DMD Fast Forward. Published on August 11, 2015 as DOI: 10.1124/dmd.115.064006
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A Screen of Approved Drugs Identifies the Androgen Receptor Antagonist, Flutamide, and its Pharmacologically Active Metabolite, 2-Hydroxy-Flutamide, as Heterotropic Activators of CYP 3A *In vitro* and *In vivo*

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Running Title: Heterotropic activation of CYP3A by Flutamide

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Text Pages: 22

Figures: 6

References: 38

Words in Abstract: 246

Words in Introduction: 744

Words in Discussion: 1366

Abbreviations: CYP, cytochrome P450; DDI(s), drug-drug interaction(s); $mGlu_5$, metabotropic glutamate receptor subtype 5; PAM, positive allosteric modulator; MDZ, midazolam; 1-OH-MDZ, 1-hydroxy midazolam; 4-OH-MID, 4-hydroxy midazolam; HAL, haloperidol; CPHP, 4-(4-chlorophenyl)-4-hydroxy piperidine; HLM, human liver microsomes; RLM, rat liver microsomes; NADPH, β-Nicotinamide adenine dinucleotide phosphate; 2-OH-Flu, 2-hydroxy-flutamide; CRC, concentration-response curve; LC-MS/MS, liquid chromatography tandem mass spectrometry; IP, intraperitoneal; PO, *per os* (by mouth)

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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 in order 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 AUC of primary rat *in vivo* MDZ 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 mutli-species hepatocytes (100% over baseline). These data serve to highlight the importance of 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.

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Introduction

The CYP3A subfamily has been the source of significant drug-drug interactions (DDIs) in the clinic through inhibition or induction of these enzymes (Lin and Lu, 1998; Hutzler et al., 2005). CY3A4 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 3A4, 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 P450 kinetics, including heterotropic effects, in acute clinical DDI scenarios (Egnell et al., 2003b; Obach, 2012; Yang et al., 2012). While heterotropic activation or inhibition of the CYP3A family enzymes (as well as the 2C 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, while the preponderance of *in vitro* atypical kinetics, such as heterotropic activation, is vast, and there is established structure-activity relationship (SAR) data within common pharmacophores (Egnell et al., 2003a; Egnell et al., 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 (HLM) that is capable of simultaneously measuring CYP metabolism and the ability

of a compound of interest to specifically and selectively inhibit or enhance the activity of CYP1A2, 2C9, 2D6 and 3A4/5 (Blobaum et al., 2013). From this approach, we uncovered a series of structurally related compounds (positive allosteric modulators of metabotropic glutamate receptor 5, $mGlu_5$ PAMs) for the potential treatment of schizophrenia that significantly activated the midazolam (MDZ) hydroxylase activity of CYP3A in human hepatic and intestinal microsomes and hepatocytes (Blobaum et al., 2013) and *in vivo* in rodents. Further investigation demonstrated that these $mGlu_5$ PAMs were able to activate the CYP3A-mediated metabolism of the neuroleptic agent haloperidol (Haldol^{TM}, HAL) by 20% over 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., 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 amongst the top classes of administered drugs, we conducted a semi-automated functional screen of the SPECTRUM chemical library via a 'cocktail' probe substrate assay design in HLM. Among the compounds identified from this screen were two androgen receptor antagonists approved for the treatment of prostate cancer, including flutamide and nilutamide (Figure 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, Figure 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-OH-MDZ and 4-OH-MDZ were observed with flutamide pre-treatment. 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. NADPH, midazolam (MDZ), 1-OH-midazolam (1-OH-MDZ), 4-OH-midazolam (4-OH-MDZ), testosterone, 6β-OH-testosterone, progesterone, 6β-OH-progesterone, phenacetin, acetaminophen, diclofenac, 4-OH-diclofenac, dextromethorphan, dextrorphan, nifedipine, oxidized nifedipine, amiodarone, desethylamiodarone, and miconazole were all purchased from Sigma-Aldrich (St. Louis, MO). VU0448187 (5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine) and VU0415089 (2,4-difluorophenyl)(2-(phenoxymethyl)-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 (2,320 compounds) were researched for availability and purchased through Sigma-Aldrich (St. Louis, MO) or LKT Laboratories (St. Paul, MN). Human liver microsomes (150-donor pool, mixed gender), human intestinal microsomes (20-donor pool, mixed gender), 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 (Baltimore, MD). All solvents used in the bioanalysis of *in vitro* and *in vivo* samples were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) and of HLPC grade.

