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CYTOCHROME P450 ENZYMES—IN VITRO, IN VIVO, AND IN SILICO STUDIES
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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in the Auditorium of the Department of Pharmacology and Toxicology, on October 20th, 2006, at 12 noon

OULUN YLIOPISTO, OULU 2006
Abstract

Metabolism is a major determinant of the pharmacokinetic properties of most drugs and is often behind bioavailability problems, drug-drug interactions, and metabolic idiosyncrasies. Cytochrome P450 (CYP) enzymes are a superfamily of microsomal hemoproteins catalysing the metabolic reactions of several exogenous compounds. The majority of crucial steps within drug metabolism are in connection with CYP enzymes.

In the present study, in vivo, in vitro, and in silico approaches were applied and characterised to evaluate the effects of chemical entities on CYP-mediated metabolism. CYP2B6 was used as a target enzyme for these studies.

For evaluation of the CYP inhibition potential of new chemical entities, a novel in vitro test system utilising the n-in-one approach was developed. This method proved to be robust and applicable to screening purposes. Validation of the n-in-one assay was done by comparing its performance to commonly used in vitro techniques using six structurally diverse drugs. All assay types yield remarkably similar results with the majority of the CYP forms tested.

Several chemicals were screened in vitro and in silico in order to find potent and selective chemical inhibitors for CYP2B6. Ticlopidine, thioTEPA and 4-(4-chlorobenzylpyridine) were found to be highly effective inhibitors of CYP2B6. The selectivity of thioTEPA proved to be very high, whereas ticlopidine and 4-(4-chlorobenzylpyridine) also inhibited other CYPs. At a concentration level of 1 μM for ticlopidine and 0.1 μM for 4-(4-chlorobenzylpyridine), the inhibitory effect towards other CYPs was negligible.

Due to wide clinical use and relevance, clopidogrel and ticlopidine were selected for further in vivo interaction studies. Both clopidogrel and ticlopidine significantly inhibited the CYP2B6-catalysed bupropion hydroxylation and patients receiving either clopidogrel or ticlopidine are likely to need dose adjustments when treated with drugs primarily metabolised by CYP2B6. The effect of impaired kidney function on CYP2B6 activity and on bupropion pharmacokinetics was also explored. In patients with kidney disease, the bupropion AUC and Cmax were significantly higher and the apparent oral clearance of bupropion was notably lower compared to healthy controls.

The present results indicate that the in silico and in vitro methods used are helpful in predicting in vivo drug-drug interactions. The effective utilisation of these models in the early phases of drug discovery could therefore help to target the in vivo studies and to eliminate metabolically unfavourable drug candidates.

Keywords: bupropion, clopidogrel, cytochrome P450 enzyme system, drug interactions, drug metabolism, enzyme inhibition, ticlopidine
To Leena and Jussi
Acknowledgements

This work was carried out at the Department of Pharmacology and Toxicology, University of Oulu and at the Department of Pharmacology, Drug Development and Therapeutics, University of Turku, during the years 2001-2006. I have been privileged to be supervised by Professor Olavi Pelkonen, *rara avis*, Head of the Department. His broad expertise, innovative ideas, and exceptional leadership have been the spark and the spirit of this thesis. Olavi’s door has always been open to enter and he has been ready to generously spend his own time offering friendly discussion, advice, and encouragement whenever needed. The blind trust he has shown to the limited capabilities of the student has been the element to carry this thesis into completion. My most sincere and deepest thanks are due to him. I feel fortunate and grateful to have gotten to know him.

I thank most sincerely my second supervisor, Docent Kari Laine from the University of Turku, for the support he has shown to this work. His open-minded attitude towards the collaboration and his impressive expertise in the field of clinical pharmacology has made it possible to carry out the clinical studies of this thesis.

I want to express my gratitude to the official referees Professor Marja-Liisa Dahl and Reader Amin Rostami-Hodjegan for their careful review and constructive comments. Sandra Hänninen, M.Sc. and Dr. Peter Cura are acknowledged for the language revision.

I am indebted to Docent Ari Tolonen and Jouko Uusitalo, M.Sc. for their contributions. Ari and Jouko have created, shared, and improved the ideas of this work and spent innumerable hours with all the original publications. Without your support, this thesis would not have been possible to make.

The fruitful and flexible collaborations with the people within the University of Oulu (Dept. of Chemistry, Division of Structural Chemistry) and between the University of Kuopio (Dept. of Pharmacology and Toxicology), the University of Turku (Dept. of Pharmacology, Drug Development and Therapeutics) and Karolinska Institutet (Institute of Laboratory Medicine) have been a pleasure. I want to thank the staff of these facilities for their warm-hearted attitude and for welcoming a stranger into their laboratories. The contributions of Professor Hannu Raunio and Professor Anders Rane are greatly appreciated and valued.

Working as a member of a team has been the best aspect of research work. I want to thank all my other co-authors, especially Päivi Taavitsainen, Ph.D., Niina Koivuuviita,
MD, Riina Nieminen, M.Sc., Stefan Lundgren, Ph.D., and Laura Korhonen, M.Sc. for their contributions. Mrs. Elina Kahra and Tuomas Korhonen, MD are acknowledged for their company and valuable help with the clinical studies. Satu Arpiainen, M.Sc., Docent Jukka Hakkola, Professor Markku Pasanen, Janne Hukkanen, MD, Ph.D., Pirkko Viitala, Ph.D., Docent Arja Rautio, and all the younger members of the Club Med -team are acknowledged for their friendship and support. My warmest thanks are due to Olli Tenhunen, MD, Ph.D., for these years and F24. The other staff at the department, especially Raisa Serpi, Ph.D., Päivi Myllynen, MD, Ph.D., Mr. Esa Kerttula, Mrs. Ritva Taurainen, Mrs. Anne Vuollo, Mrs. Raija Hanni, Mrs. Marja Räinä and Mrs. Terttu Keränen is most warmly acknowledged.

