A Screen of Approved Drugs Identifies the Androgen Receptor Antagonist Flutamide and Its Pharmacologically Active Metabolite 2-Hydroxy-Flutamide as Heterotropic Activators of Cytochrome P450 3A In Vitro and In Vivo

Anna L. Blobaum, Frank W. Byers, Thomas M. Bridges, Charles W. Locuson, P. Jeffrey Conn, Craig W. Lindsley, and J. Scott Daniels¹

Departments of Pharmacology (A.L.B., F.W.B., T.M.B., C.W.L., P.J.C., C.W.L., J.S.D) and Chemistry (C.W.L), Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical School, Nashville, Tennessee (C.W.L.)

Received February 24, 2015; accepted August 10, 2015

ABSTRACT

Once thought to be an artifact of microsomal systems, atypical kinetics with cytochrome P450 (CYP) enzymes have been extensively investigated *in vitro* and found to be substrate and species dependent. Building upon increasing reports of heterotropic CYP activation and inhibition in clinical settings, we screened a compound library of clinically approved drugs and various probe compounds to identify the frequency of heterotropism observed with different drug classes and the associated CYP enzymes thereof (1A2, 2C9, 2D6, and 3A4/5). Results of this screen revealed that the prescribed androgen receptor antagonist flutamide activated the intrinsic midazolam hydroxylase activity of CYP3A in human hepatic microsomes (66%), rat and human hepatocytes (36 and 160%, respectively), and *in vivo* in male Sprague-Dawley rats (>2-fold, combined area under the curve of primary rat *in vivo* midazolam metabolites). In addition, a screen of

the pharmacologically active metabolite 2-hydroxy-flutamide revealed that this principle metabolite increased CYP3A metabolism of midazolam in human microsomes (30%) and hepatocytes (110%). Importantly, both flutamide and 2-hydroxy-flutamide demonstrated a pronounced increase in the CYP3A-mediated metabolism of commonly paired medications, nifedipine (antihypertensive) and amiodarone (antiarrhythmic), in multispecies hepatocytes (100% over baseline). These data serve to highlight the importance of an appropriate substrate and *in vitro* system selection in the pharmacokinetic modeling of atypical enzyme kinetics. In addition, the results of our investigation have illuminated a previously undiscovered class of heterotropic CYP3A activators and have demonstrated the importance of selecting commonly paired therapeutics in the *in vitro* and *in vivo* modeling of projected clinical outcomes.

Introduction

The cytochrome P450 (CYP) 3A subfamily has been the source of significant drug-drug interactions (DDIs) in the clinic through the inhibition or induction of these enzymes (Lin and Lu, 1998; Hutzler et al., 2005). CYP3A4 and CYP3A5 enzymes are also subject to atypical (or non-Michaelis-Menten) kinetics and have been extensively investigated for differences in substrate specificity, subtype specific inhibition, and the influence of buffers, detergent, and cytochrome *b*₅ on enzymatic activity (Shou et al., 1999; Wang et al., 2000; Atkins, 2005; Jushchyshyn et al., 2005). Particularly for CYP3A4, structural and functional data exist to suggest that its active site is capable of accommodating both small and large molecules and more than one substrate or inhibitor at a time, contributing to the abundance of atypical

kinetics observed with this enzyme (Shou et al., 1994; Korzekwa et al., 1998; Hosea et al., 2000). More recently, several high-profile reports of clinical drug interactions have focused attention on the role of atypical CYP450 kinetics, including heterotropic effects, in acute clinical DDI scenarios (Egnell et al., 2003b; Obach, 2012; Yang et al., 2012). Although heterotropic activation or inhibition of the CYP3A family enzymes (as well as the CYP2C family) has received significant *in vitro* investigative attention (Hutzler and Tracy, 2002), little in the way of *in vivo* translation has been realized due to the lack of suitable animal models where the phenomenon has translated effectively (Tang et al., 1999; Hutzler et al., 2001). In addition, although the preponderance of *in vitro* atypical kinetics, such as heterotropic activation, is vast and there are established structure-activity relationship (SAR) data within common pharmacophores (Egnell et al., 2003a, 2005; Henshall et al., 2008), there are no real predictive trends *across* therapeutic drug classes (Hutzler and Tracy, 2002; Hutzler et al., 2005).

We have previously reported the use of an *in vitro* cassette methodology in human liver microsomes (HLMs) that is capable of simultaneously measuring CYP metabolism and the ability of

This work was supported by the National Institutes of Health [Grant U54MH084659].

¹Current affiliation: Sano Informed Prescribing, Franklin, Tennessee.
dx.doi.org/10.1124/dmd.115.064006.

ABBREVIATIONS: AUC, area under the curve; CYP, cytochrome P450; DDI, drug-drug interaction; HLM, human liver microsome; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDZ, midazolam; NADPH, β -nicotinamide adenine dinucleotide phosphate; 2-OH-Flu, 2-hydroxy-flutamide; 1-OH-MDZ, 1-hydroxy-midazolam; SD, Sprague-Dawley; VU0448187, 5-(4-fluorobenzyl)-2-[(3-fluorophenoxy)methyl]-4,5,6,7-tetrahydropyrazolo(1,5-a)pyrazine.

a compound of interest to specifically and selectively inhibit or enhance the activity of CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 (Blobaum et al., 2013). From this approach, we uncovered a series of structurally related compounds (positive allosteric modulators of metabotropic glutamate receptor 5) for the potential treatment of schizophrenia that significantly activated the midazolam (MDZ) hydroxylase activity of CYP3A in human hepatic and intestinal microsomes and hepatocytes and in vivo in rodents (Blobaum et al., 2013). Further investigation demonstrated that these metabotropic glutamate receptor 5 positive allosteric modulators were able to activate the CYP3A-mediated metabolism of the neuroleptic agent haloperidol (Haldol) by 20% over the baseline. Although the extent of activation of CYP3A activity was not as pronounced as what was observed for midazolam, the activation of a clinically relevant CYP3A4 substrate, such as haloperidol, suggests a potential relevance of atypical kinetic effects in this patient population and a need to consider both species and substrate selection.

