6 ml) and cooled to –100°C using a bath of liquid nitrogen in ether. *n*-Butyllithium (1.4 M in hexanes) (1.15 ml, 1.61 mmol, 1.10 eq) was added dropwise, keeping the temperature below –95°C. After the addition, the reaction was stirred for 15 minutes at –100°C, and methanol-d<sub>4</sub> (1.19 ml, 29.2 mmol, 20.0 eq) was added. The reaction was warmed to room temperature and quenched with a saturated solution of ammonium chloride. The mixture was extracted with ethyl acetate (2×), dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo. Purification using 0%–5% DCM/MeOH afforded 138 mg (51%) of the title compound as an off-white solid: ES-MS [*M* + 1]<sup>+</sup>: 186.2.

**Pyrimidin-2-d-5-ol (8).** Compound 7 (128 mg, 0.691 mmol, 1.00 eq) was dissolved in chloroform (3.5 ml) and methanesulfonic acid (449 μl, 6.91 mmol, 10.0 eq) was added. The reaction was stirred for 30 minutes and then neutralized with a saturated solution of sodium bicarbonate. The layers were separated, and the aqueous phase was extracted with 3:1 CHCl<sub>3</sub>/IPA (2×). The combined organics were dried (MgSO<sub>4</sub>), filtered, and concentrated in vacuo to afford 59 mg (88%) of the title compound as a yellow solid that was used without further purification: ES-MS [*M* + 1]<sup>+</sup>: 98.0. At this stage, remaining synthetic procedures for preparation of VU6010180 were carried out as described for VU0424238 (Felts et al., 2017).

### Biotransformation in Multispecies Hepatic S9 Fractions

Substrates (5 or 20 μM) were incubated at 37°C in borosilicate glass test tubes under ambient oxygenation for 60 minutes in a potassium phosphate-buffered reaction (100 mM, pH 7.4) containing hepatic S9 from humans, mice, rats, guinea pigs, cynomolgus monkeys, rhesus monkeys, or minipigs (2 or 5 mg/ml) and MgCl<sub>2</sub> (3 mM), with preincubation in the presence or absence of the aldehyde oxidase inhibitor hydralazine (50 μM) or the xanthine oxidase inhibitor allopurinol (100 μM). The total incubation volume was 0.5 ml. Reactions were initiated by the addition of substrate, terminated with the addition of two volumes of ice-cold acetonitrile, and subsequently centrifuged at 4000 rcf for 10 minutes. The resulting supernatants were dried under a stream of nitrogen and reconstituted in initial mobile phase in preparation for LC-MS analysis.

### Intrinsic Clearance (CL<sub>int</sub>) in Multispecies Hepatic S9 Fractions

Substrates (1 μM) were incubated at 37°C for 60 minutes in a potassium phosphate-buffered reaction (100 mM, pH 7.4) containing hepatic S9 from humans, mice, rats, guinea pigs, cynomolgus monkeys, rhesus monkeys, or minipigs (2.5 mg/ml) and MgCl<sub>2</sub> (3 mM). Reactions were initiated with the addition of substrate, and at designated times (*t* = 0, 7, 15, 30, 45, and 60 minutes), aliquots were removed and precipitated with acetonitrile containing an internal standard (carbamazepine, 50 nM). The mixture was centrifuged at 4000 rcf for 5 minutes and resulting supernatants diluted with water in preparation for LC-MS/MS analysis of substrate depletion, by monitoring the analyte/internal standard peak

area ratio. The intrinsic clearance (CL<sub>int</sub>) was calculated from the elimination rate constant, *k*, which represents the slope determined from linear regression analysis of the natural log of the percent remaining substrate as a function of incubation time (eq. 1):

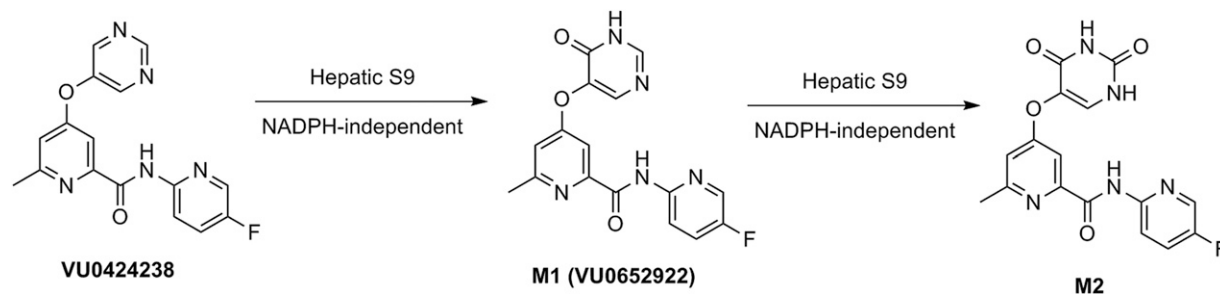
$$CL_{int} \text{ (mL/min/mg protein)} = \frac{k \text{ min}^{-1}}{2.5 \text{ mg/mL}} \quad (1)$$

A minimum of 15% substrate depletion by the terminal time point was considered a requirement to calculate CL<sub>int</sub>. If this requirement was not met, an CL<sub>int</sub> value was not reported. Data represent triplicate determinations and are reported as the mean ± S.D., obtained using GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA).

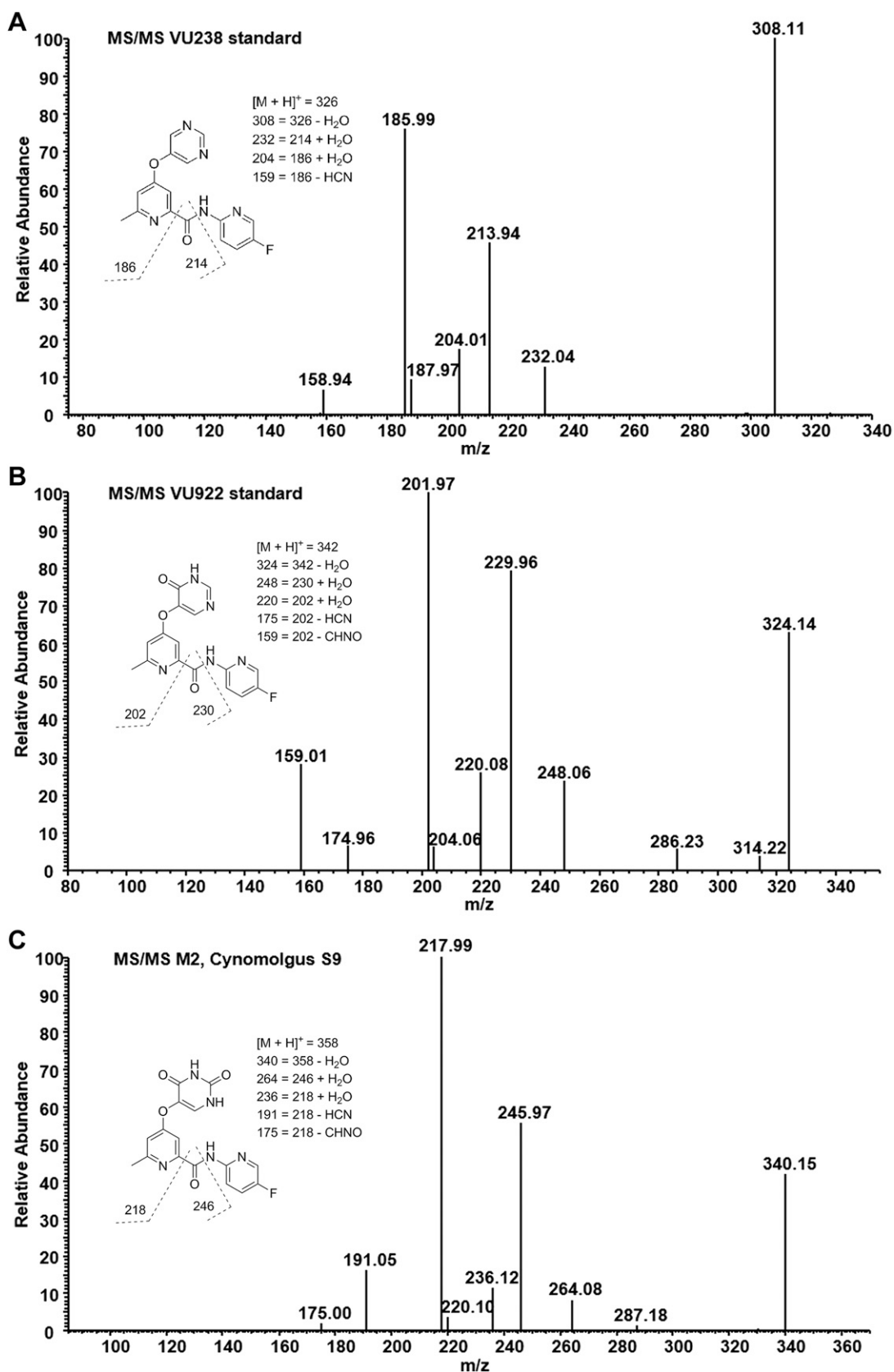
### Liquid Chromatography-UV-Mass Spectrometry Analysis

