ASSESSMENT OF ALGORITHMS FOR THE PREDICTION OF METABOLIC DRUG-DRUG INTERACTIONS

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Figure 5.1 Comparison of the predicted:observed and observed AUC ratios for 27 DDIs involving fluvoxamine. Predictions were performed in Simcyp using the time-based dynamic (●) or static (◇) model. Horizontal dashed lines represent the 2-fold margins, and vertical dashed lines represent the boundaries between weak (W), moderate (M) and strong (S) DDIs.

Figure 5.2 Comparison of predicted:observed and observed AUC ratios for 10 fluoxetine DDIs. Predictions were performed in Simcyp using either the time-based dynamic model in the presence (●) or absence (□) of the fluoxetine metabolite (norfluoxetine) or using the static model (◇). The horizontal dashed lines represent the 2-fold margins, and vertical dashed lines represent the boundaries between weak (W), moderate (M) and strong (S) DDIs.

Figure 5.3 Predicted and observed AUC ratios for the dynamic prediction of SSRI DDIs classified according to victim drug and CYP enzyme, with CYP1A2 (circle symbols): caffeine (●), clozapine (●), theophylline (●); CYP2C9 (rotated triangle): tolbutamide (◇); CYP2C19 (triangle symbols): omeprazole (△); S-mephytoin (△); CYP2D6 (square symbols): desipramine (■), imipramine (■), tolterodine (■); CYP3A4 (diamond symbols): alprazolam (◆), midazolam (◆), quinidine (◆).

Figure 5.4 Impact of the dosing time of victim drug from -10 to +24h after the final inhibitor dose on the predicted AUC ratio using the dynamic model in Simcyp. DDIs are between the inhibitor fluvoxamine and victim drugs omeprazole (○) (CYP2C19) or caffeine (■) (CYP1A2) and fluoxetine (with inclusion of the norfluoxetine active metabolite) and desipramine (△) (CYP2D6), with dosing schedules as defined in the methods.
Figure 5.5 Predicted AUC ratios obtained from 10 individual trials of 5 PM and 5 EM CYP2D6 genotyped subjects for a fluvoxamine-omeprazole (A-C) and fluvoxamine-caffeine (D-F) DDI study (Christensen et al., 2002). A and D show observed data from 10mg or 25mg fluvoxamine dosing, in the EM (twice daily dosing) or PM (once daily dosing) populations. B, C and E, F are predicted using the dynamic model in Simcyp, using fluvoxamine parameters outlined in Table 5.1, and matched dosing schedules to the clinical study. Simulations are excluding (B and E) or including (C and F) the impact of the CYP2D6 genotype (matched distribution to the clinical study). Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value, dotted line represents the mean values and the box represents the inter-quartile range boundaries. Where > 9 subjects are included the lower and upper whiskers represent the 10-90% range and outliers are represented by ●.

Figure 5.6 Predicted AUC ratios obtained from 10 individual polymorphic trials for the fluoxetine-tolterodine DDI compared to the actual distribution of AUC ratios in the three genotype groups (Brynne et al., 1999). Top left is the summary data and the PM, IM and EM boxes display data from the total of the ten trials. Predictions are using the dynamic model in Simcyp, using fluoxetine and norfluoxetine parameters outlined in Tables 5.1-5.3 and matched dosing schedules to the clinical study. Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value and the box represents the inter-quartile range boundaries. Where > 9 subjects are included, lower and upper whiskers represent the 10-90% range and outliers are represented by ●.

Figure 6.1 Schematic graph displaying the limits of the different predictive measures; the traditional 2-fold predictive measure (dashed lines) and the proposed new predictive measure (dotted lines). Observed AUC ratio include both induction and inhibition DDIs.

Figure 6.2 Limits of DDI prediction with dashed lines representing the new predictive measure with inclusion of intra-individual variability, calculated via Equations 6.1 and 6.2, with the limits defined in Equation 6.4. Prediction of DDIs involving midazolam as the victim drug, taken from 3 sources, where ■ is Einolf et al., 2007, ▲ is Fahmi et al., 2009 and ○ is the current analysis reported in Chapters 4 and 5. The new predictive measure and inclusion of intra-individual variability is utilised. Two induction DDIs are not shown (AUC ratio 0.04 and 0.05); both were successfully predicted with all methods. The vertical lines represent the limits between potency classifications, where I, NI, W, M and S represents induction, no interaction, weak, moderate and strong inhibition interaction, respectively.

Figure 6.3 The dynamic prediction of DDIs where inhibitor clearance has been characterised using in vivo clearance from oral data (with the exception of recombinant in vitro data for itraconazole DDIs) (A), or in vitro clearance from the conventional assay in human hepatocytes (Chapter 3) (B). DDIs involved fluoxetine (●), fluvoxamine (○), itraconazole (▲) or ketoconazole (▼) as the inhibitors. All other parameters were consistent between the predictions. Dashed lines representing the new predictive measure with inclusion of variability, calculated via Equations 6.1 and 6.2, with the limits defined in Equation 6.4. The vertical lines represent the limits between potency classifications, where I, NI, W, M and S represent induction, no interaction, weak, moderate and strong inhibition interaction, respectively.
Abstract

The aim of this work was to assess the ability of the static and dynamic (incorporating the time-course of the inhibitor) prediction models to predict drug-drug interactions (DDIs) using a population-based ADME simulator (Simcyp). This analysis focused on fluconazole, ketoconazole, itraconazole, fluoxetine and fluvoxamine, as CYP inhibitors. The rationale for their selection was an abundance of reported DDI studies, involving a wide range of victim drugs.

Preliminary analysis focused on the individual victim drug and inhibitor parameters that are utilised in the DDI prediction models. The victim drug properties included in the DDI prediction models are calculated intrinsically in the Simcyp simulator from in vitro data; these values were compared to estimates obtained by different in vivo methods. Estimations of the fraction metabolised by CYP enzymes were generally consistent with <20% difference between all methods for 15/23 victim drugs. No relationship was observed per CYP enzyme or per inhibitor utilised for phenocopying methods. Estimates of fraction of drug escaping metabolism in the gut were variable across methods with up to 60% coefficient of variation in the case of saquinavir. In vitro assessment of potential liver uptake of the inhibitors was identified for further investigation due to inconsistency in available literature data and sensitivity of the model to this parameter.

Extent of liver uptake of selected inhibitors was assessed via comparison of clearance obtained in hepatocytes and microsomes (conventional depletion assay) and values obtained by the conventional depletion and media loss assays in hepatocytes. Clearance was determined at a low concentration (0.1μM) and both rat and human hepatocytes and microsomes were used. The clearance ratios ranged from no difference to >1500 (fluvoxamine from the media loss assay in human hepatocytes). No consistency was observed between methods and human or rat source for any of the inhibitors investigated; therefore, the inclusion of liver uptake into the prediction of DDIs for the current inhibitors was not supported.

A database was collated from literature reports of DDIs involving the above named CYP inhibitors (n=97) and used to assess the inclusion of the time-course of inhibition into DDI prediction using the Simcyp simulator. In addition, the impact of active metabolites, dosing time and the ability to predict inter-individual variability in DDI magnitude were investigated using the dynamic prediction model. Simulations comprised of 10 trials with matching population demographics and dosage regimen to the in vivo studies. The predictive utility of the static and dynamic models was assessed relative to the inhibitor or victim drug investigated; both models were employed within Simcyp for consistency in parameters. Use of the dynamic and static models resulted in comparable prediction success, with 67 and 70% of DDIs predicted within two-fold, respectively. Over 60% of strong DDIs (>five-fold AUC increase) were under-predicted by both models, particularly for fluoxetine and fluvoxamine. Incorporation of the itraconazole metabolite into the dynamic model resulted in increased prediction accuracy of strong DDIs (80% within two-fold); no difference was observed for the inclusion of the fluoxetine metabolite. Predicted inter-individual variability in the DDI magnitude was also assessed in healthy, patient and genotyped subjects using a subset of clinical interactions (n=24). Mixed prediction success was observed and the importance of reliable clinical data was highlighted. The differences observed with the dose staggering and the incorporation of active metabolite highlight the importance of these variables in DDI prediction. Finally, the traditional ‘two-fold limits’ as a measure of the prediction success were reassessed, in particular at AUC ratios approaching one. New limits proposed are applicable for both inhibition and induction DDIs and allow incorporation of the variability in pharmacokinetics of the victim drug when required. DDI predictions were refined using in vitro clearance data for the inhibitors, and assessed using the new predictive measure.
List of abbreviations

ACAT  Advanced compartmental, absorption and transit
ADAM  Advanced absorption, dissolution and metabolism
ADME  Absorption, distribution, metabolism and excretion
afe   Average fold error
ATP   Adenosine triphosphate
AUC   Area under curve
C(0)  (Initial) concentration
CLH   Hepatic blood clearance
CLint  Intrinsic clearance
CLint,g Gut intrinsic clearance
CLint,H Hepatic intrinsic clearance
CLperm Permeability clearance
Cmax  Maximal plasma concentration
CLp   Plasma clearance
CLR   Renal clearance
CRcyp Contribution of a CYP to oral clearance of a drug
CV    Coefficient of variation
CYP   Cytochrome P450
D     Dose
DDI   Drug-drug interaction
Dn    Dispersion number
(c)DNA (Complementary) Deoxyribonucleic Acid
EG    Fraction extracted by gut
EH    Fraction extracted by liver
EM    Extensive metaboliser
FCCP  Carbonyl cyanide–p-trifluoromethoxyphenylhydrazone
FDA   Food & Drug Administration (USA)
F     Oral bioavailability
Fa    Fraction of dose absorbed
FG    Fraction escaping metabolism in the gut
FH    Fraction escaping hepatic metabolism
fMCYP Fraction metabolised by CYP
fu,b Fraction unbound in blood
fu,inc Fraction unbound in an incubation
fu,gut Fraction unbound in the gut
fu,hep Fraction unbound in an incubation of hepatocytes
fu,mic Fraction unbound in an incubation of microsomes
fu,p  Fraction of drug unbound in plasma
GFR   Glomerular filtrate rate
HLM   Human liver microsomes
[I]   Inhibitor concentration
[I]av Average systemic concentration
[I]in Maximal hepatic inlet concentration
[I]max Systemic total maximal concentration
ICH   International Conference on Harmonisation
IG    Inhibitor concentration in the absorption phase
i.v.  Intravenous
IRcyp Time-averaged apparent inhibitor ratio
ISEF  Inter-system extrapolation factors
IVIVE In-vitro in-vivo extrapolation
k     Rate constant
kdeg  Degradation rate constant
$k_a$ Absorption rate constant
$K_d$ Microsomal protein binding affinity
$K_i$ Inhibition constant
$K_I$ Concentration of inhibitor at half of the inactivation rate constant
$k_{\text{inact}}$ Inactivation rate constant
$K_m$ Michaelis-Menten constant
$K_p$ Hepatocyte to medium partition coefficient
$KHB$ Krebs-Henseleit Buffer
LC-MS/MS Liquid chromatography-mass spectrometry
LogP/D Descriptors of drug lipophilicity
MeOH Methanol
NADPH Nicotinamide adenine dinucleotide phosphate
NCE New chemical entity
NTCP Na/Taurocholate cotransporting polypeptide
OATP Organic anion transporting polypeptide
P Microsomal protein concentration
$P_{\text{eff}}$ Effective permeability
$P_{\text{gp}}$ P-glycoprotein
pKa Acid dissociation constant
PM Poor metaboliser
$Q_{\text{ent}}$ Enterocytic blood flow
$Q_{\text{gut}}$ Hybrid parameter of intestinal blood flow and drug permeability
$Q_H$ Hepatic blood flow
$R_B$ Blood to plasma ratio
rmse Root mean squared prediction error
RNA Ribonucleic acid
S.D. Standard deviation
SSRI Selective serotonin reuptake inhibitor
t Time
$t_{1/2}$ Half-life
$T_{\text{max}}$ Time to maximal plasma drug concentration
UM Ultrarapid metaboliser
V Volume of incubation
$V_{\text{cell}}$ Cell volume
$V_{\text{max}}$ Maximum rate of metabolism
$V_R$ Volume ratio of hepatocytes to medium
Declaration

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The Author

The author graduated from The University of Leeds in 2007 with a First Class BSc (Honours) degree in Pharmacology. One year of this degree (2005-6) was spent undertaking an industrial placement year within the department of International Regulatory Affairs at GlaxoSmithKline in Greenford, U.K. The final year dissertation for the degree related to *in vitro* work in cell physiology.

The author started the PhD within the Centre for Applied Pharmacokinetic Research at the University of Manchester in September 2007. The PhD project was under the supervision of Dr. Aleksandra Galetin and Prof. J. Brian Houston, and part funded by Simcyp, under the supervision of Prof. Amin Rostami-Hodjegan and Prof. Geoffrey T. Tucker. The PhD program was completed in September 2010.
CHAPTER 1: INTRODUCTION
1 INTRODUCTION

1.1 Impact of Drug-Drug Interactions
Concomitant drug administration can result in adverse drug-drug interactions (DDIs), causing clinically important alterations in the pharmacokinetic action of a drug during the absorption, distribution, metabolism and excretion processes. The majority of DDIs result from the inhibition of the metabolism of one drug by another, increasing the exposure of the drug (greater area under the plasma concentration curve over time (AUC)). Poor pharmacokinetic characteristics were one of the major reasons why drugs fail in the clinic (Bachmann and Ghosh, 2001); currently, failures are increasingly due to efficacy rather than pharmacokinetic characteristics due to advances in predictions and PBPK modelling in the past decade (Rowland et al., 2011). Cytochrome P450 (CYP)-related DDIs have resulted in the withdrawal of a number drugs from the market including terfenadine (withdrawn in 1998 due to the prolongation of the cardiac QT interval when co-administered with CYP3A4 inhibitors) and the hyperlipidaemia agent cerivastatin (withdrawn in 2001 due to toxicity) (Greenblatt, 2009; Wienkers and Heath, 2005). The United States Food and Drug Administration (FDA) provides guidelines for the design and analysis of DDI studies (Bjornsson et al., 2003; Grimm et al., 2009; Huang et al., 2007) in pre-clinical and clinical development. This process allows early assessment of the DDI potential of a drug in development in order to increase patient safety and the cost effectiveness of the drug development process.

Over the past decade, there have been increasing numbers of studies assessing the prediction of DDIs from in vitro data. The prediction models used have increased in complexity, for example incorporating the parallel pathways of metabolism through inclusion of the fmCYP, the fraction of drug escaping metabolism in the gut in the presence and absence of the inhibitor and use of alternative estimates of the inhibitor concentration at the enzyme active site (Brown et al., 2006; Brown et al., 2005; Fahmi et al., 2009; Galetin et al., 2006; Galetin et al., 2007; Galetin et al., 2005; Hinton et al., 2008; Ito et al., 2004; Ito et al., 2005; Obach, 2009; Obach et al., 2006; Wang, 2010).

1.1.1 Metabolic DDIs and the Cytochrome P450 enzyme family
DDIs are reported between a wide range of drug, food and herb combinations, representing a range of pharmacokinetic and pharmacodynamic interactions. This Thesis reports on the metabolic pharmacokinetic interactions between drug combinations. These interactions often result from the inhibition of CYP enzymes. The CYP enzymes are largely
responsible for the metabolism of drugs through Phase I oxidative metabolism, facilitating elimination by forming metabolites with increased hydrophilicity (Venkatakrishnan et al., 2001; Wilkinson, 2005). This process is generally the rate-limiting step in the metabolism and elimination of drugs (Fukasawa et al., 2007; Wienkers and Heath, 2005; Wilkinson, 2005).

The subfamilies of CYP enzymes are named according to their amino acid sequence, as the substrate specificities are often broad ranging and overlapping (Wilkinson, 2005). A gene similarity of under 40% is classified into different gene families (e.g. CYP1, CYP2), similarities of 40 – 55% are classified to different subfamilies (e.g. CYP1A, CYP1B), and a similarity of over 55% is classified into the same subfamily and numerically named (e.g. CYP1A1, CYP1A2) (Fukasawa et al., 2007). Out of the total 481 genes that code for the CYP enzymes there are 57 that code for human CYP isoforms (Redlich et al., 2008; Wilkinson, 2005). Despite this number, approximately 90% of CYP mediated drug metabolism is due to six main isoforms (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5) (Figure 1.1, Einolf, 2007; Huang et al., 2007; Lynch and Price, 2007; Venkatakrishnan et al., 2001; Wilkinson, 2005). Interactions involving these CYP enzymes will therefore be considered in this Thesis.

![Figure 1.1 The routes of elimination of the top 200 most prescribed drugs in 2002.](image)

Metabolism represents the elimination mechanism of 73% of all drugs, with approximately 75% of this metabolism as a result of CYP activity. Approximately half of CYP-mediated metabolism is a result of CYP3A. (Figure adapted from Wienkers and Heath, 2005).

Other routes of elimination aside from CYP enzymes include renal and faecal (via bile) elimination and other microsomal and cytosolic enzymes including esterases, UDP-
glucuronyl transferases and sulfotransferases. The interaction with efflux transporters such as P-glycoprotein (P-gp) (Zhang et al., 2006) and uptake transporters such as OATP1B1 (Hinton et al., 2008; Niemi et al., 2005) and NTCP (Ho et al., 2006) can also alter factors determining the pharmacokinetics of a drug and DDI prediction. Transporters will directly influence the intracellular drug concentration and kinetic parameters of a transporter substrate (Huang et al., 2008). It is also reported that the interplay between P-gp mediated efflux at the luminal membrane of intestinal mucosa modulates the efficiency of intestinal CYP3A-dependent first-pass metabolism (Benet, 2009; Benet et al., 2004), and there is a significant overlap between the substrate and inhibitor affinities for CYP3A and P-gp (Bachmann and Ghosh, 2001; Lin et al., 1999; Yu, 1999). These factors complicate DDI predictions and increase the potential for introducing error.

1.1.1.1 Distribution of CYP enzymes

The main site of CYP mediated metabolism is the liver, with the highest CYP enzyme content and widest distribution of the different isoforms (Lin et al., 1999; Nishimura et al., 2003; Rowland-Yeo et al., 2004). The relative abundance of the isoforms in the liver is shown in Figure 1.2A. Some CYP enzymes are also present in the enterocytic cells of the small intestine, particularly CYP3A that accounts for over 80% of the CYP abundance (Figure 1.2B, Paine et al., 2006). CYP enzymes located here contribute to the first-pass effect, where the drug is metabolised and eliminated before reaching systemic circulation, contributing to the low and variable bioavailability of a drug. In addition, the DDI potential is increased when the drug is metabolised in both the intestine and liver (Galetin et al., 2008; Galetin et al., 2007; Wrighton et al., 2000). The total CYP3A content in the average small intestine is approximately 1% (70.5 nmol) of that in an average liver. The expression and activity along the intestinal length varies, with the levels decreasing from the duodenum to the ileum (de Waziers et al., 1990; Galetin et al., 2008; Paine et al., 1997; von Richter et al., 2004).
There are many intrinsic factors that can alter the susceptibility of an individual to DDIs including age, race, gender and genetic polymorphisms. These factors generally result in differences in the distribution, expression levels or CYP enzyme involved (Inoue et al., 2006; Lin and Lu, 2001; Rostami-Hodjegan and Tucker, 2007) and will be further considered below. If a drug is found to inhibit a CYP enzyme it does not always lead to DDIs once the drug is in vivo due to other compounding factors. These factors can include the in vivo pharmacokinetic parameters and therapeutic index, the fraction unbound in plasma \( (f_u) \) and resultant concentration differences at the active site of the enzyme, the sole reliance on one CYP enzyme for metabolism and elimination and other inter-individual factors (Bachmann et al., 2003; Rostami-Hodjegan and Tucker, 2004). The likelihood of co-administration of two drugs, extent of distribution and risk-to-benefit ratio relating to the severity of the disorder will also have important consequences on the importance of DDIs during the development of a drug (Scripture and Figg, 2006).

1.1.1.2 Enzyme polymorphisms
A polymorphism is the occurrence of two or more alleles (alternate forms of a gene) at one locus (Fukasawa et al., 2007) occurring in at least 1% of the population (Rogers et al., 2002). The CYP enzymes CYP2C9, CYP2C19 and CYP2D6 display polymorphisms
(Rodrigues and Rushmore, 2002) with alleles of reduced or defective metabolic activity resulting in poor metaboliser (PM) phenotypes or homozygous functional alleles resulting in the extensive metaboliser (EM) phenotype. The intermediate metaboliser (IM) phenotype also results from the heterozygous combination of one functional and one non-functional allele. The decreased ability of PMs to metabolise may result in higher plasma levels of drug increasing the likelihood of toxicity. Ultrarapid phenotypes of CYP2D6 can also occur as a result of multiple copies of the wild-type allele (Lynch and Price, 2007) and result in a higher chance of therapeutic failure from the rapid elimination rate. Genetic variants of CYP3A4 are uncommon and have limited functional significance. CYP3A5 is a polymorphic enzyme and the overlapping substrate profiles between CYP3A4 and CYP3A5 result in difficulty in the selection of specific probe drugs in order to assess the relative metabolic contributions of the two enzymes (Hustert et al., 2001; Perera, 2010; Xie et al., 2004). Differences in polymorphisms can be related to ethnicity (Lynch and Price, 2007) with the CYP2C19 PM phenotype occurring in 3-6% of the Caucasian population and 15-30% of the Asian population (Fukasawa et al., 2007). The CYP2D6 PM phenotype occurs in 5-10% of the Caucasian population, 0-1% of the Oriental population and has a wide range of 0-19% of the African population with a two to five-fold difference in metabolising capacity (Hasler et al., 1999). Studies have also assessed the homozygous expression of non-functional CYP3A5*3 gene, which was found in 15 and 87% of the African and Caucasian population, respectively (Xie et al., 2004). The polymorphisms are likely to have maximal effect where a therapeutic agent is solely metabolised by the polymorphic enzyme (Fukasawa et al., 2007).

### 1.1.1.3 Types of interactions with CYP enzymes

The interaction mechanism of CYP enzymes is an important factor in DDIs, and early in vitro assessment allows the safer and more strategic design of the later in vivo clinical trials. The mechanism will either be inhibition or induction of the CYP, where the inhibition can further be classified as reversible (competitive, non-competitive or mixed competitive) or irreversible (time-dependent and mechanism based) (Houston and Galetin, 2010; Houston and Galetin, 2003; Wienkers and Heath, 2005). Uncompetitive inhibition also exists theoretically but this situation is rarely observed in vivo (Lin and Pearson, 2002). Reversible interactions consist of a non-covalent attachment of the inhibitor to the CYP and can be overcome by increasing concentrations of the substrate. The interaction can either be competitive, with direct competition for the active site, non-competitive where the inhibitor binds to a different site to the substrate, uncompetitive where the inhibitor only binds after the substrate is bound, and mixed competitive where the inhibitor
either binds to both the active site and another site or the inhibitor binds to the active site but does not stop the substrate from binding (Houston et al., 2003). Irreversible inhibition is due to either the covalent attachment of the inhibitor to the CYP active site, or through the catalytic activation of an inhibitor that subsequently binds to the CYP active site and prevents the metabolism of a substrate (Obach et al., 2007; Wang et al., 2004b; Yang et al., 2005). These complexes often occur with the CYP enzymes due to the presence of the one electron reduced heme group that is present (Bachmann et al., 2003). Recovery from irreversible inhibition is dependent on new synthesis of the CYP enzyme and therefore determined by the rate of enzyme turnover (Silverman, 1995; Yang et al., 2008). Compounds that result in the induction of CYP enzymes can lead to the therapeutic failure of another drug. CYP induction occurs by either increasing enzyme synthesis through increased DNA transcription or reducing the degradation rate through protein stabilisation. It has been reported that induction can result in a two- to eight-fold increase in enzyme levels or catalytic activity; however induction interactions are less likely to cause clinically significant DDIs than inhibition interactions (Fahmi et al., 2008; Fahmi and Ripp, 2010; Shou et al., 2008; Thummel et al., 2000) and will not be the main focus of this Thesis.

1.2 Metabolic inhibition models
The metabolic clearance of a substrate by an enzyme in the absence and presence of a reversible inhibitor are described in Equations 1.1 and 1.2, respectively. It is valid for both competitive and non-competitive inhibitors as the substrate concentration at the active site is assumed to be lower than the Michaelis-Menten constant (K\textsubscript{m}) (Brown et al., 2005; Houston and Galetin, 2010; Ito et al., 2004). These terms are combined to produce Equation 1.3, allowing the fold change in substrate clearance to be predicted from inhibitor parameters. Equation 1.3 additionally has the ability to incorporate the contribution of multiple inhibitors, including situations where there are active metabolites of the parent drug (for example with norfluoxetine, the potent metabolite of fluoxetine (Hasler et al., 1999)) which can be included in order to maximise the prediction accuracy.

\[ \text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_{\text{m}}} \]

\[ \text{CL}_{\text{int}}' = \frac{V_{\text{max}}}{K_{\text{m}} \left( 1 + \frac{[I]}{K_{i}} \right)} \]

\[ \frac{\text{CL}_{\text{int}}}{\text{CL}_{\text{int}}'} = 1 + \sum_{j = 1}^{m} \frac{[I]}{K_{i}} \]

Equation 1.1
Equation 1.2
Equation 1.3
Where $CL_{\text{int}}$ relates to the intrinsic clearance in the presence (denoted by ‗) and absence of the inhibitor, $V_{\text{max}}$ is the maximum rate of velocity, $K_m$ is the Michaelis-Menten constant relating to the concentration of substrate at $0.5V_{\text{max}}$, $j$ relates to multiple inhibitors ($m$), $[I]$ relates to a single *in vivo* inhibitor concentration at the enzyme active site, and $K_i$ is the potency of CYP inhibition found from the *in vitro* inhibition constant.

The inhibition constant describes the affinity for binding of the inhibitor to the enzyme and is only valid when the following assumptions are made: the dissociation is assumed to be the rate-limiting factor, and the concentration of the specific enzyme is assumed to be negligible when compared to the concentration of the substrate or inhibitor (Lin and Pearson, 2002). Several other factors can lead to error in the calculation of $K_i$, including protein binding, multiple binding sites and the *in vitro* system that was used for prediction (Houston et al., 2003); these *in vitro* factors are further discussed in Section 1.4. Assumptions of the model in Equation 1.3 are that the extent of inhibition is determined by the concentration of drug at the enzyme active site, that the *in vitro* constants are directly applicable to the *in vivo* interaction and that the *in vivo* inhibitor concentration at the active site is equal to the unbound plasma concentration. It is also assumed that the inhibitor concentration is time-invariant and that the substrate is exclusively cleared by a single hepatic CYP metabolism pathway (Einolf, 2007; Ito et al., 2004; Ito and Houston, 2004; Wrighton et al., 2000).

The alteration of pharmacokinetic parameters of a substrate by an inhibiting drug results in a change in the area under the curve (AUC) of a plasma concentration-time plot in the presence and absence of the inhibitor after multiple oral dosing *in vivo*. The ratio of this change represents the extent of inhibition, and is therefore used to assess the extent of DDIs (Tucker et al., 2001b). The classification of the DDI potential of CYP3A inhibitors has been reported by the FDA (Huang et al., 2007). In the case when the AUC ratio in the presence and absence of inhibitor is above 5-fold, then the compound is defined as a strong inhibitor of CYP3A4. An AUC ratio of above 2-fold but less than 5-fold is defined as a moderate inhibitor, and above 1.25-fold and below 2-fold is defined as weak. An AUC ratio below 1-fold indicates an induction interaction, with increased metabolism of the victim drug in the presence of the interacting drug. Although this classification is described for inhibitors of CYP3A, the FDA have reported in their guidance document (FDA, 2006) that it can also be applied for other CYP enzymes.
The AUC ratio relates to the clearance ratio and the [I]/K_i as described in Equation 1.4. The relationship assumes the conditions of the well-stirred model, including that the inhibitor concentration does not affect protein binding or fraction absorbed and that the substrate dosing schedule is identical in the absence and presence of the inhibitor (Rowland and Matin, 1973). The well-stirred model will be considered further in Section 1.4.2.

\[
\frac{\text{CL}_{\text{int}}}{\text{CL}_{\text{int}^{'}}} = \frac{\text{AUC}'}{\text{AUC}} = 1 + \sum_{j=1}^{m} \frac{[I]}{K_i}
\]

Equation 1.4

Where AUC relates to the area under the plasma concentration-time curve in the presence (denoted by ‘) and absence of the inhibitor.

1.2.1 Irreversible inhibition

Additional parameters are required for the prediction of the AUC ratio for irreversible inhibitors after it was reported that the reported risk was insufficient when calculated from the ‘[I]/K_i’ equation (therefore assuming reversible inhibition, Equation 1.4) or the ratio of the inactivation rate constant (k_{inact}) to the concentration of inhibitor at 0.5k_{inact} (K_i) (Grimm et al., 2009; Wang et al., 2004b). The additional parameters are used for prediction as per Equation 1.5.

\[
\frac{\text{AUC}''}{\text{AUC}} = 1 + \sum_{j=1}^{m} \left( \frac{k_{\text{inact}, j} \cdot [I]_j}{k_{\text{deg}} \cdot (K_{I,j} + [I]_j)} \right)
\]

Equation 1.5

Where k_{inact} is the inactivation rate constant, K_i is the concentration of inhibitor at half of the inactivation rate constant and k_{deg} is the first order rate constant of in vivo degradation of the affected CYP enzyme.

Assumptions of this model are the same as for reversible inhibition with the addition that the loss of enzyme is solely due to inactivation by the inhibitor (Wang et al., 2004b). The k_{inact} and K_i are derived from in vitro incubations using human liver microsomes (HLM) or hepatocytes. The slopes of the initial linear decline in enzyme activity over time against the inhibitor concentration are utilised to find the maximal rate of metabolite formation (k_{inact}) and inhibitor concentration at half-maximal rate (K_i) (Galetin et al., 2006; Ghanbari et al., 2006; Grimm et al., 2009; Mayhew et al., 2000; Silverman, 1995; Wang et al., 2004b). The active enzyme concentration is determined by the rate of de novo synthesis of enzyme and the k_{deg} (Galetin et al., 2006; Grimm et al., 2009; Mayhew et al., 2000; Wang et al.,
A 7-fold range (17-146 hours; mean, 72 hours) was observed for the liver degradation half-lives assessed from different methods and sources (Galetin et al., 2006; Yang et al., 2008). Use of this mean value was found to result in increased DDI prediction accuracy when compared to 24 or 144 hours (Galetin et al., 2006) from use of the static equation. Alternative studies have found the mean to be 23 hours from in vivo data (Wang et al., 2004c); this value resulted in increased prediction accuracy of irreversible DDIs when compared to 90 hours (Wang, 2010). Therefore, there is currently no consensus in the literature for the value of $k_{\text{deg}}$, and additional studies performing predictions of the AUC ratio of time dependent inhibition have utilised values corresponding to degradation half-lives of 23-90 hours in the liver (Einolf, 2007; Fahmi et al., 2009; Galetin et al., 2006; Galetin et al., 2010; Wang, 2010). This lack of consensus in recommended values is likely to result from differences in the remaining parameters used in the predictions. The intestinal $k_{\text{deg}}$ corresponds to a degradation half-life of 23 hours (range, 13-33 hours) (Gertz et al., 2008a; Obach et al., 2007; Yang et al., 2008) from the rate of enterocyte turnover, and this value has been consistently utilised in the prediction of time-dependent inhibition.

1.2.2 Fraction of drug metabolised by a CYP enzyme ($f_{\text{mCYP}}$)

The $f_{\text{mCYP}}$ is the fraction of a substrate drug that is metabolised via a specific CYP enzyme. It has been found that the DDI potential of a compound will increase dramatically if the $f_{\text{mCYP}}$ is over 30%, due to the increased impact following inhibition (Bachmann et al., 2003). The inclusion of $f_{\text{mCYP}}$ in the prediction of the AUC ratio results in a higher prediction accuracy regardless of the inhibitor concentration variant and remaining parameter values used in the predictions (Brown et al., 2006; Brown et al., 2005; Einolf, 2007; Galetin et al., 2005; Ito et al., 2005). Sensitivity of the prediction model positively correlates to the $f_{\text{mCYP}}$ value, as demonstrated from the observed difference in the predicted AUC ratio from simulation across a 0.5-1 $f_{\text{mCYP}}$ range (Ito et al., 2005).

The following equation is used for reversible inhibitors to include the $f_{\text{mCYP}}$ in the AUC ratio prediction for reversible inhibition (Brown et al., 2005; Galetin et al., 2005; Ito et al., 2005). The equation for incorporating $f_{\text{mCYP}}$ into irreversible inhibition is analogous to Equations 1.5 and 1.6 (Obach et al., 2007; Wang et al., 2004b). The ability to include metabolism via multiple CYP enzymes is included in Equation 1.6; the rate of clearance and therefore AUC depends on the fractional contribution of the affected pathway (Hinton et al., 2008).
Where \( fm_{CYP} \) represents the fraction metabolised by the individual CYP enzyme subject to inhibition, \( i \) represents multiple CYP enzymes \( (n) \). The \((1-fm_{CYP})\) section of the equation corresponds to renal clearance or clearance via other pathways.

### 1.2.3 Intestinal Availability \( (F_G) \)

As discussed in Section 1.1.1.1, the expression of CYP3A4 enzymes in the intestine can contribute to the first-pass effect of a drug and result in a low and variable bioavailability if not included in the DDI prediction model. Gut metabolism is physiologically relevant and recent investigations have assessed the inclusion of this parameter into DDI prediction models (Einolf, 2007; Galetin et al., 2006; Galetin et al., 2010; Galetin et al., 2007; Obach et al., 2006; Wang et al., 2004b; Wang, 2010). The fraction of a drug that is available systemically after oral administration is a mathematical product of the fraction of drug that escapes loss in each successive organ as represented by Equation 1.7, or rewritten in extraction ratios (Equation 1.8, Hall et al., 1999):

\[
F_{oral} = F_a \cdot F_G \cdot F_H
\]

\[
F_{oral} = F_a \times (1-E_G) \times (1-E_H)
\]

Where \( F_{oral} \) is the oral bioavailability, \( F_a \) is the fraction of an oral dose absorbed intact across the apical membrane of the epithelial layer, \( F_G \) and \( F_H \) are the fractions that escape elimination by the gut and liver respectively and \( E_G \) and \( E_H \) are the fractions that are extracted by the gut and liver respectively.

To include the intestinal interaction into DDI prediction equations, the \( F_G \) ratio is used to account for the ratio of intestinal availability in the absence \( (F_G) \) and presence \( (F_G') \) of an inhibitor (Rostami-Hodjegan and Tucker, 2004; Wang et al., 2004b). This intestinal availability can be included into the reversible DDI prediction model (Equation 1.9). The equation for incorporating the \( F_G \) ratio into irreversible inhibition is analogous to Equations 1.5 and 1.9.
Where \( F_G \) represents the fraction of drug escaping metabolism in the gut, in the absence or presence (denoted by ‘‘) of an inhibitor,

**1.3 Previous prediction of drug-drug interactions**

Many publications have assessed the predictions of DDIs using the static equations in varying complexity (Equations 1.3 – 1.9), and a two-fold prediction error margin is generally used as the acceptable level (Tucker et al., 2001b). Improvements in the prediction success have been reported with the increasing complexity of the equation and the majority of reversible interactions can be predicted within a 2-fold level from large databases of published DDI studies (Brown et al., 2006; Einolf, 2007; Fahmi et al., 2009; Obach et al., 2006). The increasing complexity of the equation has incorporated the use of *in vitro* data refined to include assessment of the inhibitor concentration utilised, the incorporation of the \( \text{fmCYP} \) and \( F_G \) ratio values, and multiple inhibitors and metabolic pathways (Brown et al., 2006; Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2007; Galetin and Houston, 2006; Galetin et al., 2005; Hinton et al., 2008; Ito et al., 2004; Ito et al., 2002; Ito et al., 2005; Kanamitsu et al., 2000; Obach et al., 2006). The prediction of irreversible inhibitors has been described (Galetin et al., 2006; Ito et al., 2004; Mayhew et al., 2000; Wang et al., 2004b; Yamano et al., 2001) but often have limited success due to the complex nature of the *in vitro* studies required and the large range of parameters, some of which are subject to *in vitro–in vivo* extrapolation (IVIVE) error. There has been an increasing interest in the prediction of induction interactions, considered in isolation (Shou et al., 2008) or in combination with inhibition interactions (Fahmi et al., 2009), and assessing the impact of intestinal induction (Galetin et al., 2010); however, this Thesis focuses on inhibition DDIs.

**1.4 The *in vitro* and *in vivo* estimation of parameters**

**1.4.1 Inhibitor concentrations**

The most accurate concentration of inhibitor for use in any prediction model would be the inhibitor concentration at the enzyme active site. This concentration cannot be experimentally determined *in vivo*, therefore calculations from experimentally measured
parameters are used (Blanchard et al., 2004; Brown et al., 2006; Einolf, 2007; Ito et al., 2004, Equations 1.10-1.12).

1. The systemic total maximal concentration of the inhibitor after repeated oral administration at the highest clinical dose

\[
[I]_{\text{max}} = \frac{[I]_{\text{av}} k \tau}{1 - e^{(-k \tau)}} \quad \text{Equation 1.10}
\]

Where \([I]_{\text{av}}\) is the average systemic plasma concentration after repeated oral administration, \(k\) is the elimination rate constant and \(\tau\) is the dosing interval.

2. The maximum hepatic inlet concentration after repeated oral administration

\[
[I]_{\text{in}} = [I]_{\text{av}} + \frac{k_a \cdot F_a \cdot D}{Q_H} \quad \text{Equation 1.11}
\]

Where \([I]_{\text{av}}\) is the average systemic plasma concentration after repeated oral administration, \(k_a\) is the absorption rate constant, \(F_a\) is the fraction absorbed, \(D\) is the dose and \(Q_H\) is the hepatic blood flow.

3. The average systemic concentration after repeated oral administration

\[
[I]_{\text{av}} = \frac{D}{\frac{\tau}{\text{CL} / F}} \quad \text{Equation 1.12}
\]

Where \(D\) is the dose, \(\tau\) is the dosing interval, \(\text{CL}\) is the clearance and \(F\) is the fraction of the dose that is systemically available.

All three of the above values can be multiplied by the fraction unbound in plasma (fu\(_{\text{p}}\)) to get the unbound variants of each \([I]\) concentration. The inhibitor concentration is considered as per the unbound drug hypothesis. This assumption may be confounded through the action of hepatic transporters increasing the concentration of unbound drug at the enzyme active site (Obach et al., 2007). In addition, circulating concentrations of active metabolites can impact on the inhibition effect, for example as observed with itraconazole (hydroxy-itraconazole, keto-itraconazole and \(N\)-desalkyl-itraconazole) (Isoherranen et al., 2004; Templeton et al., 2008). Early literature reports advocated use of the total concentration (Tran et al., 1997; von Moltke et al., 1994b), however current consensus is for use of the unbound concentration (Blanchard et al., 2004; Ervine et al., 1996; Grime and Riley, 2006; Sugita et al., 1981). There is additional discrepancy between the three variants of \textit{in vivo} inhibitor concentration (Equations 1.10-1.12) with reports advocating use of the unbound \([I]_{\text{in}}\) (Einolf, 2007; Obach et al., 2006; Obach et al., 2005) and unbound...
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[I]_{max} for irreversible interactions (Obach et al., 2007). In contrast, studies also report highest prediction accuracy from use of the total [I]_{in} (Brown et al., 2005; Ito et al., 2004; Ito et al., 2005) after refinement of the fmnCYP and k_{a} values. A further study by Brown et al., 2006 supported use of the total [I]_{av} after the in vitro K_{i} values were refined.

1.4.2 Methods for the estimation of intrinsic clearance

IVIVE is currently used in drug discovery and development, where the preliminary in vitro results are used to determine the priority of subsequent clinical in vivo studies. In vitro techniques have a wide range of advantages over in vivo methods, including reduction of cost, high throughput and reduced risk to human subjects (Bjornsson et al., 2003). The most commonly used in vitro systems for investigating the clearance and DDI potential of drugs are recombinant CYP enzymes, human liver microsomes (HLM) and human hepatocytes (Bjornsson et al., 2003; Brown et al., 2010; FDA, 2006; Houston and Galetin, 2008; Huang et al., 2007; Tucker et al., 2001b). Rat liver microsomes and hepatocytes have traditionally been used for the assessment of clearance, however human material is becoming more readily available and increasingly the preferential source despite an observed under-prediction trend (Brown et al., 2007b; Hallifax et al., 2010; Houston and Galetin, 2008). In addition, in vivo models such as transgenic mice expressing human CYP enzymes have been reported, but are not yet widely used (Cheung and Gonzalez, 2008). The use of in vitro systems from animal species allows consistency in genetic and environmental factors, and have been validated in a large range of studies (as described in Houston and Galetin, 2008). All systems allow the prediction of pharmacokinetic parameters that can be used to extrapolate to the prediction of in vivo clearance and DDIs. NADPH cofactors are required in microsomal incubations for the CYP enzymes to be active. In contrast, hepatocytes are structurally intact, containing the full complement of drug metabolising enzymes, cytosolic enzymes, uptake transporters and maintenance of the concentration gradients. In addition, the cells can be cryopreserved with minimal loss of enzyme activity, viability and structural integrity (Brown et al., 2007b; de Sousa et al., 1991; Li et al., 1999). For these reasons, hepatocytes are considered superior to the microsomes, although the use of microsomes remains high due to the convenience of their use (Brown et al., 2007b; Brown et al., 2010; Houston and Galetin, 2008; Lu et al., 2006).

The metabolism of a compound is determined over a wide range of substrate concentrations to calculate the enzyme kinetic parameters of biotransformation for reversible interactions. The parameters recorded include the K_{m} (substrate concentration at half maximal velocity reflecting the affinity of substrate for enzyme) and V_{max} (maximal
velocity). The determination of these parameters is from the metabolite formation; the estimation of intrinsic clearance is given by the relationship given in Equation 1.13 (assuming that the substrate concentration is lower than the $K_m$ (Houston and Galletin, 2003; Jones et al., 2004; Jones and Houston, 2004; Obach, 1999).

$$\text{CL}_{\text{int}} = \sum_{i=1}^{n} \left( \frac{V_{\text{max},i}}{K_{m,i}} \right) = \frac{0.693 \cdot V}{t_{1/2}}$$  \textbf{Equation 1.13}

Where $V_{\text{max}}$ represents the maximal velocity, $K_m$ is the substrate concentration at half maximal velocity for a specific metabolic pathway ($i$), $V$ is the incubation volume and $t_{1/2}$ is the half-life.

Intrinsic clearance values are corrected for the fraction unbound in the incubation ($f_{u_{\text{inc}}}$). This parameter can be experimentally determined or predicted based upon the lipophilicity (Gertz et al., 2008b; Hallifax and Houston, 2006; Kilford et al., 2008). Initially, no difference between the $f_{u_{\text{mic}}}$ and $f_{u_{\text{hep}}}$ was assumed, whereas this theory was later disputed (Kilford et al., 2008) and the determination of $f_{u_{\text{hep}}}$ proposed to be predicted from previously determined $f_{u_{\text{mic}}}$ values or directly from lipophilicity (further discussed in Chapter 3). The strategy taken for the scaling of 	extit{in vitro} clearance data exists to express the clearance in terms of total liver weight, including physiological processes such as binding to the blood and hepatic blood flow. The scaling of rat and human data has been well discussed, and the currently utilised values are given in Table 1.1 (Davies and Morris, 1993; Hakooz et al., 2006; Houston and Carlile, 1997; Houston and Galletin, 2008; Ito and Houston, 2004; Ito and Houston, 2005; Jones and Houston, 2004; Parker and Houston, 2008).

\textbf{Table 1.1} Scaling, blood flow and liver weight used for the scaling of rat and human clearance

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microsomal recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg protein/g liver)</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td><strong>Hepatocellularity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mcells/g liver)</td>
<td>120</td>
<td>99</td>
</tr>
<tr>
<td><strong>$Q_H$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mL/min/kg)</td>
<td>100</td>
<td>20.7</td>
</tr>
<tr>
<td><strong>Liver weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g liver/kg)</td>
<td>43.6</td>
<td>21.4</td>
</tr>
</tbody>
</table>
The unbound intrinsic clearance (CL\textsubscript{int,u}) can then be converted to hepatic clearance through the use of physiologically-based liver models, including the well-stirred, parallel tube and the dispersion model. All of the models assume perfusion rate limited distribution of drug into the liver, the free drug hypothesis where only unbound drug will be available to perfuse and that the distribution of enzymes in the liver is homogenous. Additionally, the well-stirred model assumes that the unbound concentration at the enzyme is equal to the unbound concentration in emergent venous blood (Houston and Carlile, 1997; Houston and Galetin, 2008; Ito and Houston, 2004; Pang and Rowland, 1977). Differences between the concentration gradient are accountable for the differences between the models, as defined in the relationships given in Equations 1.14-1.16.

Well-stirred model:

\begin{equation}
\text{CL}_H = \frac{Q_H \cdot f_{ub} \cdot \text{CL}_{int,u}}{Q_H + f_{ub} \cdot \text{CL}_{int,u}}
\end{equation}

Parallel tube model:

\begin{equation}
\text{CL}_H = Q_H \left[ 1 - e^{-\left(\frac{f_{ub} \cdot \text{CL}_{int,u}}{Q_H}\right)} \right]
\end{equation}

Dispersion model:

\begin{equation}
\text{CL}_H = Q_H \left[ 1 - \frac{4a}{(1 + a)^2 e^{[(a-1)/2Dn]} - \left(1 - a^2 \right)^{2/2Dn}} \right]
\end{equation}

Where:

\[ a = \sqrt{1 + 4Dn \left( \frac{f_{ub} \cdot \text{CL}_{int,u}}{Q_H} \right)} \]

Where CL\textsubscript{H} is hepatic blood clearance, Q\textsubscript{H} is hepatic blood flow, f\textsubscript{ub} is the fraction unbound in blood, CL\textsubscript{int,u} is the unbound intrinsic clearance and Dn is the dispersion number (0.17).

The well-stirred model is most commonly used as a result of its simplicity. A number of studies have assessed the use of the models (Bayliss et al., 1999; Cross and Bayliss, 2000; Hallifax et al., 2010; Houston and Galetin, 2008; Ito and Houston, 2004; Ito and Houston, 2005) and use of hepatocytes and microsomes (Brown et al., 2007a; Brown et al., 2007b; Ito and Houston, 2004; McGinnity et al., 2004; Riley et al., 2005; Stringer et al., 2008) in the prediction of clearance. Reports have suggested that the parallel tube model results in a reduction in prediction bias (Kilford et al., 2009; Stringer et al., 2008). A study assessing
the use of models in the prediction of \( \text{CL}_{\text{int}} \) using a large database (Hallifax et al., 2010) containing data from both human microsomes \((n=67)\) and hepatocytes \((n=89)\) (including the aforementioned studies) found no significant loss of prediction accuracy from the use of the well-stirred model, despite being the least physiological in nature. Comparison between the \( \text{CL}_{\text{int}} \) predicted from both hepatocytes and microsomes identified a trend described by least squares regression analysis (Hallifax et al., 2010); this topic will be further assessed in Chapter 3.

1.4.3 In vitro assessment of inhibitor potency

The potency of a reversible inhibitor is determined from the \( K_i \) value. This parameter is estimated from the co-incubations of a range of inhibitor and substrate concentrations, and is equal to the concentration of inhibitor that increases the apparent \( K_m \) value of the substrate by 2-fold. The simpler experiment to determine the concentration of inhibitor required to reduce the substrate \( V_{\text{max}} \) to 50\% (IC\(_{50}\)) are often utilised as an alternative to the \( K_i \). The IC\(_{50}\) value is equal to 2\( K_i \) when the substrate concentration is equal to the \( K_m \).

The catalytic activity of the specific CYP enzyme is determined over time in order to experimentally determine the potential of an inhibitor to cause mechanism-based interactions. A time-dependent decrease in metabolic clearance after multiple doses, where the compound shows dose proportional pharmacokinetic characteristics after the administration of single doses indicates mechanism-based inhibition (Wrighton et al., 2000), or the presence of a time dependent loss in the initial product formation rate (Tucker et al., 2001b). Kinetic parameters that are determined for prediction purposes include the \( k_{\text{inact}} \) (inactivation rate constant of the CYP by the substrate), the \( K_i \) (concentration of inhibitor at half of the inactivation rate constant) and the \( k_{\text{deg}} \) (degradation rate constant).

1.4.4 In vitro and in vivo estimation of \( f_{\text{mCYP}} \)

The fraction of drug that is metabolised can be estimated from subtraction of the amount of drug that can be recovered unchanged from renal excretion from the total drug amount. In the absence of further information into the relative contributions of each contributing CYP enzyme, it can be assumed that all clearance is mediated by a singular CYP enzyme and that no biliary excretion occurs (Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2005). Alternatively, regressional analysis can be used. This method documents a number of DDI studies involving the same victim drug and a range of inhibitors, and solves the static model equation for \( f_{\text{mCYP}} \) (Equation 1.9) using the \( [I]/K_i \) and AUC ratio for each DDI. This method usually excludes the incorporation of the gut metabolism. Another
method is to use phenocopying to estimate the \( fm_{\text{CYP}} \), comparing the amount of drug that is recovered unchanged in the presence and absence of potent and selective CYP inhibitors that have inhibited the metabolism of the victim drug (Ito et al., 2005; Obach et al., 2006; Obach et al., 2005). Variants of this method are presented in Shou et al., 2008 and Ohno et al., 2007. In Shou et al., 2008, the increase in AUC or decrease in clearance in the presence of a selective CYP3A4 inhibitor (e.g. ketoconazole) was used for an estimate of \( fm_{\text{CYP}} \) (Equation 1.17). The reported \( fm_{\text{CYP}} \) estimates in this publication for each victim drug were based upon single DDI studies.

\[
fm_{\text{CYP3A4}} = 1 - \frac{CL'}{CL} = 1 - \frac{AUC}{AUC'}
\]

Equation 1.17

Where CL is the clearance and AUC is the area under the concentration-time curve in the presence (denoted by ’) or absence of an inhibitor.

