Evaluating the Disposition of a Mixed Aldehyde Oxidase/Cytochrome P450 Substrate in Rats with Attenuated P450 Activity

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Running Title: consequences of P450 inhibition on mixed AO/P450 substrate

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Abbreviations: ABT, 1-aminobenzotriazole; AO, aldehyde oxidase; AUC, area under the time-concentration curve; C_max, maximum plasma concentration; Cl_int, intrinsic clearance; CL_p, plasma clearance; DDI, drug-drug interaction; DMPK, drug metabolism and pharmacokinetics; FDA, Food and Drug Administration; HPLC, high performance liquid chromatography; IP, intraperitoneal; IV, intravenous; K_m, Michaelis constant, LC/MS, liquid chromatography mass spectrometry; LC/MS/MS, liquid chromatography tandem mass spectrometry; NCE, new chemical entity; P450, cytochrome P450; PK, pharmacokinetics; SD, Sprague Dawley; T_max, time to reach maximal plasma concentration; V_max, maximal reaction velocity; XO, xanthine oxidase
Abstract

Marketed drugs cleared by aldehyde oxidase (AO) are few, with no known clinically relevant pharmacokinetic drug interactions associated with AO inhibition, while cytochrome P450 inhibition or induction mediates a number of clinical drug interactions. Little attention has been given to consequences of co-administering a P450 inhibitor with a compound metabolized by both AO and P450. Upon discovering that VU0409106 (1) was metabolized by AO (to M1) and P450 enzymes (to M4-M6), we sought to evaluate the in vivo disposition of 1 and its metabolites in rats with attenuated P450 activity. Male rats were orally pretreated with the pan-P450 inactivator, 1-aminobenzotriazole (ABT), prior to an intraperitoneal dose of 1. Interestingly, the plasma AUC of M1 was increased 15-fold in ABT-treated rats, indicating a metabolic shunt towards AO resulted from the drug interaction condition. The AUC of 1 also increased 7.8-fold. Accordingly, plasma clearance of 1 decreased from 53.5 to 15.3 mL/min/kg in ABT-pretreated rats receiving an intravenous dose of 1. Consistent with these data, M1 formation in hepatic S9 increased with NADPH-exclusion to eliminate P450 activity (50% over reactions containing NADPH). These studies reflect possible consequences of a drug interaction between P450 inhibitors and compounds cleared by both AO and P450 enzymes. Notably, increased exposure to an AO metabolite may hold clinical relevance for active metabolites or those mediating toxicity at elevated concentrations. The recent rise in clinical drug candidates metabolized by AO underscores the importance of these findings and the need for clinical studies to fully understand these risks.
Introduction

The 2012 US Food and Drug Administration (FDA) draft guidance on defining the drug interaction potential of new chemical entities (NCEs) in drug discovery and development (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf) focuses primarily on human in vitro and nonclinical in vivo approaches to model clinical drug-drug interactions (DDIs) involving cytochrome P450s and drug efflux proteins (Prueksaritanont et al., 2006; Di et al., 2013; Prueksaritanont et al., 2013). However, sparse attention has been paid to the potential for drug interactions involving compounds metabolized by enzymes falling outside these two classes of drug disposition proteins. One such enzyme receiving recent attention is the molybdenum-containing flavoprotein, aldehyde oxidase (AO). In particular, significant strides have been made towards understanding the structure-activity-relationships of AO binding and metabolism (Beedham et al., 1995; Dalvie et al., 2012; Coelho et al., 2015), species differences (Beedham et al., 1987; Garattini and Terao, 2012; Dalvie et al., 2013), human AO variability (Hartmann et al., 2012; Hutzler et al., 2014), and inhibition of AO in vitro (Obach et al., 2004; Barr and Jones, 2011), while studies defining the importance of this enzyme in an in vivo drug interaction scenario are lacking, perhaps owing to a deficiency of well-established specific AO inhibitors considered suitable for in vivo pharmacokinetic studies.

To date, limited pharmacokinetic drug interactions involving the few marketed [known] AO substrates have been recognized, despite identification of several clinical drugs demonstrating AO inhibitory activity in vitro (Obach et al., 2004). Rather, the few reported clinical interactions for AO-cleared drugs involve inhibition or induction of a secondary non-AO metabolism pathway (Ramanathan et al., 2016). For example, FDA labeling for zaleplon recommends a dose adjustment when co-administered with cimetidine, which inhibits not only AO, but also P450
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3A4, the secondary route of zaleplon metabolism (http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/020859s011lbl.pdf). Additionally, changes in exposure to the chemotherapeutic idealisib were noted with co-administration of the P450 3A inhibitor ketoconazole, or the inducer rifampin (Ramanathan et al., 2016). A noteworthy report recently indicated an important role for AO in a drug interaction between BILR 355 and the P450 3A inhibitor ritonavir, where co-administration resulted in a metabolic “switch” from P450 3A metabolism of BILR 355 to gut bacterial and subsequent AO metabolism (Li et al., 2012a; Li et al., 2012b). Furthermore, Li’s studies monitoring formation of this AO metabolite in human S9 in the presence and absence of NADPH were consistent with a metabolic shunt towards AO when the P450 pathway was inactive. This report brought attention to the possibility of a drug interaction leading to a metabolic switch and the potential for AO to contribute to such an event.

