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Integrated in vitro analysis for the in vivo prediction of cytochrome P450 mediated drug-drug interactions

Dermot F. McGinnity, Nigel J. Waters[1], James Tucker and Robert J. Riley

Discovery DMPK, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire. LE11 5RH. U.K. (D.F.M., N.J.W., J.T. and R.J.R.)

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Running Title

Integrated analysis of CYP mediated drug-drug interactions

Corresponding Author

Dr. Dermot McGinnity, Discovery DMPK, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire. LE11 5RH. U.K. Telephone (01509) 644261. Fax (01509) 645576.

E.mail: dermot.f.mcginnity@astrazeneca.com

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770 words in Introduction

1872 words in Discussion

Abbreviations

cytochrome P450 (CYP), recombinant cytochrome P450 (rCYP), intrinsic clearance (CL_{int}), HPLC/MS (high-performance liquid chromatography/mass spectrometry), metabolic clearance (CL_{met}), unbound fraction in incubation (fu_{inc}), unbound fraction in plasma (fu_p), unbound IC_{50} ($IC_{50,u}$), human serum albumin (HSA), bovine serum albumin (BSA), robotic sample processor (RSP), inhibitor concentration ([I]), average systemic plasma concentration after repeated oral administration ($[I]_{av}$), maximum hepatic input concentration ($[I]_{in}$), unbound maximum hepatic input concentration ($[I]_{in,u}$), inhibition constant (K_i), area under the plasma concentration time curve (AUC), drug-drug interactions (DDIs), fraction absorbed (F_{abs}), absorption rate constant (k_a), fraction of total clearance metabolised by CYP*x* (fm_x), new chemical entities (NCEs), *In vitro-in vivo* extrapolation (IVIVE).

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Abstract

Unbound IC_{50} ($IC_{50,u}$) values of 15 drugs were determined in 8 recombinantly expressed human CYPs and human hepatocytes, and the data used to simulate clinical area under the plasma concentration-time curve changes (δAUC) upon co-administration with prototypic CYP2D6 substrates. Significant differences in $IC_{50,u}$ values between enzyme sources were observed for quinidine (0.02 μM in rCYP2D6 *vs.* 0.5 μM in hepatocytes) and propafenone (0.02 *vs.* 4.1 μM). The relative contribution of individual CYPs towards the oxidative metabolism of clinical probes desipramine, imipramine, tolterodine, propranolol and metoprolol were estimated via determinations of CL_{int} using rCYPs. Simulated δAUC were compared to those observed *in vivo* via the ratios of unbound inhibitor concentration at the entrance to the liver to inhibition constants determined against rCYPs ($[I]_{in,u}:K_i$) and incorporating parallel substrate elimination pathways. For this dataset, there were 20% false negatives (observed δAUC ≥ 2, predicted δAUC < 2), 77% correct predictions and 3% false positives. The $[I]_{in,u}:K_i$ approach thus appears relatively successful at estimating the degree of clinical interactions and can be incorporated into drug discovery strategies. Using Simcyp ADME simulator® there were 3% false negatives, 94% correct simulations and 3% false positives. False negative predictions were rationalised as a result of mechanism-based inhibition, production of inhibitory metabolites and/or hepatic uptake. Integrating inhibition and reaction phenotyping data from automated rCYP screens has shown applicability to predict the occurrence and degree of *in vivo* DDI and such data may identify the clinical consequences for candidate drugs as both 'perpetrators' and 'victims' of CYP mediated interactions.

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Inhibition of cytochrome P450 (CYP) metabolism is recognised to be one of the more prevalent mechanisms of clinical drug-drug interactions (DDIs) and may result in serious clinical and toxicological consequences (Nelson, 1998). Over the last two decades, both *in vitro* and *in vivo* assessments of the CYP inhibition potential and disposition of drugs have led to a relatively thorough appreciation of the underlying reasons for certain drug combinations resulting in significant clinical outcomes. Application of this knowledge has led researchers to propose strategies which assess the potential of new chemical entities (NCEs) to cause clinical DDIs via inhibition of CYP metabolism. As a result, in the last decade or so, *in vitro* screens that determine the degree of CYP inhibition have become commonplace in drug discovery screening cascades. These screens are used to evaluate and optimise potential candidate drugs and to prioritise and design suitable clinical studies.

In vitro-in vivo extrapolation (IVIVE) strategies employed for CYP inhibition mediated DDIs range from simple but useful 'rule of thumb' alerts, such as if inhibition constant (K_i) of <1 μM, DDIs are probable; K_i between 1-10 μM, DDIs are possible and K_i >10 μM DDIs are unlikely (Lin and Pearson, 2002; Obach et al., 2005), to more quantitative approaches. There exists a broad consensus as to the common principles underlying the predicting the magnitude of an *in vivo* DDI from *in vitro* data. The increase in the AUC of a substrate when co-administered in the presence of an inhibitor of the substrates' elimination pathway is a function of the ratio of inhibitor concentration ([I]) to inhibition constant (K_i) (Ito et al., 1998; Rostami-Hodjegan and Tucker, 2004; Shou, 2005; Brown et al., 2005; Obach et al., 2006, Einolf, 2007).

Although the basic tenets of IVIVE for CYP inhibition mediated DDIs are widely accepted, the specific methodology used can vary. The 'unbound drug hypothesis' is a widely accepted fundamental principle of pharmacokinetics and there is increasing support for the use of *unbound* maximum inhibitor concentration entering the liver after oral administration ($[I_{in,u}]$) as the most appropriate surrogate for inhibitor concentration at the enzyme active site (Ito et al., 2002; McGinnity et al., 2005; Obach et al., 2006). Use of

$[I]_{total}$ rather than $[I]_u$ has been proposed, usually for pragmatic reasons, to retrospectively account for observed interactions (Brown et al., 2006; Venkatakrishnan et al., 2003) or as a cautious strategy in early drug discovery to avoid underestimation of *in vivo* interactions (Ito et al., 2002; McGinnity et al., 2005). The impact of non-specific binding on estimating unbound K_i is increasingly understood (Grime and Riley, 2006) and the sensitivity of IVIVE predictions to both the absorption rate constant of the inhibitor (k_a) and fraction metabolised by the inhibited pathway (f_m) of the substrate has been exemplified (Brown et al., 2005; McGinnity et al., 2005). The wide array of input parameters applied to the *retrospective* IVIVE of clinical DDI studies in the literature somewhat obfuscate a comprehensive and systematic *ab initio* IVIVE approach for NCEs.

Estimating K_i values of inhibitors traditionally used human liver microsomes but more recently recombinant human CYPs (rCYPs): 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5 have been frequently employed. Clinical interactions are usually classified as being dependent on one of the individual CYP enzymes and indeed this is appropriate for the more selective substrates. As part of a more systematic and holistic approach to IVIVE and with the appropriate assays now available, it is possible to propose an integrated assessment of the inhibitory potential of the perpetrator against all 8 CYPs whilst similarly evaluating the contribution of individual CYPs to the elimination of the victim substrate.

The aims of this work were firstly to assess the contribution of individual CYPs towards the total clearance of classic CYP2D6 substrates imipramine, desipramine, tolterodine, propranolol and metoprolol and to estimate K_i values for a range of marketed drugs in all 8 rCYPs. Human hepatocytes, the closest *in vitro* model to human liver, were evaluated as an alternate CYP source. This article provides further assessment of the $I_{in,u}$: $K_{i,u}$ approach for the *in vivo* prediction of CYP mediated DDIs from *in vitro* data and highlights both successes and limitations of this method within a drug discovery setting.