My beloved Mervi Turpeinen, Kim Paqvalin, and Leena & Jussi Turpeinen are acknowledged for their care and support. Finally, I want to thank Mika Ilves for his unfailing patience and regarding me worthy of his love.

The collaboration and support of Novamass Analytical, Juvantia Pharma, Hormos Medical Corporation, and Orion Pharma are appreciated. This work was funded by the grants from the National Technology Agency of Finland (TEKES), the Clinical Drug Research Graduate School, Turku University Hospital (EVO13390), the Finnish Cultural Foundation, the Finnish Medical Society Duodecim, the Finnish Medical Foundation, Juliana von Wendt Foundation, the Emil Aaltonen Foundation, the University of Oulu Science Foundation, the Research Foundation of the Orion Corporation, the Finnish Pharmacological Society, the European Federation for Pharmaceutical Sciences, the Finnish Drug Research Foundation, and the Farmos Research and Science Foundation.

Oulu, October 2006

Miia Turpeinen
Abbreviations

ACE  angiotensin I converting enzyme
ADME  Absorption, Disposition, Metabolism, Excretion
ADR  adverse drug reaction
AHR  aromatic hydrocarbon receptor
AUC  area under the plasma concentration-time curve
BMI  body mass index
BP  4-benzylpyridine
Cmax  maximum concentration in plasma
95% CI  95% confidence interval
CAR  constitutively active receptor
CBP  4-(4-chlorobenzylpyridine)
CLint  intrinsic clearance
CL/F  apparent oral clearance
CL/F/Wt  weight-corrected apparent oral clearance
CoMFA  comparative molecular fields analysis
CYP  cytochrome P450
3D-QSAR  three-dimensional quantitative structure activity relationship
DDI  drug-drug interaction
DME  drug metabolising enzyme
DMSO  dimethyl sulphoxide
ESI  electrospray ionization
F  bioavailability
Fabs  absorbed fraction of a drug
fgi  the fraction of a drug after gastrointestinal metabolism
fh  the fraction of a drug after hepatic metabolism
GFR  glomerular filtration rate
GSH  L-glutathione (reduced form)
hCG  human chorionic gonadotrophin
HLM  human liver microsomes
HMG-CoA  3-hydroxy-3-methylglutaryl-coenzyme A
HPLC  high-performance liquid chromatography
HTS  high-throughput screening
[I]  inhibitor concentration (unbound)
IC$_{50}$  concentration of inhibitor corresponding to a 50 % decrease in reaction velocity compared to the control reaction
K$_i$  inhibition constant
K$_m$  Michaelis-menten constant for a substrate
LC  liquid chromatography
m/z  mass-to-charge ratio
MS  mass spectrometry
NADPH  nicotinamide adenine dinucleotide phosphate (reduced form)
NBP  4-(4-nitrobenzylpyridine)
NCE  new chemical entity
NSAID  non-steroidal anti-inflammatory drug
OATP  organic anion transporting polypeptide
P450  cytochrome P450
P-gp  P-glycoprotein (MDR1, ABCB1)
pIC$_{50}$  log P of the concentration of inhibitor corresponding to a 50 % decrease in reaction velocity compared to the control reaction
PAH  polycyclic aromatic hydrocarbon
PAPS  3′-phosphoadenosine 5′-phosphosulfate
PK  pharmacokinetics
PM  poor metaboliser
PXR  pregnane X receptor
r  Pearson’s correlation coefficient
[S]  substrate concentration
SD  standard deviation
SEM  standard error of mean
SNP  single nucleotide polymorphism
$\text{t}_{\text{max}}$  time to maximum concentration
$\text{t}_{1/2}$  elimination half-life
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
thioTEPA  N,N,N′,N″-Triethylenethiophosphoramide
$v_{\text{max}}$  maximal reaction velocity
TOF  time-of-flight detector
UDPGA  uridine 5′-diphosphoglucuronic acid
UGT  UDP-glucuronosyltransferase
UM  ultra-rapid metaboliser
List of original articles

The present thesis is based on the following publications, which are referred to in the text by Roman numerals I to VI:


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1 Introduction

Xenobiotics are chemical entities foreign to the normal composition of the human body. These compounds cover a wide range of drugs, pesticides, food additives, and environmental pollutants. Mechanical, cellular, and enzymatic systems act as defence mechanisms against the entry of substances into the body or help the body to eliminate the foreign compounds. Elimination can take place by several different means, the most important being excretion into urine or bile. In order for a compound to be feasibly eliminated, most chemicals are modified to be more hydrophilic than the parent compound through metabolic transformation reactions. As a consequence, metabolism is beneficial for the body.

In some cases, metabolism results in the formation of reactive forms from inactive parent compounds – a phenomenon known as metabolic activation. Classical examples of this are the toxic metabolites produced from benzo[a]pyrene and acetaminophen; compounds which may further lead to cancer formation or organ toxicity (Park et al. 1995, Lin & Lu 1998). Metabolism contributes further to the bioavailability of compounds, and in the case of drugs, to the pharmacological effect and therapeutic response. The pharmacokinetic properties of drugs are determined to a great extent by metabolic reactions and therefore, metabolism is often behind bioavailability problems, interindividual variation, metabolic interactions and idiosyncrasies (White 1998, Boobis et al. 1999, Pelkonen et al. 2002). Furthermore, the lack of detailed knowledge of the metabolic fate and interactions of a drug can result in adverse effects, therapeutic failure, and toxicity from unanticipated overdose or metabolic reactions.

