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The Development of a Cocktail CYP2B6, CYP2C8 & CYP3A5 Inhibition Assay and a Preliminary Assessment of Utility in a Drug Discovery Setting

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Running title: An automated cocktail CYP2B6, 2C8 and 3A5 inhibition assay

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Abbreviations

CYP	Cytochrome P450
DDI	Drug Drug Interaction
DMSO	Dimethyl Sulphoxide
MeOH	Methanol
MRM	Multiple Reaction Monitoring
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCE	New Chemical Entity

Abstract

Tools for studying the roles of CYP2B6, CYP2C8 and CYP3A5 in drug metabolism have recently become available. The level of interest in these enzymes has been elevated because investigations have revealed substrate promiscuity and/or polymorphic expression. This study aimed to develop a single cocktail inhibition assay for the three enzymes and assess its utility in drug discovery. Bupropion hydroxylation, amodiaquine N-deethylation and midazolam 1'-hydroxylation were chosen as probe reactions for CYP2B6, CYP2C8 and CYP3A5 and were analysed using LC-MS-MS. Kinetic analyses were performed to establish suitable conditions for inhibition assays, which were subsequently automated. CYP2B6, CYP2C8 and CYP3A5 IC_{50} values were determined for marketed drugs and almost two hundred AZ discovery compounds from 16 separate discovery projects. For the marketed drugs, results obtained were comparable with literature values. Data were also compared with IC_{50} values determined for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. In this dataset, the majority of compounds were more potent inhibitors of CYP2C9, CYP2C19, CYP2D6 and CYP3A4 than CYP2B6, CYP2C8 or CYP3A5. The potential impact of these findings on CYP inhibition strategy is discussed.

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Introduction

Drug discovery programs traditionally study CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 when investigating the metabolism and enzyme inhibition potential of new chemical entitities (NCEs). The tools for studying other P450s have more recently become available and the roles of CYP2B6, CYP2C8 and CYP3A5 in drug metabolism have come to the fore because they have been implicated in the metabolism of a number of marketed drugs and physiologically important endogenous molecules. Additionally, increased variability in the pharmacokinetics of drugs metabolised by CYP2B6, CYP2C8 and CYP3A5, as a consequence of polymorphic expression, makes consideration of these enzymes important.

CYP2B6 is estimated to make up at least 5% of the total hepatic P450 content (Code et al., 1997; Ekins et al., 1998; Stresser and Kupfer, 1999). It metabolises a range of drugs including efavirenz (Ward et al., 2003) and cyclophosphamide (Huang et al., 2000) as well as drugs of abuse such as cocaine and ecstasy (Aoki et al., 2000; Kreth et al., 2000). Additionally large inter-individual variations in hepatic CYP2B6 have been demonstrated due to its highly inducible nature and the existence of polymorphisms (Ariyoshi et al., 2001; Lang et al., 2001).

CYP2C8 is a major human hepatic P450, constituting about 7% of total microsomal content in the liver (Rendic and Di Carlo, 1997; Shimada et al., 1994). It has been shown to be involved in the metabolism of a variety of drugs including amiodarone, amodiaquine, diclofenac and troglitazone (Ohyama et al., 2000; Tang, 2003; Walsky and Obach, 2004; Yamazaki et al., 1999), as well as physiologically important endogenous molecules such as arachidonic acid (Rifkind et al., 1995). CYP2C8 is polymorphic, the most common variant alleles are CYP2C8*2 and CYP2C8*3, both of which result in a functional change of the enzyme (Dai et al., 2001). The CYP2C8*2 variant occurs in African Americans populations with an allele frequency of 18% but is relatively uncommon in caucasian populations, whereas CYP2C8*3 is more common in caucasian individuals (23%) and quite rare in African Americans subjects (Totah and Rettie, 2005).