In addition the use of the Simcyp ADME Simulator® (Simcyp Ltd., Sheffield, UK) to simulate clinical δAUC changes of drugs when co-administered with CYP inhibitors was also evaluated. Simcyp incorporates a physiologically based method that simulates the change in inhibitor and substrate concentrations over time, the generation of inhibitory metabolites, the inhibition of gastrointestinal metabolism, active uptake of the inhibitor into the liver and the impact of population variability.

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Materials and Methods

Materials

Amitriptyline, chlorpromazine cimetidine, desipramine, diltiazem, diphenhydramine, (±)fluoxetine, imipramine, labetalol, (±)metoprolol, mexiletine, (±)norfluoxetine, propafenone, propranolol, quinidine, ritonavir, tolterodine, verapamil, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) and trichloroacetic acid were purchased as the highest grade available from Sigma-Aldrich Chemical (Gillingham, UK). Fluconazole, and sertraline were purchased from Sequoia Research Products Ltd. (Oxford, UK). Omeprazole was synthesised at AstraZeneca R&D Wilmington. Dimethylsulfoxide and acetonitrile were purchased from Fisher Scientific (Loughborough, UK) and methanol was purchased from Romil Ltd. (Cambridge, UK).

rCYPs and human hepatocytes

Bactosomes prepared from *E.coli* cells co-expressing recombinant human NADPH-P450 reductase and human CYPs (CYP1A2LR, CYP2B6LR, CYP2C8R, CYP2C9R, CYP2C19R, CYP2D6LR, CYP3A4LR and CYP3A5LR) were purchased from Cypex (Dundee, UK). Fresh human hepatocytes were prepared from an isolated lobe of human liver (obtained from local hospitals with ethical approval) using a procedure described previously (McGinnity et al., 2004). For convenience, human hepatocytes were cryopreserved and thawed before use (McGinnity et al., 2004). Briefly, aliquots (20 ml) of hepatocyte suspension buffer (with no added albumin) were pre-warmed to 37 ºC. Cryopreserved cells were removed from liquid N_2 and immediately immersed in a water bath that had been pre-heated to 37 ºC. The vials were shaken gently until the contents were completely free of ice crystals and were then emptied into the pre-warmed hepatocyte suspension buffer. The cells were centrifuged at 40 g for 5 min at 19 ºC, the supernatant was removed by aspiration and the resultant pellet suspended in hepatocyte suspension buffer. The concentration and viability ($\geq$ 85%) of the hepatocytes was

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determined using trypan blue exclusion and the cells were re-suspended at a concentration of 2 million cells/ml.

Inhibition assays

Inhibition assays using rCYPs were performed on a robotic sample processor (RSP) (Genesis RSP 150, Tecan, Reading, UK). Bufuralol 1-hydroxylation (Weaver et al., 2003) was used as a probe reaction for CYP2D6 based on methods previously described. The CYP isoform selectivity of bufuralol (Weaver et al., 2003) has been previously established. All reactions were conducted under conditions shown to be linear with respect to time and protein concentration. IC_{50} determination was based on seven and five inhibitor concentrations for the rCYPs and human hepatocytes, respectively.

Determination of IC_{50} using bufuralol 1'-hydroxylase activity and rCYP2D6

The assay was carried out as described by Weaver et al., (2003). Using bufuralol as a substrate, the product of the reaction is 1'-hydroxybufuralol, which is separated from the incubation mixture using HPLC. Interaction with the CYP2D6 by an inhibitor will result in a decrease in the amount of 1'-hydroxybufuralol produced. Assays were performed at a substrate concentration equivalent to the apparent K_m (10 μM) of the CYP2D6 dependent bufuralol 1'-hydroxylase activity. The amount of rCYP2D6 used in the bufuralol 1'-hydroxylation assay was 5 pmol/ml (0.015 mg protein/ml of incubate; one batch of rCYP2D6 used for all experiments). The following volumes are used in each incubation-well; 20 μl 10 mM NADPH in 0.1M phosphate buffer pH 7.4, 178 μl CYP/substrate and 2 μl of the pre-dilution stocks of inhibitor were spiked directly into the incubation micro-titre plate. The final incubation volume was 200 μl giving 100 fold dilutions of the solvent stocks of the inhibitor generated in the pre-dilution micro-titre plate and a final concentration of 1 mM NADPH. The dilution of test compounds for a seven point IC_{50} determination by the RSP were programmed as follows: the primary stock of each compound (e.g. 5 mM) was prepared manually in dimethyl sulfoxide and serially diluted by the RSP (using dimethyl

sulfoxide) to give 6 secondary solutions (e.g. 5 mM to 20 μM). Each of these secondary solutions was further diluted 1:5 in 100 mM phosphate buffer pH 7.4 to generate tertiary solutions (e.g. 1 mM to 4 μM). Finally 10 μl of each of the tertiary solutions were spiked into the incubation mix (200 μl total volume to give final concentrations (e.g. 50 μM to 0.2 μM). An incubation-containing vehicle alone allowed calculation of control activity. The final organic solvent concentration in all incubations was 1% (v/v).

Incubations were conducted for 15 min and reactions quenched with the addition of 200 μl methanol. Samples were chilled at –20°C for 2 h, spun at 3500 rpm for 15 min at 4 °C and the supernatants transferred to vials for analysis as described below.

Determination of IC_{50} using bufuralol 1'-hydroxylase activity and human hepatocytes

This assay was performed using manual pipetting in a shaking water bath at 37 °C. An appropriate amount of bufuralol (final assay concentration 10 μM) was aliquoted and the solvent evaporated under nitrogen to dryness before re-suspension in hepatocyte suspension buffer (2.2 g $NaHCO_3$, 2.34 g Na HEPES, 1 L powder equivalent of DMEM (Sigma, Gillingham, UK) diluted in 1 L of water and adjusted to pH 7.4 with 1 M HCl) to give a concentration of 200 μM. Drug stocks were prepared in dimethyl sulfoxide at 100-fold incubation concentration (e.g. 5000, 1500, 100, 15, 5 μM). 10 μl of this x100 stock were added to a vial containing 490 μl of hepatocyte suspension buffer containing substrate. A 7 ml glass bijou vial containing 250 μl of hepatocytes at a concentration of 2 million cells/ml was pre-incubated for 5 min in a shaking (80 oscillations/min) water bath at 37 °C along with the vial containing the drug/buffer/substrate mix. Reactions were started by adding 250 μl of drug/buffer/substrate mix to the 250 μl of hepatocytes giving, in the example shown, final inhibitor concentrations of 50, 15, 1, 0.15 and 0.05 μM. The final concentration of organic solvent in all incubations was 1 % (v/v). The samples were incubated for 30 min and quenched with an equivalent volume of ice-cold methanol before being frozen for 2 h at -20 °C and then centrifuged for 3500 rpm for 15 min at 4 °C. The

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supernatants were removed and transferred into vials and analysed as described below. Assays were performed in triplicate.

