CYTOCHROME P450 ISOFORM-SPECIFIC *IN VITRO* METHODS TO PREDICT DRUG METABOLISM AND INTERACTIONS

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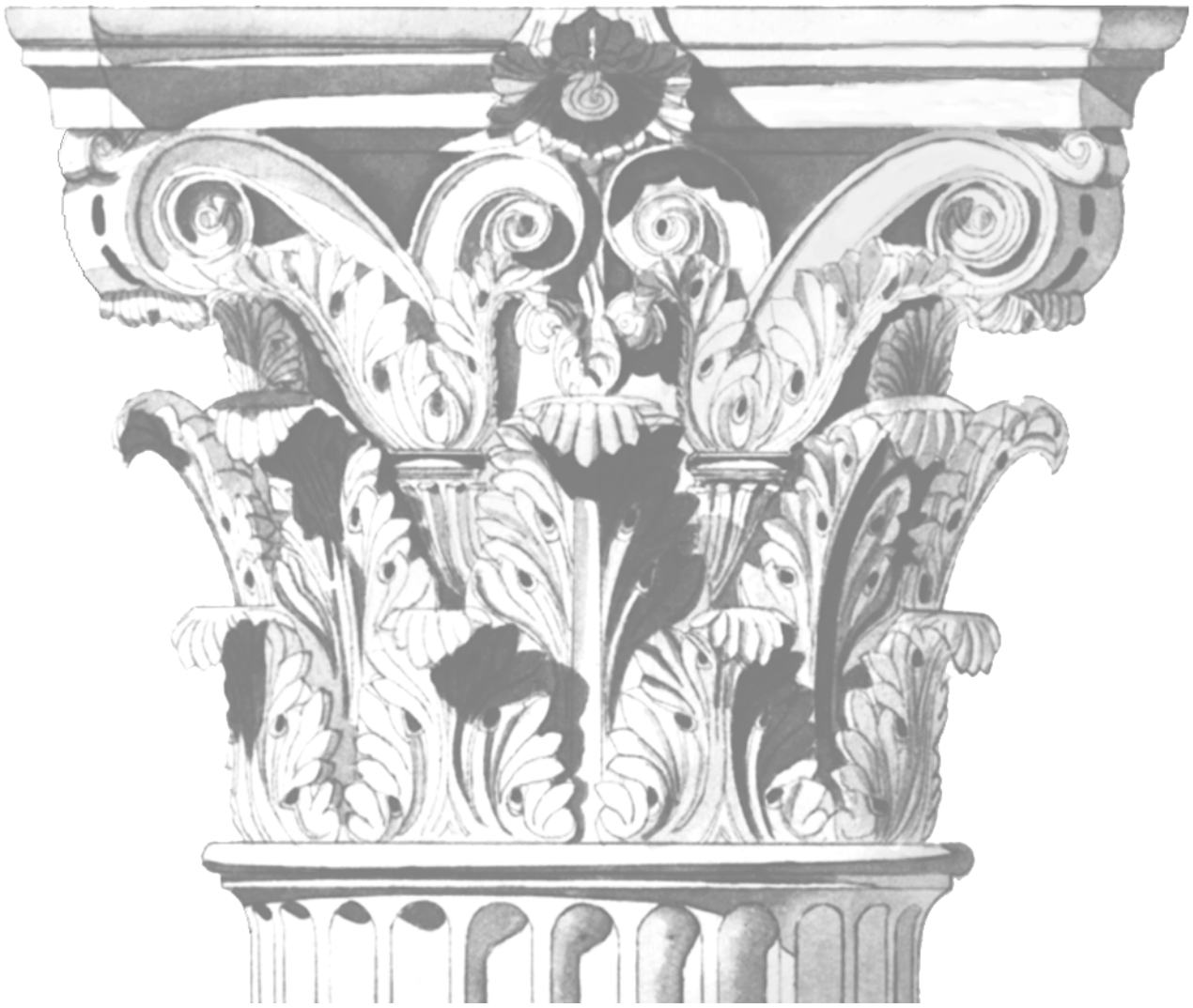

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Abstract

Cytochromes P450 (P450, CYP) are a superfamily of enzymes that participate especially in the oxidative metabolism of various xenobiotics and endogenous compounds.

The major goal of this study was to characterise suitable methods for routine preclinical *in vitro* testing of new chemical entities (NCE) and to test the methods for the affinity screening of selected drugs.

In vitro methods used involve the utilisation of human liver microsomes for studies with P450-selective reference inhibitors, inhibitory antibodies and cDNA-expressed enzymes in cytochrome P450-catalysed activities and for studying the reactions of selegiline and entacapone.

In this project, the CYP-catalysed oxidative *in vitro* biotransformation of selegiline into its primary metabolites desmethylselegiline and *l*-methamphetamine and the transformation of entacapone into its *in vitro* metabolite *N*-desethylentacapone were studied. The affinities of selegiline, desmethylselegiline, *l*-methamphetamine, entacapone, candesartan, eprosartan, irbesartan, losartan and valsartan to P450 enzymes were also elucidated, and the selectivity of tranylcypromine as a CYP2A6-selective reference inhibitor was characterised.

The most important findings were that the methodology developed during this work is suitable for preclinical *in vitro* testing of NCEs and that the results obtained for the studied compounds are in line with the available *in vivo* data.

By the *in vitro* testing methodology, it is possible to target the *in vivo* interaction studies to the relevant groups of compounds. The *in vitro* methods presented in this thesis could also make the early phases of drug development more cost-effective. Further, the number of animals used for *in vivo* testing in preclinical metabolism and interaction studies can be markedly reduced by effectively using this methodology.

Keywords: drug metabolism, cytochrome P450, inhibition, in vitro.

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Abbreviations

ADME	Adsorption, Distribution, Metabolism, Excretion
cDNA	complementary deoxyribonucleic acid
Cl_{int}	intrinsic clearance
CYP	cytochrome P450
DME	drug-metabolising enzyme
ER	endoplasmic reticulum
FMO3	flavin-containing monooxygenase type 3
HPLC	high-performance liquid chromatography
HTS	high-throughput screening
[I]	concentration of inhibitor in enzymatic reaction
IC_{50}	inhibitor concentration decreasing the enzymatic activity by 50% compared to the control reaction
K_i	inhibition constant
K_m	Michaelis-Menten constant for a substrate
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NADPH	nicotine amide diphosphate (reduced form)
NCE	new chemical entity, a drug under development
P450	cytochrome P450
PAH	polycyclic aromatic hydrocarbon
PM	poor metaboliser
r	correlation coefficient
[S]	concentration of substrate in enzymatic reaction
SER	smooth endoplasmic reticulum
$t_{½}$	*in vitro* half life of the compound under study
UDP	uridine diphosphate
V	velocity of metabolite formation in enzymatic reaction
V_{max}	maximal reaction velocity

List of original papers

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Taavitsainen P, Anttila M, Nyman L, Karnani H, Salonen JS & Pelkonen O (2000) Selegiline metabolism and cytochrome P450 enzymes: the *in vitro* study in human liver microsomes. Pharmacology & Toxicology 86: 215-221.

II Taavitsainen P, Kiukaanniemi K & Pelkonen O (2000) *In vitro* inhibition screening of human hepatic P450 enzymes by five angiotensin II receptor antagonists. Eur. J. Clin. Pharmacol. 56: 135-140.

III Taavitsainen P, Juvonen R & Pelkonen O (2001) The *in vitro* inhibition of cytochrome P450 enzymes in human liver microsomes by *trans*-2-phenylcyclopropylamine (tranylcypromine), a potent inhibitor of CYP2A6, and its non-amine containing analog, cyclopropylbenzene. In press.

IV Taavitsainen P. Wikberg T & Pelkonen O (2001) CYP3A4 participates in deethylation of entacapone in human liver microsomes. Manuscript.

Contents

1. Introduction

Xenobiotics are chemical compounds that do not belong to the normal composition of the human body. These compounds enter the body via the diet, air and medication. The principal route of elimination of xenobiotics from the body is biotransformation. They are eliminated by microsomal phase I and microsomal and cytosolic phase II drug-metabolising enzymes. These enzymes add functional groups to make lipophilic molecules more hydrophilic and hence easier to eliminate. The oxidative reactions are mainly catalysed by cytochrome P450 (CYP or P450) enzymes (phase I metabolism) and, after that, by conjugating enzymes (phase II metabolism), such as UDP-glucuronosyl transferases and *N*-acetyl transferases (Wrighton & Stevens 1992; Wrighton *et al.* 1993b). On the other hand, some drugs (called "prodrugs") need to be metabolically activated before they are pharmacologically active. This activation usually occurs via CYP or hydrolytic enzymes.

The CYP superfamily of microsomal hemoproteins catalyses the monooxygenation of a large number of endogenous and exogenous compounds. They play a key role in the metabolism of a wide variety of xenobiotics, such as drugs, pesticides and (pre)carcinogens (Pelkonen & Breimer 1994; Pelkonen *et al.* 1998). The CYP superfamily is divided into families and subfamilies on the basis of their nucleotide sequence homology. Members of the subfamilies exhibit quite strict specificity in metabolising xenobiotics with a wide variety of substrates as a whole family. Some CYPs play a role in both the formation and the elimination of endogenous compounds, while some other CYPs, especially those belonging to the families 1-3, seem to be there principally for xenobiotic metabolism purposes (Nelson *et al.* 1996).

The early knowledge about the metabolism of a new chemical entity (NCE) and its affinity to certain drug-metabolising enzymes helps in the drug development process by providing important information for the selection of a lead compound from among a number of substances pharmacologically equally effective in their therapeutic response (Obach *et al.* 1997). *In vitro* metabolic studies give information about the metabolic stability and possible interactions between compounds that have affinity for the same drug-metabolising enzymes (Pelkonen *et al.* 1998; Wrighton *et al.* 1993b). *In vitro* metabolic studies and affinity screenings have a role in determining the reasonable dose ranges in *in vivo* studies of test species and human volunteers (Obach *et al.* 1997).

When several drugs are used simultaneously or in sequence, there is always a risk of metabolic interactions in case these compounds are metabolised by the same CYP enzyme or one compound affects the metabolism of the other compound (Boobis 1995). Before an NCE becomes a drug candidate and is subjected to clinical trials, it has to be tested for safety and the possibility of drug-drug interactions. These preclinical experiments must be reliable and give relevant information about the studied properties of an NCE. Safety and interactions are, even today, examined to a considerable extent by animal tests. Extrapolation of the results from animal studies to the human situation is usually difficult and contains many sources of errors. The most important reasons for this are the species-specific differences in drug-metabolising enzymes, both qualitative (different metabolic pathways) and quantitative (different intrinsic clearances), between the human being and the test species. The current tendency is to increasingly make *in vitro* preclinical trials, which makes it possible to use human-derived cell organelle fractions, primary cultured cells or cDNA-expressed proteins as a source of drug-metabolising enzymes. This would enable more reliable results with fewer animal trials in the early preclinical phase.

The effects of an NCE on drug-metabolising CYP enzymes can be tested by using CYP isoform-specific model substrates and reactions. The effects of the studied compound on metabolite formation in the selected model system (human liver microsomes, hepatocytes, etc.) are evaluated by incubating the substrate and the studied compound with the enzymes and by observing the metabolite formation in incubations with the studied compound and by comparing it to the formation in incubations without the tested compound (Boobis 1995; Pelkonen *et al.* 1998; Wrighton *et al.* 1993b).

Testing the effects of an NCE on CYP-specific model activities and the effects of CYP-specific reference inhibitors on the metabolism of an NCE in human liver microsomes *in vitro* gives information about the affinity of an NCE for CYP enzymes and permits *in vivo* predictions about the behaviour of the NCE in man (metabolic pathways, intrinsic clearance, etc.), which helps to design *in vivo* studies for revealing possible interactions (Yuan *et al.* 1999). The determination of IC_{50} (the concentration causing 50% inhibition compared to the control activity) and K_i values (the affinity of the compound for the enzyme at the initial velocity conditions) for the studied compound produces information about the inhibitory effect of an NCE on CYP isoforms, and enzyme kinetic studies can be made to evaluate the possibilities of drug-drug interactions (Yuan *et al.* 1999).

Many different models for the prediction of drug metabolism and drug-drug interactions *in vitro* have been introduced recently. One of the best characterised models is the use of the microsomal fraction derived from the human liver tissue samples. The collection of human liver tissue has become easier globally, although there are some practical and especially ethical questions associated with their use. However, studies using human liver preparations produce a “golden standard” for other studies, given naturally their inherent limitations, and they should only be exploited to the extent feasible (Boobis 1995; Guillouzo *et al.* 1995; Pelkonen *et al.* 1998; Yuan *et al.* 1999).

Fig. 1 presents an approximate time course of drug development. As one can see, preclinical studies start at the very beginning of a lead compound selection and continue up to the time of the first phase I clinical studies. Metabolic stability assays employing different test species and human liver make it possible to select species that best represent

the human in the metabolic fate of an NCE. These results can be utilised in, for example, selecting test species for toxicological tests.

