Minimizing Polymorphic Metabolism in Drug Discovery: Evaluation of the Utility of in Vitro Methods for Predicting Pharmacokinetic Consequences Associated with CYP2D6 Metabolism

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ABSTRACT:

Minimizing interindividual variability in drug exposure is an important goal for drug discovery. The reliability of the selective CYP2D6 inhibitor quinidine was evaluated in a retrospective analysis using a standardized approach that avoids laboratory-to-laboratory variation. The goal was to evaluate the reliability of in vitro metabolism studies for predicting extensive metabolizer (EM)/poor metabolizer (PM) exposure differences. Using available literature, 18 CYP2D6 substrates were selected for further analysis. In vitro microsomal studies were conducted at 1 μM substrate and 0.5 μM P450 to monitor substrate depletion. An estimate of the fraction metabolized by CYP2D6 in microsomes was derived from the rate constant determined with and without 1 μM quinidine for 11 substrates. Clearance in EM and PM subjects and fractional recovery of metabolites were taken from the literature. A nonlinear relationship between the contribution of CYP2D6 and decreased oral clearance for PMs relative to EMs was evident. For drugs having <60% CYP2D6 involvement in vivo, a modest difference between EM and PM exposure was observed (<2.5-fold). For major CYP2D6 substrates (>60%), more dramatic exposure differences were observed (3.5- to 53-fold). For compounds primarily eliminated by hepatic P450 and with sufficient turnover to be evaluated in vitro, the fraction metabolized by CYP2D6 in vitro compared favorably with the in vivo data. The in vitro estimation of fraction metabolized using quinidine as a specific inhibitor provided an excellent predictive tool. Results from microsomal substrate depletion experiments can be used with confidence to select compounds in drug discovery using a cutoff of >60% metabolism by CYP2D6.

Minimizing interindividual variability in drug exposure is an important goal in drug discovery. One major source of interindividual variability in drug exposure involves clearance. The variable expression of drug-metabolizing enzymes can result in profound differences in drug exposure across a patient population. The genetic basis for interindividual variability has been described for numerous drug-metabolizing enzymes. Some common examples of polymorphic drug-metabolizing enzymes include thiopurine methyltransferase, glutathione S-transferase M1-1, CYP2C9, CYP2C19, CYP2D6, CYP3A5, N-acetyltransferase 1, UGT1A1, and flavin monooxygenase 3 (Haining and Yu, 2003).

The consequences of polymorphic enzymes on drug metabolism in relation to efficacy and side effects has been the focus of numerous studies (Vandel et al., 1999; Dandara et al., 2001). For instance, individuals lacking the expression of a polymorphic drug-metabolizing enzyme (commonly referred to as “poor metabolizers” or PMs) will have higher drug exposure if those drugs are metabolized by those polymorphic enzymes, which could lead to exaggerated pharmacology or enhanced side effects relative to the intermediate metabolizer and extensive metabolizer (EM) subjects given the same dose (Mahgoub et al., 1977). Alternatively, if a polymorphic enzyme forms a particular metabolite that contributes to the activity of a drug, then different efficacy profiles might be observed in EM and PM subjects (Poulsen et al., 1996).

Of the identified polymorphic enzymes involved in drug metabolism, CYP2D6 is considered one of the most important, with a substrate specificity typical of many new chemical entities (broadly speaking, lipophilic bases). An estimated 20 to 25% of all drugs in clinical use are metabolized at least in part by CYP2D6 (Evans and Relling, 1999). The frequency of CYP2D6 PMs in the population depends on race and is reported to be approximately 1% of Asians and 5 to 10% of Caucasians (Shimizu et al., 2003). The primarily hepatic expression of this enzyme governs first pass metabolism after oral drug administration, whereas the low levels of intestinal expression do not appear to be important (Madani et al., 1999). Numerous studies have characterized the impact of CYP2D6 polymorphism on substrate area under the curve (AUC) in EM and PM subjects, and a recent article by Dorne et al. (2002) provides a useful database of human clearance and metabolite recovery data.