CYP3A5 shows considerable substrate crossover with CYP3A4 (Williams et al., 2002), the importance of which in drug metabolism has been acknowledged for many years (Wrighton and Stevens, 1992). CYP3A5 has been reported to be expressed in the livers of a third of Caucasians and up to 60% of African Americans (Kuehl et al., 2001) in addition to other organs such as the intestine (Lin et al., 2002; Paine et al., 1997) and kidney (Haehner et al., 1996). Whereas individuals with the CYP3A5*3 allele have a greatly reduced translation of functional protein, individuals who possess at least one CYP3A5*1 allele have functional CYP3A5. The amount of hepatic CYP3A comprised of CYP3A5 remains under dispute and has been cited as ranging from 4 % (Koch I et al., 2002; Westlind-Johnsson et al., 2003) to 50 % (Kuehl et al., 2001).

Investigating the potential to inhibit CYP2B6, CYP2C8 and CYP3A5 dependent metabolism is important in the development of safe therapeutic agents. This laboratory has previously developed a screen using recombinant CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 and an LC-MS-MS endpoint (Weaver et al., 2003). Using similar methodology, the inhibition assay described here uses a cassette incubation of bupropion (CYP2B6), amodiaquine (CYP2C8) and midazolam (CYP3A5) at concentrations equivalent to their K_m values with a cocktail of the three human P450s expressed in *E.coli*. An automated version of the assay was established and its impact on P450 inhibition screening strategy assessed.

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Materials and Methods

E.coli co-expressing the relevant P450s and human P450 reductase were purchased from Cypex (Dundee, UK). Desethylamodiaquine and hydroxy-bupropion were purchased from Synfine Research (Ontario, Canada) and BD Gentest (Massachusetts, US) respectively. Amodiaquine, bupropion, NADPH and all other cited chemicals were purchased from Sigma Chemicals (Poole, Dorset, UK).

Incubation Conditions. The automated assay was based on the methodology described by Weaver *et al.* (2003). Incubations were performed in phosphate buffer (0.1M, pH 7.4) and P450 concentrations were 18, 1 and 5 pmol/ml for CYP2B6, CYP2C8 and CYP3A5 respectively. All incubations (controls, probe and test inhibitors) were 200 µl, contained DMSO at 1 % v/v and were terminated following 10 min incubations at 37 ˚C by the addition of ice cold MeOH (200µl). Samples were placed at –20 ˚C for 2 h, centrifuged at 2,200 *g* for 15 min and the supernatant (120 µl) transferred to 96 well microtitre plates (MTP) (Agilent Technoligies, UK) for LC-MS-MS analysis.

Analytical Conditions. Mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in MeOH). Samples (20 µl) were injected onto a Hypersil Gold column (C_{18} ,5 µm, 150 x 2.1 mm) (Thermo Electron Corporation, Cambridge, UK). The 5 min gradient was as follows: 97 % A (0-0.5 min), 0 % A (0.5-1.5 min), 0 % A (1.5-2.7 min), 97% A (2.8 min). The flow rate was 0.4 ml/min and the column temperature was 50 ºC.

LC-MS-MS was performed using an Agilent HP1100 HPLC system (Agilent Technologies, UK) coupled to a triple quadrupole Quattro Platinum mass spectrometer (Micromass, Manchester, UK) operating in ESI+ mode, with Masslynx 4.0 running in MRM mode (3 MRMs simultaneously: hydroxy bupropion, 256.19 > 184.13; desethyl amodiaquine, 328.36 > 282.99; 1'-hydroxy midazolam, 342.17 > 202.90; dwell time of 0.2 s).

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Enzyme Kinetics

Time and CYP Concentration Linearity. Incubations were performed using substrate concentrations approximating to K_m/10 and 5xK_m (based on K_m values from the literature: Gibbs et al., 1999; Hesse et al., 2000; Li et al., 2002) and P450 concentrations of 0.25, 0.5, 1, 2, 5, 10, 20, 50, and 100 pmol/ml. The substrates were prepared manually as follows: aliquots of methanolic stocks (0.1 or 1 mg/ml) of bupropion, amodiaquine and midazolam were pipetted into a 20 ml glass scintillation vial and evaporated to dryness under nitrogen gas. The dried substrates were then reconstituted in phosphate buffer (0.1 M, pH 7.4; 4.4 ml) by vortexing and sonication (20 min in a sonic bath at 37 ˚C). Initially LC-MS-MS analysis of methanolic standards was used to confirm the complete reconstitution of the dried down substrates.

