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Heterotropic Activation of the Midazolam Hydroxylase Activity of CYP3A by a Positive Allosteric Modulator of $mGlu_5$: In Vitro to In Vivo Translation and Potential Impact on Clinically Relevant Drug-Drug Interactions[S]

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ABSTRACT

Allosteric modulation of G protein-coupled receptors has gained considerable attention in the drug discovery arena because it opens avenues to achieve greater selectivity over orthosteric ligands. We recently identified a series of positive allosteric modulators (PAMs) of metabotropic glutamate receptor 5 ($mGlu_5$) for the treatment of schizophrenia that exhibited robust heterotropic activation of CYP3A4 enzymatic activity. The prototypical compound from this series, 5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrazine (VU0448187), was found to activate CYP3A4 to >100% of its baseline intrinsic midazolam (MDZ) hydroxylase activity in vitro; activation was CYP3A substrate specific and $mGlu_5$ PAM dependent. Additional studies revealed the concentration-dependence of CYP3A activation by VU0448187 in multispecies hepatic and intestinal microsomes and hepatocytes, as well as a diminished effect observed in the presence of ketoconazole. Kinetic analyses of the effect of VU0448187 on MDZ metabolism in recombinant P450 or human liver microsomes resulted in a significant increase in V_{max} (minimal change in K_m) and required the presence of cytochrome b_5. The atypical kinetics translated in vivo, as rats receiving an intraperitoneal administration of VU0448187 prior to MDZ treatment demonstrated a significant increase in circulating 1- and 4-hydroxy- midazolam (1-OH-MDZ, 4-OH-MDZ) levels compared with rats administered MDZ alone. The discovery of a potent substrate-selective activator of rodent CYP3A with an in vitro to in vivo translation serves to illuminate the impact of increasing intrinsic enzymatic activity of hepatic and extrahepatic CYP3A in rodents, and presents the basis to build models capable of framing the clinical relevance of substrate-dependent heterotropic activation.

Introduction

The neurotransmitter, L-glutamate, modulates cell excitability and synaptic transmission via neuromodulatory glutamate receptors, called metabotropic glutamate (mGlu) receptors, in the mammalian central nervous system. The mechanisms by which mGlu receptors are activated, the proteins with which they interact, and the downstream second-messenger pathways involved in the signaling of these receptors have been extensively investigated. Both orthosteric and allosteric small molecule ligands

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ABBREVIATIONS: 1-OH-MDZ, 1-hydroxy midazolam; 4-OH-MDZ, 4-hydroxy midazolam; 9EP, 9-ethynylphenanthrene; CPHP, 4-(4-chlorophenyl)-4-hydroxy piperidine; DDI, drug-drug interaction; HAL, haloperidol; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MDZ, midazolam; $mGlu_5$, metabotropic glutamate receptor subtype 5; P450, cytochrome P450; PAM, positive allosteric modulator; RHAL, reduced haloperidol; VU0448187, 5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrazine; VU0464797, 2-(phenoxymethyl)-*N*-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridin-4-amine).

can modulate the activity of the mGlu receptors; however, greater selectivity, and often potency, can be achieved with positive allosteric modulators (PAMs) or negative allosteric modulators of each mGlu receptor. Due to the widespread distribution of mGlu receptors in the central nervous system, these receptors represent attractive targets for therapeutic intervention into a wide variety of neurologic and psychiatric disorders such as Alzheimer's disease, anxiety, depression, Parkinson's disease, and schizophrenia (Niswender and Conn, 2010). We and others have developed selective PAMs for $mGlu_5$ that have potential utility in the treatment of schizophrenia and other disorders that involve impaired cognitive function (Moghaddam, 2004; Conn et al., 2009), as evidenced by the effects these compounds produce in animal models of psychosis and cognition (Kinney et al., 2005; Darrah et al., 2008; Liu et al., 2008).

Similar to other disease states, patients receiving treatment for schizophrenia are commonly subjected to polypharmacy approaches intended to alleviate the variety of symptoms of their disease. Many of these drugs, including haloperidol (HAL), are metabolized either exclusively or to a great extent by CYP3A4 (Fang et al., 2001; Kalgutkar et al., 2003; Avent et al., 2006). CYP3A4 is predominantly expressed in the liver, and to a lesser extent in the intestine, and is involved in the metabolism of a wide variety of drugs, xenobiotics, and steroids (Thummel and Wilkinson, 1998; Guengerich, 2006). Significant clinical drug-drug interactions (DDIs) have been observed in humans due to the inhibition of CYP3A4 by other concomitantly administered drugs (Lin and Lu, 1998) or naturally occurring components of herbal remedies or fruits (i.e., the grapefruit juice effect). CYP3A4 is also subject to induction by a wide variety of ligands (Lin and Lu, 1998), resulting in a significant increase in the levels of protein that are expressed in tissues. Generally, the inhibition or induction of CYP3A4 has received the most attention with regard to clinically relevant DDIs (Hutzler et al., 2005; Hafner et al., 2010). However, the intrinsic enzymatic activity of the cytochrome P450 (P450) family, including CYP3A4, has also been reported to be significantly affected in vitro (and, in only a few examples, in vivo) through homotropic or heterotropic activation of the enzyme (Hutzler and Tracy, 2002; Obach, 2012). A wide variety of compounds and known drugs have been shown to be activators of CYP3A4 enzymatic activity, including 7,8-benzoflavone (Shou et al., 1994), quinidine (Ngui et al., 2000), and steroids (Henshall et al., 2008). The activation of CYP3A4 metabolic activity in vivo may represent an acute DDI scenario that could increase the clearance of a parent drug and/or increase levels of circulating, and perhaps toxic or active, metabolites.

Herein, we report the discovery of a novel $mGlu_5$ PAM, 5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyrazine (VU0448187), that significantly activated the midazolam (MDZ) hydroxylase activity of CYP3A4 in multispecies hepatic and intestinal microsomes and hepatocytes. Interestingly, this effect appears to be substrate dependent, as the CYP3A4-mediated hydroxylation of testosterone and progesterone was unaffected by VU0448187. In addition, the effects of these $mGlu_5$ PAMs on MDZ hydroxylase activity by CYP3A4 appear to be structure dependent, as an $mGlu_5$ PAM from a second distinct structural series was shown to be an inhibitor of CYP3A4-mediated metabolism of MDZ to its 1-hydroxy metabolite (1-OH-MDZ). Furthermore, ketoconazole, a potent and selective inhibitor of CYP3A4, can block the ability of VU0448187 to activate CYP3A MDZ hydroxylase activity in vitro. Kinetic studies of MDZ metabolism with human liver microsomes and recombinant CYP3A4 and CYP3A5 demonstrated that VU0448187 increases V_{max} (with minimal changes in K_m) and requires the presence of cytochrome b_5. Importantly, we were able to show that the activation of CYP3A MDZ metabolism to either 1-OH-MDZ or 4-OH-MDZ by VU0448187 could be translated to an in vivo rodent model. This observation, along with preliminary data suggesting that VU0448187 can activate CYP3A4-mediated metabolism of haloperidol in human liver microsomes, suggests a potential to elicit a clinically relevant DDI that is a result of an acute increase in the enzymatic activity of a drug-metabolizing enzyme.