The affinities to CYP enzymes and the enzymes that participate in the biotransformation of an NCE are valuable information for the selection of lead compounds and for the planning of early clinical studies. On the basis of *in vitro* studies, a tentative prediction of the clearance and interaction potential of an NCE can be made, and the first clinical studies can be based on these results.

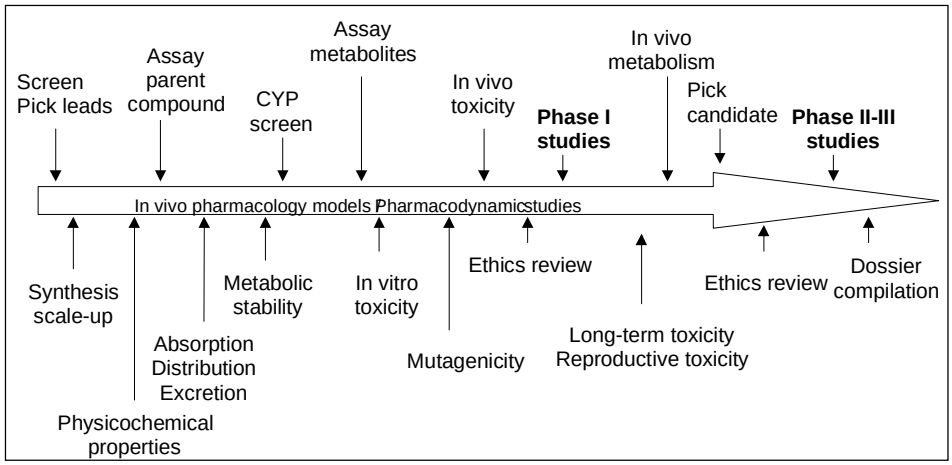


Fig. 1. Time course of the drug development process (by the courtesy of Dr H. Raunio).

2. Review of the literature

2.1. Cytochrome P450 enzymes involved in xenobiotic metabolism

The superfamily of cytochromes P450 (CYPs) consists of microsomal hemoproteins that catalyse the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds. This superfamily is divided into families and subfamilies according to homologies in their nucleic acid sequences (Nelson *et al.* 1996). Most biotransformation of xenobiotics is done by enzymes from the families CYP1, CYP2 and CYP3. Other families are mainly involved in the metabolism of endogenous compounds, such as fatty acids, bile acids, and hormones (Gonzalez 1989). The CYP2 family has been under intensive study using the rat, mouse and rabbit as model systems. The CYP2 family includes seven subfamilies in mammals. In the human, the most important CYPs from the point of view of drug metabolism are CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. The role of CYP2B6 in drug metabolism has not yet been clarified thoroughly. Although it represents only about 1% of the total P450 content in the human liver, there is some evidence to suggest significant participation of CYP2B6 in the metabolism of certain drugs. About 70% of the CYP enzymes in the human liver belong to the families which participate in drug metabolism as determined immunochemically. Of these, CYP3A4 represents about 30% and CYP2C about 20% of the total CYP enzymes. These enzymes are the major P450 forms in human liver microsomes (Shimada *et al.* 1994). Of the CYP2 enzymes, CYP2F1 has not been found to be expressed in adult liver (Hakkola *et al.* 1994), and it seems to be expressed only in lungs (Raunio *et al.* 1995).

The expression of CYP enzymes varies between individuals due to genetic and environmental factors and some diseases. These factors produce inter-individual variation in the rate and metabolic pathways of xenobiotics. One example of genetic factors influencing the inter-individual variation is the polymorphic expression of at least CYP2A6, CYP2C9, CYP2C19 and CYP2D6 among the population. The frequency of poor metabolisers (PMs) varies between races and ethnic groups. Some dietary compounds, cigarette smoking, alcohol and drugs may cause induction or diminution of the expression of certain CYPs (Pelkonen & Breimer 1994; Pelkonen *et al.* 1998).

When several drugs are administered simultaneously, there is always a risk of pharmacokinetic interactions. One drug can modulate the CYP-mediated metabolism of another if the drugs are metabolised by the same enzyme. On the other hand, one drug can inhibit the metabolism of another drug by binding to the same enzyme without being itself metabolised. These interactions can be studied by *in vitro* methods using cultured hepatocytes or subcellular organelles derived from the human liver (Boobis 1995; Pelkonen *et al.* 1998).

Experimental animals represent genotypically and phenotypically homogenous populations; i.e. they do not exhibit large inter-individual variation in the activities of drug-metabolising enzymes, which is typical of the human population. The use of animal-derived *in vitro* models in preclinical drug research is restricted by the fact that the human and test species often employ different enzymes for the same metabolic pathway, and even orthologous enzymes usually show quantitative and qualitative differences (Pelkonen & Breimer 1994). Therefore, the evaluation of human tissue-derived *in vitro* systems is of importance.

Table 1 summarises the xenobiotic-metabolising human hepatic CYPs. The relative amounts of P450 proteins in liver are highly variable among people.

Table 1: Summary of xenobiotic-metabolising human hepatic CYPs.

CYP	Relative amount in liver (%)	Substrates (reaction in parenthesis)	Selective inhibitors	Other characteristics
1A2	~10	Ethoxyresorufin (*O*-deethylation) Phenacetin (*O*-deethylation)	Furafylline	Inducible
2A6	~10	Coumarin (7-hydroxylation)		Polymorphic
2B6	~1	*S*-Mephenytoin (*N*-demethylation)	Orphenadrine	
2C8	<1	Paclitaxel (6α-hydroxylation)	Quercetin	
2C9	~20	Tolbutamide (methyl hydroxylation) Diclofenac (hydroxylation) *S*-Warfarin (7-hydroxylation)	Sulfaphenazole	Polymorphic
2C19	~5	*S*-mephenytoin (4'-hydroxylation) Omeprazole (oxidation)		Polymorphic
2D6	~5	Dextromethorphan (*O*-demethylation) Debrisoquine (4-hydroxylation) Bufuralol (1'-hydroxylation)	Quinidine	Polymorphic
2E1	~10	Chlorzoxazone (6-hydroxylation) Aniline (4-hydroxylation)	Pyridine	Inducible
3A4	~30	Midazolam (1'- and 4-hydroxylation) Testosterone (6β-hydroxylation) Nifedipine (dehydrogenation)	Azole antimycotics	Inducible

Data adapted from Pelkonen & Breimer (1994) and Pelkonen *et al.* (1998; 2000).

Table 2. The structures of commonly used CYP-specific substrates and inhibitors.

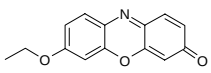
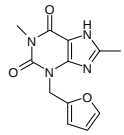
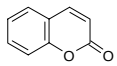
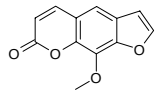
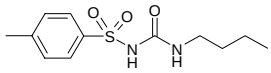
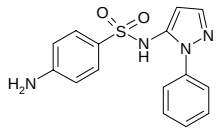
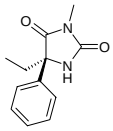
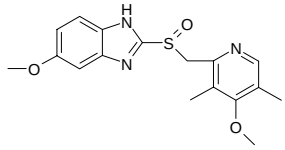
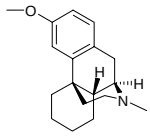
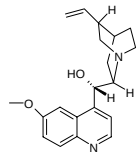
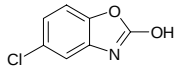
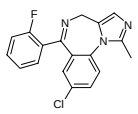
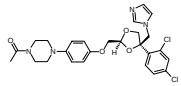
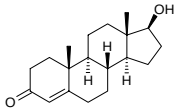
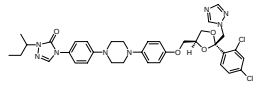
Enzyme	Substrate	Inhibitor
CYP1A2	ethoxyresorufin 	furafylline 
CYP2A6	coumarin 	methoxsalen 
CYP2C9	tolbutamide 	sulfaphenazole 
CYP2C19	*S*-mephenytoin 	omeprazole 
CYP2D6	dextromethorphan 	quinidine 
CYP2E1	chlorzoxazone 	pyridine 
CYP3A4	midazolam 	ketoconazole 
	testosterone 	itraconazole 

Table 2 presents the chemical structures of commonly used CYP-selective *in vitro* substrates and inhibitors.

2.1.1. CYP1A subfamily

The human CYP1A subfamily consists of two members, CYP1A1 and CYP1A2. CYP1A2 is mainly expressed in the liver, while CYP1A1 is primarily expressed in extrahepatic tissues. CYP1A1 is induced by cigarette smoking and polycyclic aromatic hydrocarbon (PAH) -type inducers in extrahepatic tissues, mainly the lung and placenta. In the liver, CYP1A1 is expressed at a very low level (Guengerich 1991; McKinnon *et al.* 1991; Pasanen & Pelkonen 1994; Raunio *et al.* 1995; Pelkonen *et al.* 1998). CYP1A1 has activity towards some PAHs (Guengerich 1991). The participation of CYP1A1 in drug metabolism *in vivo* is not considered here.

The constitutively expressed CYP1A2 in human liver is inducible by, for example, environmental compounds, such as constituents of cooked meat, cruciferous vegetables, cigarette smoke, PAHs and polychlorinated biphenyls (Adams *et al.* 1997). The metabolic activation of some arylamines, such as 4-aminobiphenyl, 2-aminoanthracene and 2-naphthylamine, is done by CYP1A2 (Guengerich 1991).

CYP1A2 represents about 15% of total CYP enzymes in the human liver (Pelkonen and Breimer 1994; Shimada *et al.* 1994). There is some variability between individuals in the CYP1A2 enzyme levels in the human liver (Shimada *et al.* 1994). Because of the large interindividual variation in CYP1A2 activities, the *in vivo* testing of this enzyme has been quite difficult to evaluate (Kunze and Trager 1993). CYP1A2 substrates among the currently used drugs include theophylline, caffeine, olanzapine, ondansentron, paracetamol, phenacetin and propranolol. Caffeine and theophylline have been considered *in vivo* diagnostic probes. The usually employed *in vitro* substrate is ethoxyresorufin (Table 2), which seems to be quite specific to CYP1A2 (Burke *et al.* 1985). The substrates and their enzyme kinetic parameters have recently been reviewed by Pelkonen *et al.* (1998).

Furafylline (Table 2) and fluvoxamine have been the most widely used *in vitro* diagnostic inhibitors. Furafylline is a potent and selective mechanism-based inactivator of CYP1A2 (Sesardic *et al.* 1990), but it has no function as an *in vivo* inhibitor because of its interactions with caffeine (Tarrus *et al.* 1987).

Fluvoxamine is also used as an *in vivo* diagnostic inhibitor for CYP1A2 (Brosen *et al.* 1993; Rasmussen *et al.* 1995). Fluvoxamine is known to inhibit moderately CYP2D6 and CYP2C19, though at a higher concentration range than CYP1A2 (Jeppesen *et al.* 1996), but not CYP1A1, CYP2A6, CYP2E1 or CYP3A4 *in vitro*. Venkatakrishnan and co-workers have shown that fluvoxamine inhibits CYP2C19 equally potently as CYP1A2 in lymphoblast-expressed human CYPs and, to a slightly lesser extent, CYP2C9 and CYP3A4 (von Moltke *et al.* 1995; 1996; Venkatakrishnan *et al.* 1999).

2.1.2. CYP2A subfamily

In humans, there are three genes in the CYP2A subfamily: *CYP2A6* codes the enzyme catalysing coumarin 7-hydroxylation (about 10% of total P450), while the product of *CYP2A7* is inactive and *CYP2A13* is not expressed in the liver (Pelkonen *et al.* 2000). In the olfactory mucosa, CYP2A13 is highly expressed as a catalytically active protein (Gu *et al.* 2000). Recently, CYP2A6 has been reported to be polymorphically expressed in the human liver (Oscarson *et al.* 1998). No endogenous substrate for CYP2A6 has been found.

It has been recently shown that CYP2A6 participates in the metabolism of nicotine (Cashman *et al.* 1992; Nakajima *et al.* 1996b) and its metabolite cotinine (Nakajima *et al.* 1996a). This enzyme also catalyses the metabolic activation of several procarcinogens and promutagens, such as aflatoxin B_1 (Yun *et al.* 1991; Salonpää *et al.* 1993) and several nitrosamines (Yamazaki *et al.* 1992; Tiano *et al.* 1994). Some drugs and chemicals, including halothane and coumarin (Table 2) – which is widely used as a probe substance for CYP2A6 both *in vitro* and *in vivo* - are also metabolised by this enzyme (Rendic & Di Carlo 1997; Pelkonen *et al.* 1998).

Draper and coworkers (1997) have extensively studied the commonly employed P450 substrates and inhibitors with regard to their effect on CYP2A6-catalysed coumarin 7-hydroxylation. They also screened the effects of some commonly used solvents on the same reaction. They found that many of the studied chemicals were inhibitors of CYP2A6, although earlier presumed to be selective for some other CYPs. For example, ketoconazole (Table 2) is used as a CYP3A4-specific inhibitor, but it was also found to inhibit CYP2A6 quite potently. Also, many commonly used organic solvents inhibit coumarin 7-hydroxylation at substrate concentrations near K_m.