LC/UV/MS analysis of substrates and metabolites generated in vitro was performed with an Agilent 1290 Infinity HPLC system coupled to a LTQ XL ion trap mass spectrometer (ThermoFisher Scientific). Analytes were separated by gradient elution using a Supelco Discovery C18 column (5 μm, 2.1 × 150 mm; Sigma-Aldrich). Solvent A was 10 mM (pH 4.1) ammonium formate, and solvent B was acetonitrile. The initial mobile phase was 85:15 A-B (v/v) for analysis of VU238, VU180, and VU922 and 90:10 A-B (v/v) for BIBX1382. Beginning at 5 minutes, the mobile phase was transitioned by linear gradient to 20:80 A-B over 20 minutes, held for 2 minutes, and transitioned back to starting conditions over 2 minutes for a total run time of 30 minutes. The flow rate was 0.400 ml/min. The HPLC eluent was first introduced into an Agilent 1290 diode array detector (254 nm), followed by electrospray ionization-assisted introduction into a LTQ XL ion trap mass spectrometer operated in positive ionization mode. Ionization was assisted with sheath and auxiliary gas (ultrapure nitrogen) set at 50 and 20 psi, respectively. The electrospray voltage was set at 5 kV with the heated ion transfer capillary set at 350°C and 31 V. Data were analyzed using Thermo XCalibur 2.2 software.

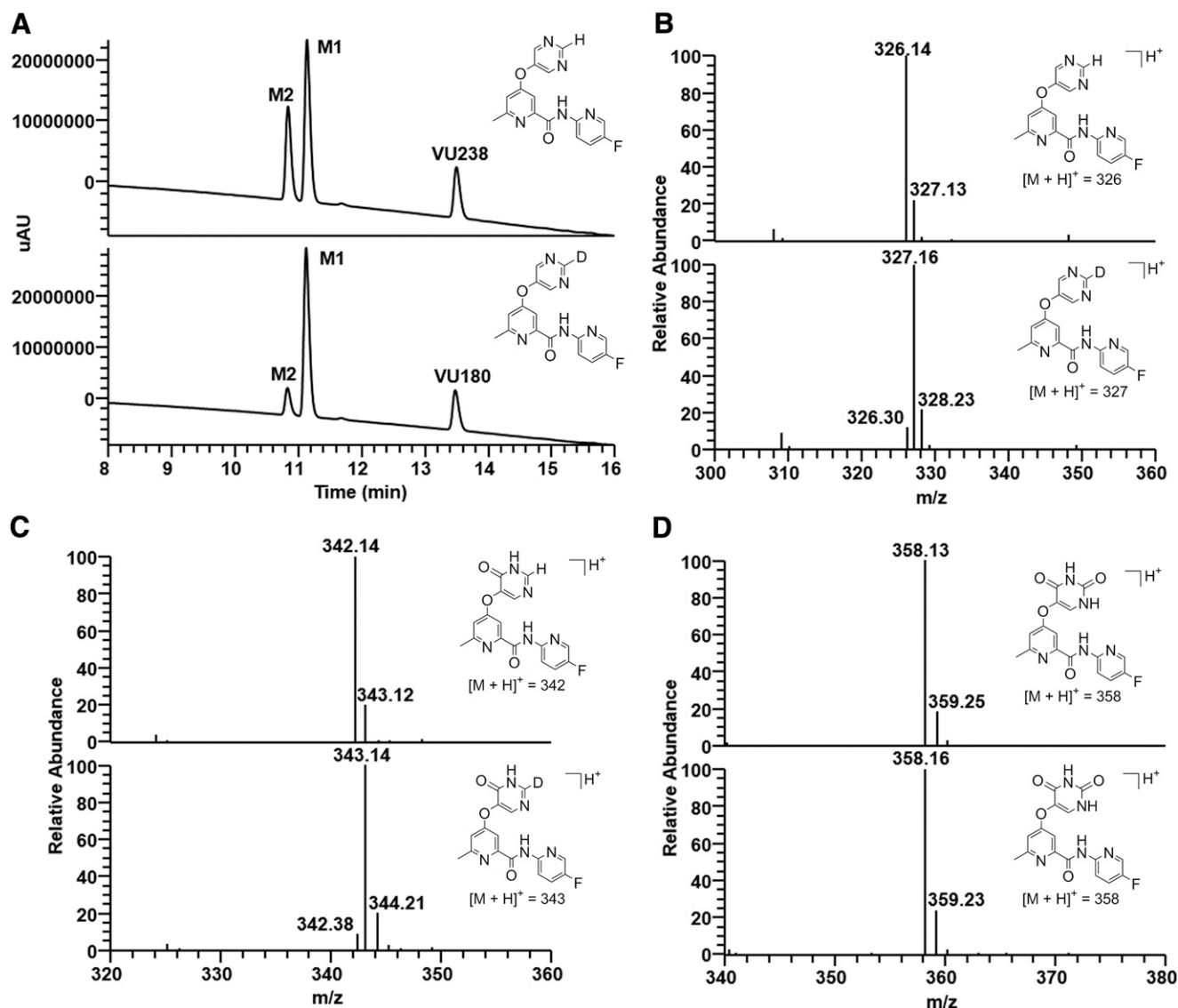
The extent of substrate depletion in S9 fractions was determined employing LC-MS/MS analysis with an electrospray ionization enabled Sciex API-4000 triple quadrupole instrument (Sciex, Foster City, CA) that was coupled to Agilent 1290 Infinity HPLC pumps (Agilent Technologies, Santa Clara, CA) and a CTC PAL autosampler (Leap Technologies, Carrboro, NC). Analytes were separated by gradient elution using a Kinetex C18 column (2.1 × 50 mm, 5 μm; Phenomenex, Torrance, CA) warmed to 40°C. Mobile phase A was 0.1% formic acid in water (pH unadjusted), and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 5% B after a 0.2-minute hold and was linearly increased to 90% B over 1.0 minute, held at 90% B for 0.4 minute, and returned to 5% B in 0.1 minute, followed by a re-equilibration (0.3 minute). The total run time was 2.0 minutes, and the HPLC flow rate was 0.5 ml/min. Mass spectral analyses were performed using multiple reaction monitoring, with transitions and voltages specific for each analyte using a Turbo Ion Spray source (source temp 500°C) in positive ionization mode (5.0 kV spray voltage). Multiple reaction monitoring transitions were the following: VU0424238 (*m/z* 326 → 186), VU0652922 (*m/z* 342 → 159), and carbamazepine (*m/z* 237 → 194). Data were analyzed using Sciex Analyst 1.5.1 software.



**Fig. 1.** Proposed metabolism of VU238 to major metabolites M1 and M2 in hepatic S9 (absent NADPH) of the human, monkey, rat, mouse, guinea pig, and minipig. M2 was not detected in extracts from incubations of VU238 with human and minipig S9 but was detected only in extracts from direct incubations with M1 (VU922).



**Fig. 2.** LC-MS/MS spectra of (A) VU238 and metabolites (B) M1 (VU922) and (C) M2 obtained from either synthetic standard solutions (A and B) or extract from male cynomolgus monkey hepatic S9 incubation with VU238 (C).



**Fig. 3.** LC-UV chromatograms and mass spectra of VU238, VU180, M1, and M2 obtained from extracts of incubations with VU238 or VU180 (20  $\mu$ M) in male cynomolgus monkey hepatic S9 (5 mg/ml). (A) LC-UV chromatograms obtained from extracts of incubations with VU238 (top) or VU180 (bottom). (B) Mass spectra of VU238 (top) or VU180 (bottom). (C) Mass spectra of M1 obtained from extracts of incubations with VU238 (top) or VU180 (bottom). (D) Mass spectra of M2 obtained from extracts of incubations with VU238 (top) or VU180 (bottom).