CYP CL_{int} Determination

CYP CL_{int} determination assays were performed using a RSP as described previously (McGinnity et al., 2000). In brief, the primary stock of all substrates was prepared in dimethyl sulfoxide at x100 the final incubation concentration. The final concentration of organic solvent in the incubation was 1% v/v. All substrates were incubated at 1 µM. Compound stocks were pre-diluted in 100 mM potassium phosphate buffer, pH 7.4 and eight rCYPs (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5) added to separate incubation tubes (100 pmol CYP/ml final) located in a 96-well block at 37 °C. A sub-aliquot was removed to produce a 0 min time point and the assay initiated via addition of NADPH (1 mM final). Aliquots (50 µl) were removed at 5, 10, 15 and 20 min and quenched in 100 µl acetonitrile. Samples were subsequently frozen for 2 h at –20 °C and then centrifuged at 3500 rpm at 4 °C for 15 min. The supernatants were removed and transferred into vials and analysed as described below.

Determination of fu_p and fu_{inc} in rCYP2D6 and human hepatocytes

Human blood was obtained from volunteers at AstraZeneca R&D Charnwood after local ethical approval and written informed consent. The extent of binding of compounds to rCYP2D6, human plasma and human hepatocytes were determined using equilibrium dialysis at 37 °C as described by Austin et al., (2005). Briefly, plasma was prepared by centrifugation of the blood, stored in EDTA tubes, at 350 g for 15 min. The amount of rCYP2D6 used was the same as for the bufuralol and dextromethorphan inhibition assays (0.015 mg and 0.06 mg mg protein/ml respectively in 100 mM phosphate buffer pH 7.4). The amount of human hepatocytes used was the same as for the inhibition assays (1 million cells (left to die for 24 h before use) / ml hepatocyte suspension buffer). Plasma and microsomal binding are normally independent of compound concentration at typical

therapeutic levels (0.1 μM – 50 μM) (Austin et al., 2005) and so binding was determined at a single concentration (10 μM for plasma and 1 μM for rCYP and hepatocytes). Compounds were solubilised in dimethyl sulfoxide and the final organic solvent concentration was 1% (v/v). Plasma was dialysed against Dulbecco's phosphate buffered saline pH 7.4 (Sigma, Gillingham, UK), rCYP against 0.1M phosphate buffer pH 7.4 and hepatocytes against hepatocyte suspension buffer, overnight at 37 °C. Samples were quantified using HPLC/MS as described below. The free fraction of each compound was determined from the ratio of buffer to sample concentrations, each interpolated from a six-point calibration curve.

HPLC/MS

All HPLC/MS used electrospray ionization and multiple reaction monitoring conducted on a Micromass Quattro Ultima triple quadrapole and an Alliance HT Waters 2790 HPLC system. Aliquots (30 μl) were analysed by HPLC-MS/MS for 1'-hydroxybufuralol appearance. A Devosil C30 column, (Phenomenx, Cheshire, UK) and mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used for the chromatography. The gradient was as follows 97% A (0-0.3 min), 5% A (0.55-1.55 min), 97% A (1.6 min). The stop time was 2.5 min, the flow rate was 1.2 $ml.min^{-1}$ and column temperature 40°C. All other analysis was performed using a Symmetry C8 (5 μm x 3.9 mm x 20 mm column, Waters, Milford, MA) and a gradient of 1% acetonitrile/99% 0.05% aqueous ammonium acetate to 99% acetonitrile/1% 0.05% aqueous ammonium acetate at a flow rate of 2 ml/min over 3.5 min and column temperature 40°C.

Data Analysis

Microsoft Excel (Redmond, WA) was used to calculate IC_{50} estimates by linear transformation of the raw data. The data were corrected for both background and control activities.

All rCYP assays were performed at a substrate concentration equivalent to the K_m of the CYP2D6 reaction as under these conditions, irrespective of the type of reversible inhibition, IC_{50} should be within 2-fold of the K_i (Cheng and Prusoff, 1973). For hepatocytes, inhibition assays were performed at a bufuralol concentration of 10 μM to ensure selectivity for CYP2D6 (apparent K_m of the CYP2D6 reaction in human hepatocytes was 100 μM - data not shown) as under these conditions, IC_{50} should be within 2-fold of the K_i, for competitive, non-competitive and linear mixed-type inhibition and only differ significantly for uncompetitive inhibition (Cheng and Prusoff, 1973) which is a rare inhibition type for CYPs (Zhang and Wong, 2005). The inhibition type has not been fully elucidated for all the inhibitors studied in this work and so in the absence of this information, $K_i = IC_{50}/2$ was uniformly applied. As a generic strategy, this is appropriate, for in the drug discovery environment, IC_{50} rather than K_i values are generated initially and the precise type of reversible inhibition is usually defined later.

The theoretical basis underlying the quantitative predictions of drug interactions associated with reversible inhibition have been covered comprehensively in the literature (Ito et al., 1998; Rostami-Hodjegan and Tucker, 2004; Shou, 2005; Brown et al., 2005; Obach et al., 2006, Einolf 2007). In brief, the ratio change of AUC in the presence or absence of a CYP inhibitor can be approximated by equation 1, according to the 'well-stirred model' (Ito et al., 1998). In clinical situations, the substrate concentration is usually much lower than the K_m and so equation 1 is valid for competitive and non-competitive inhibitors. It does not account for CYP inhibition in the gastrointestinal tract (less significant in this dataset due to the lack of CYP3A substrates) and disregards the change in inhibitor concentration during the dosing interval.

$$\delta AUC = \frac{AUC_{(inhib)}}{AUC_{(uninhib)}} = \frac{1}{\sum_{x=1}^{n}\left(\frac{fm_x}{1+I_{in,u}/K_{ix}}\right)+\left(1-\sum_{x=1}^{n} fm_x\right)} \qquad (1)$$

where fm_x is the fraction of substrate clearance mediated by CYP*x*, K_{ix} is the inhibition constant for CYP*x* and $I_{in,u}$ is maximum unbound hepatic input concentration.

$[I]_{in,u}$ was estimated as follows:

$$[I]_{in,u} = \left([I]_{av} + \frac{K_a \, F_a \, D}{Q_h}\right).fu_b \qquad (2)$$

where $[I]_{av}$ is the average inhibitor concentration ($[I]_{av} = (D/\tau)/(CL/F)$), D and τ is the dose and the dosing interval of the inhibitor respectively, k_a is the absorption rate constant. F_a is the fraction absorbed from the gastrointestinal tract, Q_h is hepatic blood flow and fu_b is the fraction unbound in blood. Blood:plasma (b:p) ratios have been sourced for several of the compounds and the value included in the predictions. For those compounds were b:p ratio has not been collated, a value of 1 has been used.

Simcyp ADME Simulator®

Relative to the approach described by equation 1, the Simcyp ADME Simulator® (Simcyp Ltd., Sheffield, UK) is a more physiologically based DDI prediction method (Rostami-Hodjegan and Tucker, 2004; Einolf, 2007). In addition to the considerations incorporated into equation 1, the Simcyp approach uses a physiological based pharmacokinetic model (PBPK) and so incorporates the change in inhibitor and substrate concentrations over time, the generation of inhibitory metabolites, the inhibition of gastrointestinal metabolism, the ability to simulate active uptake of the inhibitor into the liver and the impact of population variability. Therefore, whereas the approach outlined via equation 1 can only predict a mean outcome, Simcyp can simulate the expected variability within a population and importantly identify those individuals at most risk (Rostami-Hodjegan and Tucker, 2004). Simulations were generated for substrates desipramine, imipramine and metoprolol using the default input parameters that were within the existing library that is supplied with

the program, and for propranolol using the CL_{int} and relative contribution of the individual rCYPs towards the oxidative metabolism determined in these studies. All input parameters for the inhibitors were as detailed in the Results section.