One of the most important reasons for the failure of a new drug in development is often due to poor pharmacokinetics and metabolic properties (Kennedy 1997, Eddershaw et al. 2000, Roberts 2003). Recent market withdrawals due to metabolism-associated interactions include cerivastatin (Jamal et al. 2004, Maggini et al. 2004), mibefradil (Siepmann et al. 1995, Krayenbuhl et al. 1999), cisapride (Michalets & Williams 2000), and terfenadine (Monahan et al. 1990, Kivistö et al. 1994). Elucidation of the metabolic properties of candidate molecules in the early phases of drug development and discovery is therefore crucial.

Elimination of unfavourable drug candidates and highlighting the more potential ones in early phases of development speeds up the process and ultimately leads to cost savings.
and increased profits through earlier marketed products. Traditionally, a majority of preclinical studies have been accomplished utilising in vivo testing with experimental animals. However, in addition to high expenses and low turnover, the intra-species extrapolation has proven to be a major challenge in drug development. Public opinion concerning animal rights and the general guidelines from authorities pursuing the reduction, refinement, and replacement of animal studies have strengthened during the past years. These factors are the overpowering rationale for in vitro and in silico methods to assess drug metabolism and metabolic interactions in conjunction with drug discovery and development (Tucker et al. 2001, Bjornsson et al. 2003, Pelkonen et al. 2005, Wienkers & Heath 2005).

The present study was aimed to explore the possibilities to study drug metabolism and drug-drug interactions utilising in vivo, in vitro and in silico approaches and to develop and validate novel methods for these purposes. Cytochrome P450 (CYP) 2B6 was used as the target enzyme for these studies.
2 Review of the literature

2.1 Drug metabolism

The principal route of elimination of xenobiotics from the human body is enzymatic biotransformation. This process can be divided into two phases: to the functionalisation of the parent by oxidative reactions in phase I and a further increase in the hydrophilicity of the parent after conjugative reactions in phase II. Phase I metabolism is mainly catalysed by a group of cytochrome P450 (CYP) enzymes (Rendic & DiCarlo 1997, Parkinson 2001, Guengerich 2002) and phase II reactions by a diverse group of conjugating enzymes, especially UDP-glucuronosyltransferases and glutathione S-transferases (Krishna & Klotz 1994, Parkinson 2001).

The principal site of drug metabolism is the liver, which contains the vast majority of metabolising enzymes (Rendic & DiCarlo 1997, Guengerich 2002). As all the drugs absorbed from the small intestine are transferred to the liver by portal circulation, a drug is a subject of metabolism before entering the systemic circulation and arriving at the site of action. This phenomenon is called first-pass metabolism. Drugs having a high first-pass rate (or poor absorption) are referred to have a low bioavailability (Figure 1).

Fig. 1. The concept of first pass metabolism and bioavailability (modified from Rowland & Tozer 1994).
2.2 Cytochrome P450 (CYP) enzyme system

Cytochrome P450 is a superfamily of heme-containing mono-oxygenase enzymes, crucial for the oxidative, peroxidative, and reductive metabolism of a diverse group of substances (Wrighton & Stevens 1992, Nelson et al. 1996, Yan & Caldwell 2001). The basic reaction catalysed by CYPs is the addition of one oxygen atom to the substrate, while the other oxygen atom is further reduced to water with reducing equivalents derived from NADPH (Ortiz de Montellano 1999, Parkinson 2001, Guengerich 2001) (Figure 2).

Fig. 2. Catalytic cycle of Cytochrome P450 (modified from Parkinson 2001).

Human CYPs are located intracellularly in the endoplasmic reticulum (microsomes) (Lin & Lu 1998). The metabolism of over 90% of all drugs is estimated to be mediated, at least partially, via CYPs (Evans & Relling 1999, Rendic 2002, Pelkonen 2004, Wienkers & Heath 2005). The CYP superfamily has been divided on the basis of structural homology into families (enzymes with > 40% aminoacid sequence identity), which are then further classified into subfamilies (enzymes within family having > 55% aminoacid identity). Currently there are 18 different known CYP-families and 42 subfamilies in humans covering 57 CYP genes and 58 pseudogenes (Lewis 2004, Nelson et al. 2004, http://drnelson.utmem.edu/CytochromeP450.html). Enzymes in families 1-3 have been traditionally thought to function mostly in the metabolism of a wide variety of xenobiotics, while other families are involved mainly in physiological functions such as the biosynthesis of fatty acids, steroid hormones, and bile acids (Venkatakrishnan et al. 2001, Nembert & Russell 2002).

The expression of most xenobiotic-metabolising CYPs is complex and varies between individuals due to genetic, host, and environmental factors as well as certain pathophysiological conditions. These factors contribute to wide inter-individual variation in the metabolic rate and pathways of drugs. Several external factors, such as dietary compounds, alcohol, concomitant medication, and cigarette smoking may lead to
induction or repression of the expression of certain CYPs (Pelkonen & Breimer 1994, Pacifici & Pelkonen 2001). Genetic factors influencing the inter-individual and population level variation are often due to the polymorphic expression of many CYPs in the population (Mizutani 2003, Solus et al. 2004, http://www.imm.ki.se/CYPalleles/). This results in variability in the expression, activity, function, and stability of the enzyme and to its responsiveness to regulators (Kalow 2001, Daly 2004). The polymorphic forms of CYPs have been estimated to contribute significantly to the idiosyncratic adverse drug reactions (ADRs) (Phillips et al. 2001, Rodrigues & Rushmore 2002).

2.3 Human hepatic CYP enzymes involved in drug biotransformation

The following chapters will focus on human CYPs expressed mainly in liver. As the number of CYPs is large, only the most important enzymes for drug metabolism will be discussed in more detail. Many individual members of CYP families exhibit distinct, but often overlapping, selectivity towards certain substrates and inhibitors. The most commonly used probe substrates and diagnostic inhibitors for each CYP isoform are collected in Table 1 and discussed below.