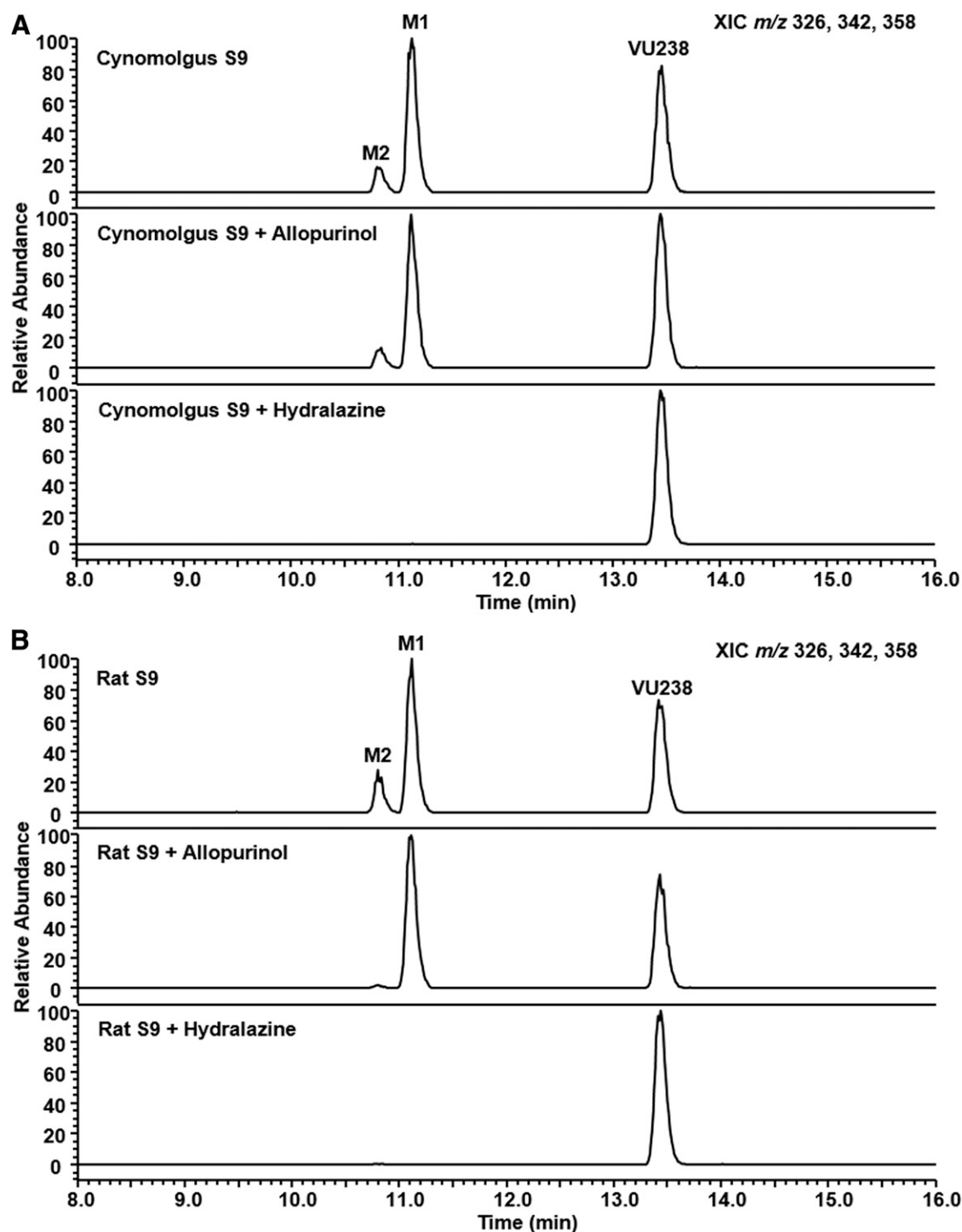
## Results

### Characterization of VU238 and NADPH-Independent Oxidative Metabolites by LC-MS/MS

VU0424238 (VU238) and the principal metabolites M1 and M2 produced *in vitro* in hepatic S9 of multiple species (Fig. 1) were characterized by LC-MS/MS (Fig. 2). Two major oxidative metabolites were produced in the absence of NADPH and thus represent metabolism via NADPH-independent (e.g., non-P450) pathways. These two metabolites were also major metabolites observed in the plasma of rats and monkeys administered VU238 *in vivo* (data not shown). A minor metabolite likely representing the carboxylic acid product of amide hydrolysis was also detected in extracts from NADPH-independent S9 experiments, and additional minor oxidative metabolites were detected in hepatic S9 extracts from incubations conducted in the presence of NADPH (data not shown). The following experiments presented herein focus solely on the NADPH-independent metabolism of VU238.

**VU0424238.** The protonated molecular ion  $[M + H]^+$  for VU238 was observed at  $m/z$  326 with a retention time of 13.5 minutes. MS/MS fragmentation of VU238 produced predominant fragment ions at  $m/z$  308, corresponding to a loss of water,  $m/z$  214, corresponding to fragmentation of the amide bond, and  $m/z$  186, corresponding to fragmentation adjacent to the amide bond, liberating the biaryl ether moiety (Fig. 2A). A fragment ion at  $m/z$  159 corresponds to the loss of HCN from the fragment ion at  $m/z$  186.

**VU0652922 (M1).** The protonated molecular ion  $[M + H]^+$  for the major oxidative metabolite produced in the S9 of all species, M1, was observed at  $m/z$  342 with a retention time of 11.1 minutes. Fragment ions produced at  $m/z$  324, 230, 202, and 175 each correspond to the aforementioned fragment ions produced by VU238 with a mass shift of +16 Da (Fig. 2B). The fragment ion at  $m/z$  159 corresponds to the loss of CHNO from the fragment ion at  $m/z$  202. Structural assignment of M1 was confirmed by LC-MS/MS analysis of a synthetic standard (VU0652922, VU922), which revealed an MS/MS fragmentation pattern and a retention time consistent with that observed for M1 from S9 extracts (data not shown). Refer to Supplemental Fig. 1 for the NMR spectra of VU922.



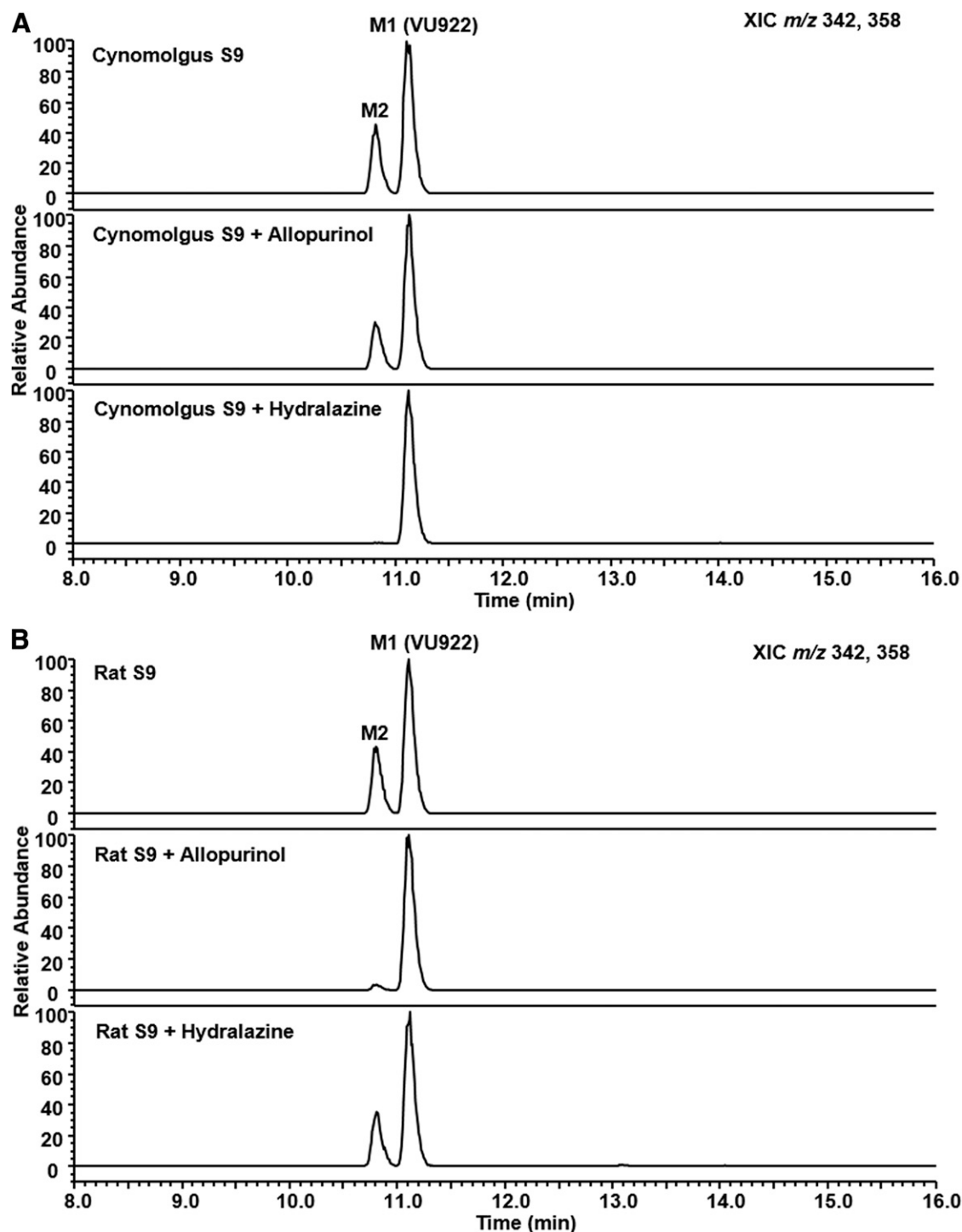
**Fig. 4.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with VU238 (5  $\mu$ M) in (A) male cynomolgus monkey hepatic S9 (2 mg/ml) and (B) male rat hepatic S9 (2 mg/ml) demonstrating the formation of M1 and M2 in the presence or absence of the XO inhibitor allopurinol (100  $\mu$ M) or the AO inhibitor hydralazine (50  $\mu$ M).