Literature in vivo drug interaction data

All literature clinical interaction studies used a crossover design and between 5-24 subjects and were at steady state for inhibitor with the exception of Bergstrom et al., (1992) and at steady state for substrate except for the following studies: Brosen and Gram 1989, Hamelin et al., 2000, Johnson and Burlew 1996, Tateishi et al., 1989, Spina et al., 1993, where the interaction was determined following a single dose. CYP2D6 poor metabolisers were excluded via either genotyping or phenotyping assessment except in the following studies where CYP2D6 status was not determined: Bergstrom et al., 1992, Donn et al., 1984, Keech et al., 1986, Kirch et al., 1982, 1984, Kowey et al., 1989, Hermann et al., 1992, Henry et al., 1987, Hunt et al., 1990, Murdoch et al., 1991, Vestal et al., 1979, Tateishi et al., 1989, von Moltke et al., 1998.

Results

Determination of $IC_{50,u}$ values for probe inhibitors in rCYPs

Table 1 shows the mean $IC_{50,u}$ values against the eight major human hepatic CYPs (rCYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5) for a selection of compounds that have been investigated in clinical studies as perpetrators of DDIs against predominantly CYP2D6 substrates. The *in vitro* CYP2D6 potency ranged from 20 nM for propafenone and quinidine to 91 μM for cimetidine. All compounds were also assessed for time-dependent inhibition of CYP2D6 and none was observed (data not shown).

CYP reaction phenotyping of prototypic CYP2D6 in vivo substrates

Table 2 shows the CL_{int} of eight individual human rCYPs to the oxidative metabolism of desipramine, imipramine, tolterodine, propranolol and metoprolol. Substrates were incubated at a low substrate concentration of 1 μM with rCYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5 respectively as described in Materials and Methods. The range of CYP CL_{int} determined was 0.01 – 10 μl/min/pmol P450. The contributions of individual CYPs towards oxidative metabolism of compounds in human liver were estimated (Table 3), considering the average % content of the eight major isoforms in human hepatic microsomes as outlined in Materials and Methods. Desipramine was estimated to be predominantly cleared by CYP2D6 (fraction metabolised by CYP2D6, 0.97) and to a small extent by CYP2C19 (0.03); imipramine by CYP2D6 (0.55), CYP2C19 (0.31), CYP3A4 (0.09) and CYP1A2 (0.02); tolterodine by CYP2D6 (0.79), CYP2C19 (0.08), CYP2C8 (0.02), CYP2B6 (0.01) and CYP3A5 (0.01); propranolol by CYP2D6 (0.73), CYP2C19 (0.15) and CYP1A2 (0.12) and metoprolol was metabolised exclusively by CYP2D6 (1.00). Up to 10% of the clearance of metoprolol is via renal elimination (Regardh and Johnsson, 1980); therefore the value of fm_{CYP} used for metoprolol was 0.9. The other substrates were assumed to be cleared exclusively by hepatic CYP (i.e. fm_{CYP} = 1), although the fm_{CYP} of propranolol may be somewhat less than unity owing to the contribution of glucuronidation

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to overall clearance (Tateishi et al., 1992).

Comparison of $IC_{50,u}$ values in human hepatocytes and rCYP2D6.

Figure 1 shows the $IC_{50,u}$ values determined using bufuralol 1'-hydroxylase activity in human hepatocytes and rCYP2D6 for seven compounds. $IC_{50,\ apparent}$ values were converted to $IC_{50,u}$ values, correcting for the unbound fraction determined in the appropriate milieu (fu_{inc}). For fluoxetine, norfluoxetine, fluvoxamine, ritonavir, sertraline and labetalol the $IC_{50,u}$ values determined in human hepatocytes were within 6-fold of the value determined in rCYP2D6. More significant differences in $IC_{50,u}$ values between the rCYPs and hepatocytes were observed for quinidine (mean $IC_{50,u}$ ± sd was 0.02 ± 0.01 μM in rCYP2D6 vs. 0.5 ± 0.4 μM in human hepatocytes) and propafenone (0.02 ± 0.01 μM vs. 4.1 ± 2.4 μM).

Predicting the magnitude of clinical DDIs from in vitro IC_{50} values

The success of predicting clinical δAUC changes of desipramine, imipramine, tolterodine, propranolol or metoprolol when co-administered with other drugs via IC_{50} values using rCYPs was evaluated (Table 4). From clinical studies, the dosing size and interval of potential DDI perpetrators were recorded and the relevant human pharmacokinetic parameters (clearance, CL; bioavailability, F; half-life, $T_{1/2}$; absorption rate constant, K_a and fraction absorbed, F_a) were collated for all the inhibitors. $[I]_{in}$ values, estimates of inhibitor concentrations at the entrance to the liver, were calculated using equation 2. Fraction unbound in plasma (fu_p) for all the inhibitors were determined as described in Materials and Methods and together with $[I]_{in}$ values, used to estimate $[I]_{in,u}$.

The predicted *in vivo* δAUC was determined using the $[I]_{in,u}$:K_i approach (equation 1) and by inputting the appropriate values for the inhibitor and substrate into the Simcyp ADME simulator. Figure 2 shows the plot of predicted δAUC, using equation 1, versus observed δAUC, based on K_i values estimated from (A) rCYPs and (B) human hepatocytes

(CYP2D6 activity only). Several inhibitors, amitriptylline, chlorpromazine, cimetidine, diltiazem, diphenhydramine, labetolol, mexiletine, omeprazole, sertraline and verapamil were correctly predicted by both the $[I]_{in,u}$:K_i method and Simcyp to cause insignificant DDIs (predicted and observed δAUC <2). Of the significant interactions (defined as observed δAUC > 2), using rCYP as the enzyme source, the interaction of quinidine with desipramine, metoprolol and imipramine (observed δAUC values of 7.5, 3.2 and 1.5 respectively) was correctly identified yet systematically over estimated using the $[I]_{in,u}$:K_i method, (predicted δAUC was 29, 9.1 and 2.3 respectively). Simcyp similarly over estimated the quindine interaction with desipramine and imipramine (observed vs. simulated δAUC 7.5 vs. 19, (range 11 - 34) and 1.5 vs. 4.8 (1.8 - 10) respectively) but estimated well the degree of interaction with metoprolol (3.2 vs. 3.5 (1.9 - 6.5)). The interaction of propafenone with propranolol (observed δAUC of 2.1) was correctly identified but over estimated using the $[I]_{in,u}$:K_i, method (δAUC 5.5) compared to Simcyp (δAUC 2.6 (1.6 - 4.6)). The interaction of fluvoxamine with imipramine (observed δAUC 3.6) was under predicted using the $[I]_{in,u}$:K_i method (δAUC 1.7), but was correctly classified if still under predicted using Simcyp (δAUC 2.1, range 1.7 – 2.8). The interaction of ritonavir with desipramine (observed δAUC 2.5) was somewhat under-predicted using $[I]_{in,u}$:K_i (δAUC 1.4) and Simcyp (δAUC 1.2, range 1.1 – 1.5), whereas the interaction of fluoxetine with all substrates was significantly under-predicted using $[I]_{in,u}$:K_i (Table 4). Using Simcyp to simulate the *in vivo* contribution of the major human metabolite of fluoxetine, norfluoxetine by inputting the rCYP K_i values (Table 1), the mechanism-based inhibition of CYP3A4 and 2C19 by fluoxetine (Mayhew et al., 2000; McGinnity et al., 2006) and using the maximum active uptake factor of 1000 of both drug and metabolite into the liver, resulted in a good estimation of the degree of interaction with imipramine (observed δAUC 3.3 vs. simulated δAUC 3.8 (2.0 – 7.7)) but the interaction with desipramine was still somewhat underestimated (Table 4).