Materials and Methods

Chemicals and Enzyme Sources. NADPH, MDZ, 1-OH-MDZ, 4-OH-MDZ, HAL, reduced haloperidol (RHAL), 4-(4-chlorophenyl)-4-hydroxy piperidine (CPHP), testosterone, 6β-OH-testosterone, progesterone, 6β-OH-progesterone, phenacetin, acetaminophen, diclofenac, 4-OH-diclofenac, dextromethorphan, dextrorphan, ketoconazole, and miconazole were all purchased from Sigma-Aldrich (St. Louis, MO). VU0448187 and VU0464797 (2-(phenoxymethyl)-*N*-(pyridin-3-ylmethyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridin-4-amine) (Fig. 1A) were synthesized internally at the Vanderbilt Center for Neuroscience Drug Discovery (Supplemental Schemes 1 and 2) with purity and structural characterization confirmed by nuclear magnetic resonance. Human liver microsomes (150-donor pool, mixed sex), human intestinal microsomes (20-donor pool, mixed sex), male murine microsomes, and rat liver microsomes were all purchased from BD Biosciences (Woburn, MA). Human (20-donor pool, mixed sex), male Sprague-Dawley rat, and male CD-1 mouse hepatocytes were purchased from Celsis In Vitro Technologies (Baltimore, MD). The cDNA-expressed CYP3A4 and CYP3A5 enzymes with reductase and with or without cytochrome b_5 (EasyCyps) were purchased from XenoTech, LLC (Kansas City, MO).

Inhibition or Activation Assays. A four-in-one (cocktail) assay for determining IC_{50} values against human CYP1A2, CYP2C9, CYP2D6, and CYP3A4 was originally developed based on previous reports (Youdim et al., 2008) and used for high-throughput screening of potential P450 inhibitors. Human liver microsomes (final concentration of 0.1 mg/ml) and a substrate mixture containing the P450 probe substrates phenacetin (10 μM), diclofenac (5 μM), dextromethorphan (5 μM), and MDZ (2 μM) were added to a potassium phosphate-buffered solution (0.1 M, pH 7.4) and warmed to 37°C. The reaction mixture was divided evenly into a 96-well plate and various dilutions of each inhibitor/compound of interest (in duplicate) were

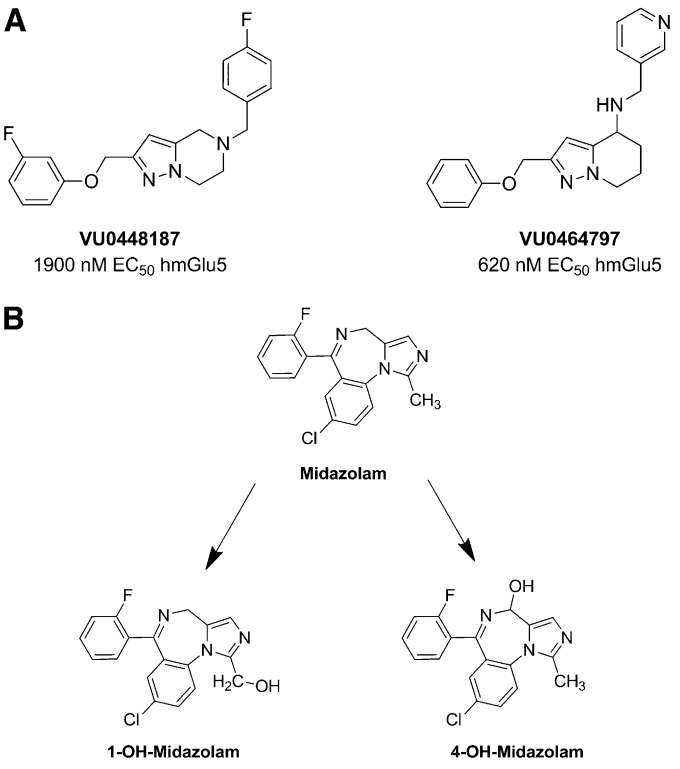

Fig. 1. (A) Chemical structures of VU0448187 and VU0464797: two structurally distinct, but active PAMs of human metabotropic glutamate receptor 5 ($hmGlu_5$). (B) The metabolism of MDZ by CYP3A4 in vitro.

then added to this reaction mixture such that the final concentration of each compound ranged from 100 nM to 30 μM. This mixture was allowed to preincubate for 15 minutes while shaking at 37°C. Buffer or NADPH (1 mM) was added and the reaction mixture was incubated for an additional 8 minutes at 37°C prior to quenching with two volumes of ice-cold acetonitrile containing 50 nM carbamazepine (internal standard). The plates were centrifuged at 4000 rpm (4°C) for 10 minutes and the supernatant was removed and diluted with water (1:4, v/v) in preparation for liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis. The IC_{50} values for each compound were obtained for the individual P450 enzymes by quantitating the inhibition of metabolite formation for each probe substrate against a 12-point standard curve specific for each metabolite: acetaminophen (CYP1A2), 4-OH-diclofenac (CYP2C9), dextrorphan (CYP2D6), and 1-OH-MDZ (CYP3A4). A 0 μM compound condition (or control) was set to 100% enzymatic activity and the effect of increasing test compound concentrations on enzymatic activity could then be calculated from the percentage of control activity. Curves were fitted using XLfit 5.2.2 (four-parameter logistic model, Eq. 201) to determine the concentration that produces half-maximal inhibition (IC_{50}). Miconazole was included as a positive control for broad spectrum P450 inhibition (pan-P450 inhibitor). Experiments were performed in duplicate unless noted otherwise.

Discrete assays for monitoring the activation or inhibition of CYP3A4 enzymatic activity in human, rat, or mouse liver and intestinal microsomes or hepatocytes were performed similarly to that described above, except that only one substrate was present in any given assay and levels of substrate-specific metabolites were exclusively monitored. Final concentrations of the substrates MDZ (2 μM), testosterone (25 μM), progesterone (50 μM), and HAL (50 μM) were chosen to be reflective of reported K_m values for CYP3A4 and the protein concentration, time of incubation with NADPH (when necessary), and reconstitution ratios for LC-MS/MS analysis were optimized for each substrate and metabolite combination. Levels of each metabolite (1-OH or 4-OH-MDZ, 6β-OH-testosterone, 6β-OH-progesterone, and CPHP) were quantitated against a 12-point standard curve and data were represented as the percentage of control activity remaining. Experiments in human liver microsomes involving ketoconazole (1 μM) were performed by preincubating ketoconazole for 5 minutes prior to the addition of VU0488187 for 15 minutes and then NADPH (1 mM) for an additional 8 minutes. Hepatocytes were thawed per the vendor's instructions, and incubations were conducted with 0.5×10^6 cells/ml.