2.3.1 CYP1 family

CYP1A2 is the only hepatic member of the CYP1 family. CYP1A1 and CYP1B1 are the other enzymes in this family, of which CYP1A1 is the major human extrahepatic CYP form (Raunio et al. 1995, Ding & Kaminsky 2003). The hepatic expression of CYP1B1 is insubstantial, but otherwise it is known to be expressed in almost every other tissue (Sutter et al. 1994, Shimada et al. 1996). No pseudogenes have been found within this superfamily (Nelson et al. 2004). Enzymes in the CYP1 family are regulated by the AHR-receptor and are inducible by TCDD, PAHs, and smoking (Hankinson 1995, Nebert et al. 2004, Shimada & Fujii-Kuriyama 2004). Besides detoxification, the CYP1 family members are often responsible for metabolic activation of PAHs and aromatic amines and thus they have been linked to chemical carcinogenesis (Boobis et al. 1994, Ioannides & Lewis 2004).

2.3.1.1 CYP1A2

Initially the expression of CYP1A2 was thought to be limited only to the liver, but recent studies have shown that it is expressed along with CYP1A1 in the lung (Wei et al. 2001, Wei et al. 2002, Liu et al. 2003). Despite the quite low quantitative proportion of CYP1A2 in liver, it is a major enzyme in the metabolism of a number of important chemicals. Ethoxyresorufin (Burke et al. 1978), caffeine (Butler et al. 1989, Tassaneeyakul et al. 1992), phenacetin (Sesardic et al. 1990b, Venkatakrishnan et al. 1998b), theophylline (Sarkar & Jackson 1994, Tjia et al. 1996), clozapine (Bertilsson et
al. 1994, Fang et al. 1998), melatonin (von Bahr et al. 2000, Facciola et al. 2001), and tizanidine (Granfors et al. 2004a, Granfors et al. 2004b) are biotransformed mainly by this CYP form.

Potent inhibitors of CYP1A2 include furafylline (Sesardic et al. 1990a), fluvoxamine (Brosen et al. 1993, Rasmussen et al. 1998), ciprofloxacin (Fuhr et al. 1992, Fuhr et al. 1993), rofecoxib (Bachmann et al. 2003, Backman et al. 2006), oral hormone replacement therapy, and contraceptives (Laine et al. 1999, Pollock et al. 1999). CYP1A2 is known to be induced by cigarette smoking (Kalow & Tang 1991, Vistisen et al. 1991, Vistisen et al. 1992), cruciferous vegetables (Vistisen et al. 1992, Lampe et al. 2000, Murray et al. 2001), and charcoal-grilled food (Kall & Klausen 1995, Boobis et al. 1996, Larsen & Brosen 2005). Smoking has been studied quite extensively in conjunction to interactions with antipsychotics and has been related to dose adjustments and/or poor outcome in the treatment of schizophrenia with atypical antipsychotics metabolised via CYP1A2 (van der Weide 2003, Bozikas 2004, de Leon 2004).

Over twenty SNPs within CYP1A2 have been reported, though most of them have been found to be very rare (Nakajima et al. 1994, Sachse et al. 1999). Certain SNPs have been associated with disturbed probe drug metabolism (Han et al. 2002, Eap et al. 2004, Chen et al. 2005, Saito et al. 2005), ADRs (Tiwari et al. 2005), and to some disease states (Moonen et al. 2005, Cornelis et al. 2006, Chen et al. 2006), but the effect of other exogenic factors has not been ruled out (Nordmark et al. 2002, Akillu et al. 2003, Eap et al. 2004).

### 2.3.2 CYP2 family

The human CYP2 family is very diverse and comprises a number of important drug metabolising CYPs. Members of this family do not share any common regulation patterns and their substrate specificities and tissue expression vary substantially. CYP2B6, CYP2D6, and CYP2E1 are the only functional enzymes in their subfamilies, whereas CYP2A contains two, and CYP2C four functional members. The clinically most important CYP polymorphisms are found within the CYP2 family (i.e. CYP2C9, CYP2C19, and CYP2D6).

#### 2.3.2.1 CYP2A6

At the quantitative level, CYP2A6 is a minor component among hepatic CYPs. Substrates of CYP2A6 are usually structurally small and planar molecules (Lewis 2004) e.g. coumarin (Miles et al. 1990, Pelkonen et al. 2000) and nicotine (Cashman et al. 1992, Hukkanen et al. 2005). In addition to pharmaceuticals, bioactivation of some toxicologically significant substances such as aflatoxin B1 and nitrosoamines are known to be mediated at least to some extent via CYP2A6 (Raunio et al. 1999, Pelkonen et al. 2000).

A number of potent inhibitors with variable selectivity against CYP2A6 have been characterised. The most used in vitro inhibitors include tranylcypromine (Draper et al.
1997, Taavitsainen et al. 2001) and methoxsalen (Mäenpää et al. 1994, Draper et al. 1997). Induction of CYP2A6 in vivo has been reported for antiepileptics (Sotaniemi et al. 1995), although the knowledge concerning the actual regulation mechanisms of this enzyme is still very restricted (Raunio et al. 1999, Pitarque et al. 2005). On the basis of studies on murine CYP2A5, an ortholog of human CYP2A6, induction mechanisms and inducer profiles display an unprecedented variation (Salonpää et al. 1997, Donato et al. 2002).

Several variant CYP2A6 alleles with distinct frequencies between ethnic populations have been characterised. Some of these SNPs have been associated with altered nicotine pharmacokinetics and furthermore to differing smoking habits in variant genotype populations (Pianezza et al. 1998, London et al. 1999, Raunio et al. 2001, Rautio et al. 2003).