This method was also utilised in Ohno et al., 2007, with the inclusion of the \( IR_{\text{CYP}} \) (time-averaged apparent inhibition ratio); a parameter that includes the inhibitor concentration at the active site of the CYP enzyme and the inhibition constant (Equation 1.18). The estimations of the contribution of a CYP enzyme to the metabolism of a substrate (\( CR_{\text{CYP}} \); analogous to the \( fm_{\text{CYP}} \)) and \( IR_{\text{CYP}} \) in the study (Hisaka et al., 2009; Hisaka et al., 2010; Ohno et al., 2007) were not assessed from \textit{in vitro} data. The \( IR_{\text{CYP}} \) of itraconazole was found from the initial assessment of an itraconazole-simvastatin DDI, assuming that the \( CR_{\text{CYP}} \) of simvastatin was equal to unity. From the itraconazole \( IR_{\text{CYP}} \), the \( CR_{\text{CYP}} \) of midazolam was derived from an itraconazole-midazolam DDI. The remaining assessment of \( IR_{\text{CYP}} \) and \( CR_{\text{CYP}} \) values were found from the substitution of either midazolam or itraconazole and the AUC ratio into Equation 1.18. Therefore, any error introduced in the early stages of this method would impact on the subsequent estimations.

\[
CR_{\text{CYP}} = 1 - \frac{AUC}{AUC'} \cdot \frac{1}{IR_{\text{CYP}}}
\]

Equation 1.18

Where \( CR_{\text{CYP}} \) is the contribution of a CYP enzyme to oral clearance (analogous to \( fm_{\text{CYP}} \)), AUC is the area under the concentration-time curve in the presence (denoted by ’) or absence of an inhibitor and \( IR_{\text{CYP}} \) is the time-averaged apparent inhibition ratio (from \( [I_{\text{app}}]/(I_{\text{app}}+K_i) \)).

The phenocopying method often utilises AUC data following oral dosing, and therefore does not include the impact of differences in the gut metabolism in the absence or presence
of the inhibitor. Phenotype data can be used to estimate the $f_{\text{mCYP}}$, from the comparison of the AUC in poor metabolisers (PM) and extensive metabolisers (EM), where this data exists for CYP2D6, CYP2C19 and CYP2C9 subjects. The fraction that is not metabolised by the polymorphic enzyme can be deduced from the ratio of the EM to PM AUC, using Equation 1.17 with the substitution of $AUC_{PM}$ for $AUC'$. In the absence of other data, ranking of an AUC ratio from a DDI to an alternative DDI involving the same inhibitor and a victim drug with known $f_{\text{mCYP}}$ can be performed, and the $f_{\text{mCYP}}$ value adjusted accordingly. Values of felodipine, nisoldipine, simvastatin and lovastatin $f_{\text{mCYP}}$ were previously obtained using this method compared to the midazolam $f_{\text{mCYP}}$ (Brown et al., 2005).

### 1.4.5 Estimation of $F_G$

The $F_G$ can only be measured directly in vivo in anhepatic patients during liver transplant operations or in patients where the portal blood circulation bypasses the liver (Paine et al., 1996; Thummel et al., 1996). The $F_G$ is therefore measured indirectly from oral and intravenous (i.v.) data, assuming that after i.v. administration there is complete absorption and the clearance of the drug is solely due to hepatic elimination with negligible intestinal metabolism (Galetin et al., 2007; Hall et al., 1999). Estimates using this method have been reported (Galetin et al., 2006), although the utilisation of this method has been challenged (Galetin et al., 2008; Galetin et al., 2010; Lin et al., 1999) as a result of the assumptions made. These assumptions include the potential for intestinal metabolism after i.v. administration (Paine et al., 1996), inter-individual variability in drug dependent parameters including the $F_a$ and blood:plasma ratio and use of the average hepatic blood flow or exclusion of drug-dependent alterations to the $Q_H$, as observed in the case of calcium channel blockers (Galetin et al., 2010). The alternative method is to estimate the $F_G$ from in vivo studies in the presence and absence of grapefruit juice, where the active furanocoumarins selectively inhibit the CYP3A4 enzymes in the intestine (Bailey et al., 1998; Dresser and Bailey, 2003; Fuhr, 1998; Gertz et al., 2008a; Kirby and Unadkat, 2007; Saito et al., 2005). The selective inhibition results because the furanocoumarins do not reach sufficient levels in the liver to inhibit hepatic CYP enzymes (Saito et al., 2005; Yang et al., 2007b). Consistency was observed between the $F_G$ estimations from the two methods for those victim drugs subject to metabolism only; however some discrepancy was observed for those victim drugs additionally subject to transport, for example with cyclosporine. This difference results from the selective inhibition of the metabolic enzymes by grapefruit juice, with little or no impact on transporters (Galetin et al., 2010; Gertz et al., 2008a).
The $F_G$ ratio ($F_G'/F_G$) can be derived from three methods. The in vivo method uses i.v. and oral data utilised for the calculation of $F_G$ when it is also available in the presence of an inhibitor (Galetin et al., 2010; Galetin et al., 2007). This data is often not available, therefore other options are often utilised. The $F_G$ ratio can be estimated assuming maximal intestinal inhibition ($F_G'$ equal to 1), and the $F_G$ control as previously estimated, or by using in vitro data as described in Equations 1.19-1.21 for reversible inhibition (Obach et al., 2006; Rostami-Hodjegan and Tucker, 2004), or for irreversible inhibition using the corresponding in vitro parameters (defined in Equation 1.5) in Equation 1.20. Estimations of the $F_G$ ratio from the in vivo and maximal inhibition methods were in good agreement for those victim drugs subject to metabolism only, for example with midazolam. However, consistency was not observed for victim drugs subject to both metabolism and transport, for example for cyclosporine (Galetin et al., 2010; Galetin et al., 2007). The inclusion of the $F_G$ ratio into DDI prediction has resulted in mixed prediction success and only has a significant effect when the intestinal extraction of the victim drug is above 50%, regardless of the inhibitor potency (Brown et al., 2006; Einolf, 2007; Fahmi et al., 2008; Galetin et al., 2006; Galetin et al., 2010; Galetin et al., 2007; Obach et al., 2007; Obach et al., 2006; Wang et al., 2004b; Wang, 2010). The differences in success levels is likely to result from different methods of $F_G'/F_G$ estimation and confounded by differences in parameter values in DDI prediction methods utilised (e.g. $f_{m_{CYP}}$, $K_i$). Inclusion of the $F_G$ ratio into the prediction of time-dependent inhibition has been assessed (Galetin et al., 2006; Galetin et al., 2010) utilising the maximal $F_G$ inhibition approach. These studies reported that the inclusion of intestinal inhibition reduced the number of false negative predictions, however it also resulted in a trend for over-prediction, notably for studies involving simvastatin, buspirone and atorvastatin. Use of the predicted $F_G$ ratio has also been utilised in the prediction of reversible and time-dependent DDIs (Einolf, 2007; Obach et al., 2007). Inclusion of the $F_G$ ratio resulted in increased prediction accuracy; however some over-prediction was again noted for predictions involving buspirone.

\[
\frac{F_G'}{F_G} = \frac{1}{F_G + (1-F_G) \cdot \left( \frac{CL_{int,g}'}{CL_{int,g}} \right)}
\]

\[
\frac{CL_{int,g}'}{CL_{int,g}} = \frac{1}{1 + \frac{I_G}{K_i}}
\]

**Equation 1.19**

**Equation 1.20**
Where $F_G$ is the fraction escaping extraction in the gut, the $I_G$ is the inhibitor concentration during the absorption phase, where $D$ is the dose, $k_a$ is the absorption rate constant, $F_a$ is the fraction absorbed and $Q_{ent}$ is the enterocytic blood flow.

### 1.5 Simcyp population based ADME simulator

Physiologically based pharmacokinetic (PBPK) models allow the comprehensive and mechanistic prediction of the *in vivo* absorption, distribution, metabolism and excretion (ADME) properties of a drug entity (Aarons, 2005; Jones et al., 2011; Jones et al., 2009; Jones et al., 2006; Nestorov, 2007; Rowland et al., 2011). The Simcyp simulator is an example of a PBPK model, allowing prediction of both ADME properties and the extent of metabolic DDIs from *in vitro* data in a virtual human population. This software incorporates the impact of demographic factors including age, sex, renal function, physiological factors including blood flow and intestinal first-pass metabolism, genetic factors including the CYP polymorphisms, and pathological factors (Einolf, 2007; Rostami-Hodjegan and Tucker, 2007). As a result, a realistic prediction of the inter-individual variability in drug exposure in human populations can be simulated. In addition, patient populations that are at a specific risk of DDIs and are unlikely to be represented in a small clinical trial population can be identified (Polasek et al., 2009; Rostami-Hodjegan and Tucker, 2004; Tucker et al., 2001b). Simulations can be performed using either a static or dynamic approach. The static model is based on the use of single time-averaged inhibitor and substrate concentrations, analogous to the principles underlying the static model of DDI prediction shown in Equation 1.9, with inclusion of the $f_{mCYP}$ and $F_G$ parameters. The dynamic model incorporates the time-course of the concentrations and hence generates a temporal profile of the inhibition process (Fahmi et al., 2009; Rowland-Yeo et al., 2010; Wang, 2010). The additional ability to incorporate multiple inhibitors, mechanisms and inhibitory metabolites in the dynamic model allows a more comprehensive and mechanistic prediction (Jamei et al., 2009a; Rostami-Hodjegan and Tucker, 2007; Rowland-Yeo et al., 2010). The individual clearance and metabolic outcomes are predicted from these models using a Monte Carlo approach to generate virtual entities with randomly assigned characteristics (Rostami-Hodjegan and Tucker, 2004; Rostami-Hodjegan and Tucker, 2007).
The kinetics of both inhibitor and victim drugs in Simcyp are described in Rowland-Yeo et al., 2010 according to the model in Figure 1.3. This model has separate compartments for the liver and gut, and the rest of the body is lumped into a single systemic compartment. Concentrations in the portal vein (\(C_{PV}\)), liver (\(C_{\text{liver}}\)) and systemic compartment (\(C_{\text{sys}}\)) are described according to differential equations derived from Figure 1.3 (Equation 1.22-1.24). The time-variant parameters of the victim drug or inhibitor are the intrinsic metabolic clearance in the liver and gut. Metabolites are treated identically to Figure 1.3 in the simulations, with the exclusion of the gut lumen compartment, and modification of the differential equations to account for the gut metabolism of parent compound (Figure 1.3, Equation 1.25-1.27). The \(k_a\) and \(D\) parameters in the metabolite differential equations relate to the parent compound (Rowland-Yeo et al., 2010).
A. Physiologically based model utilised within Simcyp to describe the kinetics of a victim drug or inhibitor (A) or a primary metabolite of either an inhibitor or victim drug (B) after oral administration. The asterisks (*) represent where the models are linked when describing the kinetics of both parent and metabolite drug. Figure taken from Rowland-Yeo et al., 2010.

![Diagram of physiologically based model](image)

**Figure 1.3**

\[
\frac{dC_{PV}}{dt} = \frac{1}{V_{PV}} \left[ (C_{sys} - C_{PV}) \cdot Q_{PV} + f_a \cdot F_{G}(t) \cdot k_D \cdot e^{-k_D t} \right]
\]

**Equation 1.22**

\[
\frac{dC_{liver}}{dt} = \frac{1}{V_{liver}} \left[ Q_{PV} \cdot C_{PV} + Q_{HA} \cdot C_{sys} - (f_{uH} \cdot CL_u_{int}(t) + Q_{PV} + Q_{HA}) \cdot C_{liver} \right]
\]

**Equation 1.23**
Chapter 1 - Introduction

Equation 1.24

\[
\frac{dC_{\text{sys}}}{dt} = \frac{1}{V} \left[ (Q_{\text{PV}} + Q_{\text{HA}}) \cdot C_{\text{liver}} - \left( \frac{CL_{\text{R}}}{fu_{\text{R}}(M)} + Q_{\text{PV}} + Q_{\text{HA}} \right) \cdot C_{\text{sys}} \right]
\]

Where \( C \) is the concentration of drug, \( V \) the volume and \( Q \) the blood flow in the portal vein (PV), liver (liver) or systemic circulation (sys) or hepatic artery (HA).

Equation 1.25

\[
\frac{dC(M)_{\text{PV}}}{dt} = \frac{1}{V_{\text{PV}}} \left[ (C(M)_{\text{sys}} - C(M)_{\text{PV}}) \cdot Q_{\text{PV}} + fa \cdot (1 - F_G(I,t)) \cdot F_G(M,t) \cdot ka(I) \cdot D(I)e^{-ka \cdot t} \right]
\]

Equation 1.26

\[
\frac{dC(M)_{\text{liver}}}{dt} = \frac{1}{V_{\text{liver}}} \left[ Q_{\text{PV}} \cdot C(M)_{\text{PV}} + Q_{\text{HA}} \cdot C(M)_{\text{sys}} + fu_{\text{liver}}(I) \cdot CL_{\text{intestine}}(I,t) \cdot I_{\text{liver}} - \left( fu_{\text{R}}(M) \cdot CL_{\text{intestine}}(M,t) + Q_{\text{PV}} + Q_{\text{HA}} \right) \cdot C(M)_{\text{liver}} \right]
\]

Equation 1.27

\[
\frac{dC(M)_{\text{sys}}}{dt} = \frac{1}{V(M)} \left[ (Q_{\text{PV}} + Q_{\text{HA}}) \cdot C(M)_{\text{liver}} - \left( \frac{CL_{\text{R}}}{fu_{\text{R}}(M)} + Q_{\text{PV}} + Q_{\text{HA}} \right) \cdot C_{\text{sys}} \right]
\]

Where \( M \) and \( I \) relate to previously defined parameters of the metabolite and parent compound, respectively.

For orally administered drugs, parameters describing the rate \((k_a)\) and extent \((F_a)\) of absorption can either be input or predicted using one of 3 absorption models (Simcyp Simulator User Manual, 2008). These models vary in complexity and can incorporate the many factors relating to both the drug (formulation and physicochemical properties) and body physiology (gastric emptying and transit time, pH, blood flow, active uptake or excretion and the fed or fasted status of the subject) which can impact on the absorption characteristics of a drug in different scenarios. The simplest model is the first order absorption model, which considers the mass balance of a compound with time within a single gut compartment. The compartmental absorption and transit (CAT) model has increased complexity, by splitting the small intestine into seven discrete compartments (Yu et al., 1996), however both models only consider drug administered as solution. The advanced dissolution, absorption and metabolism (ADAM) model is based on the advanced compartmental absorption and transit (ACAT) model (Agoram et al., 2001). It considers nine compartments from the stomach to the colon and has the additional ability to study different solid formulations and the physicochemical properties related to formulation, including dissolution and particle size (Jamei et al., 2009b, Simcyp Simulator User Manual, 2008). In all 3 models absorption into the enterocyte occurs only when the drug is in solution and is predicted according to the permeability of the drug. The measure used is effective permeability \((P_{\text{eff}})\), relating to the permeability across the enterocyte via
passive transcellular diffusion. The in vitro prediction of $P_{eff}$ utilises apparent permeability ($P_{app}$) values determined using Caco-2 or MDCK cell lines or the parallel artificial membrane permeability assay (PAMPA), or Polar Surface Area and number of hydrogen bond donors according to the relationship given in Winiwarter et al., 2003 and Winiwarter et al., 1998. (Simcyp Simulator User Handbook, 2008; Gertz et al., 2010; Sun et al., 2002; Yang et al., 2007b).

The absorption models also allow prediction of $F_G$ values. In the ADAM model it is calculated intrinsically within the model whereas for the first-order and CAT models the $Q_{gut}$ model (Yang et al., 2007b) is required. The $Q_{gut}$ model (described further in Chapter 2) requires two input parameters: fraction unbound within the enterocyte ($f_{gut}$) and a hybrid parameter of intestinal blood flow and drug permeability ($Q_{gut}$). The $f_{gut}$ determines the concentration of drug that is free and therefore available to interact within the enterocyte. The value of $f_{gut}$ utilised has been previously considered; a value of unity is considered for victim drugs as the use of $f_{gut}$ equal to $f_p$ resulted in loss of prediction success (Gertz et al., 2010; Yang et al., 2007b). The $f_{gut}$ is assumed to be equal to $f_p$ for inhibitors in DDI prediction. The $Q_{gut}$ parameter can be input directly or may alternatively be predicted from $P_{eff}$ (Yang et al., 2007b).

Distribution of drugs can be described by the input of steady state volume of distribution ($V_{ss}$) values or by using either a reduced or full (14 organ) physiological model. Elimination is either based on the input of either in vivo oral or intravenous clearance, or predicted from in vitro intrinsic clearance measured in either recombinant CYP enzymes with corresponding Inter System Extrapolation Factors (ISEF) in order to correct for differences in the intrinsic activity relative to human liver (Proctor et al., 2004), or from HLM or human hepatocytes with corresponding values of $f_{inc}$ (Jamei et al., 2009a). Values of $f_{mCYP}$ are calculated intrinsically within Simcyp. This approach uses in vitro intrinsic clearance data for each enzyme to give the fractional contribution of the CYP enzyme to total hepatic intrinsic clearance, which is then corrected for renal clearance ($CL_R$) to give $f_{mCYP}$ (Simcyp Simulator User Manual, 2008). For inhibitors, in vitro values can be added to include the reversible inhibition, mechanism based inhibition and induction of CYP enzymes (Almond et al., 2009; Jamei et al., 2009a; Simcyp Simulator User Manual, 2008).
1.5.1 Previous DDI prediction using the Simcyp simulator

The equation-based static (Equation 1.9) and Simcyp dynamic model have been previously compared in a dataset of 100 DDIs including a range of inhibitors and victim drugs metabolized by a variety of the CYP enzymes (Einolf, 2007). Use of the Simcyp dynamic model resulted in the most accurate predictions (64% within 2-fold). However, parameter values of both the inhibitor and victim drugs (e.g., $f_{dp}$, $K_i$, $F_G$, $f_{mCYP}$) differed between the two models, as well as the choice of $[I]$ value used in the equation-based static model. In addition, the impact of the time course of reversible and time-dependent inhibition and induction has recently been assessed (Fahmi et al., 2009). This analysis utilised 50 clinical DDI studies involving midazolam as the victim drug. Simulations in Simcyp utilised matched demographic and dosing details to those reported in the clinical studies, and the default input parameters provided in the software where possible. The Simcyp predicted AUC ratios for positive DDIs (AUC ratio > 2-fold) were within 2-fold for 88-90% of the studies from the static and dynamic models, respectively. The majority of comparisons of DDI prediction between the static and dynamic model used the static model equation versus the Simcyp dynamic model (Einolf, 2007), or the static model in Simcyp compared to an alternative dynamic model (Perdaems et al., 2010) rather than both models within Simcyp. Therefore, the parameters utilised have not been consistent which confounds the direct comparison between the two models. In addition, some of the studies focus on only one inhibitor (Youdim et al., 2008; Zhao et al., 2009) or one victim drug (Fahmi et al., 2009; Hyland et al., 2008; Rakhit et al., 2008) and conclusions may therefore be specific to that drug without the ability to ascertain more general trends. The use of Simcyp in these latter studies varied, including assessment of the prediction of DDIs from the *in vitro* data of compounds in clinical development (Hyland et al., 2008; Rakhit et al., 2008), assessment of alternative sources of *in vitro* data utilised in the prediction of DDIs (Youdim et al., 2008) or investigation into the impact of the inhibitor dosing regimen in the prediction of DDIs (Zhao et al., 2009). Differences in the values of the input parameters between the models are likely to result in different AUC ratio predictions, considering the sensitivity of the model to certain parameter estimates, including $f_{mCYP}$ (Brown et al., 2006; Chien et al., 2006; Galetin et al., 2006) and $F_G$ (Galetin et al., 2006; Galetin et al., 2010; Galetin et al., 2007; Gertz et al., 2008; Obach et al., 2006; Wang et al., 2004), as previously discussed. Therefore, although the results are valid for a basic comparison of the models, the assessment of the impact of specific factors such as the inclusion of the time-course is confounded by the issues outlined above.
1.6 Mechanism and pharmacokinetics of selected CYP inhibitors

1.6.1 Azole drugs

The azoles are compounds containing five-membered nitrogen-containing heterocyclic rings and are used in anti-fungal therapy. They act through the inhibition of the fungal enzyme 14-α-sterol-demethylase (CYP51A1) (Georgopapadakou, 1998; Joseph-Horne and Hollomon, 1997). This enzyme is implicated in the biosynthetic pathway of ergosterol, which is an essential molecule of the fungal cell membrane. Inhibition of this enzyme leads to accumulation of 14-α-methylsterols on the fungal surface, which results in arrest of fungal growth (Itokawa et al., 2007). This inhibition of CYP51A1 by the azole drugs is non-specific, and the implication of the azole drugs in DDIs results from inhibition of the CYP enzymes responsible for drug metabolism.

1.6.1.1 Fluconazole

Fluconazole is a triazole used for a wide variety of fungal infections, including those caused by Candida, Cryptococcal and Coccidioidal species (Brammer et al., 1990). The administration is dependent on the severity and cause of infection, ranging from single 100mg doses to chronic administration of 400mg/day dosing (British National Formulary 60, 2010). Fluconazole is completely absorbed from the gastrointestinal tract, resulting in the same plasma concentrations after oral and i.v. dosing (Brammer et al., 1990) and the absorption is not affected by food or gastric pH. Approximately 90% of fluconazole is unbound in blood plasma. Renal excretion accounts for 90% of the elimination, with the remaining 10% attributed to a minor CYP2C9 metabolic pathway (Thummel et al., 2008). Following oral administration, the half-life of fluconazole is approximately 30 hours. Fluconazole inhibits a range of CYP enzymes, including CYP2C9 and CYP2C19 (K_i < 10μM) and weaker inhibition of CYP3A4 (Brown et al., 2006; Obach et al., 2006). DDIs have been reported with a range of victim drugs across these CYP enzymes. The structure is shown in Figure 1.3.

![Structure of fluconazole](image)

**Figure 1.4 Structure of fluconazole**
1.6.1.2 Itraconazole

Itraconazole is also a triazole antifungal, and is the treatment of choice for infections caused by *Histoplasma* and *Coccidioidal* species (Zonios and Bennett, 2008). The bioavailability of itraconazole is dependent on the formulation; greatest absorption is observed in the fed state for the capsule formulation and the fasted state for the oral solution. Itraconazole is highly bound to plasma proteins (>99%) (Thummel et al., 2008). It is a substrate for and potent inhibitor of CYP3A4 ($K_i < 0.01 \mu M$), and the main metabolite (hydroxy-itraconazole) also inhibits CYP3A4 ($K_i < 0.1 \mu M$). Additional metabolites (keto-itraconazole and N-desalkyl-itraconazole) have also been characterised, and have inhibitory potential resulting from inhibition of CYP3A4 and similar circulating concentrations to the hydroxy-itraconazole metabolite. The structure of itraconazole and the metabolites is given in Figure 1.4. Numerous DDIs have been reported involving itraconazole with CYP3A4 victim drugs (Isoherranen et al., 2004). Renal excretion of the parent and metabolite are both minimal (<1%), and the half-life is 30-40 hours, regardless of formulation (Thummel et al., 2008).

![Figure 1.5 Structure of itraconazole. The circled group is sequentially altered to the inhibitory metabolites: CHCH$_3$CHOHCH$_3$ (hydroxy-itraconazole), CHCH$_3$COCH$_3$ (keto-itraconazole) and H (N-desalkyl-itraconazole).](image)

1.6.1.3 Ketoconazole

Ketoconazole is an imidazole antifungal, and is primarily used orally or topically for infections resulting from *Candida*, *Coccidioidal* and Dermatophytes with a typical daily dose of 200-400mg (Galichet, 2006). Absorption is incomplete after oral administration but increases with decreasing pH. Ketoconazole is highly bound to plasma proteins (>99%) and is a substrate for, and inhibitor of CYP3A4 ($K_i < 0.1 \mu M$) (Thummel et al., 2008). In addition, DDIs have been reported between ketoconazole and victim drugs of CYP2C9 and CYP2C19, with AUC ratios < 2. Renal excretion is minimal, and the half-life is approximately 6-10 hours (Thummel et al., 2008). The structure of ketoconazole is displayed in Figure 1.5.
1.6.2 Selective Serotonin Reuptake Inhibitor (SSRI) drugs

SSRIs are the treatment of choice for a plethora of psychological disorders, including depression, obsessive-compulsive disorders, panic disorder, alcoholism and chronic pain syndromes (Sweetman, 2009). The mechanism of action is through the inhibition of the uptake of serotonin at synapses within the median raphe nucleus, thereby increasing brain levels of serotonin (Stahl, 1998). The mechanism by which the increased serotonin level modulates mood is unclear. The inhibition of CYP enzymes by the SSRIs is not related to the mechanism of action.

1.6.2.1 Fluoxetine

Fluoxetine is well absorbed from the gastro-intestinal tract, and is highly bound to plasma proteins (>90%) (Thummel et al., 2008). It is extensively metabolized by, and is a potent inhibitor of CYP2D6 ($K_i < 0.1 \mu M$), with the active inhibitory metabolite, norfluoxetine also well characterized ($K_i < 1 \mu M$) (Alfaro et al., 2000; Galichet, 2006). Less pronounced inhibition is reported for CYP2C9 and CYP2C19 ($K_i < 10$ and $< 0.1 \mu M$, respectively). In addition, fluoxetine is reported to cause the mechanism based inhibition of CYP3A4 ($K_i < 1 \mu M$, $k_{inact} < 0.001 h^{-1}$). Despite this inhibition, the AUC ratios for DDIs involving CYP3A4 victim drugs are mostly < 2 (Fahmi et al., 2009; Obach et al., 2006; Venkatakrishnan et al., 2001). The structure of fluoxetine and norfluoxetine is given in Figure 1.6. Renal excretion of the parent compound is low, and the half-lives of both parent and metabolite are long, at 9 and 16 days, respectively (Galichet, 2006; Thummel et al., 2008).

Figure 1.7 Structure of fluoxetine. The circled group is N-demethylated to produce the inhibitory metabolite, norfluoxetine.
1.6.2.2 Fluvoxamine

The bioavailability of fluvoxamine after oral administration is moderate (0.5), and has lower protein binding in comparison to fluoxetine (80%) (Galichet, 2006). Metabolism of fluvoxamine is mediated via CYP2D6 (Carrillo et al., 1996), and no active metabolites have been characterized (Isoherranen et al., 2009). The structure is displayed in Figure 1.7. Fluvoxamine is a potent inhibitor of CYP1A2 and CYP2C19 ($K_i < 0.1\mu M$), and a weaker inhibitor of CYP2C9, CYP2D6 and CYP3A4 ($K_i > 1\mu M$) (Brown et al., 2006; Obach et al., 2006). This inhibition is reflected in the reported DDIs, where most involving the latter three CYP enzymes have AUC ratios < 2. Renal excretion of unchanged fluvoxamine is low (<2%), and it has a long half-life of approximately 15 hours (Galichet, 2006; Thummel et al., 2008).

![Structure of fluvoxamine]

Figure 1.8 Structure of fluvoxamine
AIMS

The prediction of DDIs and the assessment of the contribution of individual parameters to the prediction have been subject to increased interest (Brown et al., 2006; Brown et al., 2005; Einolf, 2007; Fahmi et al., 2009; Galetin et al., 2008; Galetin et al., 2010; Hinton et al., 2008; Isoherranen et al., 2009; Ito et al., 2004; Ito et al., 2005; Obach et al., 2005; Rowland-Yeo et al., 2010). Although some assessment of the impact of the time-varying concentration of inhibitor within DDI prediction models has been performed, the majority of comparisons of DDI prediction between the static and dynamic model used different scenarios (Einolf, 2007; Perdaems et al., 2010). The parameters utilised were not consistent which confounds the comparison. Additionally, conclusions may be specific to DDIs involving a singular inhibitor or victim drug and the ability to ascertain more general trends is yet to be fully explored.

This Thesis aims to assess the impact of individual parameters in the prediction of DDI, using the Simcyp population-based ADME simulator. The analysis will focus on five CYP inhibitors (three azole anti-fungals and two SSRI anti-depressants) and a range of victim drugs with different properties, in order to ascertain trends in prediction as a result of these parameters. Different in vivo methods of estimation of victim drug properties, fm_{CYP} and F_{G} will be compared to the in vitro methods utilised within Simcyp. Justification of values to be used for each inhibitor parameter will be given.

There is a lack of clear evidence for hepatic uptake of azole and SSRI inhibitors (Brown et al., 2007a). Therefore different in vitro systems to investigate the potential liver uptake of inhibitors will be investigated; namely microsomes and hepatocytes, isolated from both rats and humans. These experiments will consist of:

- Comparison of intrinsic clearance estimated from the depletion of the inhibitor in rat microsomes and hepatocytes
- Comparison of intrinsic clearance estimated using the depletion of the inhibitor from the full incubation in comparison to depletion from the media only, using rat hepatocytes
- Repetition of the above experiments using human microsomes and hepatocytes

The static and dynamic models will be compared using Simcyp with respect to their ability to successfully predict DDIs. This analysis will use the same or corresponding parameters in order to allow a valid assessment of the inclusion of the time-profile for inhibition in the
dynamic model. The dynamic model will also allow investigation into the impact of active metabolites in the DDI prediction (where relevant), and differences in the dosing time of inhibitor and victim drug. The prediction of inter-individual variability will also be assessed using a number of clinical DDI studies reporting data from the individual subjects. Further investigation of these parameters will be considered using individual DDI data from different genotype groups. The parameters will be assessed using DDIs with a range of potencies and involving victim drugs metabolised by the five main CYP enzymes.

Finally, different methods to assess DDI prediction success will be compared. Databases of DDIs collated in this Thesis and in previous publications will be used in order to assess the impact of alternative methodology. The use of *in vitro* intrinsic clearance of the inhibitors to potentially refine the DDI predictions will additionally be investigated.
CHAPTER 2: SELECTION AND JUSTIFICATION OF INHIBITOR AND VICTIM DRUG PARAMETERS
2 SELECTION AND JUSTIFICATION OF INHIBITOR AND VICTIM DRUG PARAMETERS

2.1 INTRODUCTION

The correct selection of individual \textit{in vitro} inhibitor and victim drug parameters has a profound impact on the prediction of DDIs. This impact has been demonstrated in many cases, involving both inhibitor properties including the $K_i$ and concentration of inhibitor utilised (Brown et al., 2006; Ito et al., 2004), and victim drug properties including the $f_{mCYP}$ and $F_G$ (Brown et al., 2005; Galetin et al., 2008; Galetin et al., 2007; Ito et al., 2005). The prediction of DDIs in the Simcyp simulator uses \textit{in vitro} parameters of both the inhibitor and victim drug in conjunction with virtual population data, as described in Section 1.5. Values of the inhibitor parameters including $K_i$ are input into the model, whereas the victim drug properties, $f_{mCYP}$ and $F_G$ are calculated intrinsically within Simcyp from the recombinant \textit{in vitro} clearance data and the use of gut models (first order or ADAM models), respectively. The first order model utilises the $Q_{gut}$ model in the prediction of $F_G$, using Equations 2.1 and 2.2 (Chalasani et al., 2002; Rostami-Hodjegan and Tucker, 2004; Yang et al., 2007b). The intestinal intrinsic clearance is calculated and subsequently utilised within the model; this parameter is derived from correction of the unbound intrinsic clearance by the CYP enzyme content in the gut.

\begin{equation}
F_G = \frac{Q_{gut}}{Q_{gut} + f_{gut} \cdot CL_{int,g}} \tag{2.1}
\end{equation}

\begin{equation}
Q_{gut} = \frac{CL_{perm} \cdot Q_{ent}}{Q_{ent} + CL_{perm}} \tag{2.2}
\end{equation}

Where $F_G$ is the fraction escaping metabolism in the gut, $Q_{gut}$ is a hybrid parameter of enterocytic blood flow and drug permeability (Equation 2.2), $f_{gut}$ is the fraction unbound in the gut, $CL_{int,g}$ is the intestinal intrinsic clearance, $CL_{perm}$ is the permeability clearance (from the intestinal surface area and permeability) and $Q_{ent}$ is the enterocytic blood flow.

The reported values of many parameters have a wide range as a result of inter-individual, inter-laboratory and inter-method variability. Inter-method variability can be rationalised, for example differences that may result from the use of different \textit{in vitro} systems such as hepatocytes and microsomes (Brown et al., 2007a; Brown et al., 2007b; Ito and Houston, 2004; Riley et al., 2005; Stringer et al., 2008), or different methods of data analysis such as the half-life and AUC methods of clearance estimation (e.g. Houston and Galetin, 2008;...
Soars et al., 2007). However, the variation of some parameters is reported despite consistency in the reported methods. For example, a study of published values of ketoconazole $K_i$ displayed a range of $0.001 - 25 \mu$M. The range was reduced to 50-fold when consistency in metabolite and probe substrate were considered, of which 20% of the variation could be attributed to differences in the incubation, duration and microsomal protein concentration utilised in the experimental procedures (Greenblatt et al., 2010).

DDI studies reported in the literature involve a large number of CYP inhibitors and victim drugs, with studies ranging from small scale investigatory trials involving a single DDI (e.g. Culm-Merdek et al., 2005), to the investigation of a number of inhibitors or victim drugs (e.g. Lam et al., 2003). A number of studies have previously collated databases of DDIs in order to predict the impact of different parameters (including, and not limited to Brown et al., 2005; Einolf, 2007; Fahmi et al., 2009; Galetin et al., 2006; Galetin et al., 2005; Ito et al., 2004; Obach et al., 2006; Wang, 2010). This analysis can be limited to a number of inhibitors or victim drugs (e.g. Fahmi et al., 2008) or assessed by the mechanism, either inhibition (e.g. Einolf, 2007) or induction (e.g. Shou et al., 2008). In the current study, a database was collated involving five CYP enzyme inhibitors; three azole anti-fungal drugs (fluconazole, itraconazole and ketoconazole) and two SSRIs (fluoxetine and fluvoxamine) were selected. The inhibitors displayed a range of inhibitory potency to a number of different CYP enzymes and therefore included representative numbers of different victim drugs, with a wide range in fm$_{CYP}$ values (Section 1.6). Further variability resulted from inhibitors that inhibited and were metabolised by the same CYP enzyme (e.g. itraconazole) and those inhibitors that inhibited and were metabolised by different CYP enzymes (e.g. fluvoxamine). Two of the selected inhibitors (fluoxetine and itraconazole) also had circulatory inhibitory metabolites, reported to increase the extent of DDIs (Aronoff et al., 1984; Bergstrom et al., 1992; Isoherranen et al., 2009; Isoherranen et al., 2004; Quinney et al., 2008; Templeton et al., 2008; Yoo et al., 2000). This effect is reported despite the lower inhibition potency of the metabolite and the fact that the same CYP enzyme is inhibited by both parent and metabolite drug in both cases (Hinton et al., 2008; Ogilvie et al., 2006; Rostami-Hodjegan and Tucker, 2004; Yeung et al., 2010).

2.1.1 Aims

The aims of this chapter were to assess the impact of different inhibitor and victim drug-related parameters on the prediction of DDI. The analysis focused on using in vitro values in the Simcyp simulator to assess each parameter. This preliminary work was performed in
order to assess the impact of using Simcyp default parameters as the victim drug parameters in the prediction model and to select and justify the source of inhibitor parameters to be utilised in DDI prediction work in further chapters. The victim drug parameters, \( \text{fm}_{\text{CYP}} \) and \( F_G \) cannot be input directly into the simulations. Therefore, this work aimed to highlight potential sources of error resulting from differences in the output values of these models compared to alternative methods of estimation. A DDI database was collated from published sources focusing on three azoles (fluconazole, itraconazole and ketoconazole) and two SSRIs (fluoxetine and fluvoxamine). These CYP inhibitors encompassed a range of properties and a range of victim drugs metabolised by the five main CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4). Sufficient DDI studies were available involving each inhibitor and victim drug and detailed information available in the clinical studies concerning the dosage regimen and demographical data in order to ascertain trends in DDI prediction.
2.2 METHODS

2.2.1 Victim drug properties

The importance of \( f_{mCYP} \) in the prediction of DDIs was highlighted in Brown et al., (2005). Prediction accuracy increased from 54 to 84% of studies within 2-fold of the actual AUC ratio after \( f_{mCYP} \) inclusion into DDI predictions. The \( f_{mCYP} \) values used in Brown et al., 2005 (and updated databases in Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008) were calculated from regression analysis from AUC ratios, recovery of unchanged drug, ranking analysis or phenotyping data (PM/EM) for polymorphic enzymes. Other in vivo methods of \( f_{mCYP3A4} \) estimation are described in Shou et al., 2008 (Equation 2.3) and Ohno et al., 2007 (Equation 2.4). These methods are based on the change in AUC or clearance in the presence of a selective and potent CYP inhibitor (e.g. ketoconazole or itraconazole). All methods of \( f_{mCYP} \) estimation were fully described in Section 1.4.4.

\[
f_{mCYP3A4} = 1 - \frac{AUC}{AUC'}
\]

Equation 2.3

\[
CR_{CYP} = 1 - \frac{AUC}{AUC'} \cdot \frac{1}{IR_{CYP}}
\]

Equation 2.4

Where \( CR_{CYP} \) is the contribution of a CYP enzyme to oral clearance (analogous to \( f_{mCYP} \)), AUC is the area under the concentration-time curve in the presence (denoted by ') or absence of an inhibitor and \( IR_{CYP} \) is the time-averaged apparent inhibition ratio.

The methods outlined in Shou et al., 2008 and Ohno et al., 2007 were investigated further in order to assess the impact of different inhibitors and dosing schedules in the AUC ratios used for estimation of \( f_{mCYP3A4} \) for midazolam. DDI studies were collated involving eight different inhibitors and the \( f_{mCYP3A4} \) calculated using Equation 2.3 and 2.4, using the \( IR_{CYP} \) values for each inhibitor reported in Ohno et al., 2007.

Additionally, \( f_{mCYP} \) can be calculated from in vitro data using Simcyp. This method is based on recombinant in vitro clearance data to give the fractional contribution of the CYP enzyme to total hepatic intrinsic clearance, which is then corrected for renal clearance to give \( f_{mCYP} \) (Simcyp Simulator User Manual, 2008). Simulations of 100 healthy subjects (10 trials containing 10 subjects each) at standard population demographics (age, weight, enzyme abundance) were used to determine the \( f_{mCYP} \). The inclusion criteria for comparison of \( f_{mCYP} \) estimates was the availability of victim drug data in the Simcyp library or where the \( f_{mCYP} \) was reported in more than one of the in vivo methods. Average in vitro estimates of \( f_{mCYP3A} \) (\( f_{mCYP3A4} + f_{mCYP3A5} \)) in Simcyp were used for consistency.
with the *in vivo* estimates, as inhibitors do not always distinguish between the two enzymes (Allqvist et al., 2007; Wang et al., 2005).

The $F_G$ can also be calculated via a number of *in vivo* methods. These methods were fully described in Section 1.4.5, and include calculation from studies reporting the i.v. and oral dosing data of the victim drug and oral dosing in the presence and absence of grapefruit juice in order to inhibit intestinal CYP3A4 (Galetin et al., 2008; Galetin et al., 2010; Galetin et al., 2007; Gertz et al., 2008a; Hall et al., 1999; Wang et al., 2004b). The former method is based upon the assumption that negligible metabolism occurs in enterocytes following i.v. administration of a drug, and latter that grapefruit juice selectively inhibits intestinal CYP3A-mediated metabolism, with no effect on hepatic metabolism (Bailey et al., 1998; Galetin et al., 2008; Galetin et al., 2010; Saito et al., 2005). The assessment utilised values of $F_G$ from the *in vivo* methods taken from Galetin et al., 2010 and Galetin et al., 2008. Models to predict the $F_G$ from *in vitro* data are incorporated into Simcyp, including the first order absorption model (based on the principles of the $Q_{gut}$ model), and the mechanistic ADAM model that allows the varying distribution of enzymes to be incorporated into $F_G$ estimations (Simcyp Simulator User Manual, 2008). The predicted $F_G$ values from the ADAM model were compared to the alternative *in vitro* and *in vivo* methods, despite the inability of Simcyp to currently implement this model in the prediction of DDIs. Simulations of 100 healthy subjects (10 trials containing 10 subjects each) at standard population demographics (age, weight, enzyme abundance) using the first order and ADAM absorption models were carried out for all victim drugs that were available in the Simcyp library and were reported from at least one of the *in vivo* methods. When using the ADAM model, the drug was assumed to be in solution and $P_{eff}$ was predicted from the default settings within Simcyp (polar surface area - alprazolam, sildenafil, simvastatin and triazolam; value input – cyclosporine; Caco-2 system – midazolam, quinidine and saquinavir; MDCK system – nifedipine and zolpidem). $P_{eff}$ was assumed to be constant through the intestinal compartments. The default CYP3A4 expression across the intestine was based on reports detailed in Jamei et al., 2009b and Paine et al., 2006 with 13.8, 54.6 and 31.6% of the total CYP3A total in the duodenum, jejunum and ileum, respectively. Default parameters were also utilised within the ADAM model for the transit time (4.6, 17.3 and 15.2% of the transit time in the duodenum, jejunum and ileum, respectively), pH across the intestine (increasing from 6.4 to 7.3 from the duodenum to ileum), the dynamic fluid volume within the gut, intestinal blood flow (with 8.8, 48.4 and 42.8% of the total villi blood flow in the duodenum, jejunum and ileum, respectively) and the fasted state of the subjects.
The prediction of victim drug profiles was also assessed for a subset of victim drugs. This analysis focused on CYP3A4 substrates (midazolam (5mg), triazolam (0.25mg), cyclosporine (2mg) and simvastatin (40mg)), although representative victim drugs were also assessed for CYP1A2 (caffeine, 100mg), CYP2C19 (omeprazole, 40mg) and CYP2D6 (tolterodine, 2mg). Simulations utilised single doses of victim drugs in 100 healthy subjects (10 trials containing 10 subjects each) at standard population demographics (age, weight, enzyme abundance) in the Simcyp simulator. The profile was assessed from determination of the $T_{\text{max}}$, $C_{\text{max}}$ and AUC in comparison to reported values (Friedman et al., 1988; Greenblatt et al., 2009; Houston and Galetin, 2008; Thummel et al., 2008) and DDI studies (in the absence of inhibitor) (Brynne et al., 1999; Christensen et al., 2002; Gomez et al., 1995; Neuvonen et al., 1996; Yasui-Furukori et al., 2004).

### 2.2.2 Sensitivity analysis of inhibitor properties

Sensitivity analysis was carried out using the Simcyp dynamic model in order to quantitatively assess the uncertainty associated with selected properties of the inhibitor. The DDI between itraconazole (200mg) and midazolam (5mg) was selected for this analysis; itraconazole is a potent, selective CYP3A4 inhibitor with a number of reported DDIs involving midazolam as the victim drug (Ahonen et al., 1995; Backman et al., 1998; Olkkola et al., 1996; Olkkola et al., 1994; Templeton et al., 2010). This analysis simulated variations in the input parameters of itraconazole and measured the impact on the AUC of midazolam in the presence and absence of itraconazole. Parameters investigated were $K_i$, $f_{up}$, $f_{gut}$, liver uptake, $F_a$ and $k_a$; these parameters were selected from investigation into the impact of different available values in the literature. Simulations utilised 100 subjects at the standard population demographics (including age, weight and enzyme abundance) with the sensitivity analysis function in Simcyp. For fractional values ($f_{up}$, $f_{gut}$, $F_a$), the full range (up to 1) was investigated, and a 10-fold range from the default values for liver uptake (0.1-10) and $k_a$ (0.06-6h$^{-1}$) was selected. The 10-fold range assessed for $K_i$ was not sufficient to describe the impact of this parameter; a 1000-fold range was therefore assessed (0.0001-0.1μM). This parameter was further assessed using the same dosing schedule but involving ketoconazole (200mg) as the inhibitor. The impact of the 50-fold range of ketoconazole $K_i$ (0.004μM and 0.18μM) reported in Greenblatt et al., 2010 on the midazolam AUC ratio was investigated to assess if the differences observed are consistent across inhibitors. Based upon this information, decisions were made regarding the selection of inhibitor parameter values for use in DDI predictions.
The prediction of inhibitor profiles in Simcyp was additionally assessed for ketoconazole, itraconazole, fluoxetine and fluvoxamine. Simulations were matched to representative DDI studies reporting inhibitor profile data, including matched dose, dosing schedules and patient demographics. The profile was assessed from determination of the $T_{\text{max}}$, $C_{\text{max}}$ and AUC in comparison to reported values (Brynne et al., 1999; Christensen et al., 2002; Greenblatt et al., 1998; Neuvonen et al., 1996).

2.2.3 Compilation of in vivo drug-drug interaction database

Five CYP inhibitors were selected for study into the impact of different properties in the prediction of DDIs. These inhibitors were fluconazole, itraconazole and ketoconazole (azoles) and fluoxetine and fluvoxamine (SSRIs). The database was compiled from a literature search involving the inhibitors listed and any victim drug currently included within the Simcyp library. The differing inhibitory potencies of the inhibitors to a range of CYP enzymes resulted in inclusion of a range of victim drugs with varying extents of victim drug $f_{\text{m,CYP}}$ per enzyme. The criterion for the inclusion of the study was the oral administration of both substrate and inhibitor. The dosing schedule and subject data (number, age and sex of subjects) was recorded, along with the ratio of the AUC in the presence of inhibitor compared to the victim drug alone. The AUC ratio was used to assess the extent of DDI, classified according to the fold change in the AUC of the victim drug as induction ($AUC$ ratio < 1), no interaction (1 to 1.25-fold increase in AUC), and weak (1.25 to 2-fold increase in AUC), moderate (2 to 5-fold increase in AUC) or strong inhibitory DDIs ($AUC$ ratio > 5), analogous to the FDA guidelines for the assessment of potential inhibitors of CYP3A4 (Huang et al., 2007). The AUC ratio used for comparison from each in vivo study was calculated from $AUC_{(0,\infty)}$. The only exception was the study between ketoconazole and itraconazole with triazolam (Varhe et al., 1994), where the AUC ratio was calculated from the last time point in the study ($AUC_{(0,t)}$). Studies involving single dosing and steady state inhibitor concentrations were both collated. Whenever available the mean $AUC_{(0,\infty)}$ ratio for each study was calculated from values reported for the individual subjects.

2.2.4 Data analysis

Predicted values of $f_{\text{m,CYP}}$ and $F_G$ from the different in vitro and in vivo methods were compared in order to assess any outliers or potential sources of error in further Simcyp simulations as a result of the use of in vitro data. Sensitivity analysis was used in order to highlight the importance of the correct selection of a number of the inhibitor parameters. Values were selected in order to reduce error in parameter estimates for use in DDI
predictions; consideration of these values was investigated in further chapters. The database was assessed per inhibitor and per victim drug after classification into the potency of DDI and $f_{mCYP}$ of victim drug involved in order to identify any trends. In addition, the potency of DDIs was compared to the physicochemical properties of the inhibitor, namely the LogP value. This value was taken from experimental data reported in ChemID Plus Advanced (http://chem.sis.nlm.nih.gov/chemidplus) for ketoconazole, itraconazole, fluoxetine and fluvoxamine or the Physical Properties Database (PhysProp) (http://www.syrres.com/esc/physdemo.htm) for fluconazole.
2.3 RESULTS

2.3.1 Comparison of simulated and in vivo victim drug data

2.3.1.1 Fraction metabolised by a CYP enzyme (fm\textsubscript{CYP})

The minimum and maximum fm\textsubscript{CYP} values for the 12 CYP3A4 victim drugs were 0.60 and 0.99 for zolpidem and buspirone, respectively for the estimates from recovery unchanged. The range of fm\textsubscript{CYP3A4} from regression analysis was 0.71 for cyclosporine and nifedipine to 0.94 for midazolam. The only fm\textsubscript{CYP3A4} that was estimated from rank analysis was simvastatin, at 0.99 (Figure 2.1, Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008; Ito et al., 2004). The in vivo method with the greatest range (0.26-0.94 for zolpidem and buspirone, respectively) was reported from the observed reduction of clearance or increase in AUC resulting from the use of selective and potent CYP3A4 inhibitors (Shou et al., 2008). An intermediate range of fm\textsubscript{CYP} values was estimated from the final method of estimation from in vivo data from the AUC ratio and IR\textsubscript{CYP} (Ohno et al., 2007, 0.40-1.00 for zolpidem and simvastatin, respectively), and estimations utilising in vitro data in Simcyp (0.38-1.00 for zolpidem and nifedipine, respectively, Figure 2.1). The overall minimum fm\textsubscript{CYP} estimate was observed for zolpidem, regardless of the method of estimation, whereas the overall maximum fm\textsubscript{CYP} value was variable between the methods. Consistency was observed between the in vivo estimations of buspirone fm\textsubscript{CYP} (>0.9), however Simcyp did not include this victim drug in the library. Of the remaining estimates with victim drug fm\textsubscript{CYP} > 0.95, 4/6 were using in vitro data within Simcyp and the remaining two estimations from recovery unchanged (saquinavir, Houston and Galetin, 2008) or from the AUC and IR\textsubscript{CYP} (simvastatin, Ohno et al., 2007). Despite this trend, no consistency was observed between the maximum fm\textsubscript{CYP} estimations observed per victim drug to one particular method of estimation. Lowest estimations of fm\textsubscript{CYP} per victim drug were observed when determined in the presence of selective inhibitors (Shou et al., 2008) for 10/12 victim drugs, with no consistency in the methods for the remaining 2/12 victim drugs (cyclosporine and simvastatin). In addition, no relationship was observed between the CYP3A4 inhibitor utilised in the estimation (reported in Shou et al., 2008) and the alternative in vivoin vitro methods. Overall, the results from the four methods were largely consistent with estimations having a < 20% difference for 7/12 victim drugs (differences were < 10% for 4/12). The coefficient of variations (CV) ranged from 2% (triazolam) to 34% (zolpidem). The biggest outliers resulted from the in vitro estimations of nifedipine and cyclosporine with differences at least 20% different to the three alternative estimates from in vivo data. No consistency was observed between those fm\textsubscript{CYP} values estimated from HLM and from recombinant in vitro
data within the Simcyp simulator. Values of \( fm_{CYP} \) cannot currently be input into Simcyp and the \textit{in vitro} values were therefore utilised in further DDI predictions; these values were largely consistent with estimates from \textit{in vivo} data and should therefore not impact on DDI predictions.