Because the safety profile of a new discovery candidate is often unknown, it is beneficial to limit the contribution of polymorphic enzymes below some cutoff to avoid the requirement of phenotyping/genotyping before the initiation of drug therapy and distinct dosing regimens in EM and PM subjects. A variety of in vitro tools are available to determine the relative contribution or percent contribution...
to metabolism by a polymorphic enzyme. These include coinubcation with specific enzyme inhibitors (chemical inhibitors/inhibitory antibodies), use of poor metabolizer in vitro reagents (either human fractions or recombinant systems), and studies in individually expressed recombinant enzymes (Bjornsson et al., 2003; Williams et al., 2003). The quantitative link between results from these assays and clinical pharmacokinetic variability has not been described.

The objective of our studies was to investigate the relationship between estimated fraction metabolized by CYP2D6 in vitro and pharmacokinetic impact observed in clinical studies. Because of the wealth of data involving in vitro and in vivo studies and the frequency of encountering CYP2D6 substrates, this polymorphism served as the basis for examining the impact of polymorphic metabolism more closely. Although less data exists on the impact of other polymorphisms, we feel that the results with CYP2D6 can be generalized to other situations in which a polymorphism contributes to individual variability in clearance.

Materials and Methods

Materials. Human liver microsomes (pooled from 60 donors) were purchased from BD Biosciences (Bedford, MA). Atomoxetine, benzirlinuranol, duloxetine, sertraline, tolterodine, and venlafaxine were synthesized at Pfizer Global Research and Development (Groton, CT and Sandwich, UK). Furafylline and (S)-methylenephenylpiperidine were obtained from Salford Ultrafine Chemicals and Research Ltd. (Manchester, UK). All other reagents were of at least Analar grade quality, obtained from Sigma Chemical Co. (Poole, UK).

Microsomal Incubations. Human liver microsomal incubations were performed at 0.5 μM P450 (1.5 mg protein/ml) and 1 μM substrate, in the presence and absence of P450 inhibitors. Each incubation (final volume 1.2 ml) comprised 50 mM potassium phosphate buffer (pH 7.4) and 5 mM MgCl2. Reducing equivalents required for P450 metabolism were provided by NADPH (1 mM), which was regenerated in situ by an isocitric acid/isocitric acid dehydrogenase system. Over the 60-min incubation period, 100-μl samples were removed and added to 100 μl of ice-cold acetoneitrile containing internal standard to terminate the reaction. Samples were centrifuged at 2000 g for 40 min, and 80 μl was directly injected onto a generic high-performance liquid chromatography-mass spectrometry system (Sciex API 2000 Mass Spectrometer with TurboIonSpray interface, with an OptiLynx Reliasil C18 liquid chromatography-mass spectrometry system (Sciex API 2000 Mass Spectrometer with TurboIonSpray interface, with an OptiLynx Reliasil C18 column). Peak responses were judged to be within the linear range for the instrument.

Specificity of Chemical Inhibitors. Incubations (n = 2) were performed as described above with the probe substrates phenacetin (CYP1A2), diclofenac (CYP2C9), S-methylenephenylpiperidine (CYP2C19), dextromethorphan (CYP2D6), and midazolam (CYP3A4). For each substrate, incubations were performed in the presence and absence of P450 inhibitors, 10 μM furafylline (CYP1A2), 10 μM sulfaphenazonate (CYP2C9), 3 μM benzirlinuranol (CYP2C19), 1 μM quinidine (CYP2D6), and 1 μM ketocmazaole (CYP3A4), and first order disappearance rate constants were determined.