We previously reported the disposition of a novel mGlu5-negative allosteric modulator, VU0409106 (1), which was discovered to be predominantly metabolized by AO (Scheme 1), to the primary metabolite, M1 (Morrison et al., 2012). Notably, we demonstrated that the in vitro scaled hepatic clearance (CL_{HEP}) of 1 was in general agreement with the in vivo plasma clearance (CL_{p}) observed in Sprague-Dawley (SD) rats and cynomolgus monkey. Our continued interest in 1 has been driven largely by the observation that P450 pathways also contribute to its biotransformation, resulting in formation of metabolites M4, M5 and M6. While Li’s report demonstrates a drug interaction that elicits a metabolic switch in a unique scenario requiring an intermediate gut bacterial metabolism step, we hypothesized that hepatic P450 inhibition may result in an elevated exposure to the AO metabolite of a drug cleared via both AO and P450 enzymes. Because AO and P450 commonly generate different metabolites due to opposing
substrate specificities (AO prefers to oxidize electron-deficient carbons whereas P450 prefers electron-rich sites), this creates a potential scenario for increased exposure to one metabolite when the other metabolic pathway is inhibited. The observation that 1 was metabolized exclusively by AO to M1 and to M4-M6 by P450 enzymes presented an opportunity to investigate the disposition of a mixed AO:P450 substrate and the corresponding AO and P450 metabolites in a drug interaction scenario of P450 inhibition. Given the similarities in rodent metabolism and clearance of 1 to that observed in human S9 and hepatocytes (Morrison et al., 2012) we designed this drug interaction scenario in vivo in SD rats, a conventional species historically employed in nonclinical pharmacokinetic investigations (Di et al., 2013), via co-administration of 1 and the pan-P450 inactivator 1-aminobenzotriazole (ABT). Observations from the present in vitro and in vivo investigations indicate evidence of metabolic shunting towards AO metabolism in the disposition of 1 in rat when P450 activity is attenuated, resulting in elevated exposure to the AO metabolite M1.
Materials and Methods

Materials. VU0409106 (1) was prepared and characterized by the Department of Medicinal Chemistry within the Vanderbilt Center for Neuroscience Drug Discovery. Potassium phosphate, ammonium formate, formic acid, magnesium chloride, 1-aminobenzotriazole (ABT), hydralazine and allopurinol were purchased from Sigma-Aldrich (St. Louis, MO). NADPH tetrasodium salt was purchased from VWR (Radnor, PA). Pooled human (150-donor, mixed gender) or male Sprague-Dawley (SD) rat hepatic microsomes and S9 were obtained from BD Biosciences (San Diego, CA). All solvents or reagents were of the highest purity commercially available.

Biotransformation of 1 in multispecies hepatic microsomes and S9 fractions.

Hepatic microsomal metabolism of 1. The in vitro metabolism of 1 was investigated in rat and human hepatic microsomal fractions. A potassium phosphate-buffered reaction (100 mM, pH 7.4) of 1 (10 μM), hepatic microsomes (1 mg/mL), MgCl₂ (3 mM) and NADPH (2 mM) was incubated at 37°C in borosilicate glass test tubes under ambient oxygenation for 60 minutes. The total incubation volume was 0.5 mL. Reactions were initiated by the addition of 1, terminated with the addition of 2 volumes of acetonitrile, and subsequently centrifuged at 3500 rcf for 10 min. The resulting supernatant was dried under a stream of nitrogen and reconstituted in 85:15 (v/v) ammonium formate (10 mM, pH 4.1):acetonitrile in preparation for LC/MS analysis.

M1 formation in multispecies hepatic S9 fractions. Similarly the metabolism of 1 and subsequent formation of M1 in rat and human hepatic S9 fractions (2.5 mg/mL; +/- 1 mM NADPH) was investigated. Total incubation volume was 200 μL. Reactions were initiated with addition of 1 (1 μM), and at designated times (t = 0, 7, 15, 30, 45, and 60 min), aliquots were removed and precipitated with ice-cold acetonitrile containing an internal standard.
(carbamazepine, 50 ng/mL). The mixture was centrifuged at 3500 rcf for 5 min and resulting supernatants diluted with water in preparation for LC/MS/MS analysis.

**Intrinsic clearance in multispecies hepatic S9 fractions.** To explore potential kinetic mechanisms responsible for our observations of M1 formation in S9, we measured the intrinsic clearance (CL\textsubscript{int}) of 1 in rat and human S9 fractions at three different concentrations of 1, using NADPH and hydralazine to isolated the NADPH-dependent (P450), NADPH-independent (AO), and total (P450 + AO) hepatic S9 CL\textsubscript{int} for each species. For measurement of total, NADPH-independent, and NADPH-dependent S9 CL\textsubscript{int}, compound 1 (0.1 μM, 1 μM, or 10 μM) was incubated as described above in rat or human S9 fractions in the presence or absence of NADPH, or in the presence of NADPH after pre-incubation with the AO-specific inhibitor hydralazine (50 μM), respectively. Rat and human S9 CL\textsubscript{int} (mL/min/kg) was estimated using the substrate depletion method and Eq. 1:

\[
CL_{\text{int}} = \frac{\ln 2}{t_{1/2 (min)}} \times \frac{mL}{2.5 \text{ mg protein}_{S9}} \times \frac{120 \text{ mg protein}_{S9}}{g \text{ liver weight}} \times \frac{(A)g \text{ liver weight}}{kg \text{ body weight}}
\]

where \(t_{1/2}\) is the substrate depletion half-life and \(A = 20\) (human) or 45 (rat).