More substrates and inhibitors currently known to be metabolised by or to interact with CYP2A6 *in vitro* and *in vivo* have been summarised by Pelkonen *et al.* (2000).

2.1.3. CYP2B subfamily

CYP2B6 is the only active member of the CYP2B subfamily in man, although the *CYP2B7* gene has also been found in the genome (Czerwinski *et al.* 1994). For a long time, it was thought that CYP2B6 would not be expressed in every human liver. For example, Mimura *et al.* (1993) assessed 50 human liver samples by immunoblotting analysis with antimonkey P4502B antibody for CYP2B6 and found only about ¼ of the livers to contain the protein (see also Yamano *et al.* 1989 and Shimada *et al.* 1994). The highest level of CYP2B6 found by Mimura and co-workers was <2% of the total P450. Today, several groups have shown that this enzyme is expressed in every human liver, but the levels of expression differ between individuals (Ekins *et al.* 1997; Stresser & Kupfer 1999). The amount of CYP2B6 protein seems to be below 1% of the total P450 present in the human liver, and the reason for the earlier reports about non-expressing individuals is probably the weak sensitivity of either the antibodies used or the detection methods. Today, there are very sensitive and specific anti-peptide antibodies available for

quantitative immunological studies (Stresser & Kupfer 1999). Similarly to the other studies on CYP2B6, they showed the expression of this enzyme to be highly variable.

CYP2B6 has been postulated to be co-regulated with CYP3A4 or at least to be inducible by the same inducers, such as rifampicin and dexamethasone, as well as by phenobarbital in cultured human hepatocytes (Strom *et al.* 1996).

The catalytic activity of CYP2B6 has recently been under efficient research by many groups. Especially Ekins and coworkers (1997, 1998) have evaluated several chemicals and drugs as specific substrates for this enzyme. Among the suggested CYP2B6-catalysed reactions are *O*-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), *S*-mephenytoin *N*-demethylation, stereoselective hydroxylation of RP 73401 [3-cyclopentyloxy-*N*-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide] to RPR 113406 (Stevens *et al.* 1997) and bupropion hydroxylation (Faucette *et al.* 2000). The availability of the other selective substrate presented above, RP 73401 and its CYP2B6-formed metabolite RPR 113406 prepared by Rhõne-Poulenc Rorer Co., is unknown. Recently, Kobayashi *et al.* (1999) evaluated the role of CYP2B6 in *S*-mephobarbital *N*-demethylation by using 10 cDNA-expressed human CYPs. They found that only CYP2B6 catalysed the reaction. Also, the activity correlated strongly with the immunodetectable CYP2B6 levels in microsomes from 10 individual human livers. This activity is probably quite usable if the substrate, *S*-mephobarbital, could be easily separated from its *R*-enantiomer. The metabolite, phenobarbital, is commonly used as a P450-inducing agent (Pelkonen *et al.* 1998).

Orphenadrine has been widely used as an inhibitor of CYP2B6, though its selectivity towards this isoform is questionable (Murray & Reidy 1990; Chang *et al.* 1993; Ekins *et al.* 1997; Guo *et al.* 1997). Although actively searched, no selective inhibitor for CYP2B6 has been found yet.

2.1.4. CYP2C subfamily

The CYP2C subfamily is the second most abundant CYP protein in the human liver, representing about 20% of the total P450 (Shimada *et al.* 1994). This subfamily consists of three active members in the human liver, namely CYP2C8, CYP2C9, and CYP2C19. Of these, CYP2C9 and CYP2C19 have been characterised to be polymorphically expressed (Goldstein & de Morais 1994; Gill *et al.* 1999).

CYP2C8 has been thought not to play an important role in drug metabolism since it is expressed at very low levels in the human liver. Still, the new, very potent anti-cancer drug, taxol, for example, is partly metabolised by CYP2C8 (Harris *et al.* 1994; Sonnichsen *et al.* 1995). CYP2C8 also participates in the metabolism of the endogenic agents retinol and retinoic acid (Leo *et al.* 1989). The involvement of this member of the CYP2C subfamily in the metabolism of NCEs has been difficult to study because there is no good, specific substrate and inhibitor for this isoform. In many cases, CYP2C8 also participates, to a small extent, in the metabolism of the CYP2C9 substrates (Wrighton *et al.* 1993b).

CYP2C9 is the major CYP2C isoform in the human liver (Goldstein & de Morais 1994), and it has been shown to be genetically polymorphic with at least three different

alleles that produce differently active protein. The functional consequences of these polymorphisms are not yet clear, although CYP2C9 has a major role in the metabolism of many clinically important, weakly acidic drugs, such as *S*-warfarin (Rettie *et al.* 1992), tolbutamide (Table 2), phenytoin (Doecke *et al.* 1991), sulphamethoxazole (Cribb *et al.* 1995) and many of the non-steroidal anti-inflammatory compounds (Leeman *et al.* 1993) as well as the new drug in this class, a cyclooxygenase-II selective inhibitor, celecoxib (Tang *et al.* 2000). The frequencies of the two variant alleles, *CYP2C9*2* and *CYP2C9*3*, have been reported to range from 7 to 19% in Caucasian populations (Furuya *et al.* 1995; Sullivan-Klose *et al.* 1996; Stubbins *et al.* 1996).

With CYP2C19, the genetic polymorphism leads to the poor metaboliser (PM) phenotypes exhibiting less active or completely inactive *S*-mephenytoin 4'-hydroxylase (Table 2). This PM phenotype is produced by at least two major and several minor variant alleles of CYP2C19 (Goldstein & de Morais 1994) and, consequently, CYP2C19 substrates are not metabolised as expected (Pelkonen *et al.* 1998). This may lead to accumulation of the drug and to *in vivo* concentrations exceeding the therapeutic level and producing unexpected toxic effects. The search for CYP2C19-selective inhibitor both *in vitro* and *in vivo* is undeer way, since omeprazole (Table 2), which is usually employed, also inhibits other CYPs (Funck-Brentano *et al.* 1997). The deficiency of the 4'-hydroxylation pathway of *S*-mephenytoin occurs in 2 to 5% of the Caucasian population (Relling *et al.* 1990).

2.1.5. CYP2D subfamily

The human genome includes only one functional gene in the CYP2D subfamily, namely *CYP2D6* (Nelson *et al.* 1996). There also exist two *CYP2D7* pseudogenes and two pseudogenes of *CYP2D8* (Heim & Meyer 1992; Nelson *et al.* 1996). More than 60 allelic variants have been reported for *CYP2D6* (Streetman *et al.* 2000; see: http://www.imm.ki.se/CYPalleles). Of these, most affect the activity of the expressed protein. CYP2D6 represents 1 to 5% of the total P450 (Pelkonen & Breimer 1994; Shimada *et al.* 1994; Pelkonen *et al.* 1998), and 7-8 % of the Caucasian population are PMs for this enzyme (Heim & Meyer 1992).

The search for endogenous substrates for CYP2D6 has recently become more interesting because it has been shown that CYP2D6 is associated with Parkinson's disease (Barbeau *et al.* 1985; Armstrong *et al.* 1992; Smith *et al.* 1992; Agúndez *et al.* 1995) and with susceptibility to develop liver and lung cancer (Idle 1981; Ayesh *et al.* 1984; Agúndez *et al.* 1994; Caporaso *et al.* 1995; Bouchardy *et al.* 1996). Martínez *et al.* (1997) suggested a neurotransmitter, tryptamine, as an endogenous substrate for CYP2D6. The metabolism of tryptamine into tryptophol was catalysed in a NADPH-dependent manner and inhibited by a specific CYP2D6 inhibitor, quinidine (Table 2).

One common feature of CYP2D6 substrates is that they contain at least one basic nitrogen atom at a distance of 5 or 7Å from the oxidation site. Secondly, there is a planar hydrophobic area near the oxidation site, and thirdly, the substrates exhibit a negative molecular electrostatic potential above the planar part of the molecule (Koymans *et al.* 1992; Strobl *et al.* 1993; de Groot *et al.* 1997). The drugs known to be substrates for this

enzyme include antiarrhythmic and other cardiovascular drugs, β-adrenergic blocking agents, tricyclic antidepressants, neuroleptics and many other commonly used therapeutical agents (Cholerton *et al.* 1992). The mostly used *in vitro* model reactions are dextromethorphan *O*-demethylation (Table 2), debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation. Of these substrates, debrisoquine is also employed in *in vivo* studies as a CYP2D6 model substance, although its use is becoming more difficult because it is not marketed any more (Pelkonen *et al.* 1998, and references therein).

As a reference inhibitor for CYP2D6, quinidine is widely used in drug metabolism studies. It is a specific and potent inhibitor with a K_i value of 0.06 μM for CYP2D6, and the CYP next in sensitivity, CYP3A4, has a K_i value around 10 μM (Broly *et al.* 1989; Bourrie *et al.* 1996).

Due to the fact that numerous drugs on the market are metabolised by CYP2D6 and the polymorphic nature of the expression of this protein, it is strongly suggested that the affinities of the NCEs on this CYP form should be characterised to predict and to possibly avoid drug-drug interactions.

2.1.6. CYP2E subfamily

The coding region of *CYP2E1* is highly conserved in different ethnic groups and species. There are significant interethnic differences in polymorphisms, but there is no clear evidence of any of the reported polymorphisms to be related to altered *in vivo* activities. All the reported polymorphisms are found in the noncoding regions of the gene, suggesting an important role in the biotransformation of endogenous substances (Ronis *et al.* 1996; Yin *et al.* 1997).

The CYP2E1 expression is regulated by many factors. For example, fasting elevates the level of the protein by increasing the transcription of the gene (Johansson *et al.* 1990) and diabetes by stabilising the mRNA (Song *et al.* 1987), while isoniazid increases the translation efficiency (Park *et al.* 1993) and affects the enzyme stabilisation similarly to ethanol and imidazole (Eliasson *et al.* 1990).

The CYP2E1 substrates include very few clinically important drugs. Actually, only paracetamol (Patten *et al.* 1989), caffeine (Gu *et al.* 1992), chlorzoxazone (Peter *et al.* 1990; Table 2) and enflurane (Thummel *et al.* 1993) are worth mentioning here. Most organic solvents and anesthetics, short-chain alcohols and many nitrosamine and azo carcinogens (Hong & Yang 1985; Koop 1992; Sohn *et al.* 1991; Yang *et al.* 1985) are also xenobiotic substrates of this enzyme. CYP2E1 participates in the metabolism of many endogenous substances, such as lipid peroxidation products (Terelius & Ingelman-Sundberg 1986), ketones (Koop & Cassazza 1985) and fatty acids, such as linoleic and arachidonic acids (Laethem *et al.* 1993). A number of other small-molecular CYP2E1 substrates are listed in the review article by Ronis *et al.* (1996).

Pyridine (Table 2) is a specific *in vitro* inhibitor and inhibits CYP2E1 at relatively low concentrations (Hargreaves *et al.* 1994; Taavitsainen *et al.*, unpublished results). The metabolite of disulfiram, diethyldithiocarbamate, has also been used as an *in vitro* inhibitor (Guengerich *et al.* 1991; Brady *et al.* 1991), though its selectivity is questionable (Yamazaki *et al.* 1992).

2.1.7. CYP3A subfamily

The CYP3A subfamily represents about 30% of the total P450 content in the human liver (Shimada *et al* 1994; Pelkonen and Breimer 1994), although the levels of the protein may vary 40-fold among individuals (Guengerich 1995a). This subfamily consists of three members (Nelson *et al.* 1996): CYP3A4 is the most abundant CYP enzyme in the human liver and it is expressed in several tissues, but the expression in the liver and in the small intestine is of major interest in view of the metabolism of drugs and other xenobiotic chemicals (Guengerich 1999), CYP3A5 is a minor, polymorphic CYP3A enzyme, which is expressed in the lungs (Kivistö *et al.* 1996; Anttila *et al.* 1997) and the colon (Gervot *et al.* 1996). About 20% of individual adults express CYP3A5 at a high level in the liver (Guengerich 1999). CYP3A7 is expressed in the fetal liver and in the adult endometrium and placenta (Schuetz *et al.* 1993; Hakkola *et al.* 1994).

The members of the CYP3A subfamily have overlapping catalytic specificities, but some selectivity exists. CYP3A4 participates in the metabolism of about half of the drugs in use today (Bertz and Granneman 1997). For example, testosterone 6β-hydroxylation (Table 2), midazolam 1'- and 4-hydroxylations (Table 2), nifedipine oxidation, and erythromycin *N*-demethylation are catalyzed by this enzyme. The known substrates of CYP3A4 vary in size from small molecules, such as acetaminophen (M_r 151), to cyclosporin A (M_r 1201) (Guengerich 1999). In addition to the substrates listed above, we could further mention physiologically important progesterone and andostenedione (Waxman *et al.* 1991), cortisol, quinidine, diltiazem, lidocaine, lovastatin, troleandomycin, warfarin, and triazolam (Guengerich and Shimada 1991; Wrighton and Stevens 1992).

CYP3A is inducible by many drugs, for example, rifampicin, dexamethasone, carbamazepine and phenobarbital type inducers (Pelkonen *et al.* 1998). The induction of CYP3A has an effect on interindividual variation and affects both bioavailability and drug-drug interactions (Guengerich 1999).