**M2.** A metabolite with a mass shift of +32 Da over the protonated molecular ion  $[M + H]^+$  of the parent VU238 was observed at  $m/z$  358 with a retention time of 10.8 minutes. Fragment ions produced at  $m/z$  340, 246, 218, and 191 each correspond to the aforementioned fragment ions that were produced by VU238 with a mass shift of +32 Da (Fig. 2C). The fragment ion at  $m/z$  175 corresponds to the loss of CHNO from the fragment ion at  $m/z$  218. Assignment of the secondary oxidation site to the 2-position of the pyrimidine ring was supported by LC-MS/MS analysis of metabolites generated during hepatic S9 incubation of a

deuterated analog of VU238 (VU6010180), where a deuterium has replaced the hydrogen atom in the 2-position of the pyrimidine ring. The results of this experiment are described as follows.

#### Incubation of VU6010180 in Cynomolgus Monkey Hepatic S9

The mechanism for oxidation of nitrogen-containing heterocycles by the molybdenum-containing hydroxylases AO and XO proceeds via hydride displacement from the carbon (adjacent to a nitrogen) being oxidized (Alfaro and Jones, 2008). Therefore, the hydrogen atom located at the site



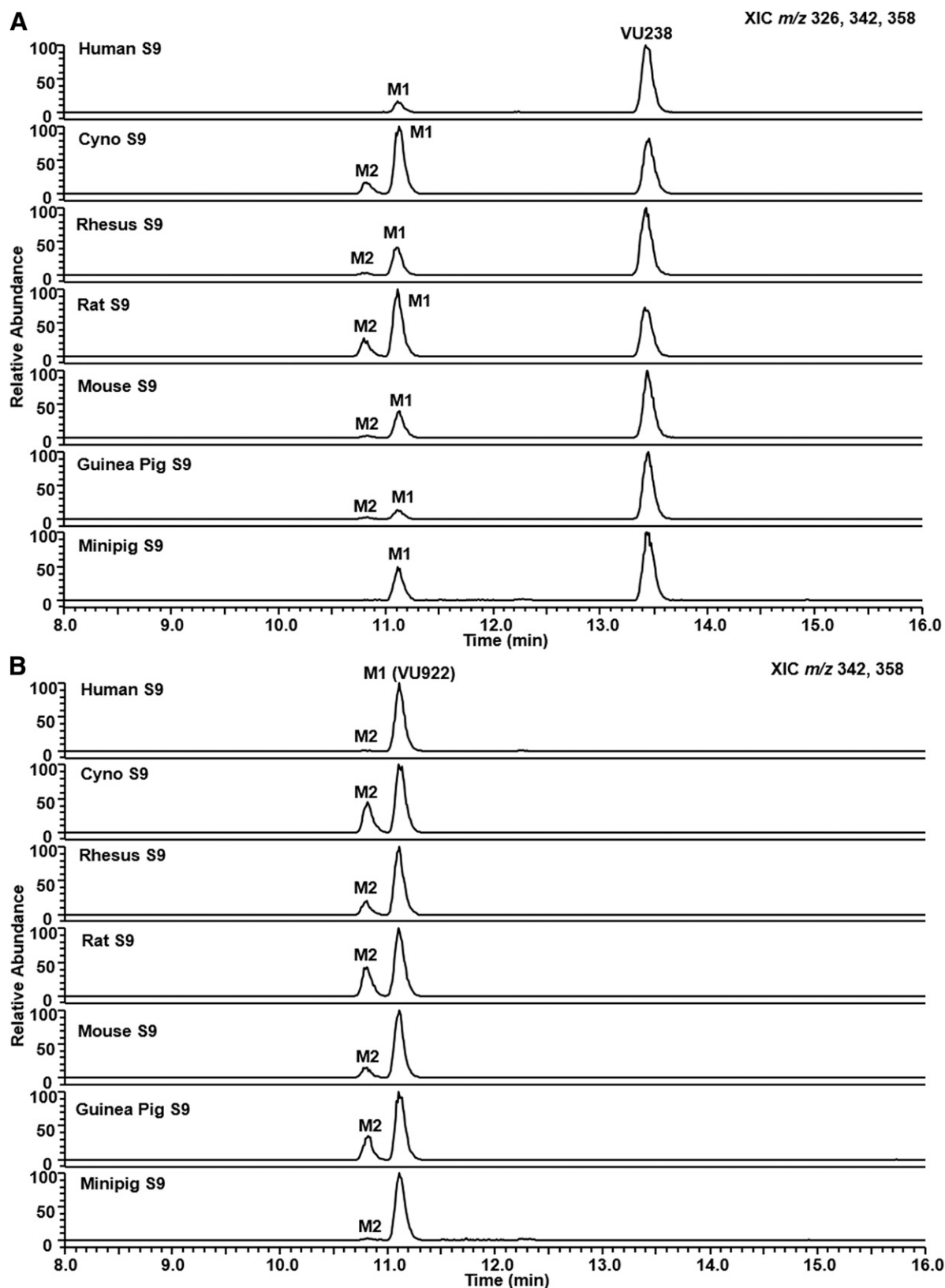
**Fig. 5.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with M1 (VU922, 5  $\mu$ M) in (A) male cynomolgus monkey hepatic S9 (2 mg/ml) and (B) male rat hepatic S9 (2 mg/ml) demonstrating formation of M2 in the presence or absence of the XO inhibitor allopurinol (100  $\mu$ M) or the AO inhibitor hydralazine (50  $\mu$ M).

of oxidation is removed during the course of oxidation. Accordingly, replacement of the hydrogen in the 2-position of the pyrimidine ring of VU238 with a deuterium (VU6010180, VU180) resulted in a kinetic deuterium isotope effect on the formation of M2, where the turnover of M1 to M2 was reduced in S9 incubations with VU180 relative to those with VU238 (Fig. 3A). In addition, while the presence of the deuterium label in VU180 and its respective M1 metabolite was evidenced by production of a protonated molecular ion  $[M + H]^+$  differing from VU238 and its M1 metabolite by +1 Da, respectively (Fig. 3, B and C), the  $[M + H]^+$  produced

by M2 was the same ( $m/z$  358) in extracts from incubations with either VU238 or VU180, indicating the deuterium label was lost during M2 formation (Fig. 3D). Together these data support assignment of the secondary oxidation to the 2-position of the pyrimidine ring.

#### Hepatic S9 Incubation of VU238 and VU922 with AO and XO Inhibitors

The NADPH-independent formation of M1 and M2 in hepatic S9 incubations, in conjunction with oxidation on the pyrimidine ring,



**Fig. 6.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with (A) VU238 (5  $\mu$ M) and (B) M1 (VU922, 5  $\mu$ M) in multiple species' hepatic S9 (2 mg/ml). Human data were obtained using mixed-gender S9. All other data were obtained using male S9.



TABLE 1

Proposed predominant metabolic pathway mediating the formation of M1 or M2 in multispecies' hepatic S9 (2 mg/ml) determined from incubations of VU238 (5  $\mu$ M) or M1 (VU922, 5  $\mu$ M) with the AO inhibitor hydralazine (50  $\mu$ M) or the XO inhibitor allopurinol (100  $\mu$ M)

Designations of AO or XO indicate that formation of the metabolite was inhibited predominantly by either hydralazine or allopurinol, respectively.

	M1	M2
Human <sup>a</sup>	AO	n.d.
Cynomolgus	AO	AO
Rhesus	AO	AO
Rat	AO	XO
Mouse	AO	XO
Guinea pig	AO	AO
Minipig	AO	n.d.
Female cynomolgus	AO	n.d.
Female rat	AO	XO

n.d., not determined.