**In vivo metabolism of 1 in SD rats.**

All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. To evaluate the pharmacokinetics of 1, 8-12 week old male SD rats (n = 2) weighing between 250 and 325 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. Similarly, in-life studies in SD rats receiving an intraperitoneal (IP) dose of 3 mg/kg (n = 3 for control group and n = 4 for ABT group) or 10 mg/kg (n = 2) of 1 were conducted at Frontage Laboratories (Exton, PA).
Administration of 1 to Sprague-Dawley Rats. Inhibitors of P450 (ABT) and/or xanthine oxidase (allopurinol) were administered orally to male SD rats at 50 mg/kg at a dose volume of 5 mL/kg (ABT, 1% methylcellulose) or 2.5 mL/kg (allopurinol, 1% methylcellulose). Two hours following inhibitor administration (approximate T_max of both ABT and allopurinol), a dose of 1 (10% ethanol/70% PEG400/20% saline) was administered at 3 or 10 mg/kg intraperitoneally (IP), or intravenously (IV) at 1 mg/kg. Blood (200 uL) was collected via the carotid artery at 0.0833, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hours post administration of compound 1. Samples were collected in chilled, EDTA-fortified tubes and centrifuged for 5 min (1700 rcf, 4°C), and the resulting plasma was stored at - 80°C until LC/MS/MS analysis. The resulting plasma samples were protein precipitated with ice-cold acetonitrile containing internal standard (carbamazepine, 50 ng/mL), centrifuged (3500 rcf for 5 min) and the resulting supernatants diluted with water in preparation for LC/MS/MS analysis.

Liquid chromatography-UV-tandem mass spectrometry analysis of 1 and metabolites.

Exposure analysis of plasma and S9 fractions. The quantitation of 1 and its metabolites (M1, M2, M4-M6) from plasma and S9 fraction incubations was conducted via electrospray ionization on an AB Sciex API-5500 QTrap (Applied Biosystems, Foster City, CA) instrument that was coupled with LC-20AD pumps (Shimadzu, Columbia, MD) or an AB Sciex API-4000 triple quadrupole instrument coupled with Shimadzu LC-10AD pumps and a CTC PAL autosampler (Leap Technologies, Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 column (3 × 50 mm, 3 μm; Fortis Technologies Ltd., Cheshire, UK) warmed to 40°C. Mobile phase A was 0.1% formic acid in water (pH unadjusted); mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 30% B after a 0.2-min hold and was linearly increased to 90% B over 1.5 min, held at 90% B for 0.5 min, and returned to 30% B in
0.1 min followed by a re-equilibration (0.4 min) with a total run time of 2.7 min, or alternatively 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 re-equilibration (0.9 min) with a total run time of 2.5 min. 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 1 or its metabolites using a Turbo Ion Spray source in positive ionization mode (5.0 kV spray voltage). Data were analyzed using Sciex Analyst 1.5.1 software. Concentrations of 1 were determined using a matrix-matched 9-point standard curve (lower limit of quantitation = 0.5 ng/mL). As no authentic standard was available to determine metabolite concentrations, a semi-quantitative analysis was expressed as the analyte peak:internal standard ratio (internal standard, carbamazepine) (Morrison et al., 2012).

Metabolite detection. LC/UV/MS analysis of metabolites generated in vitro was performed with an Agilent 1100 HPLC system coupled to a Supelco Discovery C18 column (5 μm, 2.1 × 150 mm; Sigma-Aldrich, St. Louis, MO). 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), and by linear gradient was transitioned to 20:80 A-B over 20 min for a total run time of 30 min. The flow rate was 0.400 ml/min. The HPLC eluent was first introduced into an Agilent 1100 diode array detector (254 nM) followed by electrospray ionization-assisted introduction into a LCQ Deca XPPLUS ion trap mass spectrometer (Thermoelectron Corp., San Jose, CA) operated in positive 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. Data were analyzed using Thermo XCalibur 2.0 software.

Data analysis.
GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA) was employed in the semi-quantitation of M1 resulting from the S9 fraction metabolism of 1, as well as in the generation of time-concentration profiles of 1 and its metabolites in rat plasma. Subsequent area-under-the-curve (AUC) values of M1 from S9 experiments were also generated in GraphPad Prism (trapezoid rule). Pharmacokinetic (PK) parameters of 1 were obtained using WinNonLin (noncompartmental analysis; Phoenix version 6.2; Pharsight, Mountain View, CA).

Statistical analysis.

GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA) was employed in the statistical analyses of in vivo pharmacokinetic parameters; used a two-tailed unpaired t-test with a significance level of $p < 0.05$. 
Results

A mixed AO:P450 metabolism phenotype of 1 in vitro.

Rat and Human Hepatic Microsomes. We previously reported that the primary biotransformation pathway of compound 1 was catalyzed by AO to the principal metabolite M1, plus other metabolites (e.g., M4/M5) mediated by P450 and detected in nonclinical species and human hepatic S9 fractions (Scheme 1), as well as in vivo in Sprague-Dawley (SD) rats receiving intraperitoneal administrations of 1 (Morrison et al., 2012). Furthermore, we demonstrated the in vitro:in vivo correlation of the predicted hepatic and plasma clearance of 1 in rats and nonhuman primates, a finding which established the relevancy of a principle AO mechanism of clearance in vivo, with a secondary contribution from P450 in the disposition of 1. Presently, we employed liquid chromatographic-mass spectrometry analysis (LC/UV/MS) and hepatic microsomes to define the role of P450 in the metabolism of 1 in rat and human, and to monitor P450 metabolites in the investigation of a metabolic shunt involving AO. Data from hepatic microsomal incubations of 1 indicated the NADPH-dependent formation of the hydroxylated metabolites M5 and M6. The identification and proposed structure elucidation of M6 was facilitated by LC/MS/MS analysis, with a subsequent deuterium-exchange LC/MS experiment indicating the oxidation of a carbon atom of the thiazole moiety (Table 1; Supplemental Figure 1, LC/MS data of M6); the data collected from these experiments were consistent with a P450-mediated oxidation of 1, versus the potential involvement of a flavin monooxygenase (FMO). The retention time and respective MS/MS fragmentation data for M5 was consistent with our previous report detailing the biotransformation of 1 (Morrison et al., 2012) While the thiazole-hydroxylated metabolite, M4, was observed in vivo in rat plasma, as well as in rat and human hepatocytes (attenuated with ABT pretreatment) (Morrison et al., 2012), it was below our
detection limits in rat or human hepatic microsomes in the present study. The appearance of \textbf{M1} in rat and human microsomal incubations was NADPH-independent and indicative of trace contamination of the microsomal fraction with cytosol (containing AO); this finding of background AO activity in contaminated hepatic microsomes is not uncommon (Diamond et al., 2010), and was confirmed by suppression of \textbf{M1} formation with the AO-specific inhibitor hydralazine (data not shown).

**Intrinsic clearance of 1 and relative formation rates of M1 in rat and human S9 fractions implicate a metabolic shunting mechanism mediated by AO.** When compound \textbf{1} was incubated under kinetically controlled conditions (1 \textmu M, 60 min incubation) in both rat and human hepatic S9 fractions (+/- NADPH), the observed magnitude of \textbf{M1} formation in S9 incubations, absent the P450 reducing cofactor NADPH, was greater than that observed when incubations were fortified with NADPH (Figure 1). An approximate 50\% increase in the area under the curve (AUC) of \textbf{M1} was observed in S9 incubations absent NADPH relative to those incubations containing NADPH from both human and rat experiments (Table 2). We previously demonstrated that subsequent metabolism of \textbf{M1} to \textbf{M2} is catalyzed by xanthine oxidase (XO; Scheme 1)(Morrison et al., 2012), an enzyme which does not require the reducing cofactor NADPH for catalytic activity, thus excluding P450-mediated conversion of \textbf{M1} to \textbf{M2} as the mechanism responsible for this observation. The observed increase in \textbf{M1} in S9 incubations absent NADPH may likely be due to an increase in substrate exposure to AO in the absence of NADPH-dependent P450 metabolism. To explore this possibility, we measured the CL_{int} of \textbf{1} in rat and human S9 fractions in the presence and absence of NADPH and in the presence of NADPH and the AO inhibitor, hydralazine, to estimate the CL_{int} mediated by both AO and P450, AO only, and P450 only, respectively (Table 3), and over a concentration range of \textbf{1} (0.1 \textmu M, 1
μM, and 10 μM). While NADPH-dependent CL_int in both species decreased with increasing concentration of 1, NADPH-independent CL_int remained constant in rat, with some decrease in human S9 incubations. The decrease in NADPH-dependent CL_int at higher concentrations was also reflected by a decrease in the total CL_int observed when both P450 and AO are active. These data indicate the likelihood that the overall K_m (Michealis constant) for the P450 pathways is lower than that for the AO pathway and are consistent with a mechanism of metabolic shunting towards AO under conditions of attenuated P450 metabolism and greater substrate availability for AO. While the NADPH-independent CL_int observed in the present experiments was slightly elevated than previously reported (Morrison et al., 2012), this finding is not surprising, given the potential in vitro variability of AO recently described across multiple individual hepatocyte donors, for example (Hutzler et al., 2014); variability in AO-mediated CL_int values for the same AO substrate has also been reported (Kitamura et al., 1999; Al-Salmy, 2001; Sahi et al., 2008).

Altogether, the present in vitro data indicate that SD rat represents an acceptable nonclinical model to study the in vivo disposition of 1 and its metabolites under drug interaction duress (e.g., P450 inhibition), particularly the occurrence of metabolic shunting from P450 towards AO.