The inhibitors of CYP3A have a wide variety of chemical structures. For example, azole-type fungicides, ketoconazole and itraconazole are potent inhibitors. Ketoconazole (Table 2) also inhibits other CYPs than CYP3A4, but at a concentration of 1 μM it is relatively selective for CYP3A4 (Newton *et al.* 1994; Baldwin *et al.* 1995). Gestodene, a progesterone analog with a steroid structure has been long known as a mechanism-based CYP3A inhibitor (Pelkonen *et al.* 1998). Gestodene is also a substrate for CYP3A4.

The substrate specificity and catalytic features of CYP3A4 have recently been a target of active research. It has been shown by many groups that the properties of CYP3A4 have not yet been thoroughly elucidated (Ueng *et al.* 1997; Korzekwa *et al.* 1998; Wang *et al.* 2000). For example, the role of cytochrome b_5, divalent cations and the rate-limiting steps in the catalysis are still under study (Guengerich 1999). Wang *et al.* (2000) studied the *in vitro* drug-drug interaction patterns with four commonly used CYP3A4 substrates. The results indicated that CYP3A4 is a very complex enzyme and that its interaction patterns are substrate-dependent.

Thanks to the unique properties of CYP3A4, the enzymatic processes catalysed by CYP3A4 do not always follow the typical competitive inhibition kinetics. A substrate can either inhibit or stimulate the *in vitro* metabolism of another substrate, or activate its own metabolism (Shou *et al.* 1994). The kinetics can be either cooperative or allosteric,

depending of the binding sites of the two substrate/inhibitor molecules or one molecule of two substrates each or one molecule of the substrate and an effector (Shou *et al.* 1994; Ueng *et al.* 1997; Korzekwa *et al.* 1998; Guengerich *et al.* 1999; Wang *et al.* 2000).

Although the cooperativity in CYP3A4-catalysed reactions has been known for a long time, there has not been any major progress in this field. The great majority of studies suggest that there are at least two distinct binding sites for the substrate and the effector. The location of the effector binding site – whether at the active site or at a separate allosteric site – is not yet known (Ueng *et al.* 1997). Harlow and Halpert (1998) presented a model for cooperativity which suggests that the substrate and effector molecules bind at adjacent sites and are both part of a large binding cavity. This model agrees with the one presented by Shou *et al.* (1994), with the exception that they suggested two catalytic sites both with access to the reactive oxygen.

Based on these results, it is concluded that the active site of CYP3A4 is very large and flexible, which postulation is supported by the wide substrate selectivity. Shou and co-workers (1994) also suggested that the activator, when binding to the active site, excludes water molecules from the substrate cavity and prevents hydrogen peroxide release. These models combined could together explain the atypical CYP3A4 kinetics, including activation, autoactivation, mutual inhibition, partial inhibition, substrate inhibition, biphasic saturation curves and alteration of regio-specificity.

Still, although much effort has been invested in CYP3A4 research, there may still be many properties of this enzyme that need to be studied. One should bear in mind the likelihood that because CYP3A4 plays such an important role in xenobiotic metabolism, its catalysis may not be very simple in nature. The conclusions drawn on the basis of only one substrate or inhibitor should be regarded as tentative.

2.2. Methods for studying *in vitro* metabolism of NCEs

2.2.1. Overview of preclinical studies

Preclinical studies consist of animal studies (on the pharmacokinetics and pharmacodynamics of the compound, toxicological studies) and animal and human tissue-derived *in vitro* studies. Because of the problems in extrapolating the results of animal studies to humans, various *in vitro* methods have been developed by employing human tissue-derived systems (Wrighton *et al.* 1993). Also, the authorities have begun to demand increasingly that the issues concerning metabolism and toxicity in test species compared to humans should be actively clarified in early preclinical tests. This is done by utilising liver preparations from humans and trying to find the test species that most closely resemble human metabolism and the production of toxic intermediates (Yuan *et al.* 1999).

It is important to elucidate the *in vitro* metabolism and the putative interactions at the time of planning other preclinical and early clinical studies. In the next few chapters, human tissue-derived preclinical *in vitro* systems will be briefly discussed.

2.2.2. Human-derived in vitro *systems in preclinical drug metabolism research*

There are several approaches to preclinical metabolism studies. The enzyme sources in these studies are human-derived systems currently under rapid development and evaluation. These systems consist of liver microsomes, hepatocytes and cell lines heterologously expressing drug-metabolising enzymes, liver slices and individually cDNA-expressed enzymes in host cell microsomes. Each of these will be discussed briefly here. Table 3 shows a comparison of different human-derived *in vitro* methods.

2.2.2.1. Human liver microsomes

Human liver microsomes are fractionated from subcellular organelles by differential ultracentrifugation. Microsomes are formed from smooth endoplasmic reticulum during tissue homogenisation (Boobis 1995). A microsomal fraction from human liver contains a full complement of P450 enzymes, which makes it a suitable tool for studying inhibitory interactions and CYP-catalysed metabolite formation (Kremers 1999). By employing relevant cofactors and other reaction components, one can readily investigate and distinguish between P450s, flavin-containing monooxygenases (FMO) and glucuronosyl transferases. Microsomes are relatively easy to prepare, and enzymatic activities are stable during prolonged storage (Beaune *et al.* 1986; Yamazaki *et al.* 1997), if the original tissue is correctly handled and frozen immediately after excision (Kremers 1999). The validation and harmonisation of assay procedures between different laboratories in this respect, too, would lead to less inter-laboratory variation in the same assays (Boobis *et al.* 1998; Kremers 1999).

2.2.2.2. Human hepatocytes

Hepatocytes contain the full compartment of phase I and phase II enzymes, and the whole metabolite pattern can therefore be detected in incubations with hepatocytes. Other effects of an NCE, such as the induction of drug-metabolising enzymes and possible toxic effects, can also be elucidated. (Ferrini *et al.* 1997; Li *et al.* 1997; Maurel 1996; Morel *et al.* 1990).

The utilisation of human primary hepatocytes is restricted, because sufficient liver samples are quite difficult to obtain and hepatocytes are difficult to preserve for later use. Some successful attempts to cryopreserve primary hepatocytes have been described (Guillouzo *et al.* 1995). A prolonged culture method has also been published, in which hepatocytes are maintained for over 30 days (Kono *et al.* 1997), preserving some of their drug-metabolising activities and inducibility instead of the standard for up to one week. Hepatocytes can be subjected to several test and washout periods during their lifetime. This method, together with the cryopreservation method, enables more efficient use of a single hepatocyte batch than before.

Table 3. Comparison of in vitro *enzyme sources used in preclinical research.*

Enzyme sources	Availability	Advantages	Disadvantages
Microsomes	Relatively good, from transplantations or commercial sources.	Easy to obtain. Also commercially available. Relatively inexpensive technique.	Contains only phase I DMEs and UDP-glucuronosyl transferases. Requires strictly specific substrates and inhibitors or antibodies for individual DMEs.
cDNA-expressed individual CYPs	Good, commercially available.	Can be utilised with HTS substrates. The role of individual CYPs in the metabolism of an NCE can be easily studied.	The effect of only one enzyme at a time can be evaluated.
Immortalised cell lines	Available at request, not many adequately characterised cell lines exist.	Non-limited source of enzymes.	The expression of most DMEs is poor or absent if characterised at all.
Primary hepatocytes	Relatively difficult to obtain, relatively healthy fresh tissue needed. Commercially available. Cryopreservation possible.	Contains the whole complement of DMEs. The induction effect of an NCE can be studied.	Requires specific techniques and well established procedures. The levels of many DMEs decrease rapidly during cultivation.
Liver slices	Relatively difficult to obtain, fresh tissue needed. Cryopreservation possible.	Contains the whole complement of DMEs and cell-cell connections. The induction effect of an NCE can be studied.	Requires specific techniques and well established procedures.

Data partially adapted from Wrighton *et al.* (1993) and Skett *et al.* (1995).

2.2.2.3. Permanent cell lines

Immortalised cell lines that express one or more drug-metabolising enzymes could be valuable tools in preclinical studies, but for most CYPs this approach has failed (Daujat *et al.* 1996; Gonzalez & Korzekwa 1995). When whole-cell systems are used, one must know which P450s are really expressed as functional proteins and which are liable to fade out during cultivation. Many of the cell lines used lack the complement of enzymes

present in hepatocytes *in vivo*. On the other hand, when studying direct interactions with the enzyme, whole cell systems could be too complex (Boobis 1995).

2.2.2.4. Liver slices

Of the systems presented here, precision-cut liver slices resemble most closely the *in vivo* situation, which contains not only the enzymes of the whole liver but also the connections between individual cells. The technique of maintaining liver slices is demanding. The thickness of a slice has to be minimal within the limits of the optimal number of cell layers and oxygen and nutrient transportation (Vickers *et al.* 1995). Recently, methods for cryopreservation of tissue slices have been developed (for example Fisher *et al* 1993; Ekins *et al.* 1996; de Kanter *et al.* 1998; Glöckner *et al.* 1998; de Graaf *et al.* 2000). These methods allow more flexible use of slices, because viability and CYP activities seem to be preserved quite well (de Graaf *et al.* 2000; Renwick *et al.* 2000).

2.2.2.5. cDNA-expressed enzymes

Drug-metabolising enzymes are available commercially as heterologously expressed enzyme systems. In these preparations, an individual enzyme is produced in the ER of an eucaryote host cell. The expression of human liver CYPs in different artificial systems has become easier due to the rapid development of recombinant DNA techniques (Gonzalez *et al.* 1991). The systems employed for the production of cDNA-expressed CYPs include bacteria (Fisher *et al.* 1992; Gillam *et al.* 1993), yeast (Guengerich *et al.* 1991a; Peyronneau *et al.* 1992), mammalian cell lines (Guengerich 1995b) and baculovirus systems (Asseffa *et al.* 1989). cDNA-expressed enzymes are a valuable tool in the search for the enzymes participating in the metabolism of an NCE. Because the enzymes are studied in isolation from other hepatic enzymes and because they lack the whole complement of hepatic enzymes, the *in vivo* predictive value of the data obtained from heterologously expressed enzyme systems has been debated (Rodriguez 1999).

If the inhibition of the metabolism of an unknown compound is studied in a single enzyme system, one should take into account that *in vivo* there are different amounts of individual enzymes in the human liver. The cofactor supply may also affect the relative contribution of certain CYPs. As it was pointed out above, the contribution of one enzyme to the specific metabolic route may not be so significant as it seems on the basis of cDNA-expressed enzymes (Rodriguez 1999).

As an affinity-screening tool, cDNA-expressed enzymes are valuable. It is also the most useful system allowing a high-throughput screening (HTS) technology for P450 studies today, because of the difficulties and high costs in the detection of multiple substrates and metabolites produced in the HTS applications of other techniques, such as human liver microsomes (White 2000). The detection of these multiple metabolites requires novel, highly sensitive mass spectrometry tools, whereas cDNA-expressed

systems can utilise the conventional measurement of fluorescence metabolite production for multiple enzymes (see, for example, www.gentest.com).

2.2.3. Measures of metabolism in **in vitro** *systems*

2.2.3.1. Metabolic stability of an NCE

The metabolic stability of an NCE determines, to a great extent, its future as a drug candidate (see, for example, Kuhnz & Gieschen 1998). If an NCE is rapidly metabolised in human liver preparations, its bioavailability *in vivo* is most probably too low for it to be a drug candidate. This naturally depends on the administration route of the drug. By determining the time and concentration dependence of metabolite formation from an NCE on the disappearance of the NCE *in vitro* in an appropriate system, its metabolic fate and half-life *in vivo* can be predicted. Similar studies performed in human and test species give valuable information for the selection of test species for pharmacokinetic and toxicological *in vivo* studies.

2.2.3.2. Identification of metabolites and metabolic routes

Today, there are effective methods for metabolite identification and subsequent construction of metabolic routes. Metabolite identification can be developed from incubations with human liver preparations, homogenates or microsomes (Kremers 1999). For example, mass-spectrometric (MS) methods employing HPLC as a separative tool have evolved into extremely sensitive and accurate techniques. By these methods, it is possible to determine with high accuracy the exact molecular masses and metabolite structures. As analytical methods, these techniques are at their best in skilful and highly experienced hands.

Sample preparation for MS studies is a critical step, since the available chemical information of an NCE is often limited. The incubation conditions and the reaction-terminating reagent have to be chosen so as not to alter the parent compound or the metabolites chemically and to keep the recovery of the substrate and the metabolites close to 100%. Otherwise, it is impossible to predict the pathways for biotransformation.

2.2.3.3. Identification of CYPs metabolising an NCE

After characterising the metabolic stability and metabolic routes of an NCE, the *in vivo* prediction requires clarification of the drug-metabolising enzymes that participate in the *in vitro* biotransformation of the NCE. The methods usually employed for this purpose will be presented below.

After determining the initial velocity conditions and enzyme kinetic parameters, the CYPs involved in the metabolism of an NCE can be characterised by chemical and antibody inhibitors selective or specific for respective CYPs. It is important to select the substrate concentration correctly: near or preferably below the determined K_m values, if the analytical method for metabolite detection allows it. In case the *in vivo* concentrations are known, it is recommendable to use substrate concentrations close to the therapeutic level, if at all feasible.

