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

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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. In this study, we 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 analyzed using liquid chromatography-tandem mass spectrometry. Kinetic analyses were performed to establish suitable conditions for inhibition assays, which were subsequently automated. CYP2B6, CYP2C8, and CYP3A5 IC₅₀ values were determined for marketed drugs and almost 200 AstraZeneca discovery compounds from 16 separate discovery projects. For the marketed drugs, results obtained were comparable with literature values. Data were also compared with IC₅₀ 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 of CYP2B6, CYP2C8, or CYP3A5. The potential impact of these findings on a cytochrome P450 inhibition strategy is discussed.

Drug discovery programs traditionally study CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 when the metabolism and enzyme inhibition potential of new chemical entities (NCEs) are being investigated. 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. In addition, increased variability in the pharmacokinetics of drugs metabolized 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 metabolizes 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). In addition, large interindividual variations in hepatic CYP2B6 have been demonstrated because of 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 ~7% of the total microsomal content in the liver (Shimada et al., 1994; Rendic and Di Carlo, 1997). It has been shown to be involved in the metabolism of a variety of drugs including amiodarone, amodiaquine, diclofenac, and troglitazone (Yamazaki et al., 1999; Ohyama et al., 2000; Tang, 2003; Walsky and Obach, 2004), 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-American populations with an allele frequency of 18%, but it is relatively uncommon in Caucasian populations, whereas CYP2C8*3 is more common in Caucasian individuals (23%) and quite rare in African-American subjects (Totah and Rettie, 1992).

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 one-third of Caucasians and up to 60% of African-Americans (Kuehl et al., 2001) in addition to other organs such as the intestine (Paine et al., 1997; Lin et al., 2002) 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 comprising CYP3A5 remains under dispute and has been cited as ranging from 4% (Koch 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

ABBREVIATIONS: NCE, new chemical entity; P450, cytochrome P450; LC/MS/MS, liquid chromatography/tandem mass spectrometry; DMSO, dimethyl sulfoxide; MTP, microtiter plate.
screen using recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 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 Escherichia coli. An automated version of the assay was established, and its impact on P450 inhibition screening strategy was assessed.

**Materials and Methods**

*E. coli* coexpressing the relevant P450s and human P450 reductase were purchased from Cypex (Dundee, UK). Desethylamodiaquine and hydroxybupropion were purchased from Synfine Research (Richmond Hill, ON, Canada) and BD Gentest (Woburn, MA) respectively. Amodiaquine, bupropion, NADPH, and all other cited chemicals were purchased from Sigma-Aldrich (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 (0.1 M, pH 7.4), and P450 concentrations were 18, 1, and 5 pmol/ml for CYP2B6, CYP2C8, and CYP3A5, respectively. All incubations (controls and probe and test inhibitors) were 100 μl, contained DMSO at 1% v/v and were terminated after 10-min incubations at 37°C by the addition of ice-cold methanol (200 μl). Samples were placed at −20°C for 2 h and centrifuged at 22000 g for 15 min, and the supernatant (120 μl) was transferred to 96-well microtiter plates (MTPs) (Agilent Technologies UK Ltd, South Queensferry, West Lothian, UK) for LC/MS/MS analysis.

**Analytical Conditions.** The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol). Samples (20 μl) were injected onto a Hypersil Gold column (18×5 mm, 1.5 μm; 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), and 97% A (2.8 min). The flow rate was 0.4 ml/min, and the column temperature was 50°C.

LC/MS/MS was performed using an Agilent HP1100 high-performance liquid chromatography system (Agilent Technologies UK Ltd.) coupled to a triple quadrupole Quadrupole Platinum mass spectrometer (Micromass, Manchester, UK) operating in ESI+ mode, with Masslynx 4.0 running in multiple reaction monitoring mode (three multiple reaction monitorings simultaneously: hydroxy bupropion, 256.19 → 184.13; desethylamodiaquine, 328.36 → 282.99; 1′-hydroxymidazolam, 342.17 → 202.90; dwell time of 0.2 s).

**Enzyme Kinetics. Time and P450 Concentration linearity.** Incubations were performed using substrate concentrations approximating to $K_m$ and 5× $K_m$ (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 reincubated 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 analyzed as described above.

**Determination of $K_m$ and $V_{max}$ values.** Incubations were carried out with 10 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, CYP2C8, and CYP3A5, 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 incubations containing all three pooled P450 enzymes, all three pooled substrates, and DMSO (1% v/v) to emulate the conditions of the final cocktail incubation assay.

**Materials and Methods.** 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 optimized reaction time and P450 concentration 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, CYP2C8, and CYP3A5, respectively. The incubation volumes were 200 μl and contained 1 mM NADPH. Test inhibitors (100× 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 were identical in design to the five P450 inhibition assays 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; and ketoconazole, CYP3A5). Incubations were carried out with six 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, and mifepristone). Determination of IC_{50} values for the 11 probe inhibitors were performed using single substrate, single P450 and cocktail substrate, and 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 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).