<sup>a</sup>Human data obtained using mixed-gender S9. All other data were obtained using male S9 unless otherwise indicated.

indicated the likely involvement of AO or XO in the formation of these metabolites. To determine whether AO or XO contributes to the formation of M1 and M2, VU238 was incubated in male cynomolgus monkey hepatic S9 in the presence or absence of the AO inhibitor hydralazine or the XO inhibitor allopurinol. Incubation with allopurinol resulted in little to no inhibition of M1 or M2 formation, whereas incubation with hydralazine inhibited formation of both M1 and M2 in cynomolgus monkey S9 (Fig. 4A). Hydralazine also inhibited formation of M1 and M2 in male rat hepatic S9 (Fig. 4B); however, whereas allopurinol had no effect on M1 formation in rat S9, formation of M2 was attenuated (Fig. 4B). These data suggest that XO plays a major role in the conversion of M1 to M2 in rat, but not in cynomolgus monkeys. Furthermore, incubation of synthetically prepared M1 (VU922) with allopurinol or hydralazine in cynomolgus monkey hepatic S9 revealed that hydralazine inhibited the formation of M2, whereas allopurinol had little effect (Fig. 5A). Conversely, in rat S9, allopurinol attenuated formation of M2, while hydralazine had little effect (Fig. 5B).

### Multispecies Metabolism of VU238 and VU922 in Hepatic S9

The apparent species differences in the metabolism of M1 to M2 between rats and cynomolgus monkeys prompted a multispecies evaluation to determine the potential role of AO and XO in VU238 metabolism by several species commonly used in drug metabolism and pharmacokinetics studies. In addition to male rats and cynomolgus monkeys, VU238 was incubated with hepatic S9 of humans (mixed gender), male rhesus monkeys, mice, guinea pigs, and minipigs. The relative abundance of M1 and M2 differed across species, with the greatest turnover of VU238 to M1 occurring in rats and cynomolgus monkeys. M1 and M2 were detected in S9 extracts of all species except humans and minipigs, which did not contain M2 at detectable levels (Fig. 6A); however, trace levels of M2 were detected in human and minipig S9 extracts from direct incubations with M1 (VU922) (Fig. 6B), indicating that these species can produce this metabolite. In general, although species exhibiting relatively substantial turnover of VU238 to M1 also exhibited relatively substantial turnover of VU922 to M2, minipigs exhibited considerable turnover of VU238 to M1 but very low turnover of VU922 to M2.

Multispecies S9 incubations were also conducted with allopurinol and hydralazine. These data are summarized in Table 1, where designations of AO or XO indicate that formation of the metabolite was inhibited predominantly by hydralazine or allopurinol, respectively. Conversion of VU238 to M1 was inhibited by hydralazine in all species, whereas the conversion of M1 to M2 was inhibited by hydralazine in monkeys and guinea pigs but not in rats and mice, in which cases, allopurinol inhibited

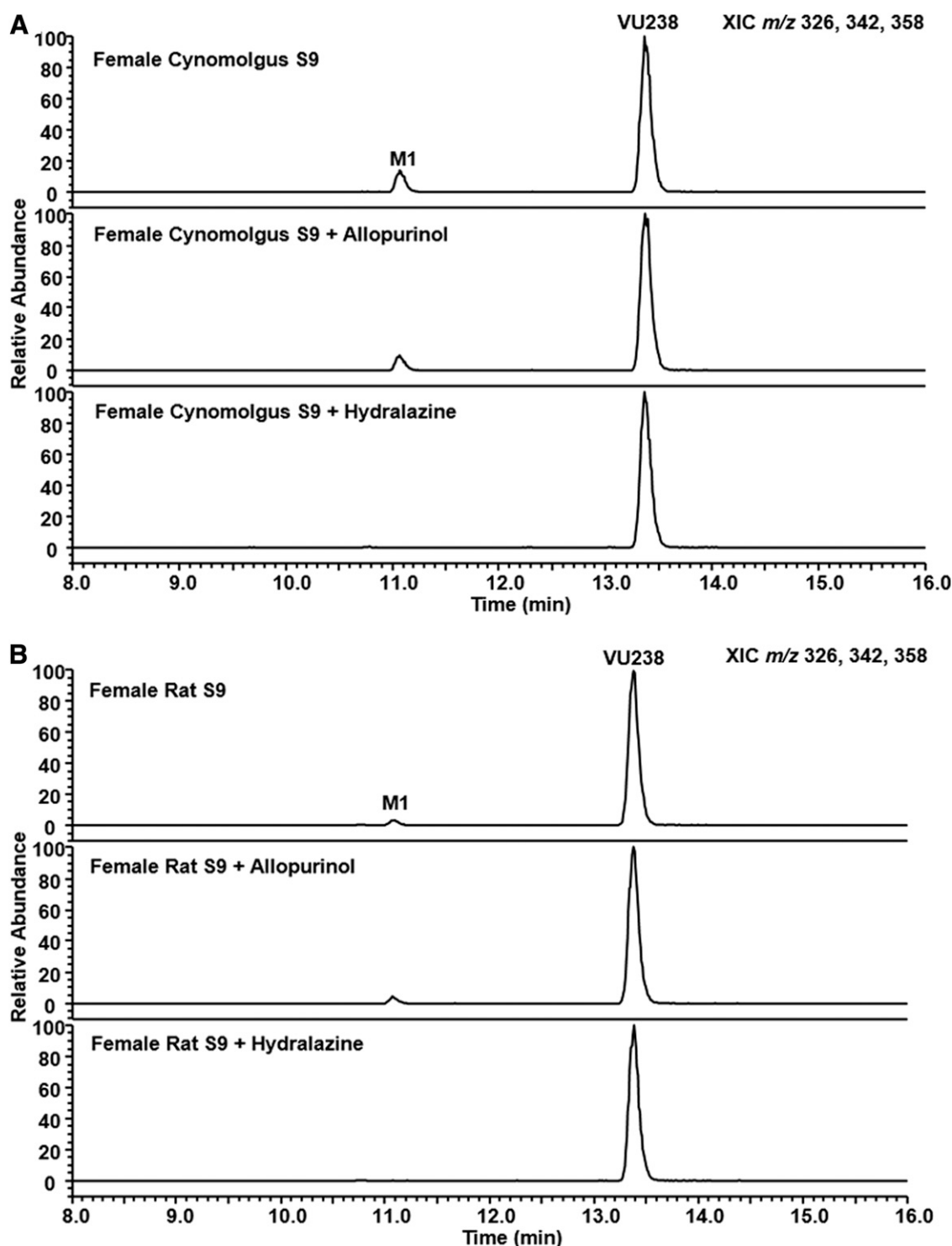
conversion of M1 to M2 (Table 1). Extracted ion chromatograms for these experiments are illustrated in Supplemental Figs. 2 and 3 along with peak area percentages in Supplemental Table 1. The predominant metabolic pathway mediating conversion of M1 to M2 in humans and minipigs could not be determined from the present experiments. Specifically, extracts of S9 incubations with hydralazine contained a trace analyte possessing a protonated molecular ion  $[M + H]^+$  at  $m/z$  357 and a retention time similar to that of M2 (10.8 minutes), which interfered with quantitation of M2 ( $m/z$  358) in hydralazine-containing extracts. As a result of the low turnover of M1 to M2 in human and minipig S9, M2 could not be distinguished from the interfering analyte in extracts of incubations with hydralazine (Supplemental Fig. 3, A and E). Although the identity of this analyte is unknown, it was also present in extracts of S9 incubations containing only hydralazine (no substrate), which suggests that it is unrelated to VU238 or its metabolites. Although a conclusion regarding the enzymatic pathway mediating conversion of M1 to M2 in humans and minipigs cannot be reliably obtained from the current data, inhibition of M2 formation was not observed in the presence of allopurinol, indicating that XO may not contribute substantially.

Barr et al. (2014) previously reported that human liver fractions may lack XO activity because of the inclusion of allopurinol in liver perfusion solutions used during the process of harvesting human livers (Barr et al., 2014). To confirm XO activity in hepatic S9 fractions of humans, as well as the other species examined herein, the exemplary XO substrate 6-thioxanthine (6-TX) was incubated with hepatic S9 in the presence and absence of allopurinol or hydralazine, and the formation of 6-thiouric acid (6-TUA) was monitored. Formation of 6-TUA was observed in S9 of all species and likewise was inhibited by allopurinol in S9 of all species (Supplemental Fig. 5; Supplemental Table 2), confirming the presence of XO activity. Whereas humans and minipigs demonstrated low turnover, all other species exhibited extensive metabolism of 6-TX to 6-TUA. Formation of 6-TUA in S9 of monkeys and guinea pigs was mildly inhibited by hydralazine, indicating the possibility that AO may contribute to metabolism of 6-TX in these species.