2.2.3.4. Utilisation of CYP-selective chemical inhibitors

Numerous compounds have been characterised for their inhibitory potency against different CYPs. Many of them are selective for only the desired enzyme at relatively low concentrations, as for example, furafylline for CYP1A2 (Sesardic *et al.* 1990; Clarke *et al.* 1994; Bourrie *et al.* 1996; Racha *et al.* 1998); CYP2C9 is selectively inhibited by sulfaphenazole (Bourrie *et al.* 1996); quinidine is potent inhibitor for CYP2D6 though metabolised *via* CYP3A4 (Bourrie *et al.* 1996); pyridine seems to be quite selective for CYP2E1 (Hargreaves *et al.* 1994; own unpublished results), and there are many selective inhibitors for CYP3A4, of which ketoconazole is the most widely used, although it also affects other CYPs (Schmider *et al.* 1995; Bourrie *et al.* 1996). For CYP2A6 and CYP2C19, the search for selective chemical inhibitors is still under way (Draper *et al.* 1997; III).

2.2.3.5. Utilisation of CYP-specific antibodies

Today, there are several commercial sources for CYP-specific inhibitory antibodies. The products are usually well characterised and not very expensive. Inhibitory antibodies are usually targeted towards a sequence in or near the substrate-binding site. The antigen can be selected in such way that the resulting antibody only inhibits the target CYP. Inhibitory antibodies raised specifically against a certain CYP form are a good tool in distinguishing between CYPs established as equally possible NCE-metabolising CYPs by other methods. Antibodies can also be used if there are no sufficiently specific chemical inhibitors available for a certain enzyme (for example CYP2B6, Stresser & Kupfer 1999).

2.2.3.6. cDNA-expressed CYPs

Similarly to CYP-specific inhibitory antibodies, cDNA-expressed enzymes are convenient tools when a specific activity or a selective chemical inhibitor cannot be used in metabolic studies. With the expressed enzymes, the relative roles of individual CYPs cannot be quantitatively estimated, mainly due to the interindividual variation in the levels of individual active CYPs in the liver (Guengerich 1995b). cDNA-expressed CYPs can

also be used in the HTS methodology when the goal is to screen large numbers of compounds (White 2000).

Recombinant enzymes can be used to ascertain the role of a certain CYP in the metabolism of an NCE. Still, the biotransformation of an NCE by a single CYP does not necessarily mean its participation in the reaction *in vivo* (IV). When taking into account the proportion of a certain CYP in the human liver, the activity obtained for the whole liver can be extrapolated to the prediction of the participation of this enzyme (Rodrigues 1999).

2.2.3.7. Correlation analysis

For correlation analysis, a well-characterised bank of human liver samples is needed. The number of individual livers should be at least ten (preferably more), to demonstrate the interindividual variation in the battery of activities. In correlation analysis, the measured CYP-specific activities are correlated against the rate of the metabolic pathway of an NCE in every individual liver sample. Another approach is to correlate the levels of an individual CYP determined by Western blot analysis against the NCE activity (Beaune *et al.* 1986; Guengerich & Shimada 1991; Guengerich 1995b). When correlating the levels of each CYP protein to the activity of an NCE, one should take into account that any inactive protein could also contribute to the estimation of protein levels in each liver. This could lead to an erroneous correlation, because the inactive CYP proteins do not participate in the metabolism of the NCE (Guengerich 1995b). If there is a sufficient number of individual samples, statistical significance can also be considered. Still, a correlation plot would give all the information needed for the evaluation of the participating CYPs. The higher the correlation between the activities, the larger the likelihood that the respective CYP enzyme is responsible for the metabolism of the NCE. For correlation analysis, the initial velocity conditions and the substrate concentrations near K_m, or preferably the concentrations found *in vivo* should be used to avoid non-specific metabolism of substrates (Guengerich 1995b).

Particularly with CYP3A4, the correlation analysis is somewhat complicated because of the peculiar nature of the CYP3A4 catalytic mechanism (Harlow & Halpert 1998; Guengerich 1999).

2.2.4. Measures of the affinities of an NCE for CYPs

2.2.4.1. Inhibition of CYP-catalysed model reactions by an NCE

From the therapeutic point of view, it is also important to know which drug-metabolising enzymes the substance under development has affinity to. For example, a compound inhibiting CYP3A4 could affect the *in vivo* concentrations of numerous drugs and other xenobiotics metabolised *via* this CYP, since over 50% of the drugs on the market are biotransformed by CYP3A4 (Bertz and Granneman 1997; Pelkonen & Breimer 1994). In

recent years, severe adverse reactions due to interactions with CYP3A4 have led to the withdrawal of some drugs from the market. For example, terfenadine coadministrated with azole antimycotics caused potentially lethal ventricular arrhythmias (Monahan *et al.* 1990). Another example is mibefradil, the plasma concentrations of which were highly elevated by CYP3A4 inhibitors, leading to serious side effects (Kleinbloesem *et al.* 1995; Siepmann *et al.* 1995). Such economically consequential decisions could have been avoided if the affinity of the new compounds to CYPs had been recognised and correctly interpreted at the early phases of the drug development process (Siepmann & Kirch 2000).

The effect of an NCE on CYP-specific activities is studied by co-incubating series of dilutions of the study substance with a reaction mixture and a specific substrate. The effect of an NCE is described as the concentration of the studied compound causing 50% inhibition of the CYP-specific activities (Boobis 1995). If there is affinity towards some CYP-enzyme, the apparent K_i can be determined. By comparing the effects of an NCE on the CYP specific-activities to the respective effects of diagnostic inhibitors, a tentative prediction of the *in vivo* situation can be made.

2.2.4.2. Spectral interaction studies

Spectral interaction studies have been used from the early years of P450 research to recognise P450 ligands. Compounds that have affinity to a CYP-active site coordinate towards the heme iron of the protein, causing a change in the spin equilibrium of heme iron. The binding can produce several types of spectral changes that can be monitored by spectrophotometric scanning according to the procedure presented by, for example, Jefcoate (1978).

2.3. *In vitro – in vivo* scaling of an NCE

2.3.1. General

The very first attempts to predict *in vivo* pharmacokinetic parameters from *in vitro* experiments were presented by Rane *et al.* at 1977. In general, *in vitro-in vivo* scaling consists of two approaches: prediction of the intrinsic clearance (Cl_{int}) of an NCE and prediction of drug-drug interactions. In the literature, methods for both purposes are extensively presented (Ito *et al.* 1998a; 1998b; Obach *et al.* 1997). Some of the methods will be reviewed briefly in the following sections.

Other pharmacokinetic parameters, such as plasma half-life, volume of distribution and oral bioavailability, have also been considered for the estimation of *in vivo* kinetics on the basis of *in vitro* studies (Ito *et al.* 1998a; 1998b; Obach *et al.* 1997). Many *in vivo* factors affect the results of extrapolation. One of these is the binding to plasma and tissue proteins and, ultimately, the distribution volume of the drug. The effect of binding with numerous different compounds has been studied, and the general conclusion seems to be

that, with highly lipophilic drugs, protein binding does not play a very important role in prediction accuracy. (Kurz & Fichtl 1983; Pacifici & Viani 1992; Obach *et al.* 1997; von Moltke *et al.* 1998).

2.3.2. Considerations regarding assay conditions for NCE studies

2.3.2.1. Initial velocity conditions

When the metabolic routes of an NCE are recognised either in liver microsomes or in liver homogenate from the human or the test species, a method for the separation and, if a reference substance is available, quantitation of metabolites is required. Determination of the initial velocity conditions is important for an accurate determination of the enzyme kinetic parameters of metabolite formation reactions. The determination of the IC_{50} values for CYP-selective diagnostic inhibitors is best performed if the substrate concentration used is selected from within the linear part of the time and protein dependence curve for the metabolite formation from an NCE. When possible, the substrate concentration should be close to the achieved concentrations *in vivo* or at least close to the K_m value (Guengerich 1995b; Yuan *et al.* 1999).

2.3.2.2. Apparent enzyme kinetic parameters K_m and V_{max}

The determination of apparent enzyme kinetic parameters in human liver preparations with different substrates has been presented in various publications and is also available as teaching material (for example, Boobis 1995), and it will not be considered in detail here. Basically, there are two approaches available for this purpose: 1) the use of graphical presentations and derivations for Michaelis-Menten kinetics and 2) the use of an iterative software for calculating the parameters from untransformed data using the Michaelis-Menten equation. The latter approach is nowadays most widely used, since it gives somewhat more accurate and more reproducible values than the graphical method. Still, the graphical method is also in use and, if properly handled, gives results accurate enough for practical purposes.

2.3.2.3. Prediction of the intrinsic clearance (Cl_{int})

For the prediction of intrinsic clearance, one should be familiar with specific information about the enzymes participating in the metabolism of an NCE. This includes the identity of the enzymes and the enzyme-kinetic parameters for the NCE with metabolising enzymes. Without this information, it is still possible to derive K_m and V_{max} by following substrate consumption. This approach does not reveal the parameters for individual

metabolic routes, but allows the prediction of overall intrinsic clearance (Cl_{int}) and produces information about the bioavailability of the drug under study.

For example, Ito *et al.* (1998a; 1998b) have studied extensively the quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* studies. Both publications would be worth an introduction, but are too extensive to be reviewed here.

Basically, the *in vitro* metabolism can be converted into its *in vivo* counterpart. From the Michaelis-Menten equation for the velocity (v) of metabolite formation,

$$v = \frac{V_{max} * [S]}{K_m + [S]},$$

when $K_m >> [S]$, the reductions of the equation lead to the general expression that the intrinsic clearance (Cl_{int}) is

$$Cl_{int} = V_{max}/K_m, \text{ where}$$

V_{max}/K_m is presented as volume/time. This constant can be compared to the respective *in vivo* value obtained from pharmacokinetic studies. Obach *et al.* (1997) presented an extensive survey, where they compared values obtained from *in vitro* studies of 50 compounds to respective *in vivo* results. In that study, 12 different methods for clearance predictions were used, of which four were employed to predict values for *in vitro* half-life ($t_{½}$) and, consequently, to predict clearance, four utilised *in vitro* enzyme kinetic data, and four were allometric methods, in which the respective human prediction was derived from animal studies. Obach *et al.* (1997) concluded that there is no single method to exactly predict human *in vivo* pharmacokinetic parameters from *in vitro* data, but many of the tested methods yielded a satisfactory level of accuracy to warrant decision-making in the drug discovery process. Generally, for the compounds that were highly protein-bound, the predicted clearance was underestimated, if the degree of binding was taken into account. Especially for the prediction of the volume of distribution, the plasma protein-binding factor must be included in the calculations. It should be noted that if an NCE binds extensively to plasma protein, it is most probable that it also binds to microsomal proteins. On the whole, the presented methods for estimating *in vivo* pharmacokinetic values from *in vitro* data provide a certain window of accuracy. For example, the estimation of $t_{½}$ from *in vitro* enzyme kinetic data gives results accurate enough for the purpose of finding out the dosage regimens for an NCE.

2.3.2.4. Extrapolation of Cl_{int} to in vivo *clearance in the whole organism*

When extrapolation of Cl_{int} to the *in vivo* clearance in the whole organism is done, some preliminary information or assumptions are needed. These are presented in, for example, reviews by Ito *et al.* (1998a, b; Obach 1997). If the studied compound is metabolised via more than one pathway, the intrinsic clearances of these pathways need to be summed up to achieve a total Cl_{int} for the study system (microsomes, whole-cell systems, liver slices):

$$Cl_{int\,1} + Cl_{int\,2} + Cl_{int\,3} \ldots + Cl_{int\,n} = Cl_{int\,tot}, \text{ where}$$

1, 2, 3, ... , n represents different metabolic pathways. The assumptions needed for scaling to the clearance of the whole organism are the amount of microsomal protein per gram of liver wet weight, the wet weight of the whole liver of the organism, the liver blood flow and the weight of the organism. Information of distribution volume is also needed. If some organ other than the liver, such as the kidneys or the intestine, will participate significantly in the elimination of the studied compound, their Cl_{organ} has to be taken into account when scaling to the clearance of the whole organism.

2.3.2.5. Apparent K_i and type of inhibition

It is important to determine the apparent inhibition constant K_i for a compound that inhibits the metabolism of an NCE, for it is used in *in vivo* interaction predictions (Ito *et al.* 1998a). The use of initial velocity conditions in this series of assays is extremely important because conditions that do not fulfil the linear velocity demand would lead to an underestimation of the inhibitory potency of an NCE (Yuan *et al.* 1999).

The determination of the apparent K_i value is done either by fitting lines by linear regression analysis into the Dixon plot or any secondary plot, or by an iterative non-linear curve fitting and calculations based on the known Michaelis-Menten kinetics for the type of inhibition (Boobis 1995).