2.3.2.2 CYP2B6

Initially CYP2B6 was regarded as a minor hepatic CYP enzyme in humans, with negligible significance in the overall xenobiotic metabolism and essentially expressed only in a few livers (Wrighton & Stevens 1992). Until recently, the research of CYP2B6 was complicated because of a lack of model substrates, inhibitors and antibodies. The view of the importance of CYP2B6 has been changing over the past few years and currently it belongs to a set of important hepatic drug-metabolising CYPs. A notable inter-individual variability in the expression of CYP2B6 has been reported, and CYP2B6 has been estimated to represent approximately 1-10 % of the total hepatic CYP content (Yamano et al. 1989, Code et al. 1997, Stresser et al. 1999, Lang et al. 2001).

The list of CYP2B6 substrates has been increased dramatically in the past few years (Ekins & Wrighton 1999, Turpeinen et al. 2006). CYP2B6 usually metabolises non-planar, neutral, or weakly basic molecules with one or two hydrogen bond acceptors (Lewis 2000). For the metabolic pathway and pharmacokinetics of bupropion (Hesse et al. 2000, Faucette et al. 2000), cyclophosphamide (Huang et al. 2000, Roy et al. 1999b), ifosfamide (Roy et al. 1999a, Roy et al. 1999b, Granvil et al. 1999), efavirenz (Ward et al. 2003), ketamine (Yanagihara et al. 2001, Hijazi et al. 2002), and propofol (Oda et al. 2001, Court et al. 2001), CYP2B6 is known to be of considerable importance. In addition, CYP2B6 appears to both bioactivate and detoxify a number of precarcinogens (Code et al. 1997, Smith et al. 2003).

CYP2B6 has been shown to be inducible via CAR- and apparently PXR-associated mechanisms (Sueyoshi et al. 1999, Goodwin et al. 2001, Wang & Negishi 2003, Faucette et al. 2004, Faucette et al. 2006); in clinical practice this is seen as increased probe drug clearance after rifampicin (Kerbusch 2001, Lopez-Cortez et al. 2002, Loboz et al. 2006), and phenytoin treatments (Robertson et al. 2005). Papers introducing selective, potent inhibitors for CYP2B6 have been very scarce. Orphenadrine has traditionally been used as an inhibitor of CYP2B6 (Chang et al. 1993), but both the selectivity and the potency of it seems to be very poor (Guo et al. 1997, Sai et al. 2000). The antiretroviral drugs ritonavir, efavirenz, and nelfinavir have demonstrated to inhibit CYP2B6 quite potently (Hesse et al. 2001). Recently an anticancer agent thioTEPA has been shown to be a
selective CYP2B6 inhibitor with good inhibitory potency (Rae et al. 2002, Harleton et al. 2004).

Several SNPs within the CYP2B6 gene with variable allelic frequencies have been characterised, e.g. CYP2B6*6, reported to contribute to increased cyclophosphamide 4-hydroxylation (Xie et al. 2003), and CYP2B6*4, associated with higher bupropion clearance (Kirchheiner et al. 2003). Besides genotype, both sex and ethnicity have been shown to affect the CYP2B6 expression (Schmidt et al. 2001, Lamba et al. 2003, Burger et al. 2006).

2.3.2.3 CYP2C8

Like in the case of CYP2B6, the importance of CYP2C8 for drug metabolism has been elucidated quite recently (Totah & Rettie 2005). Known substrates for CYP2C8 include amodiaquine (Li et al. 2002), paclitaxel (Rahman et al. 1994), cerivastatin (Backman et al. 2002), and several oral antidiabetics such as repaglinide (Niemi et al. 2003b) and rosiglitazone (Baldwin et al. 1999, Niemi et al. 2003a).

Known CYP2C8 inhibitors include quercetin (Sousa et al. 1985, Kim et al. 2005), montelukast (Walsky et al. 2005a, Walsky et al. 2005b), zafirlukast (Walsky et al. 2005b), trimethoprim (Wen et al. 2002), and the glucuronide-conjugate of gemfibrozil (Ogilvie et al. 2006). Induction of CYP2C8 has been proposed to be mediated via multiple nuclear receptors (Gerbal-Chaloin et al. 2001, Ferguson et al. 2005).

A number of functional CYP2C8 polymorphisms have been published during recent years (Dai et al. 2001, Soyama et al. 2001, Niemi et al. 2003c). Certain SNPs or their combinations have been associated with increased risk of acute myocardial infarction (Yasar et al. 2004) and cerivastatin induced rhabdomyolysis (Ishikawa et al. 2004), but more studies about the importance of SNPs and also the general role of CYP2C8 in drug metabolism are still needed.

2.3.2.4 CYP2C9

CYP2C9 is the predominant CYP2C isoform and catalyses the metabolism of a number of clinically relevant drugs such as fluoxetine, fluvastatin, glimepiride, glipizide, losartan, S-warfarin, and several NSAID’s (for reviews see Miners & Birkett 1998 and Rettie & Jones 2005), as well as the classical probe substrate tolbutamide (Knodell et al. 1987, Sullivan-Klose et al. 1996).

Among the recognised inhibitors of CYP2C9 are gemfibrozil (Wen et al. 2001), amiodarone (Heimark et al. 1992), and sulphaphenazole (Back et al. 1988, Baldwin et al. 1995). Induction of CYP2C9, as well as other CYP2C family members, seems to involve multiple regulatory elements (Ibeanu et al. 1995, Gerbal-Chaloin et al. 2001, Ferguson et al. 2005).

CYP2C9 is a polymorphically expressed enzyme, and some of the SNPs within this gene have been identified as contributors to the wide interindividual variation in the pharmacokinetics of certain drugs. In the Caucasian population, the frequencies of main
variant alleles CYP2C9*2 and CYP2C9*3 range approximately from 7% to 13% (Stubbins et al. 1996, Sullivan-Klose et al. 1996, Yasar et al. 1999). The importance of the CYP2C9 polymorphism is emphasized especially with S-warfarin, which uses CYP2C9 as a major metabolic pathway and possesses a narrow therapeutic window with a fatal side-effect profile (Aithal et al. 1999, Higashi et al. 2002, Daly & King 2003, Kirchheiner & Brockmöller 2005).