Sex differences in AO activity have been reported in some species (Beedham, 1985; Klecker et al., 2006; Akabane et al., 2011; Dalvie et al., 2013), including rats and cynomolgus monkeys, whereas reports have indicated that human AO exhibits no sex differences in activity (Al-Salmi, 2001; Klecker et al., 2006; Dalvie et al., 2013; Hutzler et al., 2014b). To explore potential sex differences in the rat and cynomolgus monkey, we conducted incubations of VU238 and VU922 in the presence and absence of inhibitors in female rat and cynomolgus monkey S9. Turnover of both VU238 to M1 (Fig. 7) and VU922 to M2 (Fig. 8) was low in both female cynomolgus and rat S9 in comparison to incubations with male S9. Accordingly, M2 was not detected in female cynomolgus and rat S9 extracts from incubations with VU238, but only those with direct incubation of VU922; however, inhibition patterns between male and female cynomolgus and rat S9 experiments were similar, where conversion of VU238 to M1 was inhibited by hydralazine in both species/sexes. Likewise, conversion of M1 to M2 was inhibited by allopurinol in incubations of M1 (VU922) with both female and male rat S9; however, low turnover and the presence of the previously described interfering analyte in hydralazine-containing extracts, which eluted at the same retention time as M2, prevented a conclusive result concerning the inhibitory activities of allopurinol versus hydralazine toward formation of M2 in female cynomolgus monkey S9 incubations.

### Multispecies Intrinsic Clearance ( $CL_{in}$ ) of VU238 and VU922 in Hepatic S9

We also evaluated the in vitro clearance of VU238 and VU922 in hepatic S9 of multiple species (male S9, with the exception of human S9,



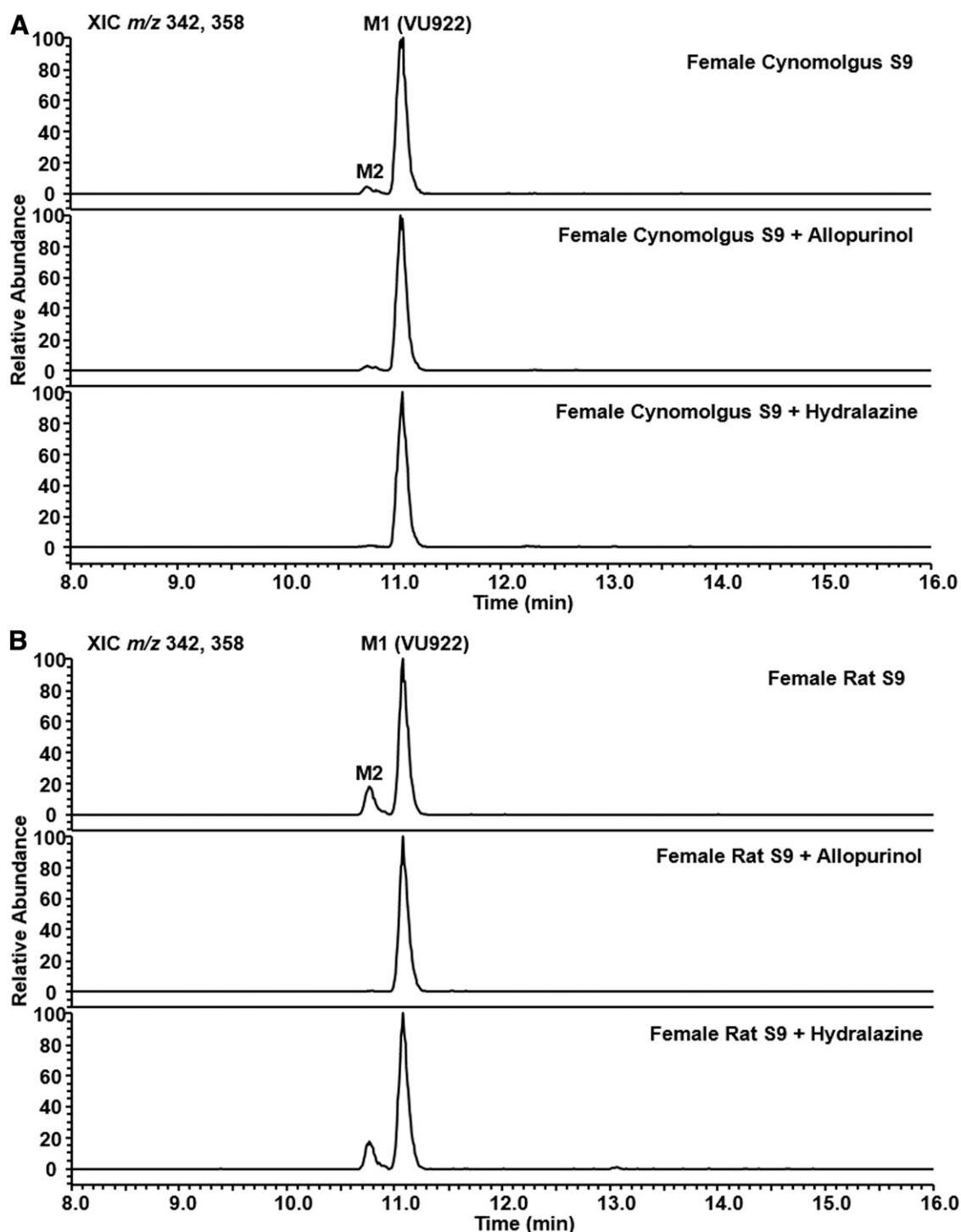
**Fig. 7.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with VU238 (5  $\mu$ M) in (A) female cynomolgus monkey hepatic S9 (2 mg/ml) and (B) female rat hepatic S9 (2 mg/ml) demonstrating the formation of M1 and M2 in the presence or absence of the XO inhibitor allopurinol (100  $\mu$ M) or the AO inhibitor hydralazine (50  $\mu$ M).

which were mixed gender). Experiments were conducted in the absence of NADPH; therefore, the values are expected to represent predominantly clearance resulting from hepatic AO/XO-mediated oxidation (although it is possible that a small contribution resulted from amide hydrolysis). These data are summarized in Table 2.  $CL_{int}$  of VU238 was highest in cynomolgus monkeys and rats, lowest in humans and guinea pigs, with rhesus monkeys, mice, and minipigs falling in the middle range.  $CL_{int}$  of VU922 was low in all species, with turnover rates so low

in human, mouse, and minipig S9 that  $CL_{int}$  could not be determined in these species. Overall, these data were consistent with the relative metabolite formation we observed across species in metabolism experiments with hepatic S9.

#### Multispecies Metabolism of BIBX1382 in Hepatic S9

BIBX1382 is another pyrimidine-containing compound reported to be oxidized at the 2- position of one of its two pyrimidine rings by



**Fig. 8.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with M1 (VU922, 5  $\mu$ M) in (A) female cynomolgus monkey hepatic S9 (2 mg/ml) and (B) female rat hepatic S9 (2 mg/ml) demonstrating formation of M2 in the presence or absence of the XO inhibitor allopurinol (100  $\mu$ M) or the AO inhibitor hydralazine (50  $\mu$ M).

human and monkey AO (Hutzler et al., 2012; Hutzler et al., 2014a). In addition to this primary oxidation, a secondary NADPH-independent oxidation has been reported (Hutzler et al., 2014a). This secondary oxidation likely occurs on BIBX1382s pyrimido-pyrimidine core but has not been confirmed. BIBX1382 is also reportedly cleared much more rapidly in humans and monkeys relative to rats and mice (Dittrich et al., 2002; Hutzler et al., 2014a). These differences were noted in the *in vitro*  $CL_{int}$  of BIBX1382 as well (Hutzler et al., 2014a; Crouch et al., 2017).

To determine whether the NADPH-independent metabolism of BIBX1382 is also inhibited by hydralazine and allopurinol in a species-specific manner (as observed with the metabolism of VU238/VU922), BIBX1382 was incubated in hepatic S9 of multiple species in the presence and absence of inhibitors. Extracted ion chromatograms for these experiments are depicted in Fig. 9, A and B for cynomolgus monkeys and rats and in Supplemental Fig. 4 for all other species. These data are summarized in Fig. 9C.

TABLE 2

Intrinsic clearance ( $CL_{int}$ , microliters per minute per milligrams protein) obtained from incubations of VU238 (1  $\mu$ M) or M1 (VU922, 1  $\mu$ M) with hepatic S9 (2.5 mg/ml) of multiple species

Data represent means  $\pm$  S.D. of triplicate determinations.

Species	VU238	VU922 (M1)
Human	1.48 $\pm$ 0.312	n.d.
Cynomolgus	12.1 $\pm$ 0.643	2.00 $\pm$ 0.040
Rhesus	5.15 $\pm$ 0.537	1.44 $\pm$ 0.139
Rat	13.7 $\pm$ 0.265	2.19 $\pm$ 0.201
Mouse	4.76 $\pm$ 0.386	n.d.
Guinea pig	1.89 $\pm$ 0.180	3.40 $\pm$ 0.069
Minipig	3.51 $\pm$ 0.306	n.d.

n.d., not determined because of low substrate turnover.

