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Minimizing polymorphic metabolism in drug discovery: evaluation of the utility of *in vitro* methods for predicting pharmacokinetic consequences associated with CYP2D6 metabolism

John P. Gibbs, Ruth Hyland, and Kuresh Youdim

Pharmacokinetics, Dynamics, and Metabolism, Pfizer, Groton, CT and Sandwich, UK

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Running title: Fraction metabolized by CYP2D6 in vitro and in vivo

Corresponding Author: John P. Gibbs

Address:
Pfizer Inc
Eastern Point Road, Mail stop 8220-4186
Groton, CT 06340
Phone: (860) 715-2432; Fax: (860) 686-1059
Email: john.p.gibbs@pfizer.com

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Abbreviations: EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; T1/2, half-life; fm, fraction metabolized; AUC, area under the concentration time curve; CL, clearance; CL/F, oral clearance.

ABSTRACT

Minimizing inter-individual 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 EM/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 monitoring 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 non-linear relationship between the contribution of CYP2D6 and decreased oral clearance for PM relative to EM 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 (3.5-53-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 utilizing 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 cut-off of >60% metabolism by CYP2D6.

INTRODUCTION

Minimizing inter-individual variability in drug exposure is an important goal in drug discovery. One major source of inter-individual 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 inter-individual 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 mono-oxygenase 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 (Dandara et al., 2001); (Vandel et al., 1999). For instance, individuals lacking the expression of a polymorphic drug metabolizing enzyme (commonly referred to as "poor metabolizers" or PM) 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 and extensive metabolizer (IM and EM, respectively) 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 metabolised at least in part by CYP2D6 (Evans and Relling, 1999). The frequency of CYP2D6 PMs in the population depends on race and are reported to be approximately 1% of Asians and 5-10% of Caucasians. (Shimizu et al, 2003). The primarily hepatic expression of this enzyme governs first pass metabolism after oral drug administration while 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. provides a useful database of human clearance and metabolite recovery data (Dorne et al., 2002).

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 prior to the initiation of drug therapy and distinct dosing regimens in EM and PM subjects. A variety of *in vitro* tools are available in order to determine the relative contribution or % contribution to metabolism by a polymorphic enzyme. These include co-incubation with specific enzyme inhibitors (chemical inhibitors/inhibitory antibodies), use of poor metaboliser *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. Though less data exists on the impact of other polymorphisms, we feel that the results with CYP2D6 can be generalized to other situations where a polymorphism contributes to inter-individual variability in clearance.

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METHODS

Materials

Human liver microsomes (pooled from 60 donors) were purchased from BD Biosciences (Bedford, MA). Atomoxetine, benzylnirvanol, duloxetine, sertraline, tolterodine and venlafaxine were synthesized at Pfizer Global Research and Development (Groton, CT and Sandwich, UK). Furafylline and (*S*)-mephenytoin 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 0.5µM CYP (1.5 mg protein/mL) and 1µM substrate, in the presence and absence of CYP inhibitors. Each incubation (final volume 1.2 ml) comprised of 50 mM potassium phosphate buffet (pH 7.4) and 5 mM $MgCl_2$. 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 ice-cold acetonitrile containing internal standard to terminate the reaction. Samples were centrifuged at 2000 *x* g for 40 min and 80 µl directly injected onto a generic HPLC-MS system (Sciex API 2000 Mass Spectrometer with TurboIonSpray interface, with an OptiLynx Reliasil C18 40µM, 15 x 2.1mm column). Peak response 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-mephenytoin (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4). For each substrate incubations were performed in the presence and absence of CYP inhibitors, 10 µM furafylline (CYP1A2), 10 µM sulphaphenazole (CYP2C9), 3 µM benzlnirvanol (CYP2C19), 1 µM quinidine (CYP2D6) and 1 µM ketoconazole (CYP3A4) and first order disappearance rate constants were determined.