The SPECTRUM library of compounds [MicroSource Discovery Systems Inc., Gaylordsville, CT; <http://www.msdiscovery.com> (Pantel et al., 2011; Kumar et al., 2014)] includes a wide range of clinically approved and structurally diverse compounds, including over 60% marketed drugs. In an attempt to understand the prevalence of CYP heterotropic activation among the top classes of administered drugs, we conducted a semiautomated functional screen of the SPECTRUM chemical library via a cocktail probe substrate assay design in HLM (Fig. 1). Among the compounds identified from this screen were two androgen receptor antagonists approved for the treatment of prostate cancer, including flutamide and nilutamide (Fig. 1) (Fig. 2) (Schmitt et al., 2001). Both compounds activated the midazolam hydroxylase activity of CYP3A in HLM. Interestingly, flutamide is rapidly metabolized by CYP1A2 to its active metabolite, 2-hydroxy-flutamide (2-OH-Flu) (Shet et al., 1997), which, when tested independently, was also shown to activate CYP3A activity. Herein, we describe the substrate- and species-specific characterization of these heterotropic effects in hepatic microsomes and hepatocytes with the probe substrate midazolam and relevant clinical concomitant

medications (such as nifedipine and amiodarone) (Fig. 2). The activation of nifedipine metabolism by flutamide is of particular interest, considering previous reports of CYP3A allostery and multiple binding sites with this substrate (Koley et al., 1997; Kenworthy et al., 1999). In addition, in vivo studies in male Sprague-Dawley (SD) rats revealed that flutamide is able to activate the midazolam hydroxylase of rat CYP3A as increases in circulating 1-hydroxy-midazolam (1-OH-MDZ) and 4-OH-MDZ were observed with flutamide pretreatment. These data reinforce the need for appropriate substrate and species selection in investigations of heterotropic effects when modeling in vitro and in vivo data to project clinical outcomes.

Materials and Methods

Chemicals and Enzyme Sources. β -Nicotinamide adenine dinucleotide phosphate (NADPH), MDZ, 1-OH-MDZ, 4-OH-MDZ, testosterone, 6 β -OH-testosterone, progesterone, 6 β -OH-progesterone, phenacetin, acetaminophen, diclofenac, 4-OH-diclofenac, dextromethorphan, dextrophan, nifedipine, oxidized nifedipine, amiodarone, desethylamiodarone, and miconazole were all purchased from Sigma-Aldrich (St. Louis, MO). 5-(4-Fluorobenzyl)-2-[(3-fluorophenoxy)methyl]-4,5,6,7-tetrahydropyrazolo(1,5-a)pyrazine (VU0448187) and 2,4-difluorophenyl[2-(phenoxy)methyl]-6,7-dihydrooxazolo(5,4-c)pyridin-5(4H-yl)methanone were synthesized internally at the Vanderbilt Center for Neuroscience Drug Discovery (Blobaum et al., 2013). Confirmed activators from the screen of the SPECTRUM collection (2320 compounds) were researched for availability and purchased through Sigma-Aldrich or LKT Laboratories (St. Paul, MN). Human liver microsomes (150-donor pool, mixed gender), human intestinal microsomes (20-donor pool, mixed gender), and male murine, rat, minipig, and guinea pig liver microsomes were purchased from BD Biosciences (Woburn, MA) or BioreclamationIVT (Baltimore, MD). Human (20-donor pool, mixed gender), male SD rat, and male CD-1 mouse hepatocytes were purchased from BioreclamationIVT. All solvents used in the bioanalysis of in vitro and in vivo samples were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA) and were of high-performance liquid chromatography (HPLC) grade.

HLM Screen of the SPECTRUM Library. SPECTRUM compounds were plated (96-well, duplicate, 10 mM dimethylsulfoxide) at the Vanderbilt University High Throughput Screening Center using an ECHO 555 automated

Fig. 1. HLM screening tree of the SPECTRUM collection of chemical compounds to identify potential heterotropic activators of different CYP enzymes.

Fig. 2. Structures of CYP3A enzyme heterotropic activators of interest identified from single-point concentration screens in HLM (top) and select CYP3A substrates (bottom).

liquid handler (Labcyte Inc., Sunnyvale, CA). The reported pan-P450 inhibitor miconazole and internal positive controls for CYP3A (VU0448187) and CYP2C (2,4-difluorophenyl[2-(phenoxyethyl)-6,7-dihydrooxazolo(5,4-c)pyridin-5(4H-yl)methanone] heterotropic activation were included in each 96-well plate. Compounds were initially screened at a single concentration (10 μ M), which afforded approximately 35 test compounds/plate, including the control activators for CYP3A and CYP2C. The compound screen employed the well described cocktail methodology executed in HLM (Zientek et al., 2008; Blobaum et al., 2013), which was designed to simultaneously monitor the propensity of a test compound to inhibit (or activate in the present account) the intrinsic enzymatic activity of CYP1A2, CYP2C9, CYP2D6, and combined CYP3A4/5. Compounds that were identified as CYP activators ($\geq 20\%$ over the baseline) were subsequently confirmed in a full concentration-response experiment in HLM using either the aforementioned cocktail or a discrete assay (wherein a single probe substrate and metabolite combination per CYP was monitored *vide infra*). Following confirmation of activation, compounds demonstrating activation of a single CYP were crossreferenced for the therapeutic area and/or patient population in which a DDI risk would be high given common drug pairings.