Kinetic Analysis of CYP3A4 Activation. Studies to monitor the effect of VU0448187 (10 μM) on the K_m and V_{max} for MDZ hydroxylation to 1-OH-MDZ were performed in potassium phosphate-buffered solution (0.1 M, pH 7.4) with 5 mM $MgCl_2$. MDZ was dissolved in methanol and diluted over a range of concentrations. Final protein concentrations were 0.5 mg/ml (human liver microsomes) and 5 pmol/ml for CYP3A4 and CYP3A5 (EasyCYPs coexpressed with reductase and with or without cytochrome b_5) and the assay was optimized for linearity or per the vendor's instructions. A premixture composed of human liver microsomes or recombinant CYP3A4 or CYP3A5 in assay buffer was distributed to tubes on ice that contained either varied concentrations of MDZ alone or MDZ with VU0448187. Every 15 seconds, a tube was placed in the 37°C water bath so that all tubes were preincubated for 5 minutes prior to the addition of NADPH (1 mM final) for an additional 5 minutes. Reactions were quenched with ice-cold acetonitrile containing 50 nM carbamazepine (internal standard). The plates were centrifuged at 4000 rpm (4°C) for 10 minutes and the supernatant was removed and diluted with water (1:1, v/v) in preparation for LC-MS/MS analysis. 1-OH-MDZ levels were quantitated against a 12-point standard curve and the data were fit to either Michaelis-Menten (Eq. 1) or substrate-inhibition (Eq. 2) equations and represented as nanomoles per minute per milligram of protein (human liver microsomes) or picomoles per minute per picomole of P450 (CYP3A4 or CYP3A5). Because MDZ metabolism undergoes substrate inhibition at higher concentrations (Martínez et al., 2000; Khan et al., 2002), it was decided to report all data using Eq. 2 for completeness. Experiments were $n = 2$ or more.

$$Y = V_{max} \times X/(K_m + X) \quad (1)$$

$$Y = V_{max} \times X/(K_m + X \times (1 + X/K_i)) \quad (2)$$

In Vivo Pharmacokinetic Studies with MDZ. MDZ is metabolized by CYP3A in vitro to 1-OH-MDZ and 4-OH-MDZ (Fig. 1B) (Kronbach et al., 1989). MDZ was prepared as a solution in 10% ethanol/50% PEG-400/40% saline and dosed intravenously (i.v.) at 1 mg/kg to dual-cannulated male Sprague-Dawley rats ($n = 2$) or male CD-1 mice ($n = 2$) (Harlan Laboratories, Indianapolis, IN). Blood was serially collected over EDTA at 0.0333, 0.117, 0.25, 0.5, 1, 3, 7, and 24 hours postdose and plasma was isolated via centrifugation (4000 rpm, 4°C) for storage at −80°C until LC-MS/MS analysis. The sample extraction of plasma was performed by protein precipitation employing three volumes of ice-cold acetonitrile containing 50 nM carbamazepine (internal standard). The samples were centrifuged (4000 rpm, 4°C) and the supernatants were transferred and diluted 1:1 (v/v) for LC-MS/MS analysis. The final pharmacokinetic parameters for MDZ (clearance, CL_p; half-life, $t_{1/2}$; volume of distribution predicted at steady state, V_{ss}) were calculated by noncompartmental analysis employing WinNonLin software (Phoenix, version 6.2; Pharsight Inc., Mountain View, CA). Alternatively, VU0448187 was prepared in 10% Tween 80 (aq) for intraperitoneal administration (i.p.) of a 10 mg/kg dose to dual-cannulated Sprague-Dawley male rats ($n = 2$) 1 hour prior to a 10 mg/kg i.p. dose of MDZ. Blood was collected at various times postdose and handled as described above for LC-MS/MS analysis. VU0448187, MDZ, 1-OH-MDZ, and 4-OH-MDZ levels were simultaneously monitored from each experiment and the levels of each analyte were quantified against a 10-point standard curve. Concentrations were reported as nanograms per milliliter.

LC-MS/MS Analysis. In vitro and in vivo samples were analyzed via electrospray ionization LC-MS/MS on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient elution using a thermostated (40°C) C18 column (Fortis 3.0 × 50 mm, 3 μ; Fortis Technologies Ltd, Cheshire, UK). Mobile phase A was 0.1% formic acid (aq) and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 10% B with a 0.2-minute hold and was then linearly increased to 90% over 1.3 minutes, followed by a return to 10% B in 0.1 minutes and subsequent re-equilibration (0.9 minutes). The total run time was 2.5 minutes and the high-performance liquid chromatography 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 specific for each compound utilizing a Turbo-Ionspray source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.5.1 software.

Results

Assessment of CYP3A-Mediated MDZ Hydroxylase Activity in Hepatic and Intestinal Microsomes and Hepatocytes. A mixed-age and mixed-sex hepatic microsomal pool of 150 human donors was used to monitor the effect of increasing concentrations of VU0448187 and VU0464797 (Fig. 1A) on the ability of four major drug-metabolizing P450 enzymes (CYP1A2, CYP2C9, CYP2D6, and CYP3A4) to metabolize their respective probe substrates. Using a cocktail (four-in-one) approach in human liver microsomes, we observed that VU0448187 displayed little to no effect on the enzymatic activities of CYP1A2 or CYP2D6 ($IC_{50} > 30$ μM) and that the activity of CYP2C9 was minimally affected ($IC_{50} = 20$ μM; inhibition of 4-OH-diclofenac formation) (Fig. 2A). Interestingly, we observed an increase in the CYP3A4-mediated metabolism of the probe substrate MDZ to its 1-OH-MDZ metabolite in the presence of VU0448187. The CYP3A4 activation discovered was concentration dependent and the maximal effect occurred at 10 μM VU0448187 (Fig. 2A). To delineate what appeared to be a CYP3A4-specific effect by VU0448187, we moved to a discrete assessment of MDZ metabolism in human liver microsomes. Importantly, a comparison of the data in Fig. 2, A and B, demonstrates that VU0448187 acts directly on the intrinsic activity of CYP3A4 to increase MDZ metabolism. In our initial metabolic assessment of a series of $mGlu_5$ PAMs, we discovered a structure-activity relationship of the substrate-specific activation of CYP3A4 in human liver microsomes. As shown in Fig. 2C, a structurally distinct $mGlu_5$ PAM (VU0464797) does not activate MDZ hydroxylase activity but, in fact, inhibits the CYP3A4-mediated conversion of MDZ to 1-OH-MDZ in human liver microsomes.

Therefore, we investigated the ability of VU0448187 to activate CYP3A metabolism of MDZ in multispecies and varied tissues (Fig. 3). Interestingly, we found that increasing concentrations of VU0448187

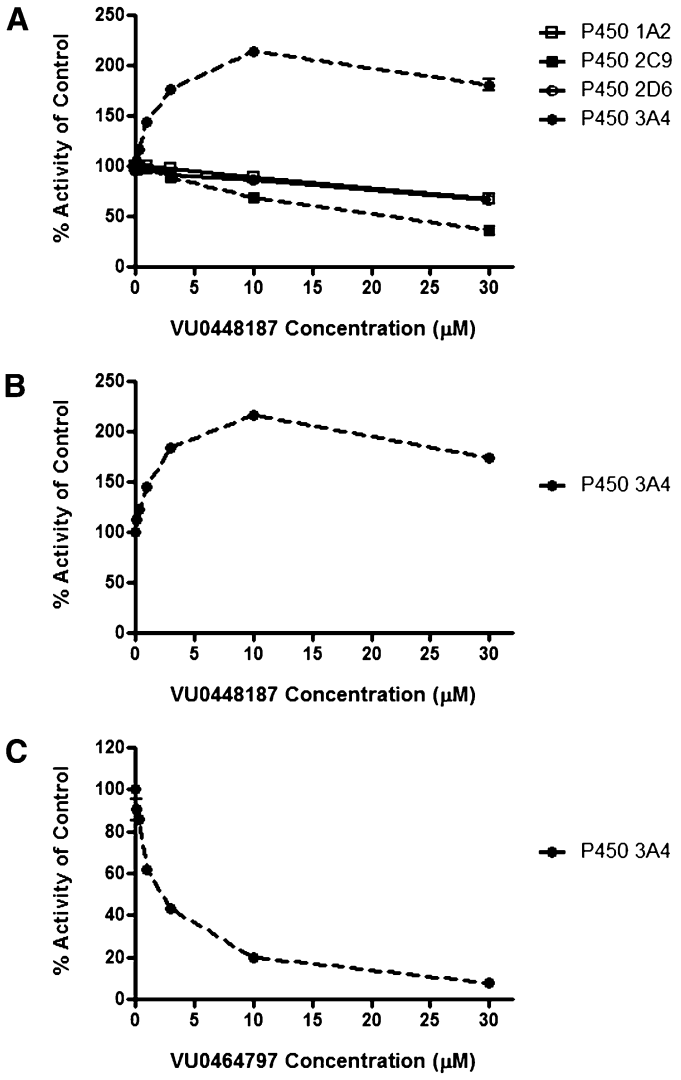

Fig. 2. The effect of increasing concentrations of VU0448187 or VU0464797 on the metabolic activity of CYP1A2, CYP2C9, CYP2D6, and CYP3A4 in human liver microsomes from a 150-donor pool using a cocktail approach to assay for enzymatic activity or inhibition (A) or a discrete assay in human liver microsomes monitoring the conversion of MDZ to 1-OH-MDZ for CYP3A4 activity (B and C).