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The measured $IC_{50,u}$ values of fluoxetine, norfluoxetine, fluvoxamine, ritonavir, labetolol or sertraline for CYP2D6 did not significantly alter between rCYP and cryopreserved human hepatocytes. However the $IC_{50,u}$ for propafenone determined in hepatocytes was higher (human hepatocytes, 4.1 ± 2.4 μM vs. rCYP2D6, 0.02 ± 0.01 μM) resulting in a closer prediction of the interaction with propranolol (using $[I]_{in,u}:K_i$ method predicted δAUC, 1.9 vs. observed δAUC, 2.1). Similarly for quinidine, the CYP2D6 $IC_{50,u}$ determined in hepatocytes was higher than in rCYP (0.5 ± 0.4 μM vs. 0.02 ± 0.01 μM respectively) resulting in a closer prediction of the interaction with metoprolol (predicted 3.2 vs. observed δAUC, 3.2), desipramine (6.2 vs. 7.5 respectively) and imipramine (2.0 vs. 1.5 respectively).

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Discussion

The prediction of DDIs mediated via reversible CYP inhibition typically relies on the use of the $[I]/K_i$ ratio. By using rCYPs as the enzyme source in the determination of IC_{50} or K_i values, very low protein levels are afforded and typically fu_{inc} approaches 1 and the experimentally generated $IC_{50,\ apparent}$ values approach $IC_{50,u}$. The need to determine the *unbound* rather than *apparent* parameter such as K_m, CL_{int}, IC_{50} and therefore K_i has been recently reemphasised (Grime and Riley, 2006; Rostami-Hodjegan and Tucker, 2004). This was the case with the CYP inhibitors studied in this work and therefore the term included in the $[I]_{in,u}:K_i$ expression equates essentially to $K_{i,u}$. The use of apparent or total *in vitro* and *in vivo* parameters and the failure to incorporate parallel substrate elimination pathways confound the IVIVE theory of DDI. Similarly, in the absence of supporting data, the use of theoretical maximum values of absorption rate (k_a) and extent (F_a) for potential DDI perpetrators (Brown et al., 2005), whilst potentially useful to avoid false negative predictions in drug discovery, obfuscates assessing the potential of IVIVE methodology to make quantitative predictions based on pharmacokinetic principles and not empirical observations alone. In this analysis, values of k_a, F_a and blood:plasma partitioning, where available, were applied. However the $[I]_{in,u}:K_i$ approach disregards the change in inhibitor concentration during the dosing interval, does not incorporate the effect of inhibitory metabolites and cannot easily assess population variability. Therefore a prediction software tool which offers an integrated population based solution to CYP mediated DDIs (Simcyp®, Rostami-Hodjegan and Tucker, 2007) was also evaluated. Simcyp incorporates physiological, genetic and epidemiological information, which, together with *in vitro* data, facilitates the modelling and simulation of the time-course and fate of drugs in representative virtual patient populations. This allows prediction of outcomes in those individuals at most risk from a DDI, not just a single value in an 'average human', a limitation of the $[I]:K_i$ approach outlined in this work.

The $IC_{50,u}$ values against the eight major human hepatic CYPs (rCYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5) were determined (Table 1). Some of the more potent CYP2D6

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inhibitors (propafenone, quinidine and chlorpromazine) were relatively selective for CYP2D6 over the remaining seven CYPs, whereas the majority of inhibitors, including fluoxetine (and its active metabolite norfluoxetine), ritonavir, sertraline, amitryptylline, fluvoxamine, omeprazole, diltiazem, verapamil, mexilitine and cimetidine showed similar or indeed greater inhibitory potency towards a range of CYPs. As none of the compounds demonstrated time-dependent inhibition against CYP2D6 the assumption was that the interaction of all compounds and CYP2D6 was of a competitive, reversible nature.

The CL_{int} and relative contribution of the individual rCYPs towards the oxidative metabolism of desipramine, imipramine, tolterodine, propranolol and metoprolol was estimated (Tables 2 & 3 respectively) considering the average content of the eight major isoforms in human liver (Rowland et al., 2004). This laboratory has previously demonstrated the kinetic parameters (including K_m and V_{max}) of the *E.coli* derived rCYP to be similar to their human liver counterparts and the application of these recombinant enzymes in estimating the enzymology of human CYP metabolism (McGinnity et al., 1999, 2000). Desipramine was cleared predominantly by CYP2D6 (CYP2D6 >> 2C19) whereas the metabolism of imipramine was less dependent on one isoform (CYP2D6 > 2C19 > 3A4 > 1A2) as was tolterodine (CYP2D6 > 2C19 > 2C8 ~ 2B6 ~ 3A5) and propranolol (CYP2D6 > 2C19 > 1A2). Metoprolol was metabolised exclusively by CYP2D6, albeit the f_{mCYP} of total clearance was estimated at 0.9 due to the minor renal component of human clearance (Regardh and Johnsson, 1980). The f_m estimates for CYP2D6 determined using rCYPs were in broad agreement with those recently calculated from independent *in vitro* and *in vivo* methods thus providing additional confidence to this approach (Ito et al., 2005; Gibbs et al., 2006).

The value of the [I]:K_i approach as an adjunct to 'rule of thumb' alerts, based on inhibition constant alone, is exemplified by chlorpromazine and CYP2D6. Chlorpromazine is a relatively potent inhibitor of CYP2D6 ($IC_{50,u}$ = 0.3 μM; Table 1) and yet due to the estimated low unbound inhibitor concentration at the entrance to the liver ($I_{in,u}$ = 0.04 μM) the predicted and observed *in vivo* δAUC are low (1.2 and 1.7 respectively; Table 4). The