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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, mexiletine, 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 4 to 6 separate days in order 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/IS) against incubation time and determining the gradient of the regression line, using data where an accurate first order decay curve could be obtained. Data were only used where; there were at least 3 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 % CYP contribution using the equation below:

$$\% \text{ contribution} = \frac{(k) - (k_{(Inhib)})}{(k)} \times 100$$

Where: k is disappearance rate constant

$k_{(inhib)}$ is disappearance rate constant in 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 (EF = enzyme function). For example, homozygous CYP2D6*6 individuals (PM) do not express functional hepatic CYP2D6 enzyme due to 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 (Vmax/Km) to ~15-20% (EF=0.15) of the wild type enzyme (Haining et al., 1996; Sullivan-Klose et al., 1996; Miners and Birkett, 1998; Kirchheiner et al., 2002). The fraction of metabolism by a polymorphic enzyme is the second key parameter that must be considered (fm(n)), which has also been referred to as relative contribution. One way to determine this parameter involves microsomal incubations and selective CYP inhibitors with the potential limitations described above.

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:

$$\mathbf{CL^{PM} = EF \cdot CL^{EM}} \qquad \text{(equation 1)}$$

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 zero 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:

$$\mathbf{CL_n^{EM} = fm_n \cdot CL^{EM}} \qquad \text{(equation 2)}$$

where fm(n) 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:

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$$\mathbf{Other\ CL} = \left[1 - \mathbf{fm_n}\right] \cdot \mathbf{CL^{EM}} \qquad \text{(equation 3)}$$

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

$$\mathbf{Total\ CL} = \mathbf{CL^{EM}} + \mathbf{Other\ CL} \qquad \text{(equation 4)}$$

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:

$$\mathbf{R_{CL}} = \frac{\mathbf{CL^{PM}}}{\mathbf{CL^{EM}}} = \mathbf{EF} \cdot \mathbf{fm_n} + \left[1 - \mathbf{fm_n}\right] \qquad \text{(equation 5)}$$

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

$$\mathbf{R_{AUC}} = \frac{\mathbf{AUC^{PM}}}{\mathbf{AUC^{EM}}} = \frac{1}{\mathbf{EF} \cdot \mathbf{fm_n} + \left[1 - \mathbf{fm_n}\right]} \qquad \text{(equation 6)}$$

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:

$$\mathbf{R_{AUC}} = \frac{\mathbf{AUC^{PM}}}{\mathbf{AUC^{EM}}} = \frac{1}{\left[1 - \mathbf{fm_{CYP2D6}}\right]} \qquad \text{(equation 7)}$$

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 CYP enzymes (Newton et al., 1995), and these have traditionally been used to assess percentage contributions to metabolism by CYP enzymes. As a preliminary investigation, the specificity of these inhibitors was re-assessed in the specific batch of human liver microsomes used for this study. A CYP concentration of 0.5 µM was chosen, which is higher than the CYP content typically used in substrate depletion assays (0.25 µM). Using 0.5 µM CYP will increase metabolic vulnerability by approximately 1.5-fold (SD = 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 CYP and 1 µM substrate on two separate days. The effect of inhibitors on both the target CYP, and cross reactivity against the other CYP's 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 in order 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 CYP and 1 µM substrate. Of the 18 compounds investigated, 5 were assessed as being too metabolically stable (T1/2 >100min) 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 an absence of 1 µM quinidine were performed for the 11 remaining CYP2D6 substrates, and the disappearance half-life 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 Figure 2 for atomoxetine and amitriptyline.

Inter-day 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 0.5µM CYP and incubations were therefore performed at 0.1µM CYP in the presence and absence of quinidine for this substrate. Propafenone was also investigated at this CYP 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 CYP (Table 3), suggesting 0.1 µM CYP 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 microsomal binding for quinidine at 0.5µM and 0.1µM, in this batch of human liver microsomes, is not likely to play a major role, with only a 2-fold change in free concentrations. In order to calculate the CYP2D6 contribution the mean disappearance rate constant 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 inter-day (see Table 2: CV of up to 63.2%) rather than intra-day. 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 intra-day 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 utilizing a positive control, to ensure added confidence, the assay need only be run over two days to give a 95% confidence interval of less than +/- 12% for the predicted percent 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 estimate mean percent CYP2D6 contribution *in vitro* for each substrate is summarized in Table 3.

