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In vitro approaches in evaluation and prediction of drug-drug interactions involving the inhibition of cytochrome P450 enzymes

by

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ACADEMIC DISSERTATION

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To Jun-Sheng and Stone

CONTENTS

ABBREVIATIONS

α	the factor by which K_m changes when an inhibitor occupies the enzyme
Ah	aryl hydrocarbon
$AUC_{(0\text{-}t)}$	area under the curve for concentration or effect versus time from 0 to t hours
CAR	constitutive androstane receptor
C_E	drug concentration around the metabolic enzyme site
Cl_h	hepatic clearance
Cl_{int}	total intrinsic clearance
$Cl_{int,\ u}$	unbound intrinsic clearance
C_{max}	peak plasma concentration
Cl_{met}	partial metabolic clearance
CYP	cytochrome P450
DDC	diethyldithiocarbamate
DMSO	dimethyl sulfoxide
E	hepatic extraction ratio
FDCL	the fractional decrement of clearance *in vivo*
FDV	the fractional decrement of reaction velocity *in vitro*
F_{oral}	oral bioavailability
f_u	unbound fraction of drug in the blood
Hlc	human liver cytosol
HPLC	high-performance liquid chromatography
Hsa	human serum albumin
i	degree of inhibition
I	inhibitor concentration
I_u	unbound concentration of inhibitor
IC_{50}	concentration of inhibitor corresponding to a 50% decrease in reaction velocity
K_i	inhibition constant
K_I	the concentration of mechanism-based inhibitor required for half-maximal inactivation
K_{inact}	the rate constant of inactivation
K_m	Michaelis-Menten constant represents the total substrate concentration at which the reaction velocity is 50% of maximum
$K_{m,\ u}$	unbound Michaelis-Menten constant represents the unbound substrate concentration at which the reaction velocity is 50% of maximum
K_{obs}	apparent inactivation rate constant
MI	metabolite intermediate
MM	Michaelis-Menten
n	Hill coefficient for cooperative substrate binding
NADPH	reduced nicotinamide adenine dinucleotide phosphate
P-gp	P-glycoprotein
PM	poor metabolism
PPAR	peroxisome proliferator activated receptor

PXR	pregnane X receptor
Q	hepatic blood flow
S	substrate concentration
SSRI	selective serotonin reuptake inhibitors
S_{50}	the substrate concentration at which half the maximal rate is attained in the Hill equation
TAO	troleandomycin
$t_{1/2}$	elimination half-life
$T_{1/2}$	apparent half-life for mechanism-based inactivation
t_{max}	time to peak plasma concentration
UGT	uridine diphosphate-glucuronosyltransferases
V_{max}	the maximum velocity of metabolite formation based on total substrate concentration
$V_{max, u}$	the maximum velocity of metabolite formation based on unbound substrate concentration
V_0	the velocity of an enzyme reaction in the absence of inhibitor
V_i	the velocity of an enzyme reaction in the presence of inhibitor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by the Roman numerals I to V.

I Wen X, Wang JS, Kivistö KT, Neuvonen PJ, Backman JT. In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). *Br J Clin Pharmacol 2001; 52:547-553.*

II Wen X, Wang JS, Backman JT, Kivistö KT, Neuvonen PJ. Gemfibrozil is a potent inhibitor of human cytochrome P450 2C9. *Drug Metab Dispos 2001; 29:1359-1361.*

III Wen X, Wang JS, Neuvonen PJ, Backman JT. Isoniazid is a mechanism-based inhibitor of cytochrome P450 1A2, 2A6, 2C19 and 3A4 isoforms in human liver microsomes. *Eur J Clin Pharmacol 2002; 57:799-804.*

IV Wen X, Wang JS, Backman JT, Laitila J, Neuvonen PJ. Trimethoprim and sulfamethoxazole are selective inhibitors of CYP2C8 and CYP2C9, respectively. *Drug Metab Dispos 2002; 30: 631–635.*

V Wang JS*, Wen X*, Backman JT, Neuvonen PJ. Effect of albumin and cytosol on enzyme kinetics of tolbutamide hydroxylation and on inhibition of CYP2C9 by gemfibrozil in human liver microsomes. *J Pharmacol Exp Ther 2002; 302: (in press) (* equal contribution).*

ABSTRACT

In vitro systems have been widely used in evaluating potential drug-drug interactions *in vivo*. In the present studies, the effects of valproic acid, gemfibrozil, isoniazid, trimethoprim and sulfamethoxazole on the CYP forms (CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) activities were examined *in vitro* using pooled human liver microsomes or recombinant CYP forms. The IC_{50} and K_i values were characterized using an apparent reversible inhibition. The K_I and K_{inact} values were characterized using a mechanism-based inhibition. The *in vivo* degrees of inhibition (i) were predicted *in vitro* using a scaling model: $i = I/(I + K_i)$. In addition, the effects of human serum albumin (Hsa) and human liver cytosol (Hlc) on the *in vitro* enzyme kinetic estimates of the formation of hydroxytolbutamide, and on the inhibitory effect of gemfibrozil on tolbutamide hydroxylase activity were evaluated using human liver microsomes.

Valproic acid preferentially inhibited CYP2C9 activity (K_i = 600 µM), while it exhibited minimal or no inhibitory effects on the other CYP forms evaluated. Gemfibrozil inhibited CYP2C9 (K_i = 5.8 µM), and CYP2C19 (K_i = 24 µM) and CYP1A2 (K_i = 82 µM) activities. Isoniazid was a mechanism-based inhibitor of CYP1A2, 2A6, 2C19 and 3A4 forms, with K_{inact} values of 0.11, 0.13, 0.09, and 0.08 min^{-1}, and K_I values of 285, 173, 112, and 228 µM, respectively. Trimethoprim (5-100 µM) selectively inhibited CYP2C8 activity (K_i = 32 µM), while sulfamethoxazole (50-500 µM) selectively inhibited CYP2C9 activity (K_i = 271 µM). Based on the aforementioned scaling model and the unbound plasma concentrations of the drugs, inhibition of CYP2C9 (7%) by valproic acid, CYP2C9 (56%), CYP2C19 (24%) and CYP1A2 (8%) by gemfibrozil, CYP2C8 (26%) by trimethoprim, and CYP2C9 (24%) by sulfamethoxazole would be expected *in vivo*. The addition of Hsa and Hlc to the incubation media distinctly changed the kinetic estimates of tolbutamide hydroxylation, with the predicted *in vivo* hepatic clearance (Cl_h) of tolbutamide hydroxylation (0.14 ml/min/kg) comparable to the actual *in vivo* value (0.15 ml/min/kg). However, the unbound K_i of gemfibrozil for CYP2C9 (6 µM in the

absence of Hsa and Hlc in the incubation media) was not markedly altered by Hsa (4 µM), Hlc (8 µM) or both Hsa and Hlc (9 µM) when the unbound substrate and inhibitor concentrations were considered.

The results of the present studies indicated that *in vitro*, valproic acid reversibly inhibits CYP2C9 and gemfibrozil inhibits CYP2C9, 2C19 and 1A2; isoniazid is a mechanism-based inhibitor of CYP1A2, 2A6, 2C19 and 3A4; trimethoprim is a selective inhibitor of CYP2C8 and sulfamethoxazole of CYP2C9. In humans, the inhibition of these CYP activities by these drugs may result in significant drug-drug interactions, and can explain some of their documented drug-drug interactions. However, in most cases, confirmation of the predicted drug-drug interactions requires further *in vivo* experiments. The addition of Hsa and Hlc to microsomal incubations may yield enzyme kinetic estimates more comparable with *in vivo* results for CYP2C9 substrates. However, further experiments are needed to clarify the effects of Hsa and Hlc on other CYP substrates and other CYP enzymes.

INTRODUCTION

The cytochrome P450 (CYP) enzymes constitute a superfamily of hemoprotein enzymes that are responsible for biotransformation of numerous xenobiotics, including therapeutic agents. In humans, the major drug-metabolising CYPs belong to enzyme families 1, 2 and 3, with the main CYP forms being 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (Shimada et al. 1994). Inhibition and induction of these CYP enzymes may result in toxicity or therapeutic failure, and are the most common causes for drug-drug interactions (see Lin & Lu 1998; Venkatakrishnan et al. 2001).

Because of limitation of *in vivo* studies and problems in extrapolating the results of animal studies to humans, *in vitro* systems using human tissues have become widely used as tools to evaluate potential drug-drug interactions in humans (see Levy et al. 2000). *In vitro* systems include enzyme-based systems (liver microsomes and cDNA-expressed enzymes systems) and cell-based systems (hepatocytes and liver slices) (Wrighton et al. 1993; Ekins et al. 2000). Among these, the human liver microsomal system has been confirmed to be the most feasible and well set up system for drug metabolism and interactions studies (see Levy et al. 2000; Venkatakrishnan et al. 2001).

Although the use of microsomal studies for quantitative or semi-quantitative prediction of *in vivo* drug inhibitions is promising, several factors can significantly affect the results of microsomal studies, resulting in an inability to accurately predict the *in vivo* situation (Newton et al. 1995; von Moltke et al. 1995a). For example, the nonspecific microsomal binding of substrates to *in vitro* incubation matrices may result in an underprediction of the *in vivo* hepatic clearance (Cl_h) due to a reduced rate of *in vitro* metabolism (Obach 1999). In addition, it was recently reported that the addition of bovine serum albumin or rat liver cytosol to the microsomal incubation medium could promote CYP2C9-mediated reactions (Ludden et al. 1997; Carlile et al. 1999; Komatsu et al. 2000a). However, the mechanisms involved in this phenomenon are

still far from clear, and require further exploration with other substrates and other CYP forms.

Valproic acid, gemfibrozil, isoniazid, trimethoprim and sulfamethoxazole are widely used therapeutic agents. These drugs can affect the pharmacokinetics of several drugs, which are metabolised by different CYP forms. For example, valproic acid affects the pharmacokinetics of phenytoin, amitriptyline, phenobarbital and diazepam (Perucca et al. 1980; Wong et al. 1996; Patel et al. 1980; Dhillon et al. 1982); gemfibrozil enhances the anticoagulant effect of warfarin, resulting in severe hypoprothrombinemia and bleeding, and it interacts with glyburide and glimepiride, resulting in hypoglycaemia (Ahmad 1990; 1991; Rindone & Keng 1998; Niemi et al. 2001). A combined therapy of gemfibrozil and statins such as atorvastatin, cervastatin, lovastatin or simvastatin in patients can result in severe myopathy and rhabdomyolysis (Murdock et al. 1999); isoniazid decreases the elimination of several drugs including carbamazepine, diazepam, triazolam, vincristine, theophylline, disulfiram, chlorzoxazone and paracetamol (Wright et al. 1982; Ochs et al. 1981; 1983; Chan et al. 1998; Samigun et al. 1990; Whittington et al. 1969; Zand et al. 1993); trimethoprim and sulfamethoxazole interact with tolbutamide, phenytoin, warfarin, and glipizide (Wing & Miners 1985; Hansen et al. 1979; O'Reilly 1980; Johnson et al. 1990). However, comprehensive studies on the inhibition of major CYP forms by these drugs have not been published.

In the present series of studies, the inhibitory effects of the aforementioned drugs on CYP activities were investigated in human liver microsomes (or recombinant CYP forms). Using selective marker reactions for the major CYP forms, prediction of the potential *in vivo* drug-drug interactions was carried out using an *in vitro-in vivo* scaling model. In addition, the effects of the addition of human serum albumin (Hsa) or human liver cytosol (Hlc) to microsomal incubation media on the enzyme kinetic estimates of the formation of hydroxytolbutamide (a marker reaction of CYP2C9), and on the inhibitory effect of gemfibrozil on tolbutamide hydroxylase activity were examined using human liver microsomes.

REVIEW OF THE LITERATURE

1. Drug metabolism and CYP enzymes

1.1 Drug metabolism

Most drugs are lipophilic compounds, which need to be enzymatically transformed into more polar, water-soluble, and excretable metabolites that can be easily eliminated from organisms (see Stockley 1999; Meyer 1996). The biotransformation of drugs can be classified into phase I and phase II reactions. Phase I metabolism includes oxidation, reduction and hydrolysis reactions, while phase II reactions include glucuronidation, acetylation, sulfation, methylationand, and glutathione and amino acids conjugation. Usually phase II reactions generate inactive and more water-soluble compounds that can be easily eliminated from an organism via urine or bile (see Gonzalez & Idle 1994; Lin & Lu 2001).

The major site of biotransformation of drugs is the liver, which contains a large number of metabolising enzymes. In addition, enzymes in the intestinal mucosa also contribute significantly to the metabolism of drugs (see Hall et al. 1999). Other extrahepatic sites of drug metabolism include the kidneys, lungs, skin, brain and nasal epithelium. However, these sites contribute to a minor extent to the systemic elimination of drugs compared with the liver and intestines (see Krishna & Klotz 1994).

1.2 CYP enzymes in man

Cytochrome P450 (CYP) enzymes, are the most important phase I enzymes that are involved in the metabolism of many endogenous compounds and a majority of clinically used drugs (see Gonzalez & Idle 1994). CYP proteins, named according to the absorption band at 450 nm of their reduced carbon-monoxide-bound forms, consist of large superfamilies of enzyme proteins (see Schenkman & Jansson 1999). The root

symbol, CYP, is followed by a number for the family (a general group of protein with more than 40% amino-acid sequence identity), a letter for the subfamily (greater than 55% identity), and a number for the gene denoting a specific CYP form. Distinct CYP forms differ from each other with respect to their chemical, immunological properties, and they have different substrate affinities (see Meyer 1996). Intracellularly, the CYP enzymes are found primarily in the endoplasmic reticulum that also serves as the locus of metabolic drug interactions (see Lin & Lu 1998). The CYP enzymes are synthesized on membrane-bound polyribosomes and inserted directly into the lipid bilayer (Gonzalez & Kasper 1980; Sabatini et al. 1982).

In mammals, at least 17 CYP gene families have been identified. Among these, the CYP1, CYP2, and CYP3 subfamilies are mainly involved in the biotransformation of pharmaceuticals and xenobiotics. The other CYP families mainly take part in the biosynthesis of steroids, metabolism of bile acids and arachidonic acid, and metabolism of other endogenous compounds (Nelson et al. 1996). The major drug metabolising CYP subfamilies in humans are CYP3A (3A4 and 3A5) (~30% of total P450 content in the liver), CYP2C (2C8, 2C9, 2C18 and 2C19) (~20%), CYP1A2 (~13%), CYP2E1 (~7%), CYP2A6 (~4%), and CYP2D6 (~2%) (Shimada et al. 1994). Among these, several CYP forms, e.g. CYP2A6, 2C8, 2C9, 2C19, 2D6, and 2E1 are polymorphic expressed in human subjects (see Raunio et al. 2001; Goldstein 2001; Rodrigues 2002; Shimada et al. 1994; Levy et al. 2000).

1.2.1 CYP1A2 enzyme

The human CYP1A subfamily comprises two members, CYP1A1 and CYP1A2. CYP1A1 is expressed at a very low level in the liver, while it is primarily an extrahepatic enzyme found in the lungs and placenta (see Pelkonen et al. 1998). There is genetic polymorphism in the inducibility of CYP1A1 by polycyclic aromatic hydrocarbons, with a high inducibility phenotype being more common in patients with lung cancer (Kouri et al. 1982; Nebert et al. 1991). CYP1A2 is the predominant enzyme of the CYP1A subfamily constituting approximately 13% of the total CYP

protein in human livers (Shimada et al. 1994). CYP1A2 activity can be induced by cigarette smoking, resulting in an almost two-fold increase of the metabolic clearances of substrates of this enzyme compared with nonsmokers (Grygiel et al. 1981). In addition, omeprazole has been shown to be a dose-dependent inducer of CYP1A2 in man (Andersson 1991).

In humans, the interindividual variability of the expression of CYP1A2 is large. For example, about 40-60 fold differences between different ethnic groups have been reported (Shimada et al. 1994). However, to date, no specific polymorphism of the CYP1A2 gene has been identified (Catteau et al. 1995).

CYP1A2 is responsible for the metabolism of several drugs, including phenacetin, caffeine, theophylline, paracetamol, olanzapine, lidocaine, and some procarcinogens (see Zevin et al. 1999; Pelkonen et al. 1998; Wang et al. 2000a). The major route of caffeine metabolism in man (*N*-3-demethylation of caffeine to paraxanthine) is mediated by CYP1A2 (Lelo et al. 1986; Butler et al. 1989). Therefore, caffeine has been used *in vivo* as a CYP1A2 probe substrate. In *in vitro* studies, ethoxyresorufin and phenacetin have been used as preferential probe substrates (Distlerath et al. 1985; Burke et al. 1994).

The selective serotonin reuptake inhibitors (SSRI) antidepressant fluvoxamine (K_i ~ 0.2 μM) (Nemeroff et al. 1996) and the fluoroquinolone antibiotics ciprofloxacin and enoxacin, are the most significant CYP1A2 inhibitors which can cause clinically significant drug-drug interactions with substrates of CYP1A2 (Schmider et al. 1997). Although fluvoxamine has been used as a potent inhibitor of CYP1A2 in reaction phenotyping studies *in vivo*, it also inhibits CYP2C19 with similar potency and may cause clinically significant drug-drug interactions with CYP2C19 substrates (von Moltke et al. 1999). Furafylline (a mechanism-based inhibitor of CYP1A2, K_I = 3 ~ 23 μM and K_{inact} = 0.07 ~ 0.87 min^{-1}) and α-naphthoflavone are used as relatively specific and potent inhibitors of CYP1A2 *in vitro* (Newton et al. 1995; Bourrie et al. 1996) (Table 1).

1.2.2 CYP2A6 enzyme

CYP2A6 is a quantitatively minor component (1-5%) of the human hepatic CYP forms. A number of compounds such as phenobarbital and pyrazole increased CYP2A6 activity at the mRNA level in human hepatocytes in primary culture (Dalet-Beluche et al. 1992; Donato et al. 2000). CYP2A6 is active in the metabolism of a few drugs, such as coumarin, methoxyflurane, halothane, valproic acid, disulfiram, losigamone, letrozole and a number of procarcingens (see Raunio et al. 1998; 2001; Pelkonen et al. 2000; Oscarson 2001). *In vitro* studies using human liver microsomes and recombinant CYP forms also indicated that CYP2A6 is the most important CYP form responsible for the *C*-oxidation of nicotine (Nakajima et al. 1996; Messina et al. 1997).

In humans, CYP2A6 is polymorphically expressed. The polymorphism of CYP2A6 has been thought to be associated with smoking habits as well as the risk of lung cancer (Pianezza et al. 1998; London et al. 1999). To date, several defective alleles of CYP2A6 have been reported with the most prevalence of them being a Leu 160 His substitution (CYP2A6*2) that yields an inactive enzyme (Fernandez-Salguero et al. 1995; Hadidi et al. 1997). By contrast, a deletion mutation (CYP2A6*4) is the most common variant (15-20%) in Asian populations (Nunoya et al. 1998). Duplication of the CYP2A6 gene also occurs and appears to be associated with increased catalytic activity (Rao et al. 2000). Overall, the frequencies of all the major variant alleles of CYP2A6 are rather uncommon in Caucasians, while some of these alleles are highly prevalent in Oriental populations (Oscarson et al. 1999a; 1999b; Chen et al. 1999; Nunoya et al. 1999).

Coumarin 7-hydroxylation is selectively catalyzed by CYP2A6, and coumarin has therefore been used as a CYP2A6 probe drug both *in vitro* and *in vivo* (Rautio et al. 1992). Compounds including 8-methoxypsoralen, menthofuran, pilocarpine and tranylcypromine have been found to be relatively potent inhibitors of CYP2A6 (Koenigs et al. 1997; Khojasteh-Bakht et al. 1998; Kinonen et al. 1995; Taavitsainen et

al. 2001). Among these, 8-methoxypsoralen is a probe and potent mechanism-based inhibitor of CYP2A6 (K_I = 1.9 µM and K_{inact} = 2 min^{-1}), which has been used as a useful *in vitro* tool for evaluation of the contribution of CYP2A6 metabolic reactions (Koenigs et al. 1997) (Table 1). In addition, tranylcypromine might be an adequately selective CYP2A6 inhibitor for *in vitro* use (Taavitsainen et al. 2001; Zhang et al. 2001).

1.2.3 CYP2B6 enzyme

CYP2B6 comprises on average only about 0.2% of the total CYP in human livers, but its expression has large interindividual variability (Levy et al. 2000). In primary cultured human hepatocytes and in different human cell lines, CYP2B6 can be induced at protein and mRNA levels by phenobarbital, and cyclophosphamide which is an anticancer drug known to be metabolised by CYP2B6 (Gervot et al. 1999). CYP2B6 can be involved in the metabolism of a number of substrates such as, nicotine, aminochrysene, tamoxifen, testosterone, diazepam, *S*-mephenytoin (*N*-demethylation), *S*-mephobarbital, cyclophosphamide and propofol (Ekins et al. 1997; Kent et al. 1999; Court et al. 2001) (Table 1). The activity of CYP2B6 can be inhibited by fluvoxamine, sertraline and paroxetine (Hesse et al. 2000).