HLM screen of the SPECTRUM library. SPECTRUM compounds were plated (96-well, duplicate, 10 mM DMSO) at the Vanderbilt University High Throughput Screening Center using an ECHO 555 automated liquid handler (Labycte Inc., Sunnyvale, CA). The reported pan-P450 inhibitor, miconazole, and internal positive controls for CYP3A (VU0448187) and CYP2C (VU0415089) heterotropic activation were included in each 96-well plate. Compounds were

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initially screened at a single concentration (10 μM), 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, 2C9, 2D6 and combined 3A4/5. Compounds that were identified as CYP activators (≥ 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-metabolite per CYP was monitored, *vide infra*). Following confirmation of activation, compounds demonstrating activation of a single CYP were cross-referenced for 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 potassium phosphate buffered solution (0.1 M, pH 7.4, 37^{o}C) subsequently fortified with midazolam (MDZ, 2 μM). The reaction mixtures were evenly distributed into 96-well plates and compounds from the SPECTRUM collection (duplicate assays, 0.1 – 30μM) were preincubated (15 min) with shaking at 37^{o}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 2 volumes of ice-cold acetonitrile containing internal standard (carbamazepine or tolbutamide). The plates were centrifuged (4000 g, 4 ^{o}C, 10 min) and the supernatant was subsequently removed and diluted in water (1:4, v/v) in preparation for LC-MS/MS analysis. The concentrations of each analyte were determined by comparison of TIC area counts (MS 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, 6β-OH-testosterone, 6β-OH-progesterone, oxidized nifedipine, and desethylamiodarone), was set to 100%. All experiments were performed in duplicate unless otherwise noted. Multi-species hepatocytes (final reaction, 0.5 x 10^6 cells/mL) were thawed and incubations with compounds carried out for 10 min prior to quenching with ice-cold acetonitrile containing 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 μM), progesterone (50 μM), nifedipine (25 μM) and amiodarone (50 μM). 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 carbamazapine; for those in negative ionization mode, the internal standard was tolbutamide. Concentrations of each metabolite (1-OH-MDZ, 4-OH-MDZ, 6β-OH-testosterone, 6β-OH-progesterone, oxidized nifedipine, and desethylamiodarone) were quantitated using a 12-point standard curve, and data were represented as % of control activity remaining (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% 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 Labs, Indianapolis, IN) 40 min prior to administration of MDZ (10 mg/kg, IP), which was formulated in 10% EtOH:50% PEG400:40% Saline (v/v; 4 mg/mL). Flutamide dose and

pre-treatment time were selected based on previously reported pharmacokinetic parameters in rat (Zuo et al., 2002). In addition to a pre-dose sampling, blood was serially collected over EDTA at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post-dose; plasma was isolated via centrifugation (1700 g, 4 ^{o}C, 5 min) and stored at -80 ^{o}C until LC-MS/MS analysis. The resulting plasma samples were protein-precipitated by employing three volumes of ice-cold acetonitrile containing internal standard. The samples were centrifuged (4000 g, 4 ^{o}C, 5 min) and the supernatants transferred and diluted 1:1 (v/v) for LC-MS/MS analysis. Flutamide, 2-OH-Flu, MDZ, 1-OH-MDZ, 4-OH-MDZ were monitored from each experiment and the concentrations (ng/mL) of each analyte determined using a matrix-matched 10-point standard curve.

LC-MS/MS analysis. *In vitro* and *in vivo* samples were analyzed via electrospray ionization (ESI) 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 auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a thermostated (40 ^{o}C) C18 column (Fortis 3.0 x 50 mm, 3 µm; 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/4-OH-MDZ, the gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 1.3 min; returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min 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 (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in 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 carbamazapine; for those in negative ionization mode, the internal standard was tolbutamide.

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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 μM) screening of compounds from SPECTRUM collection in HLM identified a number of confirmed activators, which spanned various structural motifs, drug classes, and therapeutic areas; > 90% of the compounds identified activated CYP3A activity (with the majority of remaining compounds effecting 2C9) through enhancement of the intrinsic midazolam (MDZ) hydroxylase activities of CYPs 3A4 and 3A5. The confirmed drug classes that activated CYP3A-mediated MDZ hydroxylase activity included, but were not solely limited to, antiandrogens, antibiotics, antiinflammatories, antimalarials, 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 where many paired medications would be also be metabolized by CYP3A and, therefore, may influence projected clinical outcomes.

***In vitro* characterization of CYP3A heterotropic activation of MDZ metabolism by flutamide.** Flutamide (Figure 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 (Figure 3A) in HLM following the robust activation observed at the initial single concentration (10 μM). Results of this experiment revealed flutamide to increase the intrinsic CYP3A4-mediated MDZ hydroxylase activity in HLM by 66% over 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%) (Figure 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 Figures 3B and 3D, 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.

While the substrate-dependence of atypical kinetics with CYPs has been well-defined, the species dependence of heterotropic effects have 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 mini-pig or guinea pig (Figure 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 (Figure 4A) and 2-OH-Flu (Figure 4B) was more pronounced in nonclinical species hepatocytes when compared to liver microsomes, as also demonstrated for human microsomes and hepatocytes. In the case of flutamide, the maximal activation was achieved at 3 μM in HLM and at 10 μM in hepatocytes; although, total levels of 1-OH-MDZ were higher in hepatocytes compared to HLM at all flutamide concentrations assayed. Low levels of CYP3A activation, as measured by increased metabolism of MDZ (Figure 4B), were noted for 2-OH-Flu in hepatic microsomes (30% at 10 μM), but were significantly elevated in human hepatocytes at the same concentration (100%).