Theoretical considerations

Simulations were performed to predict the ratio of AUC_{PM}/AUC_{EM} as a function of the fraction metabolized by a polymorphic enzyme. Figure 3 shows the theoretical relationship between the ratio of AUC_{PM}/AUC_{EM} (equivalent to CL_{EM}/CL_{PM}) 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 CYP metabolism relative to other elimination routes can also be considered in cases where the fraction of a dose metabolized by a particular isoform (fm_n) is less than one. For instance, if fm_n 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.

The relationship between fraction metabolized and EM/PM CL differences was almost flat at CYP2D6 contribution below 60%. For example, 30% and 60% contributions were predicted to result in 1.4- and 2.5-fold increases in PM exposure relative to EM, respectively. Above 60% contribution by CYP2D6, supra-proportional increases in the ratio of PM/EM exposure are predicted as the fm by CYP2D6 goes to unity. As noted by others, only drugs with an extremely narrow therapeutic index would require dosage adjustments for a 2-fold increase in exposure due to the inherent variability across a population (Rowland and Matin, 1973). These simulations are intended to represent the most straightforward situation where linear kinetics are

observed and "average" differences in exposure between EM and PM subjects are presented. It was assumed that increased drug exposure due to the lack of polymorphic enzyme function resulted in concentrations that were still in the linear capacity range of alternative elimination pathways.

Literature data for known CYP2D6 substrates was summarized 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 fm_{CYP2D6} from in vitro and in vivo data was calculated as the product of the fractional conversion to the recovered metabolite(s) *in vitro* and *in vivo* recovery of that specific metabolite(s). A comparison of the estimated fm_{CYP2D6} *in vivo* and *in vitro* using microsomes and the specific inhibitor quinidine was provided. 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 (Figure 4). The most extreme pharmacokinetic differences were observed with dextromethorphan and tolterodone which showed 53-and 22-fold higher oral clearances in EM subjects when compared with PM subjects. Modest differences in oral clearance (3.5–10 fold) were observed with atomoxetine, propafenone, desipramine, and venlafaxine between EM and PM subjects. For the 5 drugs that had a low estimated CYP2D6 contribution *in vivo*, differences between EM and PM oral clearance values were minor. For amitryptyline, 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 *vs*. predicted EM/PM differences were in agreement.

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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 1µM 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* 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, though 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 (inter-day 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 cut-off value for CYP2D6 metabolism can be established. Figure 4 illustrates the observed ratio of AUC^{PM}/AUC^{EM} and the estimated fraction metabolized by CYP2D6 *in vivo*. From this relationship it can be seen that for a fraction metabolized by CYP2D6 of 60% it would be anticipated that, on average, PM subjects would have a 2.5-fold increase in exposure relative to EM subjects. Due to the non-linear shape of the relationship between CYP2D6 contribution and EM/PM CL/F differences, small changes in percent contribution can resulted in large EM/PM pharmacokinetic differences when the fraction metabolized then exceeds 60%.

With more information about the safety and/or efficacy of a compound, the cutoff value can be refined. However, frequently the challenge in drug discovery is to select compounds without a full appreciation of safety profile. Given the wide inter-individual variability observed for a typical CYP3A substrate, we believe that 2.5-fold EM/PM CL/F difference should be manageable for the majority of compounds coming from drug discovery. Alternatively, with safety information or different assumptions based on

previous experience within a therapeutic class, a more precise limit could be selected to enable a single dose level appropriate for both EM and PM subjects. Until the use of pharmacogenetic tools becomes commonplace in clinical practice, it will be necessary to exclude compounds from drug discovery that do not meet preset criteria for inter-individual variability.