1.2.4 CYP2C enzymes

In human livers, the CYP2C subfamily is one of the most abundantly expressed CYP subfamilies. It includes four known members: CYP2C8, 2C9, 2C18, and 2C19. CYP2C8 and CYP2C9 are the major CYP2C forms, accounting for 35% and 60%, respectively, of the total hepatic content of human CYP2C, while CYP2C18 (4%) and CYP2C19 (1%) are the minor forms of the human CYP2C subfamily (Ged et al. 1988; Romkes et al. 1991). It has been estimated that CYP2C8, CYP2C9, and CYP2C19 are involved in the metabolism of approximately 20% of clinically used drugs (see Richardson et al. 1996; Rodrigues 1999; 2002). Although the amino-acid sequences of

CYP2C9 and CYP2C19 are 91% homologous, they exhibit relatively little overlap in their substrate specificities (Wrighton & Stevens 1992).

1.2.4.1 CYP2C8 enzyme

The importance of CYP2C8 in drug metabolism has only recently been recognized (Ong et al. 2000). CYP2C8 mRNA and protein can be induced by rifampicin and phenobarbital in primary cultures of human hepatocytes (Gerbal-Chaloin et al. 2001). CYP2C8 is primarily responsible for the metabolism of the anti-cancer drug taxol, cerivastatin, rosiglitazone, troglitazone, and is also involved in the metabolism of zopiclone, carbamazepine, verapamil, and amiodarone (Ong et al. 2000; Ohyama et al. 2000). It is also the predominant CYP responsible for the metabolism of arachidonic acid to biologically active epoxyeicosatrienoic acids in human liver and kidney (Dai et al. 2001).

CYP2C8 is polymorphically expressed in human livers (see Goldstein 2001). Two CYP2C8 alleles containing coding changes have been found. CYP2C8*2 has an Ile269Phe substitution in exon 5, and CYP2C8*3 includes both Arg139Lys and Lys399Arg amino acid substitutions in exons 3 and 8 (Dai et al. 2001). CYP2C8*2 was found only in African-Americans with a frequency of 0.18, while CYP2C8*3 occurred primarily in Caucasians with a frequency of 0.13 (Dai et al. 2001). One of the polymorphic alleles of CYP2C8 is defective in metabolising paclitaxel *in vitro* (Rifkind et al. 1995; Zeldin et al. 1996). For example, CYP2C8*2 had a two-fold higher K_m (Michaelis-Menten constant) and a two-fold lower intrinsic clearance for paclitaxel than CYP2C8*1 (wild-type) (Dai et al. 2001). Quercetin has been used *in vitro* as an inhibitor of CYP2C8, but it also significantly inhibits CYP1A2 activity (Dierks et al. 2001).

1.2.4.2 CYP2C9 enzyme

CYP2C9 is the principal CYP2C form in human liver. It metabolises many clinically

important drugs including tolbutamide, phenytoin, *S*-warfarin, losartan, ibuprofen, diclofenac, piroxicam, tenoxicam, and mefenamic acid (see Goldstein 2001). In addition, the antidiabetic drug glipizide and the diuretic torsemide have been reported to be metabolized by CYP2C9 (Kidd et al. 1999; Miners et al. 2000).

CYP2C9 has been found to be genetically polymorphic. Three naturally occurring allelic variants of CYP2C9, that showed significantly altered catalytic properties, have been identified: the wild-type Arg-144 Leu-359 (CYP2C9*1), Cys-144 Leu-359 (CYP2C9*2), and Arg-144 Ile-359 (CYP2C9*3) (see Miners et al. 1998). The polymorphism of CYP2C9 differs across various ethnic groups. White subjects have significantly higher frequencies of both CYP2C9*2 (~ 10%) and *3 (~ 10%) than Asian (0 ~ 2%) or black subjects (1~ 3%) (Sullivan-Klose et al. 1996; Inoue et al. 1997; Aynacioglu et al. 1999). The genetic polymorphism of CYP2C9 seriously affects the toxicity of drugs that are substrates of CYP2C9 with narrower therapeutic indices. For example, the CYP2C9*3 variant exhibits a lower intrinsic clearance (V_{max}/K_m) of tolbutamide, *S*-warfarin, phenytoin, piroxicam, and torsemide than the wild-type CYP2C9*1 variant (Sullivan-Klose et al. 1996; Aynacioglu et al. 1999).

S-warfarin, tolbutamide and diclofenac have been used as *in vitro* probe substrates of CYP2C9 (Doecke et al. 1991). Inhibitors of CYP2C9 include sulfaphenazole, sulfamethoxazole, sulfinpyrazone, miconazole, and fluconazole (see Miners et al. 1998). Sulphaphenazole (K_i ~ 0.3 µM) has been used as a selective inhibitor of CYP2C9 in *in vitro* studies (Newton et al. 1995; Bourrie et al. 1996) (Table 1). Inducers of CYP2C9 include barbiturates, carbamazepine, and rifampin (Treluyer et al. 1997; Gerbal-Chaloin et al. 2001).

1.2.4.3 CYP2C18 enzyme

CYP2C18 expressed at a very low level in human livers, but CYP2C18 can participate, to a small extent, in the metabolism of substrates of other CYP2C forms,

such as diazepam, imipramine, and tolbutamide (Venkatakrishnan et al. 2001; Komatsu et al. 2000b).

1.2.4.4 CYP2C19 enzyme

CYP2C19, is another important member of the CYP2C subfamily, it can be induced by barbiturates, carbamazepine, and rifampin, phenytoin in primary cultures of human hepatocytes (Gerbal-Chaloin et al. 2001). CYP2C19 is involved in the metabolism of drugs including *S*-mephenytoin, omeprazole, diazepam, propranolol, proguanil and tricyclic antidepressants, such as imipramine, clomipramine and amitriptyline (see Levy et al. 2000).

CYP2C19 exhibits genetic polymorphism, with the poor metabolism (PM) phenotype representing 2 to 6% of Caucasian populations, 12 to 23% of Oriental populations, and 2% in Black Americans (Wilkinson et al. 1989; Marinac et al. 1996; Meyer & Zanger 1997). The molecular genetic basis of the phenotypes is now well recognized. The two most common defects involving null alleles arise from G → A base pair mutations in exon 5 (CYP2C19*2) and exon 4 (CYP2C19*3), respectively, accounting for over 99% of defective alleles in Asian populations and 87% in Caucasians (de Morais et al. 1994; Brosen et al.1995). A transition mutation in the initiation codon (CYP2C19*4) accounts for an additional 3% of defective alleles in Caucasians (Ferguson et al. 1998).

S-mephenytoin and omeprazole have been used as *in vitro* probe substrates of CYP2C19 (de Morais et al. 1994; Brosen et al. 1995; Tucker et al. 2001). The inhibitors of CYP2C19 include fluvoxamine, omeprazole, fluconazole (K_i ~ 2 μM), and ticlopidine (Kunze et al. 1996; Venkatakrishnan et al. 2001). Among these, omeprazole is a relatively specific CYP2C19 inhibitor up to 10 μM, but higher concentrations inhibit CYP2C9, 3A4 and 2D6 with lower potency (Ko et al. 1997; Giancarlo et al. 2001) (Table 1).

1.2.5 CYP2D6 enzyme

CYP2D6 constitutes on average about 2% to 5% of the total hepatic CYP content, and it is also found in several extrahepatic tissues, including the gastrointestinal tract, brain, and lungs (see Levy et al. 2000). CYP2D6 accounts for the hepatic metabolism of about 30% of clinically used drugs, including antiarrhythmic agents, antihypertensives, β-blockers, monoamine oxidase inhibitors, morphine derivatives, antipsychotics, and tricyclic antidepressants (see Gonzalez & Idle 1994).

In humans, CYP2D6 is polymorphic, and at least 70 CYP2D6 alleles have been identified. Most of the known variant alleles are inactive, and produce the PM phenotype (see Meyer & Zanger 1997; Idle 2000). The prevalence of the PM phenotype shows marked ethnic differences, and it appears to be rare (~1%) in most Asian populations, common in whites (5%-10%), and varies in black populations of African descent (0%-19%) (Bertilsson 1995; Tateishi et al. 1999). The molecular genetic bases of the CYP2D6 polymorphism are now well established. Many types of null mutations result in impaired CYP2D6 activity, and homozygosity is associated with the PM phenotype, e.g., CYP2D6*3, *4, *5, and *6. Other variant alleles, such as CYP2D6*9, *10, and *17, lead to an enzyme with reduced catalytic activity compared to the wild-type allele (CYP2D6*1) (Johansson et al. 1994; Oscarson et al. 1997; Tateishi et al. 1999).

Bufuralol and dextromethorphan have been used as *in vitro* probe substrates of CYP2D6 (Schmid et al. 1985; Yamazaki et al. 1994). Inhibitors of CYP2D6 include quinidine, fluoxetine, paroxetine, perphenazine, terbinafine and ticlopidine (see Venkatakrishnan et al. 2001). Among these inhibitors, only quinidine has been widely used as a potent and selective probe inhibitor in *in vitro* studies (Ki ~ 0.06 μM) (Bourrie et al. 1996) (Table 1). In contrast to most other hepatic CYP enzymes involving in human drug metabolism, CYP2D6 seems not to be inducible (see Levy et al. 2000).

1.2.6 CYP2E1 enzyme

CYP2E1 constitutes approximately 6% of the total hepatic CYP enzymes and is present in several extrahepatic tissues, including the lungs, kidneys, nasal mucosa, bone marrow, and the white cell fraction of peripheral blood (Ding et al. 1986; 1990; Song et al. 1990). Several factors including obesity and fasting can modulate human CYP2E1 activity (O'Shea et al. 1994; Girre et al. 1994; Zand et al. 1993). In addition, ethanol is an inhibitor of CYP2E1 after transient use and an inducer after chronic use (Niemela et al. 2000). Isoniazid and imidazole can increase the translation efficiency and affect CYP2E1 enzyme stabilisation (Park et al. 1993; Eliasson et al. 1990). CYP2E1 is involved in the metabolic activation of many low molecular weight toxins and carcinogens, including *N*-nitrosamines in tobacco smoke, benzene, ethanol, and a number of drugs such as chlorzoxazone, acetaminophen, dapsone, aniline, and fluorinated general anesthetics (Yamazaki et al. 1992).

CYP2E1 is also polymorphically expressed with up to 20-fold interindividul variation among individuals (Shimada et al. 1994). Several genetic polymorphisms have been identified in the CYP2E1 gene (Uematsu et al. 1991; Hu et al. 1997; McCarver et al. 1998; Fairbrother et al. 1998), and controversial frequencies of the polymorphisms according to the racial/geographic characteristics of the study population have been described (see Rodrigues 2002). In general, CYP2E1 activity, as measured both *in vitro* and *in vivo* by the 6-hydroxylation of chlorzoxazone, does not appear to be under genetic regulation by the known allelic variants (Lucas et al. 1995, Kim et al. 1996; Fairbrother et al. 1998; Carriere et al. 1996; Powell et al. 1998). For example, the difference of chlorzoxazone's oral clearance was less than 2-fold between the homozygous wild-type individuals (c_1/c_1) and the variant c_2 alleles (Marchand et al. 1999).

Chlorzoxazone has been used both *in vivo* and *in vitro* as a probe substrate of CYP2E1 (Peter et al. 1990). Disulfiram inhibits CYP2E1 activity, but it is also an almost equally potent inhibitor of CYP2A6 (Guengerich et al. 1991). Diethyldithiocarbamate (DDC), which is a mechanism-based inhibitor of CYP2E1, and pyridine have been

used as useful *in vitro* tools for evaluation of the CYP2E1-mediated reactions (Hargreaves et al. 1994; Newton et al. 1995) (Table 1).

1.2.7 CYP3A enzymes

CYP3A enzymes are the most abundant CYP enzymes, comprising approximately 30-40% of the total hepatic CYP content (Shimada et al. 1994). CYP3A enzymes are involved in the metabolism of most clinically used drugs (40%-50%) in humans. The CYP3A subfamily contains 3 functional proteins: CYP3A4, CYP3A5 and CYP3A7 (Nelson et al. 1996).

1.2.7.1 CYP3A4 enzyme

CYP3A4 is the most abundantly CYP expressed in the human liver and intestine (see Guengerich 1999), and its activity can be induced by rifampin, barbiturates, carbamazepine, nevirapine, and dexamethasone *in vivo* and *in vitro* (Bertilsson et al. 1997; Goodwin et al. 1999). CYP3A4 plays a significant role in the metabolism of almost half of the commonly used drugs, including nifedipine, felodipine, cyclosporine, erythromycin, midazolam, alprazolam, triazolam, lovastatin, simvastatin, terfenadine, verapamil, tacrolimus, diltiazem, cicapride, terstosterone, and HIV-protease inhibitors (see Thummel et al. 1998; Rodrigues 2002).

CYP3A4 activity shows large interindividual variability (up to 40-fold in hepatic microsomes), it is affected by genetic and environmental factors (Shimada et al. 1994). In some studies, several genetic polymorphisms in the CYP3A gene were found using restriction fragment length polymorphisms detected by Southern analysis, but none of these was associated with the level of nifedipine oxidation activity among various liver samples (Beaune et al. 1986; Bork et al. 1989).

There is considerable evidence for CYP3A4 behaviour allosterically, possibly due to the simultaneous binding of two or more substrate molecules to its active site (Schwab

et al. 1988; Shou et al. 1994; Ueng et al. 1997; Korzekwa et al. 1998). Such binding can lead to atypical enzyme kinetics and inconsistent drug-drug interactions (Ekins et al. 1998; Korzekwa et al. 1998). Therefore, it was recently recommended that at least two chemically unrelated CYP3A4 substrates, such as midazolam and testosterone, should be used as probe substrates in *in vitro* studies (see Tucker et al. 2001).

A number of drugs and foreign chemicals are clinically significant inhibitors of CYP3A4 such as ketoconazole (K_i ~ 15 nM), itraconazole (K_i ~ 270 nM), clarithromycin (K_i 10 ~ 28 µM), erythromycin (K_i 13 ~ 194 µM), fluconazole (K_i 1.3 ~ 63 µM), fluvoxamine (K_i = 5.6 µM), fluoxetine (K_i 7.1 ~ 66 µM), cimetidine (K_i 36 ~ 268 µM), delavirdine (K_i = 22 µM), grapefruit juice and calcium antagonists (see Thummel et al. 1998; Guengerich 1999; Levy et al. 2000). Troleandomycin has been characterized as a selectively mechanism-based inhibitor of CYP3A4 *in vitro* (Chang et al. 1994; Newton et al. 1995). Ketoconazole is also a potent and selective inhibitor of CYP3A4 *in vitro* and *in vivo* with low concentrations (preferably 1 µM or lower) (Newton et al. 1995; Bourrie et al. 1996) (Table 1).

1.2.7.2 CYP3A5 enzyme

CYP3A5 is 83% homologous to CYP3A4, but it is expressed at a much lower lever than CYP3A4 in the liver (10-30% of CYP3A4) (see Thummel et al. 1998; Levy et al. 2000). CYP3A5 is polymorphically expressed in 30% of individuals, and it is predominantly expressed in the kidney in most individuals (Schuetz et al. 1989; Thummel et al. 1998). Also, CYP3A5 is expressed in a limited number of fetal livers (Hakkola et al. 2001). CYP3A5 can be induced by rifampicin and phenobarbital in hepatocyte cultures (Hukkanen et al. 2000; Asghar et al. 2002). CYP3A5 has been shown to be capable of metabolising most substrates of CYP3A4 (Wrighton et al. 1989; 1990). However, because of its lower expression level, the role of CYP3A5 in hepatic drug clearance has generally been regarded to be significantly smaller than that of CYP3A4, although in certain extrahepatic organs, CYP3A5 may contribute

significantly to the metabolism of some substrates, such as midazolam and endobiotics (Levy et al. 2000).

Table 1 The marker reactions and respective chemical inhibitors of the major CYP forms in man (modified from Pelkonen et al. 1998 and Rodrigues 2002)

CYP forms	Marker reactions	Selective inhibitors
CYP1A2	Phenacetin *O*-deethylation Caffeine N_3-demethylation Ethoxyresorufin *O*-deethylation	Furafylline
CYP2A6	Coumarin 7-hydroxylation	8-Methoxypsoralen
CYP2B6	*S*-Mephenytoin *N*-demethylation	Not available
CYP2C8	Paclitaxel 6α-hydroxylation	Quercetin (not fully selective)
CYP2C9	Tolbutamide hydroxylation Diclofenac 4′-hydroxylation *S*-Warfarin 7-hydroxylation	Sulfaphenazole
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	Omeprazole
CYP2D6	Bufuralol 1′-hydroxylation Debrisoquine 4-hydroxylation Dextromethorphan *O*-demethylation	Quinidine
CYP2E1	Chlorzoxazone 6-hydroxylation	Diethyldithiocarbamate (DDC)
CYP3A	Midazolam 1′-hydroxylation Testosterone 6β-hydroxylation Triazolam 1′-hydroxylation Erythromycin *N*-demethylation	Troleandomycin (TAO) Ketoconazole

1.2.7 CYP3A7 enzyme

CYP3A7 is the major CYP form detected in human embryonic, fetal and newborn liver (de Wildt et al. 1999; Hakkola et al. 2001). It may also be selectively expressed in adult livers at lower levels than CYP3A4 and CYP3A5 (Schuetz et al. 1994). The role of CYP3A7 in drug metabolism is unclear.

2. Enzyme kinetics in drug metabolism

In vitro characterization of drug biotransformation generally begins with an enzyme kinetics analysis of metabolite formation rate using human liver microsomes. A typical enzyme kinetic analysis involving a mathematical description of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent biotransformation rate as a function of substrate concentration is based on the core assumptions that substrate consumption is minimal (typically less than 5%), and that product formation rate is linearly related to microsomal protein concentration and duration of incubation (Segel 1975). Normally, if the conversion of substrate to product is catalyzed by a single enzyme, the enzyme kinetics can be well described by a Michaelis-Menten (MM) equation (one-enzyme model) as follows (Segel 1975; Schmider et al. 1996):

$$V_0 = V_{max} \cdot S / (K_m + S) \quad (1)$$

where V_0 is the rate of product formation (or substrate disappearance), S is the substrate concentration, V_{max} is the maximal velocity of the reaction, and K_m is the MM constant representing the concentration of substrate that results in half-maximal velocity.

Two or more CYP forms with distinct affinities may catalyze a given drug biotransformation. In such cases, the relationship between V_0 and S is biphasic, and may be described by a high-affinity and a low-affinity component using a two enzyme MM model (Schmider et al. 1996):

$$V_0 = V_{max1} \cdot S / (K_{m1} + S) + V_{max2} \cdot S / (K_{m2} + S) \quad (2)$$

Some CYP enzymes (CYP2B6 and CYP3A4) have been shown to exhibit kinetics consistent with allosteric interaction of the substrate with the enzyme, which is also known as substrate activation (Ueng et al. 1997; Harlow & Halpert 1998; Shou et al. 1999). These result in an *S*-shaped substrate versus rate curve and a "hook"-shaped Eadie-Hofstee plot (see Rodrigues 2002). When allosteric interactions are observed, the Hill equation can be used to calculate kinetic constants (Clarke 1998):

$V_0 = V_{max} \cdot S^n / (S_{50}{}^n + S^n)$

where n is the Hill coefficient for cooperative substrate binding, S_{50} is the substrate concentration at which half the maximal rate (V_{max}) is attained.

Another kinetic profile, substrate inhibition, occurs when an increase in substrate concentration beyond a certain value (usually greater than K_m) results in a decrease in the rate of metabolism (Haehner et al. 1996; Spracklin et al. 1997; Korzekwa et al. 1998). Although the mechanism of substrate inhibition has yet to be fully determined, it has been described by a two-site model in which one binding site is productive, whereas the other site is inhibitory and operable at high substrate concentrations, resulting in decreased velocity with increasing concentrations (Shou et al. 2001; Hutzler & Tracy 2002). In general, the magnitude of the inhibition of substrate inhibition is dependent upon the structure and concentration of the substrate, the reaction type, and CYP form examined. In most cases, substrate inhibition behaves as a partial inhibition because the inhibition of CYP does not approach zero even at very high substrate concentrations (Lin et al. 2001).

3. Mechanism of drug-drug interactions involving CYP enzymes

3.1 Inhibition of CYP enzymes

Inhibition of CYP enzymes is the most common cause of metabolism based drug-drug interactions. The inhibition of CYP enzymes is of clinical importance for both therapeutic and toxicological reasons. The mechanisms of CYP inhibition can be

categorized into reversible inhibition and mechanism-based inhibition (see Lin & Lu 1998; 2001; Levy et al. 2000).