\textbf{Figure 2.1} Comparison of \( fm_{CYP3A4} \) estimates for 12 victim drugs. Estimates were from regression analysis, recovery of unchanged drug or ranking analysis (Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008; Ito et al., 2004; •), from the change in clearance or AUC in the presence of a potent inhibitor, excluding (Shou et al., 2008; ◻) or including (Ohno et al., 2007; □) the IR\(_{CYP}\), or from \textit{in vitro} recombinant clearance data simulated using Simcyp (×).

The \textit{in vivo} methods of \( fm_{CYP} \) estimation from methods utilising selective CYP3A4 inhibitors were further considered (Equations 2.3 and 2.4). This analysis was in order to ascertain the impact of the use of different CYP3A4 inhibitors and alternative victim drug dosing methods (i.v. and oral) and inhibitor dosing schedules (single and multiple oral dosing) on the \( fm_{CYP} \) estimate. For this analysis, AUC ratios were collated for 27 DDI studies involving 8 inhibitors and midazolam as the victim drug; these studies are outlined in Table 2.1. Values of \( fm_{CYP} \) were estimated from the results for each study in the presence of a selective inhibitor using Equation 2.3 (Shou et al., 2008) or in conjunction with the IR\(_{CYP}\) reported in Ohno et al., 2007 (Table 2.1) in Equation 1.7. Results are
displayed in Figure 2.2. Estimates for midazolam fmCYP3A4 from steady state inhibitor concentrations following oral dosing using Equation 2.3 had a 0.72-0.89 range from estimation using fluconazole and ketoconazole DDIs, respectively, with a mean value of 0.80. The range was reduced to 0.89-0.96 (mean, 0.92) with the use of Equation 2.4 as a result of the IR_CYP inclusion. AUC ratios from the single dosing of inhibitors were reported for itraconazole and fluconazole only. Conflicting results were observed, with a reduction of midazolam fmCYP from the use of itraconazole to 83% of the fmCYP estimated from steady state data, whereas fmCYP estimates remained at 97% of the steady state value for fluconazole. These differences remained regardless of IR_CYP inclusion. Both values were reported from the same study, and differences did not therefore result from inter-individual variability or study protocol differences. Estimates of fmCYP from i.v. data were on average 17% lower than mean estimates from steady state oral dosing, and had a 20-30% fmCYP range from the use of Equations 2.3 and 2.4, respectively. Both methods do not include the contribution of the intestine to oral fmCYP3A4 estimates, which is significant in the case of midazolam (F_G = 0.51). Estimates from i.v. data should therefore be more adequate. However, significantly lower fmCYP3A4 values are estimated from i.v. data (regardless of the inclusion of IR_CYP) compared to those estimated from steady state oral dosing, with mean values at 0.65-0.72.
Table 2.1 Details of DDI studies used to calculate midazolam \( f_{m_{\text{CYP3A4}}^{\text{in}}} \) in the presence of selective inhibitors using methods reported by Shou et al., 2008 and Ohno et al., 2007.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( \text{IR}_{\text{CYP}} )</th>
<th>Dosing(^2)</th>
<th>Dose</th>
<th>AUC ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>1.00</td>
<td>Steady state</td>
<td>200mg</td>
<td>6.5</td>
<td>Eap et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200mg</td>
<td>6.6</td>
<td>Lam et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400mg</td>
<td>16</td>
<td>Olkkola et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200mg</td>
<td>16</td>
<td>Tsunoda et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200mg</td>
<td>5.0(^1)</td>
<td>Tsunoda et al., 1999</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.88</td>
<td>Steady state</td>
<td>1000mg</td>
<td>8.4</td>
<td>Gurley et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>250mg</td>
<td>3.6</td>
<td>Yeates et al., 1996</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>0.80</td>
<td>Steady state</td>
<td>60mg</td>
<td>3.8</td>
<td>Backman et al., 1994</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.82</td>
<td>Steady state</td>
<td>500mg</td>
<td>4.4</td>
<td>Olkkola et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500mg</td>
<td>3.8</td>
<td>Zimmermann et al., 1996</td>
</tr>
<tr>
<td>Fluconazole</td>
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<td>Single dose</td>
<td>400mg</td>
<td>3.7</td>
<td>Ahonen et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100mg</td>
<td>2.2</td>
<td>Kharasch et al., 2005</td>
</tr>
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<td>Kharasch et al., 2005</td>
</tr>
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<td>400mg</td>
<td>4.9</td>
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</tr>
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<td>Steady state</td>
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<td>3.6</td>
<td>Olkkola et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200mg</td>
<td>2.0(^{\dagger})</td>
<td>Olkkola et al., 1996</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.95</td>
<td>Single dose</td>
<td>200mg</td>
<td>3.4</td>
<td>Olkkola et al., 1996</td>
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<td>100mg</td>
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<td>200mg</td>
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<td>6.6</td>
<td>Olkkola et al., 1996</td>
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<td></td>
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<td></td>
<td>200mg</td>
<td>10.8</td>
<td>Olkkola et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200mg</td>
<td>3.2(^{\dagger})</td>
<td>Olkkola et al., 1996</td>
</tr>
<tr>
<td>Nefadozone</td>
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<td>Steady state</td>
<td>200mg</td>
<td>5.4</td>
<td>Lam et al., 2003</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.88</td>
<td>Steady state</td>
<td>1200mg</td>
<td>5.2</td>
<td>Palkama et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1200mg</td>
<td>2.5</td>
<td>Palkama et al., 1999</td>
</tr>
</tbody>
</table>

\(^{1}\) \( \text{IR}_{\text{CYP}} \) values taken directly from Ohno et al., 2007

\(^{2}\) Single dose and steady state (dose per day) refer to the oral dosing of inhibitor

\(^\dagger\) Refers to i.v. dosing of midazolam

* CL/CL’ reported
Figure 2.2 Estimation of midazolam fm\textsubscript{CYP3A4} using data from drug-drug interaction studies, and methods using the AUC ratio with the exclusion (Shou et al., 2008 (A)) or inclusion (Ohno et al., 2007 (B)) of the IR\textsubscript{CYP}. Values are estimated using a range of different CYP3A4 inhibitors, dosed by the intravenous route (●), or by single oral dosing (■) or multiple oral dosing (inhibitor at steady state) (<)).

Estimates of fm\textsubscript{CYP} for non-CYP3A4 victim drugs (CYP1A2, n=3; CYP2C9, n=3; CYP2C19, n=1; CYP2D6, n=4) were also compared when estimated from regression analysis, renal excretion data or ranking analysis (Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008; Ito et al., 2004), from the comparison of phenotyped data for CYP2D6 victim drugs (Ito et al., 2005) and using \textit{in vitro} recombinant data within Simcyp. A wide range was observed from all three aforementioned methods (0.49-0.95, 0.46-0.94 and 0.56-0.97, respectively), apparent for all CYP enzymes. No consistency was observed between the rank order of fm\textsubscript{CYP} values when assessed by the method of estimation, with the exception of the increased fm\textsubscript{CYP} given by CYP2D6 phenotype data in comparison to regression analysis in the majority (3/4) of victim drugs. Rosiglitazone fm\textsubscript{CYP2C9} was the minimal estimate in the dataset from estimation by regression analysis and Simcyp; maximal fm\textsubscript{CYP} was observed for caffeine fm\textsubscript{CYP1A2} from both regression and Simcyp methods. Simcyp also estimated high contribution (>0.95) of CYP2C9 to the clearance of S-warfarin and tolbutamide. Overall, the majority (8/11) of fm\textsubscript{CYP} estimates were within 20% of each other and 5/11 within 10% of each other.
Figure 2.3 Comparison of $fm_{\text{CYP}}$ estimates for 11 non-CYP3A4 victim drugs. Estimates were from regression analysis, ranking analysis or recovery of unchanged drug (Brown et al., 2005; Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008; Ito et al., 2004, ◆), from the change in AUC between PM/EM in CYP2D6 genotyped subjects (Ito et al., 2005, ▲), or from \textit{in vitro} recombinant clearance data simulated using Simcyp (×).

Despite the reported differences in $fm_{\text{CYP}}$ values resulting from the use of alternative methods, a good agreement was observed between all values of midazolam and triazolam $fm_{\text{CYP}}$. There are a number of reported DDIs involving these two benzodiazepines as victim drugs and differences in the $fm_{\text{CYP}}$ values should therefore not have an impact on prediction accuracy. The impact of differences for alternative victim drugs will be further considered in Chapter 4 and 5 through the assessment of DDI prediction in a large database, and will be of particular importance when differences are reported with $fm_{\text{CYP}}$ values > 0.9. This scenario is observed in the case of nifedipine, cyclosporine (CYP3A4), S-warfarin and tolbutamide (CYP2C9).

2.3.1.2 Fraction escaping metabolism in the gut ($F_{\text{G}}$)

The four methods of $F_{\text{G}}$ estimation resulted in a 5 - >10-fold range of $F_{\text{G}}$ values, with the largest overall range of 0.08 for simvastatin to 0.99 for alprazolam and quinidine, respectively from the first order model in Simcyp and smallest range 0.18-0.94 for saquinavir and alprazolam, respectively from the i.v. and oral data. No consistency was
observed between the minimal $F_G$ estimate for each victim drug and the method of estimation used. However, estimates using the ADAM model displayed a trend for producing maximal $F_G$ for 8/10 victim drugs. This method also resulted in outlying data for saquinavir and cyclosporine compared to the remaining three methods, attributed to the lack of P-gp kinetic data and drug formulation parameters for input into the model. Half of the victim drug $F_G$ values were estimated within 20% of each other, and only 2/10 within 10%. Additionally, the average coefficient of variation was 30%, and ranged from 5% (alprazolam and quinidine) to 60% (saquinavir). The $Q_{\text{gut}}$ model was selected for use in the Simcyp simulations of DDIs despite it being a minimal physiologically based model. This decision was necessary due to the inability to implement the ADAM model into DDI predictions in Simcyp (current version 10.1). The lack of input data for the ADAM model (e.g. transporter kinetic data) was an additional factor contributing to the decision not to utilise this model when predicting DDIs in Simcyp in the current analysis.

**Figure 2.4** Comparison of $F_G$ estimates for 10 CYP3A4 victim drugs. Estimates were from *in vivo* data from the comparison of i.v. and oral data (●) (Galetin et al., 2008; Galetin et al., 2010), or in the absence and presence of grapefruit juice (■) (Galetin et al., 2010; Gertz et al., 2008a), and from *in vitro* data using the first order model (utilising the principles of the $Q_{\text{gut}}$ model) (▲) or ADAM model (▲) within Simcyp.

**2.3.1.3 Prediction of victim drug profiles**

The profiles of oral midazolam (5mg) and triazolam (0.25mg) were well predicted using the default Simcyp parameters (Figure 2.5A-B). Values of $T_{\text{max}}$ were predicted within 1.5-
fold, with the actual values of 1.9h and 1.5h (Thummel et al., 2008) predicted at 1.3h and 1h for midazolam and triazolam, respectively. Imprecision was higher for $C_{\text{max}}$ predictions, with the midazolam and triazolam predictions of 0.03 and 0.002mg/L compared to the actual 0.08mg/mL (Thummel et al., 2008) and 0.004mg/L (Friedman et al., 1988), respectively. Successful prediction of the oral clearance of both midazolam and triazolam was observed (Greenblatt et al., 2009; Thummel et al., 2008), with both predicted within 1.2-fold. The combination of these parameters resulted in a small under-prediction of the AUC (80 - 82% of the actual); however, the default parameters were considered successful. The profiles of two alternative CYP3A4 victim drugs were also considered. The prediction success of the cyclosporine profile was similar to the benzodiazepine victim drugs (Figure 2.5C). Successful prediction of the $T_{\text{max}}$ (69% of the actual value) was combined with a decrease in prediction success for the $C_{\text{max}}$ value (50% of the actual value). Despite this, the AUC was predicted at 79% of the observed value (4.7mg/L/h compared to 5.9mg/L/h actual). Prediction of the simvastatin $T_{\text{max}}$ was more accurate (92% of the actual value); however, the $C_{\text{max}}$ was under-predicted at 41% of the actual. The overall AUC was predicted at 70% of the actual (0.0369 compared to 0.0525mg/L/h). For the CYP3A4 victim drugs, a higher prediction success was therefore observed for the $T_{\text{max}}$ and AUC in comparison to the $C_{\text{max}}$. Additionally, representative profiles were assessed for a CYP1A2 (caffeine), CYP2C19 (omeprazole) and CYP2D6 (tolterodine) victim drug. Successful prediction of $T_{\text{max}}$ was observed for all of these victim drugs (with the exception of caffeine, where this data was not reported). Mixed prediction success was observed for $C_{\text{max}}$ predictions, with 2-fold under-prediction of tolterodine, successful prediction of omeprazole and prediction 72% of the observed caffeine $C_{\text{max}}$. The mixed prediction success was also observed for the AUC ranging from 56% of the actual (tolterodine) to 2.9-fold over-prediction (omeprazole). The prediction of CYP3A4 victim drugs was therefore considered successful, whereas the profile prediction success of non-CYP3A4 victim drugs was reduced and warranted further investigation.
The single data points represent the observed $T_{\text{max}}$ and $C_{\text{max}}$ from dose-matched studies (Table 2.2-2.6). There were no reported $T_{\text{max}}$ values for G. Reported values of systemic concentration are displayed on the x axis if outside the scale range.

Figure 2.5 Simcyp prediction of oral midazolam (5mg) (A), triazolam (0.25mg) (B), cyclosporine (2mg) (C), simvastatin (40mg) (D), toterodine (2mg) (E), omeprazole (40mg) (F) and caffeine (100mg) (G) profiles.
2.3.2 Sensitivity analysis of inhibitor properties in simulations

The impact of six inhibitor parameters in the prediction of DDIs is displayed in Figure 2.6. This analysis used the inbuilt sensitivity analysis feature in Simcyp, and was based upon an itraconazole-midazolam DDI, as described in Section 2.2.2. The observed relationships differ between the parameters, ranging from minimal differences for the fu_{gut} and higher impact at the lower values of parameters for the fu_{p} and liver uptake, reaching a maximal level above 0.2 and 2, respectively. The liver uptake value is linked to the fu_{p} within the dynamic model in Simcyp, explaining the similarity in profiles between the two parameters. The AUC ratio decreases with k_{a} > 0.5h^{-1} with an intermediate impact; the full range of AUC ratio is 5-7 with a k_{a} range of 0.06-6h^{-1}. A direct relationship is observed with increased AUC ratio with increasing value of the F_a. Maximal impact on AUC ratio was observed from differences in the K_i value, particularly for values < 0.02μM. Use of K_i values below this level resulted in a range in the AUC ratio of 3-10. Further analysis into the impact of the K_i value utilised the reported range of ketoconazole CYP3A4 K_i reported from the same system (HLM) and metabolic pathway (α-OH-midazolam) in different studies (Greenblatt et al., 2010). This analysis was based on the same dosing schedule as the previous itraconazole-midazolam DDI analysis, utilising a 200mg ketoconazole dose. The 0.004 – 0.18μM range resulted in a difference in mean AUC ratio of 2.44 – 8.55; the K_i is therefore a critical parameter in DDI prediction and careful selection is necessary.

Preferential sources for the unbound values of the inhibition constant (and correlating fu_{inc}) were those from previous assessment in house (Brown et al., 2006); this decision therefore reduced the incorporation of error by reducing variability in the assessed metabolic pathways, substrate drugs, methods, cells and data analysis. Alternative sources for the K_i and fu_{inc} were only used when this data provided additional benefit to the in-house data. This situation was observed in the case of itraconazole and its main metabolite, hydroxyitraconazole, that were determined in the same laboratory in recombinant enzymes and HLM (Isoherranen et al., 2004) and for fluvoxamine, where the same laboratory ascertained the kinetics of the CYP1A2 and CYP2C19 pathways in recombinant expressed enzymes (Yao et al., 2001; Yao et al., 2003). In addition, sources for the fraction unbound in plasma of the inhibitors were matched to the K_i sources where available for consistency. This value would impact on the DDI predictions where the default values are low (e.g. < 0.2 for itraconazole, Figure 2.6). The default Simcyp values of the F_a for all drugs were equal to 1; differences were observed in the fu_{gut} values of the inhibitor and victim drugs, with values equal to fu_{p} and 1, respectively. These values were utilised for further DDI predictions despite this inconsistency. The use of victim drug fu_{gut} equal to unity assumes that no binding occurs due to insufficient time, no active transport occurs and that unbound
drug in the enterocyte is subject to removal by blood flow through the villi. The sensitivity analysis in Figure 2.6 indicates that the inconsistency between $f_{\text{gut}}$ assumptions between inhibitors and victim drugs would be limited in the case of itraconazole where a $f_p$ value >0.06 is utilised. This relationship should not be assumed for alternative inhibitors, where the inconsistency in utilised $f_{\text{gut}}$ values may result in a more significant impact. Default values of $k_a$ were used in simulations; however, the use of alternative $k_a$ values in the prediction of DDIs warranted further investigation, and is assessed in Chapters 4 and 5. Conflicting data were observed in the literature assessing the uptake of the five inhibitors into the liver, from a wide range of indirect methods (in vivo and in vitro). This parameter was therefore selected for further investigation in the following chapter, in order to ascertain the potential contribution of uptake to the inhibition potential of the inhibitors.

The prediction of inhibitor profiles was also assessed based on the studies previously selected for the prediction of victim drug profiles (Figure 2.7, Table 2.2-2.4; Brynne et al., 1999; Christensen et al., 2002; Greenblatt et al., 1998; Neuvonen et al., 1996). For ketoconazole, Greenblatt et al., 1998 was utilised instead of Gomez et al., 1995 due to reported parameters for ketoconazole. The remaining studies did not all report the full profile of each inhibitor, therefore profile prediction was assessed based on reported parameter values from matched dosing schedules to the studies. Ketoconazole $C_{\text{max}}$ was predicted within 1.3-fold (3.84mg/L compared to observed 5mg/L), whereas the $T_{\text{max}}$ was over-predicted by 2.5-fold (1.5h compared to 0.6h). The AUC was not reported in Greenblatt et al., 1998; Simcyp predicted 19.2mg/L/h. The profile of itraconazole had mixed prediction success, with 2-fold under-prediction of $C_{\text{max}}$ (0.234mg/L compared to 0.509mg/L), 1.6-fold over-prediction of $T_{\text{max}}$ (4.3h compared to 2.7h), and successful prediction of AUC (6.606mg/L/h compared to 6.101mg/L/h). Exposure is therefore successful despite the inconsistencies in the profile. The reported fluoxetine $C_{\text{max}}$ ranged from 0.05-0.11mg/L for different subjects; this was under-predicted in the Simcyp simulation, with average $C_{\text{max}}$ predicted at 0.046mg/L. Simcyp predicted the $T_{\text{max}}$ of 3.3h and AUC of 2.38mg/L/h; these values were not reported in Brynne et al., 1999. Fluvoxamine $C_{\text{max}}$ and $T_{\text{max}}$ were both successfully predicted, at 0.017mg/h and 4.2h compared to the reported 0.02mg/h and 4-8h, respectively. AUC data was predicted at 0.51mg/L/h, however this data was not reported for fluvoxamine in Christensen et al., 2002. Overall, mixed prediction success was observed for inhibitor profile prediction; however, the majority of predictions were predicted within 2-fold and therefore considered successful.
Figure 2.6 Sensitivity analysis for key inhibitor parameters, using an itraconazole-midazolam DDI as an example. Differences in the AUC ratio are displayed. Panels display the sensitivity of DDI predictions to inhibitor fu_p, k_a, K_i, liver uptake, fu_gut and F_a in A-F, respectively. The default value in the Simcyp simulator of each itraconazole parameter is denoted by •.
A. B. C. D.

**Figure 2.7** Simcyp prediction of oral ketoconazole (A), itraconazole (B), fluoxetine (C) and fluvoxamine (D) profiles. The single data points represent the observed $T_{\text{max}}$ and $C_{\text{max}}$ (after the final dose) with simulations matched to the reported dosing schedules (Table 2.2-2.6). Reported values of systemic concentration are displayed on the x axis if outside the scale range.

### 2.3.3 DDI database

A database was collated consisting of 97 DDI studies between the five selected inhibitors (fluconazole (n=19), itraconazole (n=18), ketoconazole (n=23), fluoxetine (n=10) and fluvoxamine (n=27)) and 16 victim drugs. Patient demographic data (including number of subjects, age and sex) and information on the dosing schedules of both the inhibitor and victim drugs were also collated. These victim drugs were metabolised by the five main CYP enzymes; CYP1A2 (11%), CYP2C9 (9%), CYP2C19 (18%), CYP2D6 (8%) and CYP3A4 (54%). The majority (70%) of the interactions in the database were positive (AUC ratio > 2), with 40% moderate (2 < AUC ratio < 5) and 30% strong (AUC ratio >5) inhibition interactions when classified in a method analogous to the FDA guidelines for the classification of an inhibitor (Huang et al., 2008). Weak inhibition interactions (1.25 < AUC ratio < 2) accounted for 25% of the database, and 4 and 1% were classified as no inhibition (1 < AUC ratio < 1.25) and induction (AUC ratio < 1) interactions, respectively.
Itraconazole was the only inhibitor where all AUC ratios were > 2; the distribution of interaction potency did not significantly differ between the remaining inhibitors (Figure 2.8A). The distribution was also investigated after the exclusion of DDIs that did not result from the main inhibited enzyme (CYP2C9 and CYP3A4 for fluconazole, CYP3A4 for ketoconazole and itraconazole, CYP2D6 for fluoxetine and CYP1A2 and CYP2C19 for fluvoxamine). The maximal classification of DDIs per inhibitor were observed for moderate DDIs for all inhibitors (with the exception of ketoconazole), with declining numbers at lower and higher potencies. The number of DDIs reported involving ketoconazole increased in frequency with increasing potency. The potency of DDIs was assessed by the physicochemical properties of the inhibitors. All inhibitors displayed low potency DDIs regardless of LogP values however the range and maximum potency of DDI increased with increasing values of LogP (Figure 2.8B). Fluoxetine did not follow this trend, potentially resulting from the comparatively low number of DDIs (n=10) or due to the low number of inhibitors included in the overall analysis. In addition, the relationship between the LogP and potency of DDI would not be expected as a result of additional factors such as the inhibitor dose and properties of the victim drug involved. The potency of DDI was also assessed in comparison to the fmCYP of the victim drug (Figure 2.8C) from regression analysis, ranking analysis or recovery unchanged (Brown et al., 2006; Galetin et al., 2006; Galetin et al., 2005; Houston and Galetin, 2008; Ito et al., 2004; Shou et al., 2008, Figures 2.1 and 2.3). The positive correlation between the DDI potency and the fmCYP corresponded to the expected relationship, where increased potency of DDIs tended to exist in DDIs with high fmCYP victim drugs.

A number of studies (n=11) in the database reported AUC data from the individual subjects in the study (representing 24 data points due to different dose, dosing schedules or subject phenotypes), with the majority of these studies containing < 10 subjects (average 9 ± 5, range 5-18). The normal distribution of the AUC ratios in each study cannot be assumed, particularly due to the low number of subjects in each study. In order to attempt to negate the impact of this potential issue, the AUC ratios were calculated from the mean of the AUC ratios calculated from each individual subject instead of from the overall average AUC in the presence and absence of inhibitor. These values can be non-consistent, for example the AUC ratios of 3.5 and 5.5 calculated from overall and individual IM subjects, respectively (Brynne et al., 1999). Studies containing reported individual data were available for all inhibitors, represented by 1 – 5 studies for fluoxetine and itraconazole, respectively. Individual data had a minimal range in some cases, for example the 1.3-fold range between minimum and maximum AUC ratio observed in the DDI between
fluvoxamine (10mg) and omeprazole. In contrast, the difference in minimum to maximum AUC ratio from the individual subjects was >10-fold in the DDI between itraconazole and triazolam (11-fold; Varhe et al., 1994) and fluoxetine and tolterodine (20-fold; Brynne et al., 1999). The predictions of the inter-individual variability of these DDIs are assessed in Chapters 4 and 5. Nine studies (representing 17 data points due to different dose or dosing schedule) in the database reported DDIs after single dose of the inhibitors and predictions of these studies were used for additional analysis and comparison with those studies reporting DDIs at steady-state inhibitor concentrations in Chapters 4 and 5.
<table>
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<tr>
<th>Inhibitor dose</th>
<th>Dosing start day &amp; time</th>
<th>Dosing interval (h)</th>
<th>No. doses</th>
<th>CYP Enzyme</th>
<th>Victim drug</th>
<th>Victim drug dose</th>
<th>Victim drug dosing time</th>
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<td>7</td>
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<td>18</td>
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<td>CYP2C9</td>
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SD = Single dose; m = male; f = female. If exact timings were not reported, the victim drug dosing time is given as plus a number of hours after the final inhibitor dose.
Table 2.3 Summary details including the dosing details and observed AUC ratio of the 18 in vivo DDI studies involving itraconazole as the inhibitor

<table>
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<tr>
<th>Inhibitor dose</th>
<th>Dosing start day &amp; time</th>
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<th>CYP Enzyme</th>
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<th>Victim drug dose</th>
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<td>SD</td>
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<td>Day 1, +4 hrs</td>
<td>6 (5m, 1f)</td>
<td>5.38</td>
<td>Templeton et al., 2010</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-4, 6pm</td>
<td>24</td>
<td>4</td>
<td>CYP3A4</td>
<td>Quinidine</td>
<td>100mg</td>
<td>Day 4, 7pm</td>
<td>9 (4m, 5f)</td>
<td>2.42</td>
<td>Kaukonen et al., 1997</td>
</tr>
<tr>
<td>100mg</td>
<td>Day 1-7, 8am</td>
<td>24</td>
<td>7</td>
<td>CYP3A4</td>
<td>Quinidine</td>
<td>200mg</td>
<td>Day 6</td>
<td>6 (6m, 0f)</td>
<td>2.58</td>
<td>Damkier et al., 1999</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-4, 7:30am</td>
<td>24</td>
<td>4</td>
<td>CYP3A4</td>
<td>Simvastatin</td>
<td>40mg</td>
<td>Day 4, 9:30am</td>
<td>10 (7m, 3f)</td>
<td>18.42</td>
<td>Neuvonen et al., 1998</td>
</tr>
<tr>
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<td>Day 1, 3pm</td>
<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 2, 3pm</td>
<td>10 (6m, 4f)</td>
<td>3.57</td>
<td>Neuvonen et al., 1996</td>
</tr>
<tr>
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<td>Day 2, 3am</td>
<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 2, 3pm</td>
<td>10 (6m, 4f)</td>
<td>4.33</td>
<td>Neuvonen et al., 1996</td>
</tr>
<tr>
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<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
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<td>0.25mg</td>
<td>Day 2, 3pm</td>
<td>10 (6m, 4f)</td>
<td>4.48</td>
<td>Neuvonen et al., 1996</td>
</tr>
<tr>
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<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 2, 3pm</td>
<td>10 (6m, 4f)</td>
<td>3.11</td>
<td>Neuvonen et al., 1996</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-4, 2pm</td>
<td>24</td>
<td>4</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 4, 3pm</td>
<td>9 (3m, 6f)</td>
<td>10.5</td>
<td>Varhe et al., 1994</td>
</tr>
</tbody>
</table>

SD = Single dose; m = male; f = female. If exact timings were not reported, the victim drug dosing time is given as plus a number of hours after the final inhibitor dose.
Table 2.4 Summary details including the dosing details and observed AUC ratio of the 23 in vivo DDI studies involving ketoconazole as the inhibitor

<table>
<thead>
<tr>
<th>Inhibitor dose</th>
<th>Dosing start day &amp; time</th>
<th>Dosing interval (h)</th>
<th>No. doses</th>
<th>CYP Enzyme</th>
<th>Victim drug</th>
<th>Victim drug dose</th>
<th>Victim drug dosing time</th>
<th>Number of subjects</th>
<th>Observed AUC Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>200mg</td>
<td>Day 1-7, 6:30am</td>
<td>24</td>
<td>7</td>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>500mg</td>
<td>Day 7, 7am</td>
<td>5 (3m, 2f)</td>
<td>1.77</td>
<td>Krishnaiah et al., 1994</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-4</td>
<td>24</td>
<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>20mg</td>
<td>Day 4</td>
<td>10 (6m, 4f)</td>
<td>1.36a</td>
<td>Bottiger et al., 1997</td>
</tr>
<tr>
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<td>Day 1-4</td>
<td>24</td>
<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>20mg</td>
<td>Day 4</td>
<td>10 (6m, 4f)</td>
<td>1.99a</td>
<td>Bottiger et al., 1997</td>
</tr>
<tr>
<td>100mg</td>
<td>Day 1-4</td>
<td>24</td>
<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>20mg</td>
<td>Day 4</td>
<td>5 (3m, 2f)</td>
<td>1.37a</td>
<td>Bottiger et al., 1997</td>
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<tr>
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<td>24</td>
<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>40mg</td>
<td>Day 4</td>
<td>5 (3m, 2f)</td>
<td>1.95b</td>
<td>Bottiger et al., 1997</td>
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<tr>
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<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>20mg</td>
<td>Day 4</td>
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<td>2.05a</td>
<td>Bottiger et al., 1997</td>
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<tr>
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<td>4</td>
<td>CYP2C19*</td>
<td>Omeprazole</td>
<td>40mg</td>
<td>Day 4</td>
<td>5 (3m, 2f)</td>
<td>1.53a</td>
<td>Bottiger et al., 1997</td>
</tr>
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<td>12</td>
<td>5</td>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>1mg</td>
<td>Day 3, 9am</td>
<td>7 (7m, 0f)</td>
<td>3.98</td>
<td>Greenblatt et al., 1998</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-3</td>
<td>12</td>
<td>5</td>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>1mg</td>
<td>Day 3</td>
<td>4 (4m, 0f)</td>
<td>1.76</td>
<td>Schmider et al., 1999</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-28</td>
<td>12</td>
<td>56</td>
<td>CYP3A4</td>
<td>Cyclosporine</td>
<td>400mg</td>
<td>Day 1-28</td>
<td>15 (14m, 1f)</td>
<td>5.15</td>
<td>Butman et al., 1991</td>
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<tr>
<td>200mg</td>
<td>Day 1</td>
<td>24</td>
<td>SS</td>
<td>CYP3A4</td>
<td>Cyclosporine</td>
<td>180mg</td>
<td>Day 1, SS</td>
<td>9</td>
<td>4.39</td>
<td>Varhe et al., 1998</td>
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<tr>
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<td>Day 1-10</td>
<td>24</td>
<td>10</td>
<td>CYP3A4</td>
<td>Cyclosporine</td>
<td>140mg</td>
<td>Day 11, + 10 hr</td>
<td>5 (2m, 3f)</td>
<td>1.30</td>
<td>Gomez et al., 1995</td>
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<tr>
<td>200mg</td>
<td>Day 1-3</td>
<td>12</td>
<td>5</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>0.075mg</td>
<td>Day 3</td>
<td>4 (1m, 3f)</td>
<td>6.47</td>
<td>Eap et al., 2004</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-12</td>
<td>24</td>
<td>12</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>10mg</td>
<td>Day 12, + 1 hr</td>
<td>10 (4m, 6f)</td>
<td>6.56</td>
<td>Lam et al., 2003</td>
</tr>
<tr>
<td>400mg</td>
<td>Day 1-4, 2pm</td>
<td>24</td>
<td>4</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>7.5mg</td>
<td>Day 4, 3pm</td>
<td>9 (2m, 7f)</td>
<td>15.9</td>
<td>Olkkola et al., 1994</td>
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<tr>
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<td>Day 1-2</td>
<td>12</td>
<td>3</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>6mg</td>
<td>Day 1, + 12hrs</td>
<td>9 (6m, 3f)</td>
<td>16.0</td>
<td>Tsunoda et al., 1999</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-2</td>
<td>12</td>
<td>3</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>5mg</td>
<td>Day 1, + 13 hrs</td>
<td>4 (2m, 2f)</td>
<td>8.10</td>
<td>Lee et al., 2002</td>
</tr>
<tr>
<td>400mg</td>
<td>Day 1-10</td>
<td>24</td>
<td>10</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>5.5mg</td>
<td>Day 9</td>
<td>10 (10m, 10f)</td>
<td>9.51</td>
<td>Chung et al., 2006</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1, 7am</td>
<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>2mg</td>
<td>Day 1, 9am</td>
<td>12 (12m, 0f)</td>
<td>4.95</td>
<td>McCrea et al., 1999</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1, 9am</td>
<td>SD</td>
<td>1</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>2mg</td>
<td>Day 1, 9am</td>
<td>12 (12m, 0f)</td>
<td>6.45</td>
<td>McCrea et al., 1999</td>
</tr>
<tr>
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<td>Day 1-3, 8am</td>
<td>12</td>
<td>5</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 3, 9am</td>
<td>7 (7m, 0f)</td>
<td>13.7</td>
<td>Greenblatt et al., 1998</td>
</tr>
<tr>
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<td>Day 1-4, 2pm</td>
<td>24</td>
<td>4</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.25mg</td>
<td>Day 4, 3pm</td>
<td>9 (3m, 6f)</td>
<td>9.17</td>
<td>Varhe et al., 1994</td>
</tr>
<tr>
<td>200mg</td>
<td>Day 1-2</td>
<td>16</td>
<td>2</td>
<td>CYP3A4</td>
<td>Triazolam</td>
<td>0.125mg</td>
<td>Day 2, + 1 hr</td>
<td>9 (7m, 2f)</td>
<td>9.16</td>
<td>von Molike et al., 1996</td>
</tr>
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</table>

SD = Single dose; m = male; f = female. If exact timings were not reported, the victim drug dosing time is given as plus a number of hours after the final inhibitor dose. * Omeprazole metabolised by CYP2C19 (major pathway) and CYP3A4 (minor pathway).

SS represents a case where both inhibitor and victim drug are at steady state (study undertaken in patients). Study in *CYP2C19 EM or ^CYP2C19 PM subjects
### Table 2.5 Summary details including the dosing details and observed AUC ratio of the 10 *in vivo* DDI studies involving fluoxetine as the inhibitor

<table>
<thead>
<tr>
<th>Inhibitor dose</th>
<th>Dosing start day &amp; time</th>
<th>Dosing interval (h)</th>
<th>Dosing interval</th>
<th>No. doses</th>
<th>CYP Enzyme</th>
<th>Victim drug</th>
<th>Victim drug dose</th>
<th>Victim drug dosing time</th>
<th>Number of subjects</th>
<th>Observed AUC Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>60mg</td>
<td>Day 1</td>
<td>SD</td>
<td>1</td>
<td>CYP2D6</td>
<td>Desipramine</td>
<td>50mg</td>
<td>Day 1, + 3 h</td>
<td>Day 1-29, 8am</td>
<td>5 (5m, 0f)</td>
<td>2.25</td>
<td>Bergstrom et al., 1992</td>
</tr>
<tr>
<td>60mg</td>
<td>Day 1-8</td>
<td>24</td>
<td>8</td>
<td>CYP2D6</td>
<td>Desipramine</td>
<td>50mg</td>
<td>Day 8, + 3 h</td>
<td>Day 1-29, 8am</td>
<td>5 (5m, 0f)</td>
<td>7.43</td>
<td>Bergstrom et al., 1992</td>
</tr>
<tr>
<td>20mg</td>
<td>Day 8-29, 8am</td>
<td>24</td>
<td>21</td>
<td>CYP2D6</td>
<td>Desipramine</td>
<td>50mg</td>
<td>Day 1-29, 8am</td>
<td>Day 8-24</td>
<td>9 (9m, 0f)</td>
<td>4.42</td>
<td>Preskorn et al., 1994</td>
</tr>
<tr>
<td>60mg</td>
<td>Day 1</td>
<td>SD</td>
<td>1</td>
<td>CYP2D6</td>
<td>Imipramine</td>
<td>50mg</td>
<td>Day 1, + 3 h</td>
<td>Day 1-29, 8am</td>
<td>5 (5m, 0f)</td>
<td>1.89</td>
<td>Bergstrom et al., 1992</td>
</tr>
<tr>
<td>60mg</td>
<td>Day 1-8</td>
<td>24</td>
<td>8</td>
<td>CYP2D6</td>
<td>Imipramine</td>
<td>50mg</td>
<td>Day 8, + 3 h</td>
<td>Day 1-29, 8am</td>
<td>5 (5m, 0f)</td>
<td>3.33</td>
<td>Bergstrom et al., 1992</td>
</tr>
<tr>
<td>20mg</td>
<td>Day 1-24</td>
<td>24</td>
<td>24</td>
<td>CYP2D6</td>
<td>Tolterodine</td>
<td>2mg</td>
<td>Day 22-24, 12h Interval 5 doses</td>
<td>9 (0m, 9f)</td>
<td>8.51</td>
<td>Brynne et al., 1999</td>
<td></td>
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<tr>
<td>40mg</td>
<td>Day 1-11</td>
<td>24</td>
<td>11</td>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>2mg</td>
<td>Day 11</td>
<td>Day 1-21</td>
<td>16 (16m, 0f)</td>
<td>1.27</td>
<td>DeVane et al., 2004</td>
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<tr>
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<td>12</td>
<td>17</td>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>1mg</td>
<td>Day 3, 9am</td>
<td>Day 1-21</td>
<td>11 (11m, 0f)</td>
<td>1.26</td>
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</tr>
<tr>
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<td>Day 1-21</td>
<td>24</td>
<td>21</td>
<td>CYP3A4</td>
<td>Alprazolam</td>
<td>1mg</td>
<td>Day 21</td>
<td>Day 1-21</td>
<td>21</td>
<td>1.32</td>
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<td>5</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>10mg</td>
<td>Day 12, + 1 h</td>
<td>Day 1-21</td>
<td>9 (4m, 5f)</td>
<td>0.84</td>
<td>Lam et al., 2003</td>
</tr>
<tr>
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<td>Day 6-12</td>
<td>24</td>
<td>7</td>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>10mg</td>
<td>Day 12, + 1 h</td>
<td>Day 1-21</td>
<td>9 (4m, 5f)</td>
<td>0.84</td>
<td>Lam et al., 2003</td>
</tr>
</tbody>
</table>

SD = Single dose; m = male; f = female. If exact timings were not reported, the victim drug dosing time is given as plus a number of hours after the final inhibitor dose.
Table 2.6 Summary details including the dosing details and observed AUC ratio of the 27 in vivo DDI studies involving fluvoxamine as the inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor dose</th>
<th>Dosing start day &amp; time</th>
<th>Dosing interval (h)</th>
<th>No. doses</th>
<th>CYP Enzyme</th>
<th>Victim drug</th>
<th>Victim drug dose</th>
<th>Victim drug dosing time</th>
<th>Number of subjects</th>
<th>Observed AUC Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mg</td>
<td>Day 1-2, 730am</td>
<td>†</td>
<td>4</td>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>250mg</td>
<td>Day 2, 9am</td>
<td>7 (6m, 1f)</td>
<td>13.7</td>
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</tr>
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<td>Day 1-7</td>
<td>12</td>
<td>14</td>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>100mg</td>
<td>Day 4, 8am</td>
<td>5 (2m, 3f)</td>
<td>2.81*</td>
<td>Christensen et al., 2002</td>
</tr>
<tr>
<td>25mg</td>
<td>Day 1-7</td>
<td>12</td>
<td>14</td>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>100mg</td>
<td>Day 4, 8am</td>
<td>5 (2m, 3f)</td>
<td>6.14*</td>
<td>Christensen et al., 2002</td>
</tr>
<tr>
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<td>Day 1-7</td>
<td>24</td>
<td>7</td>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>100mg</td>
<td>Day 4, 8am</td>
<td>5 (3m, 2f)</td>
<td>1.81*</td>
<td>Christensen et al., 2002</td>
</tr>
<tr>
<td>25mg</td>
<td>Day 1-7</td>
<td>24</td>
<td>7</td>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>100mg</td>
<td>Day 4, 8am</td>
<td>5 (3m, 2f)</td>
<td>5.76*</td>
<td>Christensen et al., 2002</td>
</tr>
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<td>Day 1-14</td>
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<td>28</td>
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<td>Clozapine</td>
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<td>Day 13</td>
<td>9</td>
<td>2.84</td>
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</tr>
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<td>9</td>
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<td>Clozapine</td>
<td>10mg</td>
<td>Day 4</td>
<td>9 (9m, 0f)</td>
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<td>Day 1 Day 2-7</td>
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<td>1 6</td>
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<td>Theophylline</td>
<td>300mg</td>
<td>Day 4</td>
<td>12 (12m, 0f)</td>
<td>3.00</td>
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<td>Theophylline</td>
<td>250mg</td>
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<td>9 (5m, 4f)</td>
<td>1.47</td>
<td>Yao et al., 2001</td>
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<td>SD 24</td>
<td>1 8</td>
<td>CYP1A2</td>
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<td>CYP2C9</td>
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<td>14</td>
<td>CYP2C19</td>
<td>Omeprazole</td>
<td>200mg</td>
<td>Day 5, 8am</td>
<td>5 (2m, 3f)</td>
<td>2.63*</td>
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## Chapter 2 – Selection of parameters

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SD = Single dose; m = male; f = female. If exact timings were not reported, the victim drug dosing time is given as plus a number of hours after the final inhibitor dose. † Fluvoxamine dosing schedule: Day 1; 8am, 4pm. Day 2; 7:30am, 5pm. Study in<sup>a</sup>CYP2D6 EM<sup>b</sup>CYP2D6 PM<sup>c</sup>CYP2C19 EM<sup>d</sup>CYP2C19 IM<sup>e</sup>CYP2C19 PM subjects.
Figure 2.8 Characteristics of the collated DDI database (n=97) involving 3 azole CYP inhibitors (fluconazole (●), itraconazole (●) and ketoconazole (●)) and 2 SSRI CYP inhibitors (fluoxetine (●) and fluvoxamine (●)). The number of DDI studies per potency classification and per inhibitor is displayed (A), divided in DDI potency categories analogous to the classification of a CYP3A inhibitor (Huang et al., 2007). The potency of DDI compared to the LogP of the inhibitor (B) and fmCYP of the victim drug (C) are also displayed.
2.4 DISCUSSION

This chapter assessed the method of victim drug $f_{mCYP}$ and $F_G$ estimation from \textit{in vivo} and \textit{in vitro} data, and the impact of different inhibitor parameters in the prediction of DDIs. This work was preliminary with the aim to consider the impact of using the default \textit{in vitro} data within the Simcyp simulator to estimate victim drug parameters in the prediction ofazole and SSRI DDIs in Chapter 4 and 5, respectively. In addition, consideration of the sensitivity of the dynamic model to different inhibitor parameters was undertaken in order to justify selection of the inhibitor parameters for the predictions in order to reduce potential sources of bias. A database of 97 published DDI studies was collated involving five inhibitors and sixteen victim drugs. The number was assessed to ensure a representative number of studies per inhibitor, per victim drug and across the potency range. Investigation into potential relationships between the distribution of the DDI potency and inhibitor and victim drug properties was also investigated.

The $f_{mCYP}$ value has been previously proven to be an important parameter in the prediction of DDI (Brown et al., 2005; Galetin et al., 2006; Ito et al., 2005), however this parameter cannot be input directly into Simcyp. The Simcyp $f_{mCYP}$ estimates are dependent on the input of recombinant or HLM \textit{in vitro} clearance values; the alteration of these values to manipulate the value of $f_{mCYP}$ to concur with alternative estimations from \textit{in vivo} data is not utilised in the current study. Reduced prediction accuracy of DDIs as a result of differences in the $f_{mCYP}$ estimates from \textit{in vitro} data will be viewed as a discussion point in further chapters. Simcyp $f_{mCYP}$ estimations are largely consistent with those from \textit{in vivo} data for all CYP enzymes. Exceptions include the $f_{mCYP}$ for cyclosporine and nifedipine, where estimates were $>30\%$ different to all values from \textit{in vivo} data. This difference potentially results from efflux that is applicable for these victim drugs (e.g. cyclosporine, Gertz et al., 2010; Saeki et al., 1993) that are not included as default within the Simcyp simulator. Addition of the efflux would reduce the $f_{mCYP}$, and improve the \textit{in vitro} predictions of $f_{mCYP}$ in line with the \textit{in vivo} estimates. The \textit{in vivo} methods reported in Shou et al., 2008 and Ohno et al., 2007 provide alternative methods to estimate the $f_{mCYP}$ from the increase in AUC, with potential inclusion of the $IR_{CYP}$ (Ohno et al., 2007), incorporating the inhibitor concentration and inhibition constant into the prediction. Midazolam $f_{mCYP}$ estimates from the Shou et al., 2008 method were lower in comparison to the alternative methods, regardless of the inhibitor used for the calculation of the AUC ratio. In addition, the value of midazolam $f_{mCYP}$ that was reported in Shou et al., 2008 (0.89) was found to be the upper limit of midazolam $f_{mCYP}$ from this method, estimated from a single DDI study. Values estimated using a range of inhibitors after steady state oral
dosing reduces the average midazolam fm\textsubscript{CYP} to 0.8 (range 0.72-0.89). The fm\textsubscript{CYP} estimations using the method reported in Ohno et al., 2007 are higher than Shou et al., 2008, rationalised by the inclusion of the IR\textsubscript{CYP}. Reported values of IR\textsubscript{CYP} were utilised in the current study, and resulted in a narrow range of midazolam fm\textsubscript{CYP3A4} values (0.89-0.96) from eight inhibitors (oral dosing at steady state). These IR\textsubscript{CYP} values were calculated based on reported clinical AUC ratios from use of Equation 2.4 and the midazolam CR\textsubscript{CYP}. This CR\textsubscript{CYP} was previously estimated from substituting values of IR\textsubscript{CYP} or CR\textsubscript{CYP} into DDIs involving alternative inhibitors or victim drugs, originating from a simvastatin-itraconazole DDI where the CR\textsubscript{CYP} for this victim drug was assumed to equal 1.00 (Ohno et al., 2007). The method therefore relied on prior data. Despite this issue with the application of this method, consistency was observed in the fm\textsubscript{CYP} estimation per inhibitor across different DDI studies, regardless of differences in the design, dosing schedule and subjects within the study.

The different methods of F\textsubscript{G} estimation from in vivo data and the resultant impact on the prediction of DDIs has been previously considered (Galetin et al., 2008; Galetin et al., 2010; Galetin et al., 2007; Gertz et al., 2008a). Values of F\textsubscript{G} cannot be input into Simcyp, and are estimated based on different models; the Q\textsubscript{gut} model or ADAM model, using intestinal intrinsic CYP3A4 clearance. Values of F\textsubscript{G} estimated from the first order model were not significantly different to estimates from in vivo data (from the comparison of i.v. and oral data and in the absence and presence of grapefruit juice) for the victim drugs assessed. This consistency was regardless of low or high intestinal extraction, in contrast to the differences observed in Gertz et al., 2010. This difference may be rationalised by the larger dataset (n=25) assessed in the study. In Gertz et al., 2010, significant differences were observed between the F\textsubscript{G} estimations for substrates with high intestinal extraction (F\textsubscript{G} < 0.5) from in vivo data and from the Q\textsubscript{gut} model (using Equations 2.1 and 2.2). In addition, differences in the prediction accuracy of F\textsubscript{G} when the P\textsubscript{eff} utilised physicochemical properties within Simcyp (alprazolam, sildenafil, simvastatin and triazolam) was not observed, in contrast to that reported by Gertz et al., 2010. The difference may be observed as a result of the lower polar surface areas (Å\textsuperscript{2} < 100 for 3/4 of the victim drugs), where the majority reported in Gertz et al., 2010 had polar surface areas greater than those previously validated (Winiwarter et al., 1998). The F\textsubscript{G} estimation via the ADAM model resulted in high estimates for the majority of victim drugs, and were significantly different (> 30%) for cyclosporine and saquinavir. This difference results from the lack of P-gp data for input into the model for these victim drugs that are subject to both metabolism and transport. The
ADAM model cannot currently be incorporated into DDI prediction, and the first order model was therefore selected for use in the prediction of DDIs.

Despite some reported differences in $f_{m_{\text{CYP}}}$ and $F_G$ estimates the overall consensus was for successful prediction. In addition, the profile of selected victim drugs was investigated, from assessment of the clearance, AUC, $T_{\text{max}}$ and $C_{\text{max}}$. Successful prediction of AUC and $T_{\text{max}}$ was observed for the majority of victim drugs, particularly CYP3A4 victim drugs. A poorer prediction of $C_{\text{max}}$ was observed in the majority of cases. The default input parameters for victim drugs were used for further assessment of DDIs in Chapter 4 and 5 based on the prediction success of the AUC, with further consideration warranted into the victim drug profiles where inter-individual DDI data is reported.