Decreased CYP3A-mediated MDZ hydroxylase activity was observed in rat hepatocytes compared to human hepatocytes; however, whereas 1-OH-MDZ is the primary human metabolite, 4-OH-MDZ is produced in equal or greater amounts compared to 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 (IP, 10 mg/kg) and serial plasma exposure of MDZ and its primary metabolites were obtained. Systemic concentrations of the two principle circulating metabolites of MDZ (1-OH- 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 min) when compared to rats pretreated with vehicle prior to MDZ administration (Figure 5A-B). Concentrations of 4-OH-MDZ were higher than 1-OH-MDZ in both treatment groups. The total combined AUC of the primary hydroxylated MDZ metabolites was approximately 2-fold higher in rodents pre-treated with flutamide compared to vehicle alone (Figure 5C). Flutamide and 2-OH-Flu concentrations were also monitored from the treatment group receiving the oral dose of flutamide (Figure 5D) to enable an understanding of *in vivo* levels of each activator in rodents. For flutamide, maximal concentrations were achieved at the 7 min 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 parent, a significantly higher C_{max} of this metabolite was observed at 2 hours post dose (T_{max}) and the systemic plasma concentrations were sustained well past 7 hours post-dose. The total C_{max} values for flutamide and 2-OH-Flu were 5.1 μM and 5.6 μM, 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 levels of 1- 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 re-administration. 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 μM, 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 towards the treatment of prostate cancer and act by competing with testosterone for binding to the androgen receptor. The antihypertensive, nifedipine, as well as the antiarrythmic, amiodarone, are CYP3A substrates and are 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 similar maximal activation of CYP3A-mediated nifedipine metabolism in human hepatocytes (~50% over baseline) at low micromolar concentration (3 μM or 1 μM, respectively) (Figure 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%, Figure 6B) and higher in mouse hepatocytes (>100% over baseline, Figure 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 (Figure 6D, rat hepatocytes).

Discussion

Heterotropic activation of CYPs 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 co-factor fortification, partner-catalytic proteins, and tissue and subcellular fraction selection, greatly impact the magnitude of activation (Hutzler and Tracy, 2002). While 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 rCYPs and/or hepatic subcellular fractions have successfully correlated in nonclinical species *in vivo*, or in human (Hutzler and Tracy, 2002). Many variables could account for this lack of *in vitro: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 to hepatocytes) (Houston and Kenworthy, 2000). As for the lack of nonclinical-to-clinical translation 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, or 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 (ER) would be of particularly high risk due to their sensitivity to increases in hepatic intrinsic clearance (CL_{int}) that 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., 3A4) 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 manifest through the induction of CYP enzymes. Drugs such as nifedipine (antihypertensive) and amiodarone (antiarrythmic) are subject to DDI scenarios in patients when co-administered with potent CYP3A4 inducers due to reductions in plasma exposures of 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 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 multi-species 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 higher activation potential when compared to rodent hepatocytes. Interestingly, in the case of the flutamide/MDZ pair of activator and substrate, mini-pig and guinea pig microsomes (Figure 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 CYP 3A5 polymorphisms, while 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, co-factor enrichment, and species differences in CYP3A expression, polymorphism and substrate specificity – all of which remain under investigation in our laboratory.

While our data in hepatocytes suggests that mouse may be a more appropriate model to investigate heterotropic effects of flutamide on CYP3A-mediated metabolism of nifedipine (Figure 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 rat is highly dependent upon the specific rat strain utilized for a given study, MDZ is generally considered to be a high clearance compound in rat (55-80 mL/min/kg) with near equal conversion to 1- 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 suprahepatic from an IV administration. To this end, we previously demonstrated that changes in MDZ and metabolite levels can be effectively obtained from IP dosing of MDZ with pre-treatment of the activator (Blobaum et al., 2013). In the present study, rats pre-treated with flutamide exhibited an increase in the total combined AUC of the primary hydroxylated MDZ metabolites (1-OH-MDZ and 4-OH-MDZ) that was approximately 2-fold higher when compared to vehicle alone (Figure 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.

Acknowledgements

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 analytical tools: Bridges

Performed data analysis: Blobaum, Byers, Daniels

Wrote or contributed to the writing of the manuscript. Blobaum, Bridges, Locuson, Conn, Lindsley, Daniels

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Footnotes

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

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DMD Fast Forward. Published on August 11, 2015 as DOI: 10.1124/dmd.115.064006
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Table 1. Representative compounds identified as heterotropic activators of CYP activity during single-point screening of the SPECTRUM library in HLM – Percent activation over baseline