3.1.1 Reversible inhibition

Reversible inhibition is the most common type of enzyme inhibition. Reversible inhibition is transient and reversible, and the normal functions of CYPs continue after the inhibitor has been eliminated from the body (see Lin & Lu 2001). Reversible inhibition can be further classified into competitive, uncompetitive, mixed-type and non-competitive inhibition (see Rodrigues et al 2002). Competitive inhibition is when the binding of an inhibitor to an enzyme prevents a further binding of a substrate to the active sites of the enzyme. In uncompetitive inhibition, an inhibitor does not bind to the free enzyme, but binds to the enzyme-substrate complex, resulting in a nonproductive enzyme-substrate-inhibitor complex. Mixed-type inhibition is when an inhibitor binds either to the free enzyme or to the enzyme-substrate complex (see Lin & Lu 2001; Rodrigues 2002). In the case of noncompetitive inhibition, an inhibitor binds to a nonactive binding site of the enzyme, and the binding has no effect on the binding of substrate, but the enzyme-substrate-inhibitor complex is nonproductive. Noncompetitive inhibition is a specific case of mixed-type inhibition. Mathematically, the velocity of an enzymatic reaction in the presence of an inhibitor (V_i), can be described by the following equations (3), (4), (5) and (6) for competitive, uncompetitive, mixed-type and non-competitive inhibition, respectively (Segel 1975) (Table 2) (Fig 1).

$$V_i = V_{max} \cdot S / [\, K_m (1 + I / K_i) + S \,] \quad (3)$$

$$V_i = V_{max} \cdot S / [\, K_m + S (1 + I / K_i) \,] \quad (4)$$

$$V_i = V_{max} \cdot S / [\, K_m (1 + I / K_i) + S (1 + I / \alpha K_i) \,] \quad (5)$$

$$V_i = V_{max} \cdot S / [\, K_m (1 + I / K_i) + S (1 + I / K_i) \,] \quad (6)$$

where K_i is the inhibition constant, (I) is the inhibitor concentration, and α is the factor by which K_m changes when an inhibitor occupies the enzyme.

3.1.2 Mechanism-based inhibition

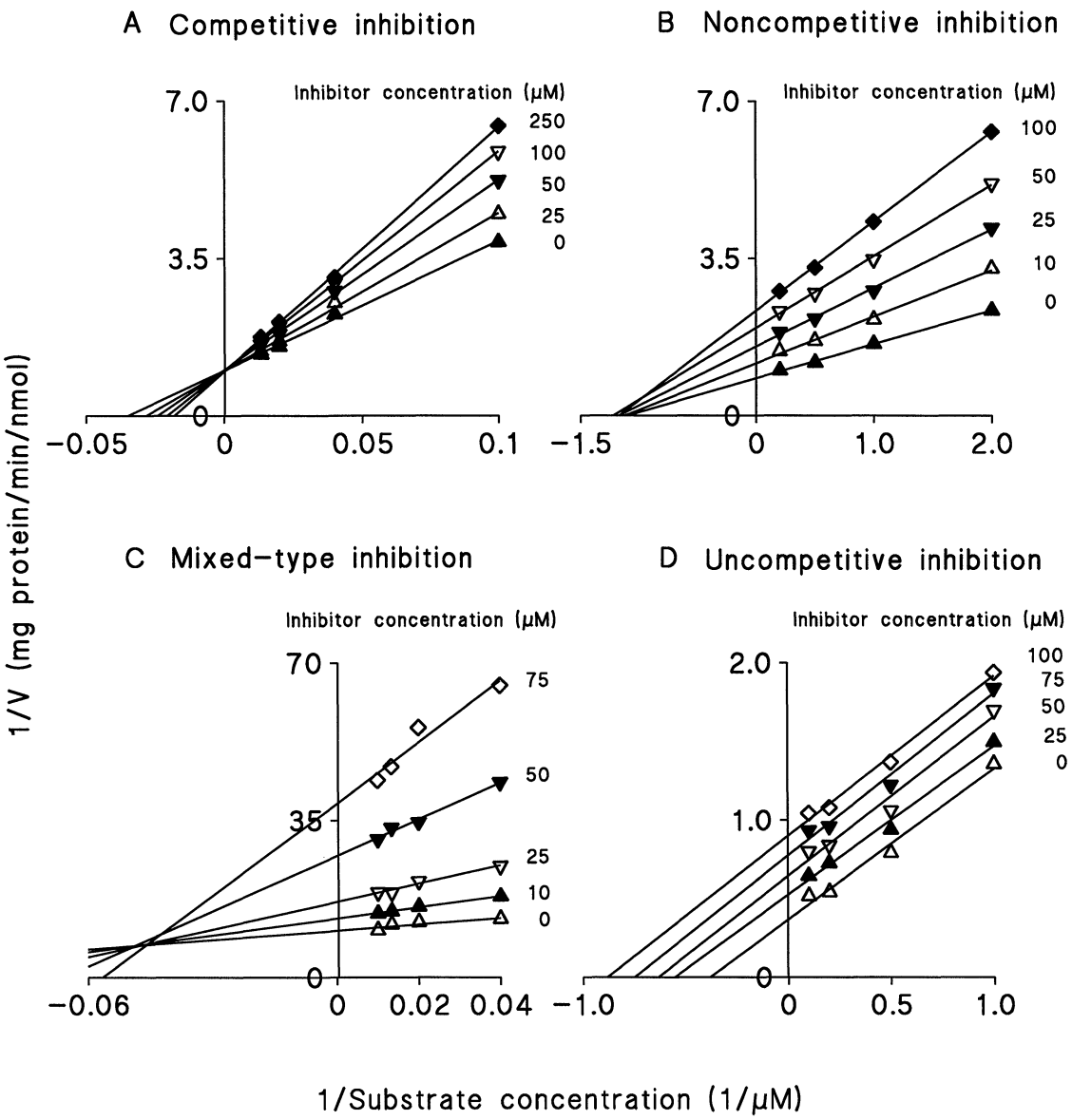

Figure 1. Representative Double-reciprocal plots of competitive (A), noncompetitive (B), mixed-type (C) and uncompetitive (D) inhibition. Data adopted from Study III.

Mechanism-based inhibition can be mediated by covalent modification of a pyrrole nitrogen in the prosthetic heme group of CYP or by direct modification of the heme moiety or the apoprotein (Halpert 1995). The mode of inhibition is highly specific because the inhibitor must both bind to and be metabolised by the enzyme (Lehman-Mckeeman et al. 1997). The inhibitory effect of mechanism-based inhibition is terminated by enzyme resynthesis rather than inhibitor washout (Mayhew et al. 2000).

One mode of mechanism-based inhibition is the formation of metabolite intermediate (MI) complexes. Compounds forming MI complexes can be catalytically oxidized to intermediate or product metabolites that uncovalently bind to the prosthetic heme of the CYP. In the case of MI complexation, the CYP activity can be restored under nonphysiological experimental conditions e.g. using potassium ferricyanide or by the *in vitro* dialysis method (Regal et al. 2000; Ma et al. 2000). However, in real *in vivo* situations, the MI complexes are so stable that resynthesis of new enzyme is the only means by which the enzyme activity can be restored (Mayhew et al. 2000). A classic example of the MI complexation is the inhibition of CYP3A4 by troleandomycin (Newton et al. 1995).

Another mode of mechanism-based inhibition is the so-called enzyme inactivation (or suicide inhibition). Suicide inhibition results from covalent binding of reactive intermediates to the heme and /or protein of CYP (see Lin & Lu 1997; Levy et al 2000). Typical examples of suicide inhibition are inactivation of CYP1A2 by furafylline, and inactivation of CYP3A4 by delavirdine (Kunze & Trager 1993; Voorman et al. 1998).

The most important phenomena of mechanism-based inhibition are time-, concentration-, and NADPH-dependent loss of the enzyme activity (see Lin et al. 1995; Lin & Lu 1997). *In vivo*, the inhibitory effect of a mechanistic inactivator is thought to be more prominent after repeated dosing and last longer than that of a reversible inhibitor (see Lin & Lu 1998). Many drugs have been identified as mechanism-based inactivators *in vitro*, and have considerable form specificity. These

inhibitors include furafylline (CYP1A2 inhibitor; K_I = 23 µM, K_{inact} = 0.87 min^{-1}) (Kunze & Trager 1993), menthofuran (CYP2A6 inhibitor; K_I = 2.5 µM, K_{inact} = 0.22 min^{-1} in human liver microsomes, and K_I = 0.84 µM, K_{inact} = 0.25 min^{-1} in purified expressed CYP2A6) (Khojasteh-Bakht et al. 1998), tienilic acid (CYP2C9 inhibitor; K_I = 4.3 µM, K_{inact} = 0.21 min^{-1}) (Lopez-Garcia et al. 1994; Jean et al. 1996), halothane (CYP2E1 inhibitor; data for K_I and K_{inact} not available) (Madan & Parkinson 1996), gestodene (CYP3A4 inhibitor; K_I = 46 µM, K_{inact} = 0.39 min^{-1}) and delavirdine (CYP3A4 inhibitor; K_I = 22 µM, K_{inact} = 0.59 min^{-1}) (Guengerich 1990; Voorman et al. 1998).

3.2 Induction of CYP enzymes

Enzyme induction is less frequently encountered and its development is a slower process in clinical practice than enzyme inhibition. Enzyme induction can occur by means of ligand stabilisation of the enzyme (ethanol-type induction) or by increased enzyme synthesis involving intracellular receptors such as the aryl hydrocarbon (Ah) receptor, the peroxisome proliferator activated receptor (PPAR), the constitutive androstane receptor (CAR, Phenobarbital induction) and the pregnane X receptor (PXR, rifampicin induction) (see Pelkonen et al. 1998; Fuhr 2000). Normally, enzyme induction may attenuate therapeutic efficacy as a result of a decrease in plasma active parent drug concentrations (Park et al. 1996). Human CYP1A1/2, CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 are known to be inducible (Ronis et al. 1999). Many drugs including rifampicin, dexamethasone, and anticonvulsants (such as phenytoin, carbamazepine, phenobarbital and primidone) are important inducers of CYP3A4 and some other forms (see Pelkonen et al. 1998).

Ethanol-type induction appears to be limited to the target enzyme CYP2E1. Four major mechanisms have been proposed for the regulation of CYP2E1 by xenobiotics and these include transcriptional activation occurring in the nucleus, stabilization of 2E1 transcripts in the cytosol, enhanced polyribosomal messenger RNA translational efficiency, and protein stabilization occurring in the endoplasmic reticulum (Koop et

al. 1990). Any or all of these mechanisms may cause enhanced 2E1 expression when a cell is targeted by a drug or chemical. The xenobiotic inducers of CYP2E1 include ethanol, isoniazid, imidazole, pyridine, pyrazole, halogenated hydrocarbons, and acetone (Lieber 1997; Raucy et al. 1993; Koop et al. 1990).

The induction of CYP1A1 is regulated by the ligand binding of polycyclic aromatic hydrocarbons, which are found in tobacco smoke, to the cytosolic Ah receptor to increased protein synthesis, and to the initiation of transcriptional activation of the CYP1A1 gene (Okey 1990; Porter & Coon 1991). However, CYP1A induced by omeprazole is not a direct ligand for the Ah receptor, but the induction process is mediated by enhanced translocation of the Ah receptor to the nuclei and binding to the regulatory elements upstream of the CYP1A coding genes (Quattrochi & Tukey 1993).

In contrast to the induction of CYP1A genes, the mechanism of the induction involving CAR, PXR and PPAR, which belong to the nuclear receptor/steroid receptor superfamily, is via foreign chemical inducers binding to receptors (CAR, PXR and PPAR) and transcription factors to induce CYP gene expression (see Waxman 1999; Fuhr 2000). For example, phenobarbital and many other phenobarbital-like lipophilic chemicals induction of CYP2B gene via CAR, PXR activates CYP3A genes in response to diverse chemicals including certain natural and synthetic steroids, and PPAR mediates the induction of the fatty acid hydroxylases of the CYP4A family by many acidic chemicals classified as non-genotoxic carcinogens and peroxisome proliferators (Bertilsson et al. 1998; Issemann & Green 1990; Gonzalez et al. 1998).

4. *In vitro* systems in evaluating and predicting metabolic clearance and drug-drug interactions

In vitro systems can be placed into two broad categories. The first type of *in vitro* systems is the enzyme-based systems, which include liver microsomes, and cDNA-expressed enzymes. In addition, purified P450s reconstituted with cytochrome P450 reductases, b5, and other cofactors also belong to this system. However, due to the

extremely difficuly for procurement, the purified enzymes are rarely used in practice, and largely been replaced by cDNA-expressed CYP enzymes. The second and more complex system is the cell-based systems, which include the hepatocytes and liver slices (see Wrighton et al. 1993; Levy et al. 2000).

4.1. Enzyme-based systems

4.1.1 Human liver microsomes

The microsomal cellular fraction is the postmitochondrial supernatant that is separated by centrifugation at 100,000 g to 250,000 g for 60 to 120 minutes and it consists of "pinched-off" closed vesicles of fragments of endoplasmic reticular membrane (Lake 1987). Microsomes prepared from human livers are the primary tools used for *in vitro* studies of metabolic-based drug-drug interactions and CYP-catalyzed metabolite formation. The systems have several advantages over other *in vitro* systems. As the ratio of NADPH-cytochrome P450 reductase to CYP in human liver microsomes and the amount of cytochrome b_5 and the type of lipids are the same as those in the intact liver, the metabolism data obtained using human liver microsomes appear to have greater relevance to the *in vivo* situation than data obtained through the use of isolated enzymes (see Lin & Lu 1997; Levy et al. 2000). In addition, the relative importance of different routes of metabolism obtained following liver microsomal incubations more closely approximates those observed *in vivo* than the information obtained by isolated enzyme preparations (see Levy et al. 2000). Furthermore, the microsomes are easy to obtain and store, the enzymes activities can be kept in crystal (frozen) forms for many years, and the experimental methods and the mathematical models used in human liver microsomal studies are well established (Clarke 1998; von Moltke et al. 1998).

Despite its prominent advantages, the microsomal system has its own drawbacks. For example, the microsomes contain only phase I drug metabolising enzymes and uridine diphosphate-glucuronosyl transferases, thus the metabolic environment and cofactors are different from those *in vivo* (see Levy et al. 2000). In addition, the *in vitro*

incubation conditions used such as the ionic strength, pH value of the incubation medium and the effect of organic solvents used can affect the outcomes of microsomal studies (see Venkatakrishnan et al. 2001).

4.1.2 cDNA-expressed enzymes

Advances in molecular biology have resulted in that the recombinant DNAs (cDNAs) encoding the drug-metabolising CYP enzymes can be isolated and transfected into host cells, such as bacteria, yeast, insect, human and other mammalian cells, to express the CYP protein (Ekins et al. 2000). The generally used cDNA-expressed CYP forms express only a single enzyme activity, thus they are very useful in studies of CYP form-specific drug metabolism and interactions (see Levy et al. 2000). In addition, the cDNA-expressed enzymes are commercially available and easy-to-use in *in vitro* studies. However, the amounts of individual enzymes and cofactors in expressed enzymes are different from those in human liver. Therefore, the contribution of an enzyme to a specific metabolic route may not be as significant as it seems on the basis of cDNA-expressed enzymes (see Levy et al. 2000). For example, the variable expression of cytochrome b_5 and/or NADPH-cytochrome P450 reductase can affect the turnover number (V_{max}) for a given enzyme (Shaw et al. 1997; Yamazaki et al. 1999), although the "affinity" (K_m) of CYP enzymes toward marker substrates is generally comparable between recombinant enzymes and human liver microsomes (McGinnity et al. 1999). In addition, transfection into nonhuman, nonhepatic celluar hosts may result in the K_m and V_{max} values of the expressed enzymes substantially differing from those observed in the native enzymes (Shaw et al. 1997).

4.2 Cell-based systems

Cell-based systems include human liver slices and hepatocyte systems, which are useful *in vitro* tools for drug metabolism and interaction studies. Both systems retain the physiological conditions of enzymes and co-factors of both phase I and phase II reactions, and therefore, they can well simulate the *in vivo* situation (see Lin & Lu

1997). Furthermore, these systems can also be used for studying xenobiotic-mediated induction of drug-metabolising enzymes (see Venkatakrishnan et al. 2001). However, operation with cell-based systems needs fresh tissues and requires specific techniques and established procedures. In addition, kinetic parameters obtained from liver slices may be different from those obtained in hepatocytes or liver microsomes, since the distribution equilibrium of the drug or compound may not be well achieved between all the cells within the liver slice and the incubation media (see Lin & Lu 1997). Moreover, the results obtained from hepatocytes should be interpreted with caution, because the enzyme activities decline spontaneously during hepatocyte isolation or culture (see Skett et al. 1995; Ekins et al. 2000; Pelkonen et al. 2001). To date, the specific CYP forms sensitive to the decline has not been identified.

Because each approach mentioned above has its own advantages and limitations, a combination of several approaches undoubtedly provides the most convincing evidence for drug metabolism and interactions studies.

5. *In vitro* approaches in the prediction of metabolic clearance

5.1 Intrinsic clearance

Intrinsic clearance (Cl_{int}) is the cornerstone for extrapolation of *in vitro* data to the *in vivo* situation (see Pelkonen et al. 1998). Cl_{int} is a direct measure of enzyme activity toward a drug and is not influenced by other determinants such as hepatic blood flow or drug binding within the blood matrix. Cl_{int} acts as a proportional constant between rate of drug metabolism and drug concentration around the metabolic enzyme site (C_E). If the process is consistent with a MM model, and if C_E is less than 10% of the K_m, Cl_{int} is equal to the V_{max}/K_m ratio (see Houston 1994) i.e.:

$$Cl_{int} = V_{max} / K_m \qquad (7)$$

In the cases of two-enzyme kinetics, the net intrinsic clearance is the sum of the low- and the high-affinity clearance (see Thummel et al. 1997; Venkatakrishnan et al.

2001):

$$Cl_{int} = V_{max1} / K_{m1} + V_{max2} / K_{m2} \quad (8)$$

5.2 Prediction of *in vivo* metabolic clearance based on *in vitro* data

In many cases, *in vivo* metabolic clearance (Cl_{met}) can be predicted using *in vitro* drug metabolism data based on the assumption that only the unbound drug can cross through the membranes, and that there is a homogenous distribution of enzymes within the liver (see Houston 1994; Iwatsubo et al. 1997; Lin & Lu 2001). Based on these assumptions, the hepatic clearance (Cl_h) can be predicted *in vitro* using the well-stirred model (see Houston 1994; Ito et al. 2000a):

$$Cl_h = Q \cdot E = Q \cdot f_u \cdot Cl_{int} / (Q + f_u\, Cl_{int}) \quad (9)$$

or the parallel-tube model:

$$Cl_h = Q \cdot E = Q\,(1 - e^{-fu \cdot Clint/\, Q}) \quad (10)$$

where Q is the hepatic blood flow (20 ml/min/kg) (Lin & Lu 1997), E is the hepatic extraction ratio calculated as Cl_h/Q, Cl_{int} is the *in vivo* intrinsic clearance, and f_u is the unbound fraction of a drug in the blood.

Kinetically, drugs can be classified as low- (enzyme limited, $E < 0.5$, such as ketoconazole and ritonavir) or high-clearance (flow limited, $E > 0.9$, such as indinavir, saquinavir, and nisoldipine) compounds (see Wilkinson 1987). When the Cl_{int} of a drug is very small relative to the hepatic blood flow ($Q >> f_u \cdot Cl_{int}$), the hepatic clearance is low and Cl_h is directly related to f_u and Cl_{int}, i.e. $Cl_h \approx f_u \cdot Cl_{int}$. Thus, a decrease in the Cl_{int} caused by inhibition will result in an almost proportional change in the clearance of low-clearance drugs. However, if the Cl_{int} is high ($f_u \cdot Cl_{int} >> Q$), then the hepatic clearance is limited by the hepatic blood flow, i.e. $Cl_h \approx Q$, thus, a decrease in the Cl_{int} caused by inhibition has little effect on the Cl_h of high-clearance drugs (see Lin 1998; Rodrigues 2002).

5.3 Considerations in the prediction of metabolic clearance

Nonspecific substrate binding to microsomal matrices may influence the estimation of Cl_{int} *in vitro*. As microsomal binding can reduce enzyme-available substrate concentrations and increase the estimated apparent K_m of the process, yielding an underestimation of the V_{max}/K_m ratio (see Venkatakrishnan et al. 2000; Mclure et al. 2000). Thus, the use of free *in vitro* intrinsic clearance (that is, Cl_{int} divided by the free fraction in the incubation matrix), rather than total intrinsic clearance, improves a prediction of *in vivo* pharmacokinetic clearance of extensively bound drugs (Obach 1997; 1999). This is more suitable for the lipophilic basic drugs, such as amiodarone, desipramine, imipramine, nortriptyline, amitriptyline, and propranolol that are extensively bound to microsomal matrices (Carlile et al. 1999; Obach 1999; Mclure et al. 2000).

A change in the hepatic blood flow will result in a substantial change in the *in vivo* hepatic clearance of a high-clearance (flow-limited) drug because the hepatic clearance of a high-clearance drug is highly dependent on the hepatic blood flow (Lin & Lu 1998). But for a low-clearance (enzyme-limited) drug, a change in the hepatic blood flow will have little effect on its hepatic clearance (see Bertz & Granneman 1997).