Incubations with CYP2D6 Substrates. In a preliminary investigation, incubations (n = 2) were performed as described above, for the following CYP2D6 substrates: amitriptyline, atomoxetine, desipramine, dextromethorphan, duloxetine, flecainide, fluoxetine, fluvoxamine, imipramine, metoprolol, mexiteline, nortriptyline, propafenone, propranolol, sertraline, sparteine, tolterodine, and venlafaxine. Substrates exhibiting sufficient metabolic vulnerability in human liver microsomes were further investigated. Incubations were performed in the presence (n = 4) and absence (n = 4) of 1 μM quinidine. Each substrate was assayed over four to six separate days to assess variability of the assay.

Data Analysis. The first order disappearance rate constant was determined for each incubation by plotting ln substrate conc. (peak area ratio, drug/internal standard) against incubation time and determining the gradient of the regression line, using data for which an accurate first order decay curve could be obtained. Data were only used when there were at least three time points collected per incubation, substrate had been depleted by >20% by the final time point, and regression of the log-linear substrate declination plot gave a correlation coefficient of >0.9.

The mean disappearance rate constant data generated on each day in the presence and absence of inhibitor was used to calculate the percentage P450 contribution using the equation below:

\[ \% \text{ contribution} = \frac{(k) - (k_{\text{inh}})}{(k)} \times 100 \]

where k is the disappearance rate constant and k_{inh} is the disappearance rate constant in the presence of inhibitor.

Theoretical Considerations. For this analysis, two key parameters were included in the prediction of CL or AUC in EM and PM subjects. The first parameter characterized the degree of functional impairment that the polymorphism causes and was termed EF (enzyme function). For example, homozgyous CYP2D6*6 individuals (PM) do not express functional hepatic CYP2D6 enzyme caused by a gene deletion (EF = 0). Contrary to the situation with CYP2D6, a polymorphism in CYP2C9 (CYP2C9*3) has been shown to reduce the capacity of the enzyme (V_{max}/K_{m}) to ~15 to 20% (EF = 0.15) of the wild-type enzyme (Haining et al., 1996; Sullivan-Klose et al., 1996; Miners and Birkett, 1998; Kirchheimer et al., 2002). The fraction of metabolism by a polymorphic enzyme is the second key parameter that must be considered (fm_{PM}), which has also been referred to as relative contribution. One way to determine this parameter involves microsomal incubations and selective P450 inhibitors with the potential limitations described below.

Equations were adapted from the literature based on the predictions of dose adjustments required in patients with renal impairment (Rowland and Matin, 1973; Shaw and Houston, 1987; Rowland and Tozer, 1989). More recently, the same principles have been applied to improve the prediction of metabolism-based drug-drug interactions (Venkatakrishnan et al., 2003; Ito et al., 2005).

For the purposes of this analysis, hepatic clearance in poor metabolizers (CL_{PM}) was defined as follows:

\[ \text{CL}_{PM} = \text{EF} \cdot \text{CL}_{EM} \]  \hspace{1cm} (1)

where EF (enzyme function) is the ratio of polymorphic enzyme function in a PM relative to an EM subject. As described above, EF values greater than 0 and less than 1 could represent the degree of impairment in enzyme function resulting from the polymorphism.

For many drugs, clearance by P450 represents one of several elimination pathways. The clearance by a specific P450 enzyme in an EM subject was calculated by the equation below:

\[ \text{CL}_{EM} = f_{m} \cdot \text{CL}_{EM} \]  \hspace{1cm} (2)

where fm is the fraction metabolized to a particular metabolite and CL_{EM} is the hepatic clearance in EM subjects. For example, the fraction metabolized by a specific P450 enzyme was denoted as fm_{CYP2D6}.

The relative importance of other elimination pathways is calculated by the equation below:

Other CL = [1 - fm] \cdot \text{CL}_{EM} \hspace{1cm} (3)

and the total clearance is the sum of all clearance pathways.