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. Though less data exists on the impact of other polymorphisms, we feel that the results with CYP2D6 can be generalized to other situations where a polymorphism contributes to inter-individual 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 Morais, 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 at 22%, 21% and 18% of normal individuals, respectively (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 (enzyme function (EF) =0.15) and differences in EM and PM exposures (refer to Figure 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, 1984) 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 as similar CYP inhibitor specificities can be achieved in cryopreserved hepatocytes as assessed by probe substrate incubations, to 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 as a significant challenge for metabolically stable compounds. Other methods based upon metabolite formation, rather than substrate depletion and/or utilizing recombinant CYP isoforms and scaling factors may be useful. A combination of these approaches has been evaluated using amitriptyline as probe substrate (Venkatakrishnan et al., 2001b). One caveat to this approach is the potential for even greater over-estimates of fraction metabolised by an individual enzyme, as in addition to non-CYP pathways being ignored, the estimates will be based solely upon the metabolites chosen to be investigated, rather than the overall CYP 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 due to degradation of the CYP enzymes. However, it has been demonstrated that CYP 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* fm. If CYP2D6 is a high affinity enzyme in EM subjects, its deletion in PM subjects could give rise to a situation where the higher exposure saturates alternative clearance mechanisms. Equally the *in vitro* assay has been performed under standard conditions of 1µM substrate and 0.5µM CYP 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 may 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 suggested that compounds that have more than 60% of their metabolism by CYP2D6 were likely to exhibit large differences in CL/F when comparing EM and PM subjects.

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Figure Legend

Figure 1. Metabolic vulnerability of CYP2D6 substrates[a] in pooled human liver microsomes (0.5 µM P450).

[a]Half-life could not be determined for fluvoxamine, metoprolol, mexiletine, nortriptyline, and sparteine due to high metabolic stability (> 100 min). Half-life of flecainide and fluoxetine were not determined due to analytical issues.

Figure 2. Representative examples of human microsomal substrate depletion data (+/- 1 µM quinidine). 2a : Atomoxetine: a substrate with high CYP2D6 contribution. 2b: Amitriptyline: a substrate with moderate CYP2D6 contribution.

Figure 3. Simulated relationship between AUC_{PM}/AUC_{EM} and enzyme function.

The x-axis shows the degree of functional impairment resulting from a genetic polymorphism in a metabolic enzyme. The different lines represent simulations based on equation 6 where the relative contribution by a polymorphic enzyme (fm(n)) to the overall clearance is varied between 0.5 and 1 as denoted in the graph.

Figure 4. Relationship between observed ratio of AUC_{PM}/AUC_{EM} and the fraction metabolized by CYP2D6 *in vivo*.

The line represents the theoretical relationship between ratio of AUC_{PM}/AUC_{EM} and fraction metabolized by CYP2D6 based on equation 7.

Table 1. Selectivity of chemical inhibitors against probe substrates.

Substrate (1μM)	Effect of inhibitor (mean % inhibition)				
	10μM furafylline	**10μM sulphaphenazole**	**3μM benzylnirvanol**	**1μM quinidine**	**1μM ketoconazole**
phenacetin	**>81.3**	15.2	20.5	1.4	17.0
diclofenac	20.6	**>92.9**	7.5	0	12.9
S-mephenytoin	6.1	5.8	**98.6**	7.0	5.0
dextromethorphan	17.4	5.7	7.4	**90.4**	15.6
midazolam	0	0	4.3	3.3	**87.4**

Table 2. Between day variability in mean control half-life data

Drug	Mean	SD.	CV (%)	Sample size
Amitriptyline	58	2	3	6
Atomoxetine	8.1	1.0	13	4
Desipramine	49	3	7	6
Dextromethorphan	10	1	14	4
Duloxetine	32	4	13	5
Imipramine	34	3	8	6
Propafenone	4.7	0.3	7	6
Propranolol	23	2	7	6
Sertraline	54	15	29	5
Venlafaxine	50	7	14	5
Propafenone*	18	11	63	6
Tolterodine*	17	8	44	6

* : 0.1μM substrate

Table 3. Summary of inter-day variability in the % CYP2D6 contribution.

Substrate	Estimated Mean	St.Dev.	95% CI limits	
			2.5%	97.5%
Amitriptyline	52.8	3.4	46.3	59.4
Atomoxetine	96.7	4.0	88.9	105
Desipramine	80.1	7.9	65.3	94.4
Dextromethorphan	92.7	3.5	86.0	99.7
Duloxetine	87.4	5.8	76.1	98.5
Imipramine	67.4	3.3	61.0	74.1
Propafenone	74.0	3.1	67.8	80.2
Propranolol	75.7	3.2	69.5	82.1
Sertraline	37.3	3.9	29.7	45.1
Venlafaxine	85.0	6.5	71.6	96.9
Propafenone*	75.2	3.1	69.0	81.3
Tolterodine*	78.7	3.1	72.7	84.9

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Table 4. *In vitro* literature data defining the role of CYP2D6 metabolism for selected drugs.