could dose-dependently activate the metabolism of MDZ to its 1-hydroxy (1-OH) metabolite in both mixed donor pools of human intestinal microsomes (Fig. 3B) and human hepatocytes (Fig. 3C). In fact, the activation of CYP3A activity in hepatocytes by VU0448187 was significantly greater than that originally observed in human liver microsomes (Fig. 3A) with activation continuing out to 30 μM (approximately 400% activity of control) and increased levels of 1-OH-MDZ were measured at the lowest concentration of VU0448187 tested (100 nM). The CYP3A activation effect observed with VU0448187 in hepatocytes versus that in human liver microsomes may be a direct result of a more complete physiologic test system (all proteins, cofactors at physiologically relevant expression and concentrations, respectively). The concentration-dependent effects of VU0448187 on CYP3A activity were also observed in rat hepatocytes (Fig. 3D) and hepatic microsomes (data not shown) and in murine liver microsomes (Fig. 3E) and hepatocytes (data not shown). These in vitro data provided confidence that our observations were not species specific, and that VU0448187 was membrane permeable and targeted CYP3A activity in hepatocytes from multiple species and distinct tissues. Together, these results provided support for the selection of an appropriate preclinical model for examining the effect of VU0448187 on CYP3A activity in vivo.

As VU0464797 was found to be an inhibitor of CYP3A4 in human liver microsomes (Fig. 2), we examined the effect of increasing concentrations of this $mGlu_5$ PAM in human and rat hepatocytes. Data provided in Fig. 4 demonstrate that VU0464797 dose-dependently inhibits the conversion of MDZ to 1-OH-MDZ in hepatic microsomes and hepatocytes from multiple species. Validating VU0464797 as a structurally distinct $mGlu_5$ PAM (negative control) that has a different reaction phenotype to VU0448187 demonstrates that not all $mGlu_5$ PAMs exhibit this CYP3A4 activation phenomenon and that any potential acute effects caused by the activation of the intrinsic enzymatic activity are likely drug and/or compound specific.

The Effects of a Specific CYP3A4 Inhibitor on VU0448187-Mediated Enhancement of Enzymatic Activity and Observations with Alternative Probe Substrates. We investigated the ability of ketoconazole, a potent and selective inhibitor of CYP3A4, to inhibit the activity of this enzyme in human liver microsomes in the presence of increasing concentrations of VU0448187. Data in Fig. 5A show that ketoconazole (at 1 μM) can significantly block the VU0448187-mediated activation of MDZ hydroxylation, indicating that CYP3A4 and/or CYP3A5 are the targets of this heterotropic activator in human liver microsomes and not other P450 enzymes. Although we cannot confirm that the effects of the activator are mediated through CYP3A4 or CYP3A5 alone, our data are consistent with historical accounts that the effects of a heterotropic activator (e.g., VU0448187) are likely mediated through the enzyme and not simply through a direct interaction of the activator with other partner proteins (e.g., reductase) (Hutzler and Tracy, 2002; Keubler et al., 2012) In addition, the blockade of VU0488187-mediated CYP3A4 activation with MDZ as the probe substrate can be titrated with varying concentrations of ketoconazole (data not shown).

Understanding that CYP3A4 may catalyze the oxidation of multiple substrates at topologically distinct sites within the protein, we investigated the ability of VU0448187 to activate the metabolism of testosterone and progesterone, two substrates known to bind to distinct sites within CYP3A4. The results displayed in Fig. 5 demonstrate that the enhancement of CYP3A4 enzymatic activity in human liver microsomes by VU0448187 is substrate-dependent, because the testosterone (Fig. 5B) and progesterone (Fig. 5C) hydroxylase activities of CYP3A4 (conversion to 6β-OH metabolites) were not activated by increasing concentrations of VU0448187. In fact, we observed a slight dose-dependent *inhibition* of testosterone and progesterone metabolism by VU0448187 in human liver microsomes. These data underscore the importance of testing multiple potential perpetrators and victims when examining atypical enzyme kinetics in vitro and further confirm previous literature reports supporting the notion that CYP3A allostery is substrate dependent (Wang et al., 2000).

The Kinetics of CYP3A4 Activation of MDZ 1-Hydroxylase Activity in cDNA-Expressed CYP3A4 and CYP3A5 and Human Liver Microsomes. Previous studies examining the effect of efavirenz on the metabolic activation of CYP3A4 MDZ metabolism found that the K_m of MDZ was relatively unaffected by the presence of efavirenz, but that the V_{max} significantly increased (Keubler et al., 2012). Therefore, we investigated the impact of VU0448187 on the binding affinity (K_m) and catalytic efficiency (V_{max}) of MDZ 1-hydroxylation in recombinant CYP3A4 and CYP3A5 (with and without cytochrome b_5), as well as in human liver microsomes. Not surprisingly, the kinetics of MDZ metabolism (1-OH-MDZ formation) was linear at concentrations up to, but not exceeding, 10 μM; as expected, we observed substrate-inhibition kinetics at higher concentrations (100 μM) (Martínez et al., 2000; Lin et al., 2001; Khan et al., 2002). Consistent with previous accounts of