Total CL = \text{CL}_{EM} + \text{Other CL} \hspace{1cm} (4)

To calculate the ratio of CL in PM relative to EM subjects, the following equation takes into account the relative importance of P450 metabolism to the overall elimination of a drug and the enzyme function in PM subjects:

\[ R_{CL} = \frac{\text{CL}_{PM}}{\text{CL}_{EM}} = \text{EF} \cdot f_{m} + [1 - f_{m}] \]  \hspace{1cm} (5)

An inverse relationship exists between CL and AUC. Equation 6 was used to calculate differences in mean AUC between PM and EM subjects:

\[ R_{AUC} = \frac{\text{AUC}_{PM}}{\text{AUC}_{EM}} = \frac{1}{\text{EF} \cdot f_{m} + [1 - f_{m}]} \]  \hspace{1cm} (6)

For the specific case where CYP2D6 is the polymorphic enzyme of interest and EF = 0, it has been shown (Ito et al., 2005) that R_{AUC} can be simplified to:

\[ R_{AUC} = \frac{1}{f_{m}} \]
TABLE 1

Selectivity of chemical inhibitors against probe substrates

<table>
<thead>
<tr>
<th>Substrate (1 μM)</th>
<th>10 μM Furaflurine</th>
<th>10 μM Sulphinpyrazone</th>
<th>3 μM Benzylnirvanol</th>
<th>1 μM Quinidine</th>
<th>1 μM Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phencetin</td>
<td>&gt;81.3</td>
<td>15.2</td>
<td>20.5</td>
<td>1.4</td>
<td>17.0</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>20.6</td>
<td>&gt;92.9</td>
<td>7.5</td>
<td>0</td>
<td>12.9</td>
</tr>
<tr>
<td>S-Mephenytoin</td>
<td>6.1</td>
<td>5.8</td>
<td>98.6</td>
<td>7.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>17.4</td>
<td>5.7</td>
<td>7.4</td>
<td>90.4</td>
<td>15.6</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0</td>
<td>0</td>
<td>4.3</td>
<td>3.3</td>
<td>87.4</td>
</tr>
</tbody>
</table>

\[ R_{\text{AUC}} = \frac{\text{AUC}_{\text{PM}}}{\text{AUC}_{\text{EM}}} = \frac{1}{1 - f_{\text{M,CYP2D6}}} \]  

**Results**

**Specificity of Chemical Inhibitors.** The success of the in vitro methodology relies upon the availability of specific inhibitors for the enzymes of interest and sufficient metabolic vulnerability of the test compound. Specific inhibitors are reported in the literature for many of the P450 enzymes (Newton et al., 1995), and these have traditionally been used to assess percentage contributions to metabolism by P450 enzymes. As a preliminary investigation, the specificity of these inhibitors was reassessed in the specific batch of human liver microsomes used for this study. A P450 concentration of 0.5 μM was chosen, which is higher than the P450 content typically used in substrate depletion assays (0.25 μM). Using 0.5 μM P450 will increase metabolic vulnerability by approximately 1.5-fold (S.D. = 0.45, n = 45) (data not shown), thereby increasing the ability to investigate more slowly metabolized compounds.

Incubations (n = 2) were performed at 0.5 μM P450 and 1 μM substrate on two separate days. The effect of inhibitors on both the target P450 and cross-reactivity against the other P450s was assessed. Of the probe substrates investigated, substrate loss could be monitored for all, with the exception of S-mephenytoin, where over the course of the 60-min incubation, substrate depletion was insufficient to determine a metabolic rate (i.e., <20% substrate depletion after 60 min). Thus, effects on CYP2C19 activity were assessed based on 4-OH mephenytoin metabolite formation. In this instance, only data points describing the initial linear reaction velocity were used. Results are summarized in Table 1 and clearly demonstrate that although some cross-reactivity is observed, the inhibitors demonstrate sufficient selectivity (>80% for target substrate, <20% for other substrates) at the inhibitor concentrations selected.

**Incubations with CYP2D6 Substrates.** An initial screening of metabolic stability for 18 CYP2D6 substrates was performed to establish those compounds with appropriate metabolic stability in human liver microsomes. Figure 1 summarizes the initial disappearance half-life estimates performed at 0.5 μM P450 and 1 μM substrate. Of the 18 compounds investigated, 5 were assessed as being too metabolically stable (t_{1/2} >100 min) to investigate further using this approach, and 2 had analytical issues and were not investigated further. The half-lives of the remaining 11 CYP2D6 substrates ranged from 4.7 to 58 min in human liver microsomal stability studies.