ABT pretreatment results in increased exposure to parent 1 and the AO metabolite M1 in vivo in SD rats.

To evaluate the impact of P450 inhibition on the disposition of 1 and its metabolites, SD rats received an IP administration of 1 (3 mg/kg) with or without oral ABT (50 mg/kg) pretreatment. We then generated standard plasma time-concentration profiles (Figure 2) employing contemporary LC/MS/MS quantitation, reporting standard PK parameters (e.g., C_max, AUC_0-inf, CL_p, V_ss, t_{1/2}, Table 4-6). Statistically significant changes were observed in the plasma clearance (CL_p), AUC, and maximal concentration (C_max) of rats pretreated with ABT, versus vehicle...
pretreatment. In rats pretreated with ABT, a 7.8-fold increase in the plasma AUC of 1 was observed (Figure 2A, Table 4). Likewise, the C\textsubscript{max} was increased 3.1-fold. The PK of 1 was also obtained (Table 4), following a parenteral administration of 1 to rats, where an increase in AUC of 1 was again observed (3.5-fold), along with a corresponding reduction in the average CL\textsubscript{p} from 53.5 to 15.3 mL/min/kg in rats pretreated with ABT.

We also observed an increase in the exposure to the AO metabolite M1 in rats receiving the ABT pretreatment, with a 15-fold and 7.3-fold increases in the average AUC and C\textsubscript{max} values, respectively (Figure 2B, Table 5). We submit that this finding is consistent with the contributions of a shunting mechanism towards the AO pathway when P450 activity is attenuated by ABT (as was observed in hepatic S9 incubations of 1 that were absent NADPH). We previously reported that M1 is converted to M2 via XO, followed by an oxidative-defluorination to M3 (Scheme 1) (Morrison et al., 2012). Consequently, a substantial increase in M2 was also observed in rats as a result of ABT pretreatment (11-fold and 14-fold increase in mean C\textsubscript{max} and AUC, respectively) (Figure 2D, Table 5). While the increase in M2 can be explained by an increase in its precursor metabolite, M1, we considered the possibility that accumulation of M2 in ABT-pretreated rats occurred as a result of reduced P450-mediated conversion of M2→M3, with the potential to reduce the rate of M1 conversion to M2 (e.g., product inhibition). In order to investigate contributions of secondary P450-mediated metabolism of M2 to the observed plasma levels of M1, rats were orally administered either ABT (50 mg/kg), the XO inhibitor allopurinol (50 mg/kg), or a combination of the two inhibitors prior to the IP injection of compound 1 (10 mg/kg). Similar to rats receiving the 3 mg/kg dose of 1, rats receiving the 10 mg/kg dose displayed a 14-fold increase in the AUC of M2 when pretreated with ABT (Figure 3A, Table 6). M2 was below the detection limits in rats pretreated with the XO inhibitor allopurinol and was
detectable only at the latter time points collected from rats pretreated with both allopurinol and ABT (data not shown). Pretreatment with allopurinol revealed a relatively small increase of 2.6-fold in the AUC of M1 compared to a 9.1-fold increase following ABT pretreatment (Figure 3B, Table 6). Importantly, when rats were pretreated with both allopurinol and ABT, the AUC of M1 increased 15-fold (Figure 3B, Table 6; see Supplemental Figure 2 for ABT and allopurinol exposure). These data indicate that the accumulation of M1 from inhibition of the M2→M3 pathway is likely a minimal contributing factor towards the increase in M1 exposure in rats experiencing the ABT-induced DDI duress.

The P450-mediated metabolites M4 and M6 were monitored to ascertain the impact of ABT pretreatment in vivo (Figure 2C). Due to the complexity in the chromatographic resolution of M4 and M6, their isobaric mass, as well as their identical MS/MS transitions, LC/MS/MS peak areas of these metabolites were grouped accordingly, for the purpose of determining a semi-quantitative plasma-time concentration profile and exposure analysis as a measure of the contribution of P450 in the metabolism of 1 in vivo. While a decrease was observed in the C_max of M4-M6 (0.64-fold), a 2.1-fold increase was observed in the AUC of these metabolites in the ABT pretreated rats (Table 5). This observation may be due to alterations in the secondary metabolism and clearance mechanisms acting on M4 and/or M6.
Discussion

While successful approaches have been developed to evaluate the impact of enzyme inhibitors and inducers on P450-mediated drug clearance and subsequent changes in drug exposure for prediction of clinical drug interactions (Zhang et al., 2009; Di et al., 2013), approaches towards predicting drug interaction potential of NCEs undergoing non P450 metabolism are less well-established, much less those exhibiting both P450 and AO clearance routes.