Aliquots (178 µl) were added to wells of a 96 well MTP containing DMSO (2 µl; 1 % v/v to represent later incubation conditions when inhibitors would be present in DMSO). The plate was pre-incubated for 10 min at 37 ˚C in a warmed-air shaking incubator. The reactions were initiated by the addition of NADPH (20 µl; 10 mM) and the MTP was incubated at 37 ˚C. The total volume of each incubation was 200 µl. Aliquots (15 µl) were taken at 5, 10, 15 and 20 min and quenched by the addition of an equal volume of ice-cold methanol. The samples were prepared and analysed as described above.

Determination of K_m and V_{max} Values. Incubations were carried out with ten substrate concentrations: bupropion (5 - 240 µM), amodiaquine (0.2 - 6 µM) and midazolam (0.15 - 30 µM). Appropriate volumes of methanolic stocks (1 mg/ml) were added to 7 ml incubation vials and evaporated to dryness under nitrogen gas. The substrates were dissolved in a volume of phosphate buffer (0.1 M, pH 7.4) to give the required final concentration. The previously determined optimum incubation time and P450 concentrations were used (10 min and 18, 1 and 5 pmol/ml for CYP2B6, 2C8 and 3A5 respectively). Incubations were initiated by the addition of NADPH (20 µl; 10 mM). Additionally, V_{max} and K_m values were determined for each P450 in

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incubations containing all three pooled P450 enzymes, all three pooled substrates and DMSO (1 % v/v), in order to emulate the conditions of the final cocktail incubation assay.

Substrate Specificity. Each of the three P450s was incubated separately with the individual substrates (bupropion, amodiaquine and midazolam) at concentrations equal to their respective K_m values using the previously optimised reaction time and P450 concentration, in order to check for substrate selectivity.

Automated IC_{50} Determination of Inhibitors. All incubations were carried out for 10 min using substrate concentrations at the measured K_m values and 18, 1 and 5 pmol/ml CYP2B6, 2C8 and 3A5 respectively. The incubation volumes were 200 µl and contained 1 mM NADPH. Test inhibitors (100x incubation concentration) were added in DMSO so that the final incubation contained 1 % v/v DMSO. Control incubations also contained 1 % v/v DMSO but no inhibitor. The ability of the DMSO to inhibit any of the reactions in question was also investigated, in all cases there was no evidence of inhibition by the 1% DMSO included in the incubations. The assays were performed using a Tecan Genesis robot (Tecan, Reading, UK) running Gemini software and was identical in design to the five P450 inhibition assay described previously (Weaver et al., 2003). IC_{50} values were established for each P450 using prototypic inhibitors of the relevant P450s (ticlopidine, CYP2B6; quercetin, CYP2C8; ketoconazole, CYP3A5). Incubations were carried out with 6 inhibitor concentrations chosen to define known (literature) IC_{50} values as follows: ticlopidine (0.003 - 1 µM), quercetin (0.02 - 8.3 µM), and ketoconazole (0.004 - 1.3 µM). Additional compounds implicated previously as inhibitors were also tested (erythromycin, diltiazem, tranylcypromine, paclitaxel, montelukast, ritonavir, nifedipine, mifepristone). Determination of IC_{50} values for the 11 probe inhibitors were performed using single substrate, single P450 and cocktail substrate, cocktail P450 (24 pmol/ml total) incubations. AstraZeneca Charnwood Drug Discovery compounds were selected from 16 separate and chemically distinct series (70 acids, 70 bases, 40 neutrals and 16 zwitterions) with molecular weights ranging from 254 to 748, log P values ranging

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from –1.4 to 6, the number of rotatable bonds ranging from 2 to 22 and the number of hydrogen bond donors and acceptors ranging from 0 to 5 and 3 to 14 respectively.