Incubations in the presence and absence of 1 μM quinidine were performed for the 11 remaining CYP2D6 substrates, and the disappearance half-life was determined. Four replicate incubations were performed (with and without quinidine) over four to six separate days and the data used to calculate a percentage CYP2D6 contribution. Representative examples of the human microsomal substrate depletion data (±1 μM quinidine) are provided in Fig. 2 for atomoxetine and amitriptyline.

**Interday Variability in the Data.** Table 2 summarizes the variability in the control half-life data collected for up to 6 days for the 11 CYP2D6 substrates. Tolterodine was rapidly metabolized at 0.5 μM P450, and incubations were therefore performed at 0.1 μM P450 in the presence and absence of quinidine for this substrate. Propafenone was also investigated at this P450 concentration to assess the validity of this as an approach. Similar calculated percentage CYP2D6 contributions were obtained for propafenone at both 0.5 μM and 0.1 μM P450 (Table 3), suggesting that 0.1 μM P450 in combination with 1 μM quinidine could be used to assess CYP2D6 contributions for rapidly metabolized compounds. In-house data would suggest that differences in micromolar binding for quinidine at 0.5 μM and 0.1 μM, in this batch of human liver microsomes, is not likely to play a major role, with only a 2-fold change in free concentrations. To

![Fig. 1. Metabolic vulnerability of CYP2D6 substrates in pooled human liver microsomes (0.5 μM P450). Half-life could not be determined for fluvoxamine, metoprolol, mexiletine, nortriptyline, and sparteine because of high metabolic stability (>100 min). Half-life of flecainide and fluoxetine was not determined because of analytical issues.](image-url)
calculate the CYP2D6 contribution, the mean disappearance rate constants from the four replicate incubations on a single day, in the presence and absence of quinidine, were used. The experimentally determined percentage CYP2D6 contributions are provided in Table 3.

Statistical analysis of the half-life data indicates that the greatest variability is observed interday (see Table 2: CV of up to 63.2%) rather than intraday. However, since for each determination the final reported result (% CYP2D6 contribution) is the relationship between the plus and minus inhibitor incubation on a single day, the intraday variability in absolute metabolic stability is automatically corrected, as demonstrated by the low variability observed in Table 3. Further statistical analysis of the data indicates that by performing the assay in the configuration used for this analysis, and using a positive control, to ensure added confidence, the assay need only be run over 2 days to give a 95% confidence interval of less than ±12% for the predicted percentage CYP2D6 contribution.

The percent contribution data were analyzed by assuming normal errors, common variance for each substrate, and the censored values were also bound to below 100. A Bayesian analysis assuming negligible prior information was implemented using the WinBUGS program, with the mean and its standard deviation of each substrate’s percent CYP2D6 response being reported. The estimated mean percent CYP2D6 contribution in vitro for each substrate is summarized in Table 3.

Theoretical Considerations. Simulations were performed to predict the ratio of AUCPM/AUCEM as a function of the fraction metabolized by a polymorphic enzyme. Figure 3 shows the theoretical relationship between the ratio of AUCPM/AUCEM (equivalent to CLEM/CLPM) and the degree of enzyme functional impairment on the x-axis. As the degree of enzyme impairment becomes more severe (and closer to zero) the distinction between PM and EM exposure was predicted to become larger. The extent of P450 metabolism relative to other elimination routes can also be considered in cases where the fraction of a dose metabolized by a particular isoform ($f_{mn}$) is less than 1. For instance, if $f_{mn}$ is 0.5 and the degree of enzyme function is reduced to 0, the analysis suggested that a 2-fold increase in AUC would be observed in PM subjects relative to EM subjects.