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need to incorporate parallel pathways of drug elimination and define the enzymology of CYP metabolism has been recently recognised as an important consideration for IVIVE (Yao and Levy, 2002; Ito et al., 2005; Rodrigues et al., 2001; Rostami-Hodjegan and Tucker, 2004; Obach et al., 2006). Indeed, the CYP2D6 mediated DDIs of desipramine, metoprolol and imipramine, substrates cleared to varying extents by CYP2D6, exemplify this concept. When co-administered with the selective CYP2D6 inhibitor quinidine (200 mg), desipramine, with the highest $f_{mCYP2D6}$ of 0.97, generated the highest δAUC using the $[I]_{in,u}$:K_i prediction (29) and observed (7.5) values. Quinidine (100 mg) and metoprolol ($f_{mCYP2D6}$ 0.90) generated the next highest predicted δAUC (9.1) and observed (3.2) interaction, whilst quinidine (200 mg) and imipramine (with the lowest $f_{mCYP2D6}$ 0.55) was the lowest predicted (2.3) and observed (1.5) interaction (Table 4, Figure 2A). This is consistent with the analysis performed by (Ito et al., 2005) where incorporation of f_m for CYP2D6 interactions improved the identification of true positive and negative DDIs from 54% to 84%. Although the ranking of the quinidine interactions with alternate substrates is correct the magnitude is somewhat over-predicted (Table 4, Figure 2A). This contrasts somewhat with a previous report (Grime and Riley, 2006) which estimated correctly the *in vivo* magnitude of the quinidine interaction with desipramine, metoprolol and imipramine using an external database (Ito et al., 2004). Interrogation of the two datasets indicate that a minor (~2-fold) difference in both fu_b and K_i values results in the ~4-fold difference in δAUC prediction. In addition, compared to the $[I]_{in,u}$:K_i method, the physiologically based Simcyp approach allows the simulation of the both the magnitude and range of an interaction within a defined population. Together these aspects advocate that databases and algorithms used for IVIVE should record variances of both *in vitro* and *in vivo* measurements and, via error propagation steps, provide estimates of the confidence intervals for predicted pharmacokinetic parameters, which are functions of variability in both *in vitro* and *in vivo* data and physiological factors.

For this dataset, using rCYPs to derive inhibition parameters and the $[I]_{in,u}$:K_i approach there were 6 (20%) false negatives (observed δAUC ≥ 2, predicted δAUC < 2), 23 (77%)

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correct predictions and 1 (3%) false positive. Simcyp simulations resulted in 1 (3%) false negative, 27 (94%) correct predictions and 1 (3%) false positive. The extent of the interactions of fluoxetine with desipramine, (predicted δAUC 1.5 vs observed δAUC 7.4), imipramine, (1.5 vs 3.3) and tolterodine (1.1 vs 4.8) were all significantly under estimated using the $[I]_{in,u}$:K_i method (Table 4). There are several reports of under-predicting fluoxetine DDIs (Obach et al., 2006; Ito et al., 1998; Grime and Riley, 2006) but the reason(s) are as yet unclear. It is noteworthy that the use of $[I]_{in,\ total}$:$K_{i,u}$ predicts the fluoxetine interactions well (data not shown), albeit perhaps coincidentally. Fluoxetine is a mechanism-based inhibitor of both CYP3A4 (Mayhew et al., 2000) and CYP2C19 (McGinnity et al., 2006) but not CYP2D6 (Bertelsen et al., 2003). In addition the major human metabolite norfluoxetine is approximately equipotent against CYP2D6 (Table 3), although the reported unbound plasma concentrations of norfluoxetine seem unlikely to account for the reported interactions (Jannuzzi et al., 2002). The total liver concentrations of fluoxetine (and norfluoxetine) are reported to be ~30-fold higher than plasma (Vermeulen, 1998) and this information has been used to make a successful retrospective prediction of the fluoxetine clinical interaction with CYP2D6 substrate mexiletine (Hara et al., 2005). Simcyp was used to simulate the contribution of mechanism-based inhibition, norfluoxetine inhibition and hepatic uptake of both fluoxetine and metabolite to the clinical interaction. This resulted in a good approximation of the fluoxetine - imipramine interaction (observed δAUC 3.3 vs Simcyp δAUC 3.8 (2.0 – 7.7)) but the observed magnitude of the fluoxetine - desipramine interaction could still not be simulated (Table 4). Despite the challenges, simulating and understanding such complex interactions will facilitate the future ability to make *a priori* predictions of compounds with analogous properties.

Primary hepatocytes provide the closest *in vitro* model to human liver and as such may afford advantages when predicting clinical DDI. Similar to a set of CYP2C9 inhibitors (McGinnity et al., 2005), the majority of compounds (fluoxetine, norfluoxetine, fluvoxamine, ritonavir, sertraline and labetalol), demonstrated comparable $IC_{50,u}$ values for CYP2D6 whether the enzyme source was rCYPs or human hepatocytes. However there were

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significant differences in $IC_{50,u}$ values for quinidine (0.02 ± 0.01 μM in rCYP2D6 vs. 0.5 ± 0.4 μM in human hepatocytes) and propafenone (0.02 ± 0.01 μM vs. 4.1 ± 2.4 μM respectively). Interestingly, propafenone and quinidine are both high affinity Pgp substrates (Schmid et al., 1999; Neuhoff et al., 2003). Compounds such as these which may be actively transported by Pgp into the bile *in vivo*, may be sequested out of the suspended hepatocytes into the media thus reducing the free concentration inside the cell and raising the *apparent* $IC_{50,u}$ value in hepatocytes versus that observed for rCYP. Such a hypothesis is worthy of further investigation, as is the predictive power of hepatocytes versus rCYPs or microsomes for substrates of hepatic efflux and uptake transporters. In these examples, the apparent $IC_{50,u}$ values generated in hepatocytes did appear to better simulate the clinical interaction of quinidine and propafenone with CYP2D6 substrates (Figure 2B).

Fluoxetine and fluvoxamine have been shown to extensively partition in the liver yet there was no evidence of these compounds having higher unbound hepatocellular concentrations using this protocol, a result consistent with these compounds exhibiting similar unbound K_i values in rat hepatocytes and microsomes (Brown et al., 2007). The observed drug accumulation in the liver may simply result from intracellular binding or sequestration for these lipophilic bases (Brown et al., 2007). However, in a drug discovery setting, CYP inhibition in intact hepatocytes may still be warranted for late-stage clinical candidates especially for compounds that concentrate in the liver as a result of cellular transport. In addition, as hepatocytes contain a comprehensive set of cofactors and drug-metabolising enzyme pathways, metabolites of one pathway may lead to inhibition of another, a phenomenon indiscernible using single rCYPs. However the lack of a regular supply of good quality fresh human tissue likely precludes the routine use of human hepatocytes for such a purpose in many drug metabolism laboratories.

The incorporation of inhibition and reaction phenotyping data from simple and rapid rCYP screens may identify the clinical consequences for candidate drugs as ‘perpetrators’ and/or ‘victims’ of CYP mediated interactions and provide the basis for DDI avoidance

strategies in our laboratory. The $[I]_{in,u}$:K_i approach appears relatively successful at estimating the degree of such clinical interactions and can be readily incorporated into early drug discovery. The commercially available software, Simcyp has been shown to be the most predictive model in this work (Einolf, 2007), and is becoming a useful simulation tool for use within drug discovery, development and in the rationale and design of clinical DDI studies. Furthermore, evaluation of human hepatocytes as the enzyme source and mechanistic analysis of erroneous predictions should facilitate further refinements to *in vitro* DDI assays and IVIVE strategies.

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Footnotes

Send reprints to Dr. Dermot McGinnity, Discovery DMPK, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire. LE11 5RH. U.K.

[1]Current address: Metabolism and Pharmacokinetics Group, Novartis Institute for Biomedical Research, Horsham, West Sussex, RH12 5AB. U.K.

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Legends for Figures

Figure 1. $IC_{50,u}$ comparisons using bufuralol as a CYP2D6 substrate in recombinant enzyme and human hepatocytes

The $IC_{50,\ apparent}$ values were corrected for fu determined in rCYP2D6 (hashed bars) and human hepatocytes (solid bars) to generate $IC_{50,u}$ values as outlined in Materials and Methods. The bar height represents the mean value from three separate determinations and the error bar represents the standard deviation from the mean.