In Vitro Assessment of Heterotropic Activation. Sprague-Dawley rat (male) and human (mixed gender, 150-donor pool) hepatic microsomes (final concentration of 0.1 mg/ml) were suspended in a potassium phosphate buffered solution (0.1 M, pH 7.4, 37°C) subsequently fortified with MDZ (2 μ M). The reaction mixtures were evenly distributed into 96-well plates, and compounds from the SPECTRUM collection (duplicate assays, 0.1–30 μ M) were preincubated (15 minutes) with shaking at 37°C. The reactions were initiated with the addition of NADPH (1 mM) and allowed to incubate for an additional 8 minutes prior to quenching with two volumes of ice-cold acetonitrile containing an internal standard (carbamazepine or tolbutamide). The plates were centrifuged (4000g, 4°C, 10 minutes), and the supernatant was subsequently removed and diluted in water (1:4 v/v) in preparation for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. The concentrations of each analyte were determined by the comparison of total ion chromatogram (TIC) area counts (mass spectrometry reaction monitoring) against a 12-point standard curve. Activation kinetics were compared with respect to buffer-only addition (control), and this enzymatic activity, as measured by metabolite formation (1-OH-MDZ, 4-OH-MDZ, 6 β -OH-testosterone, 6 β -OH-progesterone, oxidized nifedipine, and desethylamiodarone), was set to 100%. All experiments were performed in duplicate unless otherwise noted. Multispecies hepatocytes (final reaction, 0.5×10^6 cells/ml) were thawed, and incubations with the compounds were carried out for 10 minutes prior to quenching with ice-cold acetonitrile containing the internal standard and preparation for LC-MS/MS analysis as above.

Final concentrations of CYP3A substrates for microsomal and hepatocyte incubations were as follows: testosterone (25 μ M), progesterone (50 μ M), nifedipine (25 μ M), and amiodarone (50 μ M). These concentrations were chosen to be reflective of reported K_m values, and the protein concentration, time of incubation with NADPH (microsomes), and reconstitution ratios for LC-MS/MS were optimized for each substrate and metabolite combination. For analytes that required positive ionization, the internal standard was carbamazepine. For those in the negative ionization mode, the internal standard was tolbutamide. Concentrations of each metabolite (1-OH-MDZ, 4-OH-MDZ, 6 β -OH-testosterone, 6 β -OH-progesterone, oxidized nifedipine, and desethylamiodarone) were quantitated

using a 12-point standard curve, and data were represented as the percentage of the remaining control activity (*vide supra*).

In Vivo Pharmacokinetic Studies with Midazolam. Midazolam is metabolized by rat CYP3A1/2 *in vitro* and *in vivo* to two primary metabolites: 1-OH-MDZ and 4-OH-MDZ (Kronbach et al., 1989). Flutamide was prepared in 20% beta-cyclodextrin (BCD) (w/v) in water (1.5 mg/ml) for administration (15 mg/kg PO; $n = 2$) to dual-cannulated (jugular and carotid) male SD rats (Harlan Laboratories, Indianapolis, IN) 40 minutes prior to administration of MDZ (10 mg/kg i.p.), which was formulated in a 10% EtOH/50% polyethylene glycol 400/40% saline ratio (v/v; 4 mg/ml). The flutamide dose and pretreatment time were selected based on previously reported pharmacokinetic parameters in rats (Zuo et al., 2002). In addition to predose sampling, blood was serially collected over EDTA at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours postdose. Plasma was isolated via centrifugation (1700g, 4°C, 5 minutes) and stored at -80°C until LC-MS/MS analysis. The resulting plasma samples were protein precipitated by employing three volumes of ice-cold acetonitrile containing the internal standard. The samples were centrifuged (4000g, 4°C, 5 minutes), and the supernatants were transferred and diluted 1:1 (v/v) for LC-MS/MS analysis. Flutamide, 2-OH-Flu, MDZ, 1-OH-MDZ, and 4-OH-MDZ were monitored from each experiment, and the concentrations (ng/ml) of each analyte were determined using a matrix-matched 10-point standard curve.

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 (Carboron, NC). Analytes were separated by gradient elution using a thermostated (40°C) C18 column (Fortis 3.0 $\times$ 50 mm, 3 μ m; Fortis Technologies Ltd, Cheshire, UK). Mobile phase A was 0.1% formic acid (aqueous), and mobile phase B was 0.1% formic acid in acetonitrile. For MDZ and 1-OH-MDZ/4-OH-MDZ, the gradient started at 10% B after a 0.2-minute hold, was linearly increased to 90% B over 1.3 minutes, and returned to 10% B in 0.1 minutes, followed by re-equilibration (0.9 minutes). The total run time was 2.5 minutes, and the HPLC flow rate was 0.5 ml/min. For other substrate/metabolite combinations, the gradient started at 30% B and was linearly increased to 90% B. 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-ion spray source in the positive or negative ionization mode (5.0-kV spray voltage). All data were analyzed using AB Sciex Analyst 1.5.1 software. For analytes that required positive ionization, the internal standard was carbamazepine; for those in the negative ionization mode, the internal standard was tolbutamide.

Results

In Vitro HLM-Based Screen of the SPECTRUM Collection. The SPECTRUM library of compounds includes >2000 marketed drugs (60%), natural products (25%), and pharmacological probes (15%). Single-point (10 μ M) screening of compounds from the SPECTRUM collection in HLM identified a number of confirmed activators, which spanned various structural motifs, drug classes, and therapeutic areas. More than 90% of the compounds identified activated CYP3A activity

(with the majority of the remaining compounds affecting CYP2C9) through enhancement of the intrinsic MDZ hydroxylase activities of CYP3A4 and CYP3A5. The confirmed drug classes that activated CYP3A-mediated MDZ hydroxylase activity included, but were not solely limited to, antiandrogens, antibiotics, anti-inflammatories, anti-malarials, and insecticides (Table 1). Two androgen receptor antagonists clinically used in the treatment of prostate cancer, including flutamide and nilutamide, were identified from this initial screen and represent a therapeutic area and patient population, in which many paired medications would also be metabolized by CYP3A and therefore may influence the projected clinical outcomes.