DRUG	CLASSIFICATION	3A4/5	2C9	1A2	2D6
FLUTAMIDE	antiandrogen	98	-	-	-
ROSIGLITAZONE	antidiabetic	37	-	71	31
METFORMIN HYDROCHLORIDE	antidiabetic	63	23	32	63
COENZYME B12	vitamin	56	-	-	-
PYRVINIUM PAMOATE	anthelmintic	54	-	-	-
DICLOFENAC SODIUM	antiinflammatory	-	54	-	-
AVOCATIN B	antibacterial, antifungal	52	-	-	-
NILUTAMIDE	antiandrogen	49	-	-	-
DERACOXIB	antiinflammatory, antiarthritic, COX-2 inhibitor	48	-	-	-
RIBAVIRIN	antiviral	41	45	-	46
TOLBUTAMIDE	antidiabetic	45	-	-	22
TRANYLCYPROMINE SULFATE	antidepressant, MAO inhibitor	43	-	-	-
DIETHYLCARBAMAZINE CITRATE	anthelmintic	-	-	43	-
MOXIFLOXACIN HYDROCHLORIDE	antibacterial	24	41	-	41
TACRINE HYDROCHLORIDE	anticholinesterase, cognitive adjuvant, K channel blocker	40	-	-	-
CELECOXIB	antiarthritic, cyclooxygenase2 inhibitor	39	-	28	-
PROPANTHELINE BROMIDE	anticholinergic	-	38	-	-
ORPHENADRINE CITRATE	muscle relaxant (skeletal), antihistaminic	38	28	-	45
SULFANITRAN	antibacterial	37	-	-	-
CANRENONE	aldosterone antagonist; antifibrogenic	37	-	33	-

The screening concentration for each compound was 10μM. Values are expressed as % activity remaining over baseline and are the average of two replicates. Positive hits were required to have duplicate data points >/= 20% increase over baseline. Activity is calculated based on the metabolism of CYP-specific probe substrates to their respective metabolites. Compounds included in the table are representative of the diversity of 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.

Figure Legends

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

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

Figure 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 % activity of control. Mean (+/- SEM) 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.

Figure 4. Species dependency of heterotropic effects with flutamide (**A**) and 2-OH-Flu (**B**) in multi-species hepatic microsomes and hepatocytes. 1-OH-MDZ levels were quantitated and the results expressed as % 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 pool). Mean (+/-SEM) values over 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 (+/-SEM) values for 2-OH-Flu were 111% (2.67), 30% (4.29), and 36% (6.97).

Figure 5. Activation of MDZ metabolism in SD rats following pretreatment with flutamide. Flutamide was administered to male SD rats (15 mg/kg, PO) 40 min prior to of MDZ administration (10 mg/kg, IP). 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 area under the curve (AUC) for the hydroxylated MDZ metabolites is shown in (**C**). Plasma concentrations of flutamide and 2-OH-Flu are shown in (**D**). Experiments performed in duplicate. Concentrations are in ng/mL.

Figure 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 pan-P450 inhibitor, miconazole, also depicted. CYP3A activation of amiodarone was performed in SD rat hepatocytes (**D**). Maximal inhibition or activation of metabolism is expressed as % of control with the concentration producing the maximal response provided for each compound shown (μM). Mean (+/-SEM) values over baseline (defined as 100%) achieved in each species are as follows for **A**) VU0448187, 54% (9.39); Flutamide, 48% (12.3); 2-OH-Flutamide, 21% (3.70), **B**) VU0448187, 37% (0.841); Flutamide, 37% (0.388); 2-OH-Flutamide, 27% (3.32), **C**) VU0448187, 113% (12.5); Flutamide, 98% (11.9); 2-OH-Flutamide, 76% (2.93), and **D**) VU0448187, 32% (14.0); Flutamide, 27% (1.57); 2-OH-Flutamide, 37% (3.48).