The addition of albumin and cytosol to the incubation medium may also affect the *in vitro-in vivo* extrapolation. It has been noted that the addition of albumin and cytosol to microsomal incubations may substantially change the enzyme kinetic estimates of the substrates of CYP2C9 (Ludden et al. 1997; Carlile et al. 1999). For example, the addition of bovine serum albumin to microsomal incubation media has decreased the K_m estimates and increased Cl_{int} for phenytoin *p*-hydroxylation and tolbutamide hydroxylation, reactions mainly catalysed by CYP2C9, yielding predicted clearance values more comparable with the *in vivo* values (Ludden et al. 1997; Carlile et al. 1999). In addition, phenytoin and tolbutamide oxidation in human liver microsomes was substantially promoted by the addition of liver cytosol (Komastu et al. 2000a). Both albumin and cytosolic components are probably present at the metabolic enzyme site *in vivo* (Shroyer & Nakane 1987; Komastu et al. 2000a). Although several *in vitro* studies have shown good agreement between the actual *in vivo* clearance and the

predicted *in vivo* clearance based on *in vitro* kinetic estimates in the presence of albumin and cytosol (Ludden et al. 1997; Carlile et al. 1999), testing for the utility of albumin and cytosol in the prediction of *in vivo* clearance requires further extrapolation with other substrates and other CYP enzymes.

6. *In vitro* approaches in the prediction of drug-drug interactions

6.1 Determination of *in vitro* potency of inhibition

In the case of reversible inhibition, the *in vitro* inhibitory potency of a given compound is quantified by determination of its IC_{50} and K_i values (Newton et al. 1995). The IC_{50} value is defined as a concentration of inhibitor that causes 50% inhibition of an original enzyme activity. The IC_{50} value can be determined by analyzing the relationship between inhibitor concentration and decrement in reaction velocity performed at a fixed substrate concentration (around K_m) (see Rodrigues 2002). The IC_{50} value is quite useful when comparing the inhibitory potencies of different candidate inhibitors of the same chemical class, but without any knowledge of the biochemical mechanism of inhibition (von Moltke et al. 1998). However, IC_{50} values have their own important limitations in the context of *in vitro-in vivo* scaling (Brosen et al. 1993). IC_{50} value is dependent on the type of inhibitory mechanism. For example, IC_{50} is equal to the inhibition constant (K_i) only when the biochemical mechanism is noncompetitive or the substrate concentration used is much less than K_m for competitive inhibition (Venkatakrishnan et al. 2001). If the substrate concentration approaches or exceeds K_m, the IC_{50} value exceeds the competitive K_i (von Moltke et al. 1998). However, this limitation can be overcame by actual calculation of an *in vitro* K_i value based on a methodology involving coincubating varying concentrations of substrate with varying concentrations of a candidate chemical inhibitor.

K_i, which expresses or is related to the affinity of a compound to an enzyme, is one of the key parameters for prediction of *in vivo* drug-drug interactions resulting from metabolic inhibition. K_i can be estimated either graphically by using plotting methods

or by nonliear regression analysis. The Dixon plot (Fig 2A) relies on a linearized form of a nonlinear relationship of the inhibitor concentration versus the reciprocal of the rate of metabolite formation (Segel 1975). The Lineweaver-Burk plot (the double reciprocal plot), which relies on a linearized form of a nonlinear relationship of the reciprocal of the rate of metabolite formation vs the reciprocal of the substrate concentrations, is frequently used to identify the likely mechanism of the enzyme inhibition. Secondary plot (Fig 2B), which relies on a linearized form of a nonlinear relationship of slopes taken from the double reciprocal plots vs the inhibitor concentrations, is another plotting method to estimate the K_i value (Segel 1975). However, the most reliable and robust method for analyzing K_i is nonlinear regression analysis, where all data are fit simultaneously to nonlinear inhibition models [equations (3), (4), (5), and (6), see Review of the Literature, section 3.1.1] (Kakkar et al. 1999; 2000; Cleland 1989).

In the case of mechanism-based inactivation, time-, concentration- and NADPH-dependent loss of enzyme activity is an important consequence of the processes. The important kinetic parameters for mechanism-based inactivation include the half-life of enzyme inactivation ($T_{1/2}$), the rate constant of inactivation (K_{inact}) and the concentration of inactivator that produces half the maximal rate of inactivation (K_I). After preincubation of the microsomes with NADPH for an appropriate time in the presence or absence of various concentrations of an inhibitor, the $T_{1/2}$ and the apparent inactivation rate constant (K_{obs}) can be estimated from linear regression analysis of the natural logarithm of residual enzyme activity against the preincubation time (Fig 3A). The K_I and K_{inact} can be calculated from a double-reciprocal plot of the inactivation rate constant (K_{obs}) versus inhibitor concentration (Fig 3B). The intercept on the ordinate gives $1/K_{inact}$. If the line is extrapolated to the abscissa, the intercept gives $-1/K_I$ (Kent et al. 1999; Koenigs et al. 1997). In addition to the linearized plots, K_I and K_{inact} can also be estimated by fitting data to the following equation using nonlinear regression analysis (see Lin & Lu 1998):

$$T_{1/2} = 0.693\ (1 + K_I /I)/K_{inact} \qquad (11)$$

where I is the concentration of a mechanism-based inactivator.

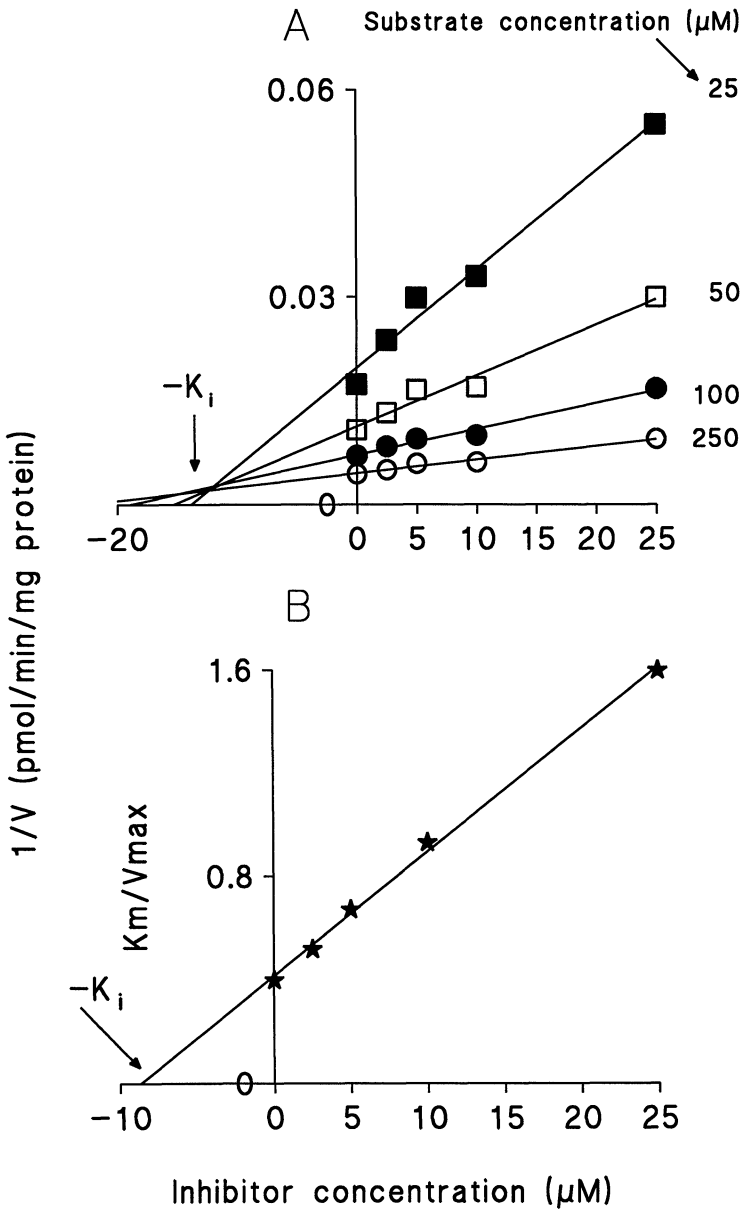

Figure 2. Representative Dixon plot (A) and Secondary plot (B) of a competitive inhibition. Data adopted from Study II.

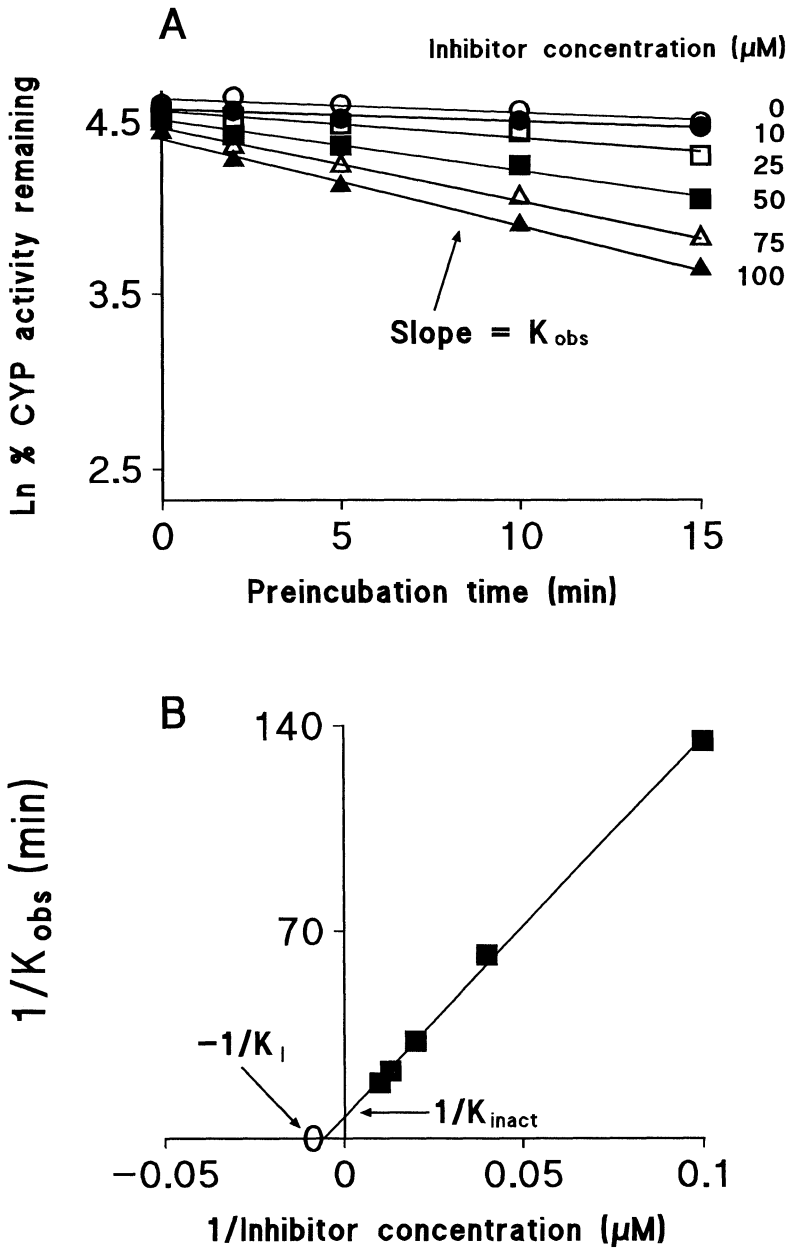

Figure 3. Preincubation time- and concentration-dependent inactivation of CYP activity by a mechanism-based inhibitor in human liver microsomes (A). (B), the double-reciprocal plot of the rate of inactivation as a function of inhibitor concentration. Data adopted from Study III.

6.2 Prediction of metabolic inhibition using an *in vitro-in vivo* scaling model

Because of limitations of *in vivo* studies and problems in extrapolating the results of animal studies to humans, *in vitro* systems using human tissues have become widely used tools to predict potential drug-drug interactions in humans. The benefit of these studies is that *in vitro* data concerning the potential for drug-drug interactions can be obtained early in the drug development phase, thereby helping researchers to focus on *in vivo* interaction studies and the prediction of pharmacokinetic variability (see Levy et al. 2000; Lin & Lu 2001).

In vitro-in vivo scaling models of pharmacokinetic interactions involving metabolic inhibition are based on the hypothesis that the fractional decrement in reaction velocity (FDV) *in vitro* in principle corresponds to the fractional decrement of clearance (FDCL) *in vivo* (von Moltke et al. 1995b; 1998). FDCL is based on the clearance of the substrate, measured with no inhibitor present, and coadministration of the inhibitor. FDV is related to the V_0 (velocity in the absence of inhibitor) and V_i (velocity in the presence of inhibitor) (von Moltke et al. 1995b; 1998). Using competitive inhibition as an example, the V_0 and V_i can be expressed using equations (1) and (3) (see Review of the Literature, section 2 and 3.1.1), and consequently, the FDCL can be calculated by the following equation:

$$FDCL = FDV = (V_0 - V_i) / V_0 = I / K_i [1+ I / K_i + S / K_m] \quad (12)$$

Thus, the potency of inhibition is dependent on both the ratios of (I/K_i) and (S/K_m). If the substrate concentration is far below its K_m ($S << K_m$), the S/K_m ratio approaches zero and the equation (12) becomes:

$$FDCL = FDV = I / (I + K_i).$$

In the case of noncompetitive inhibition, $FDCL = I / (I + K_i)$ regardless of the values of S and K_m (see Lin & Lu 1997; 1998; Von Moltke et al. 1998; Venkatakrishnan et al. 2001).

Accordingly, drug-drug interactions based on inhibition of hepatic metabolism (i) can

be roughly predicted by the K_i value and the unbound concentration of inhibitor (I) around the metabolic enzyme site in the liver using the following equation regardless of the type of inhibition (see von Moltke et al. 1998; Rodrigues 2002) (Table 2).

$$i = I / (I + K_i) \quad (13)$$

It should be kept in mind that if S approaches K_m, this equation (13) overestimates the degree of inhibition.

Table 2 Enzyme kinetic equations and in vitro-in vivo scaling models for inhibition of CYP enzymes (adopted from Rodrigues 2002)

Inhibition type	Enzyme kinetic equation	*In vitro-in vivo* extrapolation
No inhibition	$V_0 = V_{max} S/(K_m + S)$	Not applicable
Competitive	$V_i = V_{max} S/[K_m (1 + I/K_i) + S]$	$i = I/[(I + K_i (1 + S/K_m)]$ As $S << K_m$, $i = I/(I + K_i)$
Noncompetitive	$V_i = V_{max} S/[K_m (1 + I/K_i) + (1 + I/K_i) S]$	$i = I/(I + K_i)$
Uncompetitive	$V_i = V_{max} S/[K_m + (1 + I/K_i) S]$	$i = I/[(I + K_i (1 + K_m /S)]$ As $S << K_m$, $i \rightarrow 0$, as $S \rightarrow K_m$, i becomes significant.
Mixed-type inhibition (competitive-noncompetitive)	$V_i = V_{max} S/[K_m (1 + I/K_i) + (1 + I/\alpha K_i) S]$	As $S << K_m$, $i = I/(I + K_i)$

V_0: the initial rate of the reaction in the absence of inhibitor; V_i: the rate of the reaction in the presence of inhibitor; S: the substrate concentration; I: the inhibitor concentration; V_{max} and K_m: the apparent Michaelis-Menten kinetic constants; K_i: the inhibition constant; α: the factor by which K_m changes when an inhibitor occupies the enzyme; fractional inhibition (i) is the predicted inhibition of CYP forms *in vivo* in the presence of an inhibitor.

Although many drugs have been identified as mechanism-based inactivators *in vitro*, the mechanisms involved are not always clear. Prediction of *in vivo* situations based on *in vitro* data is very complex, uncertain, and susceptible to many affecting factors (Mayhew et al. 2000; Rodrigues 2002). For example, an inactivator can be released from the enzyme through reversible binding, and the degradation rates of the enzymes may be substantially affected by the inactivator (see Ito et al. 1998b; Lin & Lu 1998; Mayhew et al. 2000). Consequently, the prediction of drug-drug interactions related to mechanism-based inhibition is somewhat difficult and not straightforward, despite some successful *in vitro-in vivo* extrapolations (Mayhew et al. 2000; Rodrigues 2002).

6.3 Factors to be considered in *in vitro-in vivo* extrapolation

In the aforementioned *in vitro-in vivo* scaling model, the real *in vivo* concentration of an inhibitor around the enzyme is unknown. Based on a hypothesis stating that only the unbound concentration in plasma is available for diffusion into intrahepatic sites, the unbound plasma concentration of an inhibitor has been used to predict *in vivo* drug interactions (see Ito et al. 1998b). However, this hypothesis is not suitable for many lipophilic compounds, since the liver concentrations of these compounds may even greatly exceed their total plasma concentrations, despite extensive plasma protein binding (Bogeyevitch et al. 1987; Chou et al. 1993; Levine et al. 1994; Rodrigues 2002). In such cases, successful prediction of *in vivo* drug-drug interactions has been achieved by using the total plasma concentration of an inhibitor or multiplying total plasma concentration by a liver/plasma partition ratio (von Moltke et al. 1994a; 1994b; von Moltke et al. 1998).

In addition to the estimation of the inhibitor concentration (I), the estimation of the *in vitro* K_i value is also very critical for the scaling process. Factors such as incubation conditions, nonspecific binding to microsomal proteins, and the chemical inhibitors used may affect the accuracy of the estimation of K_i (Obach 1997). Being a substrate of certain microsomal enzymes or by binding extensively to the enzymes, an inhibitor may be significantly depleted during the incubation. Thus, the K_i value of the inhibitor

may be overestimated especially if a high microsomal protein concentration is used. For example, the K_i value for ketoconazole-CYP3A4 (midazolam α-hydroxylation) interactions in human liver microsomes was estimated to be about 8 μM using a 1.5 g/L microsomal protein concentration, whereas the K_i value was about 0.18 μM using a 0.25 g/L microsomal protein concentration (Lampen et al. 1995; Wang et al. 1999). Thus, a 6-fold increase in the microsomal protein concentration resulted in a 44-fold increase in the estimated K_i value. As a result, it may be beneficial to run experiments at the lowest possible protein concentration to help reduce the effects of nonspecific binding.

Accumulation of potent inhibitory metabolites in the incubation medium may also result in inaccurate estimation of K_i values. For example, *N*-desmethyl (K_i = 2 μM) and *N*, *N*-didesmethyl (K_i = 0.1 μM) metabolites of diltiazem are more potent in inhibiting CYP3A4-mediated testosterone 6β-hydroxylation than the parent drug (K_i = 60 μM) (Sutton et al. 1997).

The role of P-glycoprotein (P-gp) involved in the drug interactions has been recognized. P-gp is not only overexpressed in the development of multidrug resistance by tumour cells exposed to cancer chemotherapeutic agents (Gottesman et al. 1993), but is also presents in normal tissues including the intestines, liver, kidneys and brain, where it is considered to function as a drug efflux pump to reduce drug absorption, or enhance drug excretion into bile and urine (Wandel et al. 2000). P-gp and CYP3A4 are co-expressed in specific cell types, such as enterocytes and hepatocytes, and they also share a large number of substrates and modulators (Wacher et al. 1995; Kim et al. 1998). Inhibition of P-gp produces an increase in drug absorption and a decrease in hepatic and renal excretion of its substrates, resulting in elevated plasma and organ drug levels, particularly in the brain (Schinkel et al. 1996).

The choice of the inhibitor concentrations used may also affect the *in vivo* result. For example, ketoconazole, a very potent and relatively selective CYP3A4 inhibitor, can inhibit the catalytic activity of all major CYP activities when used at very high

concentrations (100 µM) (Newton et al. 1995). It is essential to consider other factors such as the choice of the substrate concentrations and the extrahepatic drug metabolism (especial for drug with substantial first-pass metabolism) when extrapolating *in vitro* results to *in vivo* situations (see Hall et al. 1999; Schinkel et al. 1996).

Although *in vitro* studies can provide valuable information, quantitative prediction of *in vivo* situations based on *in vitro* data can be foiled by numerous factors. Interpreting *in vitro* results needs a good understanding of the underlying pharmacokinetic principles, and mechanisms involved in drug interactions.

7. Clinical significance of drug-drug interactions involving CYP enzymes

In clinical practice, when two or more drugs are administered at the same or overlapping times, there is always a concern for drug-drug interactions. Although interactions can be pharmacokinetic or pharmacodynamic in nature, in many cases, the interactions have a pharmacokinetic basis (see Guengerich 1997; Lin & Lu 1998). There are many underlying mechanisms responsible for pharmacokinetic interactions that can be understood in terms of alterations of CYP-catalyzed reactions. The major reasons for drug-drug interactions involving CYP enzymes are induction, inhibition, and possibly stimulation, with inhibition appearing to be the most important in terms of known clinical problems (see Guengerich 1997; Lin & Lu 1998; Rodrigues 2002).

The inhibition of CYP enzymes can result in the undesirable elevation of plasma drug concentrations, leading to toxicity or therapeutic failure (see Lin & Lu 1998). For example, the concomitant use of terfenadine (metabolised entirely by CYP3A4) with CYP3A4 inhibitors, including the azole antifungals (ketoconazole, itraconazole) and macrolide antibiotics (erythromycin, clarithromycin, troleandromycin) can cause toxic accumulation of terfenadine in plasma and prevent the formation of antihistamine metabolite (Gillum et al. 1993; Guengerich 1997; Dresser et al. 2000; Rodrigues 2002). Coadministration of mibefradil (potent CYP3A4 inhibitor) with the substrates

of CYP3A4 such as triazolam, terfenadine, cyclosporine A, and quinidine, can result in the plasma concentrations of these drugs being substantially elevated by mibefradil, resulting in significantly adverse reactions (Welker et al. 1998; Backman et al. 1999; Wang et al. 1999; Ma et al. 2000). A good understanding of the underlying mechanisms involving in such drug-drug interactions can avoid toxicity or therapeutic failure by a corresponding reduction or increment of the therapeutic doses of a targeted drug, or close monitoring of its plasma concentration whenever a precipitant compound is added to the therapeutic regime.