*0.1 μM substrate.
A variety of methods have been used to characterize the metabolism of CYP2D6 substrates to specific products. In Table 5, the products of evaluation, and the estimated importance of CYP2D6 to product formation. In Table 4, including the major products of CYP2D6 metabolism, methods of evaluation, and the estimated importance of CYP2D6 to product formation. A variety of methods have been used to characterize the metabolism of CYP2D6 substrates to specific products. In Table 5, the estimated faction metabolized by CYP2D6 using microsomal incubations measuring substrate depletion with and without 1 μM quinidine. For drugs with CYP2D6 as the major determinant of clearance (>60%), large differences in AUC or oral clearance were observed between EM and PM subjects (Fig. 4). The most extreme pharmacokinetic differences were observed with dextromethorphan and tolterodine, which showed 53- and 22-fold higher oral clearances in EM subjects compared with PM subjects. Modest differences in oral clearance (3.5- to 10-fold) were observed with atomoxetine, propafenone, desipramine, and venlafaxine between EM and PM subjects. For the five drugs that had a low estimated CYP2D6 contribution in vivo, differences between EM and PM oral clearances were minor. For amitriptyline, and propranolol, a <3-fold difference in CL/F was observed for EM and PM subjects. The remaining drugs had <2-fold decrease in CL/F for PM subjects relative to EM subjects. In general, the observed versus predicted EM/PM differences were in agreement.

**Discussion**

A number of methodologies have been used to characterize the role of P450 isoforms in the metabolism of drugs, and chemical inhibition experiments are generally viewed as the most reliable. A preliminary investigation demonstrated that 1 μM quinidine is an appropriate concentration for investigating CYP2D6 metabolism. Thus, the fraction metabolized by CYP2D6 in microsomes was determined for 11 drugs. The fraction metabolized in vivo was estimated from available literature data by multiplying the in vitro contribution by CYP2D6 to the formation of specific metabolite(s) by the fractional metabolite recovery in EM subjects in vivo. A comparison between the in vitro

**TABLE 4**

<table>
<thead>
<tr>
<th>Drug</th>
<th>CYP2D6 Products</th>
<th>Method(s) of Evaluation</th>
<th>% CYP2D6 Contribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>10-OH</td>
<td>1, 4</td>
<td>83</td>
<td>Venkatakrishnan et al., 2001b</td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>4-OH</td>
<td>2, 3</td>
<td>Major pathway</td>
<td>Ring et al., 2002</td>
</tr>
<tr>
<td>Desipramine</td>
<td>2-OH</td>
<td>1</td>
<td>Major pathway</td>
<td>von Molke et al., 1994</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>O-Desmethyl</td>
<td>1</td>
<td>Major pathway</td>
<td>Broly et al., 1989</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>4-, 5-, or 6-OH of the naphthyl ring</td>
<td>1</td>
<td>Primary enzyme with CYP1A2</td>
<td>Skinner et al., 2003</td>
</tr>
<tr>
<td>Imipramine</td>
<td>2-OH</td>
<td>1, 4, 5</td>
<td>Major pathway</td>
<td>Kroemer et al., 1989</td>
</tr>
<tr>
<td>Propafenone</td>
<td>5-OH</td>
<td>2, 5</td>
<td>Major pathway</td>
<td>Masubuchi et al., 1994</td>
</tr>
<tr>
<td>Propranolol</td>
<td>4-OH, 5-OH</td>
<td>1</td>
<td>40</td>
<td>Kobayashi et al., 1999</td>
</tr>
<tr>
<td>Sertraline</td>
<td>5-OH</td>
<td>1</td>
<td>&gt;20–35</td>
<td>Postlind et al., 1998</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>O-Desmethyl</td>
<td>1</td>
<td>80</td>
<td>Fogelman et al., 1999</td>
</tr>
</tbody>
</table>

*1 Chemical inhibition with quinidine; 2, correlation analysis in a liver bank; 3, EM/PM livers; 4, recombinant P450 isoforms with scaling for abundance or activity; 5, inhibitory antibody.