A number of inhibitor properties were investigated using sensitivity analysis to assess the importance of each of the parameters on the prediction of DDI. Consideration was given to the source of inhibitor parameters to be used in further DDI prediction work based on these results. The impact of the inhibitor $K_i$, $f_u$, $F_a$, $f_{\text{gut}}$, liver uptake and $k_a$ on the AUC ratio were assessed, investigating the full range for fractional values and a 10- or 100-fold range above and below the Simcyp default value for other parameters. Predictions of DDI are sensitive to the $K_i$ used for both ketoconazole and itraconazole. The simulations were based on the average doses (200mg in both cases), and the impact of the $K_i$ is therefore also related to this value and the concentration of inhibitor at the active site of the enzyme. The range of reported ketoconazole $K_i$ data highlighted the issue of variability in reported values of parameters; this range persisted even when differences in the probe substrate, metabolic pathway and cell concentrations were corrected (Greenblatt et al., 2010). This range resulted in a 3.5-fold difference in the AUC ratio and the imprecision in reported values of $K_i$ therefore results in significant differences in the prediction of DDIs. In house data was therefore selected for use in further DDI predictions, in order to reduce the potential sources of variability and error, and for consistency in the methods and cell lines/concentrations utilised. Alternative results were only selected when they could offer extra data or increase consistency in the values, for example the inhibition constant for both the parent drug, itraconazole and active metabolite, hydroxyitraconazole reported in Isoherranen et al., 2004. The increased impact on AUC ratio at lower values of the $f_u$, liver uptake and $k_a$ are likely to be rationalised by the resulting increase in the inhibitor concentration up to saturation; further parameter increase will not result in change to AUC ratio beyond this point. The prediction of DDIs did not appear to be sensitive to alterations in inhibitor $f_{\text{gut}}$ values in the case of itraconazole. However, the inconsistency between the
assumptions used for the values of $f_{u_{\text{gut}}}$ for inhibitors and victim drugs within Simcyp and the use of alternative inhibitors are likely to have a much larger impact. Despite the lack of difference observed in the current analysis, the assumption of inhibitor $f_{u_{\text{gut}}}$ equal to $f_{u_{p}}$ resulted from analysis during development of the Simcyp simulator. The most successful prediction of inhibitor profiles was observed utilising this assumption. Victim drug $f_{u_{\text{gut}}}$ was maintained at unity, as use of the $f_{u_{\text{gut}}}$ equal to $f_{u_{p}}$ has previously resulted in loss of $F_G$ prediction accuracy (Gertz et al., 2010; Yang et al., 2007b). The decrease in AUC ratio as a result of reductions in $F_a$ may be relevant to the predictions of DDI, as the assumption that this value equals unity is commonly utilised (including Brown et al., 2005; Einolf, 2007). The variability in inhibitor $k_a$ used in DDI predictions resulted in altered predictions of AUC ratio, and it was therefore selected for further analysis into its impact in the prediction of azole and SSRI DDIs (Chapter 4 and Chapter 5, respectively). Liver uptake was identified as an inhibitor property that lacked clear data in the literature from both in vitro and in vivo data. In addition, the sensitivity analysis highlighted the potential impact of this factor in the prediction of DDI. It was therefore selected to be addressed through in vitro experimental work (Chapter 3). The sensitivity analysis was based on assessment of an itraconazole-midazolam DDI at one dosage level, and conclusions are therefore specific to itraconazole (with the exception of $K_i$ where ketoconazole was additionally investigated). The analysis should however provide a good example of the sensitivity, as a result of high inhibitor potency and selectivity for CYP3A4. The analysis was therefore assumed to be applicable for extrapolation to additional DDIs involving alternative dose levels and inhibitor-victim drug combinations. The Simcyp prediction of inhibitor profiles was compared to reported data for the $C_{\text{max}}$, $T_{\text{max}}$ and AUC of ketoconazole, itraconazole, fluoxetine and fluvoxamine where these data were available. The majority of predictions were within 2-fold, and therefore considered to be successful representations of the profiles. Despite this trend, some over-prediction of $C_{\text{max}}$ data was observed, consistent to those observed for the profiles of victim drugs. Further consideration of the profile prediction was limited by the availability of reported data.

A database was collated consisting of 97 DDIs involving three azole (fluconazole, itraconazole and ketoconazole) and two SSRI (fluoxetine and fluvoxamine) CYP inhibitors. The database included 16 victim drugs across the five main CYP enzymes. The DDI studies covered the full range of inhibition potency with a representative number of DDIs per inhibitor and per victim drug in order to ascertain trends. In order to investigate the impact of individual properties of the database, the azole and SSRI DDIs were assessed separately (Chapter 4 and Chapter 5, respectively). The azole database was further
subdivided into DDIs involving the benzodiazepine victim drugs (n=38), allowing detailed analysis into the interplay of inhibitor and victim drug properties.

This chapter has provided the preliminary investigation into different *in vivo* and *in vitro* methods of estimation for the \( \text{fm}_{\text{CYP}} \), \( F_G \) and profiles of victim drugs, and sensitivity analysis into the impact that inhibitor parameters have on the prediction of DDI. The victim drug parameters, \( \text{fm}_{\text{CYP}} \) and \( F_G \) are intrinsically calculated within the Simcyp simulator, and the preliminary assessment determined that there should not be significant loss of prediction accuracy from the use of default victim drug parameters in the Simcyp simulator. The source of inhibitor parameters to be used in the prediction of DDIs was justified using the sensitivity analysis data. In addition the collation of a database of *in vivo* DDIs involving azole and SSRI inhibitors and a range of victim drugs involving different CYP enzymes has provided a sufficient number of interactions to assess the prediction of DDIs in Simcyp in further chapters.
CHAPTER 3: DRUG CLEARANCE IN DIFFERENT IN VITRO SYSTEMS
3 DRUG CLEARANCE IN DIFFERENT IN VITRO SYSTEMS

3.1 INTRODUCTION

The active hepatic uptake of CYP inhibitors may increase the extent of DDIs due to higher unbound concentrations of inhibitor available at the enzyme active site. The impact is observed when the higher unbound concentration at the active site does not result in higher clearance and therefore lower exposure of the inhibitor (Parker and Houston, 2008; Shitara et al., 2006; Soars et al., 2009). The ability to assess this phenomenon in vitro represents a valuable aid in the DDI prediction models (Hinton et al., 2008; Isoherranen et al., 2009; Parker and Houston, 2008; Rostami-Hodjegan and Tucker, 2004).

3.1.1 In vitro assays for measurement of hepatic uptake

The impact of hepatic uptake on drug clearance is commonly assessed through use of the oil-spin assay, where the drug (traditionally radiolabelled) and cell co-incubation is centrifuged through an oil layer (Hirano et al., 2004; Shitara et al., 2003; Soars et al., 2009). The cells permeate as the oil density is intermediate between the density of the cells and media. The intracellular drug is then measured from radiation levels or released via methanol and measured through LC-MS/MS (if non-radiolabelled), and compared to media drug levels. Despite being the standard, this assay is not conducive to high throughput (Soars et al., 2007).

The unbound hepatocytes-to-media partition coefficient ($K_{p,u}$) can be used as an in vitro indicator of uptake. It can be estimated from the ratios of either unbound $K_i$ or clearance values obtained in microsomes and hepatocytes, as illustrated in Equation 3.1. The $K_{p,u}$ has previously been obtained from comparison of the $K_{i,u}$ ratio between rat hepatocytes and microsomes for six inhibitors (18 metabolic pathways) with $K_{i,u}$ values covering three orders of magnitude (0.05-30μM) (Brown et al., 2007a). The mean ± S.D. (range) $K_{i,u}$ ratio was 1.29 ± 0.85 (0.07-3.33), suggesting no uptake of the inhibitors investigated once the $K_i$ values were corrected for the fraction unbound. This finding was in contrast to large cell to medium concentration ratios, ranging from 4.2-6000 in the case of fluconazole and miconazole, respectively. The only exception was the 3-methoxymorphinan pathway of fluvoxamine metabolism, where the 3.3-fold difference in $K_{i,u}$ was reported. Use of the $K_{i,u}$ ratio may be confounded by clearance of the inhibitor during the assessment of $K_i$, particularly for highly cleared compounds. Use of the unbound clearance ratio may therefore be more suitable to investigate uptake using in vitro methods. The assumption is that a higher clearance observed in hepatocytes is due to a higher intracellular
concentration as a result of the presence of uptake transporters and therefore may be used as an indicator of hepatic uptake.

\[
K_{p,u} = \frac{K_{i,u \text{ microsome}}}{K_{i,u \text{ hepatocyte}}} = \frac{CL_{\text{int,u hepatocyte}}}{CL_{\text{int,u microsome}}}
\]

The media loss assay has been proposed as another potential assay for preliminary assessment of hepatic uptake (Soars et al., 2007; Soars et al., 2009). This method calculates \(CL_{\text{int,u}}\) from the rate of drug loss from the media, assuming that this loss is the inverse of the rate of drug appearance in the hepatocytes. This assay is in contrast to the conventional depletion approach in hepatocytes, where rate of drug loss is assessed from the total incubation (both the cells and media). A higher observed \(CL_{\text{int,u}}\) from the media loss assay in comparison to the conventional assay in hepatocytes is expected in the case of uptake. For example, the under-prediction of the known OATP substrates, bosentan, pravastatin and atorvastatin from the conventional assay in human hepatocytes was reduced, with predictions within 4-fold of the \(in \text{ vivo}\) clearance from use of the media loss assay in human hepatocytes (Soars et al., 2007).

### 3.1.2 Selection of drugs and \(in vitro-in vivo\) methods to assess uptake

Five inhibitors were selected for analysis based on existing clinical DDIs (Chapter 2), including fluoxetine, fluvoxamine, fluconazole, itraconazole and ketoconazole. Fluoxetine, fluvoxamine and ketoconazole were all reported to extensively partition into cells \(in \text{ vitro}\), but were also highly bound inside the cell (Brown et al., 2007a). The SSRIs were found to sequester into lysosomes once inside the cell due to the pH difference and their lipophilic amine physicochemical properties (Hallifax and Houston, 2007). This finding was ascertained through the use of the adenosine triphosphate (ATP) inhibitor, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). A range (1.3-3-fold) of unbound partitioning of ketoconazole into the liver was reported using various models, including \(in \text{ vivo}\) mouse (2-fold, von Moltke et al., 1996), \(in \text{ vivo}\) rat (3-fold, Matthew et al., 1993; 1.3-fold Yamano et al., 1999a) and \(in \text{ vitro}\) rat hepatocytes (1.3-fold, Yamano et al., 1999b). There are also a number of alternative studies documenting potential uptake for the selected inhibitors, for example the partitioning of SSRIs into mouse liver (von Moltke et al., 1994a; von Moltke et al., 1995) and the reported 10 and 40-fold increase in fluvoxamine CYP1A2 and CYP2C19 \(K_i\) from \(in \text{ vivo}\) compared to \(in \text{ vitro}\) studies (Yao et al., 2001; Yao et al., 2003).
3.1.3 Aims

The aims of this chapter were to assess different in vitro methods for the assessment of hepatic uptake and the ability of these methods to predict in vivo clearance. Additionally, in vitro methods used both rat and human hepatocytes and microsomes, allowing additional comparison of species dependent differences in the prediction of clearance. For this study, a set of five well-known inhibitors of CYP enzymes, namely fluoxetine, fluvoxamine, fluconazole, itraconazole and ketoconazole were used, encompassing a range of different properties. The \( K_{i,u} \) ratio was previously investigated as a potential method to assess uptake in rat hepatocytes and microsomes; this chapter compared the findings of the \( K_{i,u} \) ratio to the results from the following experiments:

- \( CL_{i,u} \) ratio from the depletion of inhibitors in rat hepatocytes and microsomes
- \( CL_{i,u} \) ratio from the comparison of the conventional and media loss assay in rat hepatocytes
- Repetition of the above two \( CL_{i,u} \) ratios using human hepatocytes and microsomes

Finally, reported in vivo clearance values in rat and human were collated for the five inhibitors, in order to assess the ability of the different in vitro assays to predict hepatic clearance.
3.2 METHODS

3.2.1 Source of rat and human hepatocytes and microsomes

Hepatocytes from male Sprague-Dawley rats were prepared as detailed in Appendix 2, and experiments were undertaken within 4 hours of isolation in order to preserve viability. Hepatocyte preparations with viabilities greater than 85% (average ± S.D. of 92 ± 2%, n=18) were used. Pooled rat (male Sprague-Dawley) liver microsomes were obtained from Invitrogen™ (Paisley, U.K.), at a concentration of 20mg/mL (RT039; Appendix 3). Microsomes were stored at -80°C, thawed immediately prior to the experiment and were not refrozen more than once.

Pooled human hepatocytes (20 donors, Appendix 4) were purchased from Tebu-bio (Peterborough, U.K.) at a concentration of 10Mcells/mL (HPCH20, Lot 1010270), and stored in liquid nitrogen until use. The human hepatocytes were immediately thawed following removal from liquid nitrogen in a 37°C water bath, and poured into pre-warmed William’s Media E (WME) (37°C, pH 7.4) as soon as the ice crystals had melted (<90 seconds). The cells were then centrifuged at 50g for 3 minutes, supernatant removed and cells resuspended in Krebs-Henseleit Buffer (KHB). The viability (average ± S.D. of 71 ± 4%, n=12) and concentration of the cells was determined using a haemocytometer and Trypan blue (as detailed in Appendix 2), and cells diluted to the required concentration in KHB. Pooled human microsomes (22 donors, Lot 70196) were obtained from BD Biosciences™ (Bedford, MA), at a concentration of 20mg/mL. Microsomes were stored at -80°C, thawed immediately prior to the experiment and were only thawed once. For a full list of materials and donor information, see Appendix 1, 3 and 5.

3.2.2 Depletion studies in rat and human hepatocytes

Preliminary studies were carried out in rat hepatocytes in order to optimise incubation conditions with respect to the hepatocyte concentration and drug concentration to be utilised. The 125μL hepatocytes (final concentration range: 0.1-1.5Mcells/mL) were incubated for 5 minutes in an Eppendorf Thermomixer (37°C, 900rpm). To initiate the reaction 125μL pre-warmed (37°C) drug (final concentration range: 0.01 - 10μM) was added, giving a final incubation volume of 250μL. Experiments were performed in duplicate in William’s Media E and the organic solvent concentration (methanol) in the incubation was 0.05%. At 8 specified time points (up to 60 minutes) the reaction was terminated by addition of 250μL ice cold methanol (MeOH) containing the internal standard required for LC-MS/MS (Appendix 7). Samples were centrifuged at 2500g (MSE
Mistral 3000i, London, U.K.) for 20 minutes at 4°C and supernatant analysed by LC-MS/MS. Details of LC-MS/MS methods and internal standards used are shown in Appendix 7. The selected drug concentration (0.1μM) and cell concentration (Table 3.1) were utilised to repeat the depletion profiles in rat and human hepatocytes (n=3). A lower concentration of human hepatocytes was utilised in order to preserve cells.

### 3.2.2.1 Media loss assay

The media loss assay (based on the methods by Soars et al., 2007; Soars et al., 2009) was also undertaken at the drug and cell concentration selected in Section 3.2.2, in both human and rat hepatocytes. The media loss and conventional assay were undertaken within the same experiments, to n=3. The only difference between the protocols was that at the specific time points (up to 60 minutes) in the media loss assay the incubation was centrifuged at 10g for 30 seconds, and 100μL of supernatant added to 100μL ice cold MeOH containing internal standard to terminate the reaction. Five additional time points at t < 6 minutes were assessed to fully characterise the initial stage of the concentration-time profile. The samples were then centrifuged and analysed as outlined in Section 3.2.2. The conventional and media loss assay in rat hepatocytes were also assessed in the presence of 1μM FCCP for ketoconazole; this assay was in order to ascertain whether the presence of ATP dependent processes contributed to the observed clearance differences between the assays.

### 3.2.3 Depletion studies in rat and human liver microsomes

Preliminary studies were carried out in order to determine the rat liver microsomal concentration to be utilised. Drug concentrations (0.01-10μM) were identical to those used in the hepatocyte studies (Section 3.2.2) to allow valid comparison of systems. Phosphate buffer (0.1M, pH 7.4) containing NADPH regenerating system (final incubation concentration of 1mM NADP, 7.5mM isocitric acid, 15mM magnesium chloride, 1.2units/ml isocitrate dehydrogenase) was co-incubated with 25μL of the drug (final concentration range: 0.01 - 10μM) for 5 minutes in an Eppendorf Thermomixer (37°C, 900rpm). To initiate the reaction 25μL pre-warmed (37°C) microsomes (final concentration range: 0.1 – 0.5mg/mL) were added, giving a final incubation volume of 250μL. Experiments were performed in duplicate and the organic solvent concentration (MeOH) in the incubation was 0.1%. At 8 specified time points (up to 60 minutes, as Section 3.2.2) the reaction was terminated by addition of 250μL ice cold MeOH containing the internal standard required for LC-MS/MS. Samples were centrifuged at 2500g (MSE Mistral 3000i, London, U.K.) for 20 minutes at 4°C and supernatant analysed by LC-
Details of the LC-MS/MS methods and internal standards are shown in Appendix 7. The selected drug (0.1μM) and microsomal concentration (Table 3.1) were utilised to repeat the depletion profiles (n=3), in human and rat liver microsomes.

Table 3.1 Details the incubation conditions for five inhibitors for the microsome and hepatocyte studies.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rat liver microsome concentration (mg/mL)</th>
<th>Human liver microsome concentration (mg/mL)</th>
<th>Rat hepatocyte concentration (Mcells/mL)</th>
<th>Human hepatocyte concentration (Mcells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.5</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

3.2.4 Sample analysis

A standard curve was generated with each experiment covering the range of drug concentrations (0.005-10μM for preliminary experiments and 0.025-2.5μM for subsequent experiments). Standard samples were treated identically to the experimental samples following methods for the rat and human hepatocytes (Section 3.2.2) or microsomes (Section 3.2.3). For experiments involving fluoxetine, the formation of the active metabolite, norfluoxetine, was monitored in each sample for all assays through the LC-MS/MS methods (details shown in Appendix 7). Assays with fluconazole were only undertaken in rat hepatocytes and microsomes due to the minimal clearance observed, and the resultant inability to obtain a rate constant from the data.

3.2.5 Data analysis

3.2.5.1 Clearance calculation from depletion profiles

Data were analysed using non-linear regression in GraFit 5 (Erithacus Software Ltd, Horley, U.K.) in order to obtain the rate constant, as shown in Equation 3.2.

\[ C(t) = C_0 e^{-kt} \]

Equation 3.2

Where \( C \) is the concentration at a specified time point (t) or the initial concentration (\( C_0 \)) and \( k \) is the rate constant.
The time points for the media loss assay were adjusted to account for the time taken to centrifuge the sample prior to termination of the reaction (Section 3.2.2.1). The intrinsic clearance for each experiment was determined using the depletion approach across 60 minutes using two methods; the half-life method (Equations 3.3 and 3.4) and the AUC method (Equation 3.5).

**Half-life method:**

\[
CL_{\text{int}} = \frac{0.693V}{t_{1/2}}
\]

Equation 3.3

Where:

\[
t_{1/2} = \frac{0.693}{k}
\]

Equation 3.4

Where \( V \) is volume of incubation, \( t_{1/2} \) is half-life (from Equation 3.4) and \( k \) is the rate constant (obtained via non-linear regression analysis (Equation 3.2) using GraFit 5).

**AUC method:**

\[
CL_{\text{int}} = \frac{\text{Dose}}{\text{AUC}_{0-\infty}}
\]

Equation 3.5

Where Dose is the initial concentration in the incubation multiplied by volume, and AUC is the area under the concentration-time curve calculated using the trapezoidal rule (Equation 3.6) (due to the non-monoexponential decay of the profiles).

\[
\text{AUC}_{0-\infty} = \sum_{i=0}^{n-1} \frac{C_i + C_{i+1}}{2} \cdot (t_{i+1} - t_i) + \frac{C_{\text{last}}}{k}
\]

Equation 3.6

Where \( C_i \) is the concentration at the time point, \( t_i \), and \( n \) is the number of observed data points, \( C_{\text{last}} \) is the concentration at the last time point and \( k \) is the elimination rate constant (Equation 3.2). \( C_{\text{last}}/k \) extrapolates the AUC to infinity.

The norfluoxetine concentration over time was best described by Equation 3.7 according to the first rate law (Stringer et al., 2009). The rates were determined using nonlinear regression in GraFit5.
\[ M = M^\infty \left(1 - e^{-kt}\right) \]

Equation 3.7

Where \( M \) is the concentration of the metabolite, and \( M^\infty \) is the concentration of the metabolite determined from the equation, \( k \) is the rate constant for the metabolite formation and \( t \) is time.

### 3.2.5.2 Fraction unbound in the incubation

\( \text{CL}_{\text{int}} \) values were corrected for the fraction unbound in the incubations. Values of \( \text{fu}_{\text{mic}} \) and \( \text{fu}_{\text{hep}} \) were collated from previously published rat and human data, obtained experimentally via equilibrium dialysis (Brown et al., 2007a; Brown et al., 2006; Galetin et al., 2005; Hallifax and Houston, 2007; Isoherranen et al., 2004; Kilford et al., 2008). Values were standardized for altered cell concentrations to the required \( \text{fu}_{\text{mic}} \) or \( \text{fu}_{\text{hep}} \) where necessary using Equations 3.8 and 3.9, respectively (Gertz et al., 2008b; Hallifax and Houston, 2006; Kilford et al., 2008). Clearance values obtained from the media loss assay were not corrected for the fraction unbound due to the measurement of drug loss from the media (Soars et al., 2007).

\[ \text{fu}_{\text{mic}} = \frac{1}{1 + K_a \cdot P} \]

Equation 3.8

Where \( K_a \) is the microsomal protein binding affinity and \( P \) is the microsomal protein concentration. \( K_a \) was obtained by rearranging the Equation 3.8.

\[ \text{fu}_{\text{hep}} = \frac{1}{1 + K_p \cdot V_R} \]

Equation 3.9

Where \( K_p \) is the hepatocyte/medium concentration ratio and \( V_R \) is the \( V_{\text{cell}} \) (cell volume)/\( V_{\text{inc}} \) (incubation volume) ratio. \( K_p \) was found using \( 1 - \text{fu}_{\text{hep}}/(\text{fu}_{\text{hep}} \cdot V_R) \). The \( V_R \) is 0.005 at a cell concentration of 1Mcells/mL (Brown et al., 2007b); a \( V_R \) of 0.0025 and 0.00125 was used for 0.5 and 0.25Mcells/mL, respectively.

Experimental \( \text{fu}_{\text{inc}} \) values were used for correction where available. The lack of this data for itraconazole resulted in the additional calculation of \( \text{fu}_{\text{mic}} \) and \( \text{fu}_{\text{hep}} \) values from extrapolation of experimental \( \text{fu}_{\text{mic}} \) values to \( \text{fu}_{\text{hep}} \) (Equation 3.10) and using drug lipophilicity (Equations 3.11 and 3.12) (Gertz et al., 2008b; Hallifax and Houston, 2006; Kilford et al., 2008). These alternative methods were undertaken for all inhibitors for comparison.
Chapter 3 – In vitro clearance

Equation 3.10

\[
fu_{\text{hep}} = \frac{1}{1 + \frac{K_p}{K_a} \cdot \frac{V_R}{P} \cdot \left(1 - \frac{fu_{\text{mic}}}{fu_{\text{mic}}}\right)}
\]

Equation 3.11

\[
fu_{\text{mic}} = \frac{1}{1 + P \cdot 10^{0.072 \cdot \log P / D^2 + 0.067 \cdot \log P / D - 1.126}}
\]

Equation 3.12

\[
fu_{\text{hep}} = \frac{1}{1 + 125 \cdot V_R \cdot 10^{0.072 \cdot \log P / D^2 + 0.067 \cdot \log P / D - 1.126}}
\]

Where \(K_p\) is the hepatocyte/medium concentration ratio, \(K_a\) is the microsomal protein binding affinity, \(V_R\) is the \(V_{\text{cell}}\) (cell volume)/\(V_{\text{inc}}\) (incubation volume) ratio, \(P\) is the microsomal protein concentration (mg/mL) and \(\log P\) and \(\log D_{7.4}\) are the descriptors for drug lipophilicity for bases or acidic and neutral drugs, respectively. Calculation of \(fu_{\text{hep}}\) from Equation 3.10 used a value of 125 for the \(K_p/K_a\) ratio (Hallifax et al., 2010; Kilford et al., 2008). All \(\log P\) values used in Equations 3.11 and 3.12 were from experimental data, obtained from ChemIDPlus Advanced (Bethesda, MD, USA: http://chem.sis.nlm.nih.gov/chemidplus), as shown in Table 3.2.
Table 3.2 Microsomal and hepatocyte concentration utilised in the experiments and fraction unbound in microsomes and hepatocytes for the four inhibitors; fluoxetine, fluvoxamine, itraconazole and ketoconazole. Values of fraction unbound correlate to the concentration utilised in the experiments, and corrections using experimental fu_{inc} were used where possible.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Microsomal Concentration (mg/mL)</th>
<th>Hepatocyte Concentration (Mcells/mL)</th>
<th>LogP*</th>
<th>Experimental fu_{inc}: Rat</th>
<th>Experimental fu_{inc}: Human</th>
<th>Calculated from Eq. 3.10</th>
<th>Calculated from Eq. 3.11</th>
<th>Calculated from Eq. 3.12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat &amp; Human</td>
<td>Rat</td>
<td>Human</td>
<td></td>
<td></td>
<td>Rat</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>0.1</td>
<td>0.25</td>
<td>0.5</td>
<td>4.05</td>
<td>0.51</td>
<td>0.28</td>
<td>0.63</td>
<td>0.51</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>3.21</td>
<td>0.43</td>
<td>0.54</td>
<td>0.65</td>
<td>0.38</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>5.66</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>3.54</td>
<td>0.23</td>
<td>0.15</td>
<td>0.55</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* LogP values are all from experimental data, obtained from ChemIDplus Advanced.

Standardized from $^1$ 1mg/mL, $^2$ 0.5mg/mL, $^3$ 0.25mg/mL, $^4$ 0.1mg/mL, $^5$ 0.025mg/mL, (microsomes) $^6$ 0.5Mcells/mL, (hepatocytes).
3.2.5.3 *In vivo* clearance and scaling factors

Rat $\text{CL}_{\text{int,u}}$ values were scaled to *in vivo* clearance in mL/min/kg for hepatocytes by using a hepatocellularity of 120 Mcells/g liver, and for microsomes using a recovery of 45 mg protein/g liver. Both were then scaled using a liver weight of 43.6 g liver/kg (Davies and Morris, 1993; Houston and Carlile, 1997; Houston and Galetin, 2008; Ito and Houston, 2004; Ito and Houston, 2005; Parker and Houston, 2008). Corresponding values to scale human $\text{CL}_{\text{int,u}}$ were a hepatocellularity of 99 Mcells/g liver, microsomal recovery of 40 mg protein/g liver and a liver weight of 21.4 g liver/kg (Davies and Morris, 1993; Hakooz et al., 2006; Houston and Galetin, 2008; Ito and Houston, 2005). Additional analysis was performed to compare the results from the three *in vitro* assays to collated literature reports of *in vivo* clearance in rats and humans. The mean value was taken where multiple reports were found or reports covering different dose levels, although not corrected for the number of subjects due to the lack of data. Total plasma clearance was corrected for renal clearance and blood:plasma ratio using Equation 3.13, and converted to hepatic intrinsic clearance using the well stirred model for i.v. (Equation 3.14) or oral (Equation 3.15) dosing (Pang and Rowland, 1977; Rowland and Matin, 1973; Wilkinson and Shand, 1975).

$$
\text{CL}_H = \frac{\text{CL}_p - \text{CL}_R}{R_B}
$$

Equation 3.13

$$
\text{CL}_{\text{int,h}} = \frac{\text{CL}_H}{\text{fu}_p} \left( \frac{\text{CL}_H}{R_B} \right)
$$

Equation 3.14

$$
\text{CL}_{\text{int,h}} = \frac{\text{D}}{\text{AUC} \cdot \text{fu}_b} \cdot \text{F}_G \cdot \text{F}_a
$$

Equation 3.15

Where $\text{CL}_{\text{int,h}}$ is hepatic intrinsic clearance, $\text{CL}_p$ is total plasma clearance, $\text{CL}_H$ is hepatic blood clearance, $\text{CL}_R$ is renal clearance, $R_B$ is the blood:plasma ratio, $\text{fu}_p$ is fraction unbound in plasma, $Q_H$ is hepatic blood flow, $D$ is dose, AUC is area under the plasma concentration time curve, $\text{fu}_b$ is fraction unbound in blood (from $\text{fu}_p/R_B$), $F_G$ is fraction escaping metabolism in the gut and $F_a$ is the fraction absorbed. All inhibitors were weak bases (Grime et al., 2009; Johnson, 2009), therefore the blood:plasma ratio was assumed to be 1, and the fraction absorbed was 0.59 and 0.55 for ketoconazole and itraconazole, respectively (Ito et al., 1998; Thummel et al., 2008); reported $F_a$ values for fluoxetine and fluvoxamine were > 0.9, and therefore assumed to be 1. Values for $Q_H$ were 100 mL/min/kg and 20.7 mL/min/kg for rats and humans, respectively (Brown et al., 2007b; Davies and Morris, 1993; Ito and Houston, 2004; Yang et al., 2007a), and it was assumed...
that there was no metabolism in the gut. Values of human \( f_{up} \) and \( \text{CL}_R \) were collated from the literature; \( f_{up} \) was 0.06, 0.23, 0.036 and 0.017 for fluoxetine, fluvoxamine, itraconazole and ketoconazole respectively (Brown et al., 2006; Isoherranen et al., 2004) and assumed to be consistent between the two species.

Human \( \text{CL}_R \) had a minor contribution to total clearance in all cases (<2.5% for the SSRI inhibitors and < 1% for the azoles, Thummel et al., 2008) and scaled to rat \( \text{CL}_R \) using the following equation (Lin, 1998):

\[
\text{CL}_{R, \text{rat}} = \text{CL}_{R, \text{human}} \cdot \text{GFR Ratio}
\]

**Equation 3.16**

Where the rat:human GFR (glomerular filtrate rate) ratio is 4.8 (Lin, 1998).
3.3 RESULTS

3.3.1 Preliminary work

Preliminary experiments were carried out using the conventional assay in rat hepatocytes as per Section 3.2.2 to determine linearity with cell concentration (0.1-1.5Mcells/mL) and time (0-60 minutes) (Appendix 6). Low concentrations of hepatocytes (<1Mcells/mL) were selected to minimise the impact of binding. In addition, $\text{CL}_{\text{int}}$ was assessed across a 1000-fold drug concentration range in order to select concentrations within the linear range (Figure 3.1), with clearance determined from the half-life method and corrections for fraction unbound using experimental values where possible (Section 3.2.5).

![Figure 3.1](image-url)  

**Figure 3.1** $\text{CL}_{\text{int},u}$ of fluoxetine (A), fluvoxamine (B), itraconazole (C) and ketoconazole (D) in rat hepatocytes across a 1000-fold concentration range, in order to select drug concentrations within the linear phase.

All compounds displayed linearity at concentrations < 0.1μM and therefore 0.1μM was selected for all further analysis. The same drug concentration (0.1μM) was used across all systems to allow valid comparisons, and the hepatic cell concentration kept consistent across the conventional and media loss assay. Microsomal protein linearity studies were repeated using 0.1μM in rat microsomes, and concentrations <0.5mg/mL selected to
minimise binding. The clearance of fluconazole in both rat microsomes and rat hepatocytes was negligible (<2% in all systems), as exemplified in Figure 3.2 from the conventional assay in rat hepatocytes. The rate constant could therefore not be determined and fluconazole was excluded from further analysis.

**Figure 3.2** Depletion plot for fluconazole from the conventional assay in rat hepatocytes. Data represent mean ± S.D. (n=3).

### 3.3.2 Depletion in rat hepatocytes and microsomes

Depletion plots for the four selected inhibitors from the conventional and media loss assay in rat hepatocytes and rat microsomes (n=3 for each assay) are displayed in Figures 3.3 and 3.4.
Figure 3.3 $\text{CL}_{\text{int,}u}$ obtained in different rat *in vitro* systems for fluoxetine (A-C) and fluvoxamine (D-F), where; ● represents conventional assay in rat hepatocytes (A, D), ■ media loss assay in rat hepatocytes (B, E) and ▲ rat microsomes (C, F). Data represent mean ± S.D. (n=3).
Figure 3.4 \( CL_{int,ui} \) obtained in different rat *in vitro* systems for itraconazole (A-C) and ketoconazole (D-F), where;  ● represents conventional assay in rat hepatocytes (A), ■ media loss assay in rat hepatocytes (B) and ▲ rat microsomes (C). Data represent mean ± S.D. (n=3).
Both SSRI inhibitors displayed biphasic profiles in the conventional assay in hepatocytes (Figure 3.3A and D, Figure 3.5A-D), where the depletion of the azole inhibitors was linear (Figure 3.4A and D, Figure 3.5E-F). The media loss profiles for all inhibitors were biphasic (Figure 3.3-3.4C and F, Figure 3.5). Linearity was observed in the SSRI profiles in both hepatocytes assays at time points > 6 minutes with consistent rate constants describing the metabolism phase (Figure 3.5A and C). The difference observed in the initial rate (< 6 minutes) of drug loss from the media loss assay (Figure 3.5B and D) was attributable to uptake (and binding) of the drug. Depletion in the microsomes was linear up to 40 minutes for all inhibitors; loss of linearity was only observed for the final time point (60 minutes) for all inhibitors. This profile was clearly displayed for fluvoxamine (Figure 3.3F). Variation in the extent of depletion over the time course was observed, with remaining concentration at 60 minutes ranging from 1-35% of the initial concentration for fluvoxamine (media loss assay) and itraconazole (conventional assay in hepatocytes), respectively. The extent of depletion was greater from the media loss assay than the conventional assay in rat hepatocytes. There was no overall correlation between the extent of depletion after 60 minutes between rat hepatocytes (conventional assay) and microsomes. The fraction remaining at 60 minutes from the two systems were similar for fluoxetine (5% for both systems) and itraconazole (35 and 30% remaining from hepatocytes and microsomes, respectively). Differences were observed for fluvoxamine (with 2-fold greater fraction remaining in microsomes) and ketoconazole (8-fold greater fraction remaining in hepatocytes). Overall, the differences between the fraction remaining at 60 minutes across the three assays were minimal for the SSRI inhibitors with differences < 8%, compared to the 26% difference observed for the azole inhibitors. The conventional and media loss in rat hepatocytes were repeated for ketoconazole in the presence of FCCP. No difference was observed in the profiles, therefore indicating that ATP-dependent processes are not responsible for the differences observed between the conventional and media loss assays.
Figure 3.5 Depletion profiles from the conventional assay (circles) or media loss assay (squares) in rat hepatocytes. Profiles show fluoxetine (A & B), fluvoxamine (C & D), itraconazole (E) and ketoconazole (F). B and D magnify the initial phase of the profiles (< 11 minutes). Data represent mean ± S.D. (n=3).

3.3.2.1 Calculation of rat CL_{int,u}

Values of the rat fu_{inc} from experimental data and calculated from lipophilicity are displayed in Table 3.2. The rat fu_{inc} values calculated from lipophilicity (Equations 3.11 and 3.12) were consistently higher than those from experimental values, with a mean over-prediction of 1.6-fold (range 1.5-1.6-fold) for fu_{mic} and 2.2-fold (range 1.4-3.0-fold, for fluvoxamine and fluoxetine, respectively) for fu_{hep}. The fu_{hep} estimated from experimental fu_{mic} values (Equation 3.10) were more consistent to the experimental fu_{hep} values, with estimations within 1.4-fold (with a range from 71% of the experimental fu_{hep} to 1.4-fold
over-prediction for fluvoxamine and fluoxetine, respectively. No experimental $f_{\text{inc}}$ values were available for itraconazole in rat. Calculated $\text{CL}_{\text{int}}$ values from the half-life (Equation 3.3) and AUC methods (Equation 3.5), and corrected for $f_{\text{inc}}$ are displayed in Table 3.3.
Table 3.3 Rat CL_{int,u} values for 4 inhibitors investigated calculated from the half-life or AUC method. Values represent mean ± S.D. (n=3). Corrections for fu_{inc} use experimental values (a), estimations from lipophilicity (b), fu_{hep} estimated from experimental fu_{mic} (c) or are not corrected (d) (Section 3.2.5.2). Order of preference for fu_{inc} corrections for use in further analysis was (a), (c) then (b).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hepatocyte conventional assay CL_{int,u} (µL/min/Mcells)</th>
<th>Hepatocyte media loss assay CL_{int,u} (µL/min/Mcells)</th>
<th>Microsome assay CL_{int,u} (µL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td><strong>Half-life method</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>363 ± 95.7</td>
<td>135 ± 35.7</td>
<td>254 ± 67.0</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>76.6 ± 21.3</td>
<td>58.9 ± 16.4</td>
<td>109 ± 30.3</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>-</td>
<td>101 ± 29.2</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>35.1 ± 1.13</td>
<td>16.4 ± 0.53</td>
<td>27.7 ± 0.90</td>
</tr>
<tr>
<td><strong>AUC method</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>262 ± 57.2</td>
<td>97.8 ± 21.3</td>
<td>184 ± 40.0</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>35.0 ± 22.9</td>
<td>26.9 ± 17.6</td>
<td>49.8 ± 32.5</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>-</td>
<td>99.3 ± 27.8</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>34.6 ± 1.18</td>
<td>16.1 ± 0.55</td>
<td>27.3 ± 0.93</td>
</tr>
</tbody>
</table>
Table 3.4 Coefficient of Variation (CV) (%) values from the half-life and AUC methods of rat CL_{int,u} estimation for the 4 inhibitors. Values are calculated from the overall mean ± S.D. CL_{int,u} from each assay.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hepatocyte CV (%)</th>
<th>Microsome CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional assay</td>
<td>Media loss assay</td>
</tr>
<tr>
<td></td>
<td>Half-life</td>
<td>AUC</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>28</td>
<td>65</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

No consistency was observed between the rank order of the four inhibitors from the three assays, with ketoconazole < fluvoxamine < itraconazole < fluoxetine for the conventional assay in rat hepatocytes. The media loss assay also reported fluoxetine to have the greatest CL_{int,u}, however the rank order was itraconazole < ketoconazole < fluvoxamine for the remaining three inhibitors. A difference in rank order was also observed from the microsomal assay, with fluvoxamine < ketoconazole < fluoxetine < itraconazole. Differences between the half-life and AUC methods of CL_{int,u} calculation were minimal for the conventional assay in hepatocytes and the microsomal assay, with the majority (6/8) of clearances <1.2-fold. The remaining two studies were the biphasic profiles of the SSRI inhibitors from the conventional assay in rat hepatocytes, where a 1.4- and 2.2-fold greater CL_{int,u} from the half-life method was observed for fluoxetine and fluvoxamine, respectively, attributable to the differences in rate constants as a result of the observed biphasic profile. The minimal difference observed between the half-life and AUC methods for the microsomal assay indicates that the loss of linearity observed with the final time point did not result in a significant difference in the CL_{int,u} calculation. Greater differences were observed between the half-life and AUC methods of CL_{int,u} calculation from the media loss assay for all inhibitors, with a 2.8-7.1-fold greater CL_{int,u} from use of the half-life method. The differences resulted from profiles that did not display mono-exponential decay; the half-life method uses the initial rate of drug loss in clearance calculation, attributable to the uptake (and binding) where the AUC will use the rate of metabolism. In all of the experiments the rate of uptake exceeded the rate of metabolism. Although a full profile would give a better indication of the total CL_{int,u} of the compound, the initial rate approach isolates the impact of the uptake (and binding) of the drug from the metabolism and is therefore more suitable for the media loss assay (Soars et al., 2007). Therefore, to
allow a valid comparison of clearance when comparing the media loss assay to others, the half-life method was selected for comparison to \textit{in vivo} values. The coefficient of variation (CV) was predominantly smallest in the rat microsomal assay and highest in the media loss assay in rat hepatocytes, and higher from the half-life method of $CL_{int}$ estimation than the AUC method. Exceptions to this trend were observed with fluvoxamine, where the CV from the conventional assay in rat hepatocytes was higher than the media loss assay, and CV from the AUC method of $CL_{int}$ estimation was higher than the half-life method in all assays.

Values of experimental $fu_{inc}$ were available for all inhibitors except for itraconazole, and were the preferred value to utilise for the correction of $CL_{int}$. As values of experimental $fu_{inc}$ for itraconazole were not reported in rats, $fu_{inc}$ values were estimated from lipophilicity for all inhibitors in order to assess the impact of the method of $fu_{inc}$ estimation on $CL_{int,u}$. Alternate $fu_{hep}$ corrections resulted in 1.3-2.7-fold greater $CL_{int,u}$ for fluoxetine and fluvoxamine (conventional assay in hepatocytes), respectively resulting from the use of $fu_{hep}$ from experimental values than from those estimated from lipophilicity. Correction for $fu_{hep}$ when estimated from experimental $fu_{mic}$ values resulted in $<2$-fold difference to those calculated by experimental $fu_{hep}$ values. Differences as a result of correction of microsomal $fu_{mic}$ values showed a consistent 1.6-1.7-fold greater $CL_{int,u}$ from use of experimental $fu_{mic}$ compared to those estimated from lipophilicity. Despite these differences ($<3$-fold), the high LogP values of all of the inhibitors (LogP $> 3$) resulted in reduced confidence in the $fu_{inc}$ estimated from lipophilicity (Gertz et al., 2008b), and error may have been introduced in its use for the correction of itraconazole $CL_{int}$.

3.3.2.2 Overall comparison of rat $CL_{int,u}$ from different \textit{in vitro} assays

The overall comparison of scaled $CL_{int,u}$ utilising rat microsomes and the conventional and media loss assays in rat hepatocytes for the four inhibitors is shown in Figure 3.6. All $CL_{int,u}$ values were obtained by the half-life method, corrected using the experimentally determined $fu_{inc}$ where possible, and scaled as described in Section 3.2.5.3.
Chapter 3 – In vitro clearance

Figure 3.6 Comparison of the CL_{int,u} for 4 inhibitors estimated from different rat in vitro systems, where; • represents fluoxetine, ● fluvoxamine, ▲ itraconazole and ▲ ketoconazole. A. Comparison between CL_{int,u} from rat hepatocytes (conventional assay) and microsomes. B. Comparison between estimations from the conventional and media loss assay in rat hepatocytes. Data represent mean ± S.D. (n=3).

Difference in scaled CL_{int,u} between the conventional depletion assays in rat hepatocytes and rat microsomes was minimal (1.2-fold greater in hepatocytes) for itraconazole, in contrast to the 3-fold greater clearance in rat hepatocytes for fluvoxamine and fluoxetine. Conversely, ketoconazole had a 2-fold higher CL_{int,u} in microsomes than hepatocytes. All inhibitors except for itraconazole displayed uptake, with higher CL_{int,u} from the media loss assay than the conventional assay in rat hepatocytes. This difference ranged from 2-fold for fluoxetine to 4-fold for fluvoxamine and ketoconazole. Itraconazole had a 2-fold higher CL_{int,u} from the conventional assay than the media loss assay in rat hepatocytes.

Minimal (<2-fold) difference between the conventional depletion assay in rat hepatocytes and microsomes was observed when using the AUC ratio method of CL_{int,u} estimation (instead of the half-life method) for fluvoxamine, itraconazole (1.3- and 1.2-fold greater clearance in rat hepatocytes, respectively) and ketoconazole (1.7-fold greater clearance in rat microsomes). However, fluoxetine CL_{int,u} remained 2.5-fold higher in rat hepatocytes than microsomes. Differences in the CL_{int,u} between the conventional and media loss assay in rat hepatocytes were observed when using the AUC method of CL_{int,u} calculation, including 8.3-fold higher CL_{int,u} from the conventional assay for itraconazole. No difference was observed for fluoxetine and ketoconazole (differences within 2-fold), whereas the media loss assay displayed 2.5-fold higher CL_{int,u} for fluvoxamine.
The range of the CL_{int,u} for the four inhibitors differed between the three in vitro methods. This range was minimal (4-fold) in the case of depletion from rat microsomes. A larger range was observed from the rat hepatocytes, with a 10- to 14-fold range between minimal and maximal CL_{int,u} from the conventional and media loss assays, respectively.

3.3.2.3 Formation of metabolite in the in vitro assays in rat

Formation of the metabolite of fluoxetine, norfluoxetine, was also monitored and displayed a biphasic relationship over time in all in vitro systems used (Figure 3.7). Maximum metabolite production was observed in microsomes, with norfluoxetine concentrations reaching 58 ± 11% of the administered 0.1μM fluoxetine. Lower formation was observed in rat hepatocytes, at 25 ± 2.8% and 2.1 ± 1.0% of the initial fluoxetine concentration for the conventional and media loss assays, respectively. The rate of metabolite formation in the two hepatocytes assays was double the rate observed from the microsomal assay. The initial rate of norfluoxetine formation was greater than the initial rate of fluoxetine depletion in all three assays. The maximum formation of norfluoxetine was attained at 10 minutes, compared to fluoxetine depletion rates reducing after 40 minutes. The total formation of norfluoxetine did not equal the total depletion of fluoxetine, therefore indicating the presence of alternative metabolic pathways, or experimental error in the measurement of drug concentrations.
Figure 3.7 Norfluoxetine formation in the different rat in vitro methods where; • conventional assay in rat hepatocytes (A), ■ media loss assay in rat hepatocytes (B) and ▲ microsomes (C). Data represent mean ± S.D. (n=3).
3.3.2.4 Comparison of rat CL_{int,u} values to in vivo values

Values of in vivo clearance in rat were collated from the literature for the four selected inhibitors (Caccia et al., 1990; Higashi et al., 2005; Matthew et al., 1993; Sjoberg et al., 1988; Yamano et al., 1999a) to compare to results from the in vitro assays (Table 3.5, Figure 3.8). All studies reported clearance following i.v. dosing in male rats with the exception of Sjoberg et al., 1988, where both male and female rats were dosed. All rat in vitro assays in this chapter utilised microsomes and hepatocytes from male rats, therefore the in vivo clearance values from female were excluded from the comparison. In addition, ketoconazole displayed non-linearity with increasing dose, and a high variability in clearance (e.g. 10-fold from 10mg/kg dosing), therefore clearance from dosing \leq 3mg/kg were utilised (n=3). The in vivo clearances were scaled to CL_{int,u} using the well stirred model and Equation 3.14.

Table 3.5 Details of the in vivo studies reporting rat clearance of the four selected inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose (mg/kg)</th>
<th>No. rats</th>
<th>CL (mL/min/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>8</td>
<td>4</td>
<td>5.51</td>
<td>Higashi et al., 2005</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>2.5</td>
<td>5</td>
<td>46.9</td>
<td>Caccia et al., 1990</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>45.4</td>
<td>Caccia et al., 1990</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>47.7</td>
<td>Caccia et al., 1990</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>5</td>
<td>3</td>
<td>14.0</td>
<td>Yamano et al., 1999a</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>10</td>
<td>3</td>
<td>4.40</td>
<td>Yamano et al., 1999a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>9.05</td>
<td>Sjoberg et al., 1988</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5</td>
<td>7.58</td>
<td>Sjoberg et al., 1988</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5</td>
<td>2.11</td>
<td>Sjoberg et al., 1988</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5 (^1)</td>
<td>9.37</td>
<td>Sjoberg et al., 1988</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>5-8</td>
<td>10.6</td>
<td>Matthew et al., 1993</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5-8</td>
<td>7.74</td>
<td>Matthew et al., 1993</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5-8</td>
<td>5.00</td>
<td>Matthew et al., 1993</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5-8</td>
<td>2.80</td>
<td>Matthew et al., 1993</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5-8</td>
<td>1.24</td>
<td>Matthew et al., 1993</td>
</tr>
</tbody>
</table>

\(^1\) 35 day old rats (compared to 60 days for other doses in Sjoberg et al., 1988).
Figure 3.8 Comparison of clearance estimated from different rat \textit{in vitro} assays from log predicted/observed mean \textit{in vivo} rat CL\textsubscript{int,h}. Symbols represent clearance from the conventional (circle symbols) or media loss (square symbols) assay in rat hepatocytes, or from rat microsomes (triangle symbols), where; \textbullet~fluoxetine, \textcircled{r}~fluvoxamine, \textshaded{g}~itraconazole and \textshaded{b}~ketoconazole. Error bars have been removed for clarity.

Over half (58\%) of rat \textit{in vitro} clearance values over-predicted the \textit{in vivo} clearance, by up to 40-fold (fluoxetine, media loss assay in hepatocytes). Three of the five under-predictions of \textit{in vivo} clearance were $> 2$-fold difference, and did not correlate to one assay or inhibitor. The average fold over-prediction was 5.5, 11.4 and 2.1 for the conventional assay or media loss assay in rat hepatocytes and microsomes, respectively. All of these averages were skewed by fluoxetine; the average predictions from the conventional assay in rat hepatocytes and rat microsomes were within 1.4-fold of the \textit{in vivo} clearance, and the over-prediction from the media loss assay reduced to 2-fold when it was excluded. The clearance of azole inhibitors was successfully predicted ($< 2$-fold difference) by the conventional assay in hepatocytes and microsomes, with the exception of the ketoconazole clearance from the conventional assay in rat hepatocytes, predicted at 37\% of the \textit{in vivo} clearance. With this exception, the poorest predictions were from the media loss assay in all other cases. The \textit{in vivo} rank order of clearance (fluoxetine $<$ fluvoxamine $<$ itraconazole $<$ ketoconazole) was not correctly identified in any of the assays.