Figure 1

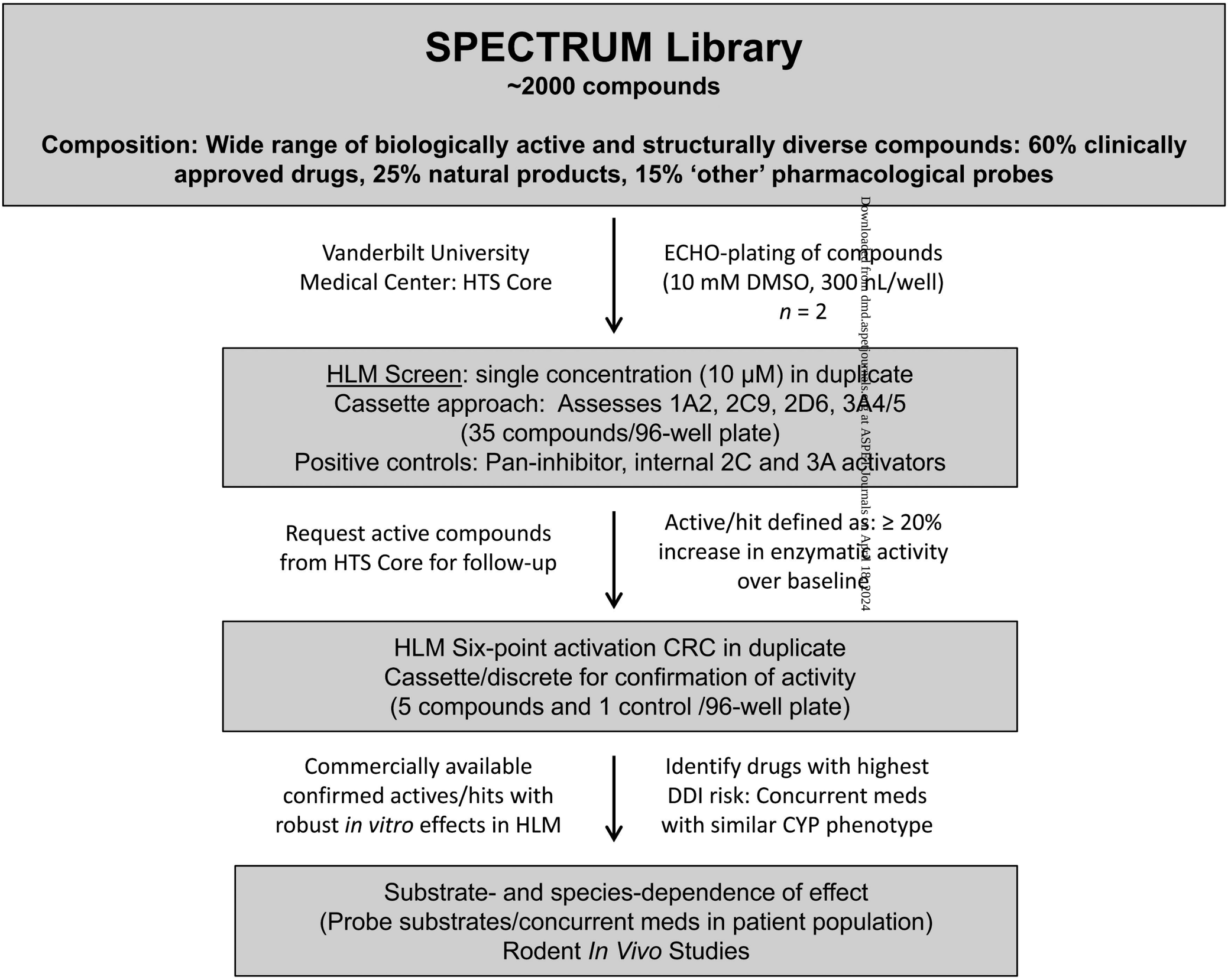
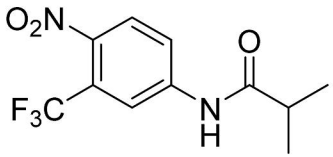
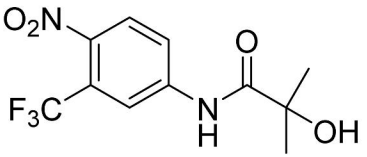