In Vitro Characterization of CYP3A Heterotropic Activation of MDZ Metabolism by Flutamide. Flutamide (Fig. 2), which was identified as a heterotropic activator of the MDZ hydroxylase activity of CYP3A in the cocktail screen of the SPECTRUM library, underwent a full concentration-response analysis (Fig. 3A) in HLM following the robust activation observed at the initial single concentration (10 μ M). Results of this experiment revealed flutamide to increase the intrinsic CYP3A4-mediated MDZ hydroxylase activity in HLM by 66% over the baseline. The pharmacologically active metabolite of flutamide, 2-OH-Flu, was also screened in a full concentration-response experiment in HLM and was found to increase the intrinsic CYP3A MDZ hydroxylase activity in vitro in HLM (~30%) (Fig. 3C). To our knowledge, these data provide a unique example of a parent drug and its metabolite that demonstrate substrate-dependent heterotropic activation at the same target CYP. As heterotropic effects are often dependent on the choice of probe substrate, other typical CYP3A substrates were examined. As depicted in Fig. 3, B and D, neither the parent, flutamide, nor its metabolite, 2-OH-Flu, increased the metabolism of testosterone (or progesterone), a finding that is consistent with the substrate dependence of the observed heterotropic activation of CYP3A.

Although the substrate dependence of atypical kinetics with CYPs has been well defined, the species dependence of heterotropic effects has not been as extensively investigated. We therefore examined the effect of species selection on the heterotropic activation of CYP3A midazolam hydroxylase activity by flutamide. Our data indicate that

flutamide activates the MDZ hydroxylase activity of CYP3A human, rat, and mouse hepatic microsomes or hepatocytes, but not in minipigs or guinea pigs (Fig. 4A). In fact, flutamide was found to inhibit the formation of 1-OH-MDZ in guinea pig hepatic microsomes. Here, species differences in CYP3A isoforms and substrate selectivity and affinity may influence the ability of an activator, such as flutamide, to elicit an effect on certain CYP3A substrates (Martignoni et al., 2006). Interestingly, the activation of MDZ hydroxylase metabolism by flutamide (Fig. 4A) and 2-OH-Flu (Fig. 4B) was more pronounced in nonclinical species hepatocytes when compared with liver microsomes, which was also demonstrated for human microsomes and hepatocytes. In the case of flutamide, the maximal activation was achieved at 3 μ M in HLM and 10 μ M in hepatocytes; however, total levels of 1-OH-MDZ were higher in hepatocytes compared with HLM at all flutamide concentrations assayed. Low levels of CYP3A activation, as measured by increased metabolism of MDZ (Fig. 4B), were noted for 2-OH-Flu in hepatic microsomes (30% at 10 μ M), but were significantly elevated in human hepatocytes at the same concentration (100%). Decreased CYP3A-mediated MDZ hydroxylase activity was observed in rat hepatocytes compared with human hepatocytes; however, whereas 1-OH-MDZ is the primary human metabolite, 4-OH-MDZ is produced in equal or greater amounts compared with 1-OH-MDZ in rats (Kotegawa et al., 2002). Taken together and with regards to this particular activator/substrate scenario, these data support the substrate and species dependency of heterotropic effects in vitro and provide confirmation for the choice of SD rat as an appropriate nonclinical species to attempt to model heterotropic activation of CYP3A activity by flutamide in vivo.

In Vivo Rodent Models of Flutamide Activation of CYP3A-Mediated MDZ Metabolism. We previously demonstrated that rodents may be a useful nonclinical species to model clinically relevant heterotropic CYP activation (Blobaum et al., 2013). In the present report, male SD rats were pretreated with flutamide (PO, 15 mg/kg) prior to administration of the probe substrate MDZ (i.p., 10 mg/kg) and serial plasma exposure of MDZ and its primary metabolites were obtained. Systemic concentrations of the two principle circulating

TABLE 1

Representative compounds identified as heterotropic activators of CYP activity during single-point screening of the SPECTRUM library in HLM

Drug	Classification	CYP3A4/5	CYP2C9	CYP1A2	CYP2D6
Flutamide	Antiandrogen	98	—	—	—
Rosiglitazone	Antidiabetic	37	—	71	31
Metformin hydrochloride	Antidiabetic	63	23	32	63
Coenzyme B12	Vitamin	56	—	—	—
Pyruvium pamoate	Anthelmintic	54	—	—	—
Diclofenac sodium	Anti-inflammatory	—	54	—	—
Avocatin B	Antibacterial, antifungal	52	—	—	—
Nilutamide	Antiandrogen	49	—	—	—
Deracoxib	Anti-inflammatory, antiarthritic, cyclooxygenase 2 inhibitor	48	—	—	—
Ribavirin	Antiviral	41	45	—	46
Tolbutamide	Antidiabetic	45	—	—	22
Tranylcypromine sulfate	Antidepressant, monoamine oxidase inhibitor	43	—	—	—
Diethylcarbamazine citrate	Anthelmintic	—	—	43	—
Moxifloxacin hydrochloride	Antibacterial	24	41	—	41
Tacrine hydrochloride	Anticholinesterase, cognitive adjuvant, K channel blocker	40	—	—	—
Celecoxib	Antiarthritic, cyclooxygenase 2 inhibitor	39	—	28	—
Propantheline bromide	Anticholinergic	—	38	—	—
Orphenadrine citrate	Muscle relaxant (skeletal), antihistaminic	38	28	—	45
Sulfantran	Antibacterial	37	—	—	—
Canrenone	Aldosterone antagonist, antifibrogenic	37	—	33	—

Percentage of activation over baseline. The screening concentration for each compound was 10 μ M. Values are expressed as the percentage of activity remaining over the baseline and are the average of two replicates. Positive hits were required to have duplicate data points $\geq 20\%$ increase over baseline. Activity is calculated based on the metabolism of CYP-specific probe substrates to their respective metabolites. The compounds included in the table are representative of the diversity of the therapeutic areas and drug classes that exhibited heterotropic activation of CYP activity. Inhibition of enzymatic activity or no observed effect is designated with a dashed line.