Recognition of AO and P450 contributions to the clearance of 1 in vitro and in vivo, provided an opportunity to investigate how P450 inhibition may impact the disposition and PK of a mixed AO:P450 substrate and its metabolites. Similarities between rat and human in vitro metabolism of 1 permitted the use of rat as a nonclinical P450 inhibition model, which revealed a trend towards increased exposure to the AO metabolite M1 in rats with ABT-attenuated P450 activity (Scheme 2). In principle, co-administration of a perpetrator drug could result in observed elevations in metabolite plasma levels as a consequence of several possible mechanisms: a) decreased metabolite clearance due to inhibition of secondary metabolism, b) metabolic activation (stimulation of enzyme activity), c) enzyme induction, or d) metabolic shunting towards the uninhibited pathway (e.g., AO) when another pathway is inhibited (e.g., P450). It is unlikely that the elevated M1 levels were due to a decrease in M1 clearance in rats pretreated with ABT, as the major M1 clearance pathway in vitro was previously determined to be XO-mediated metabolism to M2 (Morrison et al., 2012). And while the activation of AO has been previously suggested (Nirogi et al., 2014), the present increased M1 formation observed in hepatic S9 fractions absent NADPH relative to NADPH-containing reactions indicate there to be no contribution from an ABT-mediated cooperativity on AO. Furthermore, data from our
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laboratory outlining incubations of 1 with hepatocytes, revealed no increase in M1 formation when ABT was present (data not shown). Finally, while the induction of AOX1 in mice, rats, and rabbits (Garattini and Terao, 2012) has been demonstrated, an induction mechanism accounting for our observations is highly unlikely, given the single dose study design and duration thereof. Our observed increase in M1, therefore, appears to have primarily resulted from a condition of increased substrate availability to AO when the P450 pathway(s) of metabolism was attenuated. In additional support of this mechanism, the decrease in NADPH-dependent S9 CL_{int} (P450 pathway), with a maintenance of NADPH-independent S9 CL_{int} (AO pathway) with increased concentrations of 1, indicates a lower K_{m,P450} relative to the K_{m,AO}, according to the relationship:

\[ CL_{int,total} = \left( \frac{V_{max,AO}}{K_{m,AO} + S} \right) + \left( \frac{V_{max,P450}}{K_{m,P450} + S} \right) \]

where \( V_{max} \) is the enzyme’s maximum reaction velocity, \( K_m \) is the substrate concentration that yields half the maximal velocity, and \( S \) is the substrate concentration. Incidentally, our findings associate an increase in metabolite exposure with the co-administration of 1 and an enzyme inhibitor, whereas this type of DDI situation would typically be anticipated with co-administration of a victim drug and an enzyme inducer or stimulator. We might have expected this metabolic shunting observation to prevent an extensive increase in the AUC of 1, with AO compensating for loss of P450 activity. However, as ABT is a pan-P450 inactivator, we have likely forced the shunt towards a single enzyme (AO), potentially limiting the capacity for compensation (versus inhibiting one enzyme with the possibility of shunting towards multiple enzymes).

While the M4-M6 AUC unexpectedly increased in ABT-pretreated rats, the significant increase in AUC and decrease in the total body CL of parent 1 indicates ABT did inhibit P450-metabolism of 1 in vivo. A possible explanation for this observation is that ABT also inhibited
secondary metabolism of M4-M6. Alternatively, as M4 and M6 were quantified together, it is also possible that the AUC of one metabolite was increased, while that of the other was decreased, potentially resulting from differences in inhibitory activity towards enzymes responsible for formation and/or clearance of M4 and M6. Prior studies of ABT inhibitory activity towards human P450 enzymes found that P450 2C9 is only minimally impacted by ABT (60% remaining activity following 30 min pretreatment of human liver microsomes with 1 mM ABT) (Linder et al., 2009); the differential inhibitory activity of ABT towards rat P450 isoforms is unknown. Presently, we found there to be an approximate 65% decrease in M4 compared to a 25% increase in M6 in pooled plasma samples (1-6 hours) from rats pretreated with ABT relative to control rats (Supplemental Figure 3), indicating ABT may have differentially impacted the disposition of the two metabolites.

Besides significant increases in exposure to parent compound 1, mean increases observed in the AUC and C\textsubscript{max} of the AO metabolite M1 highlight the potential for a drug interaction resulting from increased exposure to a metabolite of a drug with both AO and P450 clearance routes when co-administered with a P450 inhibitor. Clinically used drugs exhibiting a major clearance pathway via AO are few, and to date, no clinically relevant DDIs resulting from AO inhibition have been recognized, despite the identification of many clinical drugs demonstrating AO inhibition \textit{in vitro} (Obach et al., 2004). However, our data indicate that inhibition of alternate clearance routes (e.g., P450) for drugs also metabolized by AO may result in elevation of a circulating AO-mediated metabolite, which importantly, could have clinical implications when metabolites exhibit pharmacological or toxicological activity (Smith and Obach, 2005). For example, cases of dose-dependent renal toxicity associated with AO-mediated formation of a low-solubility metabolite have been reported for methotrexate (Smeland et al., 1996) and the two
c-Met inhibitors SGX523 and JNJ-38877605, recently discontinued in clinical trials (Infante et al., 2013; Lolkema et al., 2015). Additionally, the primary circulating metabolite of idealisib, GS-563117, is a mechanism-based inhibitor of P450 3A, which is not the case for the parent drug. In this instance, however, both AO and P450 3A (minor) contribute to the formation of GS-563117 (Ramanathan et al., 2016).