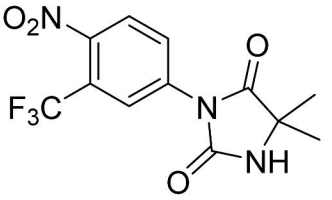
Figure 2



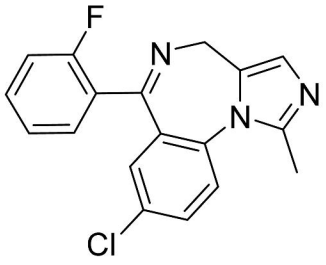
Flutamide



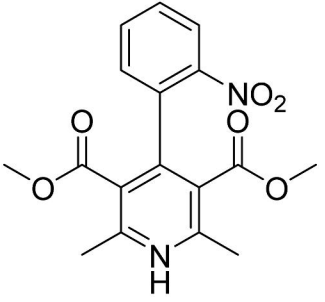
2-OH Flutamide



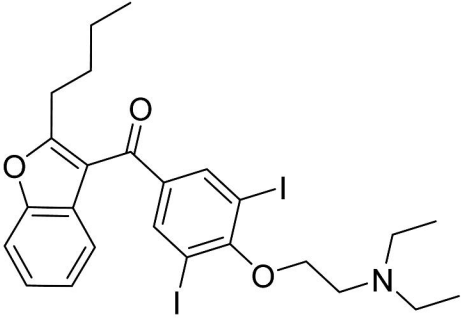
Nilutamide



Midazolam

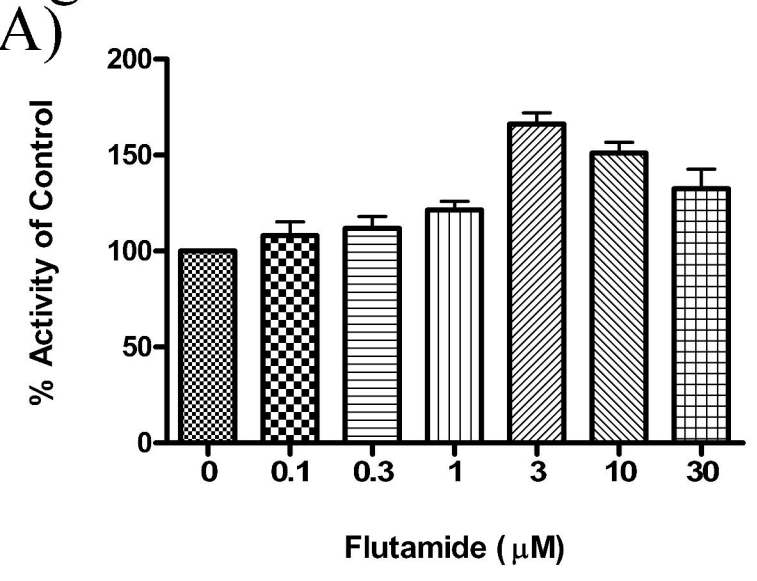


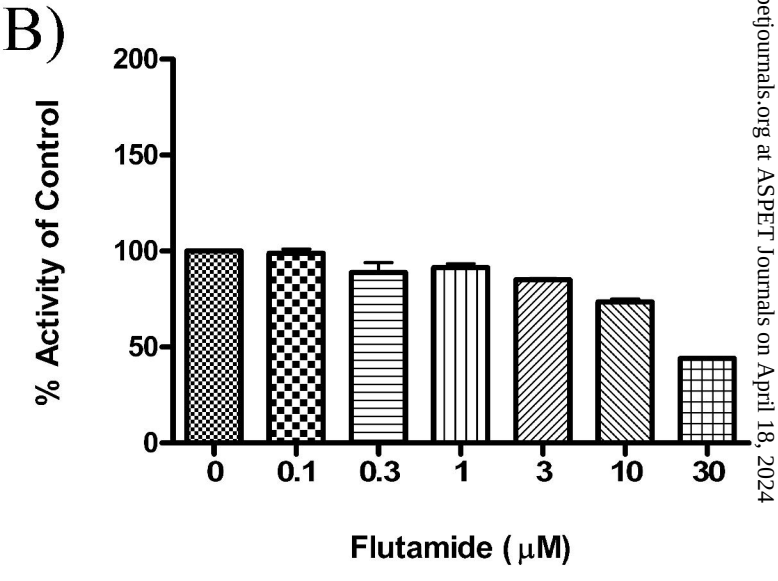
Nifedipine