3.3.3 Depletion in human hepatocytes and microsomes

Figures 3.9 and 3.10 display depletion plots for the four inhibitors from the conventional and media loss assay in human hepatocytes and from microsomes (n=3 for each assay).
Figure 3.9 $\text{CL}_{\text{int,u}}$ obtained in different human *in vitro* systems for fluoxetine (A-C) and fluvoxamine (D-F), where: ● represents conventional assay in human hepatocytes (A, D), ■ media loss in human hepatocytes (B, E), and ▲ human microsomes (C, F). Data represent mean ± S.D. (n=3).
Figure 3.10 $\text{CL}_{\text{int,u}}$ obtained in different human in vitro systems for itraconazole (A-C) and ketoconazole (D-F), where: ● represents conventional assay in human hepatocytes (A), ■ media loss in human hepatocytes (B), and ▲ human microsomes (C). Data represent mean ± S.D. (n=3).
All of the depletion profiles from the conventional assay in human hepatocytes displayed mono-exponential decay, and all of the media loss profiles in human hepatocytes were biphasic (Figures 3.9-3.10B) as expected from separation of the uptake and metabolism phases. Profiles of the SSRI inhibitors from human microsomes displayed mono-exponential decay, however biphasic profiles were evident for the azole inhibitors. The metabolism phase (terminal slope) in the media loss profiles was consistent with the conventional assay for the SSRI inhibitors as expected, but was not for the azole inhibitors. Variation in the extent of depletion over the time course was observed, with remaining concentration at 60 minutes ranging from 10-97% of the initial concentration for ketoconazole (microsomal assay) and fluoxetine and fluvoxamine (conventional assay in hepatocytes), respectively. The extent of depletion was greater from the media loss assay in human hepatocytes and in human microsomes than the conventional assay in human hepatocytes for all inhibitors. This difference was 15-64% (for ketoconazole and fluoxetine, respectively) between the media loss and conventional assay in human hepatocytes. Conversely, inhibitor dependent differences were observed between human hepatocytes (conventional assay) and microsomes, with minimal (<15%) difference observed for the SSRI inhibitors, and a 34-39% difference for the azole inhibitors. Overall, the differences between the fraction remaining at 60 minutes across the three assays were similar for all inhibitors (34-39%) with the exception of fluoxetine (64%).

3.3.3.1 Calculation of human CL_{int,u}

The different human \( fu_{inc} \) values (from experimental and calculated from lipophilicity) are displayed in Table 3.2. The \( fu_{mic} \) values estimated from lipophilicity were similar to the experimental values, with an average over-prediction of 1.2-fold (range 0.7-2.0-fold for ketoconazole and itraconazole, respectively). No experimental values were available for human \( fu_{hep} \), however the differences between the \( fu_{hep} \) estimated from lipophilicity and from human experimental \( fu_{mic} \) values were consistent with the differences observed from rat \( fu_{hep} \) values. Values of \( fu_{hep} \) from experimental \( fu_{mic} \) were therefore utilised for comparison to the human in vivo CL_{int,u}. Human CL_{int,u} values calculated from the half-life (Equation 3.3) and AUC methods (Equation 3.5) and corrected by different \( fu_{inc} \) values are displayed in Table 3.6.
Table 3.6 Human CL_{int,u} values for 4 inhibitors investigated calculated from the half-life or AUC method. Values represent mean ± S.D. (n=3). Corrections for f_{unc} use experimental values (a), estimations from lipophilicity (b), f_{unep} estimated from experimental f_{unc} (c) or were not corrected (d) (Section 3.2.5.2). Order of preference for f_{unc} corrections for use in further analysis was (a), (c) then (b).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hepatocyte conventional assay CL_{int,u} (μL/min/Mcells)</th>
<th>Hepatocyte media loss assay CL_{int,u} (μL/min/Mcells)</th>
<th>Microsome assay CL_{int,u} (μL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>b</td>
<td>d</td>
</tr>
<tr>
<td><strong>Half-life method</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>1.60 ± 1.35</td>
<td>1.00 ± 0.85</td>
<td>1125 ± 190</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>0.89 ± 0.10</td>
<td>0.71 ± 0.08</td>
<td>1411 ± 301</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>603 ± 144</td>
<td>241 ± 57.5</td>
<td>2968 ± 218</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>44.2 ± 29.2</td>
<td>67.7 ± 44.8</td>
<td>1252 ± 906</td>
</tr>
<tr>
<td><strong>AUC method</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>1.64 ± 1.36</td>
<td>1.03 ± 0.85</td>
<td>2.06 ± 1.67</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>0.86 ± 0.13</td>
<td>0.68 ± 0.11</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1054 ± 341</td>
<td>422 ± 136</td>
<td>54.7 ± 9.98</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>149 ± 140</td>
<td>228 ± 214</td>
<td>30.7 ± 15.5</td>
</tr>
</tbody>
</table>
Table 3.7 CV (%) values from the half-life and AUC methods of human CL_{int,u} estimation for the 4 inhibitors. Values are calculated from the overall mean ± S.D. CL_{int,u} for each assay.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hepatocyte CV (%)</th>
<th>Microsome CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional assay</td>
<td>Media loss assay</td>
</tr>
<tr>
<td></td>
<td>Half-life</td>
<td>AUC</td>
</tr>
<tr>
<td>CL_{int,u} estimation method:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>84</td>
<td>83</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>66</td>
<td>94</td>
</tr>
</tbody>
</table>

Consistency was seen with the rank order of CL_{int,u} from the three assays, with fluoxetine ≈ fluvoxamine < ketoconazole < itraconazole observed for the conventional assays in human hepatocytes and microsomes. Similar rank order was observed for the media loss assay in human hepatocytes, with the greatest clearance observed for itraconazole; the remaining three inhibitors were approximately equal. Differences between the half-life and AUC method of CL_{int} correction were minimal (<1.1-fold) for the conventional assay in human hepatocytes and human microsomes for the SSRI inhibitors, consistent with the linearity of the profiles. In contrast, the half-life method estimated CL_{int,u} at 546- and 1907-fold higher than the AUC method for the media loss assay for fluoxetine and fluvoxamine, respectively as a direct result of the rapid rate of initial drug loss. The azole inhibitors differed in that a 1.7- and 3.4-fold higher CL_{int,u} for itraconazole and ketoconazole, respectively resulted from the AUC method compared to the half-life method from the conventional assay in human hepatocytes. This difference was due to the discrepancy between the rates of metabolism observed from the two assays for the azole inhibitors. The half-life resulted in greater CL_{int,u}, consistent with the greater rate of uptake (and binding) than metabolism. For human microsomes, the half-life method resulted in greater CL_{int,u} than the AUC method at approximately 2-fold for both of the SSRIs and 54- and 41-fold for itraconazole and ketoconazole, respectively, as a result of the non-linearity of the profiles. No correlations were apparent between the CV from the three different assays (11-94%, 4-81% and 7-71% for the conventional or media loss assay in human hepatocytes, or human microsomes, respectively), the CV per inhibitor (17-84%, 4-71%, 7-32% and 13-94% for fluoxetine, fluvoxamine, itraconazole and ketoconazole, respectively).
or the CV from the half-life and AUC methods of $CL_{int,u}$ calculation, with a large range for both (7-84% and 4-94%, respectively).

Experimental $fu_{hep}$ values were not available for human hepatocytes, therefore $fu_{hep}$ was estimated from the experimental human $fu_{mic}$ values. All differences between $CL_{int,u}$ from the use of this $fu_{hep}$ to that estimated from lipophilicity were < 2-fold, with the exception of itraconazole (2.5-fold). Changes in $CL_{int,u}$ from the experimental and lipophilicity $fu_{mic}$ values were consistent to the $fu_{hep}$ results, with all differences < 2-fold. A minor decrease in the $fu_{inc}$ calculated from lipophilicity compared to the experimental values was observed for ketoconazole (0.37 and 0.32 from lipophilicity compared to 0.55 and 0.49 from experimental $fu_{mic}$ and $fu_{hep}$, respectively), resulting in 1.5-fold differences to the $CL_{int,u}$.

### 3.3.3.2 Overall comparison of human $CL_{int,u}$ from different in vitro assays

The overall comparison of scaled $CL_{int,u}$ from the human microsomes and human conventional and media loss assays for the four inhibitors is shown in Figure 3.11. All $CL_{int,u}$ values were obtained by the half-life method, corrected using the experimental $fu_{mic}$ and $fu_{hep}$ estimated from the experimental $fu_{mic}$, and scaled as per Section 3.2.5.3.

![Figure 3.11](image)

**Figure 3.11** Comparison of the $CL_{int,u}$ for 4 inhibitors estimated from different human in vitro methods, where; ● fluoxetine, ○ fluvoxamine, ● itraconazole and ● ketoconazole. A. Comparison between $CL_{int,u}$ from human hepatocytes (conventional assay) and microsomes. B. Comparison between estimations from the conventional and media loss assay in human hepatocytes. Data represent mean ± S.D. (n=3).

The difference in scaled $CL_{int,u}$ between the conventional depletion assays in human hepatocytes and microsomes was minimal, with an average 1.9-fold higher $CL_{int,u}$ in
human hepatocytes (and range of 1.1- to 2.6-fold for itraconazole and ketoconazole, respectively). All inhibitors displayed higher $\text{CL}_{\text{int,u}}$ from the media loss assay than the conventional assay in human hepatocytes. This difference ranged from 4.9- to 1590-fold for itraconazole and fluvoxamine, respectively, with an average difference of 580-fold.

Differences between the conventional assay in human hepatocytes and microsomes were greater using the AUC method of $\text{CL}_{\text{int,u}}$ estimation, with average difference of 6.5-fold and range of 1.8 to 17.5-fold greater in hepatocytes for fluvoxamine and ketoconazole, respectively. Conversely, the differences observed between the conventional and media loss assay in human hepatocytes were reduced for the SSRI inhibitors, with the ratios of fluoxetine and fluvoxamine now $< 1.25$-fold. Greater differences were observed for the azole inhibitors, with a 9- and 2-fold lower $\text{CL}_{\text{int,u}}$ observed from the media loss assay than the conventional assay for itraconazole and ketoconazole, respectively. These are markedly different to the differences observed when the half-life method is utilised; use of the half-life method appears to result in the loss of relationship for the media loss assay and the resultant use in ratios.

The range of the $\text{CL}_{\text{int,u}}$ for the four inhibitors differed between the three in vitro methods. This range was minimal (3-fold) in the case of depletion from the media loss assay in human hepatocytes. A significantly greater range was observed from the alternative two in vitro methods, with a 700-fold range from the conventional assay in human hepatocytes and 1000-fold range from the human microsomes.

### 3.3.3.3 Formation of metabolite in the human in vitro assays

Formation of the fluoxetine metabolite, norfluoxetine, was also monitored in the human in vitro assays. Low metabolite formation was observed in the two assays in human hepatocytes with maximal norfluoxetine concentrations reaching 1% of the initial 0.1μM fluoxetine dose in both cases (Figure 3.12A and B). The rate of formation could not be determined for the two human hepatocyte assays. The low level of norfluoxetine formation observed from the conventional assay in human hepatocytes was consistent with the observed low rate of fluoxetine depletion. The fast initial rate of fluoxetine depletion observed from the media loss assay was not observed in the formation of norfluoxetine. A higher formation of metabolite was observed in the human microsomes, however the maximum formation did not appear to be reached at 60 minutes. In this system, the norfluoxetine concentration was 3.5% of the initial 0.1μM fluoxetine dose at 60 minutes and related to the fluoxetine depletion.
Figure 3.12 Norfluoxetine formation in different human *in vitro* systems, where; ⚫ represents conventional assay in human hepatocytes (A), ■ media loss in human hepatocytes (B) and ▲ human microsomes (C). Data represent mean ± S.D. (n=3).
3.3.3.4 Comparison of human CL\textsubscript{int,u} values to in vivo values

Human in vivo plasma clearance values were collated from the literature for the four selected inhibitors for comparison with the clearance obtained from the human in vitro assays. All studies (with the exception of fluoxetine from McGinnity et al., 2004) reported clearance after oral clearance, therefore scaling to CL\textsubscript{int,u} used Equation 3.15. Clearance after multiple dosing of inhibitor was not included in the overall assessment of in vivo clearance as the in vitro data did not reflect steady state of the inhibitor. Average clearance values were calculated from itraconazole dosed at 100-200mg and ketoconazole dosed at 200mg in order to reduce the potential of non-linearity in the clearance values.
Table 3.8 Details of *in vivo* studies reporting human clearance from oral dosing of the four inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose (mg)</th>
<th>No. subjects</th>
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\(^1\) CL\(_p\) after i.v. dosing. \(^2\) Estimated from steady-state inhibitor concentration, therefore excluded from analysis. \(^3\) [www.webmd.com/drugs/mono-95-fluoxetine](http://www.webmd.com/drugs/mono-95-fluoxetine).
Figure 3.13 Comparison of clearance estimated from different human *in vitro* assays to mean *in vivo* human CL\textsubscript{int,h}. Symbols represent clearance from the conventional (circle symbols) or media loss (square symbols) in human hepatocytes, or from human microsomes (triangle symbols), where; ○ fluoxetine, ■ fluvoxamine, □ itraconazole and ● ketoconazole. Error bars have been removed for clarity.

The conventional assay in human hepatocytes and human microsomes performed similarly, with predictions 19 and 50% of the ketoconazole CL\textsubscript{int,h} and 20 and 22% of the itraconazole CL\textsubscript{int,h}, respectively. The media loss assay in human hepatocytes significantly over-predicted the *in vivo* clearance of the azole inhibitors by 25- and 14-fold for itraconazole and ketoconazole, respectively. Highest precision was observed from the media loss assay in human hepatocytes, with 14-16-fold over-prediction for fluoxetine and fluvoxamine. For the conventional assays in human hepatocytes and microsomes, predictions were 2 and 1% (conventional assay in human hepatocytes) and 1 and 0.5% (human microsomes) of CL\textsubscript{int,h} for fluoxetine and fluvoxamine, respectively. The correct rank order of *in vivo* human clearance (fluoxetine < ketoconazole < fluvoxamine < itraconazole) was not identified by any of the human *in vitro* assays.
3.4 DISCUSSION

There has been increasing interest in the ability to predict hepatic uptake from in vitro assays (Brown et al., 2007a; McGinnity et al., 2004; Riley et al., 2005; Soars et al., 2007; Soars et al., 2009). This chapter has assessed different in vitro methods for the preliminary assessment of uptake, via comparison of clearance between the conventional depletion assays in rat and human hepatocytes and microsomes, and the conventional depletion and media loss assays in rat and human hepatocytes. Fluoxetine, fluvoxamine, itraconazole and ketoconazole were selected to be investigated, consistent with the DDI database (Chapter 2). Fluconazole was excluded due to negligible clearance observed in the preliminary analysis in all rat in vitro assays.

Clearance was estimated from depletion profiles of the inhibitors at a low concentration (0.1μM) in both rat and human microsomes and hepatocytes, corrected for their respective fraction unbound in the incubation and scaled to mL/min/kg to allow a valid comparison. The difference in clearances obtained in rat hepatocytes (conventional assay) and microsomes observed for fluoxetine and fluvoxamine (2.5- and 3.2-fold, respectively) indicated the potential uptake of the SSRIs into cells. This difference was consistent with the 2-fold difference observed in the human hepatocytes (conventional assay) and microsomes for the SSRI inhibitors. Clearance estimated from the conventional assay in hepatocytes and microsomes for itraconazole were < 1.2-fold in both rat and human, and therefore did not suggest any uptake into the cell. Ketoconazole differences were not consistent, with 2-fold higher microsomal clearance in rats and 2.6-fold higher hepatocyte clearance in humans. The 2-fold difference observed in rats was contrary to the uptake hypothesis as the higher CL_{int,u} was observed in microsomes. This difference could be attributed to a higher metabolic rate than rate of uptake in rat hepatocytes with uptake therefore representing the rate limiting step in ketoconazole metabolism, as observed previously with saquinavir (Parker and Houston, 2008). However, this difference was only apparent in rat hepatocytes and it is therefore unclear if it is due to differences in transporter expression between rats and humans (Jigorel et al., 2005). The comparison of the K_{i,u} ratio from rat hepatocytes and microsomes for fluvoxamine (Brown et al., 2007a) resulted in a 3.3-fold difference for a single metabolic pathway (3-Methoxymorphinan) consistent with the observed CL_{int,u} ratio, however the other two metabolic pathways displayed K_{i,u} ratios of < 1. The K_{i,u} ratios for fluoxetine were less than the CL_{int,u} ratio, with all ratios < 2-fold. The K_{i,u} ratio was not investigated for itraconazole, and no difference was observed between the average K_{i,u} from rat hepatocytes and microsomes for four metabolic pathways for ketoconazole, despite the wide range (0.07-2). Therefore good
A number of studies have investigated differences between clearance predictions in hepatocytes and microsomes (both in human and rat) (Hallifax et al., 2010; Lu et al., 2006; McGinnity et al., 2004; Parker and Houston, 2008; Riley et al., 2005), with an observed trend of higher clearance obtained from rats than humans, and from microsomes than hepatocytes. Higher clearance was observed in rats than humans from the conventional assay in hepatocytes and microsomes, for all inhibitors except itraconazole. The discrepancy for itraconazole was minor (<3-fold) and possibly results from the use of fu_{inc} corrections derived from lipophilicity for rats and from experimental data for humans. The differences between the CL_{int,u} estimations from both rat hepatocytes (conventional assay) and microsomes were 100-600-fold greater than those from human for the SSRI inhibitors. More consistency was observed from the azole estimations, with a 2- to 10-fold greater CL_{int,u} in rats than humans for ketoconazole, and 2- to 3-fold greater CL_{int,u} in humans than rats for itraconazole. The observed relationship between the conventional assay in hepatocytes and microsomes appears to correlate well to the results reported by Hallifax et al., 2010, in a CL_{int,u} dataset of 46 drugs estimated in both human hepatocytes and microsomes. Clearance from the human hepatocytes and microsomes for the SSRI inhibitors correlate well to the log linear relationship given (from least squares regression analysis); log CL_{int,hepatocytes} = 0.53 log CL_{int,microsomes} + 0.59, deviating around 2-fold from this relationship. The azole inhibitors deviate further, with clearance 25 and 13% lower in hepatocytes than that estimated from the relationship to microsomal clearance. The trend less successfully described the CL_{int,u} from rat hepatocytes and microsomes; all deviated at least 2-fold (ketoconazole) and up to 16-fold for fluoxetine. The observed log linear relationship reported by Hallifax et al., 2010 indicates a trend of system-dependent bias between hepatocytes and microsomes; this relationship potentially undermines the assumption of obtaining the K_{p,u} from the clearance ratio between hepatocytes and microsomes, resulting in the potential for false positive indications of uptake observed at lower levels of clearance (<10 mL/min/kg). However, suggestions of uptake are still present if the deviation from the log linear relationship is assessed; for example the 4- to 8-fold difference between expected and observed clearance in hepatocytes of the azole inhibitors.
The media loss assay in rat and human hepatocytes resulted in higher $\text{CL}_{\text{int,hu}}$ than the conventional assay in hepatocytes for the majority of inhibitors. The differences in rat hepatocytes ranged from 0.5- to 4-fold, whereas the differences were more significant in human hepatocytes (from 5- to 1600-fold). This difference was as a result of the half-life method of clearance estimation, calculated using the initial loss of drug (<6 minutes) attributed to uptake as specified in Soars et al., (2007; 2009); nine time points were utilised over this time range in order to increase confidence in characterising the initial concentration changes. There are a number of reports showing a faster rate of uptake in rat hepatocytes than human hepatocytes (Sandker et al., 1994; Shitara et al., 2006), exemplified by a higher rate of initial drug disappearance (Soars et al., 2007). This trend is in contrast to the results observed in this study, where the rate of uptake from the media loss assay is similar in rats and humans for the SSRI inhibitors (<2-fold), however a 4- to 20-fold higher clearance calculated from the rate of uptake was observed for ketoconazole and itraconazole, respectively in the human hepatocytes. All compounds in this study are highly lipophilic, and the assumption that uptake would be negligible for these compounds due to high permeability (Shitara et al., 2006) was not observed. This result was consistent with Soars et al. (2007), with differences observed between the conventional and media loss assays in hepatocytes. It is assumed that the loss of drug from the media would be equal to the intracellular increase of drug; however this assumption may not be the case as a result of non-specific binding of the drug. This method does not allow for the uptake and binding components to be separated, potentially resulting in significant differences for the half-life method of clearance calculation was utilised in all comparisons in order to isolate the initial drug loss as a result of uptake, from the latter loss of drug as a result of metabolism. Differences between the methods for the conventional assay in hepatocytes and microsomes were minor, and only present for those drugs with biphasic profiles. In many cases, the biphasic profiles were only attributed to the final time point, likely as a result of the low and variable concentrations. Differences between the AUC and half-life methods of clearance calculation in these cases were generally < 20%, and were not significantly different as a result of extrapolation to infinity from the final time point (at 60 minutes), the estimation of concentration at 60 minutes from the non-linear regression fit, or from the penultimate time point (at 40 minutes). The inability to separate binding and uptake components in the media loss assay reduces the accuracy of predictions from this method, and confounds the ability to clearly attribute active uptake to any of the compounds.
Marked differences were observed between the formation of the active metabolite of fluoxetine, norfluoxetine in each of the in vitro assays. The formation was greatest in rat microsomes and the conventional assay in rat hepatocytes (to 59 and 25% of the initial fluoxetine concentration), whereas the low formation (maximal of 2.13% of the initial fluoxetine concentration) from the media loss assay in rat hepatocytes was likely to be attributable to the intracellular location of the metabolite as a result of increased polarity after metabolism. The rate of formation was not consistent with the extent, with the rate in rat microsomes approximately 2-fold greater than the conventional assay in rat hepatocytes. Both the rate and extent of formation in human microsomes and hepatocytes was low, with a maximum formation of 1-3.5% of the initial fluoxetine concentration. The results from this in vitro work were inconsistent to reported data, describing the extent of norfluoxetine formation (Ring et al., 2001). Reasons for the system-dependent formation, unequal rates of fluoxetine depletion and norfluoxetine formation and disparity between the observed in vitro results and previous literature are unclear, however they may be attributable to the significantly lower rate of fluoxetine clearance in human hepatocytes and microsomes compared to rat, the potential presence of alternative metabolic pathways or high levels of binding.

The differences between the in vivo rat clearance and the estimations from the three in vitro assays were < 25% for all inhibitors except for fluoxetine. The in vivo fluoxetine CL_{int} was determined from a single CL_{p} value; therefore inaccuracy in this value may explain the over-prediction of CL_{int} predictions from all three rat in vitro assays for this inhibitor, with differences from 6- to 40-fold. The average in vivo clearance used for ketoconazole excluded the values reported from female rats as all in vitro assays used microsomes or hepatocytes from male rats, and only included ketoconazole dosing <3mg/kg. These criteria were in order to reduce the observed variability resulting from the non-linearity in clearance with increasing doses (Matthew et al., 1993; Sjoberg et al., 1988), and resulted in the reduction of the CV from 58 to 39%. The calculation of the in vivo CL_{int,h} was based on the assumption of consistency between rat and human fu_{p}, and the blood to plasma ratio of 1. These assumptions may have contributed to error in the predictions; however, variation of the fu_{p} did not result in increased prediction accuracy for fluoxetine and deviation of the blood to plasma ratio from 0.5 -1.5 did not have a significant impact on the prediction accuracy of in vivo CL_{int,h} by the in vitro assays for all inhibitors. The prediction of human in vivo clearance from the conventional and media loss assay in human hepatocytes and human microsomes was unsuccessful with the majority of clearances <20% of the in vivo clearance. This under-prediction was significant for the
SSRI inhibitors, with predictions 0.5-2% of the in vivo clearance. Use of the rate of initial drug loss in the media loss assay in human hepatocytes negated this under-prediction and resulted in over-predicted in vivo clearance by up to 25-fold, consistent with the over-prediction observed using this assay in rat hepatocytes. The over-prediction from the media loss assay does not concur with the previous reports from Soars et al., (2007), who report the media loss assay to result in the superior prediction of clearance than the conventional assay in hepatocytes. The in vivo CL<sub>int,h</sub> was largely based on data after oral dosing, where the successful rat predictions were based on i.v. dosing. The assumption of no gut metabolism would not have had any impact on the prediction of fluoxetine and fluvoxamine CL<sub>int,h</sub> as neither inhibitors are metabolised by CYP3A4, the predominant enzyme in the gut. Bothazole inhibitors are metabolised by this enzyme; however the inclusion of any F<sub>G</sub> would have increased the extent of under-prediction and is therefore not responsible for the lack of prediction accuracy. In addition, variation of the blood to plasma ratio up to 2-fold did not improve the accuracy of predicted CL<sub>int,h</sub>. Comparisons were confounded by the highly variable nature of reported in vivo results in both rat and human, for example the 10-fold range from three studies reported clearance following 10mg/kg ketoconazole, in a similar number (n= 3-8) of Sprague-Dawley rats. This factor is of particular importance for the comparison of single studies reporting fluoxetine and itraconazole in rats and fluvoxamine in humans. Overall, no assay was significantly superior for the prediction of human clearance.

The formation of norfluoxetine (the fluoxetine active metabolite) was monitored through the time profiles of the different assays in rat and human hepatocytes and microsomes. The significant amount and rate of formation in the rat assays and previous work documenting the inhibition potential (Preskorn et al., 1994; Stevens and Wrighton, 1993) indicates that this metabolite is likely to have an important role in the inhibition after fluoxetine administration and should therefore also be included in DDI predictions. Lack of formation in human assays appeared to correlate to the significantly lower CL<sub>int,u</sub> rates. Comparison of the CL<sub>int,u</sub> calculated from the in vitro assays to those reported for rat and human in vivo displayed mixed success in prediction, with the most successful predictions of rat clearance resulting from the conventional assay in rat hepatocytes and of human clearance resulting from the media loss assay. This chapter has assessed hepatic uptake for four CYP inhibitors from three different in vitro assays in rat and human hepatocytes and microsomes; conflicting results were observed. No consistent uptake was identified from the comparison of CL<sub>int,u</sub> from rat or human hepatocytes (conventional assay) and microsomes or the media loss and conventional assay in rat hepatocytes. The clearance
ratios of fluoxetine and fluvoxamine suggested uptake, and were 2- to 4-fold in the majority of systems. Conflicting results were observed for ketoconazole, with uptake suggested from the comparisons of the conventional and media loss assay in both rat and human hepatocytes and from the hepatocytes (conventional assay) and microsomes in human. In contrast, this comparison displayed higher clearance in rat microsomes, potentially as a result of uptake being the rate limiting step in ketoconazole metabolism in rats. These results were consistent with the 3-fold $K_{i,u}$ ratio previously observed for fluvoxamine and the variable $K_{i,u}$ ratio observed for ketoconazole between rat hepatocytes and microsomes (Brown et al., 2007a). The media loss assay in hepatocytes displayed higher $CL_{int,u}$ than the conventional assay in hepatocytes in both rat (2- to 4-fold, with the exception of itraconazole) and human (5- to 1600-fold), suggesting that all of the inhibitors are subject to uptake into the cells from this assay. Overall, the different methods assessed did not result in a consistent suggestion of uptake to be attributed to any of the inhibitors.
CHAPTER 4: ASSESSMENT OF THE STATIC AND DYNAMIC PREDICTION OF DRUG-DRUG INTERACTIONS INVOLING AZOLE INHIBITORS
4 ASSESSMENT OF THE STATIC AND DYNAMIC PREDICTION OF DDIs INVOLVING AZOLE INHIBITORS

Parts of this chapter have been modified from published work (Guest et al., 2011) (Appendix 8, Publication 1).

4.1 INTRODUCTION

Previous studies have assessed the prediction accuracy of DDIs involving the azole inhibitors (Brown et al., 2006; Einolf, 2007; Fahmi et al., 2009; Ito et al., 2004; Templeton et al., 2010; Wang, 2010; Yang et al., 2003); some of which have included comparisons of the static and dynamic models of DDI prediction, as introduced in Section 1.5. The utility of different parameter values between the models resulted in differences to the prediction accuracy that could not be attributed to the time-course of inhibition. Active metabolites are an important consideration for DDI prediction models, particularly if the metabolite circulates at a sufficiently high concentration (Hinton et al., 2008; Rostami-Hodjegan and Tucker, 2004; Yeung et al., 2010) or has a potent effect on the metabolic pathways (Hinton et al., 2008; Ogilvie et al., 2006). For example, both the acyl-glucuronide metabolite of gemfibrozil and the N-desmethyldiltiazem metabolite are more potent inhibitors than the parent drugs, of CYP2C8 and OATP1B1 for the former, and CYP3A4 for the latter (Hinton et al., 2008; Rowland-Yeo et al., 2010; Zhang et al., 2009). In addition, if the metabolite shows a different interaction mechanism or metabolism pathway to the parent drug it may result in increased DDI levels, and therefore the impact of its inclusion in DDI prediction models may be significant. For example, the metabolite of nefazodone, m-chlorophenylpiperazine has different pharmacological effects to the parent and is metabolised by CYP2D6 in comparison to the CYP3A4-mediated metabolism of the parent (Kahn et al., 1988; Mayol et al., 1994; Rotzinger and Baker, 2002; Rotzinger et al., 1999). Polymorphisms in CYP2D6 or co-prescription with CYP2D6 inhibitors are therefore of importance as a result of the metabolite (Barbhaiya et al., 1996). Isoherranen et al. (2009), report that the majority of clinically important inhibitors possess circulating metabolites. However, in vitro evaluation of the inhibition potential of the circulating metabolites was only reported for approximately 30% of these studies. Multiple inhibitors are not commonly included in DDI assessment (Einolf, 2007; Galetin et al., 2006; Galetin et al., 2005; Hinton et al., 2008; Houston and Galetin, 2008; Obach, 2009; Rostami-Hodjegan and Tucker, 2004; Zhang et al., 2009) which leads to a potential under-estimation of the magnitude of DDI, as previously reported for a possible reason for the under-prediction of fluoxetine DDIs using Equation 1.9 and the dynamic model within Simcyp (Einolf, 2007).
One example of a clinically important metabolite is hydroxy-itraconazole. This metabolite has lower clearance compared with the parent and equal circulatory concentration in plasma and has been suggested to be an important contributor in overall CYP3A inhibition, despite lower potency compared with the parent ($K_i$ of 14.4nM compared to 1.3nM for the parent) (Isoherranen et al., 2004; Quinney et al., 2008; Templeton et al., 2008; Yoo et al., 2000). Inclusion of the inhibitory itraconazole metabolites into DDI prediction using the equation-based static model has previously been assessed (Templeton et al., 2010); prediction accuracy increased following the inclusion. The Simcyp simulator currently allows for inclusion of active metabolites only when the dynamic model is utilised. This selective inclusion is rationalised by the physiological relevance of the time-course of inhibition in the depletion of the parent drug and formation of the inhibitory metabolite. The impact of active metabolite inclusion into dynamic Simcyp simulations has not previously received consideration.

The assessment of DDI prediction success is often based on the average AUC ratio of the subjects within the study (Brown et al., 2006; Einolf, 2007; Fahmi et al., 2009; Ito et al., 2004; Templeton et al., 2010; Wang, 2010; Yang et al., 2003). The prediction of the inter-individual variability in the AUC ratio is rarely assessed, and when included, can often be based on the calculated S.D. based on normal distribution (Einolf, 2007). The ability of the Simcyp simulator to predict the DDI potential in a population therefore allows the range of AUC ratios to be assessed in comparison to the actual study distribution. The ability to exclude the assumption of normal distribution is paramount in cases where the study population is small (as observed in many of the studies reporting DDIs; see Section 2.3.3) and assess the prediction of outliers within the study population. The inter-individual variability in DDI prediction has not been comprehensively investigated, and the Simcyp simulator provides an ideal platform for this assessment.

4.1.1 Aims

The objectives of the current chapter were to compare directly and evaluate the static and dynamic model approaches to DDI prediction and to assess the impact of including the time-course for the inhibition process. The ability to assess both models within Simcyp allowed a valid comparison, as other factors were kept consistent. For this study, a set of 38 DDIs between three azole inhibitors (fluconazole, ketoconazole and itraconazole) and three benzodiazepine substrates (alprazolam, midazolam, triazolam) encompassing different properties was used, in order to assess a sufficient number of studies per inhibitor and per victim drug to ascertain trends. An additional 22 DDIs between the azole inhibitors
and a wider range of victim drugs were also assessed, including non-benzodiazepine CYP3A4 victim drugs and victim drugs metabolised by CYP1A2, CYP2C9 and CYP2C19 enzymes with a wide range of properties. The impact of different inhibitor and victim drug properties on DDI prediction was also assessed, namely the incorporation of an inhibitory metabolite (of itraconazole) and inhibitor dosing time relative to the victim drugs. A number of additional factors were assessed for itraconazole-triazolam interactions considering the poor prediction success observed in preliminary analysis, namely variability in itraconazole ka, use of differential triazolam in vitro clearance and permeability data. Finally, the ability of Simcyp to predict inter-individual variability in the magnitude of DDI was assessed by comparison with reported individual data in eight selected clinical studies reporting such data.
4.2 METHODS

4.2.1 Virtual trials

The static and dynamic approaches were compared using the Simcyp population-based ADME simulator (Version 8.10, SP1). The underlying concepts and principles of Simcyp have been previously described (Section 1.5; Jamei et al., 2009; Rowland-Yeo et al., 2010). The use of Simcyp allowed assessment of the ability of each model to predict the magnitude of DDI (through change in the AUC) of 38 published interactions. The selected DDIs included fluconazole, ketoconazole and itraconazole as inhibitors and alprazolam, midazolam and triazolam as victim drugs. A separate database was collated (n=22) of DDIs involving non-benzodiazepine victim drugs, and assessed identically. All DDIs were reversible inhibition interactions and the criterion for the inclusion of the study was the oral administration of both substrate and inhibitor. The DDIs in the current analysis were classified according to the fold change in the AUC of the victim drug either as a weak (AUC ratio<2), moderate (2 to 5-fold increase in AUC) or strong DDI (AUC ratio>5), analogous to the FDA guidelines for the assessment of potential inhibitor of CYP3A4 (Huang et al., 2007). Six studies (representing 13 data points due to different dose or dosing schedule) in the azole database (Ahonen et al., 1997; Kharasch et al., 2005; Lazar and Wilner, 1990; McCrea et al., 1999; Neuvonen et al., 1996; Olkkola et al., 1996) reported DDIs after single dose of the inhibitors and were used for additional analysis and comparison with those studies reporting DDIs at steady-state inhibitor concentrations. The AUC ratio used for comparison from each study was calculated from AUC(0,∞). The only exception was the study between ketoconazole and itraconazole with triazolam (Varhe et al., 1994), where the AUC ratio was calculated from the last time point in the study (AUC(0,t)). Whenever available the mean AUC(0,∞) ratio for each study was calculated from values reported for the individual subjects.

In order to keep as many variables as consistent as possible, the simulations for both the static and dynamic models were performed in Simcyp, allowing direct assessment of the impact of inclusion of the time course. For assessment of DDIs, each simulation trial had matching population demographics (number of subjects, age and male:female ratio) and dosage regimen (dose and timing of all inhibitor and victim drug doses) to the reported clinical study. Ten of these trials were performed for each study in the dataset in order to assess variability across groups of subjects. Simulations were based on the systemic plasma concentration of the inhibitor, the use of the minimal PBPK model (Rowland-Yeo et al., 2010; Zhao et al., 2009) and a population of healthy, fasted subjects assuming a
250ml fluid intake with dosing. In cases when the age range was given as a mean ± S.D.,
the distribution was estimated or if the age was not reported the default range was used
(18-65 years with the proportion of females at 0.34). If the dose timing details were not
reported, simulations were undertaken with the victim drug dosed one hour after the final
inhibitor dose, as this scenario was the most common dosing schedule for the azole
inhibitors (Chapter 2). The simulations were terminated at least two inhibitor half-lives
after the final inhibitor dose in order to allow assessment of the AUC where it is
approaching infinity. The kinetic parameters in the Simcyp model are described similarly
to those reported by Ito et al. (Ito et al., 2003), but also include intestinal wall metabolism
as described previously (Rowland-Yeo et al., 2010; Yang et al., 2007b). The gut and the
liver are represented as separate compartments and the other organs are included in a single
systemic compartment (Rowland-Yeo et al., 2010). The dynamic model is based on
analogous principles to the static model, but it allows the incorporation of the time-variant
intestinal and hepatic metabolism. Drug metabolism is described using the Michaelis-
Menten relationship and incorporates changes of active enzymes with time in both liver
and intestine. The differential equations and assumptions of these methods are described
fully in Rowland-Yeo et al., (2010) (Section 1.5).

All of the inhibition input parameters for the azole inhibitors are shown in Table 4.1;
Simcyp default values were used for all other input parameters and the coefficient of
variation for these parameters were kept as set by the software during the simulations (30% in
most cases), as performed previously (Zhao et al., 2009). The values of Ki and fu_p
utilised for fluconazole and ketoconazole were taken from Brown et al., 2006, and those
for itraconazole (and hydroxy-itraconazole when included in simulation) from Isoherranen
et al., 2004 and Templeton et al., 2008 (Table 4.1). Some of the input parameters vary from
Simcyp default values; for example, default ketoconazole values are 0.015μM for CYP3A4
Ki (Gibbs et al., 1999) and fu_p is 0.029. All the Ki values used were obtained in CYP3A4
Supersomes or HLM from Caucasian donors; hence the assumption was that the Ki
reflected the inhibition of CYP3A4. Inhibition of CYP3A5 as a potentially contributing
enzyme to the clearance of these three victim drugs (Galetin et al., 2004) was included
whenever the information on CYP3A5 inhibition by azole inhibitors was available. For
example, CYP3A5 Ki of 0.109 and 84.6μM (Gibbs et al., 1999) were incorporated in the
assessment of ketoconazole and fluconazole, respectively; no such information was
available for itraconazole. Recent studies have reported a minor contribution
(approximately 6%) of N-glucuronidation to midazolam clearance in vitro (Kilford et al.,
2009; Klieber et al., 2008). It is important to note that the glucuronidation metabolic
pathway was not considered as a pathway for midazolam in Version 8.10, SP1 of the software used for the current analysis. Klieber et al., 2008 reported no significant inhibition of midazolam N-glucuronidation in the presence of ketoconazole.

*Table 4.1* Input parameters for the azole inhibitors used in Simcyp simulations. Values for $k_a$ are Simcyp defaults, where CYP3A4 and CYP2C9 $K_i$ and $f_u$ data for fluconazole and ketoconazole were from Brown et al., 2006 and CYP3A4 itraconazole and hydroxy-itraconazole from Isoherranen et al., 2004 and Templeton et al., 2008.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{iu}$ CYP1A2 (µM)</th>
<th>$K_{iu}$ CYP2C9 (µM)</th>
<th>$K_{iu}$ CYP2C19 (µM)</th>
<th>$K_{iu}$ CYP3A4 (µM)</th>
<th>$K_{iu}$ CYP3A5 (µM)</th>
<th>$f_u$</th>
<th>$k_a$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>*</td>
<td>6.5</td>
<td>2.0</td>
<td>11.9</td>
<td>84.6</td>
<td>0.890</td>
<td>1.28</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>-</td>
<td>2.8</td>
<td>-</td>
<td>0.042</td>
<td>0.109</td>
<td>0.017</td>
<td>1.90</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0013</td>
<td>-</td>
<td>0.036</td>
<td>0.62</td>
</tr>
<tr>
<td>Hydroxy-itraconazole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0144</td>
<td>-</td>
<td>0.0045</td>
<td>-</td>
</tr>
</tbody>
</table>

* No default $K_i$ included in Simcyp, and IC50 was reported to be > 300µM (Obach et al., 2006). 500µM was therefore utilised, as previously reported (Einolf, 2007).

The fraction absorbed for all drugs was assumed to be 1. Differences between the default values of $k_a$ included within Simcyp were <2.5-fold in comparison to reported *in vivo* values (Brown et al., 2005) for all three inhibitors. In the case of ketoconazole, default hepatic uptake value of 2.07 was used, consistent with previous reports (Ito et al., 1998; von Moltke et al., 1996); for other inhibitors no uptake was assumed. Default clearances were used for all inhibitors; average oral clearance values of 13.3 and 1.5 Lh$^{-1}$ were used for ketoconazole and fluconazole, respectively (Huang et al., 1986; Thummel et al., 2008). In the case of itraconazole and hydroxy-itraconazole, *in vitro* $K_{m,ia}$ (3.9 and 27 nM, respectively) and $V_{max}$ (270 and 543 pmol/min/nmol3A4, respectively) data obtained in recombinant baculovirus CYP3A4 were used (Isoherranen et al., 2004), with $V_{max}$ corrected intrinsically within Simcyp using the corresponding intersystem extrapolation factor of 0.98 (Rostami-Hodjegan and Tucker, 2004). The impact of the inclusion of the most abundant metabolite of itraconazole, hydroxy-itraconazole in dynamic DDI prediction was also assessed. The impact of different $k_a$ values for itraconazole was explored, based on the 0.58-0.64h$^{-1}$ range calculated from the clinical 90% confidence
intervals of time to maximal plasma drug concentration ($T_{\text{max}}$) and half-life ($t_{1/2}$) (Barone et al., 1998); impact of a wider range of this parameter (0.3-1 h$^{-1}$) was additionally investigated. Simulations used the most common inhibitor dosing schedule from the database outlined in Chapter 2 (i.e. 200mg itraconazole per day for four days, with the inclusion of hydroxy-itraconazole in simulations), with administration of the substrate dose (0.5mg, 350mg, 5mg and 0.25mg of alprazolam, cyclosporine, midazolam and triazolam, respectively) one hour after the last itraconazole dose.

### 4.2.2 Impact of victim drug properties and dose timing

The data showing the accuracy of the DDI predictions were also compared for each victim drug in order to identify any trends. Values of $F_G$ and $f_{\text{m,CYP}}$ were calculated intrinsically within Simcyp; output values for $F_G$ estimated using the $Q_{\text{gut}}$ model are displayed in Table 4.2. In all of the cases fraction unbound in the intestine was assumed to be 1, consistent with previous reports (Gertz et al., 2010; Rowland-Yeo et al., 2010; Yang et al., 2007b). The Simcyp outputs displayed low levels of gut metabolism ($F_G > 0.9$) for the majority (7/10) of the victim drugs, exceptions were observed for midazolam and cyclosporine with intermediate $F_G$ values of 0.59 and 0.65, respectively, and simvastatin with significant gut metabolism ($F_G = 0.08$). The contribution of each CYP enzyme was estimated from *in vitro* clearance data (Simcyp default) after correcting the simulated plasma clearance for renal excretion. All default clearance values in Simcyp were from recombinant data, with the exception of cyclosporine, quinidine and simvastatin, where reported data was from HLM. The contribution of renal clearance to the total clearance of the victim drug was $<3\%$ for all victim drugs apart from quinidine and alprazolam, where it represented 14 and 18\% of the clearance, respectively. Proportional contribution of CYP3A5 was only reported for the benzodiazepine victim drugs and was minimal ($<0.10$) in all three cases.
Table 4.2 Simcyp output values of victim drug properties, including fm\textsubscript{CYP}, F\textsubscript{G} and the contribution of renal clearance to the overall clearance

<table>
<thead>
<tr>
<th>Victim drug</th>
<th>fm\textsubscript{CYP1A2}</th>
<th>fm\textsubscript{CYP2C9}</th>
<th>fm\textsubscript{CYP2C19}</th>
<th>fm\textsubscript{CYP3A4}</th>
<th>fm\textsubscript{CYP3A5}</th>
<th>F\textsubscript{G}</th>
<th>CL\textsubscript{R} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.77</td>
<td>0.05</td>
<td>0.99</td>
<td>18</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>0.65</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Quinidine</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
<td>0.66</td>
<td>-</td>
<td>0.99</td>
<td>14</td>
</tr>
<tr>
<td>Midazolam</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.85</td>
<td>0.10</td>
<td>0.59</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.84</td>
<td>-</td>
<td>0.08</td>
<td>0</td>
</tr>
<tr>
<td>Triazolam</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.86</td>
<td>0.09</td>
<td>0.91</td>
<td>2</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.82</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01</td>
<td>-</td>
<td>1.00</td>
<td>3</td>
</tr>
<tr>
<td>S-warfarin</td>
<td>-</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.99</td>
<td>1</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.99</td>
<td>0</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>-</td>
<td>-</td>
<td>0.73</td>
<td>0.26</td>
<td>-</td>
<td>0.93</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

In the case of triazolam, virtual trials were also performed using mean triazolam CL\textsubscript{int} from several sources of human liver microsomal data (30.3 ± 11.1 and 22.3 ± 11.6 μL min\textsuperscript{-1} mg protein\textsuperscript{-1} for 1’-hydroxy and 4-hydroxytriazolam, respectively (Galetin and Houston, 2006; Gertz et al., 2010; Patki et al., 2003; Patki et al., 2004; von Moltke et al., 1996)). Impact of variability in triazolam in vitro clearance data on the DDI prediction was assessed due to availability of data from individual donors of HLM (von Moltke et al., 1996). To investigate the impact of the timing of dose on the magnitude of itraconazole DDIs, dynamic simulations were performed with alprazolam, cyclosporine, midazolam and triazolam victim drugs using the most common inhibitor dosing schedule (i.e. 200mg itraconazole per day for 4 days, with the inclusion of hydroxy-itraconazole in simulations). The administration of the substrate dose (0.5mg, 350mg, 5mg and 0.25mg of alprazolam, cyclosporine, midazolam and triazolam, respectively) was varied from -10 to +24 h from the last itraconazole dose. Where available, observed AUC ratios from the studies were compared to those obtained by virtual trials to assess the ability of the dynamic model to predict the actual differences seen as a result of changes in the timing of the dose.

4.2.3 Inter-individual variability in the AUC ratios
Seven clinical studies (corresponding to 10 data points due to differences in the inhibitor, victim drug dose or dosing schedule) reported the AUC data from each individual subject were selected for investigation for the DDIs between either ketoconazole or itraconazole
with triazolam as a victim drug (Varhe et al., 1994), itraconazole-midazolam (Templeton et al., 2010), itraconazole-simvastatin (Neuvonen et al., 1998), ketoconazole-cyclosporine (Gomez et al., 1995), ketoconazole-triazolam (Greenblatt et al., 1998), fluconazole-S-warfarin (Black et al., 1996) and fluconazole-cyclosporine (Canafax et al., 1991). Summaries of these clinical studies are provided in Table 4.3. These individual AUC ratios were therefore used to assess the ability of the dynamic model to predict inter-individual variability in the magnitude of DDI. Ten virtual trials were simulated with the same number of subjects as in the study; median, minimum and maximum predicted AUC ratio of each individual trial and all 10 trials in total were compared to the observed data. The simulations of the DDIs involving itraconazole were performed with the inclusion of hydroxy-itraconazole, and triazolam human liver microsomal clearance data, as outlined above. To investigate variation further in the virtual trial results, the data on CYP3A5 genotype and CYP3A4 abundance in the liver and gut were studied for the individuals in the simulated trials (Varhe et al., 1994). No such data were available for the clinical studies included in the current dataset as information on the CYP3A5 status of the subjects involved in the DDI clinical studies is generally limited (Kuypers et al., 2008; Yu et al., 2004). Additionally, the profiles of poorly predicted DDIs were considered in greater detail in order to ascertain any trends in the simulations. Consideration was given to the observed and predicted values for the victim drug AUC in the absence and presence of inhibitor and the $C_{\text{max}}$ values.
Table 4.3 Summary details of the studies reporting results of the DDI study in individual subjects.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Victim Drug</th>
<th>Number and sex of subjects</th>
<th>AUC ratio Mean ± S.D.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole 400mg</td>
<td>S-warfarin 0.75mg/kg</td>
<td>n = 6 6m, 0f</td>
<td>2.92 ± 0.41</td>
<td>Black et al., 1996</td>
</tr>
<tr>
<td>Fluconazole 200mg</td>
<td>Cyclosporine 3mg/kg</td>
<td>n = 8 5m, 3f</td>
<td>1.92 ± 0.41</td>
<td>Canafax et al., 1991</td>
</tr>
<tr>
<td>Ketoconazole 200mg</td>
<td>Cyclosporine 2mg/kg</td>
<td>n = 5 2m, 3f</td>
<td>1.30 ± 0.61</td>
<td>Gomez et al., 1995</td>
</tr>
<tr>
<td>Ketoconazole 200mg</td>
<td>Triazolam 0.25mg</td>
<td>n = 7 7m, 0f</td>
<td>12.8 ± 3.72</td>
<td>Greenblatt et al., 1998</td>
</tr>
<tr>
<td>Ketoconazole 400mg</td>
<td>Triazolam 0.25mg</td>
<td>n = 9 3m, 6f</td>
<td>9.17 ± 4.60</td>
<td>Varhe et al., 1994</td>
</tr>
<tr>
<td>Itraconazole 50mg</td>
<td>Midazolam 2mg</td>
<td>n = 6 5m, 1f</td>
<td>2.04 ± 0.65</td>
<td>Templeton et al., 2010</td>
</tr>
<tr>
<td>Itraconazole 200mg</td>
<td>Midazolam 2mg</td>
<td>n = 6 5m, 1f</td>
<td>4.66 ± 1.42</td>
<td>Templeton et al., 2010</td>
</tr>
<tr>
<td>Itraconazole 400mg</td>
<td>Midazolam 2mg</td>
<td>n = 6 5m, 1f</td>
<td>5.38 ± 1.50</td>
<td>Templeton et al., 2010</td>
</tr>
<tr>
<td>Itraconazole 200mg</td>
<td>Triazolam 0.25mg</td>
<td>n = 9 3m, 6f</td>
<td>10.5 ± 11.0</td>
<td>Varhe et al., 1994</td>
</tr>
<tr>
<td>Itraconazole 200mg</td>
<td>Simvastatin 40mg</td>
<td>n = 10 7m, 3f</td>
<td>18.4 ± 3.95</td>
<td>Neuvonen et al., 1998</td>
</tr>
</tbody>
</table>

Where n is the number of subjects, and m and f represent male and female subjects, respectively.