2.3.2.6. Prediction of drug-drug interactions

Drug-drug interactions that may alter pharmacokinetics could occur at several sites, including gastrointestinal absorption, plasma and/or tissue protein binding, transporter proteins, and metabolism (Ito *et al.* 1998b). The prediction of hepatic clearance is an important prerequisite for the prediction of drug-drug interactions, and the accuracy of the prediction method is dependent on the accuracy of the clearance prediction. The interaction prediction is complicated if there are multiple CYPs participating the biotransformation of the NCE. In that case, the relative contribution of each enzyme has to be taken into account (Obach *et al.* 1997).

Also, the type of inhibition (competitive, noncompetitive or uncompetitive) of the NCE is a valuable item of information. Independently of the inhibition type - except in the case of uncompetitive inhibition - when the substrate concentration is much lower than K_m, the degree of inhibition (R) is expressed as follows:

$$R = \frac{v(\text{with inhibitor})}{v(\text{without inhibitor})} = \frac{1}{1 + [I]/K_i} .$$

This is usually the case in therapeutic use (Ito *et al.* 1998b).

The determination of the inhibitor concentration *in vivo* is very difficult (or impossible), and the unbound concentration of an inhibitor is therefore usually utilised when calculating the degree of inhibition. However, as discussed above, the total concentration may be more appropriate to use in these calculations (Obach *et al.* 1997). The interindividual variation should also be considered, though it is problematic to take into account when extrapolating *in vivo* from *in vitro* studies (Ito *et al.* 1998a, 1998b).

When the inhibition of a CYP enzyme causes interactions, it has to be examined both from the point of view how the NCE affects other drugs administered simultaneously and how the other drugs affect the metabolism or therapeutic effect of the NCE. Clinically, if the half-lives of simultaneously administered drugs were equal or nearly equal, it would be possible to schedule the dosing regimens in such a way that the drugs do not interfere with each other's effects (Obach *et al.* 1997). If an NCE inhibits the activity of some CYP enzymes, the *in vivo* concentrations of the drugs metabolised by these CYPs might increase dangerously. This was the case with mibefradil, which is a potent inhibitor of both the drug-transporting P-glycoprotein and CYP3A *in vitro* (Siepmann *et al.* 1995; Krahenbuhl *et al.* 1998; Mullins *et al.* 1998; Spoendlin *et al*. 1998; Prueksaritanont *et al.* 1999; Wandel *et al.* 2000). When the CYP enzymes catalysing the oxidative reactions of an NCE are identified, there will already exist some candidates for inhibiting the reactions. For example, if an NCE is metabolised predominantly by CYP3A4, it is most probable that classical CYP3A4 inhibitors such as azole antimycotics (for example Varhe *et al.* 1994; von Moltke *et al.* 1996) or grapefruit juice (see for example Proppe *et al.* 1995; Armeer & Weintraub 1997; Kivistö *et al.* 1999), could cause an elevation in the *in vivo* concentrations of the NCE.

2.4. Ethical issues

In all research on human beings or employing human *in vivo* studies or human tissue-derived methods, such as microsomes or isolated primary cells, one must take good care of fulfilling the demands of ethical issues. All such studies have to be accepted by the local ethics committee.

2.4.1. Human studies

Whenever performing studies on humans, it is extremely important to take into account the Declaration of Helsinki. *In vitro* studies also warrant careful consideration of what research procedures are acceptable for human-derived material. In Finland, there is currently no legislation covering such commercial purposes. However, as the techniques are widely used and there are academic or commercial groups providing preclinical studies for drug development companies, this issue will become increasingly important. Today, the use of human surplus tissue has to be approved by the local ethics committee.

2.4.2. Animal studies

Animal studies are quite expensive and because of the marked differences in the drug-metabolising enzymes between humans and the test species, it is reasonable to perform *in vitro* testing to select the species best able to characterise human metabolism for an NCE. The use of animal liver microsomes, homogenate or isolated hepatocytes for first studying the metabolic patterns of the NCE will reduce both costs and the number of animals needed for preclinical drug development.

The use of human tissue-derived *in vitro* systems in affinity screening and metabolic stability studies gives more accurate information about the *in vivo* situation than the use of test species treated with the compound under study. Still, because of the demands of the authorities, some testing for pharmacokinetics and toxicity has to be performed on animals. Human-derived *in vitro* systems are increasingly reducing the number of animals needed for such tests. For the time being, these animal tests are necessary, but careful planning and effective use of the *in vitro* methodology can reduce the use of animals in many research fields.

3. Aims of the present study

This thesis project was conducted in parallel with, and partially as a part of, the EU Biomed2 project EUROCYP. The evaluation of human tissue-derived preclinical *in vitro* tests is an on-going process in various academic and commercial research institutes throughout the world. The ultimate goal of this work was to evaluate, with the help of some model compounds, a pattern of P450-selective reactions/activities and inhibitors for routine *in vitro* affinity and metabolic studies with NCEs. The specific aims were:

1. To develop and employ a battery of methods for preclinical *in vitro* testing of NCEs in human liver microsomes. The aim was to compile a group of assays suitable for routine low-throughput analysis and to employ the assays in the metabolic studies of some relevant drugs. Also, the assays should be capable of being employed reliably and cost-effectively.
2. To study some model compounds for which *in vivo* metabolic data exist.
3. To further evaluate tranylcypromine as a CYP2A6-selective inhibitor.
4. To evaluate these *in vitro* methods in the light of the studied model compounds and their *in vivo* behaviour.

4. Materials and methods

4.1. Human liver samples

Human liver samples were obtained from the University Hospital of Oulu as waste from organ transplantation donors. The Ethics Committee of the University of Oulu has approved the use of surplus tissue for research purposes.

The livers were transferred to ice immediately after the surgical excision and cut into pieces of approximately one gram each. The pieces were snap-frozen in liquid nitrogen and stored at –80 °C until the microsomes were prepared by standard differential ultracentrifugation (Pelkonen *et al.* 1974). During the storage, no significant loss of activity took place (data not shown, see also Yamazaki *et al.* 1997). Relevant data of the human liver samples used in this work are shown in Table 4.

Table 4. The human liver samples used in this work.

Code[a]	Age (years)	Sex	Reason for sampling	Medication, other
HL15	not known	m	Transplantation donor (gun shot)	no medication
HL16	not known	f	Transplantation donor (ICH[b])	no medication
HL20	54	m	Transplantation donor (ICH)	diazepam before death
HL21	44	m	Transplantation donor (ICH)	phenytoin before death, cirrhotic
HL22	40	f	Transplantation donor (ICH)	dexamethasone, nizatidine and phenytoin before death
HL23	43	m	Transplantation donor (ICH)	diazepam before death
HL24	47	m	Transplantation donor (ICH)	no medication
HL25	33	m	Malignant brain astrocytoma	not known
HL26	70	f	Melanoma, >30% steatosis	not known
HL27	52	m	>30% steatosis	not known

[a]All individuals were of Caucasian race; [b]ICH, intracerebral hemorrhage.

4.2. Chemicals

In the study of NCEs, it is of utmost importance to ascertain that compounds interact with their targets (enzymes or receptors) in the predefined form and concentration. Consequently, the compounds employed in this study as well as the substrates, metabolites and chemical inhibitors were prepared and stored as described in Table 5 to ascertain their solubility and stability. The sources of substrates, inhibitors, reference compounds and the studied NCEs have been presented in the original publications I-IV. The other chemicals were from commercial sources and were at least of HPLC-grade purity.

Table 5. The solution and storage conditions of substrates, inhibitors and metabolites (I, II, III, IV).

Compound	Concentration of stock solution in mM	Solvent (and its content in incubation mixture)	Storage conditions and stability information[a]
Substrates			
Chlorzoxazone	10	60 mM KOH (0.6 mM)	not stable, new solution every working day
Coumarin[b]	100	50% methanol (0.5%)	+4 °C for two weeks
Dextromethorphan	100	H_2O (2 µl/200 µl)	+4 °C, stable
Entacapone[b]	100	DMSO (<0.1%), Tris-HCl, pH 8.0 (2 µl/200 µl)	not stable, stock solution in DMSO, dilutions in Tris-HCl, pH 8.0 every week, for CYP2E1 activity all solutions in Tris-HCl, pH 8.0
Ethoxyresorufin	0.5	MeOH:DMSO (0.02%)	stock solution at –20 °C, dilutions at +4 °C
rac-Mephenytoin[b]	100	Acetonitrile (<0.1%)	-70 °C, stable
Midazolam	10	Acetonitrile (<0.1%)	+4 °C for two weeks
Selegiline[b]	100	H_2O (2 µl/200 µl)	+4 °C for one week
Testosterone	100	EtOH (1%)	+4 °C for one month
Tolbutamide	1000	EtOH (<0.1%)	+4 °C for two weeks
Inhibitors			
Candesartan	60	DMSO (1%)	RT[c], for one month
Cyclopropylbenzene	100	EtOH, Acetonitrile (1%)	-20 °C, new dilutions from stock solution weekly
Desmethylselegiline[d]	100	EtOH (1%)	-20 °C, stable
Eprosartan	100	0.25 M KOH in 0.1 M phosphate buffer, pH 7.4	+4 °C, new stock solution weekly
Fluvoxamine	15	H_2O (2 µl/200 µl)	-20 °C, stable
Furafylline	1 mg/ml	DMSO (1%)	stock solution at -70 °C, others RT, stable
Gestodene	10	DMSO (1%)	stock solution at –20 °C, others RT, new dilutions from stock solution weekly
Irbesartan	100	DMSO (1%)	RT, for one month
Itraconazole	10	DMSO (1%)	stock solution at –20 °C, others RT, new dilutions from stock solution weekly

Table 5. Continued

Ketoconazole	10	MeOH (1%)	stock solution at –20 °C, others RT, new dilutions from stock solution weekly
Losartan	100	DMSO (1%)	RT, for one month
l-Methamphetamine[d]	100	EtOH (1%)	+4 °C, stable
8-Methoxypsoralen (methoxsalen)	100	DMSO (1%)	RT, for one week
Omeprazole	10	DMSO (1%)	not stable
Pyridine	100	H_2O (2 µl/200 µl)	+4 °C for one week
Quercetin	100	H_2O (2 µl/200 µl)	+4 °C for one week
Quinidine	10	H_2O (2 µl/200 µl)	+4 °C for one week
Sulfaphenazole	100	Acetonitrile (1%)	+4 °C for one week
Tranylcypromine	100	EtOH (1%)	-20 °C
Troleandomycin (TAO)	10	H_2O (2 µl/200 µl)	+4 °C for one week
Valsartan	100	0.25 M KOH in 0.1 M phosphate buffer, pH 7.4	+4 °C for one week
Metabolites			
N-Desethylentacapone	1 mg/ml	stock solutions in DMSO	stock solutions in -70 °C, standard dilutions in -20 °C
Dextrorphan	5 mg/ml	stock solutions in DMSO	stock solutions in -70 °C, standard dilutions in -20 °C
6-Hydroxy-chlorzoxazone	5 mg/ml	stock solutions in DMSO	stock solutions in -70 °C, standard dilutions in -20 °C
7-Hydroxycoumarin	1 mM	stock solutions in 50% EtOH	stock solutions in +4 °C for one month
1'-Hydroxymidazolam	1 mg/ml	stock solutions in acetonitrile	stock solutions in -70 °C, standard dilutions in -20 °C
4'-Hydroxy-mephenytoin	5 mg/ml	stock solutions in DMSO	stock solutions in -70 °C, standard dilutions in -20 °C
6β-Hydroxy-testosterone	1 mg/ml	stock solutions in EtOH	stock solutions in +4 °C, stable
Hydroxytolbutamide	5 mg/ml	stock solutions in DMSO	stock solutions in -70 °C, standard dilutions in -20 °C
Resorufin	0.5 mM	stock solutions in 1 mM NaOH (0.1 mM)	+4 °C

[a]Stability according to empirical results or references. All solutions were stored in dark; [b]also used as an inhibitor; [c]RT = room temperature; [d]also a metabolite

4.3. Antibodies and recombinant enzymes

The antibody against human CYP3A4 (Mab-3A4)(IV) was obtained from Gentest Corp. (Woburn, MA, USA), and it was employed according to the manufacturer's procedure.

The recombinant CYP2B6, CYP3A4, CYP3A5 and FMO3 (flavin-containing monooxygenase type 3) were purchased from Gentest Corp., and they were incubated with entacapone (IV) as suggested by the manufacturer.

4.4. Analysis of metabolites

The metabolites of ethoxyresorufin and coumarin were determined with the Hitachi F-4010 fluorescence spectrophotometer. In HPLC analysis, a Shimadzu *VP* series HPLC with an autoinjector was used. The columns and analysis conditions are shown summarised in Table 6 and in the original publications I-IV.

4.5. Enzymatic assays

The incubation and analysis conditions used in this work are shown summarised in Table 6 and have been presented in detail in the original publications I-IV.

4.6. Determination of enzyme kinetic parameters

The formation of metabolites was calculated and expressed as pmol*min^{-1}*mg protein^{-1}. For the determination of the apparent Michaelis-Menten constant K_m and the maximal velocity of the reaction, V_{max}, plots of the metabolite formation rate in relation to the substrate concentration and Lineweaver-Burk plots were derived. The determination of apparent K_i in the studied activities was conducted from the respective Dixon plots. The lines in the plots were fitted by linear regression analysis (Microcal Origin™ version 4.1). The intersection points were determined graphically. Each data point represented an average of at least two parallel incubations.