8. Individual drugs studied

8.1 Valproic acid

Pharmacokinetics Valproic acid is a widely used anticonvulsant agent. Valproic acid has been shown to be completely absorbed following oral administration (see Dollery 1999). Its time to peak plasma concentration (t_{max}) is 1-2 h and the elimination half-life ($t_{1/2}$) is 12 h (Klotz et al. 1977). After a 1000 mg single oral dose of valproic acid, its peak plasma concentration (C_{max}) ranged from 40 to 110 mg/l (240-662 μM) (Davis et al. 1994). In patients with normal renal and hepatic functions, the binding of valproic acid to plasma proteins (mainly albumin) is concentration-dependent (85%-94%) (see Dollery 1999). It is thus estimated that the plasma-unbound valproic acid concentration is normally lower than 15 mg/l (130 μM) after a usual therapeutic dose of valproic acid. However, the free fraction of valproic acid increases nonlinearly when its total plasma concentrations exceed 92 mg/l (550 μM) (Davis et al. 1994) (Table 3).

Metabolism Valproic acid is eliminated via extensive hepatic metabolism to several metabolites (Davis et al. 1994). Its major metabolic pathways are glucuronidation and mitochondrial β-oxidation, while CYP-dependent oxidation is only a minor pathway (Rettie et al. 1995) (Fig 4). The steady-state plasma concentrations of the metabolites are at least 100-fold lower than those of the parent compound (Levy et al. 1990). Thus,

it is unlikely that the metabolites would significantly alter CYP-dependent drug metabolism *in vivo*. However, 4-ene-valproic acid, a minor metabolite whose formation is mediated by CYP2C9 and CYP2A6 *in vitro*, is possibly involved in valproic acid-mediated hepatotoxicity (Sadeque et al. 1997) (Table 3).

Drug-drug interactions Several drug-drug interactions between valproic acid and CYP substrates have been reported. In one study, valproic acid at a serum concentration of about 400 µM reduced the systemic clearance of unbound phenytoin by 23% (Perucca et al. 1980). Valproic acid (500 mg, twice daily) increased the maximum serum levels and the area under the curve (AUC) of amitriptyline by 19% and 30%, respectively (Wong et al. 1996). In addition, valproic acid (250 mg, twice daily) prolonged the $t_{1/2}$ of phenobarbital from 96 to 142 h and decreased its metabolic clearance from 3.3 to 2.0 mg/hr/kg (Patel et al. 1980). Furthermore, the concentrations of unbound diazepam in serum were significantly increased and the clearance of unbound diazepam was significantly reduced by valproic acid 1500 mg daily (Dhillon et al. 1982). *In vitro* studies showed that 100 µM and 250 µM valproic acid had no effect on CYP3A4-mediated cyclosporin oxidation and on the CYP2D6-mediated hydroxylation of mexiletine (Broly et al. 1990; Pichard et al. 1990).

8.2 Gemfibrozil

Pharmacokinetics Gemfibrozil is a fibric acid derivative, which is used in the treatment of patients with hypertriglyceridaemia and hypercholesterolaemia (Todd & Ward 1988). Gemfibrozil is rapidly and completely absorbed following oral administration. After the usual daily doses of gemfibrozil (600 mg b.i.d.), its C_{max} is about 30-60 mg/l (120-240 µM), and the average total plasma gemfibrozil concentrations during the 12-h-dosing interval are about 5-23 mg/l (20-92 µM) (Backman et al. 2000). Its t_{max} is 1 to 2 h and $t_{1/2}$ is 1.5 h (Miller & Spence 1998). Gemfibrozil is extensively bound to serum albumin (about 95%) (Miller & Spence 1998) (Table 3).

Metabolism Gemfibrozil is metabolised to a number of compounds in man (see Dollery 1999). About 48%-70% of the administered doses are excreted in the urine, and the rest is excreted mainly as glucuronide conjugates of gemfibrozil and its oxidised metabolites (Todd & Ward 1988; Parfitt 1999) (Table 3) (Fig 5).

Drug-drug interactions Gemfibrozil has been reported to enhance the anticoagulant effect of warfarin, resulting in severe hypoprothrombinemia and bleeding (Ahmad 1990; Rindone & Keng 1998). In one case report, gemfibrozil has been reported to interact with glyburide, resulting in hypoglycemia (Ahmad 1991). Gemfibrozil also increased the AUC of glimepiride by 23%, and prolonged the $t_{1/2}$ of glimepiride from 2.1 to 2.3 h (Niemi et al. 2001). In addition, it has been documented that a combined therapy of gemfibrozil and statins such as atorvastatin, cerivastatin, lovastatin or simvastatin can result in severe myopathy and rhabdomyolysis (Murdock et al. 1999). Gemfibrozil-statins interactions may have a pharmacokinetic explanation, since gemfibrozil has markedly increased the plasma concentrations of active simvastatin acid and lovastatin acid, whereas it only slightly increased that of the parent simvastatin and lovastatin (Backman et al. 2000; Kyrklund et al. 2001). In *in vitro*, gemfibrozil has been confirmed not to be an inhibitor of CYP3A4 (Backman et al. 2000).

8.3 Isoniazid

Pharmacokinetics Isoniazid is widely used in the treatment and prophylaxis of tuberculosis. It is rapidly and completely absorbed after oral administration. The C_{max} of isoniazid is 3-7 mg/l (22-50 µM) and is achieved 1-2 h after oral administration of a normal therapeutic dose (300 mg/day) (see Dollery 1999). The $t_{1/2}$ of isoniazid exhibits a wide interindividual variability and is determined by the acetylator status. The $t_{1/2}$ of isoniazid is about 0.5-2 h in rapid acetylators, while it is about 2-6.5 h in slow acetylators (Weber & Hein 1979). Isoniazid is probably not bound to an appreciable extent to plasma proteins (Boxenbaum et al. 1975) (Table 3).

Metabolism The major metabolic pathway of isoniazid in man is acetylation by a cytosolic *N*-acetyltransferase (Weber & Hein 1979; Meyer & Zanger 1997) (Fig 4). However, an *in vitro* study suggested that CYP enzymes might contribute to the metabolism of isoniazid to isonicotinic acid and hydrazine, which constitute a small fraction of the total metabolism of isoniazid (Ono et al. 1998). Isoniazid also undergoes appreciable presystemic (first-pass) metabolism in the wall of the small intestine and liver, and therefore its concentration in the plasma of rapid acetylators is half of the concentration in slow acetylators after a normal therapeutic dose (300 mg) of isoniazid (see Dollery 1999) (Table 3).

Drug-drug interactions In humans, isoniazid (300-600 mg daily) decreased the elimination of several drugs, including carbamazepine (Wright et al. 1982), diazepam (Ochs et al. 1981), triazolam (Ochs et al. 1983), vincristine (Chan et al. 1998), theophylline (Samigun et al. 1990), disulfiram (Whittington et al. 1969), chlorzoxazone and paracetamol (Zand et al. 1993). In addition, a commonly prescribed daily dose of isoniazid (300 mg daily) markedly increased serum phenytoin concentrations, with resultant toxicity in some patients (Kutt et al. 1968; Miller et al. 1979). A recent *in vitro* study has shown that isoniazid inhibited CYP2C19 and 3A4 activities in human liver microsomes, with K_i values of 25 µM and 52 µM, respectively (Desta et al. 2001).

8.4. Trimethoprim

Pharmacokinetics Trimethoprim is frequently combined with sulfamethoxazole (cotrimoxazole) to treat a wide range of infections. Trimethoprim is rapidly and completely absorbed following oral administration, and its C_{max} is 1 to 4 h, and approximately 5.8 mg/l (about 20 µM) following oral administration of trimethoprim 200 mg twice daily (Moore et al. 1996). The $t_{1/2}$ of trimethoprim is about 10 h. Trimethoprim exhibits moderate plasma protein binding (42%-46%) (see Dollery 1999) (Table 3).

Metabolism Trimethoprim is mainly excreted unchanged in urine, while a small proportion (20%) of a dose is metabolised by the hepatic CYP forms (Gleckman et al. 1981) (Fig 6; Table 3). In some individuals with severe liver damage, the $t_{1/2}$ of trimethoprim can be lengthened by up to 2-fold (Rieder et al. 1975).

Drug-drug interactions In humans, trimethoprim and sulfamethoxazole used alone at normal therapeutic doses have inhibited the metabolic clearance of tolbutamide (by 14% and 14%, respectively) and phenytoin (by 30% and 10%, respectively) (Wing & Miners 1985; Hansen et al. 1979). The use of the combinanation of trimethoprim and sulfamethoxazole (1:5), increased the AUC of *S*-warfarin by about 20% (O'Reilly 1980), and inhibited the metabolic clearance of tolbutamide by about 25% (Wing & Miners 1985). In *in vitro* studies, sulfamethoxazole competitively inhibits tolbutamide hydroxylase activity, with K_i values of 246 µM or 283 µM in human liver microsomes (Back et al. 1988; Komatsu et al. 2000b).

8.5 Sulfamethoxazole

Pharmacokinetics Sulfamethoxazole is readily absorbed from the gastro-intestinal tract and its C_{max} is reached about 2 h postdose (Parfitt 1999). Its C_{max} is approximately 26-63 mg/l (103-250 µM) after sulfamethoxazole oral 800 mg twice daily (Kaplan et al. 1973; Dollery 1999). The $t_{1/2}$ is about 9 h, and the plasma protein binding is about 66% (Dollery 1999) (Table 3).

Metabolism Sulfamethoxazole mainly undergoes acetylation (60%) in the liver, while a minor part of sulfamethoxazole is subject to oxidative metabolised by CYP2C9 to the hydroxylamine, a metabolite that has been implicated in adverse reactions to sulphonamides (Cribb et al. 1995; Parfitt 1999) (Fig 6; Table 3).

Drug-drug interactions (see **Drug-drug interactions** in **8.4**)

Table 3 Pharmacokinetic characteristics of individual drugs studied

Compound	Drug category	F_{oral} (%)	C_{max} (μM)	t_{max} (h)	$t_{1/2}$ (h)	Plasma protein binding (%)	Metabolic enzyme	References
Valproic acid	Anticonvulsant	> 95	240-662	1-2	12	85-94	GT* (primarily), β-oxidase (primarily) and CYP (< 20%)	Dollery 1999 Davis et al. 1994 Rettie et al. 1995 Sadeque et al. 1997
Gemfibrozil	Hypolipidaemic	100	120-240	1-2	1.5	95	GT* (30-50%) and CYP (minor?)	Dollery 1999 Todd & Ward 1988 Parfitt 199 Miller & Spence 1998
Isoniazid	Anti-infective	100	22-50	1-2	0.5-2 (rapid acetylator) 2-6.5 (slow acetylator)	negligible	Acetylase (primary) and CYP (minor)	Dollery 1999 Weber & Hein 1979 Ono et al. 1998
Trimethoprim	Anti-infective	> 95	20	1-4	10	42-46	CYP (minor)	Dollery 1999 Gleckman et al. 1981
Sulfamethoxazole	Anti-infective	85	103-250	2	9	65-66	N-acetylase (primary), GT* and CYP2C9 (minor)	Dollery 1999 Cribb et al. 1995 Parfitt 1999

*: Glucuronyltransferase.

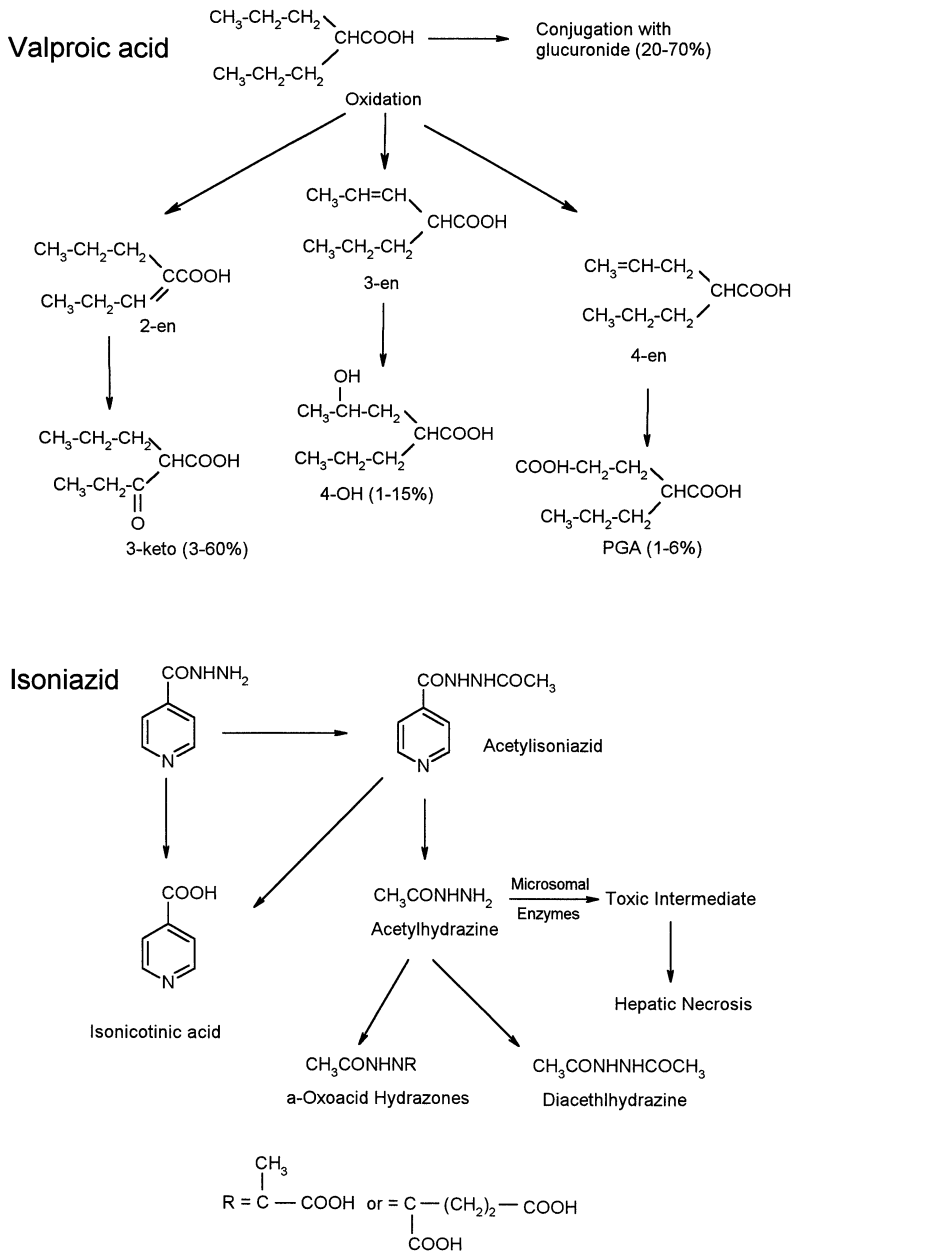

Figure 4. Chemical structures and metabolic pathways of valproic acid and isoniazid.

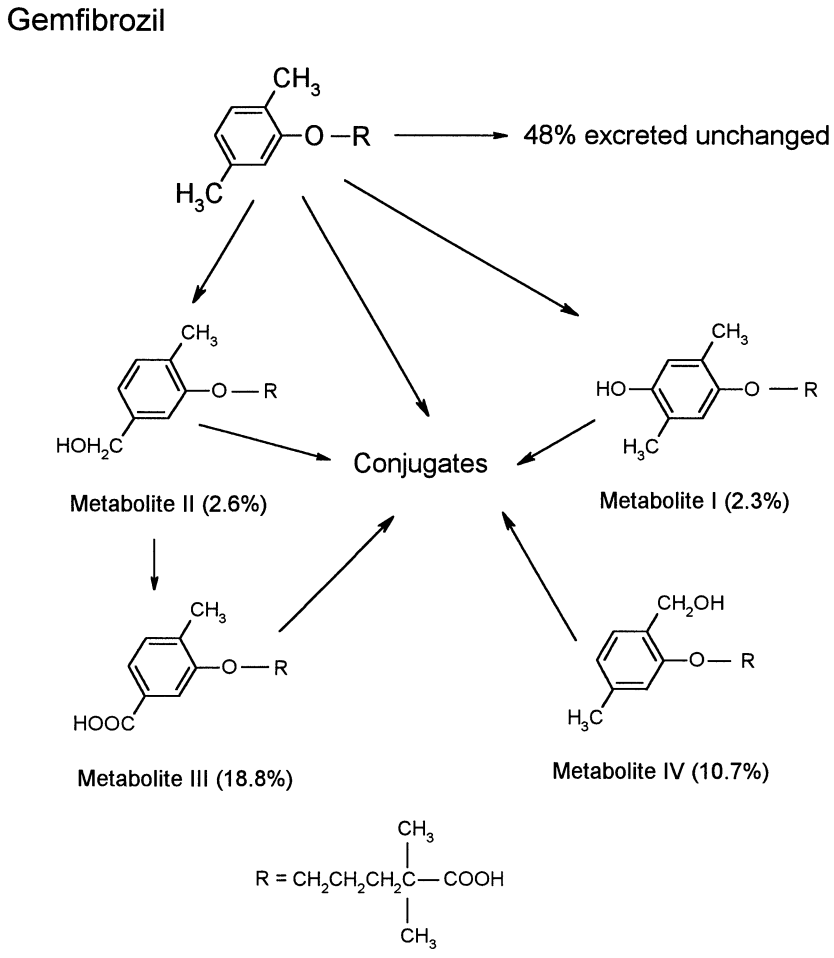

Figure 5. Chemical structure and metabolic pathway of gemfibrozil

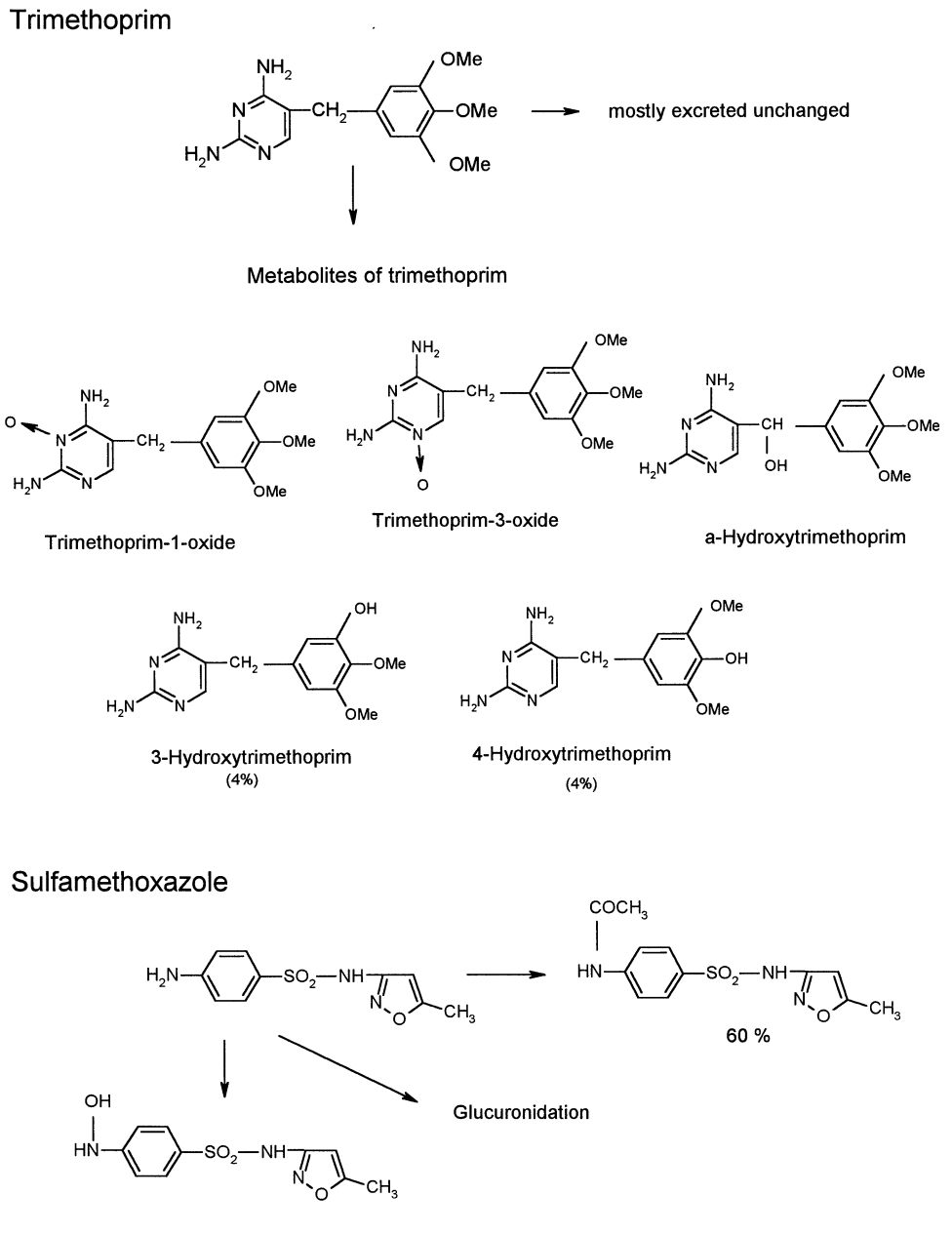

Figure 6. Chemical structures and metabolic pathways of trimethoprim and sulfamethoxazole

AIMS OF THE STUDY

The specific aims of the study were:

1. Evaluation of the effects of valproic acid, gemfibrozil, isoniazid, trimethoprim and sulfamethoxazole on CYP1A2-, CYP2A6-, CYP2C8- (only in Study IV), CYP2C9-, CYP2C19-, CYP2D6-, CYP2E1- and CYP3A4-mediated reactions (Study I-IV).