Drug	CYP2D6 products	Method(s) of evaluation	%CYP2D6 contribution	Reference(s)
Amitryptiline	10-OH	1, 4	83%	(Venkatakrishnan et al., 2001a)
Atomoxetine	4-OH	2, 3	Major pathway	(Ring et al., 2002)
Desipramine	2-OH	1	Major pathway	(von Moltke et al., 1994)
Dextromethorphan	O-Desmethyl	1	Major pathway	(Broly et al., 1989)
Duloxetine	4-, 5-, or 6-OH of the napthyl ring	1	Primary enzyme with CYP1A2	(Skinner et al., 2003)
Imipramine	2-OH	1, 4, 5	80%	(Brosen et al., 1991)
Propafenone	5-OH	2, 5	Major pathway	(Kroemer et al., 1989)
Propranolol	4-OH, 5-OH	1	40%	(Masubuchi et al., 1994)
Sertraline	N-Desmethyl	1, 4	<20 – 35%	(Kobayashi et al., 1999)
Tolterodine	5-OH	1	80%	(Postlind et al., 1998)
Venlafaxine	O-Desmethyl	1	Major pathway	(Fogelman et al., 1999)

1- Chemical inhibition with quinidine

2- correlation analysis in a liver bank

3- EM/PM livers

4- recombinant CYP isoforms with scaling for abundance or activity

5- Inhibitory antibody

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Table 5. Summary of estimated fraction metabolized by CYP2D6 *in vitro* and *in vivo*.

Drug	**fm CYP2D6[a] (literature)**	**Metabolite recovery[b] (EM)**	**Estimated fm_{CYP2D6}[c]**	**Estimated fm_{CYP2D6}[d] (*in vitro*)**
Amitryptiline	0.83	0.50	0.42	0.53 ± 0.03
Atomoxetine	>0.90	0.87	0.87	0.97 ± 0.04
Desipramine	Major[e]	0.68	0.68	0.80 ± 0.08
Dextromethorphan	Major[e]	ND[f]	0.90	0.93 ± 0.04
Duloxetine	Major[e]	0.50	0.50	0.87 ± 0.06
Imipramine	0.80	0.44	0.35	0.67 ± 0.03
Propafenone	ND	>0.70[f]	0.70	0.74 ± 0.03
Propranolol	0.40	0.42	0.17	0.76 ± 0.03
Sertraline	<0.20-0.35	ND[f]	0.32	0.37 ± 0.04
Tolterodine	Major[e]	0.70	0.70	0.79 ± 0.03
Venlafaxine	Major[e]	0.72	0.72	0.85 ± 0.07

ND – not determined.

[a]Estimated conversion to specific metabolite(s) catalyzed by CYP2D6 *in vitro*.

[b]Metabolite(s) recovery in vivo for CYP2D6 products taken directly from Table 4.

[c]Calculated as the product of the fractional conversion by CYP2D6 taken from the literature and metabolite recovery in EM subjects.

[d]Fraction metabolized by CYP2D6 using microsomal incubations measuring substrate depletion with and without 1 μM quinidine.

[e]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*.

[f]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*.