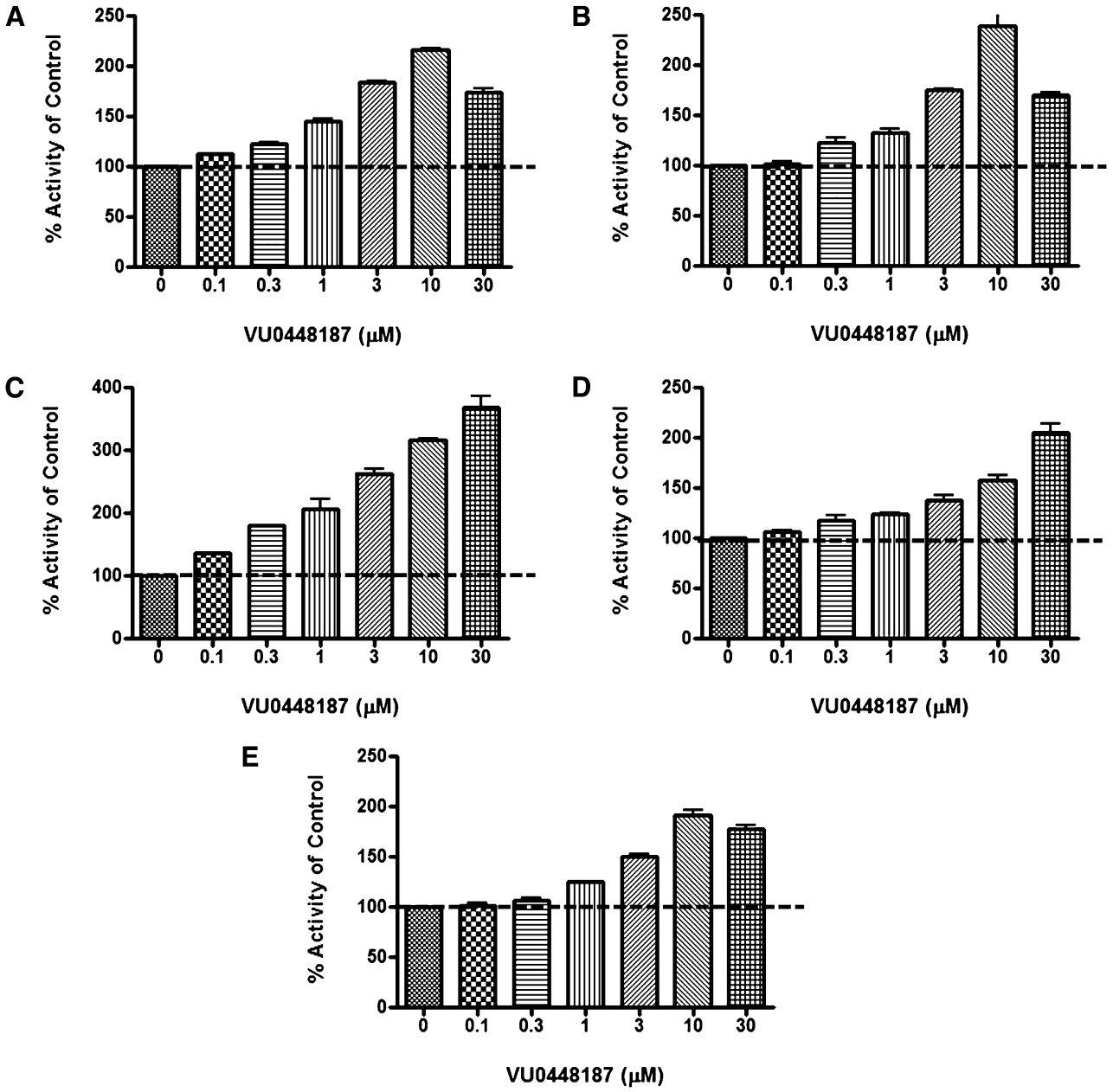

Fig. 3. The activation of MDZ 1-hydroxylase activity in pools of human liver microsomes (A), human intestinal microsomes (B), human hepatocytes (C), rat hepatocytes (D), and murine liver microsomes (E) by increasing concentrations of VU0448187. Human donor pools were mixed sex/mixed age; rat and murine pools were male Sprague-Dawley and male CD-1, respectively.

efavirenz-mediated activation, the presence of VU0448187 (10 μM) activated the maximal product formation rate (V_{max}) by 1.2- to 1.4-fold in CYP3A4 and CYP3A5 and 2.8-fold in human liver microsomes, with little to no change in K_m for cDNA-expressed CYP3A4 (Fig. 6 and Table 1), CYP3A5 (data not shown) and in human liver microsomes (Fig. 6C and Table 1). The increase in V_{max} associated with VU0448187 in the recombinant P450 assay was dependent on the presence of cytochrome b_5 (Fig. 6, A and B), and the overall intrinsic enzymatic activity of either CYP3A4 or CYP3A5 was higher with cytochrome b_5. Importantly, P450 enzymes have been reported to display compound-dependent variability in metabolic activity (or inhibition profiles) when reactions are fortified with cytochrome b_5 (Jushchyshyn et al., 2005). In fact, efavirenz activation of CYP3A4-mediated MDZ metabolism was also shown to be dependent on the presence of cytochrome b_5 (Keubler et al., 2012). Although it is unclear at this time what precise role cytochrome b_5 plays in the activation of CYP3A4-mediated MDZ metabolism by VU0448187 (or efavirenz), it is possible that cytochrome b_5 induces a conformational change in CYP3A4 or CYP3A5 (Yamazaki et al., 1996) or increases the coupling between the P450 protein and reductase (Lee et al., 1997; Locuson et al., 2007), such that the metabolism is enhanced by the presence of VU0448187.

MDZ Pharmacokinetics and In Vivo Models of VU0448187 Activation of CYP3A in Rodents. Although MDZ hepatic clearance in the rat is dependent upon the specific rat strain used for a given study, it is also well known that MDZ is generally a high-clearance compound (55–80 ml/min per kilogram) in rat (Kotegawa et al., 2002) with the predominant metabolic pathways being CYP3A-mediated conversion to 1-OH-MDZ and 4-OH-MDZ (at near equal amounts). In

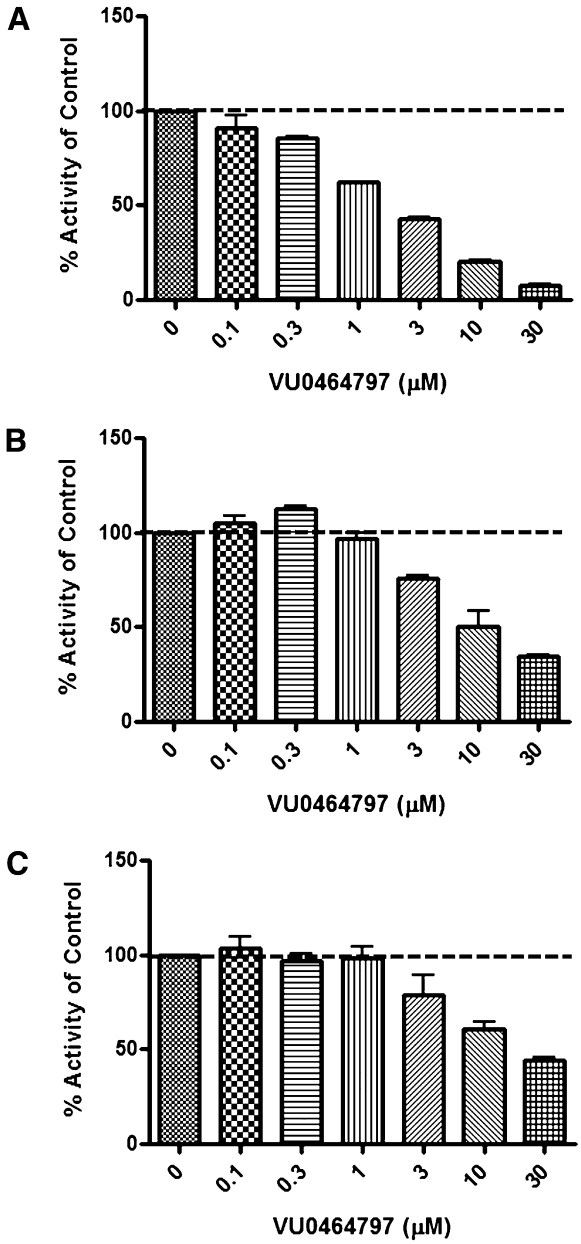

Fig. 4. The inhibition of MDZ 1-hydroxylase activity in pools of human liver microsomes (A), human hepatocytes (B), and rat hepatocytes (C) by increasing concentrations of VU0464797. Human donor pools were mixed sex/mixed age; rat pools were male Sprague-Dawley.