In accordance with the species differences in clearance rates previously reported for BIBX1382, we observed species differences in the turnover of BIBX1382 to M1b (primary oxidative metabolite), as well as in the turnover of M1b to M2b (secondary oxidative metabolite). BIBX1382 was extensively converted to M1b in all species except the rat and mouse, which demonstrated poor turnover to M1b (Fig. 9; Supplemental Fig. 4). Unlike our observations of species differences in the inhibitory activities of hydralazine and allopurinol on the conversion of VU922 to M2, we observed no species differences in the inhibition of BIBX1382 conversion to M1b. Formation of BIBX1382 metabolites M1b and M2b was inhibited by hydralazine in incubations of BIBX1382 with S9 of all species, whereas minimal inhibition resulted from the presence of allopurinol (Fig. 9; Supplemental Fig. 4; Supplemental Table 1). Inhibitory activity on the formation of M2b was not evaluated via direct incubation of M1b; thus, the contributions of AO and XO toward the formation of this secondary metabolite were not determined. The minimal impact of allopurinol on M2b levels in incubations with BIBX1382 suggests, however, that XO may only play a small role (if any) in the formation of M2b in any of the species evaluated.

## Discussion

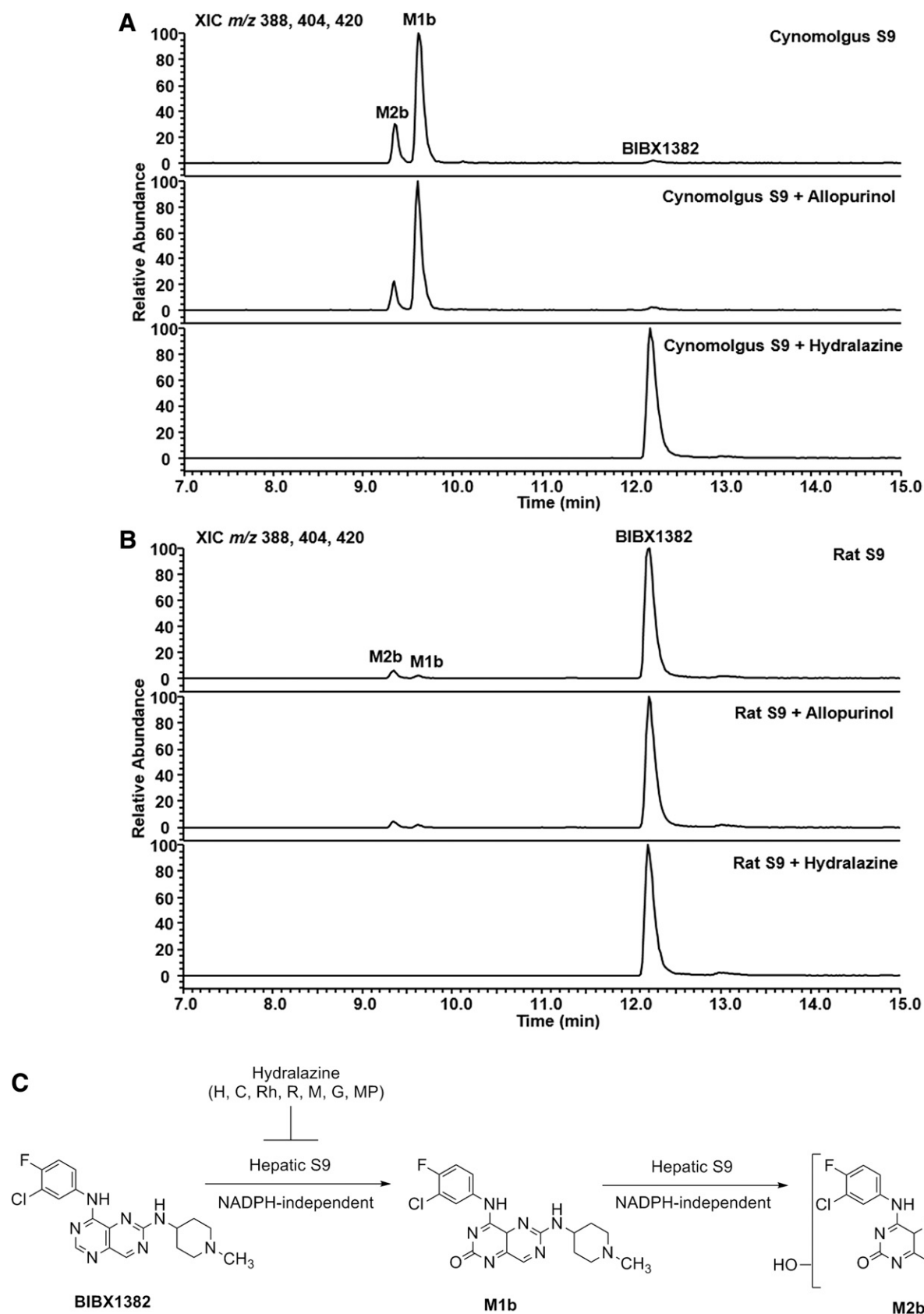
VU238 was under development by our group as a candidate drug to treat various neurologic disorders, such as Alzheimer disease, pain, fragile X syndrome, levodopa-induced dyskinesia in Parkinson disease, depression, addiction, and anxiety, by specifically targeting the mGlu<sub>5</sub> receptor via an allosteric site (Felts et al., 2017). Brain-penetrant with good oral bioavailability and low clearance in rats and monkeys ( $F$  = 0.53 and 0.44, respectively;  $CL_p$  = 19.3 and 15.5 ml/min per kilogram, respectively), VU238 was found to potently and selectively inhibit mGlu<sub>5</sub> in vitro (human mGlu<sub>5</sub> IC<sub>50</sub> = 14 nM) and demonstrate efficacy in rodent models of anxiety and depression (Felts et al., 2017). Unfortunately, advancement of VU238 into first-in-human studies was halted after a 28-day toxicologic assessment indicated species-specific toxicities in cynomolgus monkeys, which were not observed in rats. Although mechanisms responsible for these toxicities are currently under investigation, they are suspected to be metabolism-related. Consequently, using the AO and XO inhibitors hydralazine and allopurinol, we uncovered an apparent species difference between rats and monkeys in the enzymes involved in the formation of the 2,6-dioxypyrimidine metabolite (M2) of VU238. Given these observations, it is possible that species differences in the involvement of AO and XO in VU238 metabolism could play a role in the observed monkey-specific toxicity.

Experiments with allopurinol and hydralazine indicated little role (if any) for AO in the formation of M2 in rat and mouse, which could be attributed to species differences in hepatic AO expression. Humans,

monkeys, and guinea pigs, for example, express only a single liver isoform, aldehyde oxidase 1 (AOX1), whereas mice and rats express two: AOX1 and AOX3 (Terao et al., 2016). Furthermore, the AOX3 isoform is present in higher quantities than AOX1 in mouse hepatic tissue, and computational and site-directed mutagenesis studies suggest differences in the substrate specificities of these two isoforms (Coelho et al., 2012; Mahro et al., 2013; Cerqueira et al., 2015; Terao et al., 2016). Recently, a direct comparison of mouse AOX1 and AOX3 revealed substantial differences in the kinetic parameters of six substrates between the two isoforms (Kucukgoze et al., 2017). Accordingly, because our studies indicate that M1 is converted to M2 predominantly by XO rather than AO in rats and mice, it is possible that M1 (VU922) is a poor substrate for AOX3 relative to AOX1; however, because we also observed low turnover of M1 to M2 in human S9 (Supplemental Fig. 3A), structural differences in AOX1 across species, among other factors, may contribute to the observed species differences.