Data Analysis. V_{max} and K_m were determined, using the Michaelis-Menten equation, by nonlinear regression analysis using WinNonlin (Pharsight, Mountain View, CA).

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Results

Time and P450 Concentration Linearity. At both 6µM and 300µM bupropion, CYP2B6-dependent bupropion hydroxylation was linear to 15 minutes with CYP2B6 concentrations up to 100 pmol/ml. At both 0.1 and 10µM amodiaquine, the CYP2C8-dependent formation of N-desethyl amodiaquine was linear to 15 minutes with CYP2C8 concentrations up to 2 pmol/ml. At both 0.2µM and 8µM midazolam, the CYP3A5-dependent formation of 1'-hydroxy midazolam was linear to 20 min with CYP3A5 concentrations up to 10 pmol/ml. In all incubations where the formation of metabolite was linear with respect to time and P450 concentration, the amount of substrate consumed was less than 15%. For V_{max} and K_m determinations and the automated CYP2B6, CYP2C8 and CYP3A5 cocktail inhibition assay, an incubation time of 10 min was chosen with CYP2B6, 2C8 and 3A5 concentrations of 18, 1 and 5 pmol/ml.

Determination of V_{max} and K_m. V_{max} and K_m values were determined for individual substrates with individual enzymes. Incubations were carried out with a range of substrate concentrations, spanning the reported literature K_m, up to a value of 5 times K_m. The V_{max} and K_m values for CYP2B6 bupropion hydroxylation were 0.2 pmol/min/pmol P450 and 25 µM. The V_{max} and K_m values for CYP2C8 amodiaquine deethylation were 11 pmol/min/pmol P450 and 1 µM . The V_{max} and K_m values for CYP3A5 midazolam 1'-hydroxylation were 7 pmol/min/pmol protein and 2 µM. Similar V_{max} and K_m values were estimated using the P450 and substrate cocktail assay: 0.3 pmol/min/pmol and 20 µM (bupropion hydroxylation), 14 pmol/min/pmol and 2 µM (amodiaquine deethylation) and 7 pmol/min/pmol and 2 µM (midazolam 1'-hydroxylation). The Michaelis-Menten plots for bupropion, amodiaquine and midazolam are shown in Fig. 1.

Substrate Specificity. Each of the three P450s was separately incubated with the individual substrates at concentrations equal to their respective K_m values using the previously optimised reaction time and P450 concentration, in order to assess substrate selectivity. The results demonstrated that bupropion hydroxylation

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was also catalysed by CYP3A5 (1%) but not by CYP2C8, amodiaquine deethylation was catalysed by both CYP3A5 (6%) and CYP2B6 (4%) and midazolam 1'-hydroxylation was catalysed by CYP2B6 (2%) but not by CYP2C8, where the values in parentheses refer to percentage metabolism compared to the 'selective' P450. This was deemed acceptable since the impact on IC_{50} estimates would be minimal.

IC_{50} Determination of Probe Inhibitors. The IC_{50} values of the three probe inhibitors to be used in the final cocktail inhibition assay (ticlopidine, CYP2B6; quercetin, CYP2C8; ketoconazole, CYP3A5) were determined using both single P450, single substrate and combined P450, combined substrate assay formats. The IC_{50} values for all P450 isoforms were similar using either the cocktail or single P450, single substrate incubations and agreed well with values obtained from the literature (Table 1). IC_{50} determinations were performed for 8 additional drugs known to be P450 inhibitors (erythromycin, diltiazem, tranylcypromine, paclitaxel, montelukast, ritonavir, tamoxifen and nifedipine). Again the IC_{50} values determined in the cocktail assay agreed well with those determined in the single P450, single substrate assay (Table 1).