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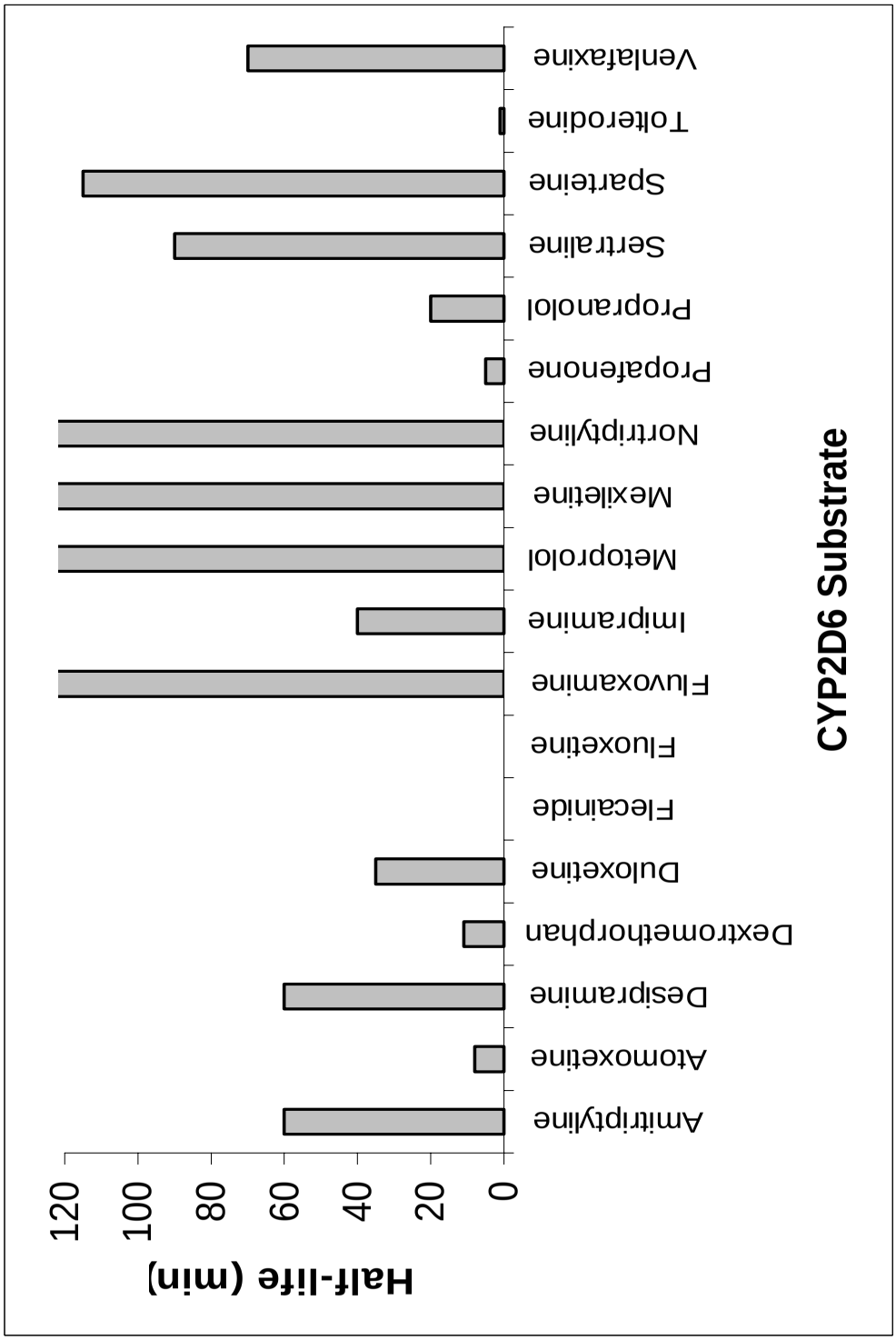
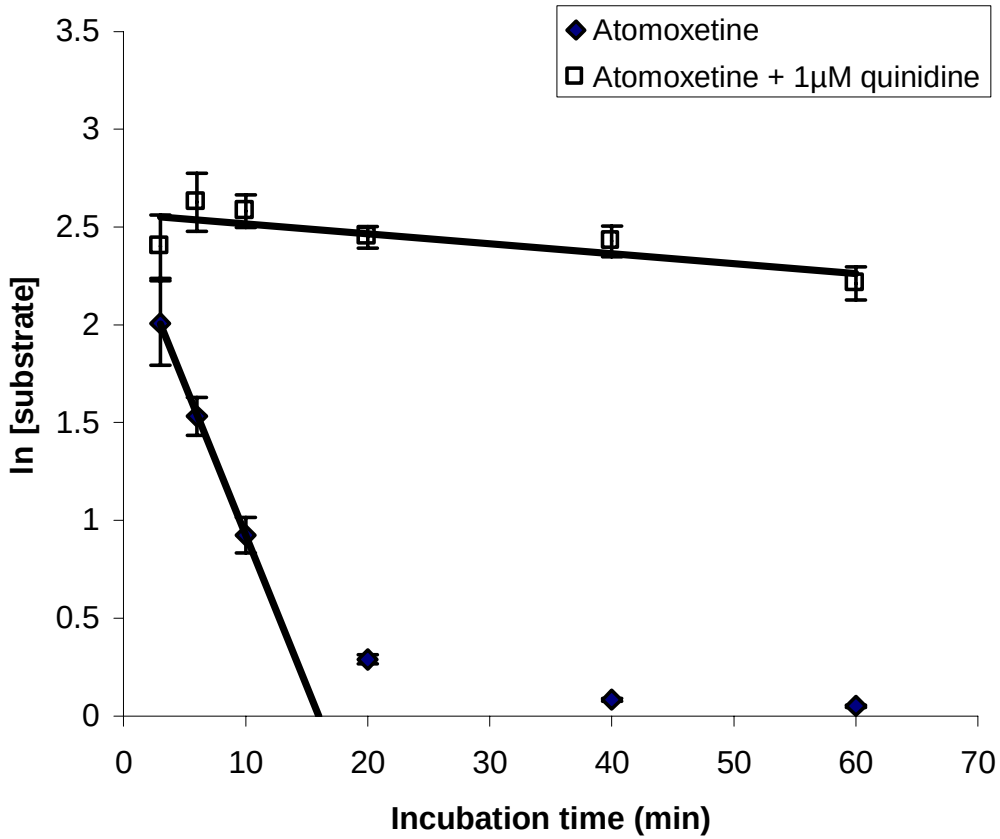