**Results**

**Time and P450 Concentration Linearity.** At both 6 and 300 μM bupropion, CYP2B6-dependent bupropion hydroxylation was linear to 15 min with CYP2B6 concentrations up to 100 pmol/ml. At both 0.1 and 10 μM amodiaquine, the CYP2C8-dependent formation of N-desethylamodiaquine was linear to 15 min with CYP2C8 concentrations up to 2 pmol/ml. At both 0.2 and 8 μM 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 in which the formation of metabolite was linear with respect to time and P450 concentration, the amount of substrate consumed was $V_{max}$ and $K_m$ determinations and the automated CYP2B6, CYP2C8, and CYP3A5 cocktail inhibition assay, an incubation time of 10 min was chosen with CYP2B6, CYP2C8, and CYP3A5 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 $K_m$ values, up to a value of 5×$K_m$. 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 represent the mean of triplicate incubation results, and error bars are the SD from the mean.

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 optimized reaction time and P450 concentration to assess substrate selectivity. The results demonstrated that bupropion hydroxylation was also catalyzed by CYP3A5 (1%) but not by CYP2C8, amodiaquine deethylation was catalyzed by both CYP3A5 (6%) and CYP2B6 (4%), and midazolam 1'-hydroxylation was catalyzed by CYP2B6 (2%) but not by CYP2C8, where the values in parentheses refer to percentage metabolism compared with the “selective” P450. This was deemed acceptable because 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; and ketoconazole, CYP3A5) were determined using both single P450, single substrate and combined P450, and 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 eight 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 physicochemical properties (see Materials and Methods) were tested in the final automated CYP2B6, CYP2C8, and CYP3A5 cocktail assay. The IC\(_{50}\) values generated were compared with 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).

Discussion

This article describes an automated, cocktail P450 inhibition screen using substrates selective for CYP2B6, CYP2C8, and CYP3A5 with an LC/MS/MS endpoint. 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 were 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 (Li et al., 2002; Kim et al., 2005; Turpeinen et al., 2005), and midazolam as a substrate for CYP3A5 (Gorski et al., 1994; Williams et al., 2002; Huang et al., 2004). Substrates cited for the analysis of CYP2B6 activity include 7-ethoxy-4-trifluoromethylcoumarin O-deethylation and 3-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, in which 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 catalyzes the hy-

![FIG. 1. Michaelis-Menten plots for single substrate, single P450 incubations: top, the metabolism of bupropion to hydroxybupropion by CYP2B6; middle, the metabolism of amodiaquine to N-desethylamodiaquine by CYP2C8; and bottom, the metabolism of midazolam to 1'-hydroxymidazolam by CYP3A5. Data points represent the mean of triplicate incubation results, and error bars are the SD from the mean.](image-url)
droxylation of bupropion (Faucette et al., 2000), albeit with a $K_m$ value at least 20-fold higher than that of CYP2B6. Both CYP2B6 and CYP3A5 catalyzed 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 catalyze this reaction in a study by Li et al. (2002). The $1/2$H11032-hydroxylation of midazolam was catalyzed not only by CYP3A5 but also to a small extent by CYP2B6 (2%). Human CYP2B6 has been shown previously to catalyze this reaction, with a $K_m$ of 41 $\mu$M (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 IC50 values would be negligible.

Enzyme-substrate affinity data provide information on the validity of the enzyme source and experimental conditions because, regardless of the 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 because 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; Kim et al., 2005), with the exception of CYP2B6-dependent bupropion which was lower, but within 3-fold of the median literature value of 75 $\mu$M (Faucette et al., 2000; Hesse et al., 2000; Walsky and Obach, 2004; Kim et al., 2005) 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 with 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., 2002). The midazolam $1/2$-hydroxylation $V_{max}$ also compares well with values determined using other recombinant systems (Williams et al., 2002; Yamaori et al., 2003; Walsky and Obach, 2004).

IC50 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 IC50 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.

A total of 196 compounds, from several distinct chemical series encompassing a wide range of physicochemical 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 IC50 values with those determined for CYP2C9 and CYP3A4. In general, compounds were more potent inhibitors of CYP3A4 than of either CYP2B6, CYP2C8, or CYP3A5 and more potent inhibitors of CYP2C9 than of CYP2C8. In
this dataset, there are very few compounds that exhibit lower IC50 values against CYP2B6, CYP2C8, and CYP3A5 than against 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 physicochemical 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 indicate that the value of routinely assessing the inhibition of CYP2B6, CYP2C8, and CYP3A5 in addition to CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 is debatable. However, as part of a thorough assessment of drug-drug interaction risk for leading compounds at key drug discovery milestones, investigating inhibition of these additional P450 enzymes is recommended.

References
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