Figure 2. Plot of observed versus predicted δAUC values using the $I_{in,u}$:K_i approach shown in Table 4 using K_i values generated in rCYPs (A) or using CYP2D6 K_i values generated in human hepatocytes (B). For (B) the K_i values from rCYPs except CYP2D6 were included in equation 1. Predicted or observed δAUC $\geq$2 data points are labelled with the name of the inhibitor. The open circles represent fluoxetine values. The solid line equals unity. The dashed lines signify 2-fold errors.

Table 1. Determination of $IC_{50,u}$ of individual human CYPs for probe inhibitors

Results are expressed as the mean IC_{50} of duplicate determinations. The IC_{50} measurements were carried out as described in Materials and Methods. Due to the very low protein concentration used in the assays (~0.01 mg/ml) fu_{inc} approached unity for all compounds (> 0.8).

Compound	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A5
				$IC_{50,u}$ (μM)				
Propafenone	12	>50	>50	>50	11	0.02	9	30
Quinidine	>50	>50	>50	>50	>50	0.02	19	4
Chlorpromazine	4	>50	>50	>50	9	0.3	2	42
Fluoxetine	90	>50	>50	20	0.3	0.7	10	>50
Norfluoxetine	14	14	>50	28	2	2	5	>50
Ritonavir	42	6	6	0.2	7	2	0.01	0.002
Sertraline	35	4	>50	41	1	2	9	18
Labetalol	>50	61	35	>50	30	6	>50	94
Diphenhydramine	>50	66	73	>50	88	6	55	>50
Amitriptyline	92	>50	>50	63	15	8	12	42
Fluvoxamine	0.1	8	>50	3	0.1	13	4	>50
Omeprazole	23	>50	>50	21	2	15	14	46
Diltiazem	>50	>50	>50	>50	>50	23	10	67
Verapamil	123	>50	>50	74	>50	24	5	10
Mexiletine	17	>50	>50	>50	>50	25	>50	>50
Cimetidine	>50	>50	>50	>50	28	91	>50	>50

Table 2. Determination of CYP CL_{int} of individual human CYPs to oxidative metabolism

Compound	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A5
				CL_{int} (μl/min/pmol)				
Desipramine	ND	ND	ND	ND	0.19 ± 0.09	7.28 ± 1.28	ND	0.01 ± 0.01
Imipramine	0.04 ± 0.01	ND	0.05 ± 0.02	ND	2.53 ± 1.13	5.79 ± 0.53	0.05 ± 0.01	0.02 ± 0.02
Tolterodine	ND	0.02 ± 0.01	0.40 ± 0.03	ND	0.85 ± 0.22	10.22 ± 1.93	ND	0.12 ± 0.02
Propranolol	0.14 ± 0.04	ND	0.01 ± 0.01	ND	0.71 ± 0.24	4.48 ± 0.44	ND	ND
Metoprolol	ND	ND	ND	ND	ND	1.02	ND	ND

ND – not detectable (< 0.01 μl/min/pmol). Mean ± sd for n=3 separate experiments

Table 3. Estimation of fraction metabolised by individual major human hepatic CYPs

The fraction metabolised by CYPs in human liver is estimated by accounting for the mean fraction abundance of each individual CYP human liver as determined by Rowland-Yeo et al., 2004 (CYP1A2 0.11, 2B6 0.07, 2C8 0.07, 2C9 0.17, 2C19 0.026, 2D6 0.02, 3A4 0.38 and 3A5 0.03).

Compound	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A5
Desipramine	-	-		-	0.03	0.97	-	-
Imipramine	0.02	-	-	-	0.31	0.55	0.09	-
Tolterodine	-	0.01	0.11	-	0.08	0.79	-	0.01
Propranolol	0.12	-	-	-	0.15	0.73	-	-
Metoprolol	-	-	-	-	-	1.00	-	-

Table 4. Summary of data inputs and outputs for δAUC predictions for a set of CYP mediated drug-drug interactions.

In vivo Substrate	Inhibitor	Dose (mg)	τ (h)	CL/F (ml/min)	$T_{1/2}$ (h)	k_a (min^{-1})	F_{abs}	$[I]_{in}$ (μM)	fu_p	b:p[b]	$[I]_{in,u}$ (μM)	Predicted δAUC using $I_{in,u}/K_i$	Simulated δAUC using Simcyp v7.1 Median (5^{th}-95^{th} percentile)	Observed δAUC	References
Metoprolol	Amitriptyline	75	24	1677	21	0.020[a]	0.9	3.6	0.01	1.0	0.03	1.0	1.0 (1.0-1.0)	1.4	Kirch et al., 1984
Propranolol	Chlorpromazine	50	8	1881	30	0.020[a]	1	2.4	0.02	1.48	0.04	1.2	1.2 (1.1-1.3)	1.7	Vestal et al., 1979
Metoprolol	Cimetidine	250	6	692	2	0.011	0.9	16.5	0.90	1.0	14.83	1.3	1.1 (1.0-1.1)	1.6	Kirch et al., 1982
Propranolol		250						19.8			17.80	1.4	1.4 (1.2-1.6)	1.9	
Propranolol		300						16.5			14.83	1.5	1.4 (1.2-1.7)	1.5	Donn et al., 1984
Metoprolol	Diltiazem	30	8	2173	4	0.028	0.9	1.1	0.41	1.0	0.43	1.0	1.1 (1.0-1.3)	1.3	Tateishi et al., 1989
Propranolol		30						3.2			1.30	1.0	1.0 (1.0-1.1)	1.5	
Propranolol		90						1.1			0.43	1.1	1.1 (1.0-1.2)	1.3	Hunt et al., 1990
Metoprolol	Diphenhydramine	75	12	711	5	0.020[a]	0.8	5.4	0.36	1.0	2.38	1.7	1.2 (1.1-1.3)	1.6	Hamelin et al., 2000
Desipramine	Fluoxetine	20	24	1120	47	0.009	1	0.5	0.06	0.55	0.05	1.2	2.1 (1.3-3.6)[d]	4.8	Preskorn et al., 1994
Desipramine		60						1.4			0.15	1.5	2.1 (1.4-3.6)[d]	7.4[c]	Bergstrom et al., 1992
Imipramine		60						1.4			0.15	1.5	3.8 (2.0-7.7)[d]	3.3[c]	
Tolterodine		20						0.5			0.05	1.1	nd	4.8	Brynne et al., 1999
Desipramine	Fluvoxamine	100	24	1498	15	0.008	1	2.0	0.28	0.55	1.03	1.2	1.4(1.3-1.7)	1.1	Spina et al., 1993
Imipramine		100						2.0			1.03	1.7	2.1(1.7-2.8)	3.6	
Imipramine	Labetalol	200	24	7350	4	0.020[a]	1	9.0	0.38	1.0	3.38	1.6	1.4 (1.1-1.8)	1.5	Hermann et al., 1992
Metoprolol	Mexiletine	75	12	531	10	0.020[a]	1	7.6	0.58	1.0	4.42	1.3	1.1 (1.1-1.2)	1.8	Sakamoto et al., 1995
Metoprolol	Omeprazole	40	24	991	1	0.1	0.7	7.1	0.03	0.60	0.24	1.0	1.0 (1.0-1.0)	1.0	Andersson et al., 1991
Propranolol		20						3.6			0.20	1.0	1.0 (1.0-1.0)	1.0	Henry et al., 1987
Propranolol	Propafenone	225	8	2380	4	0.020[a]	1	10.5	0.16	0.61	2.73	5.5	2.6 (1.6-4.6)	2.1	Kowey et al., 1989
Desipramine	Quinidine	200	24	463	11	0.014	0.9	7.4	0.23	0.92	1.81	29	19 (11-34)	7.5	Brosen and Gram, 1989
Imipramine		200						7.4			1.81	2.3	4.8 (1.8-10)	1.5	
Metoprolol		100						3.7			0.90	9.1	3.5 (1.9-6.5)	3.2	Johnson and Burlew, 1996
Desipramine	Ritonavir	250	12	147	4	0.014	0.8	11.8	0.02	0.55	0.32	1.4	1.2 (1.1-1.5)	2.5	von Moltke et al., 1998
Desipramine	Sertraline	50	24	2660	23	0.007	1	0.9	0.01	1.0	0.01	1.0	1.0 (1.0-1.0)	1.3	Preskorn et al., 1994; Alderman et al., 1997
Desipramine		100						1.8			0.02	1.0	1.0 (1.0-1.0)	1.5	
Desipramine		150						2.6			0.03	1.0	1.0 (1.0-1.0)	1.5	Kurtz et al., 1997
Imipramine		150						2.6			0.03	1.0	1.0 (1.0-1.1)	1.7	
Metoprolol	Verapamil	120	8	4773	4	0.020	1	4.0	0.12	0.77	0.60	1.1	1.1 (1.1-1.5)	1.3	Keech et al., 1986
Propranolol		120						4.0			0.60	1.0	1.0 (1.0-1.0)	1.4	McCourty et al., 1988; Murdoch et al., 1991