Figure 1.

Figure 2A.



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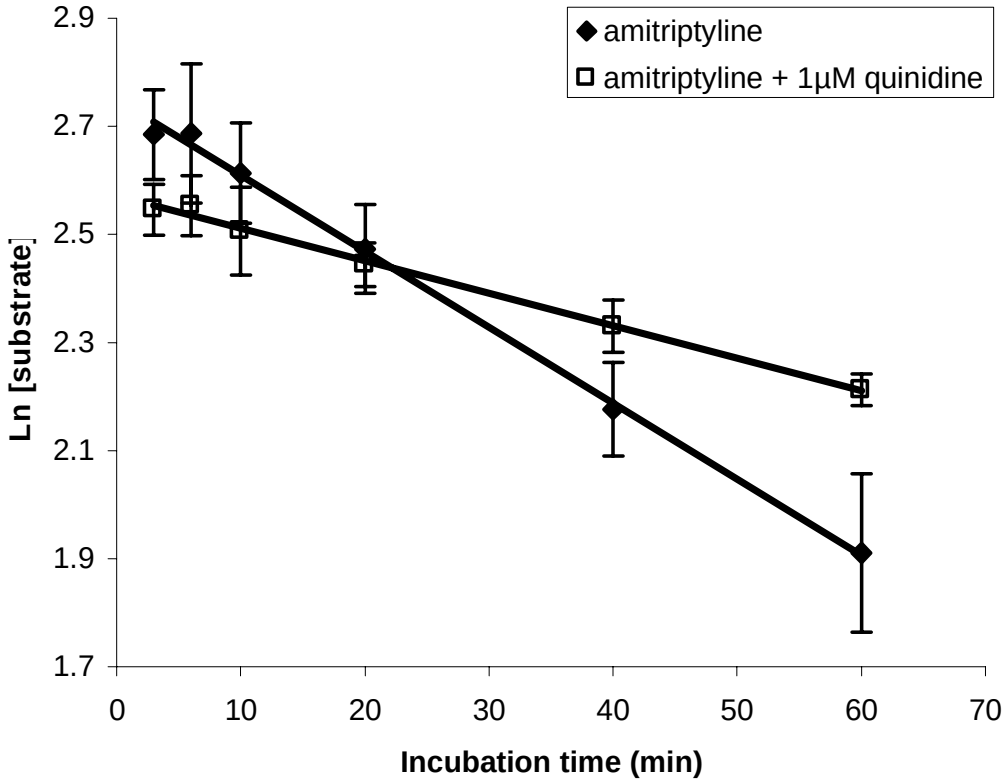

Figure 3.