4.7. Correlation analysis

The correlation of entacapone *N*-deethylation with the CYP-catalysed activities in ten individual liver samples was performed by Microsoft Excel 97 software (IV).

Table 6. Standard conditions for the determination of CYP-associated metabolic activities and other assays.

CYP	Activity	[S] μM (times K_m)	Protein content mg/ml	Incubation time min	Detection of the metabolite	Reference
1A2	ethoxyresorufin *O*-deethylation	1 (2)	0.1	5	fluorometric (530 nm/585 nm)	Burke *et al.* 1977
2A6	coumarin 7-hydroxylation	10 (2)	0.2	10	fluorometric (365 nm/454 nm)	Aitio 1978; Raunio *et al.* 1988, 1990
2C9	tolbutamide hydroxylation	200 (1)	0.75	20	UV-HPLC (236 nm)	Knodell *et al.* 1987; Sullivan-Klose *et al.* 1996
2C19	*S*-mephenytoin 4'-hydroxylation	100 (1)[a]	0.5	20	UV-HPLC (204 nm/240 nm)	Wrighton *et al.* 1993a
2D6	dextromethorphan *O*-demethylation	10 (1)	0.5	20	UV-HPLC (204 nm/280 nm)	Park *et al.* 1984; Kronbach *et al.* 1987
2E1	chlorzoxazone 6-hydroxylation	100 (2)	0.5	20	UV-HPLC (282 nm)	Peter *et al.* 1990
3A4	midazolam 1'-hydroxylation	10 (2)	0.5	10	UV-HPLC (245 nm)	Kronbach *et al.* 1989
	testosterone 6β-hydroxylation	100 (1)	0.5	20	TLC (autoradio-graphy)	Waxman *et al.* 1983
	entacapone *N*-deethylation	100 (0.5)	1.0	30	UV-HPLC (300 nm)	IV
	selegiline *N*-demethylation	100 (0.5)	0.6	45	UV-HPLC (214 nm)	I
	selegiline *N*-depropargylation	100 (0.5)	0.6	45		I

[a]The concentration of *rac*-mephenytoin corresponds to approximately 50 μM *S*-mephenytoin.

5. Results

5.1. Studies on the metabolism of two model compounds (I & IV)

In this part of the work, the CYP-catalysed *in vitro* metabolism of selegiline (I) and entacapone (IV) was studied. These compounds served as model substances for this study, although both are already in therapeutic use for the treatment of Parkinson's disease.

5.1.1. Selegiline (I)

At the beginning of these studies, knowledge about the *in vitro* metabolism of selegiline was rather scarce. There were suggestions about CYP2D6 (Grace *et al.* 1994) and CYP3A4 (Wacher *et al.* 1996) participating in the formation of *l*-methamphetamine (selegiline *N*-depropargylation) and about the formation of both desmethylselegiline (selegiline *N*-demethylation) and *l*-methamphetamine, respectively, by human liver microsomes and by cDNA-expressed CYPs.

According to this study, *in vitro* metabolism of selegiline seems to be catalysed by at least CYP1A2 (desmethylselegiline formation) and CYP3A4 (both pathways). These assignments were based on chemical inhibition studies. The only CYP inhibitors affecting the reactions were furafylline (desmethylselegiline formation), fluvoxamine (both pathways) and ketoconazole (both pathways). Of these, furafylline is a selective CYP1A2 inhibitor (Sesardic *et al.* 1990), while fluvoxamine and ketoconazole have recently been reported not to be very specific CYP1A2 and CYP3A4 inhibitors (Kashuba *et al.* 1998; Rasmussen *et al.* 1998; Pelkonen *et al.* 1998; own unpublished results). However, these studies were deemed insufficient and the assignments only tentative, because chemical inhibitors are not completely selective. Thus, further studies are required.

5.1.2. Entacapone (IV)

When CYP-mediated *in vitro* metabolism of entacapone was studied in rat liver microsomes, it was found to be both *N*-monodeethylated and *N*-dideethylated (Wikberg *et al.* 1993). In humans, glucuronidation of entacapone was found to be the major pathway *in vivo*, ϖ-hydroxylated entacapone being the minor and only oxidised metabolite.

In this study, incubations with human liver microsomes revealed only monodeethylated entacapone, and no hydroxylated product was formed. This metabolic route was catalysed predominantly by CYP3A4 on the basis of chemical and antibody inhibition studies and incubations with recombinant CYP3A4. Also, correlation analysis in ten human liver samples supported CYP3A4 being the major *in vitro* oxidative enzyme, although participation of some other CYPs cannot be excluded. Calculations of CYP3A4-mediated intrinsic clearance of entacapone confirmed the minor *in vivo* role of oxidative metabolism in the total metabolism of entacapone.

5.2. Inhibitory effects and potential interactions (I, II & IV)

In preclinical *in vitro* studies, tests of the affinity of an NCE to CYPs are usually among the first series of assays performed. The elucidation of the *in vitro* inhibitory effect towards some CYPs at clinically relevant concentrations predicts drug-drug interactions with other substances, which are metabolised by certain CYPs.

5.2.1. Selegiline and its primary metabolites (I)

Selegiline was studied at various concentrations for its inhibitory potency towards CYP-specific reactions. It was found to inhibit quite potently only CYP2C19-selective *S*-mephenytoin 4'-hydroxylase, which is polymorphically expressed in the human population. Likewise, desmethylselegiline, a primary metabolite of selegiline, inhibited markedly only CYP2C19 at the same potency as the parent compound. Another primary selegiline metabolite, *l*-methamphetamine, did not inhibit any CYP-catalysed activity at the studied concentration levels. The inhibition constant K_i for selegiline towards *S*-mephenytoin 4'-hydroxylation was determined to be 7 μM, and inhibition was of mixed type.

5.2.2. Five angiotensin-II receptor antagonists (II)

In *in vitro* inhibition screening of CYPs by five angiotensin-II receptor antagonists, candesartan, eprosartan, irbesartan, losartan and valsartan, marked differences between their inhibitory potencies were observed. Losartan, irbesartan, valsartan and candesartan inhibited CYP2C9. The rank order of inhibitory potencies in terms of K_i values was: losartan (4.1 μM), irbesartan (24.5 μM), valsartan (135 μM) and candesartan (155 μM).

Losartan and irbesartan also inhibited CYP1A2 and CYP3A4 with relatively high IC_{50} values (between 200 μM and 500 μM). Losartan also inhibited CYP2C19 with an IC_{50} value of 138 μM. Eprosartan did not show any inhibition of the studied CYP-associated activities. Valsartan and candesartan did not inhibit any other CYP than CYP2C9. The chemical structures of these five compounds are presented in Fig. 2.

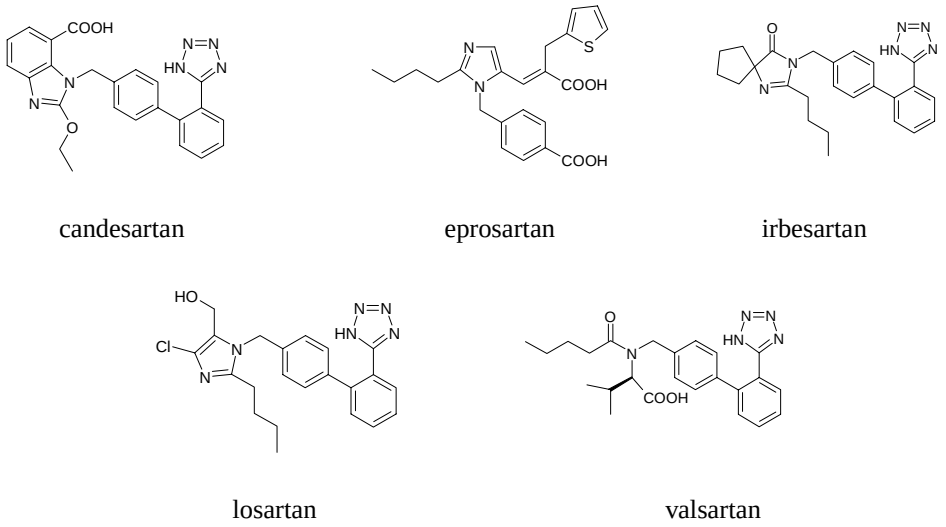


Fig. 2. Structures of five angiotensin-II receptor antagonists studied in original publication II.

5.2.3. Entacapone (IV)

Since entacapone is a new therapeutic agent for Parkinson's disease and because tolcapone, another nitrocatechol with the same inhibitory effect for catechol *O*-methyltransferase, was withdrawn from the market due to serious adverse effects, the characterisation of the inhibitory potential of entacapone towards CYPs was considered an important task.

Entacapone was found quite restricted in inhibiting CYP-catalysed activities in human liver microsomes. Actually only CYP2C9-catalysed tolbutamide hydroxylation was markedly inhibited by entacapone. The IC_{50} value obtained as an average of three individual determinations in different livers was 3.5 μM, K_i being 0.3 μM, and inhibition was of mixed type. Of the other CYP-associated activities, entacapone inhibited coumarin 7-hydroxylation with an IC_{50} value of 321 μM and *S*-mephenytoin 4'-hydroxylation with an IC_{50} value of 212 μM, while CYP3A4 catalysed midazolam 1'-hydroxylation with an IC_{50} value of 304 μM.

5.3. Evaluation of a selective inhibitor for CYP2A6 (III)

For CYP2A6, which catalyses the model activity coumarin 7-hydroxylase, no sufficiently selective chemical inhibitor has been characterised. Methoxalen (8-methoxypsoralen) (Koenigs *et al.* 1997) has been used for this purpose, but its selectivity is questionable. Actually, the CYP2A6-specific substrate coumarin can be used as a reference inhibitor, but its inhibitory selectivity has not been thoroughly elucidated.

The lack of a selective inhibitor for CYP2A6 led to a search for human CYP2A6 and mouse CYP2A5 inhibitory agents by Juvonen *et al.* (Kimonen et al. 1995; Kinonen et al. 1995; Juvonen et al. 2000). From these studies, the structural analogues *t*-2-phenylcyclopropylamine (tranylcypromine) and cyclopropylbenzene were selected for further characterisation. The original hypothesis, which was later proved correct, was that the amine group in tranylcypromine would potentiate the inhibitory effect compared to that of cyclopropylbenzene. In inhibition screening of CYP-specific model activities, tranylcypromine was a more potent inhibitor than cyclopropylbenzene. Tranylcypromine inhibited CYP2A6 quite potently with an IC_{50} value 0.42 μM as an average of four individual liver samples. The second most effective inhibition was that of CYP2E1-selective chlorzoxazone 6-hydroxylase with an IC_{50} value of 3.0 μM. The inhibition constant for coumarin 7-hydroxylation was determined to be 0.14 μM based on one microsome sample. Inhibition was of mixed type.

6. Discussion

6.1. Tools for preclinical *in vitro* studies

As it was earlier pointed out, preclinical *in vitro* studies are becoming valuable tools for predicting the *in vivo* metabolism and drug-drug interactions of NCEs. None of these methods alone is sufficient for a complete prediction because every *in vitro* model system has its advantages and disadvantages (Table 3). Liver microsomes are easy to obtain and use, and in skilful hands they are also a relatively inexpensive and reliable system for assessing the oxidative metabolism and affinity of an NCE to CYPs. Still, microsomes lack the whole complement of hepatic drug-metabolising enzymes, namely conjugating enzymes other than glucuronosyl transferases. It is relatively easy to obtain information of enzyme kinetic parameters for determining intrinsic clearance and other pharmacokinetic values from microsome studies. In this project, human liver microsomes were employed in tests reported in the original publications (I-IV). The specific results have been discussed in the respective publications.

Liver homogenate with appropriate cofactors can be used for metabolic stability studies when the biotransformation pathways of a new compound are unknown. Liver homogenate fortified with appropriate cofactors exhibits the drug-metabolising enzyme activities that are present in intact tissue. In incubations with liver homogenate, the whole spectrum of *in vitro* metabolites are formed and the metabolite identification of an unknown compound is therefore possible.

When the transport of an NCE through cell membranes is under study, whole cell systems or liver slices are a good choice, since in these preparations the transport systems into and out of the cell are present, and even the cell-cell connections are preserved in liver slices. These preparations are also suitable for induction studies.

cDNA-expressed enzymes offer a nearly infinite source of drug metabolising enzymes (DME). With recombinant enzymes, the relative levels of the respective enzymes in human liver must be kept in mind, as otherwise the participation of a low-abundance enzyme in the metabolism of the compound could be overestimated. Rodrigues (1999) presented an analysis in which he attempted to bridge the gap between cDNA-expressed enzymes and native human liver microsomes. In the original publication IV, some recombinant enzymes were utilized. The results of this work will be discussed later.