4.2.4 Data Analysis

Predictions within 2-fold of the observed AUC ratio were considered successful, although the number within 1.5-fold of the in vivo values was also assessed. The average fold error (afe) was used to assess bias in the DDI prediction, and the mean and root mean squared prediction error (mse and rmse, respectively) provided a measure of precision of the DDI prediction (Equations 4.2-4.4) (Obach et al., 1997; Sheiner and Beal, 1981). In addition,
the weighted afe of the predictions performed in the dynamic model was calculated by correction for the number of subjects in the corresponding clinical trial.

\[
\text{afe} = 10 \left| \frac{1}{n} \sum \log \frac{\text{Predicted}}{\text{Observed}} \right| \\
mse = \frac{1}{n} \sum (\text{Predicted} - \text{Observed})^2 \\
\text{rmse} = \sqrt{\text{mse}}
\]

_Equation 4.1_  
_Equation 4.2_  
_Equation 4.3_

Where \( n \) represents number of studies, predicted and observed refer to the predicted and observed AUC ratio (AUC'/AUC), respectively.
4.3 RESULTS

A database consisting of 60 studies was collated in order to assess the static and dynamic prediction of DDIs within Simcyp. This database consisted of 38 studies reporting DDIs between the azole inhibitors (fluconazole, ketoconazole and itraconazole) and CYP3A4 benzodiazepine substrates (alprazolam, midazolam and triazolam), and 22 studies reporting DDIs between azole inhibitors and CYP1A2, CYP2C9, CYP2C19 and non-benzodiazepine CYP3A4 victim drugs. The dosing details of the inhibitors and victim drugs and the observed AUC ratios for the studies investigated are listed in Chapter 2. The potency distribution of the full dataset (n=60) was 2, 20, 46 and 32% of no interaction (AUC ratio < 1.25), weak (1.25- to 2-fold increase in AUC of the victim drug), moderate (2 to 5-fold increase in AUC of the victim drug) and strong (AUC ratio > 5) interactions, respectively. This distribution differed between the two datasets, with the azole-benzodiazepine DDIs (n=38) consisting of 53% and 42% of moderate and strong interactions, respectively and the remainder (5%) were weak. Lower potencies were observed in the alternative dataset, with the percentage of no interaction, weak, moderate and strong DDIs at 9, 44, 33 and 14%, respectively.

4.3.1 Comparison between static and dynamic azole-benzodiazepine DDI predictions (n=38)

Figure 4.1 shows the comparison of the predicted AUC ratios from virtual trials obtained by the dynamic and static model within Simcyp, classified according to the azole inhibitors or benzodiazepine victim drugs in the dataset. Breakdown of studies where the predicted magnitude of DDI was greater by the dynamic model (dynamic:static ratio >1) and vice versa is illustrated in Table 4.4. The dynamic:static model ratio of predicted DDI magnitude ranged from 0.4 for an itraconazole DDI with midazolam, up to 1.73 in the case of an itraconazole DDI with triazolam. For 15/38 DDIs in the dataset, the difference between the models was minimal and predicted AUC ratios by static and dynamic model were within 20% of each other (Figure 4.1). Half of the interactions in the dataset (50%) had a dynamic:static ratio of <0.8, indicating higher AUC ratio predictions obtained using the static model. This trend was particularly apparent for DDIs involving fluconazole and ketoconazole with 70% and 54% of study ratios in this range, respectively. The only inhibitor with a tendency of increased predictions via the dynamic model was itraconazole, where the average ratio between the models was 1.22 (Table 4.4). DDIs with triazolam as the victim drug had the most comparable mean ratio between the models (0.93) despite the
wide range observed (0.51-1.73). No trend was observed between the potency of DDI and the dynamic:static ratio when assessed per victim drug (Figure 4.1).

**Figure 4.1** Comparison of the predicted AUC ratio for 38 DDIs performed using either the time-based dynamic or static model in Simcyp. The median and inter-quartile ranges are represented by the black line and box boundaries, respectively, whiskers represent 10-90% ranges and outliers are represented by •. The predictions are classified according to the inhibitor or victim drug and the pie charts underneath display the proportion of DDIs for each.

**Table 4.4** Comparison of predicted AUC ratios by the dynamic and static models according to the inhibitor and potency of DDI investigated. Weak, moderate and strong DDI refer to interactions with fold increase in victim drug AUC <2, 2-5 and >5-fold, respectively

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Weak DDI</th>
<th>Moderate DDI</th>
<th>Strong DDI</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic:static AUC ratio:</td>
<td>&gt;1 &lt;1</td>
<td>&gt;1 &lt;1</td>
<td>&gt;1 &lt;1</td>
<td>&gt;1 &lt;1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>- 1/10</td>
<td>- 9/10</td>
<td>- -</td>
<td>- 10/10</td>
</tr>
<tr>
<td>Subtotal</td>
<td>- 2/38</td>
<td>3/38</td>
<td>17/38</td>
<td>6/38</td>
</tr>
<tr>
<td>Total</td>
<td>2/38</td>
<td>20/38</td>
<td>16/38</td>
<td>38/38</td>
</tr>
</tbody>
</table>
4.3.2 Azole-benzodiazepine DDI prediction accuracy from the static and dynamic model

For the 38 azole-benzodiazepine DDIs, 74% and 79% were predicted within 2-fold using the dynamic and static model, respectively, using the parameters detailed in Table 4.1. The static model also resulted in higher precision and lower bias across the range of DDI potency (Table 4.4), although the difference was not significant (P>0.1, Student’s t-test). Fluconazole DDIs showed good prediction accuracy as either all or 9/10 studies were predicted within 2-fold when using the static or dynamic model, respectively, although all of these DDIs were either weak or moderate interactions, i.e., AUC ratio<5. A clear under-prediction trend was observed with increasing potency of DDIs with approximately 40% of strong DDIs (AUC increase >5-fold) under-predicted regardless of the model used. The prediction of ketoconazole DDIs particularly showed this trend, with 40-50% of strong DDIs under-predicted using the dynamic or static model. However, overall prediction accuracy for ketoconazole DDIs was comparable between the models with 54-62% of studies within a 2-fold margin (Table 4.5). Predicted:observed AUC ratios from virtual trials obtained using either the static or dynamic model are shown in Figure 4.2.

![Figure 4.2](image-url)

**Figure 4.2** Comparison of predicted/observed and observed AUC ratios for 38 DDIs with azole inhibitors. Predictions were performed using either the time-based dynamic (●) or static (▽) model in Simcyp. Horizontal dashed lines represent the 2-fold margins, and vertical dashed lines represent the boundaries between weak (W), moderate (M) and strong (S) DDIs.
Table 4.5 Accuracy of 38 DDI predictions using either the time-based dynamic or static model in Simcyp, classified according to either inhibitor or DDI potency (moderate and strong indicating 2-5 and >5-fold increase in AUC of the victim drug, respectively).

<table>
<thead>
<tr>
<th>Model</th>
<th>Inhibitor</th>
<th>Fluconazole (n=10)</th>
<th>Number of studies within 2-fold (%)</th>
<th>Number of studies within 1.5-fold (%)</th>
<th>afe</th>
<th>rmse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic</td>
<td>Inhibitor</td>
<td>Fluconazole (n=10)</td>
<td>90</td>
<td>50</td>
<td>1.52</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ketoconazole (n=13)</td>
<td>54</td>
<td>31</td>
<td>2.07</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itraconazole (n=15)</td>
<td>75</td>
<td>60</td>
<td>1.39</td>
<td>2.38</td>
</tr>
<tr>
<td>Potency</td>
<td>Moderate (n=20)</td>
<td>80</td>
<td>55</td>
<td></td>
<td>1.48</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>Strong (n=16)</td>
<td>63</td>
<td>31</td>
<td></td>
<td>1.89</td>
<td>5.40</td>
</tr>
<tr>
<td>Overall* (n=38)</td>
<td>74</td>
<td>47</td>
<td></td>
<td></td>
<td>1.63</td>
<td>3.66</td>
</tr>
<tr>
<td>Static</td>
<td>Inhibitor</td>
<td>Fluconazole (n=10)</td>
<td>100</td>
<td>100</td>
<td>1.06</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ketoconazole (n=13)</td>
<td>62</td>
<td>54</td>
<td>1.63</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Itraconazole (n=15)</td>
<td>80</td>
<td>40</td>
<td>1.29</td>
<td>2.93</td>
</tr>
<tr>
<td>Potency</td>
<td>Moderate (n=20)</td>
<td>95</td>
<td>70</td>
<td></td>
<td>1.01</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>Strong (n=16)</td>
<td>56</td>
<td>44</td>
<td></td>
<td>1.81</td>
<td>5.19</td>
</tr>
<tr>
<td>Overall* (n=38)</td>
<td>79</td>
<td>61</td>
<td></td>
<td></td>
<td>1.29</td>
<td>3.47</td>
</tr>
</tbody>
</table>

*Accuracy of the two weak DDIs is not displayed in the analysis according to the DDI potency but was included in the overall analysis.

No difference was observed in prediction accuracy between fluconazole DDIs resulting from steady-state inhibitor concentrations (five studies) or single dosing (five studies) as nearly all (4/5 or 5/5) were predicted within 2-fold, respectively. In the case of
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ketoconazole, two of the 13 DDIs with this inhibitor used a single inhibitor dose. However, those DDIs were predicted within 2-fold regardless of the model used, whereas either 5 or 6 of the remaining 11 multiple dosing studies were predicted within 2-fold using the dynamic or static model, respectively. Itraconazole DDIs with steady-state inhibitor concentrations (seven studies) or with single dosing regimens (eight studies), were predicted 86% and 75% within 2-fold, respectively, when using the dynamic model (regardless of metabolite inclusion). However, a difference was observed between the dosing scenarios when using the static model with 57% of steady-state and 100% of single dosing studies predicted within 2-fold. Overall, 87 and 100% of the 15 single dose studies, and 65 and 61% of the 23 multiple dose studies were predicted within 2-fold using the dynamic and static models, respectively.

4.3.3 The impact of active metabolite inclusion on DDI prediction accuracy
The inclusion of the most abundant active metabolite of itraconazole (hydroxy-itraconazole) into the dynamic model resulted in an increase in the predicted AUC ratio. Although this inclusion did not result in a difference in prediction accuracy for moderate DDIs, a significantly higher prediction accuracy was observed for strong DDIs (83% within 2-fold compared to 50% without the metabolite). The dynamic model (including metabolite) also showed improved prediction accuracy for strong DDIs when compared to the static model (83% within 2-fold compared to 67%, respectively), and had an increased overall number of studies within 1.5-fold (60% compared to 33-40% observed with other two scenarios). The overall trend of under-prediction at higher potency DDIs can be observed in Figure 4.3 for the predictions made using the static and dynamic (without metabolite) model. Overall, the dynamic (with metabolite) and static model result in comparable number of studies predicted within 2-fold (12/15). Comparison of the simulated profiles of itraconazole and hydroxy-itraconazole after 100mg single dose of itraconazole (Templeton et al., 2008) displayed successful prediction of the AUC, with itraconazole predicted within 1.6-fold (1.24mg/L.h compared to the 2.02mg/L.h actual AUC) and hydroxy-itraconazole AUC predicted at 3.11mg/L.h compared to the 3.06mg/L.h observed. This success was despite over-prediction of the T_max by approximately 2.6-fold and 17-fold with predictions of 4h and 26h for itraconazole and hydroxy-itraconazole, respectively compared to the reported 1-2h for both parent and metabolite (Templeton et al., 2008). The C_max values were under-predicted in both cases by 4.6- and 3.6-fold, respectively, however predictions were more consistent to each other at 0.056 and 0.073mg/L for itraconazole and hydroxy-itraconazole, compared to the observed C_max values of 0.256 and 0.258mg/L, respectively (Templeton et al., 2008). These
differences in the profiles may impact on DDIs involving victim drugs with short half-lives; however successful prediction of the overall AUC should negate further impact.

**Figure 4.3** Comparison of predicted/observed and observed AUC ratios for 15 itraconazole DDIs. Predictions were performed in Simcyp using either the time-based dynamic model in the presence (●) or absence (■) of the itraconazole metabolite (hydroxy-itraconazole) or using the static model (∇). The horizontal dashed lines represent the 2-fold margins.

### 4.3.4 Impact of properties of the victim drug on dynamic DDI prediction accuracy

Figure 4.4 shows the comparison of predicted and observed AUC ratios according to the benzodiazepine victim drug, obtained using either the dynamic or static model.
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Figure 4.4 Predicted and observed AUC ratios for 38 azole DDIs classified according to the benzodiazepine victim drug where ● represents alprazolam (n=3), ▲ midazolam (n=23) and □ triazolam (n=12). Predictions used the dynamic model (plus metabolite for itraconazole DDIs) (A) or the static model (B). The solid and dashed lines represent the line of unity and 2-fold margins respectively. Error bars have been removed for clarity.

From the 26 DDIs with midazolam or alprazolam, 91 and 67% predictions obtained by dynamic model were within 2-fold of the in vivo value, respectively. Bias in prediction of midazolam and alprazolam was comparable (afe of 1.48 and 1.77, respectively). However, prediction of midazolam DDIs showed higher imprecision (rmse of 3.42). In the case of triazolam, bias and imprecision were higher (afe and rmse of 1.67 and 4.29, respectively). In addition, only 42% of triazolam DDIs were predicted within 2-fold of the observed AUC ratio regardless of inhibitor. None of the interactions within 2-fold was in the class of strong DDIs and only 4/7 of the moderate DDIs were predicted successfully. Similar precision and bias was observed per substrate for predictions using the static model compared to the dynamic model.

The prediction of alternative victim drugs was assessed (n=22), including CYP3A4 victim drugs other than the benzodiazepines and those metabolised by other CYP enzymes where clinical DDIs with azoles were available (Figure 4.5). Studies were available for all threeazole inhibitors (fluconazole n=9, itraconazole n=3 and ketoconazole n=10). Successful prediction was observed for 17/21 of the DDIs with no inhibitor-dependent success rate; the unsuccessful predictions were the three strong DDIs in the database between fluconazole-omeprazole (Kang et al., 2002), itraconazole-simvastatin (Neuvonen et al., 1998) and ketoconazole-cyclosporine (Gomez et al., 1995). A third of studies (32%) involved alternative CYP3A4 victim drugs (accounting for 11%, 100% and 30% of the
additional fluconazole, itraconazole and ketoconazole studies, respectively) and the remainder were DDIs between fluconazole or ketoconazole with CYP1A2 (4.5%), CYP2C9 (32%) and CYP2C19 (32%) victim drugs. Assessment of prediction success (from 1.5-fold or 2-fold) did not result in any trends when assessed by the CYP enzyme involved, potentially as a result of the low number of studies per enzyme (n=1 for CYP1A2 to n=7 for all others). No trend was observed from the assessment of the rank order of the prediction accuracy compared to the half-life or bioavailability of the CYP3A4 victim drugs (Table 4.6). This trend was still apparent when victim drugs that were represented by < 3 studies were excluded. The trend for reduction in dynamic predictions with increasing potency of DDI was present for all DDIs. However, potent DDIs represented a low proportion of the database (3/22) and the three studies involved different CYP enzymes and inhibitors. From the total database (n=60), 100, 100, 81 and 53% of no interaction, weak, moderate and strong DDIs, respectively and 80% of the total DDIs were predicted within 2-fold using the dynamic model.

**Figure 4.5** Comparison of predicted and observed AUC ratios for 21 DDIs involving non-benzodiazepine victim drugs. Predictions were performed using the dynamic model in Simcyp, and are classified according to theazole inhibitor (A) (fluconazole (▲), ketoconazole (○) or itraconazole (■)) or victim drug (B). In B, CYP enzymes involved are CYP1A2 (circle symbols): theophylline (●); CYP2C9 (rotated triangle symbols): S-warfarin (▼) and tolbutamide (▼); CYP2C19 (triangle symbols): omeprazole (△); and CYP3A4 (diamond symbols): cyclosporine (◆), quinidine (♦) and simvastatin (●). The horizontal dashed lines represent 2-fold margins, and vertical lines represent the distinction between no interaction (NI) and weak (W), moderate (M) and strong (S) DDIs.
Table 4.6 Rank order of the prediction success of DDIs involving CYP3A4 victim drugs, and the rank order of the victim drug bioavailability and half-life.

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>High</th>
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<tbody>
<tr>
<td>Prediction success</td>
<td>Simvastatin &lt; triazolam &lt; alprazolam &lt; cyclosporine &lt; midazolam &lt; quinidine</td>
<td>Simvastatin &lt; cyclosporine = midazolam &lt; triazolam &lt; quinidine &lt; alprazolam</td>
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<tr>
<td>Victim drug parameter:</td>
<td><em>Bioavailability</em></td>
<td><em>Half-life</em></td>
</tr>
<tr>
<td>Bioavailability</td>
<td>Simvastatin &lt; cyclosporine = midazolam &lt; triazolam &lt; quinidine &lt; alprazolam</td>
<td>Triazolam &lt; midazolam &lt; simvastatin &lt; quinidine &lt; alprazolam &lt; cyclosporine</td>
</tr>
</tbody>
</table>

The impact of altered dosing times of four CYP3A4 victim drugs (from t = -10 to +24 h) on the predicted AUC ratio was assessed using a DDI involving itraconazole (Figure 4.6). A similar trend was observed for all four substrates but it was more pronounced for midazolam and triazolam DDIs with a maximum difference in the predicted AUC ratio of 4.5-fold and 3.5-fold, respectively, observed between dosing at -24 hours and simultaneously. In contrast, the impact on the lower potency DDIs with alprazolam and cyclosporine as the victim drugs was lower, with a maximum 22.6% (1.3-fold) and 31.4% (1.5-fold) difference in AUC ratio observed across the different dosing times from simultaneous dosing to dosing at +24 hours. Differences are likely to be attributable to the long half-lives of alprazolam and cyclosporine (12 and 24 h, respectively), in comparison to the shorter half-lives of midazolam and triazolam (1.9 and 1.5 h, respectively) (Thummel et al., 2008). Prediction was particularly sensitive to the dosing time of -2 to 0 h, with an increase in AUC ratio of up to 2.5-fold for midazolam. A 16% decline in AUC ratio was observed when midazolam was administered 1 h after the itraconazole dose rather than simultaneously (Figure 4.6B). This decline was in contrast to simulations with ketoconazole reported by Zhao et al., 2009 where only a minimal change in AUC ratio was observed during the same time period. Limited observed data were available to directly assess prediction success related to the dose timing. In addition, the assessment was confounded by inconsistency in observed data, for example between two 200mg single itraconazole dose studies, with a higher AUC ratio (4.66) observed after 2mg midazolam, 4 hours after itraconazole dosing (Templeton et al., 2010) compared to 7.5mg midazolam, 2 hours after dosing (AUC ratio 3.42) (Olkkola et al., 1996). Hence, proportional decreases in the AUC ratio were investigated. In the case of interactions involving midazolam, a 43 ± 4% (n=3) decrease in AUC ratio was observed between dosing at 1 or 2 h after the inhibitor dose. This decrease was also seen in simulations, but accounted to only 13%. In the case of triazolam, an increase in observed AUC ratio was noted with substrate dosing from 0-3 h after inhibitor dose (based on Neuvonen et al., 1996), whereas simulated results
display a 34% decrease. The subsequent 20% decrease observed in vivo from 3 to 24 h was over-predicted in simulations by 38%. Use of a range of itraconazole $k_a$ values between 0.3–1 h$^{-1}$ following the dosing schedule outlined in Section 4.2.1 had a minimal impact on the predicted AUC ratio for all victim drugs investigated. Profiles are displayed in Figure 4.6C, with maximal difference in AUC ratios of 8-11% for triazolam and midazolam. Maximal differences for alprazolam and cyclosporine were <1% in both cases.

Figure 4.6 Impact of dosing time from -10 to 24 hours on the predicted AUC ratio in the DDI between itraconazole as the inhibitor and four CYP3A4 substrates (A), and focus on the dosing time from -1 to 1h (B). Difference in the AUC ratio as a result of varied itraconazole $k_a$ from 0.3-1 h$^{-1}$ is displayed (C) at the 1h victim drug dosing time. The increase in AUC was predicted using the dynamic model (with the inclusion of the hydroxy-itraconazole active metabolite) and with alprazolam (◇), midazolam (▲), triazolam (□) or cyclosporine (▼) as the victim drugs, with dosing schedules as defined in the methods.

4.3.5 Assessment of inter-individual variability via the dynamic model

Eight studies reporting the AUC ratios of individual subjects in the clinical studies were selected from the azole database in order to assess the prediction of AUC ratio variability
in the prediction of DDIs. An overview of the simulation results for all studies is given in Table 4.7. Figure 4.7 shows the median and distribution of predicted AUC ratios from ketoconazole–triazolam (Figure 4.7A) and itraconazole–triazolam (Figure 4.7B) DDIs in each virtual trial compared to the actual data reported in 9 individuals (Varhe et al., 1994). In the case of ketoconazole, an under-prediction trend was apparent, as illustrated by the predicted median AUC ratio of 3.7 (obtained from 10 virtual trials, inter-quartile range of 2.9-4.7) in comparison to the observed median AUC ratio of 8.96 (inter-quartile range of 6.1-11.3). However, coefficient of variation was consistent across the virtual trials (29-57%) and with the variation observed in the actual study (50%). Fold difference between maximum and minimum predicted AUC ratios across 10 virtual trials ranged from 2.7-5.9, whereas 6.4-fold was observed in the actual study. In the case of itraconazole, predicted median AUC ratio was 4.7 (obtained from 10 virtual trials, inter-quartile range of 4.0-5.6) in comparison to the observed median AUC ratio of 6.4 (inter-quartile range of 6.3-8.9). However, virtual trials estimated only 23-39% coefficient of variation, consistent with the 2.9-fold difference between maximum and minimum predicted AUC ratio across 10 virtual trials. These results were in contrast to 105% coefficient of variation and 11-fold difference between maximum and minimum AUC ratio observed in the clinical study due to one significant outlier. If the analysis is performed without this outlier then the fold difference between the maximum and minimum AUC ratio is reduced to 2.7. Coefficient of variation is also significantly reduced (to 28%) and is therefore consistent with the observed data (Figure 4.7). In both cases, up to three CYP3A5*1/*1 subjects were included per virtual trial and the variation in CYP3A4 liver and intestinal abundance in each virtual trial is displayed in Figure 4.7C and D, respectively. The mean CYP3A4 abundance in the liver is fairly constant across the virtual trials, with a mean value of 8,200 nmol, ranging from 633 to 62,212 nmol, with coefficient of variation of 105.8%. Similarly, the CYP3A4 abundance in the gut has a consistent mean of 60nmol, but has an approximately 15-fold range and a coefficient of variation of 54.7%. No correlation was observed between CYP3A5 genotype status or liver and gut CYP3A4 abundance with the observed AUC ratio, and it was therefore not further assessed.

The reported inter-individual variability of itraconazole-midazolam DDIs was consistent between three different itraconazole dose levels (50, 200 and 400mg, single dose) from 6 subjects (Templeton et al., 2010), with CVs of 28-32% and difference between the minimum and maximum AUC ratio at 71-76% of the mean value for 50-400mg, respectively from the clinical data. The minimum AUC ratio was reported from the same subject in each dosing scenario; however, the maximum AUC ratio varied between
subjects. The consistency in observed CV was therefore not associated with linear increases in the AUC in each subject. The mean AUC ratios were successfully predicted from the virtual trials (within 1.5-fold) at all three dose levels, and the average predicted CV from ten virtual trials at each of the three dose levels was 34%. Although the overall mean CV was successfully predicted, dose dependency in the values from the ten trials at different dose levels was observed, at 20, 37 and 44% for the predictions of 50, 200 and 400mg itraconazole dose, respectively. The average simulated range (assessed using the minimum to maximum AUC ratio) was over-predicted at 92% of the mean value, and also displayed dose dependency, at 52-121% of the mean for 50-400mg, respectively. Figure 4.8 displays the median and range of predicted AUC ratios in the ten trials at the three different dose levels compared to the observed results.
Table 4.7 Overview of predicted and observed inter-individual variability of the observed clinical and simulated studies. Predicted values were obtained using 10 trials in the dynamic model in Simcyp, where simulations were matched to the reported dosing and demographic details of the clinical study. Inhibitor and victim drug parameters detailed in Table 4.1 and Section 4.2.1 were utilised.

<table>
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<tr>
<th>Study</th>
<th>Mean ± S.D AUC ratio</th>
<th>Median AUC ratio</th>
<th>CV (%)</th>
<th>Inter-quartile range of AUC ratio</th>
<th>Minimum AUC ratio</th>
<th>Maximum AUC ratio</th>
<th>Fold difference (max/min AUC ratio)</th>
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<td>3.9-5.8</td>
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<td>6.0</td>
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<td>3.21</td>
<td>39</td>
<td>2.6-4.0</td>
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<td>Itraconazole – triazolam</td>
<td>10.5 ± 11.0</td>
<td>6.36</td>
<td>105</td>
<td>6.3-8.9</td>
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Figure 4.7 Predicted AUC ratios obtained in 10 individual and combined virtual trials for the ketoconazole-triazolam (A) or itraconazole-triazolam (B) DDI compared to the actual distribution of the data from the nine individual AUC ratios reported by Varhe et al., 1994. Predictions used triazolam $\text{CL}_{\text{int}}$ from HLM (Galetin and Houston, 2006; Patki et al., 2003; Patki et al., 2004; von Moltke et al., 1996) in the dynamic model and ketoconazole and itraconazole parameters as detailed in Table 4.1. The variation in liver (C) and gut (D) content of CYP3A4 in each trial is additionally displayed (data was the same in both DDI simulations). Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value, the box represents the inter-quartile range boundaries, lower and upper whiskers represent 10-90% range and outliers are represented by •.
Figure 4.8 Predicted AUC ratios obtained in ten trials of 6 virtual subjects compared to the actual distribution of the six subjects from itraconazole-midazolam DDIs (Templeton et al., 2010). Results are from single doses of itraconazole, at 50mg (A), 200mg (B) or 400mg (C) at t = 0, with 2mg midazolam dosing at t = 4 hours. Simulations were performed using the dynamic model and include the hydroxy-itraconazole metabolite (parameters are detailed in Table 4.1). Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value and the box represents the inter-quartile range boundaries. Where >9 subjects are included, lower and upper whiskers represent 10-90% range and outliers are represented by •.
Individual results were also presented for a number of other studies and results of the prediction of variability for these studies had varying success. The mean and median AUC ratio from the fluconazole-S-warfarin DDI (Black et al., 1996) was under-predicted by two-fold, with no overlap between the inter-quartile ranges. Overlap between the minimum and maximum AUC ratios between the simulated and actual study was attributed to two subjects in the second simulated trial that were predicted within the inter-quartile range of the observed data. Approximately 2-fold over-prediction of CV was observed despite this under-prediction of mean values (Figure 4.9A). In contrast, the mean of the weak fluconazole-cyclosporine DDI (AUC ratio 1.92, Canafax et al., 1991) from the ten virtual trials was predicted within 0.9-1.1-fold, however there was some under-prediction of inter-individual variability with mean CV (± S.D., range) of 14% (± 3%, 9-21%) from the ten virtual trials, compared to the actual CV of 21% (Figure 4.9B). In addition, the observed inter-quartile range and minimum and maximum AUC ratio were successfully predicted, with differences < 1.1-fold (Table 4.7). Prediction of the ketoconazole-cyclosporine DDI was confounded by the reported differences in the cyclosporine dosing schedule in the control and inhibitor phases of the study (8mg/kg and 2mg/kg, respectively, Gomez et al., 1995). Dose-corrected control phase data were used to calculate the observed AUC ratios, and 2mg/kg dosing therefore used in the simulation. Significant 4-fold under-prediction of mean was observed from the virtual trials and no overlap between the observed and predicted range of AUC ratios. All predicted AUC ratios were < 1.8 with a mean of 1.2, compared to the observed mean of 5.2, with 2.8-9.3 range (Figure 4.9C). An under-prediction trend of 5-fold was also observed for the CV, with 10% predicted compared to the 47% actual. In comparison, the CV of the ketoconazole-triazolam DDI (Greenblatt et al., 1998) was over-predicted, at 45% compared to the observed data 29%, and over-prediction of the range (assessed by maximum/minimum AUC ratio) was >3-fold. This over-prediction was despite 3.5-fold under-prediction of the mean and the inability to predict any AUC ratios within the observed inter-quartile range. The results are consistent with the prediction of inter-individual variability of the ketoconazole-triazolam DDI (Varhe et al., 1994) discussed previously, with fair prediction of the CV but poor mean AUC ratio prediction. In contrast, the simulated DDI between itraconazole-simvastatin (Neuvonen et al., 1998) was over-predicted in comparison to the observed clinical data, in terms of mean, median, CV, and range of AUC ratios. The extent of over-prediction was 2- and 3-fold for the mean and CV, respectively. The larger range of predicted AUC ratios was due to similar predicted and observed minimum AUC ratios (within 2-fold) combined with a 7-fold greater predicted maximum AUC ratio. Overall, the prediction of inter-individual variability in the DDIs demonstrated no clear trend, with 7/10 CV values
predicted within 20% of the observed value (3/10 within 10%), and a range of over- and under-prediction in CV, minimum and maximum AUC ratios and fold difference in the range. The extent of under- or over-prediction of mean AUC ratio correlated to prediction accuracy of the CV; however, the maximum/minimum AUC ratio was predicted within 2-fold for 6/10 of the case studies, where only 3 of these 6 studies also had successful mean AUC ratio predictions.

All three DDIs involving ketoconazole as the inhibitor were under-predicted by > 2-fold; therefore the profiles were further investigated. The cyclosporine AUC in the absence of the inhibitor (at 8mg/kg) was over-predicted by 4-fold (22.01 ± 15.2 mg/L.h compared to 5.94 ± 1.2 mg/L.h observed) (Gomez et al., 1995); this difference resulted in under-prediction of the AUC ratio when combined with the 1.4-fold under-prediction of the AUC of 2mg/kg cyclosporine in the presence of ketoconazole (5.63 ± 3.5 mg/L.h compared to the 7.88 ± 4.9 mg/L.h actual). The differences were consistent when predicted AUC values were scaled up or down for the 2mg/kg and 8mg/kg dosing schedules, respectively. Values of C\textsubscript{max} were well predicted for 8mg/kg cyclosporine in the absence of ketoconazole (931µg/L predicted compared to approximately 800µg/L observed), whereas significant under-prediction of C\textsubscript{max} was observed for 2mg/kg cyclosporine in the presence of ketoconazole (218µg/L predicted compared to approximately 1500µg/L observed). In contrast, the under-prediction of the ketoconazole-triazolam DDI (Greenblatt et al., 1998) resulted from the successful prediction of the control triazolam phase (within 1.2-fold) combined with a >5-fold under-prediction of triazolam AUC in the presence of ketoconazole (0.03 ± 0.02 mg/L.h compared to 0.15 ± 0.10 mg/L.h observed). The under-prediction of the ketoconazole-triazolam DDI (Varhe et al., 1994) resulted from the combination of the 1.9-fold over-prediction of triazolam AUC in the control phase and the 1.4-fold under-prediction of triazolam AUC in the presence of ketoconazole. Prediction of the triazolam AUC was consistent to that simulated for the previous study, however a 2-fold difference in triazolam AUC was reported in the two studies (0.011 and 0.006 mg/L.h for Greenblatt et al., 1998 and Varhe et al., 1994, respectively) despite the identical dose level (0.25mg) and similar number of subjects (6 and 9, respectively). This highlights the variability that is observed during the use of clinical data, complicating the comparison of predicted data to variable observed data. Successful prediction of the triazolam T\textsubscript{max} and C\textsubscript{max} values in the absence and presence of ketoconazole was observed for both DDIs, with predictions all within 1.5-fold. No common trends were therefore identified accounting for the under-prediction of ketoconazole DDIs.
Figure 4.9 Predicted AUC ratios obtained in 10 trials of virtual subjects compared to the actual distribution from clinical studies. DDIs are between fluconazole-S-warfarin (A, Black et al., 1996), fluconazole-cyclosporine (B, Canafax et al., 1991), ketoconazole-cyclosporine (C, Gomez et al., 1995), ketoconazole-triazolam (D, Greenblatt et al., 1998) and itraconazole-simvastatin (E, Neuvonen et al., 1998). Predictions were performed using the dynamic model and were matched to the dosing schedule and patient demographics reported in the study. Inhibitor and victim drug parameters are detailed in Table 4.1, and simulations included the hydroxy-itraconazole active metabolite and utilised triazolam \( \text{CL}_{\text{int}} \) from HLM. Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value and the box represents the inter-quartile range boundaries. Where >9 subjects are included, lower and upper whiskers represent 10-90% range and outliers are represented by •.
Simcyp is an ADME simulator with the ability to include inter-individual variability within DDI predictions (Jamei et al., 2009a; Rostami-Hodjegan and Tucker, 2004; Rostami-Hodjegan and Tucker, 2007). Previously, the utility of Simcyp to predict DDIs has been investigated across a range of inhibitors and victim drugs, also assessing the impact of the time course of inhibition and induction (Fahmi et al., 2009). However, the majority of comparisons of DDI prediction between the static and dynamic model used the static model equation (Einolf, 2007), or the static model in Simcyp compared to an alternative dynamic model (Perdaems et al., 2010) rather than both models within Simcyp. Therefore, the parameters utilised have not been consistent which confounds the direct comparison between the two models. In addition, some of the studies focus on only one inhibitor (Rakhit et al., 2008; Youdim et al., 2008; Zhao et al., 2009) or one victim drug (Fahmi et al., 2009; Hyland et al., 2008) and conclusions may therefore be specific to that drug without the ability to ascertain more general trends. In contrast, the current chapter performs predictions for both the dynamic and static models in Simcyp, allowing consistency in parameters and direct assessment of the impact of the time course. This study comprehensively assessed the prediction accuracy of reversible DDIs of azole inhibitors and different victim drugs, including a representative number of weak, medium and strong interactions. Despite a plethora of research available with these combinations there is still no consensus on certain practices. In addition, a sufficient number of studies per inhibitor or victim drug allowed identification of trends and individual assessment of parameters in the current study. The impact of different parameters including the active metabolites of inhibitors, dose timing and the $k_{a}$ was investigated, as well as the assessing the prediction of inter-individual variability in the extent of DDIs using Simcyp.

The ability of Simcyp to incorporate inter-individual variability and to generate a range of output values gives an advantage over the equation-based static model (Equation 4.1). It also allows a successful level of prediction when using the unbound drug concentrations, therefore conforming to the free drug hypothesis. Use of this value is in contrast to some previous publications advocating the use of total concentration based on the prediction success and number of studies estimated within the 2-fold margin (Brown et al., 2006; Ito et al., 2005). In the current analysis, the Simcyp static model gave consistently higher results than the dynamic model (Figure 4.1), as the ratio of the predicted AUC ratios by dynamic:static model were predominantly <1. This finding is not surprising considering the consistently higher average inhibitor concentration used in static predictions. A comparable number of studies were predicted within 2-fold of the observed AUC ratio (for
the azole-benzodiazepine DDIs) regardless of the model used, with similar bias (afe of 1.63 and 1.29) and precision (rmse of 3.66 and 3.47) for the dynamic or static model, respectively. Use of the weighting according to the number of subjects in the clinical studies did not affect bias and precision of predictions performed in the dynamic model (afe and rmse of 1.61 and 3.58, respectively). Any benefit from incorporating the time-course of the inhibitor in the dynamic model was relatively minor for ketoconazole despite its short half-life (approximately 3.3 ± 1 h). The largest differences between static and dynamic model predictions were observed for interactions involving itraconazole or triazolam. The difference observed with itraconazole studies was likely to be explained by the inclusion of the active metabolite, where studies with a longer time scale included the additional action of hydroxy-itraconazole. The pie charts in Figure 4.1 indicate that the data are not skewed by a disproportionate amount of DDI studies involving any particular inhibitor or victim drug.

Good overall prediction of the azole-benzodiazepine DDIs investigated was observed using both the dynamic and static models with 74 and 79% of studies within 2-fold of the observed AUC ratio, respectively. However, a trend for under-prediction was observed (using the parameter input values detailed in Table 4.1 rather than Simcyp default values) particularly involving the strong interactions (with ketoconazole and itraconazole), regardless of the model used. Dynamic prediction accuracy was increased after inclusion of the non-benzodiazepine DDIs, with 100, 100, 81 and 53% of no interaction, weak, moderate and strong DDIs, respectively predicted within 2-fold (n=59), with an overall prediction success of 80%. The under-prediction trend at increased potency DDIs was still apparent, with no strong DDIs predicted within 2-fold. Six studies (15 data points) were included in the subset where DDIs were reported after single dosing of the inhibitor. One of the assumptions behind the prediction model shown in Equation 4.1 is that the inhibitor concentrations are at steady-state. As demonstrated by the simulations performed by Zhao et al., 2009, this assumption of steady-state would result in significant differences in the DDI magnitude for substrates with a long t$_{1/2}$. The single dose studies in this subset only involve midazolam or triazolam which both have relatively short half-lives. Predictions from virtual trials for these victim drugs support the findings by Zhao et al., 2009 and were not affected by the single dosing regimen of the inhibitor. However, the limited number of clinical studies in this range confounds any firm conclusions. No clear trend was observed to support a difference between static and dynamic DDI predictions or the accuracy against the observed clinical AUC ratio in terms of the bioavailability (0.05-0.88 for simvastatin
and alprazolam, respectively) (Galetin et al., 2008) or $t_{1/2}$ (1.5-24 h for triazolam and cyclosporine, respectively) (Thummel et al., 2008) of the victim drugs in the dataset.

The inclusion of the most abundant active metabolite of itraconazole resulted in improved prediction accuracy in the dynamic model, demonstrating the importance of this parameter in DDI predictions (Figure 4.3). This improvement was despite the itraconazole parent having a higher potency and greater concentration at the CYP enzyme active site. The average increase in AUC ratio observed from the inclusion of hydroxy-itraconazole was 1.2-fold; the increase was minor due to the same target CYP enzyme, and any increase attributed to the increased circulatory time of the metabolite compared to the parent. Simcyp currently allows the inclusion of one metabolite into DDI prediction, excluding the other two potentially relevant itraconazole inhibitory metabolites, keto- and $N$-desalkylitraconazole. $N$-desalkyl-itraconazole has a particularly long $t_{1/2}$ and a comparable ratio of steady-state average unbound concentration and $K_i$ to hydroxy metabolite (Templeton et al., 2008). Therefore, it may contribute further to the persistence of inhibition after itraconazole administration. The variation in itraconazole $k_a$ of approximately 2-fold of the clinical range in the dynamic model resulted in marginal difference in predicted AUC ratio (<11%). The impact may be seen for the larger range in $k_a$ (e.g. at 10-fold as simulated in Zhao et al., 2009), but assuming that the range reported in the clinical study was proportional to the overall patient population, this 10-fold range is unlikely to be observed.

Good prediction accuracy was observed for DDIs involving alprazolam or midazolam as the victim drug, with the majority of these DDIs predicted within 2-fold of the observed value. However, there was an under-prediction trend observed for triazolam DDIs regardless of the model used. Therefore, the impact of alternative in vitro clearance and permeability data used as input parameters was assessed. Minimal improvement in AUC ratio prediction accuracy was seen when triazolam in-house permeability data (from either Caco-2 or MDCK-MDR1 cells) (Gertz et al., 2010) were used in the $Q_{gut}$ model to estimate triazolam $F_G$ values, which were consequently used in DDI prediction. This trend was expected due to the high permeability of triazolam, and therefore low sensitivity of the $Q_{gut}$ model to this parameter (Gertz et al., 2010; Yang et al., 2007b). Values of $F_G$ from the $Q_{gut}$ model (0.91) were 16% higher than that estimated from in vivo values (0.75) (Galetin et al., 2008; Gertz et al., 2010); however, the low observed gut extraction is likely to result in low sensitivity of the DDI to this parameter (Galetin et al., 2010; Galetin et al., 2007). The triazolam $CL_{int}$ utilised in AUC ratio predictions represented a mean value from four different studies performed in HLM. However, in one of these studies (von Moltke et al.,
Chapter 4 – Assessment of azole DDI prediction

1996), a 20-fold difference in $CL_{\text{int}}$ was observed across the six donors, resulting in a maximum 5-fold difference (range of 2.20 to 9.54) in AUC ratio in the Simcyp simulations. This AUC ratio difference is not observed in individual trials, but can be observed across the whole virtual population; this difference could be due to other confounding factors rather than a direct result of the $CL_{\text{int}}$ variability. Differences in predicted AUC ratio and concentration-time profile (assessed by $T_{\text{max}}$ and $C_{\text{max}}$) resulting from the use of $CL_{\text{int}}$ from HLM and the default recombinant values in Simcyp were minor in the case of triazolam. The assessment of azole DDIs involving 22 non-benzodiazepine victim drugs covered a range of CYP enzymes, and resulted in high prediction accuracy, with only four unsuccessful predictions. The proportion of strong DDIs was significantly lower than the database used to assess the azole-benzodiazepine DDIs; this proportion is likely to explain the increase in the number of successful predictions. All DDIs followed the trend for reduced AUC ratio predictions with increasing potency, with unsuccessful prediction of the three strong DDIs. No relationships were observed from assessment of non-benzodiazepine DDIs when assessed by inhibitor or victim drug; this finding is in contrast to those observed from the azole-benzodiazepine database.

In the current study impact of different dosing regimens (staggered dosing) of the inhibitor on DDI prediction was assessed using an itraconazole interaction. In addition to midazolam (previously studied in a ketoconazole-midazolam interaction pair; Zhao et al., 2009), triazolam, cyclosporine and alprazolam were also included in the assessment. Investigation found that the most pronounced difference based solely on virtual trials was observed with midazolam, and the maximum AUC ratio was observed with simultaneous dosing. In addition, the impact of staggered dosing was maximal for victim drugs with short half-lives (<2h for midazolam and triazolam); minimal differences were observed for alprazolam and cyclosporine (half-lives > 12h). Higher AUC ratios were simulated for DDIs involving these latter two victim drugs when dosed 10 hours before the inhibitor as a result of the longer half-life and therefore increased potential for interaction. From the results of the virtual trials, the success of dose staggering in a clinical settling in order to reduce the extent of DDIs is therefore likely to be increased when involving victim drugs with short half-lives. However, the same trends were not observed when compared to the observed clinical data with different dosing schedules. For example, the maximum AUC ratio in one study with triazolam (Neuvonen et al., 1996) was actually observed with substrate dosing 3 h after the inhibitor dose. However, this observed difference may be a result of the study design, considering that food intake was included with the inhibitor dose in this scenario and not when the inhibitor and substrate were simultaneously dosed. The
decreases in the AUC for the dosing time >3 h for both midazolam and triazolam was overestimated in the simulations in comparison to the observed AUC ratios (Neuvonen et al., 1996). However, limited availability of clinical data for different dosing regimens and the wide range of DDI magnitude observed for the same dosing regimen confounds any clear trends. The differential impact of dosing time between previous work with ketoconazole (Zhao et al., 2009) and current analysis with itraconazole cannot be explained by the approximately 4-fold longer t1/2 of itraconazole (21 ± 6 h) in comparison to ketoconazole (Thummel et al., 2008).

The prediction of inter-individual variability in DDI magnitude was assessed in comparison to studies where AUC values of the victim drug ± inhibitor were reported for each individual (Canafax et al., 1991; Gomez et al., 1995; Greenblatt et al., 1998; Templeton et al., 2010; Varhe et al., 1994). In the case of the ketoconazole-triazolam DDI, ratio of median predicted:observed values across the 10 virtual trials ranged from 0.29-0.52 highlighting the under-prediction trend. However, predicted inter-individual variability in the AUC ratio reflected observed variability, as the coefficient of variation across the trials (45%) was consistent with the actual study (50%). A similar under-prediction trend was observed in the case of itraconazole-triazolam, but to a lesser extent, as the ratio of median predicted:observed values was 0.74. However, variability estimated across trials for this interaction pair (32% coefficient of variation) was considerably lower in comparison to the observed data with this inhibitor (105%), associated with one significant outlier. If this subject was excluded from the analysis of the itraconazole DDI with triazolam, the predicted variability and differences between minimum and maximum AUC ratio are comparable to the observed data. The same trend was also observed for another ketoconazole-triazolam DDI (Greenblatt et al., 1998), with successful prediction of CV and range despite a 3.5-fold under-prediction of mean. This trend was not observed for other case studies, and is potentially specific to DDIs involving triazolam as the victim drug. Assessment of other studies resulted in successful prediction of the mean but under-prediction of variability in the case of a fluconazole-cyclosporine DDI (Canafax et al., 1991), under-prediction of both the mean and variability for a ketoconazole-cyclosporine DDI (Gomez et al., 1995), or successful prediction of both the mean AUC ratios and mean variability of an itraconazole-midazolam DDI (at three different inhibitor dose levels, Templeton et al., 2010). In the latter study however, dose-dependency of the inter-individual variability was predicted from the virtual trials with a CV range of 20-44% at 50-400mg itraconazole. This increase in CV was not observed in the clinical study, with consistent CV (28-32%) across the three dose levels. The three itraconazole dosage
regimens were carried out in the same six individuals, and the minimum AUC ratio was observed from the same subject in each case. The maximal AUC ratio was not however, and therefore does not explain the consistency in observed CV values across the three dose levels. No consistency was found between the prediction of AUC in the presence and absence of inhibitor, or the prediction of the victim drug profile to rationalise the under-prediction of AUC ratio for DDIs involving ketoconazole. Variability was observed in the prediction success of victim drug AUC and \(C_{\text{max}}\) in the absence and presence of inhibitor. The successful and poor prediction of the midazolam and triazolam variability, respectively correlate to the prediction success of mean AUC ratio discussed above, however the inconsistency between the predictions of mean and variability of the cyclosporine DDIs do not conform to this trend.

Assessment of different physiological, demographic and genetic characteristics of the individual subjects in the virtual population was performed in order to rationalise the variability seen in virtual subjects’ AUC ratio, although corresponding data from in vivo trials were not available for direct comparison. This analysis included simulated CYP3A4 content, based on a meta-analyses of liver data (Rostami-Hodjegan and Tucker, 2007) or intestinal data from 31 individuals (Paine et al., 2006), which could only partially explain the inter-individual variability in AUC ratios. The prediction success of DDIs can only be validated against the reported in vivo values. However, it is advisable to consider these data very critically, considering that variability can be introduced as a result of inconsistency in study designs and data analysis. The small size of the population observed in the assessed DDI study (nine subjects, Varhe et al., 1994) or overall in the studies investigated (mean 10.0 ± 2.76, range 4-20 subjects) may result in a reduced ability to accurately predict population values, either by limiting the variability observed, or increasing the power of one subject if a significantly different result is observed, as illustrated in the case of itraconazole-triazolam.

Overall, this chapter has reinforced the value of Simcyp as a successful platform to assess the prediction of DDIs in a population based model, as high prediction accuracy was observed for 38 DDIs. An under-prediction trend was observed in the current dataset for strong DDIs, with no overall significant difference in the prediction accuracy between the static and dynamic model. This lack of major difference between the two models can be attributed to the fact that concentrations of azoles are substantially higher in comparison to their \(K_{\text{i}}\) regardless of the approach used. However, this relationship cannot be extrapolated to less potent or inhibitors administered at a low dose. Prediction accuracy of
benzodiazepines was related to the particular victim drug in the DDI, with high prediction accuracy observed for midazolam DDIs and lower prediction accuracy seen for triazolam DDIs. In contrast, no such trends were observed from analysis of non-benzodiazepine victim drugs (n=22). Incorporation of the time course of inhibition was mainly beneficial for itraconazole, due to the ability to include the contribution of the active metabolite in the prediction model. An increasing number of compounds with multiple interaction mechanisms or contributing inhibitory metabolites (Fahmi et al., 2009; Hinton et al., 2008; Isoherranen et al., 2009; Rowland-Yeo et al., 2010; Zhang et al., 2009) emphasize the importance of the dynamic model approach. Predicted inter-individual variability in DDI magnitude reflected the variability observed in vivo despite some under-prediction of mean, and highlighted the importance of critical evaluation of both the input parameters used in the simulations and the clinical data used for validation of the prediction success.
CHAPTER 5: ASSESSMENT OF THE PREDICTION OF DDIs INVOLVING SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRIs)
5 ASSESSMENT OF THE PREDICTION OF DDIs INVOLVING SSRIs

5.1 INTRODUCTION

The background into the prediction of DDIs via static and dynamic models has been previously discussed, including the demonstration of the importance of inhibitory metabolites and their inclusion into DDI prediction (Chapter 4). The SSRI inhibitors form an important group of prescribed drugs. This group is associated with high risk for DDIs due to the likelihood of poly-pharmacy, inhibition of a number of CYP enzymes, and long duration of circulating time (Hiemke and Hartter, 2000). In addition, it has been widely reported that the active metabolite of fluoxetine, norfluoxetine plays an important role in the observed persistence of inhibition after cessation of fluoxetine administration (Aronoff et al., 1984; Bergstrom et al., 1993; Isoherranen et al., 2009). The observed trend cannot be rationalised from the 10-fold lower $K_i$ in comparison to the parent, and is likely to result from the long half-life of the metabolite. The observed difference between the fluoxetine and norfluoxetine $K_i$ values are consistent to the difference for itraconazole and the major metabolite, hydroxy-itraconazole; the itraconazole metabolites (hydroxy-, N-desalkyl- and keto-itraconazole) have been found to be responsible for approximately 50% of the total inhibition after itraconazole administration (Templeton et al., 2010).

Previous work on the prediction of DDIs and comparisons between the static and dynamic models have assessed DDIs involving the SSRI inhibitors (Brown et al., 2006; Einolf, 2007; Fahmi et al., 2009; Ito et al., 2003; Wang, 2010); however these comparisons have been complicated by differences in parameter values (e.g., $fu_p$, $K_i$, $F_G$, $fm_{CYP}$), which may have led to unsupported conclusions. Matched parameter values were used in Simcyp to assess the weak mechanism-based inhibition of CYP3A4 by fluoxetine (all AUC ratios < 2-fold) in a study by Wang, 2010. The prediction of SSRI DDIs in Simcyp often includes a hepatic uptake factor, ranging from 3- (fluoxetine) to 10-fold (fluvoxamine). These values resulted from sensitivity analysis (Einolf, 2007) and inclusion was justified from a number of studies reporting uptake. These uptake studies included the 12- and 27-fold partitioning of fluvoxamine and fluoxetine between liver and water/plasma from in vivo data in mouse and rat (von Moltke et al., 1994a; von Moltke et al., 1995; Yao and Levy, 2002) and the 10- and 40-fold difference between observed in vitro and in vivo CYP1A2 and CYP2C19 $K_i$ estimates, respectively for fluvoxamine (Yao et al., 2001; Yao et al., 2003). The inconsistency in the in vitro/in vivo difference between the two CYP enzymes does not support the hypothesis of hepatic uptake. The oil-spin assay and evidence for lysosomal trapping in rat hepatocytes indicate that no significant uptake is apparent for the SSRI
inhibitors (Hallifax and Houston, 2007). In addition, no difference in \( K_{i,u} \) estimated from rat hepatocytes and microsomes was observed for either fluoxetine or fluvoxamine, with the exception of a 3-fold increase in hepatocyte potency in comparison to microsomes present from one metabolic pathway for fluvoxamine (Brown et al., 2007a).