**TABLE 5**

<table>
<thead>
<tr>
<th>Drug</th>
<th>fm CYP2D6&lt;sup&gt;a&lt;/sup&gt; (Literature)</th>
<th>Metabolite Recovery&lt;sup&gt;b&lt;/sup&gt; (EM)</th>
<th>Estimated fm CYP2D6&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Estimated fm CYP2D6&lt;sup&gt;d&lt;/sup&gt; (in Vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>0.83</td>
<td>0.50</td>
<td>0.42</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>&gt;0.90</td>
<td>0.87</td>
<td>0.87</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>Desipramine</td>
<td>Major&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.68</td>
<td>0.66</td>
<td>0.80 ± 0.08</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Major&lt;sup&gt;e&lt;/sup&gt;</td>
<td>N.D.&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.90</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>Major&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.50</td>
<td>0.50</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.80</td>
<td>0.44</td>
<td>0.35</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Propafenone</td>
<td>N.D.</td>
<td>&gt;0.70&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.70</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>Propranolol</td>
<td>0.40</td>
<td>0.42</td>
<td>0.17</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>Sertraline</td>
<td>&lt;0.20–0.35</td>
<td>N.D.&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.32</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Tolerodine</td>
<td>Major&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.70</td>
<td>0.70</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>Major&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.72</td>
<td>0.72</td>
<td>0.85 ± 0.07</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated conversion to specific metabolite(s) catalyzed by CYP2D6 in vitro.
<sup>b</sup> Metabolite(s) recovery in vivo for CYP2D6 products taken from Dome et al. (2002).
<sup>c</sup> Calculated as the product of the fractional conversion by CYP2D6 taken from the literature and metabolite recovery in EM subjects.
<sup>d</sup> Fraction metabolized by CYP2D6 using microsomal incubations measuring substrate depletion with and without 1 μM quinidine.
<sup>e</sup> For drugs with a major contribution by CYP2D6 in vitro, a value of 1 was used for the calculation of estimated fraction metabolized by CYP2D6 in vivo.
<sup>f</sup> For drugs without metabolite recovery data reported in the literature, a value of 0.90 was used for the calculation of estimated fraction metabolized by CYP2D6 in vivo.
estimate of fraction metabolized and the in vivo estimate of fraction metabolized by CYP2D6 from available literature data revealed a strong relationship (Table 5). These results, although based on a retrospective analysis, support the use of this assay in compound selection during discovery. The inhibition assay was reliable for compounds with moderate to low stability in microsomes, robust (interday variability <25%), and amenable to medium throughput.

Based on the relationship between fraction metabolized by CYP2D6 and the observed pharmacokinetic differences in CL/F between EM and PM subjects, a recommended cutoff value for CYP2D6 metabolism can be established. Figure 4 illustrates the observed ratio between AUC_{PM}/AUC_{EM} and the fraction metabolized by CYP2D6 based on eq. 7.

Because of the wealth of data available for CYP2D6, this polymorphism has served as the basis for examining the in vitro-in vivo relationship more closely. Although less data exists on the impact of other polymorphisms, we feel that the results with CYP2D6 can be generalized to other situations in which a polymorphism contributes to interindividual variability in clearance, in a similar manner. As discussed previously, it is important to consider the effect of the polymorphism on enzyme function in addition to the fraction metabolized by the enzyme. The CYP2C19 polymorphism, like CYP2D6, is a null-polymorphism and results in a complete absence of enzyme activity in homozygous PM. We believe that these similarities between the CYP2D6 and CYP2C19 polymorphisms will allow for generalization of the CYP2D6 results to CYP2C19.