IC_{50} Determination for NCEs. 196 NCEs with wide ranging physico-chemical properties (see Materials and Methods) were tested in the final automated CYP2B6, CYP2C8 and CYP3A5 cocktail assay. The IC_{50} values generated were compared to values obtained for the inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, generated as described previously (Weaver et al., 2003). In general, for this dataset, compounds were more potent inhibitors of CYP3A4 than CYP2B6, CYP2C8 and CYP3A5 and more potent inhibitors of CYP2C9 than CYP2C8 (Fig. 2).

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Discussion

This paper describes an automated, cocktail P450 inhibition screen using substrates selective for CYP2B6, CYP2C8 and CYP3A5 with an LC-MS-MS end-point. The assay was validated with known inhibitors and evaluated further with AstraZeneca Charnwood NCEs.

It was important to select specific substrates for each of the P450 enzymes to be studied. Literature data was used to select bupropion as a substrate for CYP2B6 (Faucette et al., 2000; Hesse et al., 2000; Kim et al., 2005; Turpeinen et al., 2005), amodiaquine as a substrate for CYP2C8 (Kim et al., 2005; Li et al., 2002; Turpeinen et al., 2005) and midazolam as a substrate for CYP3A5 (Gorski et al., 1994; Huang et al., 2004; Williams et al., 2002). Substrates cited for the analysis of CYP2B6 activity include 7-ethoxy-4-trifluoromethylcoumarin O-deethylation and S-mephenytoin N-demethylation but the selectivity of these reactions is questionable (Faucette et al., 2000). Paclitaxel 6α-hydroxylation, which has been frequently used to measure CYP2C8 activity, was not chosen because poor solubility prevented its use in this assay, where the dried down substrates are resuspended in buffer and the 1% v/v DMSO in the final incubation comes from the inhibitor solution. Midazolam was chosen as the CYP3A5 probe because it is a well-established substrate for both CYP3A4 and CYP3A5 and its use in the CYP3A4 inhibition assay used in this laboratory facilitates ready comparison of the inhibition of the two enzymes.

Specificity was checked for all substrates with each P450 at the concentrations to be used in the final assay. There was a small amount of CYP3A5 mediated bupropion hydroxylation detected (0.5%). This is consistent with a previous report that CYP3A4 catalyses the hydroxylation of bupropion (Faucette et al., 2000), albeit with a K_m at least 20 fold higher than that of CYP2B6. Both CYP2B6 and CYP3A5 catalysed the formation of desethylamodiaquine to a relatively small extent (4 % and 6 % respectively). Although amodiaquine deethylation by CYP3A isoforms has been reported previously (Jewell et al., 1995), CYP3A5 did not catalyse this reaction in a study by Li *et al*. (Li et al., 2002). The 1'-hydroxylation of midazolam

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was catalysed not only by CYP3A5 but also to a small extent by CYP2B6 (2 %). Human CYP2B6 has been shown previously to catalyse this reaction, with a K_m of 41 µM (Hamaoka et al., 2001), far higher than the concentration used in this assay. These analyses confirmed that the probe reactions selected were acceptable for the cocktail assay as any potential impact on IC_{50} values would be negligible.

Enzyme-substrate affinity data provides information on the validity of the enzyme source and experimental conditions since, regardless of enzyme source, K_m values should be similar once incubational binding has been accounted for. Comparison of maximal reaction velocities from various (heterologously expressed) enzyme sources is useful since it provides information on the experimental conditions and the relative catalytic efficiencies of the systems (incorporating P450 and P450 reductase expression levels and coupling). The K_m values estimated in this study were similar to values quoted in the literature (Gibbs et al., 1999; Li et al., 2002; Williams et al., 2002; Yamaori et al., 2003; Walsky and Obach, 2004; Walsky et al., 2005), with the exception of CYP2B6-bupropion which was lower, but within 3-fold of the median literature value of 75µM (Kim et al., 2005, Walsky and Obach, 2004; Faucette et al., 2000, Hesse et al., 2000) and as such can be viewed as valid (Tucker et al., 2001). The V_{max} value of 0.2 pmol/min/pmol P450 was considerably lower than those cited in the literature for CYP2B6-dependent bupropion hydroxylation (P450 expressed in lymphoblastoid cells and baculovirus-infected insect cells: Hesse et al., 2000; Faucette et al., 2000; Walsky and Obach, 2004). However, the V_{max} for diazepam N-demethylation by CYP2B6 (expressed in *E.coli*) of 1.5 pmol/min/pmol P450 measured in this laboratory compares well with the value of 0.6 pmol/min/pmol P450 quoted by the P450 supplier (Cypex) confirming that the 0.2 pmol/min/pmol P450 V_{max} value for bupropion hydroxylation is legitimate. The V_{max} value for CYP2C8-dependent amodiaquine N-deethylation was comparable to data from experiments using CYP2C8 expressed in baculovirus-infected insect cells (Walsky and Obach, 2004) and higher than V_{max} values from studies using CYP2C8 expressed in lymphoblastoid cells and yeast (Li *et al.,* 2001). The midazolam 1'-