Although the rat has been generally labeled as a species with low AO activity, our data reiterate observations in the literature indicating that relative AO activity in rats versus other species is substrate-dependent (Beedham et al., 1987; Kawashima et al., 1999; Kitamura et al., 1999; Itoh et al., 2006; Dalvie et al., 2013; Crouch et al., 2017). AO-mediated oxidation of zaleplon, for example, is reportedly higher in cynomolgus monkeys versus Sprague-Dawley rats (Kawashima et al., 1999), whereas the converse has been reported for zonisamide (Dalvie et al., 2013). Presently, we found that the  $CL_{int}$  of VU238 and its metabolism to M1 in hepatic S9 was similar between rats and cynomolgus monkeys, whereas the  $CL_{int}$  and metabolism of BIBX1382 to M1b was much lower in rats versus cynomolgus monkeys, consistent with prior reports of in vivo and in vitro BIBX1382 clearance (Dittrich et al., 2002; Hutzler et al., 2014a; Crouch et al., 2017). Despite similar AO-mediated conversion of VU238 to M1 between cynomolgus monkeys and rats, AO appeared to play little role in the secondary oxidation of M1 (VU922) to M2 in rats. Notably, the conversion of BIBX1382 to M1b and of VU922 to M2 both proceed via oxidation at the 2-position of a pyrimidine ring. Itoh et al. (2006) previously reported decreased AO-mediated 2-oxidation of the pyrimidine ring of RS-8359 in rats relative to monkey. These data all together may indicate that rat AO has low reactivity toward 2-oxidation of a pyrimidine versus monkey. This, of course, does not consider other structural components outside the pyrimidine ring, which may influence metabolism at the 2-position. Rashidi et al. (1997) previously demonstrated that both AO and XO contribute to 6-oxidation of the guanine derivative 6-deoxypenciclovir in rat liver fractions, whereas the oxidation was found to be exclusively mediated by AO in humans, guinea pigs, and rabbits. The contribution of XO, however, was evident primarily in rats reported to be "AO deficient." Alternatively, 6-oxidation of famciclovir (diacetyl ester of 6-deoxypenciclovir) was mediated exclusively by AO in all species (Rashidi et al., 1997). Investigations into factors influencing substrate specificity/reactivity of AO have been conducted (Vila et al., 2004; Fukiya et al., 2010; Pryde et al., 2010; Coelho et al., 2012; Dalvie et al., 2012; Jones and Korzekwa, 2013; Cerqueira et al., 2015; Choughule et al., 2015; Coelho et al., 2015; Terao et al., 2016; Lepri et al., 2017; Xu et al., 2017), although most of these studies focus primarily on human or mouse AO. Furthermore, after evaluation of 198 aza-aromatic compounds for susceptibility toward metabolism by human AO, Lepri et al. (2017) concluded that AO substrate prediction is not a simple task.

Although XO appears to metabolize VU922 to M2 in rats, we observed essentially complete inhibition of M2 formation by hydralazine in cynomolgus monkey S9 and minimal attenuation by allopurinol, suggesting that XO plays a very minor role, if any, in cynomolgus monkeys. Sharma et al. (2011) previously reported a species difference in the oxidation of a quinoxaline derivative by plasma xanthine oxidase,



**Fig. 9.** Extracted ion chromatograms (XICs) obtained from extracts of incubations with BIBX1382 (5  $\mu$ M) in (A) male cynomolgus monkey hepatic S9 (2 mg/ml) and (B) male rat hepatic S9 (2 mg/ml) demonstrating formation of M1b and M2b in the presence or absence of the XO inhibitor allopurinol (100  $\mu$ M) or the AO inhibitor hydralazine (50  $\mu$ M). (C) Summary of BIBX1382 hepatic S9 metabolism in humans, monkeys, rats, mice, guinea pigs, and minipigs. Formation of M1b and M2b in incubations of BIBX1382 with hepatic S9 was inhibited by hydralazine in all species. C, cynomolgus monkey; G, guinea pig; H, human; M, mouse; MP, minipig; R, rat; Rh, rhesus monkey.

where the compound was extensively metabolized in rodent (rat, mouse, and guinea pig) plasma, but it was stable in plasma of dogs, monkeys, and humans (Sharma et al., 2011). Although our studies were conducted in hepatic S9, thus representing hepatic XO activity, a similar result was obtained in which the formation of M2 was inhibited by allopurinol in rat (and mouse) S9 but was minimally affected by the XO inhibitor in monkey S9. Interestingly, experiments with guinea pig S9 indicated that conversion of M1 to M2 proceeds via an AO-mediated pathway similar to that in monkeys, with hydralazine mediating nearly complete inhibition. This observation is not altogether surprising, given the reported expression of only AOX1 (absence of AOX3) in both guinea pigs and monkeys (Garattini and Terao, 2012), although we might have anticipated more involvement from XO in guinea pig based on the observations reported by Sharma et al. Notably, M2 was not detected in extracts obtained from *in vitro* incubations of VU922 (20  $\mu$ M substrate, incubated for 90 minutes at 37 °C) in rat or cynomolgus monkey plasma (data not shown), suggesting that VU922 is not metabolized by plasma XO in either species. 6-TX was metabolized to 6-TUA (inhibited by allopurinol) in S9 of all species, demonstrating that XO activity was present; however, human and minipig S9 fractions exhibited low turnover of 6-TX relative to the other species. Although the use of allopurinol during the harvesting of human livers may explain this observation in human S9 (Barr et al., 2014), vendors that supplied S9 fractions used in these studies reported that animal livers are harvested without the use of allopurinol. It is unclear whether low XO activity contributed to the low turnover of VU922 to M2 in human and minipig S9.

Along with species differences in AO activity, substrate-specific sex differences have also been observed in certain species (Beedham, 1985; Klecker et al., 2006; Akabane et al., 2011; Dalvie et al., 2013). In addition to evaluating VU238/VU922 metabolism in male hepatic S9 of multiple species, we also evaluated metabolism in female rat and cynomolgus monkey S9. Whereas turnover in female S9 was low compared with male S9 in both species, the involvement of AO and XO appeared to be the same regardless of sex (the enzymatic pathway of VU922 to M2 could not be confirmed from the present data in female cynomolgus monkeys, although it appears that the reaction was minimally affected by allopurinol). Variability in AO activity has been observed *in vitro*, particularly in human *in vitro* preparations (Fu et al., 2013; Hutzler et al., 2014b), and differences have been noted in AO activity when comparing cytosol and S9 obtained from different vendors (Zientek and Youdim, 2015). Consequently, male and female S9 fractions used in our experiments were obtained from different vendors, which could have influenced the activity displayed in these studies. Although the AO-mediated turnover of VU238 to M1 was much lower in female rat S9 versus male (female M1:VU238 ratio = 0.03; male M1:VU238 ratio = 1.1), the difference in XO-mediated turnover of VU922 to M2 between male and female rat S9 incubations was not nearly as dramatic (female M2:VU922 ratio = 0.2; male M2:VU922 ratio = 0.4). These data are consistent with reports of sex-dependent xanthine oxidase activity in Sprague-Dawley rats indicating that males exhibit greater activity than females (Levinson and Chalker, 1980; Decker and Levinson, 1982). As prior reports indicate sex differences in AO activity are likely substrate-, species-, and even strain-dependent (Beedham, 1985; Klecker et al., 2006; Akabane et al., 2011; Dalvie et al., 2013), sex may be an important consideration when selecting species for toxicologic evaluations of AO/XO substrates.

Our observations highlight an additional complication in the use of animal models for understanding human metabolism and disposition of AO substrates. Although two species may exhibit similar AO activity toward the parent compound, profound species differences may still exist in secondary AO-mediated metabolism of a primary metabolite. Consequently, after identification of an AO- (or XO-) mediated pathway

in one species, caution should be taken not to assign the same pathway to other species without confirmation, as misidentification of the responsible enzyme could result in unexpected pharmacokinetic drug-drug interactions. Furthermore, potential differences in extrahepatic expression of AO (Terao et al., 2016) versus XO (Pritsos, 2000; Agarwal et al., 2011) may result in unanticipated metabolite exposures *in vivo* if the correct enzyme has not been identified. As metabolites generated by AO have been associated with toxicities related to poor solubility (Smeland et al., 1996; Infante et al., 2013; Lolkema et al., 2015), unanticipated metabolite exposures could also lead to unanticipated toxicity *in vivo*.

In conclusion, we have demonstrated a species difference in the secondary metabolism of VU238, in which the primary oxidative pathway was determined to be mediated by AO in humans, cynomolgus and rhesus monkeys, rats, mice, guinea pigs, and minipigs, and the subsequent oxidation was predominantly mediated by XO in rats and mice and by AO in monkeys and guinea pigs. These data will be important to consider when AO- or XO-mediated metabolism is suspected to avoid incorrect assignment of a metabolism pathway because of differences across species. As literature reports continue to indicate that species differences in AO activity are substrate-dependent, selection of preclinical species for prediction of human PK and *in vivo* toxicology should be customized to each newly identified AO substrate (Crouch et al., 2017). Continued investigation into species differences surrounding AO metabolism (e.g., substrate specificity, expression, and regulation, etc.) will be useful in guiding substrate-specific experiments to determine the most suitable species.

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#### Authorship Contributions

*Participated in research design:* Crouch, Blobaum, Felts, Lindsley.

*Conducted experiments:* Crouch.

*Contributed new reagents or analytic tools:* Felts.

*Performed data analysis:* Crouch, Felts.

*Wrote or contributed to the writing of the manuscript:* Crouch, Blobaum, Felts, Conn, Lindsley.

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