2.3.2.5 CYP2C19


No selective drug inhibitors for CYP2C19 have been found yet, but at least omeprazole (Funck-Brentano et al. 1997) and fluconazole (Kang et al. 2002, Niwa et al. 2005) have been employed for this purpose. CYP2C19 is known to be inducible by chemicals, which act as ligands/activators for PXR and CAR (Gerbal-Chaloin et al. 2001, Raucy et al. 2002).

Compared to CYP2D6, polymorphisms of CYP2C19 represent a smaller proportion and perhaps have less clinical significance in Caucasians, but in Orientals the frequency of CYP2C19 poor metabolisers (PM) has been characterised to be up to 20% of the population (Bertilsson 1995, Desta et al. 2002). Of the two main mutations, CYP2C19*2 and CYP2C19*3, the former accounts for the majority of the defective alleles (de Morais et al. 1994, Chang et al. 1995b).

2.3.2.6 CYP2D6

Since the characterisation of the interindividual differences in the oxidation of debrisoquine and sparteine in the late 70’s (Mahgoub et al. 1977, Eichelbaum et al. 1979), CYP2D6 has become the most studied CYP with respect to pharmacogenetics. The genetic polymorphism within the CYP2D6 gene causes wide and clinically important variability in CYP2D6 enzyme activity, ranging from complete deficiency causing high risk for ADRs to extensively increased activity resulting in nonresponsiveness to treatments (Bertilsson et al. 2001, Ingelman-Sundberg 2005a). Examples of the large number of allelic variants of the CYP2D6 gene include the PMs lacking the functional enzyme, and ultra-rapid metabolisers (UM) having duplications or multiplications of the gene. Approximately 7% and 5.5% of Caucasians have been genotyped for CYP2D6 PMs and UMs, respectively (Daly 2004, Zanger et al. 2004, Ingelman-Sundberg 2005a). CYP2D6 belongs to the set of most relevant target genes where genotype/phenotype testing has suggested as a useful tool in dosing and monitoring in clinical practice (Kirchheiner et al. 2001, Dahl 2002, Kirchheiner et al. 2005, Eichelbaum et al. 2006).
CYP2D6 contributes to the metabolism of dextromethorphan (Park et al. 1984, Kronbach et al. 1987), debrisoquine (Mahgoub et al. 1977, Boobis et al. 1983), and β-adrenoceptor antagonists metoprolol (Otton et al. 1988b) and propranolol (Ward et al. 1989). Metabolism of several important antidepressants such as fluoxetine and paroxetine (Hiemke & Härtter 2000, Rasmussen & Brosen 2000) and atypical antipsychotics like risperidone (Berecz et al. 2004) and aripiprazole (Swainston-Harrison & Perry 2004) is mediated via CYP2D6.

CYP2D6 is inhibited potently by a variety of different drugs, of which a large proportion belongs also to the list of CYP2D6 substrates. Traditionally quinidine has been utilised as a highly selective and efficient CYP2D6 inhibitor for metabolism studies (Speirs et al. 1986, Newton et al. 1995). Incidentally, quinidine is not a substrate of CYP2D6 (Guengerich et al. 1986, Otton et al. 1988a). In contrast to other hepatic xenobiotic metabolising CYPs, CYP2D6 expression is not regulated by any known environmental agent and so far no inducers of CYP2D6 have been reported - although physiological conditions such as pregnancy have been shown to increase the rate of CYP2D6-mediated metabolic reactions (Wadelius et al. 1997, Heikkinen et al. 2002, Heikkinen et al. 2003, Ingelman-Sundberg 2005a).

### 2.3.2.7 CYP2E1

CYP2E1 is the only gene of its subfamily (Lewis 2004, Nelson et al. 2004). Although the relative hepatic content of CYP2E1 is notable, only a few pharmaceuticals are metabolised via this enzyme. The substrates of CYP2E1 usually consist of hydrophobic and low molecular weight compounds (Koop 1992, Lewis et al. 2002). For modelling purposes, chlorzoxazone (Peter et al. 1990) is probably the most widely used, but also the metabolism of acetaminophen (Lee et al. 1996), enflurane, and halothane (Raucy et al. 1993) seems to be mediated to some extent by CYP2E1.

From the toxicology perspective, the role of CYP2E1 is indisputable. It has been studied extensively due to its pivotal role in ethanol metabolism (Kessova & Cederbaum 2003, Lieber 2004), in the bioactivation of several industrial solvents (Raucy et al. 1993), in acetaminophen-related hepatotoxicity (Rumack 2004), as an activator of chemical carcinogenesis, and as a producer of free radicals causing tissue injury (Caro & Cederbaum 2004, Gonzalez 2005).

Several substrates of CYP2E1 (e.g. ethanol, acetone and pyrazole) also act as inducing agents of this enzyme. The mechanisms of induction take place at several levels of the regulation process and seem to be very complex, including post-translational modifications and a dependency on substrates (Eliasson et al. 1988, Eliasson et al. 1990, Song 1995, Kessova & Cederbaum 2003). Inhibitors of CYP2E1 include pyridine (Hargreaves et al. 1994) and disulfiram (Kharasch et al. 1993), the latter also being utilised in clinical practice. Although a number of SNPs within the CYP2E1 gene have been characterised, any polymorphisms leading to a loss of functionality have not been found (Gonzalez 2005).
The human CYP3 family represents about 30% of the total hepatic P450 content and is considered to be the most important CYP subfamily in the biotransformation of drugs. This family contains one subfamily including three functional proteins: CYP3A4, CYP3A5, and CYP3A7, and one pseudoprotein, CYP3A34 (Ingelman-Sundberg 2005b). These enzymes have overlapping catalytic specificities and their tissue expression patterns differ.