While a small percentage of currently marketed drugs are cleared via AO, a recent study indicated the proportion of NCEs in research and development containing potentially AO-susceptible moieties is much higher (Pryde et al., 2010). Drug discovery scientists are more frequently encountering AO metabolism due to incorporation of nitrogen-containing aromatic rings for either target engagement (e.g. kinases) or mitigation of P450 metabolism (reduced lipophilicity). As many AO substrates (e.g., zaleplon and idealisib) are known to also undergo metabolism via enzymes other than AO (Strelevitz et al., 2012; Ramanathan et al., 2016), it is likely that current and future NCEs exhibiting AO metabolism will also undergo metabolism via alternate enzymes. As such, metabolic shunting may be important to consider during toxicology and DDI assessment of these compounds. Likewise, this consideration may also be important for NCEs not displaying AO metabolism without concomitant administration of an enzyme inhibitor, yet containing an AO-susceptible structural moiety (potential for metabolic switching (Li et al., 2012a; Li et al., 2012b)). The likelihood of substantial elevations in metabolite exposure (via metabolic shunting) may be increased for drugs cleared by both AO and P450 enzymes versus drugs cleared only by multiple P450 pathways, as different P450 enzymes commonly generate the same metabolite, in which case the metabolite would be expected to be generated at decreased or similar levels when one of the P450 pathways is inhibited.
In conclusion, our studies with 1 and ABT using rat as a nonclinical PK model revealed increased exposure to both the parent drug and the AO metabolite. The present investigation highlights the potential drug interactions that may occur with co-administration of a P450 inhibitor and a mixed AO/P450 substrate. The similarities we observed \textit{in vitro} between SD rat and human in the formation of AO and P450 metabolites, trends in the impact of P450 activity on the formation of M1, and in the kinetic behavior of the two enzymatic pathways indicate that our observations \textit{in vivo} in rat may translate to human \textit{in vivo}. We submit that rat may offer a nonclinical model to probe drug interactions (and mechanisms thereof) where a \textit{mixed AO:P450 substrate} experiences a clinical drug interaction duress, while underscoring the potential existence of the \textit{compound-dependent} use of nonclinical models to predict AO-mediated interactions. As investigators are more frequently encountering AO metabolism through the drug discovery and development continuum, understanding the drug interaction potential for drugs exhibiting an AO clearance component will be important in the successful advancement and safe clinical implementation of future drug candidates bearing this nonP450 phenotype.
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Authorship Contributions.

Participated in research design: Crouch, Morrison, Daniels.

Conducted experiments: Byers, Crouch, Morrison

Contributed new reagents or analytical tools: Lindsley, Emmitt.

Performed data analysis: Crouch, Morrison, Daniels.

Wrote or contributed to the writing of the manuscript. Emmitt, Lindsley, Crouch, Daniels.
References.


Footnotes.

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Scheme Legends.

**Scheme 1.** Metabolism of VU0409106 (1) *in vitro* in SD rats and human.

**Scheme 2.** Increase in M1 formation observed in rats following ABT inhibition of P450.
Figure Legends.

**Figure 1.** Formation of M1 in incubations of VU0409106 (1) with human hepatic S9 (A) or SD rat hepatic S9 (B) in the presence (closed circles) or absence (open circles) of NADPH. Data points are expressed as the peak area ratio of analyte/internal standard and represent the mean of a triplicate determination (± SD).

**Figure 2.** Mean plasma concentration-time profiles of VU0409106 (1) (A), M1 (B), M4-M6 (C), and M2 (D) following administration of 1 to control (closed symbol) or ABT pretreated (open symbol) SD rats. Each data point represents the mean (± SD; n = 2-3 (control), n = 3-4 ABT pretreated).

**Figure 3.** Mean plasma concentration-time profiles of M2 (A) or M1 (B) after intraperitoneal administration of VU0409106 (1) (10 mg/kg) to rats with or without an inhibitor. (A) Relative levels of M2 in rats pretreated with ABT (open squares) versus vehicle (closed squares). (B) Relative levels of M1 in rats pretreated with ABT (open circles), allopurinol (closed triangles), ABT + allopurinol (open triangles), or vehicle (closed circles). Data is expressed as the peak area ratio of analyte/internal standard and represent the mean (± SEM, n = 2).
Table 1. LC/MS detection of metabolites *in vivo* (SD rat) or *in vitro* in SD rat and human microsomes.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Metabolites Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
</tr>
<tr>
<td>Rat Plasma</td>
<td>✓</td>
</tr>
<tr>
<td>Rat microsomes</td>
<td>✓</td>
</tr>
<tr>
<td>Human microsomes</td>
<td>✓</td>
</tr>
</tbody>
</table>

*aCytosolic AO contamination of microsomes produced low levels of M1.*
Table 2. Exposure of M1 Formed from Human or Rat hepatic S9 incubations of 1 in the presence or absence of NADPH.