Fig. 3. The substrate-specific effects of flutamide and 2-OH-Flu on CYP3A metabolism in human liver microsomes. Increases in metabolite formation for 1-OH-MDZ (A and C) or 6β-OH-testosterone (B and D) are expressed as the percentage of activity of control. Mean ($\pm$ S.E.M.) values for the maximal activation of midazolam metabolism (over baseline, 100%) with flutamide and 2-OH-Flu were 66% (5.76) and 32% (2.29), respectively.

metabolites of MDZ (1-OH-MDZ and 4-OH-MDZ) were determined along with circulating concentrations of the CYP3A activators, MDZ, flutamide, and 2-OH-Flu. In rats receiving flutamide, higher concentrations of both 1-OH-MDZ and 4-OH-MDZ were noted (T_{max} , 7 minutes) when compared with rats pretreated with vehicle prior to MDZ administration (Fig. 5, A and B). Concentrations of 4-OH-MDZ were higher than those of 1-OH-MDZ in both treatment groups. The total

combined area under the curve (AUC) of the primary hydroxylated MDZ metabolites was approximately 2-fold higher in rodents pretreated with flutamide compared with vehicle alone (Fig. 5C). Flutamide and 2-OH-Flu concentrations were also monitored from the treatment group receiving the oral dose of flutamide (Fig. 5D) to enable an understanding of the *in vivo* levels of each activator in rodents. For flutamide, maximal concentrations were achieved at the

Fig. 4. Species dependency of heterotropic effects with flutamide (A) and 2-OH-Flu (B) in multispecies hepatic microsomes and hepatocytes. 1-OH-MDZ levels were quantitated, and the results were expressed as the percentage activity of control: HLM (150 donor pool) human hepatocytes (20 donor pool, mixed gender); SD rat, Gottingen minipig, and guinea pig hepatic microsomes and hepatocytes were obtained from male animals (10–20 pools). Mean ($\pm$ S.E.M.) values over the baseline (defined as 100%) achieved for flutamide activation of MDZ metabolism were 160% (22.9), human hepatocytes; 66% (5.76), human liver microsomes; and 35% (6.97), rat hepatocytes. The corresponding mean ($\pm$ S.E.M.) values for 2-OH-Flu were 111% (2.67), 30% (4.29), and 36% (6.97).

7-minute time point post-MDZ dose and declined to negligible levels by 4 hours. Likely due to an apparent longer extravascular plasma half-life of 2-OH-Flu (not determined) relative to the parent, a significantly higher C_{max} of this metabolite was observed at 2 hours postdose (T_{max}), and the systemic plasma concentrations were sustained well past 7 hours postdose. The total C_{max} values for flutamide and 2-OH-Flu were 5.1 and 5.6 μ M, respectively. Importantly, the *in vitro* activation of CYP3A was observed at similar magnitudes for both compounds. From these data, it may be difficult to delineate the relative contributions of flutamide versus 2-OH-Flu, with regards to increases in the levels of 1-OH-MDZ and 4-OH-MDZ since MDZ is effectively cleared within 2 hours, and differences between the two treatment groups could only be monitored in this time range from a single MDZ dose. Although it remains the subject of investigation, the extended apparent half-life of 2-OH-Flu may have contributed to an increase in circulating MDZ metabolite levels upon readministration. Combined total plasma concentrations of both flutamide and 2-OH-Flu, specifically over the first four time points, range from 7.0 to 9.0 μ M, and increases in circulating MDZ metabolites were similar over the initial blood sampling times post-dose.

Characterization of CYP3A Heterotropic Activation in Hepatocytes with Flutamide and Commonly Paired Clinical CYP3A Substrates. Flutamide and nilutamide are used toward the treatment of prostate cancer and act by competing with testosterone for binding to the androgen receptor. The antihypertensive nifedipine as well as the antiarrhythmic amiodarone are CYP3A substrates and maintenance therapies commonly paired in this patient population. Due to these agents' narrow therapeutic window, both are subject to clinical DDIs through the induction of CYP3A4 (Latini et al., 1984; Chung et al., 1987). Rodent and human hepatocytes were incubated with either a pan-P450 inhibitor (miconazole), our structurally unrelated CYP3A activator VU0448187, or flutamide and its metabolite 2-OH-Flu in a full concentration-response experiment with the commonly paired CYP3A substrates nifedipine and amiodarone. VU0448187 and flutamide achieved a similar maximal activation of CYP3A-mediated nifedipine metabolism in human hepatocytes (~50% over baseline) at low micromolar concentrations (3 or 1 μ M, respectively) (Fig. 6A). 2-OH-Flu demonstrated maximal activation (20%) over the same

concentration range. Species differences in heterotropic activation with VU0448187, flutamide, and 2-OH-Flu were observed, such that maximal activation was less in rat hepatocytes (30%; Fig. 6B) and higher in mouse hepatocytes (>100% over baseline; Fig. 6C) when compared with data generated in human hepatocytes. Amiodarone metabolism by CYP3A to desethylamiodarone was also examined in hepatocytes and was demonstrated to be increased in the presence of flutamide and 2-OH-Flu (Fig. 6D, rat hepatocytes).

Discussion

Heterotropic activation of cytochromes P450 has been extensively investigated *in vitro*, the findings of which have broadened our understanding of the variables that influence heterotropism. Besides influences of substrate and species variability in heterotropic activation, it is now appreciated that subtle alteration(s) to cofactor fortification, partner-catalytic proteins, and tissue and subcellular fraction selection greatly impact the magnitude of activation (Hutzler and Tracy, 2002). Although instances of clinical DDIs resulting from heterotropic activation and inhibition have been reported (Yang et al., 2012), we lack new approaches to adequately predict clinical outcomes of existing *in vitro* kinetic data. As was the case with the non-nucleoside reverse transcriptase inhibitor efavirenz and the report of an unexpected clinical heterotropic activation of CYP3A (Bayer et al., 2009), such findings underscore the ability of atypical kinetics to influence the pharmacokinetics of acutely administered drugs. Importantly, a retrospective investigation confirmed activation in recombinant CYP3A4/5 and HLM (Keubler et al., 2012). Efavirenz data notwithstanding, limited examples exist where heterotropic activation data generated in recombinantly expressed CYPs and/or hepatic subcellular fractions have successfully correlated in nonclinical species *in vivo* or in humans (Hutzler and Tracy, 2002). Many variables could account for this lack of *in vitro* to *in vivo* correlation, but ultimately, the correlation may depend on the catalytic efficiency (V_{max}) of CYP activation achieved, the affinity (K_m) of the substrate and/or activator for the affected CYP or the biochemical efficiency of the catalytic mechanism within varied systems (e.g., microsomes compared with hepatocytes) (Houston and Kenworthy, 2000). As for the lack of nonclinical-to-clinical translation