CYP3A5 is a minor polymorphic CYP isof orm in human liver (Westlind-Johnsson et al. 2003), but is extrahepatically consistently expressed in the kidney, lung, colon, and oesophagus (Ding & Kaminsky 2003, Burk & Wojonowski 2004). Despite a few exceptions, the substrate and inhibitor specificity of CYP3A5 seems to be highly similar to CYP3A4, albeit the catalytic capability might be somewhat lower (Wrighton et al. 1990, Yamazaki et al. 1995, Williams et al. 2002).

CYP3A7 is mainly expressed in embryonic, fetal, and newborn livers, where it is the predominant CYP form (Kitada & Kamataki 1994, Hakkola et al. 2001). In the adult liver, CYP3A7 seems to be a minor form (Komori et al. 1990, Schuetz et al. 1993). CYP3A7 has an important role during the fetal period in the hydroxylation of several endogenous substances like retinoic acid and steroid hormones, and therefore has relevance to normal embryonal development (de Wildt et al. 1999, Hines & McCarver 2002). In drug metabolism, the role of CYP3A7 is not yet clear.

### 2.3.3.1 CYP3A4

CYP3A4 is the sixth most abundant enzyme in human liver at the mRNA level and constitutes the major CYP isof orm in the liver (Shimada et al. 1994, Imaoka et al. 1996) and the small intestine (Kolars et al. 1992, von Richter et al. 1994, Kivistö et al. 1996, Paine et al. 2006). CYP3A4 has a pivotal role in xenobiotic metabolism, and it has been estimated to be involved in the metabolism of approximately half of the currently used drugs (Bertz & Granneman 1997).

The active site of CYP3A4 is very large and flexible allowing multiple small molecules to be present simultaneously in the active site. The substrate binding is principally based on hydrophobicity with some steric interactions. Also, a concept where multiple conformations of the enzyme can exist both in the presence and absence of substrate has been proposed (Atkins et al. 2001, Ekins et al. 2003, Scott & Halpert 2005). The kinetic interaction between CYP3A4 and its substrates is often atypical, making the prediction and modelling of CYP3A4-mediated drug-drug interactions troublesome (Ekins et al. 2003, Houston & Galetin 2004).

The known substrates of CYP3A4 vary widely in size and structure. Among the substrates of CYP3A4 are several clinically important drugs, e.g. acetaminophen, cyclosporine, erythromycin, nifedipine, felodipine, midazolam, triazolam, simvastatin, atorvastatin, and quinidine (Li et al. 1995, Bertz & Granneman 1997, Rendic 2002), as well as several endogenous agents including testosterone, progesterone, androstenedione, and bile acid (Waxman et al. 1991, Patki et al. 2003). Consequently, altered CYP3A4
activity can lead to notable drug-drug interactions and adverse effects. Bioactivation of some procarcinogens such as aflatoxin B1 (Aoyama et al. 1990) and PAHs (Hecht 1999) is also mediated to some extent via CYP3A4.

A relatively low degree of substrate selectivity makes CYP3A4 susceptible to inhibition by different chemicals. Inhibitors of CYP3A4 cover a broad variety of structurally unrelated substances. The most well established and clinically the most relevant inhibitors include certain azole antifungal agents (ketoconazole, itraconazole), antibacterials (clarithromycin, erythromycin), antihypertensives (verapamil, diltiazem) (Zhou et al. 2005) and several herbal and food constituents, e.g. grapefruit juice and bergamottin (He & Edeki 2004, Fujita 2004). IC₅₀ values for CYP3A4 inhibitors are highly substrate-dependent, and the use of multiple probe substrates is thus recommended (Kenworthy et al. 1999, Wang et al. 2000, Galetin et al. 2003). Usually the inhibition of CYP3A4 leads to ADRs, but in clinical use the “boosting effect” obtained through CYP3A4 inhibition with ritonavir has been successfully utilised in the treatment of HIV infection with other protease inhibitors (Corbett et al. 2002, Becker 2003, Plosker & Scott 2003).

CYP3A4 is known to be inducible by a large number of substances, for example phenobarbital type inducers, rifampicin, dexamethasone, phenytoin, carbamazepine, and St. John’s wort (Pelkonen et al. 1998, Luo et al. 2004). The CYP3A4 induction is a well known problem in clinical drug therapy, contributing to bioavailability, interactions, and interindividual variation. Unlike inhibition and induction, the role of CYP3A4 polymorphisms in variable CYP3A4-mediated metabolism is not yet fully characterised or understood (Lamba et al. 2002, Wojonowski 2004, Flockhart & Rae 2003).
Table 1. Summary of human hepatic drug metabolising CYP enzymes and their selected probe substrates and inhibitors used in vitro and in vivo studies (data adapted from Bertz & Granneman 1997; Pelkonen et al. 1998, 2000, 2005; Rendic 2002; Lewis 2003; Ingelman-Sundberg 2004 and Totah & Rettie 2005).