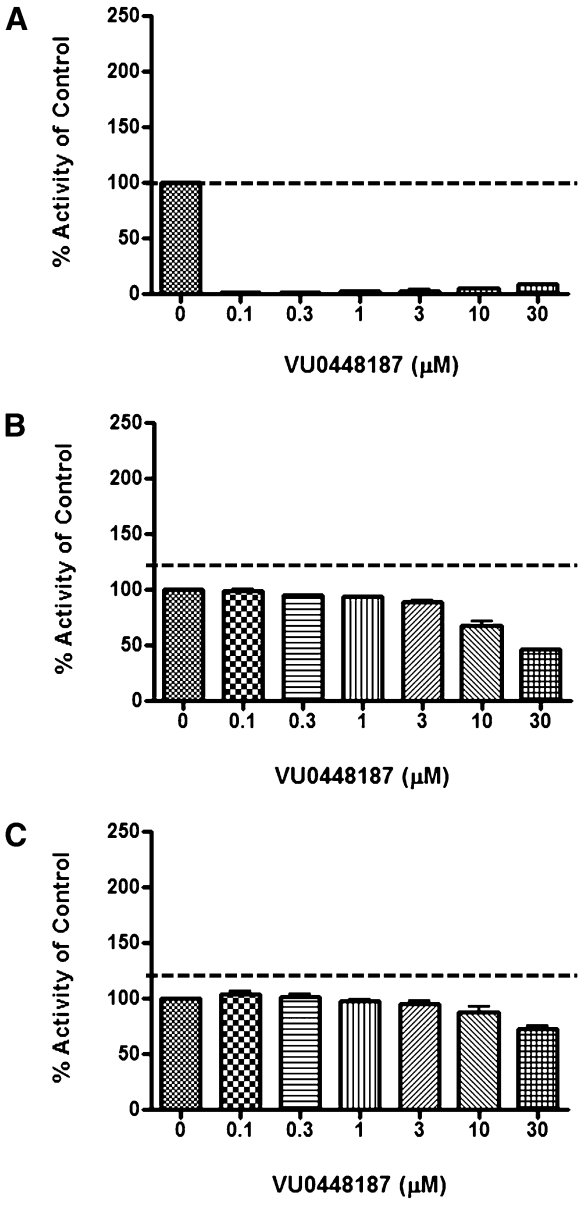

Fig. 5. (A) The effect of a specific CYP3A4 inhibitor, ketoconazole (1 μM), on the activation of MDZ 1-hydroxylase activity in human liver microsomes by VU0448187. (B and C) The effect of increasing concentrations of VU0448187 on the hydroxylase activity of alternative CYP3A4 substrates testosterone (25 μM) and progesterone (50 μM).

a single time point experiment in which rats (n = 2) were pretreated with vehicle or VU0448187 (10 mg/kg i.p., 1 hour) followed by MDZ administration (10 mg/kg i.p.), we observed a significant increase in concentrations (in nanograms per milliliter) of both 1-OH-MDZ (Fig. 7A) and 4-OH-MDZ (data not shown) at 15 minutes after MDZ treatment. The 1-OH-MDZ metabolite was observed in plasma at approximately 5 ng/ml in rats treated with MDZ alone versus concentrations approaching 125 ng/ml in rats that were pretreated with VU0448187. The increase in the metabolism of MDZ (approximately a 25-fold increase in 1-OH-MDZ production) exceeded the fold increase that was predicted from the results generated in vitro in rat liver microsomes or rat hepatocytes. Importantly, the increase in 1-OH-MDZ that we observed in rodents was not an effect stemming from the inhibition of the glucuronidation of this metabolite by the $mGlu_5$ PAM, because LC/MS analysis indicated that VU0448187 (25 μM) displayed no inhibition of the glucuronidation of 1-OH-MDZ in vitro in rat liver microsomes or rat hepatocytes (data not shown). In a follow-up experiment in which VU0448187 (10 mg/kg i.p.) pretreated rats received a single dose of MDZ (10 mg/kg i.p.), we also observed a time-dependence in the increased formation of circulating levels of 1-OH and 4-OH-MDZ (Fig. 7B). Specifically, the time-concentration data displayed in Fig. 7B indicated that compared with the rats receiving MDZ alone, 1-OH-MDZ metabolite concentrations were maximal in the plasma shortly after rats received an i.p. administration of MDZ (apparent T_{max} of 7 minutes). Importantly, the concentrations of 1-OH-MDZ returned to plasma levels that were observed in vehicle-treated rats within 2 hours, an observation that is consistent with the notion that CYP3A activation of MDZ hydroxylase activity is a transient effect dependent on VU0448187 exposure.

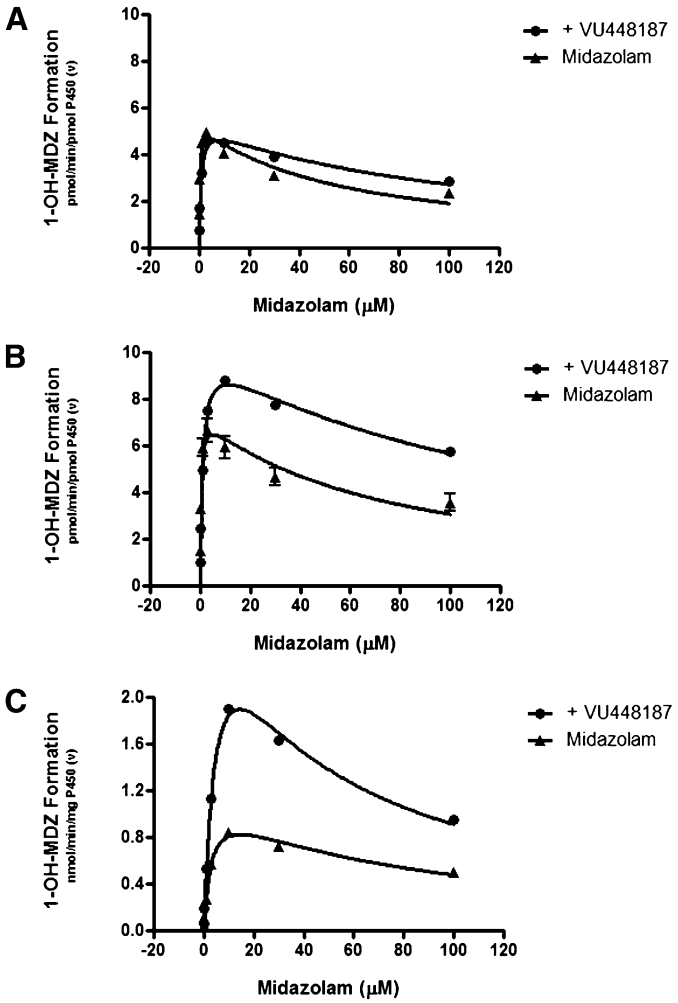

Fig. 6. The kinetics of CYP3A4 activation of 1-OH-MDZ activity by VU0448187 in recombinant systems with (B) or without (A) cytochrome b_5 or human liver microsomes (C). K_m and V_{max} parameters were determined by fitting the data to an equation for substrate inhibition. The metabolite formation rate is expressed as picomoles per minute per picomole of P450 for the recombinant P450 or as nanomoles per minute per milligram of P450 for human liver microsomes.