Fig. 5. Activation of MDZ metabolism in SD rats following pretreatment with flutamide. Flutamide was administered to male SD rats (15 mg/kg PO) 40 minutes prior to MDZ administration (10 mg/kg i.p.). Plasma concentrations of MDZ, 1-OH-MDZ, 4-OH-MDZ, flutamide, and 2-OH-Flu were determined by LC-MS/MS analysis. Time-concentration pharmacokinetic curves are shown for 1-OH-MDZ (A) and 4-OH-MDZ (B). A change in the total combined AUC for the hydroxylated MDZ metabolites is shown in (C). Plasma concentrations of flutamide and 2-OH-Flu are shown in (D). Experiments were performed in duplicate. Concentrations are in ng/mL.

Fig. 6. Activation of CYP3A metabolism of nifedipine in human (A), SD rat (B), and CD-1 mouse (C) hepatocytes by flutamide, 2-OH-flutamide, or VU0448187. Maximal inhibition by the pan-P450 inhibitor miconazole is also depicted. CYP3A activation of amiodarone was performed in SD rat hepatocytes (D). Maximal inhibition or activation of metabolism is expressed as the percentage of control, with the concentration producing the maximal response provided for each compound shown (μM). Mean ($\pm$ S.E.M.) values over baseline (defined as 100%) achieved in each species are as follows for (A) VU0448187, 54% (9.39), flutamide, 48% (12.3), and 2-OH-flutamide, 21% (3.70); (B) VU0448187, 37% (0.841), flutamide, 37% (0.388), and 2-OH-flutamide, 27% (3.32); (C) VU0448187, 113% (12.5), flutamide, 98% (11.9), and 2-OH-flutamide, 76% (2.93); and (D) VU0448187, 32% (14.0), flutamide, 27% (1.57), and 2-OH-flutamide, 37% (3.48).

of heterotropic activation, this may be simply linked to the species differences in CYP metabolism of the particular probe substrate (or victim drug). Lastly, the magnitude of an ensuing DDI may also be linked to the particular perpetrator-victim drug combination and the therapeutic window of the victim drug within a pharmacological class.

To understand the extent of heterotropic CYP activation across drug classes, we screened the SPECTRUM collection of over 2000 compounds (60% being approved drugs) in a cocktail metabolic stability assay in HLM that was modified to expose CYP activators. Of the compounds identified, the majority were classified as activators of CYP3A and belonged to different drug classes. The discovery that the antiandrogen flutamide activated CYP3A4/5 in HLM and hepatocytes is particularly noteworthy, considering that several commonly paired medications with this agent are metabolized primarily through CYP3A enzymes and display narrow clinical therapeutic margins, where alterations in their metabolism (e.g., induction) could result in a loss of medication effectiveness due to reductions in systemic exposure. Alternatively, an apparent activator may increase the hepatic clearance of a drug, which could result in a significant increase in an active metabolite, toxic reactive intermediate, or toxic metabolite that could precipitate an adverse drug reaction. Further, victim drugs cleared predominantly via hepatic metabolism and possessing low to moderate hepatic extraction ratios would be of particularly high risk due to their sensitivity to increases in hepatic intrinsic clearance (CL_{int}), which could result from CYP3A activation. It is possible that the clinical manifestation of an acute exposure to a CYP activator may resemble the multiple-dose exposure to a CYP inducer, both of which would result in an increase in the apparent enzymatic activity of a CYP (e.g., CYP3A4) and a subsequent reduction in plasma exposure. Importantly, the effects

of CYP activation on the systemic exposure of a victim drug could happen immediately following a single administration and therefore precipitate an *acute* DDI for those (victim) drugs that are metabolized by the target CYP.

A multitude of clinical DDIs are manifested through the induction of CYP enzymes. Drugs such as nifedipine (antihypertensive) and amiodarone (antiarrhythmic) are subject to DDI scenarios in patients when coadministered with potent CYP3A4 inducers due to reductions in plasma exposures of the parent drug (nifedipine) (Chung et al., 1987) or increases in the formation of pharmacologically active and hepatotoxic metabolites (amiodarone) (Latini et al., 1984). Both drugs are often prescribed to prostate cancer patients receiving a polypharmacy approach to treatment (Fabre et al., 1993; Patki et al., 2003) and are metabolized primarily by CYP3A4. We therefore selected these clinically relevant alternative CYP3A substrates to investigate the potential heterotropic effects by flutamide in vitro. Interestingly, both flutamide and its pharmacologically active metabolite, 2-OH-Flu, increased the CYP3A-mediated metabolism of nifedipine and amiodarone in multispecies hepatocytes, resulting in the increased production of their primary metabolites (oxidized nifedipine or desethylamiodarone).

Importantly, in this current report, significant matrix and species differences in CYP3A activation by flutamide (and 2-OH-Flu) were observed for midazolam, nifedipine, and amiodarone when levels of substrate-specific metabolites were measured in hepatic microsomes and hepatocytes. Higher overall levels of activation were achieved in hepatocytes relative to liver microsomes and, generally, human hepatocytes were observed to have a higher activation potential when compared with rodent hepatocytes. Interestingly, in the case of the flutamide/MDZ pair of activator and substrate, minipig and guinea pig

microsomes (Fig. 4) and hepatocytes (not shown) did not activate MDZ metabolism at any concentration of flutamide or its metabolite. It is of note that the human donor pools were mixed age and gender and consisted of either 150 donors (microsomes) or 20 donors (hepatocytes), with variations in CYP3A activity and CYP3A5 polymorphisms, whereas all other species microsomes/hepatocytes were small pools of only male animals. The species and gender differences in the heterotropic activation of CYP3A activity appear to be related to multiple factors, including the choice of activator and substrate, subcellular fraction or cellular source examined, cofactor enrichment, and species differences in CYP3A expression, polymorphism, and substrate specificity, all of which remain under investigation in our laboratory.