The information about *in vitro* metabolism is of value when planning the dose and timing of a drug administered in Phase I clinical studies. Although real-life metabolism is not always the same as that assessed from *in vitro* experiments, these investigations certainly give relevant data for the prediction of possible drug-drug interactions. An example of the discrepancy between *in vivo* and *in vitro* situations is presented in the original publication IV, where the *in vitro* oxidative metabolite of entacapone is introduced, a desethyl entacapone, as *in vivo* the only oxidative metabolite found is ϖ-hydroxylated entacapone. The *in vitro* study showed no sign of this hydroxylated entacapone.

6.2. Methods used in *in vitro* studies

The methods used in *in vitro* studies are inhibition of CYP-catalysed model activities, CYP enzyme-selective chemical inhibitors and inhibitory antibodies specifically raised against individual CYPs, correlation analysis, and the metabolism of an NCE by the use of human liver preparations and recombinant expressed CYPs. In the original publications (I-IV), all these methods were employed. None of them alone is adequate to warrant conclusions about NCE-metabolising enzymes, but the conclusions drawn by utilising the consistent results from the whole battery of approaches is needed (Halpert 1994).

The exact results of each part of this work have been discussed in the original papers and are not considered here in detail.

6.2.1. Practical issues

6.2.1.1. Model activities (I-IV)

Utilisation of model activities for CYP studies requires that the enzyme kinetics of reactions are well known. Especially the enzyme content of the incubation mixture and the reaction time should be in the range of the linear velocity of metabolite formation.

During this project, seven model activities were identified from the literature and developed to suit routine laboratory work. A summary of the final methods is presented in Table 1 of the original article III. The goal of the development was to simplify the methods as much as possible without losing sensitivity or accuracy. First, the incubation procedure for each of the model activities was checked for linear velocity conditions. In the works I and II, testosterone 6β-hydroxylation was used to represent CYP3A4 with thin-layer chromatographic separation and subsequent autoradiographic detection. This method took approximately one week to perform, which led to a decision to search for an easier and faster method. A comparison of possible substrates and analysis methods was made, and midazolam 1'-hydroxylation was selected for CYP3A4 model activity. This activity has been employed in the publications III and IV.

Substrate concentrations were originally selected according to the methods presented in the literature or by the detection limit of the HPLC system used. When a more sensitive HPLC apparatus was obtained, the substrate concentrations could be adjusted close to the K_m values of the respective reactions. At the same time, the extraction procedures originally used in HPLC methods could be abandoned because the new apparatus appeared to be about five times more sensitive than the originally used one. This led to more consistent results, as the samples could be injected into HPLC directly after protein precipitation and centrifugation. Because of the inhibitors and NCEs used, the calibration of each method had to be performed by external standardisation instead of internal standardisation. The physico-chemical properties - especially the UV absorption spectrum and solubility - for an NCE are not always known and the amount of substance is too small (about 5 to 100 mg) for more extensive studies, and interference by the studied compounds could not be avoided in every case. Internal standardisation was omitted to simplify the procedures.

6.2.1.2. Chemical CYP inhibitors (I, III & IV) and anti-CYP antibodies (IV)

The solution of chemical inhibitors used in this study is presented in Table 5 (p. 40). When evaluating the effect of chemical inhibitors on the activities studied, one must always also evaluate the effect of solvent used. It is a well recognised fact that every organic solvent has some effect, either inhibitory or activating, on P450-catalysed activities (Chauret *et al.* 1997; Hickman *et al.* 1998). This has to be taken into account when interpreting the results of inhibitory studies. The content of organic solvent should be kept under one, and preferably down to a half, percent of the total incubation volume, to avoid inhibition or activation by the solvent system (Chauret *et al.* 1997; Hickman *et al.* 1998; Rodrigues 1999).

The selectivity and inhibition mechanisms of chemical inhibitors must be clarified before a compound is suitable to be used as a reference inhibitor. This approach was used in original publication III, where tranylcypromine was characterised for a CYP2A6-selective inhibitor. For this P450, there has been no sufficiently selective chemical inhibitor, although there are inhibitory antibodies. Tranylcypromine was studied in a non-mechanism-based manner, although it has long been used as a mechanism-based inhibitor for CYP2C19 (Inaba *et al.* 1985; Chauret *et al.* 1997). The most important findings were that tranylcypromine inhibits CYP2A6-catalysed coumarin 7-hydroxylation to a greater extent than any other P450-catalysed activities studied. Only CYP2E1-catalysed chlorzoxazone 6-hydroxylation was inhibited in such a manner that the possible inhibition of this CYP should be taken into account when using over one micromolar concentrations of tranylcypromine. This problem could be overcome by the simultaneous use of pyridine, a selective CYP2E1 inhibitor (Hargreaves *et al.* 1994; Taavitsainen *et al.* unpublished results), which does not inhibit CYP2A6 even at the highest concentration studied, 1000 μM (Taavitsainen *et al.* unpublished results).

Chemical inhibitors have some advantages over anti-CYP antibodies. Although the availability of inhibitory antibodies is currently better than, say, some five years ago,

chemical inhibitors are even easier to obtain. Another advantage of chemical inhibitors is that they can be used in intact cells and some also *in vivo* (Halpert *et al.* 1994).

6.2.1.3. Correlation analysis (IV)

Correlation analysis is performed in a bank of individual human liver microsomes by correlating the known activities of each microsome sample to the studied activity in the same samples. At least ten microsome samples should be used to reveal interindividual variation. However, a good statistical analysis requires more samples, as ten individual values are far too few to allow statistical evaluation (R. Bloigu, personal information).

Correlation analysis gives information about the possible extent of the contribution of certain P450s to the reaction under study. The values of the correlation coefficient (r) may range from –1 to 1, with 0 referring to an absent correlation and 1 to a perfect positive correlation between the two activities.

Evaluation of a correlation by only the correlation coefficient gives no information about the distribution of data points. The graphical presentation of a correlation is visually more illustrative, and with small numbers of different microsomes, it might be a more informative way of presenting the results. Correlation analysis is not an absolute measurement of the participation of certain CYP enzymes. A marked correlation can also be due to similar regulation of enzymes (for example, CYP3A4 and CYP2B6, Strom *et al.* 1996; Rodriguez 1999).

6.2.1.4. cDNA-expressed enzymes (IV)

The participation of CYP3A4 in entacapone *N*-deethylation in human liver microsomes was confirmed by recombinant CYP3A4 catalysis. The potentials of CYP3A5, CYP2B6 and FMO3 were also investigated in this context, because there are no selective, fully characterised chemical inhibitors for these enzymes. Antibodies against CYP2B6 exist (Stresser and Kupfer 1999; Gentest Corp.), but were not available during this work. The results obtained by cDNA-expressed P450s in this work supported the participation of CYP3A4 in *N*-desethylentacapone formation.

The P450 enzymes expressed in host cell microsomes are always artificial systems, and that should be kept firmly in mind. For example, the participation of cytochrome b_5 in the catalysis is needed for some enzymes and substrates. Also, the need for additional lipid systems and the ratio of cytochrome P450-NADPH-reductase to recombinant P450 protein need to be studied. There have been attempts to scale the activities presented by recombinant enzymes to human liver microsomes by estimating the relative contents of the respective CYPs in native hepatic microsomes and by presenting the recombinant activities in proportion to those estimates (Rodrigues 1999). Still, microsomes are superior in that the level of each CYP participating in the metabolism of an NCE can be estimated with the help of inhibition studies by chemical or antibody inhibitors (Halpert *et al.* 1994).

6.3. *In vitro* versus *in vivo*

When extrapolating *in vitro* data to *in vivo* situations, one should be extremely careful with such things as the therapeutic concentrations of the studied substance in relation to the *in vitro* substrate concentration and the inhibitory values (IC_{50} and K_i), possible co-medicated drugs, the relative content(s) of P450 enzyme(s) participating in the metabolism of an NCE and the other possible pathways of metabolism, such as conjugating enzymes, gut wall P450s and other enzymes, elimination as an unchanged compound, binding to serum protein or the effect of active transport systems in and out of the cell. All these factors may have to be taken into consideration in the extrapolation from *in vitro* metabolic clearance to *in vivo* clearance in patients.

In original publication I, selegiline was found to be metabolised at least partly by CYP1A2 and CYP3A4. The participation of CYP3A4 in selegiline metabolism might explain some *in vivo* findings. For example, Laine *et al.* (1999) studied the effect of oral contraceptives on selegiline pharmacokinetics and found that these drugs markedly increased the selegiline plasma concentration in healthy volunteers. This can be also explained by the inhibition of CYP1A2, which is affected by ethinylestradiol, a common component in oral contraceptives.

Among the angiotensin-II receptor antagonists, called “sartans”, studied in original publication II, eprosartan and valsartan are eliminated practically completely by excretion (Markham & Goa 1997; McClellan & Balfour 1998). Losartan is oxidised into an active metabolite by CYP2C9 and CYP3A5 (Goa & Wagstaff 1996), irbesartan is partially glucuronidated and oxidated, most probably by CYP2C9 (Gillis & Markham 1997), and CYP2C9 produces a minor oxidation product from candesartan (McClellan & Goa 1998). Of these, losartan, irbesartan, valsartan and candesartan inhibited CYP2C9 dependent activity to some extent. Losartan has been shown to demonstrate a slight, but not clinically important, inhibition of losartan metabolism by fluconazole, an inhibitor of CYP2C9, in healthy volunteers (Kazierad *et al.* 1997). In the same study, no effect on eprosartan pharmacokinetics was observed. Due to the resulting low free concentration (sartans are highly protein-binding agents) of each of the studied sartans, it is most likely that only losartan and irbesartan are possible candidates for drug-drug interactions with compounds that inhibit or are metabolised by CYP2C9.

With entacapone (IV), there has been no observations about the *in vivo* effects on drugs metabolised by CYP2C9 (Orion Pharma, unpublished results, Jorga *et al.* 2000), although it quite potently inhibits tolbutamide hydroxylation in human liver microsomes.

Because the deethylation of entacapone does not seem to be active *in vivo,* and because entacapone is efficiently glucuronidated *in vivo,* it seems irrelevant to speculate about the effect of CYP3A4 inhibitors in entacapone elimination on the basis of this work. Entacapone is a good example of a situation where *in vitro* oxidative metabolism differs markedly from *in vivo* biotransformation. Entacapone is efficiently metabolised into glucuronide, the *in vitro* Cl_{int} being 15225 ml/min, while the Cl_{int} for CYP3A4-catalysed entacapone *N*-deethylation is only 52.5 ml/min. It is strongly suggested that appropriate caution should be exercised in case no *in vivo* data are available of the NCE and some predictions are based on *in vitro* studies, whether done with human liver microsomes, hepatocytes or liver slices. Whether the latter two tools could have given

more correct metabolites than microsome in this work, since there is the whole spectrum of DMEs present in both of them, remains to be studied.

During this work, no calculations to predict the *in vivo* pharmacokinetic parameters of drug-drug interactions were performed, although this approach was used for selegiline (I) during the EUROCYP project (unpublished data, manuscript under preparation).

6.4. Decreasing the use of test animals

Previously, a great deal of preclinical studies have been accomplished by *in vivo* animal testing. The development and validation of *in vitro* methodologies have made it possible to give up animal testing or to markedly decrease the number of animals used for some early ADME studies. This development is in line with the public opinion concerning animal rights and the demand for fewer animal studies.

P450 affinity studies have increasingly utilised the possibility to substitute liver microsomes from specifically induced or control animals for human liver microsomes. This has led to the current situation, where the common protocol is to conduct affinity screening of an NCE towards P450s participating in xenobiotic metabolism.

Pharmacology and especially toxicology have traditionally been the areas where most of the test animals have been used principally to fulfil the regulatory requirements. Authorities strictly regulate toxicology testing, and it takes much more time to find appropriate *in vitro* tests for long-term toxicity studies. The *in vitro* methodology can already give a partial answer to those who are actively seeking to cut down the number of animals used for toxicity testing. For an NCE, it is possible to conduct a metabolite search by liver preparations from the test species of choice. Using a pool of liver preparations, it is possible to avoid the use of many intact animals for metabolite searching. Also, the selection of test species for toxicity tests can be made by comparing the metabolite profiles produced by human liver microsomes and by microsomes from different test species. After identification of the formed metabolites, a comparison between the metabolites formed by human liver preparations and the test species can be done. This procedure can be used as an aid in selecting the species that most closely resemble humans in the metabolite profile. On the other hand, if the metabolic pathways differ considerably, this knowledge is still very useful in assessing the results of animal toxicity studies.

7. Conclusions

1. The methods for seven P450 specific activities, the use of chemical and antibody inhibitors and some cDNA-expressed enzymes were characterised for use in routine low-throughput preclinical *in vitro* testing of NCEs in human liver microsomes.
2. These approaches were tested during this work for eleven different activities. The results from the different approaches were shown to be relevant, repeatable and reliable, and it is proposed that these methods are suitable for this kind of work.
3. Tranylcypromine was evaluated to be a selective chemical inhibitor for CYP2A6 in a certain concentration range under one micromolar level.
4. The results of each study have been discussed in separate publications in the light of the existing *in vivo* data. The *in vitro* results for the studied angiotensin II receptor antagonists are in line with the available *in vivo* data, but the participation of CYP3A4 *in vivo* in selegiline and entacapone metabolism remains to be clarified.

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