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hydroxylation V_{max} also compares well with values determined using other recombinant systems (Walsky and Obach, 2004; Williams et al., 2002; Yamaori et al., 2003).

IC_{50} values were determined for 11 inhibitors using the single P450, single substrate and the cocktail assay formats. In all cases the values obtained from single or cocktail P450 experiments agreed well and were found to be consistent with IC_{50} values found in the literature (Table 1). Based on these findings and the similarity of V_{max} and K_m values for the three probe reactions in the single P450, single substrate and cocktail assays, the cocktail assay was considered acceptable for use.

196 compounds, from several distinct chemical series encompassing a wide range of physico-chemical properties (see Materials and Methods) were tested for inhibitory potency against CYP2B6, CYP2C8 and CYP3A5 using the cocktail assay. The same compounds were tested for inhibitory potency against CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 as described previously (Weaver et al., 2003). The purpose of this study was in part to evaluate the utility of the screen for inhibition of CYP2B6, CYP2C8 and CYP3A5 in a drug discovery setting. Figure 2 shows the comparison of CYP2B6, CYP2C8 and CYP3A5 IC_{50} values with those determined for CYP2C9 and CYP3A4. Generally compounds were more potent inhibitors of CYP3A4 than either CYP2B6, CYP2C8 or CYP3A5 and more potent inhibitors of CYP2C9 than CYP2C8. In this dataset there are very few compounds which exhibit lower IC_{50} values against CYP2B6, CYP2C8 and CYP3A5 than for CYP2C9, CYP2C19, CYP2D6 and CYP3A4. It would be wrong to claim that this finding would hold true for all compounds. However, the compound set chosen here is reasonably large (almost 200), from 16 separate and chemically distinct series and with a broad range of physico-chemical properties. Consequently, the trends observed in this study may prove to be the rule rather than the exception.

In conclusion, the methods presented here offer a single inhibition assay for CYP2B6, CYP2C8 and CYP3A5. The data indicates that the value of routinely assessing the inhibition of CYP2B6, CYP2C8 and CYP3A5 in addition to CYP1A2,

CYP2C9, CYP2C19, CYP2D6 and CYP3A4 is debateable. However, as part of a thorough assessment of DDI risk for leading compounds at key drug discovery milestones, investigating inhibition of these additional P450 enzymes is recommended.

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Figure 1. Michaelis-Menten plots for single substrate, single P450 incubations: (a) the metabolism of bupropion to hydroxy bupropion by CYP2B6, (b) the metabolism of amodiaquine to N-desethyl amodiaquine by CYP2C8, (c) the metabolism of midazolam to 1'-hydroxy midazolam by CYP3A5. Data points represent the mean of triplicate incubation results and error bars are the standard deviation from the mean.

Figure 2. Plots showing the relationship between IC_{50} estimated for (a) CYP3A4 and CYP2B6, (b) CYP3A4 and CYP2C8, (c) CYP3A4 and CYP3A5 and (d) CYP2C9 and CYP2C8. Data was generated using the CYP2B6, CYP2C8 and CYP3A5 cocktail inhibition assay described in this publication and using the CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 cocktail inhibition assay described by Weaver et al., 2003. Solid lines are unity and dashed lines are 5-fold from unity.