2. Prediction of the potential *in vivo* drug-drug interactions using the *in vitro* enzyme kinetic data and a scaling model (Study I-II, IV) and evaluation of their clinical relevance.

3. Examination of the effects of Hsa and Hlc on the *in vitro* enzyme kinetics of the formation of hydroxytolbutamide (CYP2C9 marker reaction), and on the inhibitory effect of gemfibrozil on tolbutamide hydroxylation using human liver microsomes (Study V).

MATERIALS AND METHODS

1. *In vitro* enzyme-assay systems

1.1 Human liver microsomal studies

Pooled human liver microsomes (prepared from five male and five female human liver microsomal samples) known to contain high levels of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 were obtained from Gentest Corp. (Woburn, MA). The liver samples were obtained from several organ procurement organizations, which collect tissues in accordance with all the pertinent regulations and obtain permission from the donors' families prior to organ collection. The procedures of these organizations have all been reviewed and approved by their respective institutional Human Subjects Committee. The same pooled batch was used in all of the *in vitro* studies (Study I-V). The microsomes were frozen and kept at -80°C until used.

Eight (CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) CYP form-specific marker reactions were studied in the present studies (Table 4). Phenacetin *O*-deethylation was used to probe for CYP1A2, coumarin 7-hydroxylation for CYP2A6, paclitaxel 6α-hydroxylation for CYP2C8 (used in Study IV only), tolbutamide hydroxylation for CYP2C9, *S*-mephenytoin 4′-hydroxylation for CYP2C19, dextromethorphan *O*-demethylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1, midazolam 1′-hydroxylation and testosterone 6β-hydroxylation (used in Study IV only) for CYP3A4.

All incubations were performed using an incubation medium containing 0.1 M sodium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, and 20 µg human liver microsomes (original concentration 20 mg/ml) in a final incubation volume of 0.2 ml. The candidate inhibitors were preincubated with the incubation medium at 37°C for 15 min, either in the presence or absence of 1.0 mM NADPH. After the preincubation, the probe substrates were added either with or without 1.0 mM NADPH for measurement of the

corresponding marker activities. Concentrations of the probe substrates used were chosen around appropriate apparent K_m values. After a specific incubation time, the reactions were terminated by adding the appropriate chemicals to precipitate the proteins. The detailed assay conditions for each CYP marker reaction are described in Table 4. All incubations were performed in duplicate and in the linear range with respect to the microsomal protein concentration, the substrate concentration ranges used and the incubation time.

In Study I, six form-selective CYP inhibitors were used as positive controls at appropriate concentrations, i.e. furafylline (1 and 5 µM) for CYP1A2, sulfaphenazole (3.6 and 100 µM) for CYP2C9, tranylcypromine (50 and 100 µM) for CYP2C19, quinidine (1 and 5 µM) for CYP2D6, pyridine (20 and 80 µM) for CYP2E1 and troleandomycin (20 and 100 µM) for CYP3A4. The final concentrations of each inhibitor were chosen according to previous publications (Hargreaves et al. 1994; Newton et al. 1995; Eagling et al. 1998; Hickman et al. 1998). As furafylline and troleandomycin are mechanism-based inhibitors, they were preincubated for 15 min with the incubation medium in the presence of NADPH before adding the corresponding marker substrates (Fig 7).

1.2 Recombinant human CYP form studies

Microsomes from baculovirus-infected insect cells engineered to express the cDNA encoding human CYP2C8 and CYP2C9 (Gentest Corp., Woburn, MA) were used in Study IV. The supersomes of the expressed enzymes were frozen and kept at -80°C until used.

Incubations with the recombinant CYP2C8 and CYP2C9 forms were performed using the same conditions as the corresponding assays for CYP2C8 and CYP2C9 activities in human liver microsomes, except that the incubation mixture contained 100 µg/ml of CYP2C8 and CYP2C9 supersomes (original concentrations of 3 mg/ml for CYP2C8, and 2.1 mg/ml for CYP2C9), and was incubated for 20 min (CYP2C8), and 30 min

Table 4 Summary of the reaction conditions of the CYP form-selective marker reactions in the studies (I-V)

CYP	Substrate / solvent (final concentration in the incubations)	Substrate concentration in assay (range) (μM) [a]	Incubation time (min)	Quenching of 200 μl incubation	K_{m1} (K_{m2}) (μM) [b]	V_{max1} (V_{max2}) (pmol mg^{-1} min^{-1}) [b]	Positive control inhibitor concentration (μM) / solvent (final concentration in the incubations)
CYP1A2	Phenacetin / methanol (1%)	10 (5-250)	30	100 μl acetonitrile	19 (556)	836 (824)	Furafylline (1, 5) / methanol (0.5%)
CYP2A6	Coumarin / methanol (0.1%)	0.5, 1, 5 (0.1-50)	10	20 μl 70% perchloric acid	1.1	1303	NA[c]
CYP2C8	Paclitaxel / methanol (1%)	1, 2, 5 (0.5-10)	20	100 μl acetonitrile	4.3	510	NA[c]
CYP2C9	Tolbutamide / ethanol (1%)	25, 50, 100, 250 (10-250)	60	20 μl 85% phosphoric acid	65	194	Sulfaphenazole (3.6, 100) / methanol (0.2%)
CYP2C19	*S*-mephenytoin / methanol (0.5%)	25, 50, 75, 100 (10-250)	60	20 μl 85% phosphoric acid	39	102	Tranylcypromine (50, 100) / water
CYP2D6	Dextromethorphan / water	1.5 (0.5-50)	20	20 μl 70% perchloric acid	1.5	227	Quinidine (1, 5) / methanol (0.2%)
CYP2E1	Chlorzoxazone / methanol (1%)	30 (5 -250)	30	20 μl 85% phosphoric acid	27	807	Pyridine (20, 80) / water
CYP3A4	Midazolam / acetonitrile (1%)	2, 5, 10, 15 (0.5-50)	6	200 μl methanol	1.4	1858	Troleandomycin (20, 100) / methanol (0.5%)
	Testosterone / methanol (1%)	25 (2.5-250)	6	100 μl acetonitrile	32	6158	

[a] Concentrations used in the inhibition studies were chosen around appropriate apparent K_m values; values in parenthesis are the substrate concentration ranges used for characterization of K_m and V_{max}.

[b] A two-enzyme Michaelis-Menten equation was best fitted to data for phenacetin *O*-deethylation (the apparent kinetic data for CYP1A2 is shown without parentheses); in agreement with previous studies, a single Michaelis-Menten equation was best fitted to the other marker reactions.

[c] Chemical (8-methoxypsoralen) as the inhibitor of CYP2A6 not available; a control inhibitor for CYP2C8 also not available.

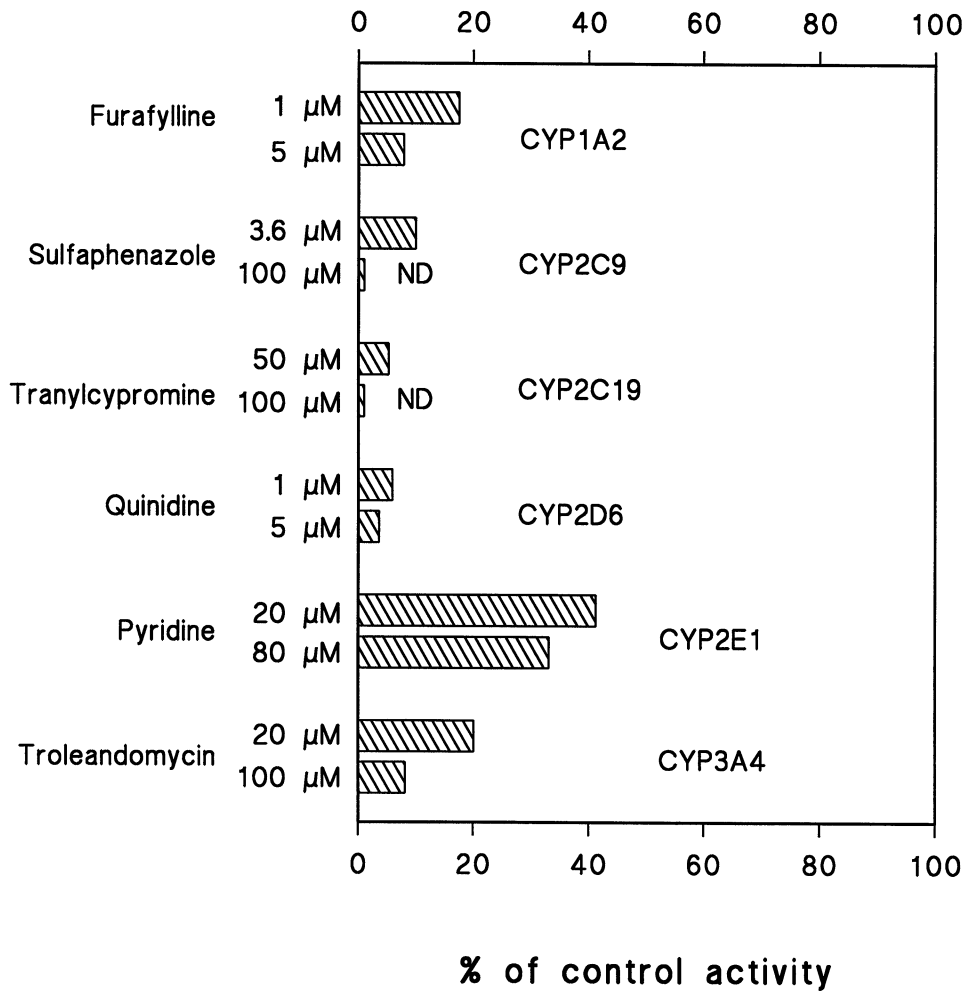

Figure 7. Six form-selective CYP inhibitors used as positive controls at appropriate concentrations. Data adopted from Study I.

(CYP2C9) with the probe substrates, respectively. All incubations were performed in duplicate and in linear range with respect to the microsomal protein concentration, the substrate concentration ranges used and the incubation time.

2. Design of the inhibition studies

In order to estimate the inhibitory potencies of the candidate compounds (Study I-IV) against the major human CYP form activities, a fixed concentration of the marker substrates (around their corresponding K_m values) was coincubated with various concentrations of a candidate inhibitor. The IC_{50} and K_i values were characterized when the tested drugs produced dose dependent inhibitory effects on the probe reactions. To determine the values of IC_{50} and K_i, the concentration of the marker substrates were chosen around their corresponding K_m values, and about 0.5- 4 fold of their corresponding K_m values, respectively. Additionally, to test whether the inhibition of CYP forms by the candidate compounds could be mechanism-based, the compounds were preincubated with the incubation medium at 37°C for 15 min, either in the presence or absence of 1.0 mM NADPH. In the case of NADPH-dependent inhibition (in Study I and III), the inhibitor was preincubated with the incubation medium for 0, 2, 5, 10 and 15 min in the presence or absence of 1.0 mM NADPH before adding corresponding marker substrates.

The range of concentrations used for each compound tested were as follows: valproic acid, 50 to 25000 µM (dissolved in 0.1 M sodium phosphate buffer, pH 7.4); gemfibrozil, 2.5 to 250 µM [dissolved in acetonitrile, then acetonitrile was evaporated to dryness and the residue was reconstituted using the incubation medium (it has been validated by HPLC assay that gemfibrozil with concentrations up to 250 µM was totally dissolved in the incubation medium)]; isoniazid, 2.5 to 250 µM [prepared in water with a minimal (0.2%) use of methanol]; trimethoprim, 5 to 500 µM, and sulfamethoxazole, 50 to 1000 µM (stock solutions of trimethoprim and sulfamethoxazole were dissolved in methanol, and 2 µl of each stock solution was added to the incubation medium with a final volume of 200 µl, i.e. the final

concentration of methanol was 1%). The same concentrations of the solvents used were added to the corresponding control samples, to minimize the effects of the organic solvents.

3. Effects of Hsa and Hlc on the enzyme kinetics of tolbutamide hydroxylation and on the inhibition of CYP2C9 by gemfibrozil

Pooled Hlc (prepared from 5 male and 5 female human liver samples) obtained from Tebu International Co. (Paris, France), and Hsa (analytical grade, fatty acid free) purchased from Sigma Chemical Co. (St. Louis, MO) were used in Study V.

Unbound fractions of tolbutamide and gemfibrozil in incubation media containing microsomal protein (0.1 mg/ml), Hsa (0.5-5 mg/ml) and Hlc (0.5-5 mg/ml) were separated by ultrafiltration (Sallustio et al. 1997; Carlile et al. 1999). In brief, tolbutamide (5-1000 μM) and gemfibrozil (10-100 μM) were prepared in each of the media used in the microsomal incubations, but without NADPH. After preincubation at 37 °C for 30 min in the absence of Hsa and Hlc, and in the presence of Hlc, and for 120 min in the presence of Hsa, and Hsa plus Hlc (the preincubation times used were validated by pilot tests to attain equilibrium binding), the samples were centrifuged for 30 min at 37 °C in a micropartition filter (Centrifree, part no 4104; Amicon Co, MA). After the centrifugation, an aliquot of the ultrafiltrate was subjected to analysis of the unbound drug concentration by HPLC.

The various concentration ranges of the marker substrates used for the kinetic analysis (Study I, IV-V) as well as their assay conditions have been specified in Table 4.

In Study V, tolbutamide (5-1000 μM) was preincubated with the incubation medium at 37°C for 30 min in the absence of Hsa and Hlc, and in the presence of Hlc (0.5-5 mg/ml), and for 120 min in the presence of Hsa (0.5-5 mg/ml), and Hlc plus Hsa. The reaction was initiated by the addition of 1.0 mM NADPH followed by 0.1 mg/ml microsomes. The time of incubation and concentration of microsomal protein used in

the assay were determined to be in the linear range for the rate of hydroxytolbutamide formation. After incubation for 60 min, the reactions were terminated by adding 20 µl phosphoric acid (85%) to precipitate the proteins.

4. HPLC analysis

Assays for the products of the CYP marker reactions were carried out using HPLC, as described previously (Chauret et al. 1997; Ko et al. 1997; Wester et al. 2000; Raucy et al. 1997; Ha et al. 1993; Stewart et al. 1986; Harris et al, 1994; Wang et al. 2000b). The HPLC system consisted of a Pharmacia LKB 2150 pump (LKB, Uppsala, Sweden), a Hewlett-Packard 1050 autosampler (Hewlett Packard), a Hewlett-Packard 3396 integrator (Hewlett Packard), a SPD-10AV Shimadzu UV-detector (Shimadzu, Kyoto, Japan) (for analysis of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 activities), a RF-551 Shimadzu fluorescence detector (Shimadzu; for analysis of CYP2A6 and CYP2D6 activities) or Model 5100A Coulochem electrochemical detector (ESA Inc., Bedford, MA; for analysis of the inhibitory effect of sulfamethoxazole on CYP2E1 activity). The intraday and interday coefficients of variation for all assays were less than 7% at the relevant concentrations (n = 6). The analytical methods for the *in vitro* assays are summarized and listed in Table 5.

5. Data Analysis

The IC_{50} values were determined graphically. The total apparent K_m and V_{max} (or the unbound apparent kinetics, i.e. $K_{m,\,u}$ and $V_{max,\,u}$, determined only in Study V for the formation of hydroxytolbutamide) for the CYP form-selective marker reactions, and the total K_i (or the unbound K_i determined in Study V for the inhibition of formation of hydroxytolbutamide by gemfibrozil) values of the tested drugs were calculated by nonlinear regression analysis. Different models of enzyme kinetics [equations (1) and (2), see Review of the Literature, section 2] or enzyme inhibition [equations (3), (4), (5) and (6), see Review of the Literature, section 3.1.1], i.e. competitive, noncompetitive, uncompetitive and mixed-type inhibition, were fitted to the data

Table 5 Summary of the HPLC methods for the studies I-V

Marker reaction	Column	Mobile Phase	Flow rate (ml/min)	Detector	Internal Standard
Phenacetin *O*-deethylation (CYP1A2)	Waters Symmetry-C_8 3.5 µm (4.6 × 150 mm)	6 mM CH_3COONH_4:CH_3CN 78:22	0.5	UV: 243 nm	-
Coumarin 7-hydroxylation (CYP2A6)	Waters Symmetry-C_8 3.5 µm (4.6× 150 mm)	1% CH_3COOH:CH_3OH 55:45	0.5	Fluorescence (excitation: 371 nm, emission: 454 nm)	-
Paclitaxel 6α-hydroxylation (CYP2C8)	ODS Hypersil 3 µM (4.6 × 100 mm)	CH_3CN:H_2O 40:60	1.0	UV: 230 nm	-
Tolbutamide hydroxylation (CYP2C9)	Waters Symmetry-C_8 3.5 µm (4.6 × 150 mm)	10 mM CH_3COONH_4 (pH 5.4):CH_3CN 64:36	0.5	UV: 230 nm	Chlorpropamide (2.5 µg/ml)
	*Supelco 5 µM (4.6 × 150 mm)	*1M NaH_2PO_4 (pH 3.4) :CH_3OH 60:40			*Carbutamide (5 µg/ml)
S-mephenytoin 4-hydroxylation (CYP2C19)	Waters Symmetry-C_8 3.5 µm (4.6 × 150 mm)	0.05 M KH_2PO_4 (pH 4.0):CH_3CN 71:29	0.5	UV: 211 nm	5-(p-hydroxyphenyl)-5-phenylhydantoin (1µg/ml)
	*X Terra RP18 3.5 µm (3.9 × 100 mm)	*0.05 M Na_2HPO_4:CH_3CN 81:19			*4-methyprimidone (5 µg/ml)
Dextromethorphan *O*-demethylation (CYP2D6)	Hypersil BDS-C_{18} 3 µm (4.0 × 100 mm)	2 mM $HClO_4$:CH_3CN 75:25	0.5	Fluorescence (excitation: 270 nm, emission: 312 nm)	-
Chlorzoxazone 6-hydroxylation (CYP2E1)	Waters Symmetry-C_8 3.5 µm (4.6 × 150 mm)	0.05% H_3PO_4 :CH_3CN 70:30	0.5	UV: 287 nm	8-chlorotheophylline (2 µg/ml)
	*Inertsil ODS 25 µm (4.0 × 150 mm)	*0.05 M Na_2HPO_4:CH_3CN 75:25	*0.6	*Electrochemical detector (oxidative potential + 0.6 V)	*Phenacetin (10 µM)
Midazolam α-hydroxylation (CYP3A4)	Waters Spherisorb S5 CN 4.6 × 250mm	3.5 mM $HClO_4$: $C_3C(OH)HC_3$:CH_3CN18:25:57	1.5	UV: 245 nm	Methoxydiazepam (50 ng/ml)
Testosterone 6β-hydroxylation (CYP3A4)	Waters Symmetry-C_8 3.5 µm (4.6 × 150 mm)	15 mM CH_3COONH_4: (CH_3CN: CH_3OH 9:1) 65:35	0.5	UV: 240 nm	-

*Only used for the analysis of the inhibition of CYP forms by sulfamethoxazole. -: internal standard not available.

(Segel 1975). An assessment of goodness of fit of the models was made using the size of the residual sum of squares, the random distribution of the residuals, the standard error, and the 95% confidence interval of the parameter estimates (Motulsky & Ransnas 1987).

In the case of preincubation time dependent CYP inactivation (Study I and III), $T_{1/2}$ was estimated from linear regression analysis of the natural logarithm of residual enzyme activity against preincubation time. The concentrations required for K_I and K_{inact} were estimated by fitting data to the equation (11) (see Review of the Literature, section 6.1).

The *in vivo* (i) values of valproic acid, gemfibrozil, trimethoprim and sulfamethoxazole, which showed no preincubation time dependent inhibition, were predicted by the scaling model [equation (13), see Review of the Literature, section 6.2], where (I) represents the inhibitor concentration around the metabolic enzyme site. Because the real *in vivo* (I) is unknown, the free or total drug concentration in plasma (Study I, II, and IV) or the total plasma drug concentration multiplied by the liver/plasma partition ratio taken from animal experiments (Study IV) was used in the calculation.