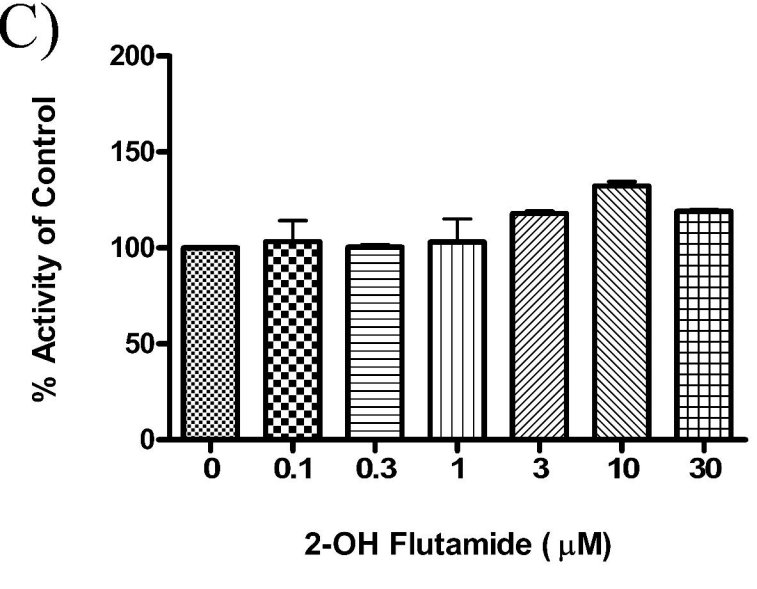


Amiodarone

Figure 3







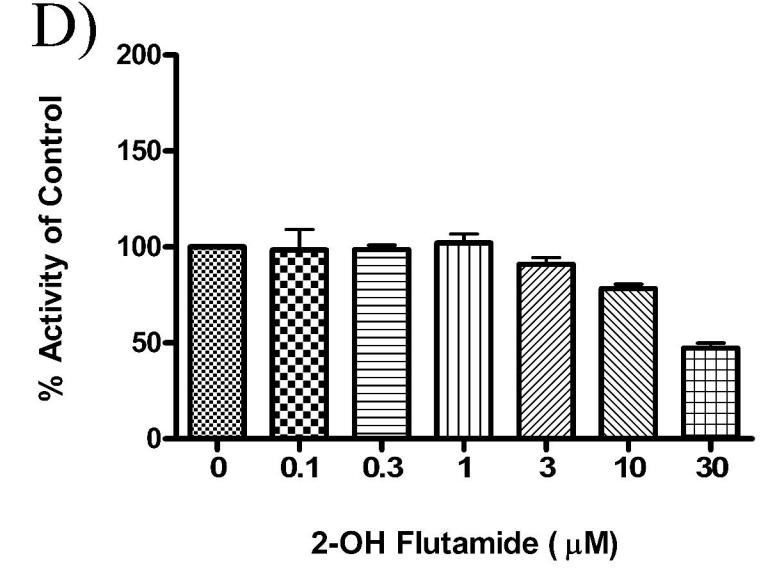
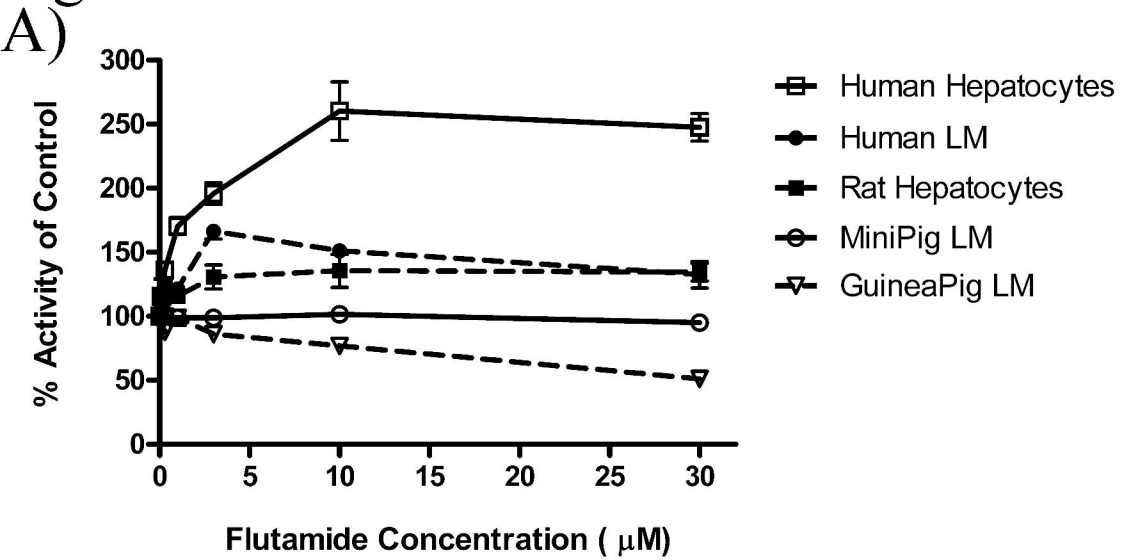


Figure 4

A)


% Activity of Control
300
250
200
150
100
50
0
0
5
10
15
20
25
30
Flutamide Concentration (μM)
Human Hepatocytes
Human LM
Rat Hepatocytes
MiniPig LM
GuineaPig LM

B)