<table>
<thead>
<tr>
<th>In vitro Incubation</th>
<th>+ NADPH</th>
<th>- NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human S9</td>
<td>7.7 ± 0.66</td>
<td>12.2 ± 0.68</td>
</tr>
<tr>
<td>Rat S9</td>
<td>10.4 ± 0.90</td>
<td>15.7 ± 0.68</td>
</tr>
</tbody>
</table>

AUC expressed as the peak area ratio analyte/IS*min and represent means of triplicate determinations (± SD).
Table 3. Total, NADPH-dependent, and NADPH-independent rat and human hepatic S9 intrinsic clearance of 1 at concentrations of 0.1 μM, 1 μM, and 10 μM

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+NADPH</td>
<td>+NADPH</td>
</tr>
<tr>
<td></td>
<td>+hydralazine</td>
<td>-hydralazine</td>
</tr>
<tr>
<td></td>
<td>CL_{int}</td>
<td>CL_{int}</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>41.5 ± 4.6</td>
<td>99.6 ± 8.3</td>
</tr>
<tr>
<td>1 μM</td>
<td>22.4 ± 1.7</td>
<td>52.1 ± 6.1</td>
</tr>
<tr>
<td>10 μM</td>
<td>&lt;4.9</td>
<td>24.7 ± 2.0</td>
</tr>
</tbody>
</table>

Data represent means of triplicate determinations (± SD).
**Table 4.** Pharmacokinetic (PK) parameters of VU0409106 following the IV (1 mg/kg) IP (3 mg/kg) administration of 1 to control rats or rats pretreated with ABT.

<table>
<thead>
<tr>
<th>Compound Dosed and Quantitated</th>
<th>Dose (route)</th>
<th>PK Parameter</th>
<th>Control</th>
<th>ABT</th>
<th>Mean Fold Over Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/kg (IV)</td>
<td>CL (mL/min/kg)</td>
<td>53.5±1.12</td>
<td>15.3±1.19 **</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V_{ss}$ (L/kg)</td>
<td>0.994±0.052</td>
<td>0.946±0.005</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t_{1/2}$ (h)</td>
<td>0.215±0.016</td>
<td>0.721±0.061*</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>3 mg/kg (IP)</td>
<td>$C_{max}$ (ng/mL)</td>
<td>787±782</td>
<td>2453±589 *</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$AUC_{0-\infty}$ (h*ng/mL)</td>
<td>311±6.52</td>
<td>1099±85.7 *</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$AUC_{0-\infty}$ (h*ng/mL)</td>
<td>482±247</td>
<td>3742±970 **</td>
<td>7.8</td>
</tr>
</tbody>
</table>

$AUC = \text{area under the plasma concentration-time curve}; \ C_{max} = \text{peak plasma concentration}; \ CL = \text{plasma clearance}; \ t_{1/2} = \text{half-life} (t_{1/2} = \text{MRT} \times \ln 2); \ V_{ss} = \text{volume of distribution at steady-state}. \text{Data for control and ABT groups represent a mean of } n = 2 (\pm \text{SEM}). \text{Data for control (}n = 3\text{) and ABT (}n = 4\text{) groups represent a mean (}\pm \text{SD}). \text{Statistical analysis performed using a two-tailed unpaired t test. } *p < 0.05, **p < 0.01$
Table 5. Pharmacokinetic (PK) parameters of metabolites M1, M2, and M4-M6 following an IP administration of 1 (3 mg/kg) to control rats or rats pretreated with ABT.

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>M1</th>
<th>M2</th>
<th>M4-M6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ABT</td>
<td>Mean Fold Over Control</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>0.282 ± 0.223</td>
<td>2.07 ± 1.74</td>
<td>7.3</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-infinity&lt;/sub&gt;</td>
<td>0.432 ± 0.269</td>
<td>6.65 ± 6.41</td>
<td>15</td>
</tr>
</tbody>
</table>

AUC and C<sub>max</sub> of all metabolites reported as peak area ratio analyte/IS*h and analyte/IS, respectively. Data represent a mean (± SD). Statistical analysis performed using a two-tailed unpaired t test. *p < 0.05, **p < 0.01
Table 6. Systemic exposure of metabolites M1 and M2 following an IP administration of 1 (10 mg/kg) to control rats or rats pretreated with ABT, allopurinol, or allopurinol + ABT.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>ABT</th>
<th>Mean Fold Over Control</th>
<th>Allopurinol</th>
<th>Mean Fold Over Control</th>
<th>Allopurinol + ABT</th>
<th>Mean Fold Over Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>5.10 ± 3.79</td>
<td>46.3 ± 24.6</td>
<td>9.1</td>
<td>13.4 ± 3.98</td>
<td>2.6</td>
<td>74.9 ± 1.74 **</td>
<td>15</td>
</tr>
<tr>
<td>M2</td>
<td>1.20 ± 1.10</td>
<td>17.1 ± 11.9</td>
<td>14</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

AUC reported as peak area ratio analyte/IS*h, as no authentic metabolite standards were available. Data represent a mean of n = 2 (± SEM). Statistical analyses performed using a two-tailed unpaired t test. *p < 0.05, **p < 0.01
Scheme 1
Scheme 2

M4, M5, M6 ← ABT

P450

M1 ← AO

VU0409106 (1)
Figure 1

A

- Human S9 (+ NADPH)
- Human S9 (- NADPH)

B

- Rat S9 (+ NADPH)
- Rat S9 (- NADPH)
Figure 2