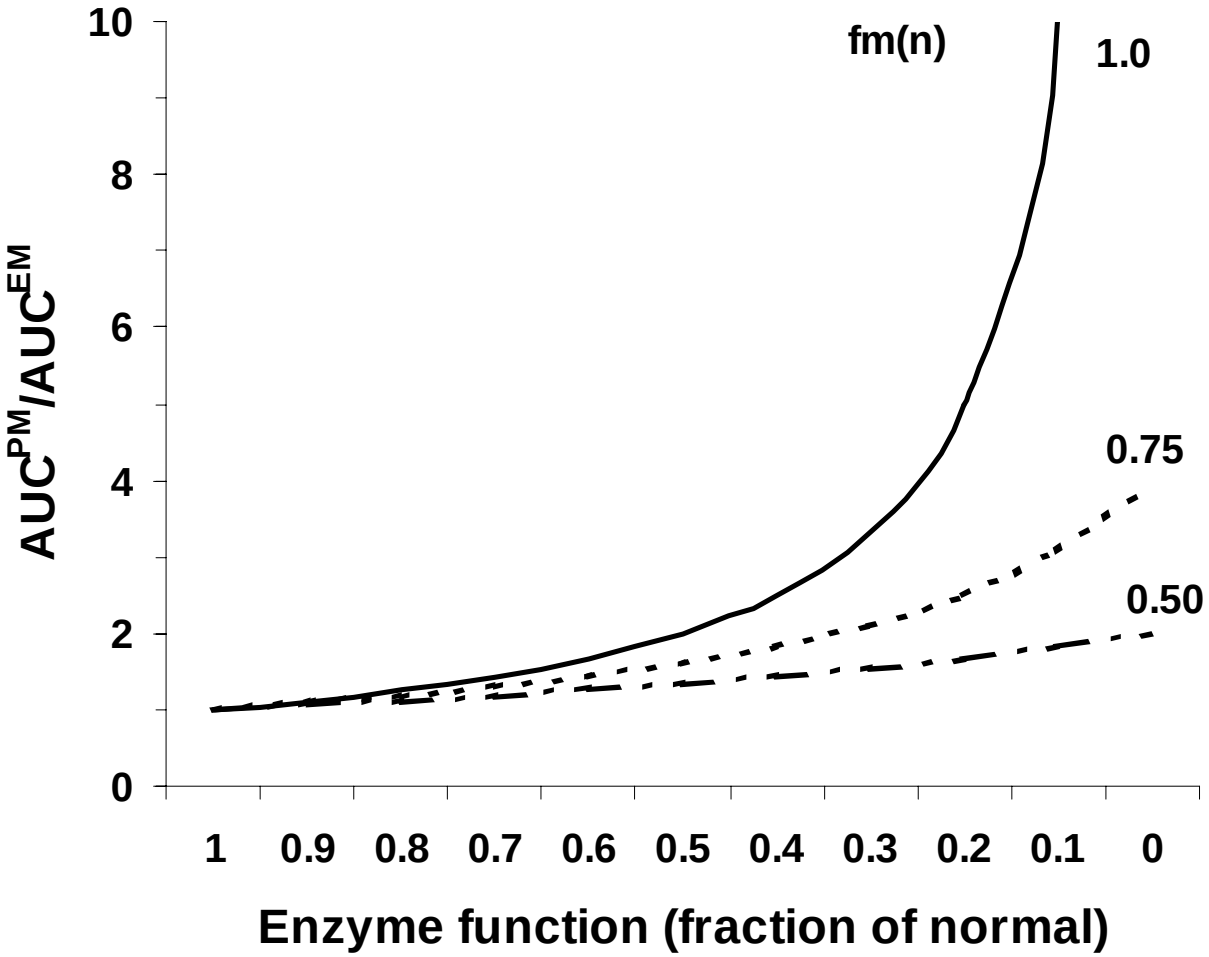

Figure 4.