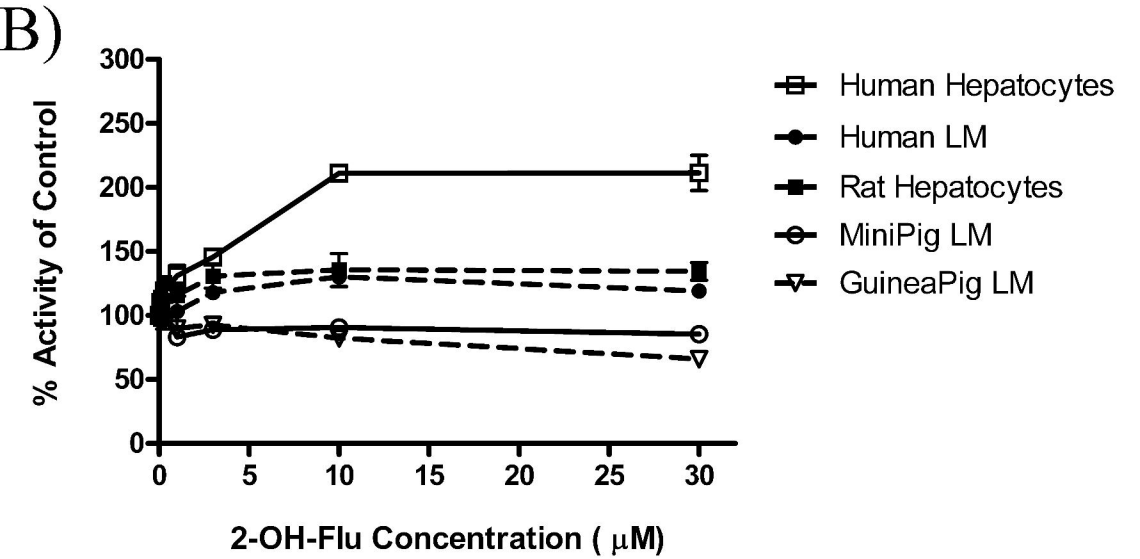
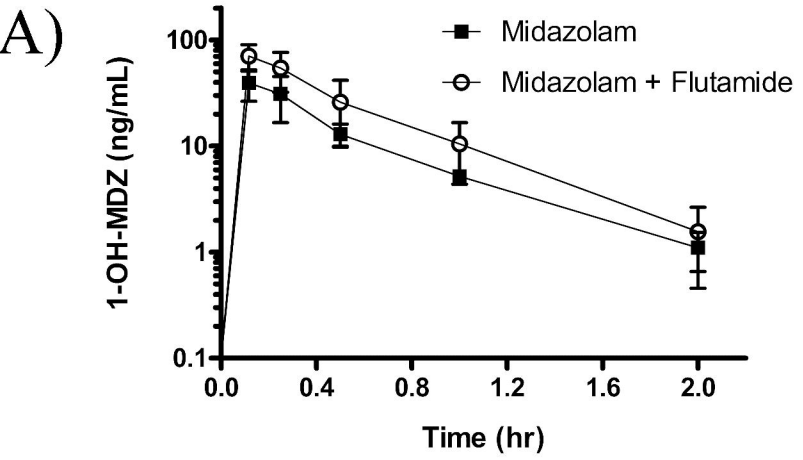
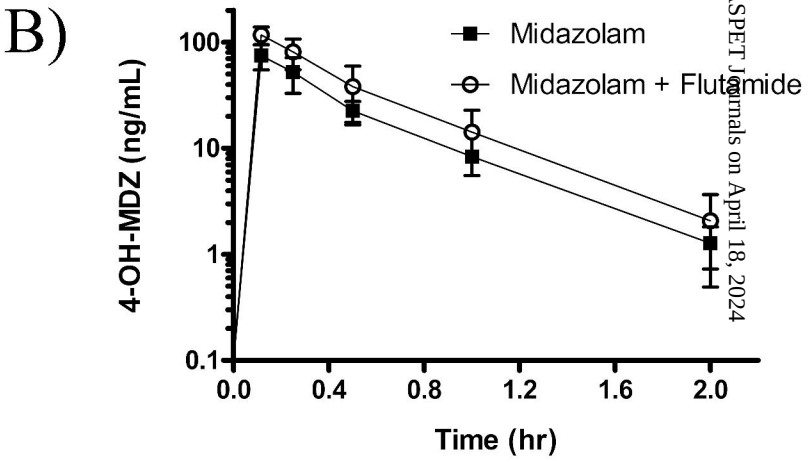
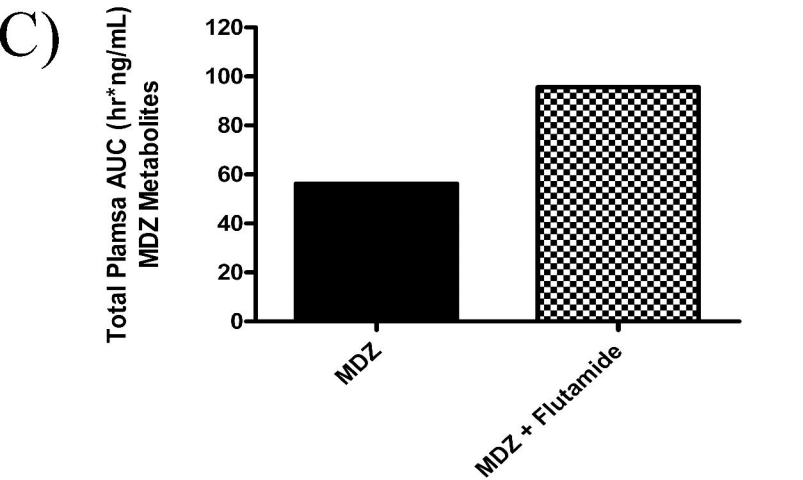

% Activity of Control
300
250
200
150
100
50
0
0
5
10
15
20
25
30
2-OH-Flu Concentration (μM)
Human Hepatocytes
Human LM
Rat Hepatocytes
MiniPig LM
GuineaPig LM

Figure 5







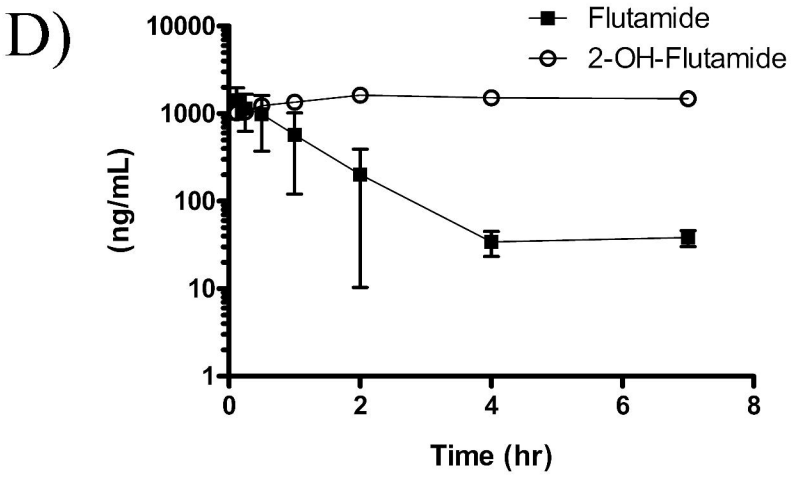


Figure 6