Although our data in hepatocytes suggest that mouse may be a more appropriate model to investigate the heterotropic effects of flutamide on CYP3A-mediated metabolism of nifedipine (Fig. 6), SD rat was a more favorable preclinical species for examining the potential in vivo consequences of CYP3A activation of midazolam metabolism by flutamide. Although MDZ hepatic clearance in a rat is highly dependent on the specific rat strain used for a given study, MDZ is generally considered to be a high clearance compound in rats (55–80 ml/min per kg), with near equal conversion to 1-OH-MDZ and 4-OH-MDZ (Kotegawa et al., 2002). For this reason, changes in MDZ clearance in the presence of a potential activator may be negligible or supra-hepatic from an i.v. administration. To this end, we previously demonstrated that changes in MDZ and metabolite levels can be effectively obtained from i.p. dosing of MDZ, with pretreatment of the activator (Blobaum et al., 2013). In the present study, rats pretreated with flutamide exhibited an increase in the total combined AUC of the primary hydroxylated MDZ metabolites (1-OH-MDZ and 4-OH-MDZ), which was approximately 2-fold higher when compared with vehicle alone (Fig. 5). Notably, we observed no change in T_{max} , but a nearly 2-fold decrease in plasma C_{max} and 1.5-fold decrease in AUC of parent MDZ levels (data not shown). Although it is possible that flutamide may be affecting other clearance pathways of MDZ in vivo (e.g., inhibition of glucuronidation that would result in increases in metabolite levels), we did not observe these effects with the CYP3A activator VU0448187 when examined in previous studies (Blobaum et al., 2013). However, the in vivo results with flutamide may be complicated by the presence of its active metabolite (2-OH-Flu). To our knowledge, the ability of 2-OH-Flu to activate CYP3A metabolism in vitro represents a significant finding in the heterotropism field, as metabolites of parent activators are often found to be inactive. The relative contribution of 2-OH-Flu to any changes in MDZ or metabolite levels in vivo is unknown at this time, but further studies are warranted.

Appropriate in vitro assessment and choice of suitable in vivo models are paramount in predicting clinical DDIs where CYPs represent the primary route of metabolic clearance. The potential immediate and transient effects on CYP activity following a single clinical exposure to a heterotropic activator serves to underscore the need for innovative models in the prediction of this category of DDI. The results of our investigation have not only illuminated a previously undiscovered class of heterotropic CYP3A activators through the SPECTRUM collection HLM screen, but have also demonstrated the importance of appropriate substrate and species selection in the in vitro and in vivo modeling of the projected clinical outcome.

Acknowledgments

The Microsource SPECTRUM collection was obtained through the assistance of Dr. Paige Vinson and the Vanderbilt High Throughput Screening Center, which receives institutional support through the Vanderbilt Institute of Chemical Biology.

Authorship Contributions

Participated in research design: Blobaum, Bridges, Byers, Daniels.
Conducted experiments: Blobaum, Byers.
Contributed new reagents or analytic tools: Bridges.
Performed data analysis: Blobaum, Byers, Daniels.
Wrote or contributed to the writing of the manuscript: Blobaum, Bridges, Locuson, Conn, Lindsley, Daniels.