It is widely reported that genotypes have a large impact on the disposition of drugs, particularly when the polymorphic enzyme is the predominant route of metabolism (Bertilsson, 2007; Kirchheiner et al., 2004; Rostami-Hodjegan and Tucker, 2004; Rostami-Hodjegan and Tucker, 2007; Sachse et al., 1997; Spina et al., 1997; Xie et al., 1999). Further to this impact, a number of differences in DDI potency have been reported involving polymorphic enzymes as a result of altered metabolism of the inhibitor or victim drug (e.g. Brynne et al., 1999; Christensen et al., 2002; Yasui-Furukori et al., 2004); Simcyp has the ability to predict DDIs in different genotyped populations. Health- and disease-induced changes in drug disposition have also been incorporated into Simcyp, allowing ADME properties to be described in specified populations. A number of DDI studies report from patient populations instead of investigative trials in healthy subjects, for example involving cyclosporine (in transplant patients, Canafax et al., 1991) or fluoxetine (in depression patients, Brynne et al., 1999), however not all disease states have obvious or reported physiological changes. Despite the plethora of research, the effects that different clinical populations have on the prediction of DDIs have not been comprehensively investigated, including the impact of different phenotypes and disease status.

5.1.1 Aims

The objectives of this chapter were to assess the static and dynamic method of DDI prediction involving SSRI inhibitors in order to extend the previous comparison (Chapter 4) to the potent inhibition of CYP enzymes other than CYP3A4. Both models were assessed within Simcyp to keep input parameters consistent and therefore allow a valid comparison. A database was collated consisting of 37 DDIs involving inhibition of the five main CYP enzymes by two SSRIs, fluoxetine and fluvoxamine. Predictions were further assessed by inhibitor and victim drug properties. This analysis included the incorporation of active metabolites into DDI prediction and the relative dosing times of the inhibitor and victim drug. The ability of the Simcyp dynamic model to predict the inter-individual variability of clinical studies was also investigated, including assessment of the impact of different subject populations with regards to the health and genotype status of the subjects.
5.2 METHODS

5.2.1 Comparison of the static and dynamic models in SSRI DDI prediction

The static and dynamic approaches to DDI prediction were assessed using the 37 SSRI DDIs in the database (Chapter 2) in Simcyp V9.30 using methods analogous to those in Chapter 4. In brief, a simulation of ten trials was performed, with each trial consisting of matching population demographics and dosing regimen to the clinical study. One clinical study in the database reported the fluvoxamine-omeprazole DDI in a Japanese population (Yasui-Furukori et al., 2004). Simcyp allows selection of this population for simulations, with altered physiological parameters including differences in the distributions of height and body weight, and genetic factors including differences in the abundance and polymorphism frequency for CYP enzymes (Inoue et al., 2006). DDI prediction accuracy was compared between simulations in the Japanese and Caucasian populations to investigate the impact of this matched population. Inhibitor input values are displayed in Table 5.1, with the mechanism-based inhibition of fluoxetine to CYP3A4 included. The mechanism-based parameters were not included for CYP2C19 as no CYP2C19-based DDIs were available for fluoxetine. The default rate of CYP3A4 $k_{\text{deg}}$ was equivalent to a half-life of 23 hours in the intestine (0.03 h$^{-1}$) (Obach et al., 2007); the default liver value correlating to a CYP3A4 degradation half-life of 90 hours (0.0077 h$^{-1}$) was compared to 72 hours (0.0096 h$^{-1}$) (Galetin et al., 2006; Galetin et al., 2010) and 23 hours (0.03 h$^{-1}$) (Wang, 2010). The CYP2D6 $K_i$ and $f_u$ values for both inhibitors in Table 5.1 were taken from Brown et al., 2006 and fluvoxamine CYP1A2 and CYP2C19 $K_i$ values from Yao et al., 2001; Yao et al., 2003. All other parameters (including fluvoxamine $K_i$ for CYP2C9 and CYP3A4) remained as Simcyp defaults.

Table 5.1 Input parameters for the SSRI inhibitors used in Simcyp simulations

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<th>$K_i$ (µM)</th>
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<th>$K_i$ (µM)</th>
<th>$K_i$ (µM)</th>
<th>$f_u$</th>
</tr>
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<tbody>
<tr>
<td>Fluvoxamine</td>
<td>0.038</td>
<td>8.43</td>
<td>0.076</td>
<td>2.47</td>
<td>17.89</td>
<td>0.23</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.075</td>
<td>*</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Mechanism-based inhibition included, with $K_{L_{\text{inu}}}$ of 0.5 (±0.02) µM and $k_{\text{inact}}$ of 0.0002 (±0.0) h$^{-1}$ obtained using midazolam 1’-hydroxylation as a probe substrate in HLM (Mayhew et al., 2000; McGinnity et al., 2006). $K_i$ values were corrected using the reported value of $f_{u_{\text{mic}}}$ estimated from LogP (McGinnity et al., 2006).
No uptake was included as default for fluoxetine or fluvoxamine in Simcyp, and the fraction absorbed was assumed to be 1. The default oral clearances in Simcyp (from *in vivo* data) were 182.7Lh\(^{-1}\) and 36.8Lh\(^{-1}\) for fluvoxamine and fluoxetine, respectively (McGinnity et al., 2004; Thummel et al., 2008). The use of *in vivo* clearance data in Simcyp does not allow active metabolite inclusion into simulations; recombinant CL\(_{int}\) was therefore collated for fluoxetine from Margolis et al., 2000 (Table 5.2) in order that the active metabolite, norfluoxetine could be incorporated into predictions. Norfluoxetine was not in the Simcyp library therefore corresponding parameters were collated (Table 5.3). Dynamic predictions of fluoxetine DDIs with the inclusion or exclusion of the active metabolite were compared in order to assess the impact of norfluoxetine to DDI prediction accuracy.

**Table 5.2** \(V_{\text{max}}\) and \(K_{\text{m}}\) values for racemic fluoxetine in baculovirus-expressed recombinant human CYP enzymes as input parameters for Simcyp. Values of \(K_{\text{m}}\) and \(V_{\text{max}}\) were taken from Margolis et al., 2000, and corrected by inter-system extrapolation factors (ISEF) in Simcyp (Rostami-Hodjegan and Tucker, 2004).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(K_{\text{m}}) (μM)</th>
<th>(V_{\text{max}}) (pmol/min/pmolCYP)</th>
<th>ISEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>501</td>
<td>37.9</td>
<td>1.17</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>246</td>
<td>1.64</td>
<td>0.98</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>30.7</td>
<td>19.6</td>
<td>1.04</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>172</td>
<td>39.0</td>
<td>0.55</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>2.20</td>
<td>6.32</td>
<td>0.56</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>19.3</td>
<td>8.36</td>
<td>0.98</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>89.4</td>
<td>8.67</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Table 5.3 Norfluoxetine input parameters for the dynamic prediction of fluoxetine DDIs in Simcyp

<table>
<thead>
<tr>
<th>Input parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D6 K&lt;sub&gt;i&lt;/sub&gt; (μM)</td>
<td>0.493 ± 0.33</td>
<td>1</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>331.8</td>
<td>Sigma Aldrich Ltd.</td>
</tr>
<tr>
<td>Log&lt;sub&gt;P&lt;/sub&gt;O&lt;sub&gt;W&lt;/sub&gt;</td>
<td>4.05</td>
<td>Vrakas et al., 2006</td>
</tr>
<tr>
<td>pKa</td>
<td>9.05</td>
<td>Zorita et al., 2007</td>
</tr>
<tr>
<td>CL (L h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>8.50</td>
<td>2</td>
</tr>
<tr>
<td>R&lt;sub&gt;B&lt;/sub&gt;*</td>
<td>0.55</td>
<td>Mills et al., 2010</td>
</tr>
<tr>
<td>fu&lt;sub&gt;p&lt;/sub&gt;*</td>
<td>0.06</td>
<td>Brown et al., 2006</td>
</tr>
</tbody>
</table>

* indicates values that were assumed to be the same as fluoxetine, as was previously observed for fluoxetine and norfluoxetine in rats and sheep (Caccia et al., 1990; Kim et al., 2004).

1 Mean value from K<sub>i</sub> values in human liver microsomes: 0.596μM (Brosen and Skjelbo, 1991), 0.33μM (Skjelbo and Brosen, 1992), 0.19μM (Otton et al., 1993), 1.1μM (Belpaire et al., 1998), 0.43μM (Crewe et al., 1992), 0.31μM (Stevens and Wrighton, 1993).

2 Weighted mean value from 40mg oral clearance: 8.71L h<sup>-1</sup> (healthy subjects (n=6) from Aronoff et al., 1984) and 8.40L h<sup>-1</sup> (healthy subjects (n=12) from Schenker et al., 1988).

Simcyp default k<sub>a</sub> values for fluoxetine and fluvoxamine were 0.97 and 0.39 h<sup>-1</sup>, respectively. These values did not differ from those calculated from literature references for the time to maximal plasma drug concentration (T<sub>max</sub>) and half-life (t<sub>1/2</sub>), which were 0.91 ± 0.47 h<sup>-1</sup> for fluoxetine (Keller et al., 2005; Pan et al., 2002; Thummel et al., 2008) and 0.54 ± 0.15 h<sup>-1</sup> for fluvoxamine (Hrdina, 1991; Spigset et al., 1999). The impact of approximately 50% variation in the k<sub>a</sub> value (0.2-0.8 and 0.5-1.5 h<sup>-1</sup> for fluoxetine and fluvoxamine, respectively) on prediction outcome was additionally investigated.

5.2.2 Impact of victim drug dose timing on the prediction of SSRI DDIs

The predictions for the SSRI DDIs were assessed by CYP enzyme and victim drug involved in order to ascertain any trends. Table 5.4 displays the contribution of renal excretion to the total clearance and the intrinsically calculated Simcyp values of F<sub>G</sub> (estimated using the Q<sub>gut</sub> model) and fm<sub>CYP</sub> for the five CYP enzymes where SSRI DDIs were identified (Chapter 2), calculated from recombinant data and corrected for renal clearance. All CYP enzymes were represented by at least one victim drug where the fm<sub>CYP</sub> contributed to >50% of the metabolism. The majority of victim drugs had minor renal clearance (<3% contribution to overall clearance), exceptions were the CYP3A4 substrates...
alprazolam and quinidine, with 14-18% renal contribution. Values of $F_G$ were mostly $>0.9$, with the exception of the significant gut metabolism observed for midazolam and tolterodine ($F_G$ of 0.59 in both cases). Simulations of altered victim drug dosing were performed using typical victim drugs for the main CYP enzymes inhibited by the two SSRI inhibitors. These doses were 20mg omeprazole for CYP2C19 and 150mg caffeine for CYP1A2 (fluvoxamine), and 50mg desipramine for CYP2D6 (fluoxetine). The victim drugs were selected where the specific CYP enzyme represented the single or major metabolic pathway in Simcyp, and did not always correlate to the preferred substrates proposed in the FDA guidelines (FDA, 2006) and Tucker et al., 2001a. Simulations were performed using the dynamic model over a four-day period with the inhibitor administered once daily (100mg and 40mg for fluvoxamine and fluoxetine, respectively, with the inclusion of the norfluoxetine active metabolite in simulations) with victim drug administration from -10 to +24 hours after the final inhibitor dose.

### Table 5.4 Simcyp output values of victim drug properties, including $fm_{CYP}$, $F_G$ and the contribution of renal clearance to the overall clearance.

<table>
<thead>
<tr>
<th>Victim drug</th>
<th>$fm_{CYP1A2}$</th>
<th>$fm_{CYP2C9}$</th>
<th>$fm_{CYP2C19}$</th>
<th>$fm_{CYP2D6}$</th>
<th>$fm_{CYP3A4}$</th>
<th>$F_G$</th>
<th>$CL_R$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.77</td>
<td>0.99</td>
<td>18</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01</td>
<td>1.00</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Clozapine</td>
<td>0.56</td>
<td>0.13</td>
<td>0.10</td>
<td>0.02</td>
<td>0.19</td>
<td>0.94</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Desipramine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.87</td>
<td>-</td>
<td>0.95</td>
<td>3</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.07</td>
<td>-</td>
<td>0.15</td>
<td>0.71</td>
<td>0.05</td>
<td>0.82</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Midazolam</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.85</td>
<td>-</td>
<td>0.59</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>-</td>
<td>-</td>
<td>0.73</td>
<td>-</td>
<td>0.26</td>
<td>0.93</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Quinidine</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>0.66</td>
<td>0.99</td>
<td>14</td>
</tr>
<tr>
<td>S-mephytoin</td>
<td>-</td>
<td>-</td>
<td>0.93</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.82</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01</td>
<td>1.00</td>
<td>3</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.99</td>
<td>0</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.71</td>
<td>0.22</td>
<td>0.59</td>
<td>3</td>
</tr>
</tbody>
</table>

5.2.3 Assessment of inter-individual variability in the AUC ratios; impact of disease status and genotype on DDI prediction

Clinical studies reporting the AUC data from the individual subjects in the study were selected in order to further assess the ability of Simcyp to predict inter-individual variability (as Section 4.2.3), including the fluvoxamine-tolbutamide DDI (Madsen et al.,
A number of studies also reported AUC ratios in CYP2C19 or CYP2D6 genotyped subjects, in addition to (Brynne et al., 1999; Christensen et al., 2002) or instead of (Yasui-Furukori et al., 2004) individual AUC results. The ability of Simcyp to predict DDI differences across different phenotype groups was therefore also assessed.

The analysis was performed via 10 trials with matched population demographics and dosing schedules; median, minimum and maximum predicted AUC ratios were compared to the observed data, as per Section 4.2.3. Profiles of the victim drug AUC and $C_{\text{max}}$ in the presence and absence of inhibitor were also assessed to attempt rationalisation of the level of prediction success. When assessing DDIs in different genotype populations, no difference was observed between trials solely consisting of the different genotype populations (EM, IM or PM), or trials containing the matched proportions of each genotype as the clinical trial. Therefore, the latter case was usually selected using simulations consisting of 10 trials. The proportions of genotypes from subjects in the clinical studies and therefore used in the simulations were 0.33 EM, 0.44 IM and 0.22 PM of CYP2D6 (Brynne et al., 1999), 0.5 EM and 0.5 PM of CYP2D6 (Christensen et al., 2002), and 0.33 EM, 0.33 IM and 0.33 PM of CYP2C19 (Yasui-Furukori et al., 2004), where IM represents the subject having one functional allele.

CYP2D6 is responsible for the metabolism of fluvoxamine. Christensen et al., 2002 report the fluvoxamine-caffeine and fluvoxamine-omeprazole DDIs in subjects genotyped for CYP2D6. The default Simcyp parameters are based on in vivo clearance which is not attributed to any specific CYP enzymes. Therefore, in vitro CYP2D6 data from human liver microsomes ($K_m$ of 76.3μM and $V_{\text{max}}$ of 37.5 pmol min$^{-1}$ mg prot$^{-1}$ (Miura and Ohkubo, 2007)) were utilised in Simcyp to assess the impact of different CYP2D6 genotypes on fluvoxamine DDI predictions. Use of these values resulted in a 10- and 2-fold greater $C_{\text{max}}$ and $T_{\text{max}}$, respectively in comparison to the Simcyp default in vivo clearance. One study reported individual data from a fluoxetine-tolterodine DDI in a patient group, in contrast to healthy subjects (Brynne et al., 1999). This study was in elderly female, depression/anxiety patients with urinary incontinence. This population was not featured in Simcyp and physiological attributes of the population were not given, therefore the simulation was assessed in healthy subjects and compared to the actual data. The impact of the assumption of a healthy state was therefore assessed, in addition to the reported genotype data. Analysis of the simulations was performed in the presence and absence of the norfluoxetine inhibitory metabolite, to assess the impact of its inclusion on the DDI prediction accuracy.
5.2.4 Data analysis
Data was analysed as described in Section 4.2.4. Briefly this analysis considered prediction within a 2-fold range of the observed AUC ratio as successful, although the number within 1.5-fold was also considered. The prediction of DDI studies was assessed per inhibitor and per victim drug, and after classification of the potency in a method analogous to the FDA guidelines for the assessment of potential inhibitors of CYP3A4 (Huang et al., 2007). Predictions of the range and CV, in addition to the median and mean AUC ratios from matched populations, were compared to observed data from the individual subjects where this data were available. The afe and rmse were used as measures of bias and precision, respectively, as outlined in Section 4.2.4, Equations 4.2-4.4.
5.3 RESULTS

DDI studies involving fluvoxamine (n=27) or fluoxetine (n=10) as the inhibitor were collated (Chapter 2) and used to further assess the prediction of DDIs using the static and dynamic model. These inhibitors were assessed separately due to the difference in CYP enzymes inhibited by the two SSRI inhibitors. In addition, four studies (representing 14 data points) in patient populations or with genotyped subjects were identified to use as case studies for the prediction of the inter-individual variability of DDIs.

5.3.1 Prediction of fluvoxamine DDIs via the static and dynamic models

The dynamic:static ratio for the predictions of DDIs involving fluvoxamine as the inhibitor did not exceed 1 in any prediction (range 0.51-0.99). Differences between predictions made using the static and dynamic models were minimal with only 7/27 studies having differences greater than 20%. This difference appeared to correlate to the victim drug, with 5 of these 7 studies attributable to S-mephytoin (CYP2C19) and theophylline (CYP1A2). The two remaining studies with differences >20% were the imipramine DDI, and one of the five DDIs involving caffeine. This difference did not, however, extend to the correlation of significant difference to the CYP enzyme involved, as the prediction accuracy of fluvoxamine DDIs involving other victim drugs of CYP2C19 and CYP1A2 (omeprazole and caffeine, respectively) were similar using the static and dynamic models. Figure 5.1 shows the prediction of the fluvoxamine DDIs using the static and dynamic model.
Chapter 5 – Assessment of SSRI DDI prediction

Figure 5.1 Comparison of the predicted:observed and observed AUC ratios for 27 DDIs involving fluvoxamine. Predictions were performed in Simcyp using the time-based dynamic (●) or static (▽) model. Horizontal dashed lines represent the 2-fold margins, and vertical dashed lines represent the boundaries between weak (W), moderate (M) and strong (S) DDIs.

The number of fluvoxamine DDI studies predicted within 2-fold are displayed in Table 5.5. The trend of decreasing accuracy of the predictions with increasing DDI potency is observed, with none of the strong DDIs predicted within 2-fold of the actual AUC ratio by the static or dynamic model. The overall accuracy differences between the static and dynamic model were small, with one additional study successfully predicted using the static model (14/27 compared to 13/27 for the dynamic model). This study was unsuccessfully predicted by the dynamic model, despite the similar predictions between the two models (dynamic:static ratio of 0.93). Precision and bias as assessed by the afe and rmse were 2.4 and 3.6, respectively for dynamic predictions and 2.1 and 3.2 for static predictions. All fluvoxamine studies were multiple-dosing studies, and all would therefore be assumed to feature the inhibitor at steady state concentrations, complying with the assumptions of the DDI prediction model. No difference was observed from the assessment of the number of static and dynamic studies predicted within 1.5-fold of the actual DDI, with the same 10/27 (37%) studies successfully predicted in both cases.
Table 5.5 Percentages of fluvoxamine DDI studies predicted within 2-fold from use of the
time-based dynamic or static model in Simcyp. Studies are divided according to potency,
via methods analogous to the FDA guidelines for the assessment of potential inhibitors of
CYP3A4 (Huang et al., 2007).

<table>
<thead>
<tr>
<th>Model</th>
<th>No interaction (%)</th>
<th>Weak (%)</th>
<th>Moderate (%)</th>
<th>Strong (%)</th>
<th>Overall (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=7)</td>
<td>(n=9)</td>
<td>(n=8)</td>
<td>(n=27)</td>
</tr>
<tr>
<td>Dynamic</td>
<td>100</td>
<td>100</td>
<td>33</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>Static</td>
<td>100</td>
<td>100</td>
<td>44</td>
<td>0</td>
<td>52</td>
</tr>
</tbody>
</table>

5.3.2 Prediction of fluoxetine DDIs via the static and dynamic models

The dynamic:static prediction ratios for fluoxetine DDIs were ≤ 1 in all cases. Half of the
studies were more than 20% different between the models. The range of dynamic:static
ratios ranged from 0.32-0.81 for the DDIs involving CYP2D6 victim drugs (6/10) and
0.99-1.00 for CYP3A victim drugs (4/10). The differences observed are greater than those
from fluvoxamine DDI predictions, and the ranges observed per CYP enzyme did not
correlate between the two inhibitors. All CYP2D6 interactions with fluvoxamine had a
<20% difference, compared to only 1/6 of the CYP2D6 DDIs with fluoxetine. The
prediction accuracy of fluoxetine DDIs using the static or dynamic models is displayed in
Figure 5.2.
Figure 5.2 Comparison of predicted:observed and observed AUC ratios for 10 fluoxetine DDIs. Predictions were performed in Simcyp using either the time-based dynamic model in the presence (●) or absence (■) of the fluoxetine metabolite (norfluoxetine) or using the static model (▽). The horizontal dashed lines represent the 2-fold margins, and vertical dashed lines represent the boundaries between weak (W), moderate (M) and strong (S) DDIs.

Table 5.6 shows the number of fluoxetine DDI studies predicted within 2-fold using the static and dynamic models according to their potency. The trend of reduced prediction accuracy with increasing potency is again displayed and is more pronounced for dynamic predictions. All DDIs with AUC ratios < 4-fold were successfully predicted and all interactions above this level (3/3) were under-predicted. The static model also displayed reduced AUC ratio predictions at higher DDI potencies. However, there were two over-predicted DDIs, both with an approximate 2-fold observed AUC ratio. The inclusion or exclusion of the norfluoxetine active metabolite resulted in no difference in predictions or prediction accuracy in the dynamic model. Precision and bias for these studies were 1.7 and 2.6, respectively for dynamic predictions and 1.2 and 2.2 for static predictions. The dynamic model performed marginally better than the static model (regardless of metabolite inclusion) when predictions within 1.5-fold of the actual AUC ratio were assessed, with 70% success for the dynamic model, compared to 60% with the static predictions.
Table 5.6 Percentages of fluoxetine DDI studies predicted within 2-fold by the time-based dynamic or static model in Simcyp. Studies are divided according to potency, via methods analogous to the FDA guidelines for the assessment of potential inhibitors of CYP3A4 (Huang et al., 2007).

<table>
<thead>
<tr>
<th>Model</th>
<th>Induction (%)</th>
<th>Weak (%)</th>
<th>Moderate (%)</th>
<th>Strong (%)</th>
<th>Overall (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=1)</td>
<td>(n=4)</td>
<td>(n=3)</td>
<td>(n=2)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td>Dynamic (-metabolite)</td>
<td>100</td>
<td>100</td>
<td>67</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Dynamic (+metabolite)</td>
<td>100</td>
<td>100</td>
<td>67</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Static</td>
<td>100</td>
<td>75</td>
<td>67</td>
<td>50</td>
<td>70</td>
</tr>
</tbody>
</table>

One study reported the effect of desipramine and imipramine after single and multiple dosing of fluoxetine. This resulted in a wide potency range of DDIs with AUC ratios from 1.89 to 7.43 from the imipramine DDI after single fluoxetine dosing, and the desipramine DDI after multiple fluoxetine dosing, respectively (Bergstrom et al., 1992). The static model did not predict either single dosing scenario within 2-fold, but successfully predicted the multiple dosing scenarios. This difference may result from the assumptions of steady-state inhibitor concentration which are not correct in the single dosing scenarios. The dynamic model performed better than the static model with 3/4 predictions within 2-fold of the actual AUC ratio. The unsuccessful prediction was a 3.5-fold under-prediction of the interaction with desipramine following multiple fluoxetine dosing, and is therefore not due to the steady-state hypothesis. Therefore, the fluoxetine DDIs do not support a reduction in prediction accuracy resulting from the assumption of steady state inhibitor concentrations in single dosing scenarios. Figure 5.2 also displays the results of dynamic simulations with the inclusion and exclusion of the fluoxetine active metabolite, norfluoxetine. The inclusion of norfluoxetine resulted in no difference to prediction accuracy or precision compared to fluoxetine alone.

5.3.3 Impact of victim drug properties on the prediction of DDIs

The SSRI database included victim drugs metabolised by five CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4), and twelve victim drugs. CYP2C9 was represented by one victim drug; all others had two (CYP2C19) or three (CYP1A2, CYP2D6 and CYP3A4). The database of DDIs used to assess fluvoxamine predictions consisted of victim drugs metabolised by all five different CYP enzymes. Dynamic
predictions when assessed per CYP enzyme varied from 10% within 2-fold (CYP2C19, n=10), to 50-60% of CYP2D6 and CYP1A2 interactions (n=2 and 10, respectively), and 100% of CYP2C9 and CYP3A4 interactions (n=2 and 3, respectively). All CYP2C9 and CYP3A4 studies were weak interactions. The poorly predicted CYP2C19 studies were moderate (4/10) or strong (5/10) interactions. Static prediction success was similar, except for CYP1A2 DDIs (70% were predicted within 2-fold). The database for fluvoxamine included DDI studies with at least 2 victim drugs per CYP enzyme (with the exception of CYP2D6). These results should therefore not be skewed by successful or poor predictions arising from DDIs involving one victim drug. The database of fluoxetine DDIs involved victim drugs metabolised by two CYP enzymes; CYP2D6 and CYP3A4. All of the CYP3A4 interactions were weak inhibition interactions, with the exception of a fluoxetine-midazolam DDI that had an AUC ratio of 0.84, and therefore classified as induction. All CYP3A4 interactions were predicted within 2-fold, regardless of use of the static or dynamic model. This success included the fluoxetine-midazolam induction interaction that was predicted with an AUC ratio (± S.D.) of 1.00 (± 0.00) in both cases. This prediction was independent of liver CYP3A4 $k_{\text{deg}}$; no difference in predicted AUC ratio was observed between utilisation of degradation half-lives of 23, 72 or 90 hours. The static and dynamic model both predicted 50% of the six CYP2D6 DDIs within 2-fold. There was no clear relationship between the three different victim drugs and prediction accuracy. The dynamic prediction of SSRI DDI predictions divided by victim drug is displayed in Figure 5.3.
Figure 5.3 Predicted and observed AUC ratios for the dynamic prediction of SSRI DDIs classified according to victim drug and CYP enzyme, with CYP1A2 (circle symbols): caffeine (●), clozapine (●), theophylline (●); CYP2C9 (rotated triangle): tolbutamide (▼); CYP2C19 (triangle symbols): omeprazole (▲); S-mephysto (△); CYP2D6 (square symbols): desipramine (■), imipramine (■), tolterodine (■); CYP3A4 (diamond symbols): alprazolam (◇), midazolam (◇), quinidine (◇).

Figure 5.4 Impact of the dosing time of victim drug from -10 to +24h after the final inhibitor dose on the predicted AUC ratio using the dynamic model in Simcyp. DDIs are between the inhibitor fluvoxamine and victim drugs omeprazole (○) (CYP2C19) or caffeine (■) (CYP1A2) and fluoxetine (with inclusion of the norfluoxetine active metabolite) and desipramine (▲) (CYP2D6), with dosing schedules as defined in the methods.

The impact of altered dosing times (from -10 to +24h) on the predicted AUC ratios of SSRI DDIs are displayed in Figure 5.4. Representative victim drugs were selected for the
CYP enzymes most potently inhibited by fluvoxamine (caffeine and omeprazole for CYP1A2 and CYP2C19, respectively) and fluoxetine (desipramine for CYP2D6). Similar trends were observed for all victim drugs, with highest consistency between the two fluvoxamine DDIs. The AUC ratio-time profile of SSRI DDIs appeared to be symmetrical from -5 to +5h around simultaneous dosing (Figure 5.4). The decline in AUC ratio between 0 and 1h was 7% for omeprazole and caffeine and 6% for desipramine. Approximately half of fluvoxamine (14/27) and fluoxetine (4/10) DDIs in the database did not report the dosing times for victim drugs. The assumed 1 hour dosing in simulations may have resulted in a <10% prediction error when compared to simultaneous dosing; larger errors may result from larger time differences. In addition, actual percentage differences between dosing schedules could not be calculated as a result of the lack of corresponding clinical data. Variation in the fluoxetine and fluvoxamine \( k_a \) had a minimal impact on the predicted AUC ratio for all three interactions, resulting in a maximal difference of 8% (for fluvoxamine with CYP1A2). Maximal differences for fluvoxamine (CYP2C19) and fluoxetine (CYP2D6) were 2 and 4%, respectively.

### 5.3.4 Prediction of inter-individual variability in DDIs involving SSRI inhibitors, in healthy and genotyped populations

The prediction of inter-individual variability in DDIs was assessed for studies that reported individual AUC data from healthy subjects (fluvoxamine-tolbutamide DDI; Madsen et al., 2001), from genotyped healthy subjects (fluvoxamine-caffeine and fluvoxamine-omeprazole DDIs; Christensen et al., 2002) or in a patient population (fluoxetine-tolterodine DDI; Brynne et al., 1999). In addition, one DDI reported data from each phenotype group for a fluvoxamine-omeprazole DDI (Yasui-Furukori et al., 2004). The inter-individual variability of a fluvoxamine-tolbutamide (Madsen et al., 2001) DDI in healthy subjects was under-predicted by both the static and dynamic model. Clinical data from the study reported weak interactions at two different fluvoxamine dose levels, with mean AUC ratios of 1.24 and 1.93 and ranges of 0.94-1.59 and 0.99-3.35 for 75 or 150mg/day fluvoxamine, respectively. Simcyp did not successfully predict any interaction between fluvoxamine and tolbutamide regardless of model selection. Mean AUC ratios were close to unity when predicted by either model. These results display the inability of Simcyp to predict the interaction and the inter-individual variability for this study, with the range of predicted AUC ratios for all subjects 1.00 – 1.02. The predicted AUC ratio was within the observed range in both dosing cases, and the mean AUC ratio was within the 2-fold prediction boundaries.
One case study involved ten subjects that were genotyped for CYP2D6 in a fluvoxamine DDI (Christensen et al., 2002). The two victim drugs, 100mg caffeine and 200mg omeprazole, were substrates of CYP1A2 and CYP2C19, respectively. Fluvoxamine (10mg and 25mg) was dosed once (PM subjects) or twice (EM subjects) daily, resulting in a similar circulating level of the inhibitor in both phenotype groups. Results of the simulations involving the two interactions in the different genotype groups (containing 5 EM subjects or 5 PM subjects) are shown in Figure 5.5. Simulations utilised the in vivo clearance of fluvoxamine (Figure 5.5B and E) or in vitro data from HLM (Miura and Ohkubo, 2007) and thereby included the impact of different CYP2D6 genotypes on fluvoxamine in the DDI predictions (Figure 5.5C and F). Differences observed between the genotype groups in Figure 5.5B and E are attributed to the inhibitor dose level in the study design. The use of in vivo clearance in simulations that would not have specified the fluvoxamine clearance to CYP2D6 and CYP2D6 genotype would therefore have no impact on the DDI predictions. The under-prediction of the AUC ratio for omeprazole was observed regardless of the dosing level or whether or not the genotype was included. The extent of under-prediction was greater when the genotype was not included. For example, the mean AUC ratio for 10mg dosing in PM subjects is under-predicted by 2.4-fold when genotype was not included compared to when genotype was accounted for (2.0-fold). A 4.5-fold under-prediction reduced to 2.8-fold when genotype was included in the simulation of the 25mg dose in EM subjects. Prediction of the range of AUC ratios was successful at the lower fluvoxamine dose level (with genotype inclusion), with minimum and maximum values predicted within 2.3-fold. At the higher fluvoxamine dose level, all minimum and maximum AUC ratio values were unsuccessfully predicted with differences from 2.4 to 4.7-fold. Despite the inability to predict these values, the CV was predicted within 2-fold for both dosing levels and genotype groups with the exception of the 10mg fluvoxamine dose level in PM subjects where a 3.0-fold under-prediction of CV was observed. In all cases, the omeprazole AUC in the absence and presence of inhibitor was over-predicted for both dose levels, although the AUC in the presence of inhibitor was over-predicted to a lesser extent. This discrepancy between the over-predictions rationalised the under-prediction of the overall AUC ratio. The actual Cmax data in the absence or presence of inhibitor was not reported for the omeprazole-fluvoxamine interaction. The caffeine DDI was successfully predicted (< 2-fold difference in all dosing and genotype cases) once the genotype was included. However, the 1.4-1.5-fold difference between the predicted median values of the two genotype groups was over-predicted at both dose levels (actual difference 1.1-fold). The range of AUC ratios (with genotype inclusion) was successfully predicted, with differences between observed and predicted
minimum and maximum AUC ratio < 1.6-fold for both dosing schedules and genotypes. The exception to this trend was a 3.0-fold under-prediction of the minimum AUC ratio in PM subjects at the 10mg dose level. This subject appeared to be an outlier with an AUC ratio 3-fold lower than any other PM subjects. Under-prediction of the inter-individual variability of the studies was observed despite the successful prediction of the minimum and maximum AUC ratios, with CV from 0.3 to 0.4 of that observed from the clinical study for the two polymorphism groups at the lower 10mg fluvoxamine dose level. The CV at the higher 25mg fluvoxamine dose level was improved, with predictions from 0.5 to 0.7 of the observed for PM and EM subjects, respectively. The difference between the median and mean values of the Simcyp simulations was negligible, in contrast to the actual data, where both mean and median values are shown (Figure 5.5). The AUC of caffeine in the absence and presence of fluvoxamine was successfully predicted at both dose levels in EM subjects, with all differences < 1.9-fold than the actual values. The under-prediction of the AUC ratio of the previously mentioned PM subject resulted from under-prediction of the AUC in the absence of fluvoxamine (34% of the actual value) combined with successful prediction of AUC in the presence of fluvoxamine (1.12-fold over-prediction).

All other predictions in PM subjects were within 1.4-fold. The prediction of caffeine Cmax values in EM and PM subjects in the absence and presence of inhibitor were within 2.4-fold of the actual values in all cases, with the exception of the Cmax of the outlier subject, predicted at 27% of the actual value. Overall, the mean and range of the fluvoxamine-caffeine DDIs were successfully predicted in both phenotype populations and at both dose levels.

Section 2.3.3 displayed the under-prediction of CYP2C19 DDIs involving fluvoxamine as the inhibitor. One study reported an interaction between fluvoxamine and omeprazole in CYP2C19 genotyped subjects (6 EM, 6 IM and 6 PM subjects) in a Japanese population (Yasui-Furukori et al., 2004). The predicted AUC ratios in the PM and IM subjects were 1.00 (S.D. = 0.00), regardless of whether the simulation was predicted in virtual Caucasian or Japanese subjects. The mean AUC ratio in EM subjects (5.62) was under-predicted, at 22% and 18% of the observed in Caucasian (AUC ratio 1.24 ± 0.18) and Japanese (AUC ratio 1.03 ± 0.03) subjects, respectively. No individual results were available for comparison to the Simcyp predicted values. In Caucasian subjects this under-prediction of AUC ratio can be rationalised by the over-prediction of the control, i.e., omeprazole only phase (AUC of 3700 ngml⁻¹h compared to the observed 1483 ngml⁻¹h) and an under-prediction of omeprazole in the presence of fluvoxamine (4310 ngml⁻¹h instead of the observed 8340 ngml⁻¹h). In contrast, the under-prediction of the AUC ratio when using
Japanese subjects resulted from AUC over-prediction in both arms of the study; over-prediction was over 11-fold in the control phase (17500 ng/ml h) and over 2-fold in the omeprazole plus fluvoxamine phase (18080 ng/ml h). This difference is likely to be due to the lower abundance of CYP2C19 in Japanese subjects than Caucasian (1 (CV of 98%) vs. 14 (CV of 106%) pmol CYP mg\textsuperscript{-1} microsomal protein, respectively) that is utilised in Simcyp (Inoue et al., 2006). Both C\textsubscript{max} and AUC values were under-predicted in the absence of fluvoxamine for PM and IM subjects (regardless of Caucasian or Japanese subjects), at 36-48% of the actual values. Differences in predicted values were minimal in the absence and presence of fluvoxamine (within 1.1-fold), therefore greater under-prediction was observed in the presence of inhibitor (20-37% of the actual). The relative prediction accuracy of the C\textsubscript{max} in the absence and presence of fluvoxamine was consistent to the observed accuracy of the AUC values in both Caucasian and Japanese subjects. EM C\textsubscript{max} values in Caucasian subjects were over-predicted by 2.1-fold, and under-predicted in the presence of fluvoxamine (74% of the actual), whereas C\textsubscript{max} values in the absence and presence of fluvoxamine were over-predicted by 4.6- and 2.4-fold, respectively in Japanese EM subjects.

The effect of genotype on a patient population was studied from a DDI case study of fluoxetine-tolterodine in elderly depression/anxiety patients with urinary incontinence (Brynne et al., 1999, Figure 5.6). The lack of interaction in PM subjects was predicted successfully (mean AUC ratio of 1.00 compared to actual of 1.24), but the CV was under-predicted by 4.5-fold (0.4% compared to the 1.7% actual). Both the AUC and AUC in the presence of inhibitor were under-predicted by a similar extent (2.6- and 3.2-fold, respectively), therefore the ratio was unaffected. Under-prediction of the mean and median increased with the increasing AUC ratio i.e. for the IM and EM subjects, with under-predictions of 2.6-fold and 5.8-fold, respectively. This under-prediction is largely due to the AUC in the presence of inhibitor, which was under-predicted by approximately 7-fold in both cases, combined with a 4-fold under-prediction of control AUC in IM subjects and good prediction of the control AUC in EM subjects. In contrast to the mean AUC ratio, the CV accuracy increased. The CV was under-predicted in the IM subjects (26% compared to the 63% observed), however, in EM subjects the CV was over-predicted at 49% compared to the actual 24%. Prediction of C\textsubscript{max} in the absence of fluoxetine was predicted within 1.8- and 2.6-fold in EM and IM subjects, respectively. The C\textsubscript{max} in PM subjects was predicted at 36% of the actual value. Values of C\textsubscript{max} in the presence of fluoxetine were under-predicted in all subjects, with predictions <50% of the reported C\textsubscript{max,i} values for all
subjects. Simcyp simulations did show the distinction between the mean and variation of DDI in the different genotype groups, despite the overall under-prediction of mean.
Figure 5.5 Predicted AUC ratios obtained from 10 individual trials of 5 PM and 5 EM CYP2D6 genotyped subjects for a fluvoxamine-omeprazole (A-C) and fluvoxamine-caffeine (D-F) DDI study (Christensen et al., 2002). A and D show observed data from 10mg or 25mg fluvoxamine dosing, in the EM (twice daily dosing) or PM (once daily dosing) populations. B, C and E, F are predicted using the dynamic model in Simcyp, using fluvoxamine parameters outlined in Table 5.1, and matched dosing schedules to the clinical study. Simulations are excluding (B and E) or including (C and F) the impact of the CYP2D6 genotype (matched distribution to the clinical study). Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value, dotted line represents the mean values and the box represents the inter-quartile range boundaries. Where > 9 subjects are included the lower and upper whiskers represent the 10-90% range and outliers are represented by ⋄.
Chapter 5 – Assessment of SSRI DDI prediction

Figure 5.6 Predicted AUC ratios obtained from 10 individual polymorphic trials for the fluoxetine-tolterodine DDI compared to the actual distribution of AUC ratios in the three genotype groups (Brynne et al., 1999). Top left is the summary data and the PM, IM and EM boxes display data from the total of the ten trials. Predictions are using the dynamic model in Simcyp, using fluoxetine and norfluoxetine parameters outlined in Tables 5.1-5.3 and matched dosing schedules to the clinical study. Box and whisker plots illustrate the distribution in the prediction success; the black line represents the median value and the box represents the inter-quartile range boundaries. Where > 9 subjects are included, lower and upper whiskers represent the 10-90% range and outliers are represented by ●.
5.4 DISCUSSION

This chapter has assessed the prediction of DDIs involving two SSRI inhibitors, fluoxetine and fluvoxamine. The impact of the inclusion of the time course was investigated further to Chapter 4 by increasing the number of DDIs investigated and assessing the impact across a number of different victim drugs and CYP enzymes, covering a representative number of weak, moderate and strong interactions. In addition, a sufficient number of studies were available per inhibitor and per victim drug to assess the impact of individual parameters, including active metabolite inclusion, dose timing and the $k_a$, allowing comparison to those relationships observed in Chapter 4 with the azole inhibitors. Further investigation into the ability of Simcyp to predict the inter-individual variability of DDIs was undertaken, with clinical data available in different subject groups including patients and subjects divided by CYP2C19 or CYP2D6 phenotype status.

The static model in Simcyp gave consistently higher results than the dynamic model for all DDI predictions involving both of the SSRI inhibitors, with all dynamic:static ratios ≤1. This result is consistent with the relationship observed with azole inhibitors, as a result of the single, maximal concentration of inhibitor that is utilised in the static model, in comparison to the time-dependent inhibitor concentration in the dynamic model. Differences between the static and dynamic predictions were greater than 20% for a quarter and half of the fluvoxamine and fluoxetine DDIs, respectively. Some CYP-dependent differences were observed for fluoxetine DDIs with the majority (5/6) of CYP2D6 DDIs in this category, compared to none of the CYP3A4 DDIs. This CYP-based difference did not correlate to fluvoxamine DDIs involving the same CYP2D6 victim drugs, although some alternative victim drug-dependent differences were observed with S-mephénytoïn and theophylline. Differences may be attributable to the differences in inhibitor potency to the CYP enzymes, for example the >30-fold difference between the CYP2D6 $K_i$ for fluoxetine and fluvoxamine. Prediction of all SSRI DDIs from use of the static and dynamic model is comparable, with accuracy at 57 and 54% within 2-fold, precision (assessed using afe) of 1.8 and 2.2, and bias (assessed using rmse) of 3.0 and 3.4, respectively. The prediction of fluoxetine DDIs had a higher prediction success rate (via both the static and dynamic model) with higher precision and less bias than fluvoxamine DDI prediction, although this result is based on a smaller database (n=10 compared to n=27) and a smaller proportion of strong DDIs (20% compared to 30%). Both SSRI inhibitors have long half-lives (approximately 50-60 and 18-22 hours, for fluoxetine and fluvoxamine, respectively (Thummel et al., 2008)), and the impact of the time-course inclusion is therefore expected to be limited. In addition, neither database was skewed by a
high proportion of any particular victim drug (Chapter 2). The under-prediction of AUC ratios with increasing DDI potency was observed, with reduction of prediction accuracy within 2-fold from 100% for no and weak interactions to 0% for strong interactions using the dynamic model, and 93% to 10% using the static model. This conclusion was regardless of inhibitor or victim drug, and consistent to that observed with the azole DDIs. The prediction of SSRI DDIs involving the most potently inhibited CYP enzymes (CYP1A2 and CYP2C19 for fluvoxamine and CYP2D6 for fluoxetine) was variable at 50-60% in all cases; prediction success correlated to the potency of the DDI rather than the most potently inhibited CYP enzyme. A number of DDI predictions highlighted the inability of Simcyp to successfully predict any interaction (with maximum predicted AUC ratios of 1.01 ± 0.01) using both the static and dynamic model. These were weak DDIs (AUC ratio < 2) involving victim drugs metabolized by CYP enzymes where the inhibitor Ki value > 5μM (fluvoxamine CYP3A4 (17.89μM) and CYP2C9 (8.43μM)). In addition, no interaction was predicted for the weak CYP3A4 based DDIs involving the mechanism-based inhibition by fluoxetine. No difference in predicted AUC ratio was observed when the hepatic CYP3A4 degradation rate was varied from being equal to, or greater than intestinal CYP3A4 kdeg (correlating to half-lives of 23, 72 and 90 hours) for the fluoxetine DDIs. This result is consistent with previous reports (Wang, 2010), where the impact of kdeg was most evident when the AUC ratio > 2-fold. Both CYP3A4 victim drugs assessed (alprazolam and midazolam) had fmCYP3A values > 0.8, increasing the sensitivity to kdeg values; however, the lack of impact was rationalised by the combination of the low CYP3A4 time-dependent inhibitory potency with the low simulated maximal concentration of fluoxetine (0.2μM) (Galetin and Houston, 2006). Despite the AUC ratio predictions of 1.00-1.01 for all CYP3A4 DDIs, all of these interactions were predicted within 2-fold of the observed AUC ratio.

All fluvoxamine DDIs followed multiple dosing of the inhibitor, whereas one study reported the DDIs of fluoxetine with the victim drugs desipramine and imipramine after single and multiple dosing of the inhibitor. The dynamic model successfully predicted the DDIs after single inhibitor dosing, where the static model failed to do so for both victim drugs. The static model successfully predicted both DDIs after the multiple dosing of fluoxetine, therefore indicating that the assumption of steady-state inhibitor concentrations could reduce the prediction accuracy after single inhibitor dosing. However, the under-prediction of the DDI with desipramine following multiple inhibitor dosing using the dynamic model does not support this hypothesis. Differences observed between these results and those involving the azole inhibitors correlate to the findings observed in Zhao et
al., 2009, where increased impact on the DDI magnitude is expected with longer victim drug half-lives. Half-lives for desipramine and imipramine are 13 and 17h, respectively (Sutfin et al., 1984), in comparison to the relatively shorter half-lives for midazolam and triazolam, and hence the lower impact of single or multiple dosing (Chapter 4). The limited number of clinical studies reporting such data confounds firm conclusions, as observed with the azole inhibitors. The lack of trend between prediction accuracy or difference between static and dynamic model with the bioavailability (0.32-1.00 for midazolam and caffeine, respectively) and half-life (1.1-17h for omeprazole and desipramine, respectively) were also observed with the SSRI DDIs. The 50% variation in $k_a$ for fluvoxamine and fluoxetine resulted in marginal differences in AUC ratio (<8%), consistent with the lack of difference observed for itraconazole (Chapter 4). The differences observed in AUC ratio from the same fluvoxamine $k_a$ range (0.2-0.8 h$^{-1}$) were not consistent between the interactions involving CYP1A2 or CYP2C19; differences are therefore likely to be attributable to the 2-fold difference in inhibition potency between the two enzymes and not as a direct result of the $k_a$.

The incorporation of the active metabolite of fluoxetine, norfluoxetine did not have any impact on the prediction of DDIs. This result would be expected in the two studies reporting single dosing of fluoxetine, where the time scale would restrict the impact of the contribution of the metabolite. The impact of the inclusion of the metabolite may be reduced by the long half-life (53h, Thummel et al., 2008) and 10-fold higher potency of the parent to the same CYP enzyme (CYP2D6). There is also the possibility that the formation of norfluoxetine in vivo inhibits further metabolism of fluoxetine by inhibiting CYP2D6. This was not reflected in Simcyp, however, as it would be result in an increased DDI potential following the norfluoxetine inclusion in predictions. Despite these factors, some impact of metabolite would be expected as a result of the long time-scales of the multiple fluoxetine dosing DDIs (8-24 days). The inclusion of hydroxy-itraconazole (Chapter 4) into dynamic predictions results in a much greater difference. This result is despite the same 10-fold difference in potency (for CYP3A4 in this case) and potential inhibition of itraconazole metabolism by hydroxy-itraconazole, therefore the difference may be attributable to the approximately 2-fold longer half-life of fluoxetine than itraconazole.

DDIs involving 12 victim drugs were assessed in the SSRI database; these drugs were metabolised by the five major CYP enzymes. Prediction success (when assessed within 2-fold of the observed) appeared to correlate to the CYP enzyme, ranging from 10% for CYP2C19 to 100% for CYP2C9 and CYP3A4. Intermediate success rate, and therefore
poor correlation with the CYP enzyme was observed for CYP2D6 and CYP1A2 (50 and 60% successfully predicted, respectively). This correlation is confounded by the potency of DDIs however, as all of the CYP2C9 and CYP3A4 DDIs had AUC ratios < 2, and 90% of the CYP2C19 DDIs had AUC ratios > 2. All predictions correlated to the potency of DDI; no DDIs across the range of potency correlated to prediction success when assessed per victim drug. The impact of staggered dosing on the prediction of DDIs involving SSRI inhibitors was investigated using representative victim drugs from the database for the CYP enzymes that were inhibited with a \( K_i < 0.1 \mu M \) (CYP2D6 for fluoxetine and CYP1A2 and CYP2C19 for fluvoxamine). The profiles observed from the simulated data were similar to each other, particularly for the two involving fluvoxamine as the inhibitor, and to those observed for the CYP3A substrates (Chapter 4, Zhao et al., 2009), with maximum AUC ratio resulting from simultaneous dosing. The profile of AUC ratios between -10 to -2h differed for fluoxetine (CYP2D6); this difference is likely to be attributable to the long half-life of desipramine as a victim drug compared to caffeine and omeprazole. Differences between the profiles of the azole and SSRI inhibitors resulted from the maximum AUC ratio being approximately 2.5- and 6.0-fold lower for all SSRI interactions compared to the DDIs involving itraconazole and ketoconazole, respectively. The symmetrical nature of the AUC ratios around simultaneous dosing was in contradiction to the increased sensitivity of the model dosing between -2 to 0h for the itraconazole DDI, potentially implicated in the lower AUC ratio for SSRI inhibitors at simultaneous dosing. However, this finding cannot be a result of a longer inhibitor half-life, as this would only be correct for fluoxetine. In addition, the observed 6-7% decline in AUC ratio between simultaneous victim drug and inhibitor dosing and victim dosing 1h after the inhibitor was intermediate between the 16% decline for itraconazole (Chapter 4) and a minimal change for ketoconazole (Zhao et al., 2009). This difference also cannot be attributed to the half-lives of the inhibitors. No observed data for the SSRI inhibitors were available from clinical studies to compare to the findings from the simulations. Despite the differences that are discussed, results from these simulations concur with a number of studies (Fang et al., 2000; Neuvonen et al., 1996; Yang et al., 2003) to indicate that the extent of drug-drug interaction can be significantly reduced in a clinical setting from differences in the dosing schedule.