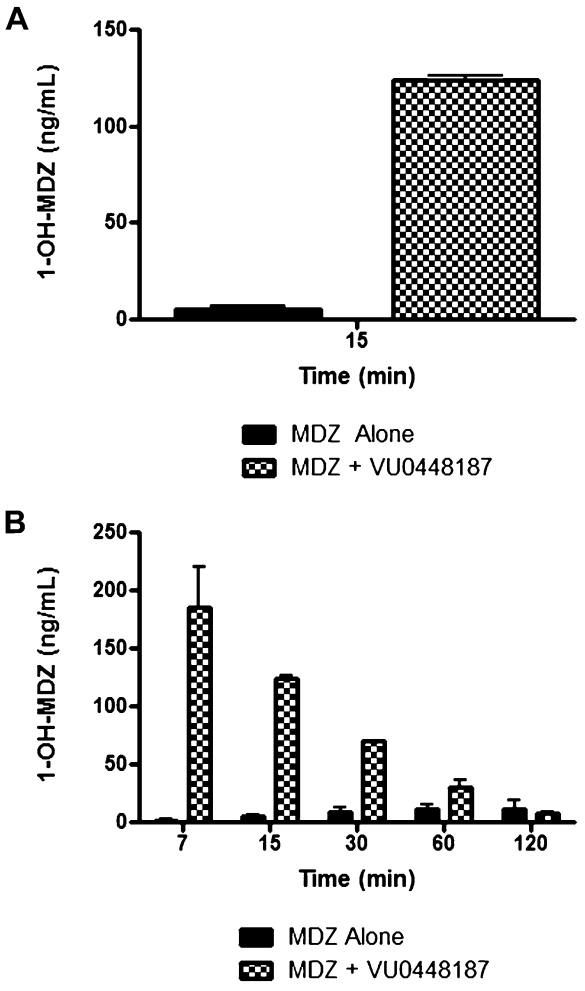

Fig. 7. Increases in 1-OH-MDZ levels in Sprague-Dawley rats after pre-treatment with VU0448187. Single time point measurement (A) or time course (B) of 1-OH-MDZ levels in male Sprague-Dawley rats (n=2) that were administered a 10 mg/kg i.p. dose of MDZ after a 1-hour pretreatment with either vehicle or a 10 mg/kg i.p. dose of VU0448187.

Clinical Relevance of Heteroactivation of CYP3A4 Activity in Human Liver Microsomes. The neuroleptic agent HAL is metabolized nearly exclusively by CYP3A4 to multiple metabolites, including RHAL, CPHP, HP^+ (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium), and RHP^+ (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-pyridinium) (Fang et al., 2001; Kalgutkar et al., 2003; Avent et al., 2006). Importantly, HAL was also found to be back-oxidized from RHAL in vitro. To determine the impact of VU0448187-mediated CYP3A4 activation on the metabolism of HAL, we monitored the conversion of HAL to its primary oxidized metabolite, CPHP, in human liver microsomes. Because our data utilizing MDZ as the CYP3A4 probe substrate (victim) indicated that the resulting enzymatic activation was perpetrator specific, we conducted the experiments with HAL in human liver microsomes in the presence or absence of VU0448187 (CYP3A4 activator), VU0464797 (CYP3A4 inhibitor), and miconazole (pan-P450 inhibitor). We observed that both VU0464797 (10 μM) and miconazole (1 μM) significantly inhibited the metabolism of HAL to CPHP (Fig. 8), whereas VU0448187 (10 μM) enhanced CPHP product formation at an approximate 20% increase over the control experiment. Although the extent of activation of CYP3A4 activity was not as significant as what was observed for MDZ hydroxylation, the observed activation of a clinically relevant CYP3A4 substrate is noteworthy. Likewise, compounds that inhibited MDZ hydroxylation in human liver microsomes also showed inhibitory profiles with HAL. These data suggest a substrate dependence to heteroactivation and imply that drugs displaying heteroactivation of enzymatic activity in vitro may mediate this effect in vivo.

TABLE 1

Kinetic effects of VU0448187 heteroactivation of CYP3A4 MDZ activity

Assay Condition	V_{max}
CYP3A4	5.30 ± 0.26
CYP3A4 + VU0448187	5.31 ± 0.14
CYP3A4/b_5	7.35 ± 0.43
CYP3A4/b_5 + VU0448187	10.1 ± 0.21
Human liver microsomes	1.16 ± 0.08
Human liver microsomes + VU0448187	3.29 ± 0.16

Changes in V_{max} for the conversion of MDZ to 1-OH-MDZ in the absence and presence of VU0448187 (10 μM) for cDNA-expressed CYP3A4 and reductase with and without cytochrome b_5 and in human liver microsomes. The metabolite formation rate is expressed as picomoles per minute per picomole of P450 for the recombinant P450 or as nanomoles per minute per milligram of P450 for human liver microsomes.

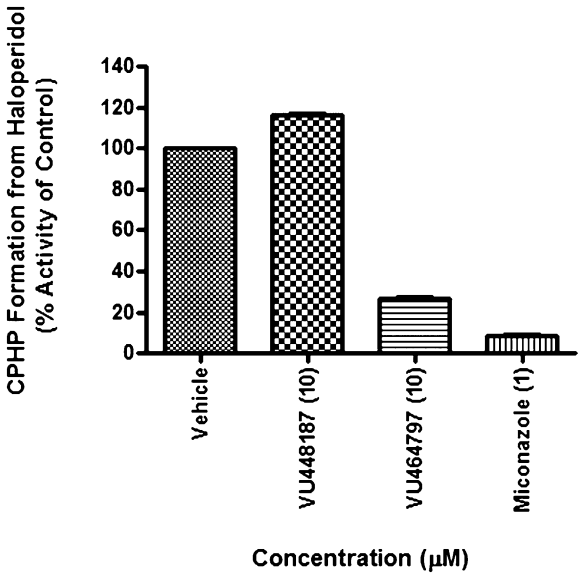

Fig. 8. The activation or inhibition of CYP3A4 HAL metabolism in human liver microsomes. VU0448187 (10 μM), VU0464797 (10 μM), or miconazole (1 μM) were incubated with HAL (50 μM) and NADPH for 40 minutes and the conversion of HAL to its metabolite, CPHP, was monitored.

Discussion

Although the metabolism of a particular substrate by a P450 enzyme is best described employing Michaelis-Menten kinetics, there are many instances of substrate-enzyme interactions that follow more atypical kinetic profiles, such as homotropic or heterotropic activation, substrate inhibition, partial inhibition, or biphasic metabolism (Korzekwa et al., 1998; Hutzler and Tracy, 2002; Atkins, 2005; Egnell et al., 2005). One of the earliest demonstrations of the ability of a P450 activator (i.e., perpetrator) to enhance the metabolism of a concomitantly administered compound (i.e., victim) was the CYP3A4-mediated hydroxylation of benzo[*a*]pyrene by α-naphthoflavone reported by Shou et al. (1994). Although multiple P450 enzymes experience heterotropic activation, the majority of examples of this phenomenon to date have involved CYP3A4. Due to the plethora of liganded/nonliganded x-ray crystal structures available with this enzyme (Williams et al., 2004; Yano et al., 2004), as well as a predominance of kinetics, mutagenesis, and various spectral titration studies or mathematical models (Domanski et al., 1998, 2000; Harlow and Halpert, 1998; Shou et al., 1999; Hosea et al., 2000; Kenworthy et al., 2001; Khan et al., 2002), multiple hypotheses exist to explain the ability of a perpetrator to enhance the metabolism of a victim substrate. The perpetrator may either bind in a separate or overlapping binding site in the enzyme active site or it may bind to a distinct allosteric site of the enzyme to influence the metabolism of the victim substrate; however, it is generally accepted that the victim drug must bind close to the center heme prosthetic and in close proximity to the activated-oxygen species. Although evidence exists to support both theories, it is well accepted that the mechanism of heteroactivation may be dependent upon the specific victim-perpetrator combination and the enzyme itself (due to the relative size of the active site).