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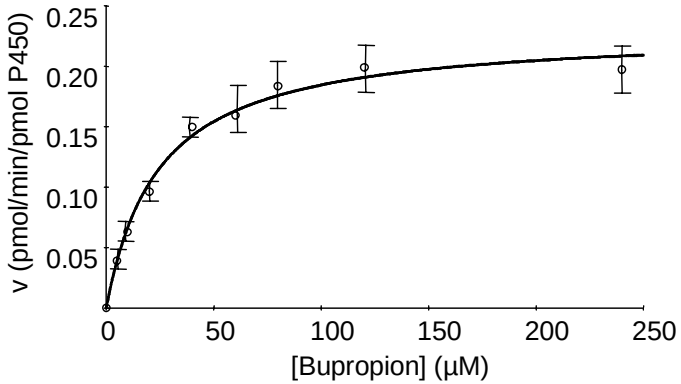
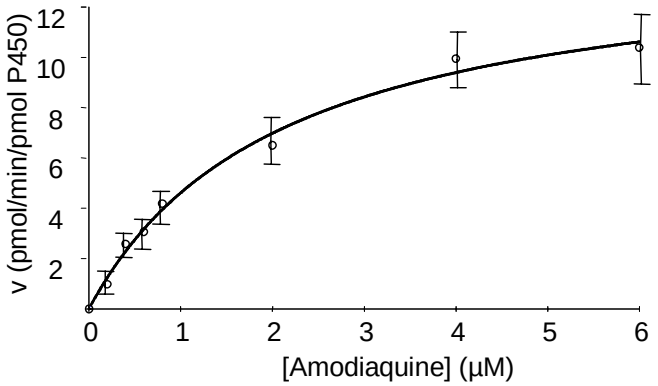
Table 1. IC_{50} values estimated using the 'cocktail' and single substrate, single P450 inhibition assays described in this publication and comparisons to data available in the scientific literature

Enzyme	Compound	IC_{50} μM		
		Single P450	P450 'Cocktail'	Literature
CYP2B6	Ketoconazole	4	4	3.5[1]
	Tranylcypromine	10	6	3.2[1]
	Ticlopidine	0.06	0.05	0.1[1]
CYP2C8	Ketoconazole	4	4	5.5[2]
	Tranylcypromine	103	113	12[2]
				>100[1]
	Quercetin	3.3	5.6	4[2]
	Paclitaxel	24	23	13.3[2]
	Montelukast	0.01	0.009	0.009[2]
	Ritonavir	1	2	3[2]
	Tamoxifen	10	3	3.3[2]
	Nifedipine	23	20	9.7[2]
CYP3A5	Ketoconazole	0.3	0.23	0.15[3]
				0.11[3]
	Erythromycin	15	17	64[3]
	Diltiazem	37	48	117[3]

[1]Turpeinen et al., 2005; [2]Walsky et al., 2005; [3]Gibbs et al., 1999

Figure 1





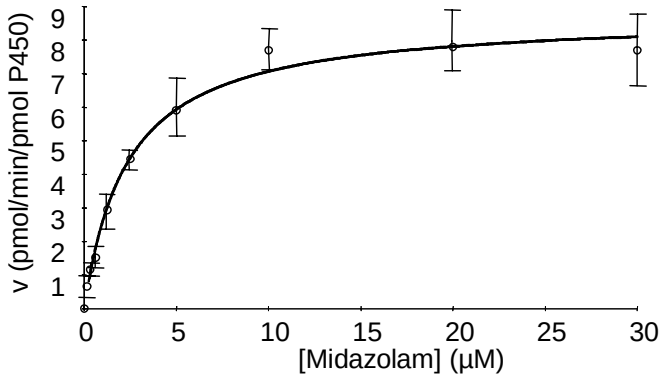


Figure 2