Dose size / interval and observed δAUC were retrieved from the listed references. Human PK parameters (CL, F, $T_{1/2,}$ k_a and oral F_{abs}) were sourced from several key compendiums including Goodman and Gilman's The Pharmacological Basis of Therapeutics 9^{th} & 10^{th} ed; Dollery's Therapeutic Drugs 2^{nd} ed; Taeschner and Vozeh's Drug Data Handbook 3^{rd} ed and Bertz and Granneman, 1997. [a] Where K_a could not be retrieved from the literature the mean value of 0.02 min^{-1} was used. $[I]_{in}$ was calculated using equation 2. $[I]_{in,u}$ values = $[I]_{in}$ x fu_p determined in this laboratory. [b] Blood:plasma ratios of inhibitors were sourced as follows: Amitriptyline (Rollins et al., 1980), chlorpromazine (Shibata et al., 2000), diltiazem (Naritomi et al., 2003), fluoxetine, fluvoxamine, omeprazole, ritonavir (SimCYP Simulator v7.1), propafenone (Trenk et al., 1989), quindine and verapamil (Shibata et al., 2002). For those compounds where b:p ratio has

not been obtained a value of 1 has been used. All $IC_{50,u}$ values of the inhibitors are reported in Table 1 and $K_{i,u}$ values were determined as described in Materials and Methods. Where an $IC_{50,u}$ value of >100 μM is reported a nominal $K_{i,u}$ value of 50 μM was used. $[I]_{in,u}$ and $K_{i,u}$ values for the inhibitors together with the estimates of fm_{CYP} for desipramine, imipramine, tolterodine, propranolol and metoprolol (Table 3), were used in Equation 1 to generate predicted δAUC values. [c] steady-state of inhibitor not reached. [d] using active uptake factor into hepatocytes of 1000 for fluoxetine. nd not determined

Figure 1

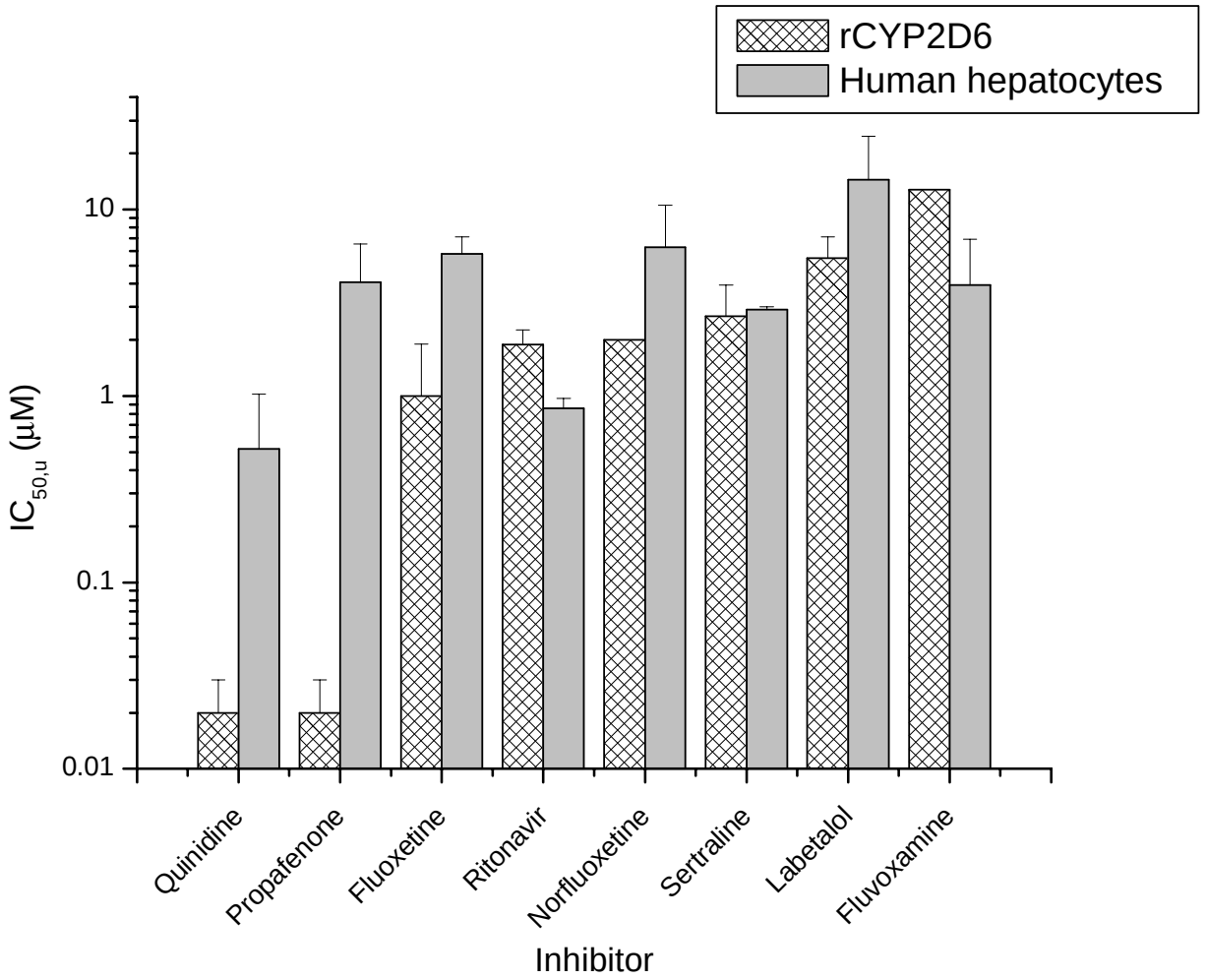

Figure 2