References

- Atkins WM (2005) Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions. *Annu Rev Pharmacol Toxicol* **45**:291–310.
- Bayer J, Heinrich T, Burhenne J, Haefeli WE, and Mikus G (2009) Potential induction of CYP3A4 after a single dose of efavirenz using midazolam pharmacokinetics as a marker. *Basic Clin Pharmacol Toxicol* **104**:515.
- Blobaum AL, Bridges TM, Byers FW, Turlington ML, Mattmann ME, Morrison RD, Mackie C, Lavreysen H, Bartolomé JM, and Macdonald GJ, et al. (2013) Heterotropic activation of the midazolam hydroxylase activity of CYP3A by a positive allosteric modulator of mGlu5: in vitro to in vivo translation and potential impact on clinically relevant drug-drug interactions. *Drug Metab Dispos* **41**:2066–2075.
- Chung M, Reitberg DP, Gaffney M, and Singleton W (1987) Clinical pharmacokinetics of nifedipine gastrointestinal therapeutic system. A controlled-release formulation of nifedipine. *Am J Med* **83**:10–14.
- Egnell AC, Eriksson C, Albertson N, Houston B, and Boyer S (2003a) Generation and evaluation of a CYP2C9 heteroactivation pharmacophore. *J Pharmacol Exp Ther* **307**:878–887.
- Egnell AC, Houston B, and Boyer S (2003b) In vivo CYP3A4 heteroactivation is a possible mechanism for the drug interaction between felbamate and carbamazepine. *J Pharmacol Exp Ther* **305**:1251–1262.
- Egnell AC, Houston JB, and Boyer CS (2005) Predictive models of CYP3A4 heteroactivation: in vitro-in vivo scaling and pharmacophore modeling. *J Pharmacol Exp Ther* **312**:926–937.
- Fabre G, Julian B, Saint-Aubert B, Joyeux H, and Berger Y (1993) Evidence for CYP3A-mediated N-deethylation of amiodarone in human liver microsomal fractions. *Drug Metab Dispos* **21**:978–985.
- Henshall J, Galetin A, Harrison A, and Houston JB (2008) Comparative analysis of CYP3A heteroactivation by steroid hormones and flavonoids in different in vitro systems and potential in vivo implications. *Drug Metab Dispos* **36**:1332–1340.
- Hosea NA, Miller GP, and Guengerich FP (2000) Elucidation of distinct ligand binding sites for cytochrome P450 3A4. *Biochemistry* **39**:5929–5939.
- Houston JB and Kenworthy KE (2000) In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metab Dispos* **28**:246–254.
- Hutzler JM, Frye RF, Korzekwa KR, Branch RA, Huang SM, and Tracy TS (2001) Minimal in vivo activation of CYP2C9-mediated flurbiprofen metabolism by dapsone. *Eur J Pharm Sci* **14**:47–52.
- Hutzler JM and Tracy TS (2002) Atypical kinetic profiles in drug metabolism reactions. *Drug Metab Dispos* **30**:355–362.
- Hutzler M, Messing DM, and Wienkers LC (2005) Predicting drug-drug interactions in drug discovery: where are we now and where are we going? *Curr Opin Drug Discov Devel* **8**:51–58.
- Jushchyshyn MI, Hutzler JM, Schrag ML, and Wienkers LC (2005) Catalytic turnover of pyrene by CYP3A4: evidence that cytochrome b5 directly induces positive cooperativity. *Arch Biochem Biophys* **438**:21–28.
- Kenworthy KE, Bloomer JC, Clarke SE, and Houston JB (1999) CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. *Br J Clin Pharmacol* **48**:716–727.
- Keubler A, Weiss J, Haefeli WE, Mikus G, and Burhenne J (2012) Drug interaction of efavirenz and midazolam: efavirenz activates the CYP3A-mediated midazolam 1'-hydroxylation in vitro. *Drug Metab Dispos* **40**:1178–1182.
- Koley AP, Robinson RC, Markowitz A, and Friedman FK (1997) Drug-drug interactions: effect of quinidine on nifedipine binding to human cytochrome P450 3A4. *Biochem Pharmacol* **53**:455–460.
- Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37**:4137–4147.
- Kotegawa T, Laurijsens BE, Von Moltke LL, Cotreau MM, Perloff MD, Venkatakrisnan K, Warrington JS, Granda BW, Harmatz JS, and Greenblatt DJ (2002) In vitro, pharmacokinetic, and pharmacodynamic interactions of ketoconazole and midazolam in the rat. *J Pharmacol Exp Ther* **302**:1228–1237.
- Kronbach T, Mathys D, Umeno M, Gonzalez FJ, and Meyer UA (1989) Oxidation of midazolam and triazolam by human liver cytochrome P450III_{A4}. *Mol Pharmacol* **36**:89–96.
- Kumar KK, Lowe EW, Jr, Aboud AA, Neely MD, Redha R, Bauer JA, Odak M, Weaver CD, Meiler J, and Aschner M, et al. (2014) Cellular manganese content is developmentally regulated in human dopaminergic neurons. *Sci Rep* **4**:6801.
- Latini R, Tognoni G, and Kates RE (1984) Clinical pharmacokinetics of amiodarone. *Clin Pharmacokinet* **9**:136–156.
- Lin JH and Lu AY (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet* **35**:361–390.
- Martignoni M, Groothuis GM, and de Kanter R (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol* **2**:875–894.
- MicroSource Discovery Systems, Inc.
- Obach RS (2012) Heterotropic effects on drug-metabolizing enzyme activities: in vitro curiosity emerges as a clinically meaningful phenomenon (perhaps?). *Clin Pharmacol Ther* **91**:385–387.
- Pantel J, Williams SY, Mi D, Sebag J, Corbin JD, Weaver CD, and Cone RD (2011) Development of a high throughput screen for allosteric modulators of melanocortin-4 receptor signaling using a real time cAMP assay. *Eur J Pharmacol* **660**:139–147.
- Patki KC, Von Moltke LL, and Greenblatt DJ (2003) In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. *Drug Metab Dispos* **31**:938–944.

- Schmitt B, Wilt TJ, Schellhammer PF, DeMasi V, Sartor O, Crawford ED, and Bennett CL (2001) Combined androgen blockade with nonsteroidal antiandrogens for advanced prostate cancer: a systematic review. *Urology* **57**:727–732.
- Shet MS, McPhaul M, Fisher CW, Stallings NR, and Estabrook RW (1997) Metabolism of the antiandrogenic drug (Flutamide) by human CYP1A2. *Drug Metab Dispos* **25**: 1298–1303.
- Shou M, Grogan J, Mancewicz JA, Krausz KW, Gonzalez FJ, Gelboin HV, and Korzekwa KR (1994) Activation of CYP3A4: evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. *Biochemistry* **33**:6450–6455.
- Shou M, Mei Q, Ettore MW, Jr, Dai R, Baillie TA, and Rushmore TH (1999) Sigmoidal kinetic model for two co-operative substrate-binding sites in a cytochrome P450 3A4 active site: an example of the metabolism of diazepam and its derivatives. *Biochem J* **340**:845–853.
- Tang W, Stearns RA, Kwei GY, Iliff SA, Miller RR, Egan MA, Yu NX, Dean DC, Kumar S, and Shou M, et al. (1999) Interaction of diclofenac and quinidine in monkeys: stimulation of diclofenac metabolism. *J Pharmacol Exp Ther* **291**:1068–1074.
- Wang RW, Newton DJ, Liu N, Atkins WM, and Lu AY (2000) Human cytochrome P-450 3A4: in vitro drug-drug interaction patterns are substrate-dependent. *Drug Metab Dispos* **28**: 360–366.
- Yang J, Atkins WM, Isoherranen N, Paine MF, and Thummel KE (2012) Evidence of CYP3A allosterism in vivo: analysis of interaction between fluconazole and midazolam. *Clin Pharmacol Ther* **91**:442–449.
- Zientek M, Miller H, Smith D, Dunklee MB, Heinle L, Thurston A, Lee C, Hyland R, Fahmi O, and Burdette D (2008) Development of an in vitro drug-drug interaction assay to simultaneously monitor five cytochrome P450 isoforms and performance assessment using drug library compounds. *J Pharmacol Toxicol Methods* **58**:206–214.
- Zuo Z, Tam YK, Diakur J, and Wiebe LJ (2002) Hydroxypropyl-beta-cyclodextrin-flutamide inclusion complex. II. Oral and intravenous pharmacokinetics of flutamide in the rat. *J Pharm Pharm Sci* **5**:292–298.

Address correspondence to: Dr. Anna L. Blobaum, Drug Metabolism and Pharmacokinetics, Vanderbilt Center for Neuroscience Drug Discovery, Cool Springs Life Sciences Center, 393 Nichol Mill Lane, Franklin, TN, 37067. E-mail: annie.blobaum@vanderbilt.edu