Four studies (representing 14 data points due to multiple dose levels or phenotype populations) reported data from individual subjects or phenotype populations (Brynne et al., 1999; Christensen et al., 2002; Madsen et al., 2001; Yasui-Furukori et al., 2004); the prediction of AUC ratio variability, victim drug profile and DDIs in different populations.
was investigated. The variability of the weak interactions between fluvoxamine and tolbutamide (Madsen et al., 2001) was not predicted by Simcyp at the two dose levels, despite mean prediction within 2-fold of the observed. No other studies reporting a potential DDI implicating CYP2C9, and it is therefore unclear whether the inability to predict was due to the weak inhibition of CYP2C9 by fluvoxamine (8.4μM). The hydroxylation pathway of tolbutamide was the single metabolic pathway included within Simcyp, and the lack of interaction is therefore not due to parallel pathways of metabolism. The lack of impact of the CYP2D6 genotype on the variability of fluvoxamine DDIs with omeprazole and caffeine were successfully predicted, using the in vitro clearance of fluvoxamine from human liver microsomes (Miura and Ohkubo, 2007). The lower predicted AUC ratio in the PM subjects than the EM subjects indicated that the lower dose level in the PM subjects had more impact in the prediction of DDI than the impact of the genotype on the metabolism of fluvoxamine, as reported in Christensen et al., 2002. The reduced clearance and therefore increased circulating levels of fluvoxamine in PM subjects would have resulted in increased AUC levels if genotype had been the predominant factor. The dose-dependency of the CYP2D6 genotype on fluvoxamine metabolism has been demonstrated (Watanabe et al., 2008), with greatest impact of the genotype at <50mg fluvoxamine dosing. These results are therefore inconsistent with the results observed in Christensen et al., 2002 and those predicted by Simcyp simulations and cannot rationalise the lack of impact observed. In addition, the CYP2D6 pathway was the only metabolic pathway included within Simcyp contributing to the fluvoxamine clearance. A greater impact was observed from inclusion of the genotype with the CYP1A2 substrate, caffeine. This difference may be due to the higher fm_{CYP1A2} of caffeine (0.97) compared to that of omeprazole (fm_{CYP2C19}, 0.73), and therefore the increased impact that fluvoxamine inhibition would have on the metabolism of these victim drugs. Successful prediction accuracy was observed for the victim drug AUC or C_{max} in the absence and presence of inhibitor across the different genotypes, with the exception of the single outlying patient. The profile in the absence of inhibitor was under-predicted in terms of AUC and C_{max}, resulting in significant over-prediction of AUC ratio when combined with successful profile prediction in the presence of inhibitor. The results from this study successfully predicted that the CYP2D6 genotype does not have a profound effect on fluvoxamine DDIs, consistent with the results from the in vivo study.

The poor prediction of CYP2C19 DDIs was discussed above; prediction of a fluvoxamine-omeprazole DDI in genotyped Japanese subjects (Yasui-Furukori et al., 2004) followed this trend. This difference was not as a result of a virtual Japanese population, as the 5-fold
under-prediction of both mean and range of DDI in EM subjects was observed regardless of simulations in Caucasian or Japanese subjects. No variability was predicted for the DDI in IM and PM subjects, and distinction between the DDI in these phenotype populations was not observed. This lack of impact is in contrast to the DDI between fluoxetine and tolterodine (Brynne et al., 1999), where distinction in the AUC ratio between the different phenotypes was observed despite the under-prediction of mean. The prediction of this DDI could be confounded by a number of factors in the clinical study including the age range of the subjects (63 ± 9 years), as the upper age limit in Simcyp is 65 years. In addition, the subjects were depression/anxiety patients with urinary incontinence, unattributed to physiological factors that could be included within the simulation. These factors may contribute to the poor prediction of variability in both IM and EM subjects, with an approximate 2-fold under- and over-prediction of CV, respectively. These results could also be observed when assessed across 10 trials of solely EM, IM or PM subjects, and did not relate to the methods used to assess variability. No consistency was observed between studies in the success of profile prediction in the absence and presence of inhibitors or in different genotype populations, with variable under- and over-prediction observed. Overall, results for the prediction of inter-individual variability are inconsistent, with some evidence for successful prediction of the variability in SSRI DDI prediction, including prediction in different phenotype groups; however under-prediction of both mean and the range in some cases is evident, which is of concern particularly for potent DDIs, and the interaction is often missed in DDIs involving a CYP enzyme where the SSRI inhibits with a $K_i > 0.1\mu M$.

This chapter has provided further data in order to assess the Simcyp method of DDI prediction in a population based model, with moderate prediction accuracy observed for 37 SSRI DDIs. The under-prediction of strong DDIs was reinforced, along with minimal difference overall between the static and dynamic model. Prediction accuracy appeared to correlate to the CYP enzyme that was involved in the DDI; however this difference was actually related to the potency of interaction, with DDIs involving CYP enzymes that correlated to weak DDIs resulting in high prediction success, and vice versa. Inclusion of the fluoxetine active metabolite had negligible effect on DDI prediction, potentially related to the long half-life of the parent. Prediction success of inter-individual variability in SSRI DDI magnitude was variable, confounded by clinical data in patient populations or involving CYP enzymes where the SSRI inhibitory effect is not potent. Despite these issues, some distinction between DDI predictions in different genotype groups was
identified. Further evidence to support critical evaluation of the simulation input parameters used and clinical data for prediction validation was highlighted.
CHAPTER 6: FINAL DISCUSSION
Chapter 6 – Final Discussion

Parts of this chapter have been modified from published work (Guest et al., 2011) (Appendix 8, Publication 2).

Metabolic DDIs are clinically important due to the impact that they can have on the safety profile of drugs (Grimm et al., 2009; Huang et al., 2007; Wienkers and Heath, 2005). The prediction of metabolic inhibition and induction DDIs from in vitro data and the contribution of individual parameters to the prediction have therefore been subject to increased interest. The prediction models utilised to assess DDIs have increased in complexity through the inclusion of multiple inhibitors, parallel pathways of metabolism and multiple inhibition mechanisms (Brown et al., 2006; Brown et al., 2005; Einolf, 2007; Fahmi et al., 2009; Galetin et al., 2006; Galetin et al., 2008; Galetin et al., 2007; Hinton et al., 2008; Isoherranen et al., 2009; Ito et al., 2004; Ito et al., 2005; Obach, 2009; Obach et al., 2005; Rowland-Yeo et al., 2010; Wang, 2010). Recent studies have also investigated the impact of the time-varying concentration of inhibitor within the prediction of DDIs using static and dynamic models; however, the majority of these comparisons used different parameters within the two scenarios (Einolf, 2007; Perdaems et al., 2010). The lack of consistency in the parameters has confounded the direct assessment of the impact of time-course inclusion. Additionally, conclusions may be specific to DDIs involving a certain inhibitor or victim drug without the ability to ascertain more general trends (Fahmi et al., 2009; Hyland et al., 2008; Rakhit et al., 2008; Youdim et al., 2008; Zhao et al., 2009). The physiologically-based Simcyp simulator allows the prediction of the ADME properties of drugs and DDIs from in vitro data. The ability to predict drug properties and DDIs in a virtual population allows the assessment of inter-individual variability and thus the identification of individuals at increased risk of toxicity and outliers in a normal population distribution. The aims of this Thesis were therefore to investigate the value of including the time course on the prediction of DDIs from the direct comparison of the static and dynamic models within the Simcyp simulator. The contribution of individual victim drug and inhibitor parameters to the DDI predictions and simulation of the inter-individual variability in AUC ratios were also assessed using a large database of metabolic DDIs.

The collated literature database comprised of 97 DDIs involving five CYP inhibitors (three azole anti-fungals and two SSRI anti-depressants) and a range of victim drugs. The selection of these inhibitors was based upon the availability of DDI studies in the literature that covered a wide range of inhibition potency, inhibition of a range of CYP enzymes and
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inclusion of a variety of victim drugs with differential properties. Initial analysis in this
Thesis assessed the default Simcyp parameters of victim drug properties (e.g. fm\text{CYP}) in
comparison to alternative methods. This comparison was important considering the
inability to input fm\text{CYP} and F_G values directly into the Simcyp simulator, and to assess
sources of error that may therefore be introduced from differences between the methods.

Preliminary sensitivity analysis was utilised in order to highlight inhibitor parameters with
the greatest impact in DDI prediction in order to justify the selection of parameter values.
The DDI database was then utilised in Chapter 4 and 5 to compare the prediction success
of metabolic DDIs using the static and dynamic models in the Simcyp simulator. The same
parameters were utilised in both models to allow a valid assessment of the inclusion of the
time-profile of inhibition. The Simcyp dynamic model also allowed investigation into the
impact of active metabolite inclusion into the DDI prediction, and differences resulting
from different dosing times of the inhibitor and victim drug. The prediction of inter-
individual variability was assessed in comparison to a number of clinical DDI studies
reporting data from the individual subjects. Further assessment was considered using
individual DDI data from different genotype groups. Findings from these investigations
found that the estimates of fm\text{CYP} and F_G from Simcyp were within 20% of the alternative
methods of estimation in the majority of cases; default victim drug parameters were
therefore considered to be successfully predicted by Simcyp and suitable for use in further
predictions using the Simcyp simulator. The ability to include active metabolites and
specify dosing times in the dynamic model resulted in increased prediction accuracy in
comparison to the Simcyp static model for the studies investigated, and the utility of these
methods gives Simcyp advantages over other commercially available PBPK models.

However, the overall increase in prediction accuracy from use of the dynamic model was
marginal (4%) in comparison to the static model. The ability of Simcyp to predict the inter-
individual variability varied between the studies, with no inhibitor- or subject-related
trends ascertained. Success appeared to correlate to the potency of the DDI rather than the
aforementioned variables; however, the two-fold method used to assess prediction success
warranted further consideration.

6.1 In vitro methods for the assessment of uptake

Further investigation into the liver uptake of SSRI (fluoxetine and fluvoxamine) and azole
(itraconazole and ketoconazole) inhibitors was proposed, based on the lack of clear
literature evidence (Brown et al., 2007a; Hallifax and Houston, 2007; Matthew et al., 1993;
von Moltke et al., 1995; von Moltke et al., 1994b; von Moltke et al., 1996; Yamano et al.,
1999a; Yamano et al., 1999b; Yao et al., 2001; Yao et al., 2003). Different preliminary in
vitro methods for the investigation of hepatic uptake were assessed, based on the comparison of the clearances of inhibitors either in different systems (microsomes and hepatocytes), or between different methods (the conventional and media loss assay in hepatocytes). Methods were assessed using rat and human material; the former for comparison with previous preliminary analysis of the extent of uptake obtained from the hepatocyte to microsomes $K_{i,u}$ ratio (Brown et al., 2007a) and the latter for comparison of the two species and for subsequent inclusion into the DDI prediction model.

No consistency was observed from results from the in vitro assessment of uptake using different methods (Chapter 3) from results using both rat and human derived material. Some inhibitors displayed no difference in clearance, therefore no uptake is assumed. In contrast, the same drug could display a large difference in clearance when assessed using an alternative method or cell source (Table 6.1). For example, the ketoconazole clearance ratio from the conventional to media loss assays in human hepatocytes was 7.8- and 10.8-fold higher than the same assays in rat hepatocytes or the human hepatocyte to microsome ratio, respectively. This inconsistency was also observed in the case of itraconazole, where a 10-fold range of ratios was observed, ranging from lower clearance observed in the media loss assay than the conventional assay in rat hepatocytes, to a 5-fold increase from the same comparison in human hepatocytes. The media loss < conventional clearance was only observed for itraconazole, and may be rationalised from experimental error in one, or both of the assays, or from non-specific binding of itraconazole. Previous studies have investigated preliminary methods for assessing liver uptake; however they have not reported results from multiple methods in order to compare the results of the $K_i$ ratio, clearance ratio and the media loss assay, or compare the results to those from the oil spin assay (Brown et al., 2007b; Soars et al., 2007). Results from the $K_i$ and clearance ratio in rat hepatocytes and microsomes from Brown et al., 2007a and the current analysis, respectively, were consistent for fluvoxamine and ketoconazole. The clearance ratios of 2.47 and 0.49 were within the reported 0.59-3.33 and 0.07-2 range of $K_i$ ratios (resulting from the use of different pathways in $K_i$ assessment) for fluvoxamine and ketoconazole, respectively (Brown et al., 2007a). Results for fluoxetine were inconsistent between the two methods; the clearance ratio was >2.5-fold greater than the average $K_i$ ratio (3.15 vs. 1.22). The $K_i$ ratio for itraconazole has not been reported and therefore could not be compared to the clearance ratio. The majority of clearance ratios were > 2, with 3/4 of the assays for fluoxetine, fluvoxamine and ketoconazole. Itraconazole did not display this trend, with only 1 assay displaying a clearance ratio > 2. The highest variability was observed from the ratio of the media loss:conventional assay in human hepatocytes, where
a >300-fold range was observed across the inhibitors. Soars et al., 2007 reported improvement in clearance prediction based on estimates from the media loss assay in hepatocytes, in comparison to the conventional assay in hepatocytes. Therefore, this assay has been proposed as the first line assessment of the potential uptake of compounds (Soars et al., 2009). In the current study, there has been no consistent improvement in clearance estimations from the media loss assay compared to different methods and from different sources. The assessment of clearance from the media loss assay is potentially confounded by non-specific binding and utility of the media loss assay (without further characterisation of the unbound drug) is therefore limited. Values were not corrected for fraction unbound in the incubation as the drug concentration that was measured in this assay was extracellular; however, the initial loss of drug from the media would be a combination of uptake and binding. The importance of non-specific incubation binding has previously been described in a number of studies (Austin et al., 2002; Austin et al., 2005; McLure et al., 2000; Obach et al., 2006). The uptake and binding cannot easily be distinguished from each other without further studies assessing the appearance of drug in the cell, for example using the oil spin assay, in order to allow for modelling of the free drug concentrations (Paine et al., 2008). Although the addition of this step to the protocol would solve the issue of the uptake and binding of drug, it would additionally increase the complexity of the protocol. This would therefore decrease the ability of this method to be used as a first line, high-throughput method to assess potential uptake, and not meet the objectives of the study. No method assessed in this study was therefore conclusively applicable for the high-throughput assessment of hepatic uptake, as no consistency was observed from the comparisons. The use of the high-throughput methods to assess drug uptake also do not have the ability to fully characterise the drug activity, for example, the lysosomal binding of SSRIs as identified from the oil uptake studies with FCCP (Hallifax and Houston, 2007) could not be identified using the clearance or Ki ratios. Use of the high-throughput methods may be useful for a first line assessment; however, in isolation they are not suitable for uptake assessment and the traditional methods to assess uptake such as oil uptake or studies incorporating the intracellular drug concentration (Poirier et al., 2008) are recommended based upon the results of this study. Additionally, the use of human hepatocytes is advocated for the IVIVE of human predictions considering the exclusion of species-related differences; the increasing availability and reducing costs of human hepatocytes facilitates this use.
Table 6.1 Clearance ratios for uptake assessment using *in vitro* data. Method 1 refers to the ratio of clearance from hepatocytes:microsomes, and method 2 to the ratio of clearance from the media loss:conventional assays in hepatocytes. Both methods were assessed in rat and human-derived material.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Method</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>1</td>
<td>3.15</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.03</td>
<td>702</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>1</td>
<td>2.47</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.87</td>
<td>1590</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1</td>
<td>1.20</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.52</td>
<td>4.92</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1</td>
<td>0.49</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.65</td>
<td>28.3</td>
</tr>
</tbody>
</table>

6.2 Parameter values utilised within DDI prediction and the Simcyp simulator

The default output values for victim drugs in the Simcyp library were found to be sufficient for analysis in comparison with alternative methods of estimation. An increasing number of studies have used the Simcyp simulator with default victim drug parameters (Einolf, 2007; Fahmi et al., 2009; Youdim et al., 2008; Zhao et al., 2009), and the correct estimation of these parameters is therefore essential. Individual parameters were assessed using DDIs with a range of potencies and involving victim drugs metabolised by the five main CYP enzymes. Differences in the input values of inhibitor parameters resulted in large differences in the predicted AUC ratio when combined into the overall model. This result can rationalise differences observed in a number of previous studies utilising the equation-based static model, where different studies have recommended the use of different parameter variants in DDI prediction. For example, different values of parameters (e.g. \( f_{\text{m,CYP}} \), \( F_G \), the use of unbound variants and \( K_i \) values) were utilised across studies investigating the different inhibitor concentration variants at the active site of the enzyme (Brown et al., 2006; Brown et al., 2005; Ito et al., 2004; Ito et al., 2005; Obach et al., 2007; Obach et al., 2006; Obach et al., 2005). In addition, different *in vivo* databases were used in the assessment of the inhibitor concentration variants, further complicating the comparisons. Differences in individual parameters across the studies can rationalise the overall differences observed and hence, the recommendations made based upon the findings. Consideration should therefore be given to the specifics of the conclusions when
extrapolating findings to alternative DDI databases. High variability in the reported values of inhibitor parameters increases the potential for the inclusion of error into predictions, as observed with the ketoconazole $K_i$ (Greenblatt et al., 2010). Despite this potential, the use of Simcyp ‘as is’ for retrospective analysis has been observed in a plethora of studies. These studies have often adopted an alternative approach to the current study, as new compounds in development have been added to Simcyp and DDIs predicted based upon inhibitors within the Simcyp library (casopitant, Motta et al., 2011; efavirenz, Rekic et al., 2010; linezolid, Gandelman et al., 2011; zolpidem, Polasek et al., 2009; maraviroc, Hyland et al., 2008; erlotinib, Rakhit et al., 2008), where the current study has assessed the impact of altered parameter values to existing victim drugs within the Simcyp library. Profile assessment of the victim drugs in the absence and presence of an inhibiting drug has been reported in the majority of published reports, focusing on the prediction of the $T_{\text{max}}$, $C_{\text{max}}$ and AUC of compounds (for example, Hyland et al., 2008; Polasek et al., 2009; Rakhit et al., 2008). The profiles of midazolam and triazolam were successfully predicted in this Thesis after oral dosing at 5mg and 0.25mg, respectively, despite the small under-prediction of AUC. Profile parameters were also successfully predicted in the presence of an inhibitor, however, the under-prediction of AUC’ resulted in the overall under-prediction of the AUC ratio in a number of cases. The profile success validates the use of the Simcyp simulator for DDI prediction, however deviation from this trend when considering alternative victim drugs and dosing schedules may rationalise altered prediction success for non-benzodiazepine victim drugs.

Assessment of a database of DDIs identified a number of trends. The dynamic model is identified as a more mechanistic approach than the more commonly utilised static model, despite the marginal improvement to DDI prediction accuracy. The dynamic model was able to identify differences resulting from the inclusion of active metabolites, and the ability to include differences in the dosage regimen of the inhibitor and victim drug doses in order to allow a more physiological prediction. Previous studies have reported an approximate 10% improvement in the prediction accuracy of DDIs using the dynamic model in Simcyp (Einolf, 2007; Fahmi et al., 2009), however this reported improvement was marginal (approximately 2%) in the latter study when only positive (AUC ratio > 2) DDIs were analysed. Differences in results were observed between Einolf (2007), Fahmi et al., (2009) and the current analysis despite overlap between included in vivo studies as a result of alternative parameter values; the potential impact of these differences was discussed above. In addition, the ability of the Simcyp simulator to predict DDIs in a population-based model gives advantage over a single, average predicted value. Prediction
success of the AUC ratios of individuals varied; this success was often linked to the potency of the reported DDI, with decreasing success observed with the increasing DDI potency. The majority of studies reporting the individual AUC ratios involved < 9 subjects, and the inability to successfully predict the AUC ratios could be linked to potential outliers within the reported data (e.g. Varhe et al., 1994). Previous studies have not assessed the prediction of the range of AUC ratios, with the exception of Einolf et al. (2007), where the standard deviations were compared. This method provides a preliminary assessment, but does not provide the ability to identify outliers in a non-standard distribution population. Dynamic DDI prediction is considered superior to static prediction from the assessment of conclusions across all previously reported studies.

Prediction of DDIs in genotyped populations has received little consideration, and has been limited to static prediction models of the victim drug exposure increase following inhibition of non-polymorphic pathways in PM subjects (Collins et al., 2006). This lack of investigation is despite the potential for the extent of observed DDI to be dependent on the genotype of the subject, as observed with the 5-fold difference between CYP2C19 PM and EM subjects in the DDI between fluvoxamine and omeprazole (Yasui-Furukori et al., 2004). In the current study, differences in the AUC ratio resulting from the genotype status of the clinical population were predicted successfully in the majority of cases. The prediction of a DDI in a genotyped population was assessed for one study reported in Einolf et al. (2007) where similar prediction success was observed in both genotype populations. The ability to identify DDI risk in different genotyped populations represents an additional advantage for use of the Simcyp simulator.

The moderate difference observed in DDI prediction success when investigating the impact of individual parameters using the Simcyp simulator could often not account for the differences in observed DDI prediction accuracy reported from a number of different literature sources (Brown et al., 2006; Brown et al., 2005; Ito et al., 2004; Ito et al., 2005; Obach et al., 2006). However, the combination of the different parameters could. Therefore, the impact of particular parameters could potentially be negated by the selection of a parameter with equal opposing impact, or magnified by the selection of a parameter with additional impact. For example, approximately equal AUC ratio predictions were observed for an itraconazole-simvastatin DDI between the current analysis and Brown et al., 2005. This observation was despite the differences in itraconazole potency (0.0013µM and 0.91µM) and simvastatin fmCYP (0.82 and 0.99 for the current analysis and Brown et al., 2005, respectively). In addition to parameter differences, the studies selected to assess
parameters are likely to impact on prediction accuracy. Midazolam parameters are well defined and prediction accuracy is usually high when assessing DDIs involving this victim drug. The inclusion of multiple, less defined inhibitors or victim drugs often reduces the prediction accuracy, for example 25% of victim drugs include in the current study had fm\textsubscript{CYP} values < 0.75. The increased number of victim drugs may have contributed to the reduction in prediction accuracy from 90 to 64% in the comparison of Fahmi et al., 2009 (midazolam only) and Einolf, 2007 (14 victim drugs), in combination with differences in the parameter values utilised in the models. In addition, the inclusion of a higher proportion of low potency DDIs is likely to increase prediction success, resulting from the trend for a reduction in prediction accuracy with increasing DDI potency, as observed in Chapter 4 and 5. Conclusions from each study should therefore be assessed based on all of the parameter values that were selected and characteristics of the DDI database used to assess trends.

6.2.1 Importance of the ‘two-fold’ criterion for investigating DDI prediction success
The conclusions in DDI prediction result from the use of the ‘2-fold’ level to define prediction success; the level accepted in the current consensus (Brown et al., 2006; Einolf, 2007; Galetin et al., 2006; Galetin et al., 2005; Teitelbaum et al., 2010; Wang, 2010). Further consideration of this two-fold criterion in the assessment of DDI prediction success is warranted. While a two-fold range may be appropriate for absolute values, the application of this method to the prediction of a ratio has not been fully considered in the literature. The wide two-fold range at lower AUC ratio values can lead to false impression of high prediction accuracy and therefore a potential bias in prediction trends. For example, for an actual AUC ratio of 1 (classified as no interaction), the traditional two-fold measure accepts predicted ratios ranging from 0.5 (induction) to 2.0 (border of weak/moderate inhibition interaction), as successful. Many publications assessing DDI prediction accuracy have been based on databases containing almost half of studies with AUC ratios < 2 (e.g. 42%: Einolf, 2007, 46%: Fahmi et al., 2009) and conclusions drawn may have been skewed by this proportion. This trend was evident in the analysis performed by Obach et al. (2006), where the inclusion of DDIs with < 2-fold increase in AUC resulted in increased accuracy and precision of DDI prediction.

In addition, application of the two-fold range at lower AUC ratio can lead to misclassification of DDI potential. Table 6.2 shows predicted AUC ratios for a range of midazolam DDIs (in all cases observed AUC ratio < 2) obtained using the dynamic DDI prediction model in the Simcyp simulator, as reported by Einolf (2007) and Fahmi et al.,
(2009). All DDIs were reported to be successfully predicted when assessed via the traditional two-fold measure. However, correct classification of the observed interaction (i.e., induction, no interaction or weak inhibition) was successfully predicted for less than 50% of the studies, often as a result of under-prediction of weak DDIs and subsequent classification as no interaction. The induction interaction with fluoxetine (AUC ratio 0.84, Lam et al., 2003) was predicted as weak inhibition (AUC ratio 1.28) and concluded as successful, despite this pertinent difference in classification.
Table 6.2 Accuracy in classification of midazolam DDIs (AUC ratio < 2) based on predictions obtained using dynamic model. All studies were reported as successfully predicted when assessed via the traditional two-fold measure approach.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Actual AUC ratio and DDI classification</th>
<th>Correct classification of predicted DDI using dynamic model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomoxetine</td>
<td>1.0</td>
<td>✓</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>1.4</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Chlorzoxazone</td>
<td>1.7</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>1.4</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>1.4</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>1.5</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.9</td>
<td>✓</td>
</tr>
<tr>
<td>Flumazenil</td>
<td>0.97</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>1.7</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>1.1</td>
<td>✓</td>
</tr>
<tr>
<td>Parecoxib</td>
<td>1.1</td>
<td>× (I)</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>1.2</td>
<td>✓</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>1.3</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>1.7</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>1.3</td>
<td>✓</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.04</td>
<td>✓</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.05</td>
<td>✓</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>0.93</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>1.1</td>
<td>✓</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>0.76</td>
<td>× (NI)</td>
</tr>
<tr>
<td>Valdecoxib</td>
<td>1.1</td>
<td>✓</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>0.84</td>
<td>× (W)</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>1.39</td>
<td>× (NI)</td>
</tr>
</tbody>
</table>

I, NI and W represent induction, no interaction and weak inhibition interactions, respectively. ✓ represents the correct classification predicted and × represents incorrect prediction, with the incorrect classification in parenthesis.
Prediction of DDIs associated with highly variable drugs (HVDs) represents an additional concern. These victim drugs (e.g. chlorpromazine and cyclosporine (Shah et al., 1996)) have a high within-subject variability in either $C_{max}$ and/or AUC (CV > 30%) (Davit et al., 2008; Tothfalusi et al., 2009) and a low observed AUC ratio in a DDI study could therefore be a result of either DDI or variability. The difference between the two is indistinguishable, emphasizing again that the prediction within traditional two-fold limits may be inadequate for this scenario. Therefore, a new measure of prediction accuracy applicable for both induction and inhibition DDIs is warranted, with the additional ability to incorporate the variability in pharmacokinetics of the victim drug when required.

New limits shown in Equations 6.1-6.3 coalesce when the observed ratio is one and approach the traditional two-fold limits as the ratio becomes large (Figure 6.1).

Upper limit: $R_{obs} \times \text{Limit}$

Lower limit: $R_{obs} / \text{Limit}$

$\text{Limit} = \frac{1+2(R_{obs}-1)}{R_{obs}}$ \hspace{1cm} \text{Equation 6.1} \hspace{1cm} \text{Equation 6.2} \hspace{1cm} \text{Equation 6.3}$

Where $R_{obs}$ represents $\frac{AUC_{inhibitor}}{AUC_{control}} \geq 1$, i.e. in the case of inhibition DDIs. The new predictive measure is also applicable for induction DDIs ($\frac{AUC_{inducer}}{AUC_{control}} < 1$) if the reciprocal of the observed AUC ratio, $\frac{AUC_{control}}{AUC_{inducer}}$, is used.

Figure 6.1 shows the differences in the limits of successful prediction for the traditional two-fold measure compared to the new predictive approach implemented using Equations 6.1-6.3; the corresponding observed data cover a 10-fold induction and inhibition range. The largest difference between methods is observed for AUC ratios ranging from 0.3-3, whereas the differences at 0.3 > AUC ratio > 3 are minimal (Figure 6.1). This range is particularly important from a regulatory point of view, as it represents the distinction between a positive and negative DDI (AUC ratio $\geq 2$) and therefore the decision on future follow-up clinical DDI trials will be based on the small scale studies and/or prediction from \textit{in vitro} data using DDI models or prediction software such as the Simcyp simulator (Hyland et al., 2008; Zhao et al., 2010).
Figure 6.1 Schematic graph displaying the limits of the different predictive measures; the traditional 2-fold predictive measure (dashed lines) and the proposed new predictive measure (dotted lines). Observed AUC ratio include both induction and inhibition DDIs.

The new proposed analysis in Figure 6.1 allows only a small deviation for successful prediction of AUC ratios at the level of no interaction (AUC ratio 1-1.25). However, this level is the area where there may be deviation in the victim drug AUC as a result of variability. Upper and lower limits in this case are as defined in Equations 6.1 and 6.2, respectively, but the variability is now introduced into the limit (δ) as shown in Equation 6.4 and Figure 6.2. The CV reported for midazolam AUC after i.v. dosing (Kharasch et al., 1999; Kharasch et al., 2007) was approximately 20%. This correlates to a value of δ=1.25, where the limits on R are between 0.80 and 1.25 when $R_{obs}=1$. This range corresponds to the conventional 20% limits used in bioequivalence testing (FDA, 2003). Maximal impact of the variability is expected at the level of no interaction, whereas at higher AUC ratios the impact of variability is minimal in comparison to the increase in AUC ratio in the presence of an inhibitor and the limit approaches 2-fold.

$$\text{Limit} = \frac{\delta + 2(R_{obs} - 1)}{R_{obs}}$$  \hspace{1cm} \textbf{Equation 6.4}

Where δ is a parameter that accounts for variability. If δ=1, there is no variability and limits revert to those defined by Equation 6.3.
In order to assess the new predictive measure, DDI predictions were collated from two publications (Einolf, 2007; Fahmi et al., 2009) and the analysis in Chapters 4 and 5 focusing on the prediction of DDIs involving midazolam as the victim drug. In all studies, predictions were obtained using the dynamic model in Simcyp Simulator (n=94) and input parameters were as defined in the respective papers. Use of different parameter inputs (for example for $K_i$ and $f_{up}$) resulted in different predictions even though around half of the DDIs overlapped between the two publications and the current analysis. Classification of the predicted DDI and the conclusions drawn in each study were compared using the conventional two-fold method and new measure of prediction accuracy (Equations 6.1-6.3). The impact of inclusion of the variability into the predictive measure was also assessed (Equation 6.4).

The impact of the application of the new predictive measure on the conclusions previously made in three existing large DDI databases (Einolf, 2007; Fahmi et al., 2009 and the analysis in Chapters 4 and 5) was assessed, focusing in particular on the analysis of the DDI prediction success involving midazolam as the victim drug (Figure 6.2). The previous analysis covered a wide range of AUC ratios (0.84-15.9 involving 8 inhibitors for Einolf, 2007), 0.04-16 involving 30 inhibitors for Fahmi et al., (2009), and 0.84-15.9 involving 5 inhibitors for the analysis in Chapters 4 and 5), and involved reversible inhibitors in all studies. Time-dependent inhibitors were additionally included in the analysis reported by Einolf, (2007) and Fahmi et al., (2009), with the latter study also reporting induction interactions. Considerable overlap (approximately 50%) was also observed between the three publications in the midazolam DDIs involving the azole and SSRI inhibitors. Table 6.2 displays the prediction accuracy resulting from the traditional or the new predictive measure with or without inclusion of the variability. Notable trends include the 21% reduction in the overall predictive accuracy using the new predictive measure compared to the traditional measure in all three studies; this decrease was apparent in particular at the level of no or weak interactions (50-61% decrease in accuracy). The inclusion of variability into the new predictive measure resulted in a 2-fold increase in prediction accuracy for these particular studies. The overall difference for all studies was not as pronounced (13%) due to the low proportion of no and weak interactions considered in the subset (19/94). The overall conclusions on the performance of both static and dynamic models within the three publications did not change. However, all studies also reported the trend of reduced prediction accuracy and higher bias at higher potency/positive (AUC ratio $\geq 2$) inhibition DDIs with both over- and under-predictions reported depending on the study. However, reanalysis with the new predictive measure shows a more consistent level
of prediction accuracy across the different DDI potencies, with no clear relationship between DDI potency and prediction accuracy (Table 6.2). The initial trend of higher accuracy at the lower AUC ratios was likely to be due to the wide two-fold boundaries at this range based on the traditional DDI prediction measure.

**Figure 6.2** Limits of DDI prediction with dashed lines representing the new predictive measure with inclusion of intra-individual variability, calculated via Equations 6.1 and 6.2, with the limits defined in Equation 6.4. Prediction of DDIs involving midazolam as the victim drug, taken from 3 sources, where ■ is Einolf et al., 2007, ▲ is Fahmi et al., 2009 and ○ is the current analysis reported in Chapters 4 and 5. The new predictive measure and inclusion of intra-individual variability is utilised. Two induction DDIs are not shown (AUC ratio 0.04 and 0.05); both were successfully predicted with all methods. The vertical lines represent the limits between potency classifications, where I, NI, W, M and S represents induction, no interaction, weak, moderate and strong inhibition interaction, respectively.
Table 6.3 Prediction accuracy of 94 DDI studies involving midazolam as the victim drug, collated from two publications (Einolf, 2007; Fahmi et al., 2009) and the current analysis in Chapters 4 and 5. Prediction accuracy is assessed by the following methods: the traditional 2-fold measure, the new predictive measure and the new predictive measure with the incorporation of limits to allow for the variability of midazolam.

<table>
<thead>
<tr>
<th>Predictive Measure (Number of studies)</th>
<th>Induction (8)</th>
<th>No interaction (6)</th>
<th>Weak (13)</th>
<th>Moderate (31)</th>
<th>Strong (36)</th>
<th>Total (94)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional 2-fold measure</td>
<td>88%</td>
<td>100%</td>
<td>92%</td>
<td>84%</td>
<td>81%</td>
<td>85%</td>
</tr>
<tr>
<td>New predictive measure</td>
<td>63%</td>
<td>50%</td>
<td>31%</td>
<td>68%</td>
<td>75%</td>
<td>64%</td>
</tr>
<tr>
<td>New predictive measure + variability</td>
<td>75%</td>
<td>100%</td>
<td>69%</td>
<td>74%</td>
<td>78%</td>
<td>77%</td>
</tr>
</tbody>
</table>
The 20% value used here for the inclusion of variability was taken from the limits currently used in bioequivalence testing (FDA, 2003). This value was in agreement with the reported variability in midazolam AUC (Kharasch et al., 1999; Kharasch et al., 2007). The CV used was based on i.v. dosing and would therefore exclude aspects of variability that may result after oral dosing - e.g., variability in intestinal first-pass (Galetin et al., 2008) and differences in GI tract physiology (e.g. gastric emptying) with the added impact of fasted/fed states in subjects (Shah et al., 1996). The use of 20% is proposed as a generic value when extending the methodology to other drugs in the absence of specific variability data. The proposed new methodology is appropriate for the assessment of ratios and allows tighter prediction boundaries for low AUC ratios, applicable across different interaction mechanisms (induction and inhibition). Importance of prediction accuracy and performance in the region below 2-fold change in AUC from a regulatory point of view has been addressed. In addition, this refined approach allows inclusion of variability into DDI predictions.

6.3 Refinement of DDI prediction using in vitro clearance

The unbound in vitro clearance data from the conventional assay in human hepatocytes (Chapter 3, scaled as described in Section 3.2.5.3) was utilised to assess the accuracy of DDI predictions following the input of in vitro clearance parameters within the Simcyp simulator. Prediction of the database of DDIs involving itraconazole, ketoconazole, fluoxetine and fluvoxamine are displayed below; the database described in Chapter 2 was utilised. Fluconazole was excluded from this analysis as the clearance was not measured. Comparison of the in vitro clearance data for the inhibitors was with the in vivo clearance from oral data or recombinant in vitro data for itraconazole as per the previous analysis in Chapters 4 and 5. All other parameters in the prediction were consistent to the analysis in Chapters 4 and 5. The prediction success was assessed using the new predictive measure using 20% variability (Section 6.3).
A.

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<table>
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<tr>
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<th>NI</th>
<th>W</th>
<th>M</th>
<th>S</th>
</tr>
</thead>
</table>

Observed AUC Ratio

Figure 6.3 The dynamic prediction of DDIs where inhibitor clearance has been characterised using in vivo clearance from oral data (with the exception of recombinant in vitro data for itraconazole DDIs) (A), or in vitro clearance from the conventional assay in human hepatocytes (Chapter 3) (B). DDIs involved fluoxetine (●), fluvoxamine (■), itraconazole (▲) or ketoconazole (▼) as the inhibitors. All other parameters were consistent between the predictions. Dashed lines representing the new predictive measure with inclusion of variability, calculated via Equations 6.1 and 6.2, with the limits defined in Equation 6.4. The vertical lines represent the limits between potency classifications, where I, NI, W, M and S represent induction, no interaction, weak, moderate and strong inhibition interaction, respectively.

The itraconazole, ketoconazole, fluoxetine and fluvoxamine DDIs (n=78) from the database described in Chapter 2 were reassessed using unbound clearance data from the conventional assay in human hepatocytes in Chapter 3. No hepatic uptake was assumed for any of the inhibitors, as per the observed lack of consistency in uptake results. Incorporation of the in vitro clearance improved the predictions (using the refined prediction success model; Section 6.3) from 50 to 72% within the new ‘2-fold’ boundaries (Figure 6.3). The use of in vitro data resulted in a 30% improvement in prediction accuracy for DDIs involving fluoxetine (n=10) and ketoconazole (n=23). Minimal improvement was observed for itraconazole DDIs (6%), resulting from the use of in vitro data in Chapter 4; differences were from the use of hepatocyte data instead of recombinant data. The increase in prediction accuracy was accompanied by an increase in precision (mse of 1.99 and 1.26 for in vivo and in vitro data, respectively) and a decrease in bias (rmse of 3.56 and 2.87 for in vivo and in vitro data, respectively). The difference in prediction accuracy was minimal where the AUC ratio < 2; the prediction accuracy for moderate and strong inhibition interactions both increased by 27% from use of in vitro data. In addition, the prediction of the midazolam profile in the presence of itraconazole and ketoconazole was assessed. Values of T_max were successfully predicted, C_max predictions improved in comparison to
those predicted from the \textit{in vivo} data. An association between the number of studies successfully predicted and inhibitor involved in the DDI was observed for fluvoxamine; no such relationship was observed for the alternative inhibitors. In the case of fluvoxamine, the number of moderate interactions successfully predicted increased by 2-fold, and 50% of strong interactions were successfully predicted from use of the \textit{in vitro} data, compared to none of the DDIs from \textit{in vivo} data. No trends were ascertained from the use of \textit{in vitro} data resulting from the CYP enzyme involved in the DDI. The observed difference in overall prediction accuracy cannot be rationalised by a trend of under-prediction of \textit{in vivo} inhibitor clearance by the \textit{in vitro} data. The \textit{in vivo} clearances of fluoxetine and fluvoxamine were under-predicted by the conventional assay in human hepatocytes; however, ketoconazole was successfully predicted and therefore the observed increase in prediction accuracy is inconsistent to the prediction success of inhibitor clearance. It is possible that the observed difference in overall prediction accuracy may be rationalised by the use of $K_{\text{inhu}}$ and clearance values that were estimated from \textit{in vitro} experiments within the same laboratory. This single source of data is in comparison to the parameters utilised within Chapters 4 and 5 that were a combination of in house and Simcyp library values. Prediction of the DDIs from \textit{in vitro} data is important considering that the data is likely to be available early in development, in the case of prospective analysis. Human hepatocyte data was selected based on the recommendations made in Section 6.1; the expression of the full complement of enzymes and structural integrity results in increased physiological relevance in comparison to microsomes. The clearance in human hepatocytes was often 2-3-fold higher than that observed in the microsomes, and it is therefore unlikely that use of microsomal data would have increased DDI prediction accuracy in the refined analysis. This supports the recommendation for the use of human hepatocytes for \textit{in vitro} data.

6.4 Final conclusions

PBPK models are increasingly being utilised in the prediction of pharmacokinetic parameters and DDIs from human \textit{in vitro} data, with implications in the design of clinical studies in drug development (Aarons, 2005; Jones et al., 2011; Jones et al., 2006; Rowland et al., 2011). This study has utilised an example of a PBPK model, the Simcyp simulator, in order to assess the prediction of DDIs, focussing on the inclusion of the time profile of inhibition using the dynamic model, and the impact of individual parameters on the prediction. The findings suggest that the dynamic model results in a more physiological prediction in complex scenarios, for example in the presence of inhibitory metabolites or with alterations to the inhibitor or victim drug dosing schedules. In addition, the ability of Simcyp to predict inter-individual variability in different demographic populations was
demonstrated, including the impact of different genotype groups. The ability to identify
groups of patients with increased DDI risk is important during drug development and the
regulatory process. Despite these outlined advantages, the overall use of the dynamic
model in Simcyp did not result in a significant improvement to DDI predictions in
comparison to the static model in Simcyp and previous equation-based DDI predictions.
Additionally, a number of limitations have been identified in this Thesis including
inconsistencies between assumptions for victim drugs and inhibitors, and the inability to
modify all equations that are used in the program. The use of PBPK analysis to support
regulatory processes is increasing as an alternative to clinical studies in subset populations.
This demonstrates the importance of PBPK models, and highlights the need for thorough
consideration of conclusions. Analysis into the impact of different parameters in the
prediction of DDIs found that the model sensitivity to individual parameters often would
not result in large differences in AUC ratio; these differences resulted from the
combination of all parameters within the model. This finding contradicts many current
analyses that aim to investigate the impact of a singular parameter in the prediction of
DDIs, and highlights the importance of considering overall DDI prediction. The validation
of predicted DDIs to reported *in vivo* data was critiqued, considering the variability
introduced as a result of low numbers of subjects and inconsistency in study designs and
data analysis. The trend for loss of prediction accuracy correlating to increasing potency of
DDI was found to result from the method traditionally used to assess the prediction
accuracy of DDIs. The proposed new predictive measure is applicable for the assessment
of ratios, can be applied for both inhibition and induction interactions and allows the
incorporation of variability where necessary.
REFERENCES


References


References


References


References


determining rates, and implications for the prediction of drug interactions. *Curr Drug Metab* **9**: 384-94.


7 Appendices

7.1 Appendix I - Materials

Dulbecco’s phosphate buffered saline (DPBS), Earls Balanced Salt Solution (EBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Ethylene glycol-bis-(3-aminoethyl ether) N, N, N’, N’-tetraacetic acid (EGTA), Sodium hydroxide (NaOH), Calcium chloride (CaCl$_2$), Trypsin inhibitor, Collagenase H, Williams Media E (WME), Sodium bicarbonate (NaHCO$_3$), Trypan blue, Krebs-Henseleit Buffer, Itraconazole, Ketoconazole, Fluconazole, Fluoxetine, Norfluoxetine, Fluvoxamine, Dextromethorphan, Verapamil, FCCP, Magnesium chloride, isocitric acid and isocitrate dehydrogenase were all purchased from Sigma Aldrich (Dorset, U.K.). Hydroxy-itraconazole was purchased from Fitzgerald Industries International (MA, USA). All solvents were of high analytical grade.
Male Sprague-Dawley rats were obtained from the Biological Sciences Unit, Medical School, University of Manchester. They were housed in pairs in opaque boxes with sawdust bedding. Temperature was maintained at 20 ± 3°C, relative humidity at 40-70% and in a 12 hour light/dark cycle. The rats had access to a standard laboratory pelleted CRM diet and drinking water *ad libitum*. Rat liver hepatocytes were isolated via a method based on Berry and Friend, 1969. Male Sprague-Dawley rats (250g) were sacrificed by cervical dislocation, after acclimatisation to surroundings for at least one week. The two largest lobes of the liver (left and medial) were removed and placed in cold Dulbecco’s phosphate buffered saline (DPBS). They were both cannulated and perfused with Earls Balanced Salt Solution (EBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.25mM ethylene glycol-bis-(3-aminoethyl ether) N, N, N’, N’-tetraacetic acid (EGTA) in sodium hydroxide (NaOH), at 37°C, pH7.4 for 4 minutes at a rate of 6-8ml/min/lobe. The EGTA is a chelating agent and removes calcium from the tight junctions in order to decrease calcium dependent cell adhesion, and HEPES acts as a buffering agent to maintain the pH. The lobes were then perfused for 4 minutes with EBSS only to remove the EGTA from the system. Finally, the lobes were perfused with EBSS containing 1mM calcium chloride (CaCl\(_2\)), collagenase H (0.68mg/ml) and trypsin inhibitor (0.07mg/ml) for 8-12 minutes. The calcium-dependent hydrolytic cleavage of collagen molecules by collagenase results in matrix break up, with excess trypsin removed by the trypsin inhibitor. The lobes were then placed in Williams Media E (WME) containing sodium bicarbonate (NaHCO\(_3\)) and gently agitated to release the cells. The solution was then passed through a nylon 64µM mesh (Clarcor, Warrington, UK) and centrifuged for 3 minutes at 300g (4°C) (Heraeus Labofuge 400R, Kendro Laboratory Products, Germany). The supernatant was removed, pellet resuspended in WME and centrifugation step repeated twice. To count the cells, 50µL cells, 150µL WME and 200µL 0.4% trypan blue were mixed (Jauregui et al., 1981), transferred to a Neubauer haemocytometer counting chamber (Kova® Glasstic Slide, Hycor Biomedical Ltd, CA, USA) and observed under x 400 magnification (Gillert and Silbert Ltd, London, UK). Live cells appeared yellow due to exclusion of trypan blue from cells, where dead cells appeared blue. Five chambers were counted, and viability was calculated by dividing the number of viable cells to total cells for each chamber. Preparations with an average viability level below 85% were not used. The number of cells/mL was calculated by multiplying the average number of viable cells per chamber by 80,000 (dilution factor).
7.3 Appendix III – Rat microsomes

Pooled rat microsomes (Lot number RT039)
Purchased from Invitrogen™, Paisley, U.K. (Catalogue number CZDRTMCPL)
Supplied at 20mg/mL

**Donor information:**
Pool of 200 Healthy, Sprague-Dawley rats

**P450 Content:**
0.579 nmol P450/mg protein
7.4 Appendix IV – Human hepatocytes

CryostaX single freeze cryopreserved human hepatocytes (Lot number 1010270). Purchased from Tebu-bio, Peterborough, U.K. (Catalogue number HPCH20). Supplied at 10Mcells/mL, 73.8% viability.

Donor information:
Pool of 20 donors (10 male, 10 female); 32-76 years of age.

Race:
Caucasian (13), African American (3), Hispanic (2), Indian (1), Pacific Islander (1).

Cause of death:
Anoxia (5), Head trauma (3), Cerebrovascular accident (11), Aortic aneurysm (1).

Relevant enzyme activity:

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<th>Enzyme</th>
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<th>Reaction (pmol/Mcells/min)</th>
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<tr>
<td>CYP1A2</td>
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<td>Diclofenac 4’-hydroxylation</td>
<td>355 ± 51</td>
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<td>Midazolam 1’-hydroxylation</td>
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7.5 Appendix V - Human microsomes

Pooled human microsomes (Lot number 70196).
Purchased from BD Biosciences™, Bedford, MA (Catalogue number 452161)
Supplied at 20mg/mL.

Donor information:
Pool of 22 donors (15 male, 7 female); 10-70 years of age.

Race:
Caucasian (20), African American (1), Hispanic (1).

Cause of death:
Cerebrovascular accident/stroke (6), Intracerebral haemorrhage (5), Anoxia (3), Head trauma (4), Subarachnoid haemorrhage (3), Closed head injury/motor vehicle accident (1).

Relevant enzyme activity:

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<th>Reaction (pmol/min/mg)</th>
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<td>Testosterone 6β-hydroxylation</td>
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7.6 Appendix VI – Preliminary in vitro data

Preliminary experiments were carried out using the conventional assay in rat hepatocytes as per Section 3.2.2 to determine linearity of depletion with increasing cell concentration (0.1-1.5Mcells/mL, Figure A.1). Inhibitor concentrations of 0.1μM were used over a time period of 0-60 minutes. From the linearity studies a cell concentration of 0.25Mcells/mL was selected for fluoxetine, and 1Mcells/mL for fluvoxamine, itraconazole and ketoconazole. Average calibration curves for n=3 experiments in rat hepatocytes and microsomes and human hepatocytes and microsomes are also given (Figure A.2).

![Figure A.1](image)

Figure A.1 Linearity plots for fluoxetine (A), fluvoxamine (B), itraconazole (C) and ketoconazole (D) CL<sub>int,u</sub> in rat hepatocytes.
Figure A.2 Calibration curves for fluoxetine (A), fluvoxamine (B), itraconazole (C) and ketoconazole (D) in rat hepatocytes (○) or microsomes (●), or human hepatocytes (△) and microsomes (▲). Points represent mean ± S.D. (n=3 for each system).
7.7 Appendix VII – LC-MS/MS Assay

Fluconazole, ketoconazole, itraconazole and fluoxetine samples were analysed using high performance liquid chromatography (HPLC) (Waters 2795 Separations Module, Alliance HT) to separate the peaks, and tandem mass spectrometry (MS/MS) (Micromass Quattro Ultima) to ionize the parent compound. The HPLC and MS/MS for the fluvoxamine analysis used Waters 2695 and Micromass Quattro Micro, respectively. Detection was performed by multiple reaction monitoring (MRM) of the parent and selected daughter ion.

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<td>C</td>
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<td>D</td>
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**Fluoxetine**

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**Fluconazole, Itraconazole, Ketoconazole**

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**Fluvoxamine**

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**MRM:**

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7.8 Appendix VIII - Publication information

**Publication 1:**

**Publication 2:**