For CYP2C9, a number of allelic variants exist (Goldstein and de Moraes, 1994; Bhasker et al., 1997); however, there appear to be only three naturally occurring in Caucasians: the wild type (CYP2C9*1), CYP2C9*2, and CYP2C9*3 (Sullivan-Klose et al., 1996). The CYP2C9*2 and CYP2C9*3 polymorphisms are associated with a reduction in enzyme function rather than complete ablation of enzyme activity. Homozygous variants for both CYP2C9*2 and CYP2C9*3 have been shown to have a poor metabolizer phenotype (Sullivan-Klose et al., 1996). The clearance of tolbutamide, phenytoin, and glipizide in individuals with the CYP2C9*3 variant has been reported to be at 22%, 21%, and 18%, respectively, of that in normal individuals (Miners et al., 1985; Kidd et al., 1999). Using simulations, it is possible to predict the relationship between fraction cleared through an enzyme with reduced function (EF = 0.15) and differences in EM and PM exposures (refer to Fig. 3). In this instance, the percentage contribution increases to around 70% before a 2.5-fold difference in clearance between EMs and PMs is observed. A similar analysis performed using celecoxib as a probe for CYP2C9 suggested a similar EM/PM CL/F difference using simulations (Tang et al., 2001).

There was strong agreement between the literature values for CYP2D6 contribution as determined in vivo through a variety of methods and data generated in vitro using the 1 μM quinidine/microsomal substrate depletion approach. The large disconnect (greater than 4-fold) between propranolol literature and in-house data underscores the challenge of incorporating information on other routes of metabolism into the assessment of polymorphic enzyme contribution. In vivo, other routes of elimination beyond NADPH-dependent microsomal metabolism are important to the clearance of propranolol (Walle et al., 1985), resulting in an overestimation of the importance of CYP2D6 from microsomes. The use of cryopreserved human hepatocytes may serve as a better tool for compounds with known non-P450 routes of metabolism since similar P450 inhibitor specificities can be achieved in cryopreserved hepatocytes as assessed by probe substrate incubations and those achieved in microsomal assays (Li et al., 1999).

In the initial assessment, a number of drugs that were reported to be partially metabolized by CYP2D6 could not be evaluated using the microsomal depletion approach. This remains a significant challenge for metabolically stable compounds. Other methods based upon metabolite formation, rather than substrate depletion and/or using recombinant P450 isoforms, and scaling factors may be useful. A combination of these approaches has been evaluated using amitriptyline as probe substrate (Venkatakrishnan et al., 2001a). One caveat to this approach is the potential for even greater overestimates of fraction metabolized by an individual enzyme since, in addition to non-P450 pathways being ignored, the estimates will be based solely upon the metabolites chosen to be investigated, rather than the overall P450 metabolism. An alternative approach may be to increase the incubation time for particularly stable substrates. Traditionally, it was not recommended that microsomal incubations proceed for greater than 60 min because of degradation of the P450 enzymes. However, it has been demonstrated that P450 lability can be reduced by introducing reactive oxygen species scavengers, such as superoxide dismutase, into the incubation (Foti and Fisher, 2004). Hence, this method has a potential application for characterization of more slowly metabolized compounds.
There are several additional caveats to the analysis that should also be highlighted. For compounds metabolized through multiple pathways, no consideration has been made for potential saturation of elimination pathways due to accumulation of substrate in the estimate of in vivo %F. If CYP2D6 is a high-affinity enzyme in EM subjects, its deletion in PM subjects could give rise to a situation in which the higher exposure saturates alternative clearance mechanisms. Likewise, in the in vitro assay has been performed under standard conditions of 1 μM substrate and 0.5 μM P450, which may not reflect free concentrations in vivo. If the stability of the compound was measured at concentrations that were above the Km for CYP2D6, and lower concentrations were present after oral administration, an underestimation of the role of CYP2D6 might result.

The use of microsomes as a predictive tool for clearance estimation has been well documented in the literature. For new compounds in drug discovery, it is possible to select compounds with consideration of variability arising from the complement of P450 isoforms contributing to the overall metabolic profile. Our results suggest that compounds that have more than 60% of their metabolism by CYP2D6 are likely to exhibit large differences in CL/F when comparing EM and PM subjects.

References