In study V, a single enzyme MM model [equation (1), see Review of the Literature, section 2] was found to be the best fit enzyme model relating hydroxytolbutamide formation rates to the concentrations of either total or unbound tolbutamide (S). Additionally, competitive inhibition [equation (3), see Review of the Literature, section 3.1.1] was found to be the best fit enzyme model for the inhibition of CYP2C9 by gemfibrozil either in the presence or absence of Hsa and/or Hlc. The *in vitro* total (or unbound) intrinsic clearance Cl_{int} (or $Cl_{int,\ u}$) values was calculated using V_{max}/K_m (or $V_{max,\ u}/K_{m,\ u}$). The *in vitro* Cl_{int} (or $Cl_{int,\ u}$) values were scaled to *in vivo* using the standard scaling factors of 45 mg microsomal protein/g liver and 20 g liver/kg body weight for humans (Obach 2000). The Cl_h values were calculated using the well-stirred model:

$Cl_h = Q \cdot f_u \cdot Cl_{int} / (Q + f_u \cdot Cl_{int})$

where Q is 20 ml/min/kg (Lin & Lu 1997), and f_u is the unbound fraction of tolbutamide in the blood (0.03; Dollery 1999).

6. Statistical analyses

In Study V, the t-test was used to compare the observed *in vivo* metabolic clearance of tolbutamide with that predicted from literature microsomal data. The unbound fractions of tolbutamide and gemfibrozil in incubation media in the presence or absence of 5 mg/ml Hsa, and 0.5 mg/ml Hlc were compared using the Wilcoxon test (in Study V).

RESULTS

1. Enzyme kinetics and known inhibitors of human liver CYP enzymes

The apparent K_m and V_{max} values for each CYP marker reaction (Table 4) were in agreement with previous literature results. The effects of form-selective inhibitors on these CYP activities were similar to those reported previously (Fig 7).

2. Inhibition studies

2.1 Valproic acid

In vitro, valproic acid preferentially and competitively inhibited CYP2C9-catalysed tolbutamide hydroxylation with an apparent K_i value of 600 μM, while it was a mixed-type inhibitor for CYP2C19 (K_i = 8553 μM) and a competitive type inhibitor for CYP3A4 (K_i = 7975 μM) activities (Table 6). In addition, inhibition of CYP2A6-catalysed coumarin 7-hydroxylation by valproic acid was found to be preincubation time- and concentration-dependent with a K_{inact} of 0.048 min^{-1} and K_I of 9150 μM (Table 6). However, only minimal inhibition of CYP1A2, CYP2D6 and CYP2E1 activities by valproic acid was observed.

2.2 Gemfibrozil

Gemfibrozil competitively inhibited CYP2C9-catalysed tolbutamide hydroxylation with a K_i (IC_{50}) value of 5.8 (9.6) μM (Table 6). In addition, gemfibrozil exhibited a modest but significant inhibitory effect on CYP2C19 and CYP1A2 activities with an apparent K_i (IC_{50}) values of 24 (47) μM and 82 (136) μM, respectively (Table 6). The pattern of inhibition of CYP2C19 and CYP1A2 by gemfibrozil was compatible with mixed-type inhibition. With concentrations ranging from 5 to 250 μM, gemfibrozil showed no remarkable effects on CYP2A6, CYP2D6, CYP2E1, and CYP3A4 activities.

2.3 Isoniazid

Isoniazid preferentially inhibited CYP2C19- and CYP3A4-mediated reactions after a 15 min preincubation without NADPH. The double-reciprocal plots and Dixon plots consistently indicated that isoniazid competitively inhibited CYP2C19 activity with a K_i of 36 μM, and uncompetitively inhibited CYP3A4 with a K_i value of 73 μM (Table 6). However, isoniazid had only very weak inhibitory effects on CYP1A2, CYP2A6, CYP2C9, CYP2D6 and CYP2E1 activities (K_i > 110 μM). After a 15 min preincubation with NADPH, isoniazid showed an increased inhibitory potency toward CYP1A2, CYP2A6, CYP2C19 and CYP3A4 activities. The estimated K_i values of isoniazid for CYP1A2, CYP2A6, CYP2C19 and CYP3A4 activities (K_i = 56, 60, 10 and 36 μM, respectively) were markedly lower in the presence of NADPH than in the absence of NADPH during preincubation (Table 6). In addition, the inactivation of these CYP forms by isoniazid was found to be preincubation time-, and concentration-dependent, and was characterized by K_{inact} values of 0.11, 0.13, 0.09 and 0.08 min^{-1}, and K_I values of 285, 173, 112 and 228 μM, respectively (Table 6).

2.4 Trimethoprim

With concentrations ranging from 5 to 100 μM, trimethoprim exhibited a selective and competitive inhibitory effect on CYP2C8-mediated paclitaxel 6α-hydroxylation with an apparent IC_{50} (K_i) value of 54 μM (32 μM) in human liver microsomes, and an IC_{50} of 75 μM in recombinant CYP2C8 (Table 6). However, trimethoprim lost its form-selectivity at concentrations higher than 100 μM. As much as 20%-50% of CYP1A2-, CYP2C9-, CYP2C19-, CYP2D6- and CYP3A4 activities were inhibited at 250 μM or 500 μM of trimethoprim.

2.5 Sulfamethoxazole

With concentrations ranging from 50 to 500 μM, sulfamethoxazole selectively and competitively inhibited tolbutamide hydroxylase activity in human liver microsomes

Table 6 Summary of the in vitro inhibitory effects of the tested drugs on CYP form activities in human liver microsomes [a]

CYP forms	Valproic acid	Gemfibrozil	Isoniazid	Trimethoprim	Sulfamethoxazole
CYP1A2	NI [d]	82 (136) mixed, α [b] = 7.3	[285, 0.11] [c] mechanism-based {111 competitive, 56 mixed α [b] = 1.9} [e]	NI [d]	NI [d]
CYP2A6	[9150, 0.048] [c] mechanism-based	5.8 (9.6) competitive	[173, 0.13] [c] mechanism-based {221 mixed α [b] = 0.8, 60 noncompetitive} [e]	NI [d]	NI [d]
CYP2C8	NI [d]	NI [d]	NI [d]	32 (54) competitive	NI [d]
CYP2C9	600 competitive	NI [d]	633 competitive	NI [d]	271 (544) competitive
CYP2C19	8553 mixed, α [b] = 2.5	24 (47) mixed, α [b] = 7.3	[112, 0.09] [c] mechanism-based {36 mixed α [b] = 2.1, 10 mixed α [b] = 1.6} [e]	NI [d]	NI [d]
CYP2D6	NI [d]	NI [d]	399 mixed, α [b] = 0.9	NI [d]	NI [d]
CYP2E1	NI [d]	NI [d]	238 competitive	NI [d]	NI [d]
CYP3A4 (midazolam 1'-hydroxylation)	7975 competitive	NI [d]	[228, 0.08] [c] mechanism-based {73 uncompetitive, 36 uncompetitive} [e]	NI [d]	NI [d]

[a] The K_i (μM) values of reversible inhibition were estimated using nonlinear regression analysis, while the IC_{50} (μM) values (with the parentheses) were calculated graphically.

[b] The factors by which K_m changes when inhibitor occupies the enzyme site.

[c] Data in the square brackets represent the K_I (μM), and K_{inact} (min^{-1}) values of mechanism-based inhibition, which was derived using nonlinear regression analysis.

[d] no inhibition or insignificant inhibition.

[e] Data in the braces represent the K_i (μM) value and the types of reversible inhibition based on preincubation without (prior) or with (behind) NADPH. The K_i values were estimated using nonlinear regression analysis.

and recombinant CYP2C9, with apparent IC_{50} (K_i) values of 544 μM (271 μM) and 456 μM, respectively (Table 6). Very little (< 20%) or no inhibition of other CYP forms was found in this concentration range. However, at concentrations higher than 500 μM, sulfamethoxazole showed a modest (30-40%) inhibitory effect on CYP2A6-mediated coumarin 7-hydroxylation, and CYP3A4-mediated midazolam 1′-hydroxylation. Interestingly, sulfamethoxazole only very slightly inhibited CYP3A4-mediated testosterone 6β-hydroxylation which was as high as 1000 μM.

3. Prediction of drug-drug interactions of valproic acid, gemfibrozil, trimethoprim and sulfamethoxazole *in vivo*

Based on the scaling model, assuming that the concentration of valproic acid at the enzyme site equals its mean peak total plasma concentration (about 450 μM), valproic acid would cause approximately 40% inhibition of the hepatic clearance of CYP2C9 substrates (Table 7). However, using the mean unbound plasma concentration (about 45 μM) of valproic acid in the calculation, valproic acid would cause approximately 7% inhibition of the metabolic clearance of CYP2C9 substrates (Table 7).

The predicted *in vivo* inhibitory effects of gemfibrozil, trimethoprim, and sulfamethoxazole on CYP form activities are summarized in Table 7.

4. Effects of Hsa and Hlc on the enzyme kinetics of tolbutamide hydroxylation and on inhibition of CYP2C9 by gemfibrozil

4.1 Survey of the *in vitro* and *in vivo* literature data regarding the metabolism of tolbutamide

Six separate *in vitro* studies on the formation of hydroxytolbutamide (Doecke et al. 1991; Hemeryck et al. 1999; Shin et al. 1999; Komatsu et al. 2000a; Hickman et al. 1998; Miners et al. 1988) including 20 individual human liver microsomal samples

were summarized. The inclusion of these studies was based on the critical criteria (see the original manuscript, Study V, for details). The predicted *in vivo* Cl_h values of tolbutamide based on these data were considerablely lower than the values of actual metabolic clearance of hydroxytolbutamide (Cl_{met}) (0.059 ml/min/kg vs 0.15 ml/min/kg), adopted from a clinical pharmacokinetic study on 10 healthy subjects [Gross et al. 1999, the Cl_{met} values were calculated by dividing the sum amount of hydroxytolbutamide and carboxytolbutamide excreted during a 32 hours interval by the total area under the tolbutamide plasma concentration time curve from 0 to 32-hour (AUC_{0-32}), and were normalized by body weight in kg].

Table 7 Summary of the predicted in vivo inhibition (%) of the metabolism of coadministered CYP substrates by valproic acid, gemfibrozil, trimethoprim, and sulfamethoxazole, respectively, from in vitro data using the scaling model: $I/(I + K_i)$ $^{\alpha}$

CYP forms	Valproic acid	Gemfibrozil	Trimethoprim	Sulfamethoxazole
CYP1A2	-	64 (8)	-	-
CYP2C8	-	-	80 [26]	-
CYP2C9	40 (7)	96 (56)	-	13 [24]
CYP2C19	-	86 (24)	-	-

$^{\alpha}$: The model supposes that the substrate concentration for CYP is much lower than its K_m value. In the cases of prediction with valproic acid and gemfibrozil, the I values used in the model were the total (or unbound) C_{max} of valproic acid [450 (45) µM, Davis et al. 1994; Dollery 1999] and gemfibrozil [150 (2) µM, Backman et al. 2000; Dollery 1999]. In the cases of prediction with trimethoprim and sulfamethoxazole, the I values were the total hepatic concentrations of trimethoprim (130 µM) and sulfamethoxazole (40 µM), estimated using the total plasma concentration of trimethoprim (20 µM) and sulfamethoxazole (250 µM) multiplied by the liver/plasma partition ratios of monkeys (6.5 and 0.15 for trimethoprim and sulfamethoxazole, respectively) or [free plasma concentrations of trimethoprim (11 µM) and sulfamethoxazole (85 µM)] (see the original publications for details).

- : not calculated, negligible inhibition.

4.2 Effects of Hsa and Hlc on the unbound concentrations of tolbutamide and gemfibrozil

Tolbutamide or gemfibrozil at 50 μM did not bind with the incubation buffer including 0.1 mg/ml microsomal proteins. Concentrations of 0.5-5 mg/ml Hsa and Hlc dose-dependently decreased the unbound fractions of tolbutamide and gemfibrozil, but the effects of Hlc were much smaller than those of Hsa. The lowest, 0.5 mg/ml concentration of Hlc had no statistically significant effect on the unbound fractions of tolbutamide (5-250 μM) and gemfibrozil (10-100 μM) ($P > 0.05$). However, at a fixed 5 mg/ml concentration of Hsa, the unbound fractions of tolbutamide (5-250 μM) and gemfibrozil (10-100 μM) increased with increasing drug concentrations. The co-addition of Hsa and Hlc yielded no apparent difference ($P > 0.05$) in the unbound fractions of tolbutamide and gemfibrozil compared to Hsa alone.

4.3 Effects of Hsa and Hlc on the enzyme kinetics of tolbutamide hydroxylation

The MM and Eadie-Hofstee plots consistently indicated that a single enzyme played a predominant role in the formation of hydroxytolbutamide in all the incubation mixtures. The apparent K_m, and V_{max} values in the standard incubation media were 123 μM, and 282 pmol/mg/min, respectively. Based on these estimates, the predicted $Cl_{h,u}$ value for tolbutamide (0.06 ml/min/kg) was 2.5 fold lower than the actual *in vivo* data (0.15 ml/min/kg). The addition of 5 mg/ml Hsa or 0.5 mg/ml Hlc to the microsomal incubation media greatly decreased the unbound K_m of the formation of hydroxytolbutamide (73 μM or 88 μM, respectively), making the predicted $Cl_{h,u}$ values much closer to the *in vivo* data (0.11 or 0.09 ml/min/kg, respectively). The co-addition of Hsa and Hlc yielded the predicted $Cl_{h,u}$ value (0.14 ml/min/kg) which was closest to the *in vivo* data (0.15 ml/min/kg).

4.4 Effects of Hsa and Hlc on inhibition of CYP2C9 by gemfibrozil

Gemfibrozil dose-dependently inhibited tolbutamide hydroxylase, with an IC_{50} value of 13 μM in the absence of Hsa and Hlc in the incubation media. Considering the total gemfibrozil concentrations, the inhibitory effects of gemfibrozil on tolbutamide hydroxylase were greatly decreased by the addition of 5 mg/ml Hsa, 0.5 mg/ml Hlc, or both to the incubation media, with the IC_{50} values of 67 μM, 24 μM or > 100 μM, respectively. Consistently, the K_i (6 μM) of gemfibrozil for CYP2C9 calculated using the total drug concentrations was increased by Hlc (8 μM), Hsa (40 μM) or both (72 μM). However, considering the unbound tolbutamide and gemfibrozil concentrations in the calculation, the K_i (6 μM, in the absence of Hsa and Hlc in the incubation media) was not significantly altered by the addition of Hsa (4 μM), Hlc (8 μM) or both Hsa and Hlc (9 μM) (Table 8).

Table 8 The inhibitory effects of gemfibrozil for CYP2C9-mediated tolbutamide hydroxylation in human liver microsomes in the absence and presence of 5 mg/ml Hsa and 0.5 mg/ml Hlc

Incubation	K_i (μM)	
	Total [a]	Unbound [b]
Without Hsa and Hlc	6	6
With Hlc	8	8
With Hsa	20 (40)[c]	4
With Hsa and Hlc	36 (72)[c]	9

The K_i values were estimated using nonlinear regression analysis.

[a] Total K_i of gemfibrozil estimated using the competitive inhibition model: $V_i = V_{max} \cdot S_u / [K_m (1 + I / K_i) + S_u]$, where S_u is the unbound tolbutamide concentration, and I is the total gemfibrozil concentration.

[b] Unbound K_i of gemfibrozil estimated using $V_i = V_{max} \cdot S_u / [K_m (1 + I_u / K_i) + S_u]$, where S_u is unbound tolbutamide, and I_u is unbound gemfibrozil.

[c] Total K_i of gemfibrozil estimated using $V_i = V_{max} \cdot S / [K_m (1 + I / K_i) + S]$, where S is total tolbutamide, and I is total gemfibrozil.

DISCUSSION

1. Methodological considerations

1.1 *In vitro* enzyme-assay system

In the present studies, pooled human liver microsomes were used for evaluating the effects of the selected drugs on the major CYP activities. Although the human liver microsomal system is probably the most feasible system for the study of drug metabolism and drug-drug interactions *in vitro* (Levy et al. 2000), this system has its limitations (see Review of the Literature, section 4). To circumvent the interindividual variability in CYP activities, a large pooled human liver microsome batch (5 male and 5 female individual donors) was used in the present studies to provide average data about the evaluated compounds as inhibitors of CYP forms. A range of values rather than an average value is often preferred in *in vitro-in vivo* extrapolation studies, especially when studying the contributions of different forms to a metabolic reaction (Tucker et al. 2001). However, due to the inability to match healthy volunteer subjects with human tissue donors, interindividual variability cannot be totally avoided even using individual microsomal samples (Carlile et al. 1999). Therefore, the use of pooled liver microsomes more precisely represents the population average than the use of a limited amount of individual microsomal samples, and is more appropriate for scaling *in vitro* clearance data to *in vivo* data (Obach 1997). In addition, the pooled microsomal batch used had been confirmed to contain representative activities of individual CYP forms. For example, the characterized K_m and V_{max} values for the individual CYP forms from the pooled microsomes conformed well to those previously reported in the literature (Study I, IV and V). In any case, it should be noted that any conclusions about interindividual variability of the inhibitory potency of the drugs studied could not be drawn from the present studies due to the use of pooled microsomes.

Recombinant CYP forms were used in Study IV. Because recombinant enzymes used

expressed only a single CYP form activity, they can be used for further evidence that the inhibition of a certain reaction is due to inhibition of a specific CYP form. However, it should be noted that recombinant CYP forms expressed in different chimeras might have different enzyme activities, and that the enzyme activities expressed may substantially differ from those of the native human enzymes (Yamazaki et al. 1997). In addition, this system has serious limitations in inhibition studies, and is not suitable for the study of mechanism-based inhibition when the target enzyme studied is not responsible for the metabolism of the mechanism-based inhibitor (Moody et al. 1999). Therefore, it can only be used as a complementary and screening tool for human liver microsomes.

The choice of incubation conditions such as ionic strength, and the pH value of the incubation medium may influence the outcome of *in vitro* studies (Venkatakrishnan et al. 2001). We routinely use 0.1 M sodium phosphate buffer (pH 7.4) as the incubation medium, which in principle parallels the ionic strength and pH of hepatic cytoplasma *in vivo*. In addition, the effects of organic solvents on CYP activities are also of great concern. As most drugs have poor aqueous solubility in incubation media, organic solvents are commonly used to dissolve them. However, organic solvents can potently and selectively inhibit CYP enzymes (Hickman et al. 1998; Chauret et al 1998; Busby et al. 1999). For example, the 1% (v/v) methanol used in an incubation did not inhibit CYP2C19 and 3A4 activities, but markedly inhibited CYP2E1 and CYP2C9 activities by about 30% (Chauret et al. 1998). The other commonly used organic solvents, i.e. accetonitrile, ethanol and dimethyl sulfoxide (DMSO), have also somewhat significant inhibitory affects on certain CYP activities (Hickman et al. 1998; Chauret et al 1998; Busby et al. 1999). Accordingly, the final concentration of the organic solvent used in the *in vitro* incubations should be minimised as much as possible. In the present studies, the stock solutions of the tested compounds were dissolved in methanol, acetonitrile or ethanol, and subsequently diluted using the reaction buffer. The final concentrations of the organic solvents in the incubations were kept as low as possible and constant in the incubation series. The final solvent concentration in incubation did not exceed 1% (v/v). Furthermore, the control samples were made with an equal

concentration of the respective solvent. The final organic solvent concentrations used in the incubations are summarised and listed in Table 4.

Besides the factors mentioned above, the amount of microsomal protein and incubation time used are also very important for the *in vitro* studies. Drugs can bind to the microsomal protein in a manner that influences their ability to inhibit the enzyme being evaluated (Rodrigues 2002). Furthermore, a long incubation time and a large amount of protein may cause the drug to be significantly metabolised during the incubation. The consequence of both excessive binding and excessive metabolism is that the concentration of the drug available for metabolism or inhibition of the CYP mediated reaction may be significantly reduced during the incubation (Gibbs et al. 1999; Obach 1996; 1997; 1999). Accordingly, in our studies, a low microsomal protein concentration (100 µg/ml) was used in all of the incubations. In addition, the time of incubation (Table 4) and the concentration of microsomes or supersomes used (100 µg/ml) were determined to be in the linear range for the rate of metabolite formation for each substrate.

In the present series of studies, to determine whether the inhibition of CYP forms by drugs could be mechanism-based, the studied drugs were preincubated with the incubation medium at 37°C for 15 min, either in the presence or absence of NADPH. An excessive inhibitory effect (more than 30%), after preincubation of drugs with NADPH, may suggest a potential mechanism-based inhibition (Rodrigues 2002). These kinds of studies are important to avoid underestimation of the *in vitro* inhibitory potencies of the studied drugs.

Finally, six form-selective CYP inhibitors were used as positive controls at appropriate concentrations to validate the present *in vitro* enzyme-assay systems (Fig 7). The results clearly indicated that each positive control inhibitor inhibited their targeted enzyme activity in agreement with previous reports (Hargreaves et al. 1994; Newton et al. 1995; Eagling et al. 1998; Hickman et al. 1998). Tranylcypromine was used as an

inhibitor of CYP2C19. However, it also potently inhibits CYP2A6 (Taavitsainen et al. 2001; Zhang et al. 2001).