The contribution of allosteric binding is not limited to atypical enzyme kinetics, such as heterotropic activation, but, in fact, has been linked to the mechanism-based inactivation of CYP2B4 by 9-ethynylphenanthrene (9EP) (Zhang et al., 2013). Zhang and colleagues found that quenching of 9EP fluorescence by unmodified or 9EP-modified CYP2B4 revealed two binding sites with distinct affinities, with the high-affinity site located on the protein periphery. The suggested location of this high-affinity site is at the entrance of a substrate access channel surrounded by the F' helix, β1–β2 loop region, and β4 loop; these and other protein coordinates were previously described as a potential allosteric site involved in the heterotropic activation of substrate-specific P450 enzymatic activity (Harlow and Halpert, 1998; Davydov et al., 2012; Shah et al., 2012). In the example of 9EP and the mechanism-based inactivation of CYP2B4, the high-affinity site functions as an allosteric site to enhance the efficiency of activation of the acetylenic group of 9EP and subsequent covalent modification of the active site Thr302 residue. Considering the well known consequences of P450 inhibition or mechanism-based inactivation in vivo (adverse drug interactions and toxicity) and the magnitude of the effects reported here, it is now prudent that the potential for allosteric effects of a drug molecule either on activation and/or inhibition of a P450 must also be considered during a preclinical DDI assessment. The methods and studies described here served as an example of an approach to define these types of interactions in vitro and in animal models prior to clinical advancement.

A recent perspective has served to reintroduce the importance of the kinetic phenomena of heterotropic activation (and atypical kinetics in general) and its impact to clinical pharmacokinetics of coadministered P450-metabolized drugs (Obach, 2012). Moreover, the perspective highlighted the importance of establishing new approaches toward the prediction of DDIs associated with atypical kinetics; particularly noteworthy was the notion of identifying preclinical models capable of mirroring the clinical setting. Importantly, there have been relatively limited examples of translating observed in vitro heteroactivation to in vivo animal models or humans. Tang et al. (1999) found an in vitro to in vivo translation for the stimulation of diclofenac metabolism by quinidine in monkeys; however, follow-up studies by Hutzler et al. (2001a,b) on the observed robust in vitro activation of CYP2C9-mediated flurbiprofen metabolism by dapsone showed only minimal effects in vivo. More importantly, Yang et al., (2012) demonstrated evidence for CYP3A allostery in healthy human volunteers by examining the interaction between fluconazole and MDZ. In a clinical study, Bayer et al. (2009) found that efavirenz rapidly increased MDZ metabolism, suggesting an acute activation of CYP3A-mediated MDZ metabolism that did not occur in a time frame suitable for induction of the enzyme via mRNA transcription. Subsequent in vitro analyses confirmed that the heteroactivation of CYP3A MDZ hydroxylase activity in human liver microsomes and in recombinant CYP3A4 and CYP3A5 (Keubler et al., 2012) was a V_{max}-driven effect linked to the presence of cytochrome b_5.

CYP3A4 is expressed in a variety of tissues, but is predominantly localized to the liver and intestine. Recent focus has also been placed on the metabolism and inhibition of CYP3A5, which is often expressed in the same tissues as CYP3A4, but may or may not have overlapping substrate specificities and/or show subtype-specific inhibition in vitro (Gorski et al., 1994). In addition, rat and murine CYP3A catalytic activity (or inhibition thereof) does not always translate to human CYP3A4 and CYP3A5 activity (Komura and Iwaki, 2008). For example, there have been reports (Kronbach et al., 1989; Perloff et al., 2000) indicating that murine metabolism of MDZ correlates more closely to human metabolism (MDZ to 1-OH-MDZ) than rat (MDZ to 1-OH-MDZ and 4-OH-MDZ in equal quantities). More recently, studies in humanized CYP3A4 transgenic mice using triazolam have shown stimulation of intestinal and hepatic CYP3A activity (van Waterschoot et al., 2009). In addition, Yamazaki et al. (2013) investigated the in vivo drug interaction between MDZ and thalidomide in mice with humanized livers and found a significant increase in the area under the curve for the production of 1-OH-MDZ in mice that were coadministered MDZ (intravenously) and thalidomide (orally). In an attempt to find alternative rodent models to examine the activation of CYP3A activity in vivo by VU0448187, we examined the hepatic clearance of MDZ in male CD-1 mice (n = 2; 1 mg/kg i.v.). The hepatic clearance of MDZ was moderate at 50 ml/min

per kilogram (hepatic blood flow ≈90 ml/min per kilogram) and 1-OH-MDZ was readily detected in mice at this dose employing LC-MS/MS analysis (data not shown). Due to the similarities in the metabolism of MDZ in humans and mice, these preliminary data will serve to direct future studies in which potential changes in MDZ clearance and exposure as well as circulating metabolite exposures can be monitored in the presence of VU0448187.

Historical data surrounding the homotropic and heterotropic activation of CYP3A4 enzymatic activity in vitro indicate that these observations are likely to be compound (or perpetrator) specific, as well as substrate (or victim) specific (Shou et al., 1994; Ngui et al., 2000; Wang et al., 2000; Kenworthy et al., 2001). CYP3A4 and CYP3A5 metabolize over half of all known drugs on the market today (Thummel and Wilkinson, 1998). The likelihood of patients receiving drugs and herbal remedies that are metabolized by this same enzymatic pathway is high (antibiotics, antidepressants, birth control, etc); in particular, the chance to precipitate a clinically relevant DDI in patients is significantly increased with disease states (e.g., neurologic disorders, tissue malignancies, and AIDS) that require concomitant administration of multiple medications, the metabolism of which may be catalyzed at topological sites within the CYP3A protein that are distinct from the MDZ binding site. Understanding the potential off-target activities of a drug or its metabolite(s) is crucial for drug development and clinical success (Hutzler et al., 2005). The discovery and development of $mGlu_5$ PAMs represents a novel approach in the treatment of schizophrenia. With the understanding that many patients are subjected to polypharmacy, in addition to the deleterious issues of patient compliance and drug overdose, the potential for DDIs (target- or off-mediated) is increased in this particular patient group. Although it is uncertain at this time whether the heterotropic activation of P450 enzymes is clinically relevant in terms of DDI potential, it is important to understand the effects of this phenomenon on increasing the clearance of parent drug and/or increasing circulating metabolite levels that may have pharmacological or toxicological significance. We have shown here that an in vitro observation of potent activation of CYP3A in multispecies liver and intestinal fractions by a novel $mGlu_5$ PAM can be translated to rodents in vivo, subsequently enabling the modeling of atypical clinical pharmacokinetics with relevant therapeutic agents.

Authorship Contributions

Participated in research design: Blobaum, Bridges, Byers, Mackie, Lavreysen, Steckler, Daniels.

Conducted experiments: Blobaum, Byers.

Contributed new reagents or analytic tools: Turlington, Mattmann, Morrison, Bartolomé, MacDonald, Stauffer.

Performed data analysis: Blobaum, Morrison, Mackie.

Wrote or contributed to the writing of the manuscript. Blobaum, Bridges, Morrison, Bartolomé, Jones, Niswender, Conn, Lindsley, Stauffer, Daniels.

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