1.2 Experimental designs

Eight different CYP form-specific marker reactions were studied in the present studies. In the case of CYP3A4, the potency of inhibition of a drug has been shown to be dependent on the choice of the marker substrate, because the active site of CYP3A4 is sufficiently large and flexible to allow simultaneous binding of two or more molecules of which one can be an activator or inhibitor (Schrag & Wiekers 1999; Rodrigues 2002). In order to achieve a better evaluation of the effects of a drug on CYP3A4 activity and to be able to extrapolate the *in vitro* findings to the clinical situation, it is probably safer to study CYP3A4 inhibition using several marker substrates. Therefore, both midazolam 1′-hydroxylation and testosterone 6β-hydroxylation were used as CYP3A4 marker reactions in Study IV.

The selection of appropriate concentrations of the marker substrates is another important issue in design of *in vitro* studies. The selection of the concentrations of a marker substrate can largely affect the kinetic parameters of the enzyme reaction (K_m, V_{max} and K_i values) and the type of inhibition. The "appropriate range" of the substrate concentrations is between 0.5 K_m and 4 K_m (Rodrigues 2002). Consistent with this, the substrate concentrations used in the present studies were in the range of 0.5 ~ 4 K_m (Table 4). In addition, the selection of the concentrations of a chemical inhibitor should be carefully considered. Normally, the concentrations of the drug were selected on the basis of the known *in vivo* C_{max} of the drug. However, considering that many lipophilic drugs are concentrated in the liver, the concentration of a lipophilic inhibitor at the active site of the CYP enzymes may be higher than its plasma C_{max} (von Moltke et al. 1998). Also, a significant portion of lipophilic drugs may bind to microsomal proteins and lipids in the incubation media, thus reducing the "free" drug available to inhibit the enzyme (Obach 1996; 1997; 1999). Therefore, in the present studies, the lipophilic drugs evaluated, i.e. valproic acid, gemfibrozil, trimethoprim and

sulfamethoxazole (Parfitt 1999), were used at very high concentrations (i.e. approximately 10-fold their C_{max}) in the inhibition studies, to exclude the possibility of false negative results.

A relatively low Hsa concentration (5 mg/ml, 0.5%) was used in Study V. The concentration of albumin in cultured rat hepatocyte *S*-9 fractions (contains the cytosolic and microsomal factions) has been about 65 mg/g proteins, i.e. 6.5% of total protein weight (Milosevic et al. 1999). Although the real *in vivo* concentration of albumin around the metabolic enzyme site is not known, it is probably clearly lower than the concentration of albumin in serum (about 4%). Thus, the addition of 0.5% Hsa to *in vitro* microsomal incubations may more accurately reflect the physiological conditions *in vivo*. Consistent with this postulation, a higher albumin concentration (2%) used in the incubations, resulted in an overestimation of the *in vivo* metabolic clearance of tolbutamide (Carlile et al. 1999).

1.3 *In vitro-in vivo* extrapolations

A scaling model, i.e. $i = I/(I + K_i)$ was used to extrapolate the *in vitro* results to *in vivo* ones. The use of this model is based on an assumption that the concentration of substrate is substantially less than K_m i.e. $S << K_m$ in the case of competitive inhibition (see Review of the Literature, section 6.3). However, in some cases, this approach overestimates the inhibitory potency, because S is actually near the K_m, or even higher than the K_m. In some cases, the "free" plasma concentration of an inhibitor was used as (I) in the scaling model, owing to an assumption that only the free drug can perfuse into the liver. However, for lipophilic compounds, their liver concentrations may far exceed their "free" plasma concentrations (von Moltke et al. 1998). For example, valproic acid would cause approximately 40% (or 7%) inhibition of the hepatic clearance of CYP2C9 substrates using the mean (450 µM) (or unbound, 45 µM) plasma concentration to predict *in vivo* situation. However, *in vivo*, valproic acid at a serum concentration of about 400 µM, reduced the systemic clearance of unbound phenytoin by 23% (Perucca et al. 1980). The decrease is about half or three times

larger than what would be expected on the basis of total or unbound valproic acid plasma concentrations. Simply put the total or unbound plasma concentrations of an inhibitor may not directly relate to its hepatic concentration around the metabolic enzyme sites. Predictions based on the predictive model is not straightforward.

In Study IV, the liver/plasma partition ratios of drugs in monkeys were used to estimate the total hepatic trimethoprim and sulfamethoxazole concentrations. However, due to the different species used, the estimated results may still be different from the real values. Therefore, it should be kept in mind that although the predicted results based on the above model can provide valuable information, they are only rough estimations of the *in vivo* situations. The confirmation of such inhibition needs more *in vivo* evidence.

2. Valproic acid, gemfibrozil, isonizaid, trimethoprim and sulfamethoxazole as inhibitors of CYP forms

In our *in vitro* study, valproic acid preferentially inhibited CYP2C9 activity with the K_i value of 600 μM, while it exhibited weak or no inhibitory effects on other CYP forms. Although the plasma unbound valproic acid concentration is lower than 130 μM after a normal therapeutic dose (1000 mg daily), the concentrations of valproic acid at the enzyme site in the liver could be disproportionately higher when its plasma level approach or exceed the upper limit of the therapeutic range of valproic acid (about 700 μM) to achieve seizure control (Davis et al. 1994). Thus, a significant interaction between valproic acid and CYP2C9 substrates may occur *in vivo*. Inhibition of CYP2C9 activity by valproic acid may explain the interactions between valproic acid and phenytoin, diazepam, phenobarbitone and amitriptyline (Perucca et al. 1980; Patel et al. 1980; Dhillon et al. 1982; Wong et al. 1996). Although valproic acid is an inhibitor of CYP3A4 (K_i = 7975 μM) *in vitro*, the concentration of valproic acid may be very high in the enterocytes during absorption. Consequently, inhibition of intestinal CYP3A4 activity cannot be completely excluded. The observation that inactivation of CYP2A6 activity by valproic acid involves time-, concentration-, and

NADPH- dependence suggests that it is a mechanism-based inactivator of this CYP form, a conclusion supported further by the finding that formation of 4-ene-valproic acid, a minor metabolite of valproic acid, was mediated by CYP2A6 and CYP2C9 *in vitro* (Sadeque et al. 1997). However, as the inhibitory effect was weak, it is unlikely that valproic acid significantly inactivates CYP2A6 at clinically relevant concentrations. With the absence of an inhibitory effect on CYP1A2, CYP2D6 and CYP2E1 activities, and the weak inhibition of CYP2C19 activity, valproic acid is unlikely to produce clinically relevant interactions by inhibiting these CYP forms. This conclusion is also in agreement with studies showing that valproic acid had no effect on the plasma concentrations of the CYP1A2 substrates caffeine (Wietholtz et al. 1989) and clozapine (Facciola et al. 1999), and the CYP2D6 and CYP3A4 substrate haloperidol (Hesslinger et al. 1999).

Gemfibrozil inhibited CYP2C9 activity with a K_i value of 5.8 µM, while it inhibited CYP2C19 (K_i = 24 µM) and CYP1A2 (K_i = 82 µM) activities to a reduced extent in human liver microsomes. The K_i values for CYP2C9, 2C19 and 1A2 fall in the range of the total plasma concentrations of gemfibrozil (approximately 120-240 µM) observed in humans after 600 mg gemfibrozil b.i.d. (Backman et al. 2000). Based on the *in vitro-in vivo* scaling model: $i = I/(I + K_i)$, using peak total (or unbound) plasma concentration of gemfibrozil, 96% (56%), 86% (24%) and 64% (8%) inhibition of the clearance of CYP2C9, 2C19 and 1A2 substrates by gemfibrozil could be expected, respectively. Thus, inhibition of CYP2C9 activity by gemfibrozil is the likely mechanism of the gemfibrozil-warfarin and gemfibrozil-glyburide interactions (Rindone & Keng 1998; Ahmad 1990; 1991). *In vivo*, gemfibrozil increased the AUC of glimepiride (a CYP2C9 substrate) by 23% (Niemi et al. 2001). It seems that the predicted inhibition (24%) based on the unbound mean plasma concentration of gemfibrozil (1.9 µM) is the most relevant data to the *in vivo* situation. On the other hand, as gemfibrozil is not a CYP3A4 inhibitor *in vitro*, the gemfibrozil-simvastatin and gemfibrozil-lovastatin interactions cannot be explained by inhibition of the CYP3A4-mediated simvastatin and lovastatin metabolism, as is the case with itraconazole-lovastatin and itraconazole-simvastatin interactions (Backman et al. 2000;

Kyrklund et al. 2001). However, inhibition of some other pathways such as glucuronidation (Prueksaritanont et al. 2002) that regulate the levels of the statin acids by gemfibrozil cannot be ruled out.

In study III, isoniazid was found to be a mechanism-based inhibitor of CYP1A2, 2A6, 2C19 and 3A4 forms in human liver microsomes, with K_{inact} values of 0.11, 0.13, 0.09 and 0.08 min^{-1}, and K_I values of 285, 173, 112 and 228 µM, respectively. Because of the difficulty to predict the *in vivo* inhibition of a mechanism-based inactivator, these parameters were compared with those of other mechanism-based inactivators of CYPs such as erythromycin (inactivated CYP3A4 activity, K_I = 19 µM and K_{inact} = 0.064 min^{-1}) (Ohyama et al. 2000), furafylline (inactivated CYP1A2 activity, K_I = 23 µM and K_{inact} = 0.87 min^{-1}) (Kunze & Trager 1993), and delavirdine (inactivated CYP3A4 activity, K_I = 22 µM and K_{inact} = 0.59 min^{-1}) (Voorman et al. 1998). Considering that the peak plasma concentrations of isoniazid (up to 50 µM) are higher than those of erythromycin (5 µM) (Dollery 1999), furafylline (8 µM) (Dollery 1999) and delavirdine (10 µM) (Tarrus et al. 1987), isoniazid may also be a mechanism-based inactivator of human CYP2C19, 3A4, 1A2 and 2A6 *in vivo* although the K_I values of isoniazid are higher than those of above mentioned mechanism-based inactivators. Consequently, inactivation of CYP1A2, CYP2A6, CYP2C19 and CYP3A4 activities by isoniazid may explain the documented drug-drug interactions between isoniazid and phenytoin (metabolised by CYP2C9 and 2C19), theophylline (metabolised by CYP1A2 and 3A4), diazepam (metabolised by CYP2C19 and 3A4), triazolam (metabolised by CYP3A4), paracetamol (metabolised by CYP2E1, 1A2, 3A4 and 2A6), chlorzoxazone (metabolised by CYP2E1 and 1A2), carbamazepine (metabolised by CYP3A4), vincristine (metabolised by CYP3A4) and disulfiram (metabolised by CYP2A6 and 3A4) (Kutt et al. 1968; Samigun et al. 1990; Ochs et al. 1981; 1983; Zand et al. 1993; O'Shea et al. 1997; Wright et al. 1982; Chan 1998; Whittington et al. 1969).

In vitro, trimethoprim (5-100 µM) and sulfamethoxazole (50-500 µM) selectively inhibited CYP2C8 (K_i = 32 µM) and 2C9 (K_i = 271 µM) activities, respectively. Thus,

trimethoprim and sulfamethoxazole can be used as selective inhibitors for CYP2C8 and CYP2C9, respectively, in *in vitro* studies. With trimethoprim concentrations higher than 100 µM and sulfamethoxazole concentrations higher than 500 µM, both drugs lost their selectivity for the CYP forms. Based on the estimated total hepatic concentrations (or free plasma concentrations) of the drugs and the scaling model, one would expect *in vivo* in humans 80% (26%) and 13% (24%) inhibition of the metabolic clearance of CYP2C8 and 2C9 substrates by trimethoprim and sulfamethoxazole, respectively. Consistent with this prediction, trimethoprim and sulfamethoxazole used alone inhibited the metabolic clearance of tolbutamide (14% and 14%, respectively) and phenytoin (30% and 10%, respectively) (Wing & Miners 1985; Hansen et al. 1979). In addition, when trimethoprim and sulfamethoxazole were coadministered, the AUC of *S*-warfarin was increased by 20% (O'Reilly 1980), and the inhibition of the metabolic clearance of tolbutamide was more prominent (about 25%) than when trimethoprim, and sulfamethoxazole were used alone (14% and 14%, respectively) (Wing &Miners 1985).

3. Effects of albumin and cytosol on the enzyme kinetics of tolbutamide hydroxylation and on inhibition of CYP2C9 by gemfibrozil

3.1 Effects of Hsa and Hlc on the enzyme kinetics

Addition of 0.5% Hsa (5 mg/ml) and Hlc (0.5 mg/ml) to the incubation media significantly changed the enzyme kinetic parameters of tolbutamide, yielding the *in vivo* Cl_h of tolbutamide, based on the co-addition of Hsa and Hlc to the incubations, compable with the actual *in vivo* clearance values. Consistent findings have been observed also with phenytoin, another CYP2C9 substrate (Ludden et al. 1997; Carlile et al. 1999; Komatsu et al. 2000a). For example, addition of 2% or 4% bovine serum albumin to microsomal incubation media changed the K_m or Cl_{int} of phenytoin *p*-hydroxylation, resulting in more accruate predictions of the *in vivo* values (Ludden et al. 1997; Carlile et al. 1999). In addition, rat liver cytosol has been shown to stimulate phenytoin oxidation in human liver microsomes (Komatsu et al. 2000a). To date, the

mechanisms underlying these phenomena associated with albumin and cytosol are unclear. It has been suggested that the binding affinity between the substrate and the enzyme may be increased by albumin and cytosol, due to the conformational change of the metabolic enzyme, or by sequestering some endogenous compounds that inhibit CYP2C9. In any case, the mechanism of stimulation of CYP2C9 by cytosol seems not to be related to binding of the substrate by cytosol, because the cytosol at 0.5 mg/ml concentrations used in the present study, did not change the unbound fraction of tolbutamide.

3.2 Effects of Hsa and Hlc on inhibition of CYP2C9 by gemfibrozil

The *in vitro* K_i value (6 μM) of gemfibrozil for CYP2C9 calculated using total drug concentrations was substantially increased by Hlc (8 μM), Hsa (40 μM) or both (72 μM). However, considering the unbound substrate and inhibitor concentrations in the calculation, the K_i (6 μM in the absence of Hsa and Hlc) was not markedly altered by Hsa (4 μM), Hlc (8 μM) or both Hsa and Hlc (9 μM). Interestingly, although the addition of Hsa may increase the binding affinity of tolbutamide to the enzyme (as suggested by the K_m values), it did not greatly change the unbound K_i value of gemfibrozil. The underlying mechanism is unclear, it is assumed that both the binding affinity of the substrate and the inhibitor to the enzyme were increased, resulting in counterbalance, and minor changes of K_i.

As the present study cannot provide any mechanistic explanation for the observed kinetic changes, and it is not known whether the effects of Hsa and Hlc are substrate- or CYP form-specific, other substrates and other CYP forms need to be further explored.

4. General discussion

Over the past decade, considerable progress has occurred in the development and application of *in vitro* approaches using human tissues for assessing drug metabolism

and interactions. As shown by the present studies, the effects of drugs on CYP forms can be evaluated using microsomes and/or recombinant enzymes, and the *in vivo* potential drug interactions can be quantitatively or qualitatively predicted based on the *in vitro* data (Lin & Lu 1997; 2001; von Moltke et al. 1998). This information about the evaluation of drugs safety is very useful in minimizing the economic losses of pharmaceutical companies, as well as more effectively safeguarding the welfare of patients. However, the complexity of human physiology contributes to the inaccuracy of quantitative prediction of drug interactions. Therefore, it is important to carefully set up the *in vitro* experimental systems and conditions that simulate the *in vivo* situations. For example, optimal experimental conditions need to be established for accurate estimation of the K_i value and suitable techniques need to be developed to enable direct measurement of unbound inhibitor concentrations around the site of target enzymes.

In general, one probe substrate, which always is the "preferred" probe reflecting literature preference and extended period of use, is chosen for use in characterizing the inhibition (determination of IC_{50} and K_i values) of each of the various CYPs *in vitro* (Tucker et al. 2001). Considering that a weak correlation exists among some CYP3A marker substrates (Kinirons et al. 1993; Stein et al. 1996; Thummel & Wilkinson 1998; Kenworthy et al. 1999), multiple structurally unrelated CYP3A4 probes, such as midazolam and testosterone, have been recommended for used in the *in vitro* assessment of CYP3A4-mediated drug interactions (Kenworthy et al. 1999; Tucker et al. 2001). Furthermore, the correlations among other CYP marker substrates also need to be considered in a comprehensive characterization of inhibition of CYP enzymes.

Drug metabolism and interactions are complex processes, which very often involve several pathways and various enzyme systems. Besides CYP enzymes, phase II enzymes such as the uridine diphosphate (UDP)-glucuronosyltransferases (UGT), which catalyse the covalent addition of glucuronic acid to a wide range of lipophilic chemicals, play a major role in the detoxification of many exogenous and endogenous compounds (Burchell et al. 1995; 1998; Meech et al. 1997). Interactions involving

glucuronidation mediated by UGT have been reported in a number of clinical and *in vitro* studies (Markowitz et al. 2002; Hachad et al. 2002; Liston et al. 2001; Zhou et al. 2001; Ito et al. 2001). UGT is involved in the metabolism of valproic acid, gemfibrozil and sulfamethoxazole, as shown in Table 3. It is well known that valproic acid can inhibit the glucuronidation of drugs such as zidovudine, lamotrigine, phenobarbitone, and 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (Trapnell et al. 1998; Hachad et al. 2002; Bernus et al. 1994; O'Leary et al. 1981). However, the effects of other drugs tested on the activity of UGT are unknown, and have not been tested in previous studies. Therefore, the role of UGT in drug metabolism and interactions could also be considered.

In assessing the consequences of drug interaction, the identity of CYP forms responsible for metabolising the involved drugs and the relative contribution of the metabolic pathways being inhibited to the overall elimination of the drug must be considered. The discrepancy of *in vitro* and *in vivo* may be caused by the differences in the drug concentration used *in vitro* and observed *in vivo* (Lin & Lu 1998). Therefore, the use of clinically relevant concentrations of inhibitor and substrate is very important in the *in vitro* study. Other factors such as the *in vitro* systems chosen and mechanism involved in drug interactions may also affect *in vitro*-*in vivo* extrapolations.

As the liver is the major site of the biotransformation of drugs, disease states, especially diseases with hepatic involvement influence the metabolic capacity of the individual and alter CYP-dependent drug elimination (Murray 1992; Tanaka 1998; Rodighiero 1999; Sotaniemi et al. 2002). Furthermore, owing to extrahepatic drug metabolism, drug transportation, interindividual variation and interethnic differences in drug disposition, and numerous other complexities, the interpretation of *in vitro* results to *in vivo* situations needs a good understanding of pharmacokinetic principles and clinical considerations. Also there is a need for much more *in vivo* pharmacokinetic data to validate *in vitro* data in the study of drug interactions.

SUMMARY AND CONCLUSIONS

In the present study, the *in vitro* inhibitory effects of valproic acid, gemfibrozil, isoniazid, trimethoprim and sulfamethoxazole on CYP forms were evaluated. In addition, their potential *in vivo* drug-drug interactions were predicted. In Study V, the effects of albumin and cytosol on enzyme kinetics of tolbutamide hydroxylation and on inhibition of CYP2C9 by gemfibrozil in human liver microsomes were examined. The major findings and conclusions are as the follows:

1. Valproic acid inhibits the activity of CYP2C9 at clinically relevant concentrations in human liver microsomes. Inhibition of CYP2C9 can explain some of the effects of valproic acid on the pharmacokinetics of other drugs, such as phenytoin. Co-administration of high doses of valproic acid with drugs that are primarily metabolised by CYP2C9 may result in significant drug-drug interactions.

2. Gemfibrozil inhibits the activity of CYP2C9 at clinically relevant concentrations, and this is the likely mechanism by which gemfibrozil interact with CYP2C9 substrate drugs, such as warfarin and glyburide. Gemfibrozil may also impair the clearance of CYP2C19 and CYP1A2 substrates, but inhibition of other CYP forms is unlikely.

3. Isoniazid at clinically relevant concentrations reversibly inhibits CYP2C19 and CYP3A4 activities, and mechanistically inactivates CYP1A2, CYP2A6, CYP2C19 and CYP3A4 in human liver microsomes. Co-administration of isoniazid and drugs that are primarily metabolised by these CYP forms, particularly by CYP2C19 and CYP3A4, may result in significant drug-drug interactions.

4. Trimethoprim can be used as selective inhibitors of CYP2C8 in *in vitro* studies. In humans, trimethoprim may inhibit the activities of CYP2C8.

5. Sulfamethoxazole is a selective inhibitor of CYP2C9 *in vitro*. The inhibition of CYP2C9 activity by sulfamethoxazole may be the mechanism involved in the drug-drug interactions between sulfamethoxazole and the substrates of CYP2C9.

6. Addition of Hsa and Hlc to microsomal incubations substantially changed the enzyme kinetic estimates of tolbutamide. The predicted value of the *in vivo* Cl_h of tolbutamide, based on the co-addition of Hsa and Hlc to the incubations, showed good agreement with actual *in vivo* clearance values. However, addition of Hsa and Hlc to the microsomal incubations only slightly altered the *in vitro* inhibitory effect of gemfibrozil on tolbutamide hydroxylase activity when unbound drug concentrations were considered. The present findings suggest that addition of both substrates to the microsomal incubations may yield predictions which are more comparable with the *in vivo* results.

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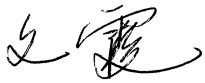
Xia Wen



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