<table>
<thead>
<tr>
<th>CYP</th>
<th>% in liver *</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Other characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>2</td>
<td>Ethoxyresorufin</td>
<td>Furafylline</td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melatonin</td>
<td>Fluvoxamine</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caffeine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phencetin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A6</td>
<td>2</td>
<td>Coumarin</td>
<td>Tranleypromine</td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicotine</td>
<td></td>
<td>Polymorphic</td>
</tr>
<tr>
<td>2B6</td>
<td>2-4</td>
<td>Bupropion</td>
<td>Thio-Tepa</td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Efavirenz</td>
<td>Ticlopidine</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>1</td>
<td>Paclitaxel</td>
<td>Montelukast</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amodiaquine</td>
<td>Quercetin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rosiglitazone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C9</td>
<td>10</td>
<td>S-warfarin</td>
<td>Sulphaphenazole</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diclophenac</td>
<td></td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tolbutamide</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Losartan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C19</td>
<td>5</td>
<td>Omeprazole</td>
<td>Fluconazole</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S-mephenytoin</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Proguanil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2D6</td>
<td>2-3</td>
<td>Dextromethorphan</td>
<td>Quinidine</td>
<td>Polymorphic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Debrisquione</td>
<td>Paroxetine</td>
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<tr>
<td></td>
<td></td>
<td>Bufaralol</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Propranolololol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2E1</td>
<td>2-4</td>
<td>Chlorozoxazone</td>
<td>Pyridine</td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>Disulphiram</td>
<td></td>
</tr>
<tr>
<td>3A4</td>
<td>45-50</td>
<td>Midazolam</td>
<td>Ketoconazole</td>
<td>Inducible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>Itraconazole</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simvastatin</td>
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<tr>
<td></td>
<td></td>
<td>Nifedipine</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Erythromycin</td>
<td></td>
<td></td>
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</tbody>
</table>

* The relative amounts of hepatic P450 proteins vary highly among people. Values are based on a meta-analysis of Rowland Yeo et al. 2004 and rounded.
2.4 Drug-drug interactions involving CYP enzymes

Harmful drug-drug interactions (DDIs) are a major concern in pharmacotherapy. The incidence of serious and fatal ADRs has been estimated to be one of the leading causes of death in the Western world (Lazarou et al. 1998) and to contribute to significant economical losses and increased hospitalisation (Moore et al. 1998, Lazarou et al. 1998, Juurlink et al. 2003). The frequency of DDIs as a cause of ADRs varies from 10% to 26% depending on the study population (Kelly 2001, McDonnell & Jacobs 2002). The amount of concomitant medication is known to be crucial for the likelihood of DDI to occur (Weidelman et al. 1998). With deeper understanding, the drug-induced ADRs could usually have been predicted and avoided beforehand (Juurlink et al. 2003).

Drug interactions are generally classified as pharmacodynamic or pharmacokinetic. Pharmacokinetic interactions can take place at any level of the absorption, distribution, metabolism, or excretion (ADME) process (Stockley 1998). The most clinically relevant interactions occur principally either in the absorption or metabolism phase. As CYPs are often the rate-limiting enzymes in the biotransformation process, they have a crucial role in pharmacokinetics and DDIs. Consequences of CYP-associated DDIs resulting from reduced (inhibition) or increased (induction) rate and extent of the biotransformation will be discussed in more detail in following sections.

2.4.1 Inhibition of CYP enzymes

Inhibition of CYP enzymes is the most common cause of DDIs and has led to the removal of several drugs from the market during the past years (Friedman et al. 1999, Lasser et al. 2002). Inhibition can lead to increased bioavailability of the parent compound normally subject to extensive first-pass elimination or to decreased elimination of compounds dependent on metabolism for systemic clearance. If a drug is metabolised via a single pathway, inhibition may result in on increased accumulation ratio, increased steady-state concentration, and non-linear kinetics as a consequence of the saturation of enzymatic processes. Especially with prodrugs, where the drug is administered in an inactive form, inhibition may result in a decrease in the amount of the active drug form. Thus, inhibition of CYPs may lead to the toxicity or reduced efficacy of a drug.

The type of CYP inhibition can be either irreversible (mechanism-based inhibition) or reversible. The distinction is relative and can be hard to determine if the inhibitor binds tightly to the enzyme and is released slowly (Wienkers & Heath 2005). Irreversible inhibition requires biotransformation of the inhibitor, while reversible inhibition can take place directly, without metabolism. Reversible inhibition is the most common type of enzyme inhibition and can be further divided into competitive, non-competitive, uncompetitive, and mixed-type inhibition (Lin & Lu 1998, Hollenberg 2002, Madan et al. 2002, Copeland & Anderson 2002).
2.4.1.1 Mechanism-based inhibition

Mechanism-based inhibition can occur via the formation of metabolite intermediate complexes or via the strong, covalent binding of reactive intermediates to the protein or heme of the CYP. Mechanism-based inhibition is terminated by enzyme resynthesis and is therefore usually long-lasting (Halpert 1995, Ito et al. 1998, Kent et al. 2001). In some cases, the metabolic product inactivates the enzyme completely. This is referred to suicide inhibition. The most important phenomenon of mechanism-based inhibition is the time-, concentration-, and NADPH-dependent enzyme inactivation (Halpert 1995, Lin & Lu 1998). Classical mechanism-based inhibitors include furafylline (CYP1A2) (Sesardic et al. 1990a, Kunze & Trager 1993) and gestodene (CYP3A4) (Guengerich 1990, Back et al. 1991).

2.4.1.2 Reversible inhibition

Reversible inhibition occurs as a result of competition at the active site of the enzyme and probably involves only the first step of the catalytic cycle of CYP. Binding to the enzyme takes place usually with weak bonds, which are both formed and broken down easily. Consequently, reversible inhibitors act fast, but do not permanently destroy the enzyme (Lin & Lu 1998, Hollenberg 2002, Madan et al. 2002). A majority of enzyme inhibitors used as drugs are reversible inhibitors of their target enzymes (e.g. ACE inhibitors and HMG-CoA reductase inhibitors) (Copeland & Anderson 2002).

In competitive inhibition, there is competition between the substrate and inhibitor to bind to the same position on the active site of the enzyme. In the noncompetitive type of inhibition, the active binding site of the substrate and inhibitor is different from each other. In the case of uncompetitive inhibition, the inhibitor binds to the enzyme-substrate complex, but not to the free enzyme entity. In practice, mixed-type inhibition displaying elements of both competitive and noncompetitive inhibition mode is frequently observed (Madan et al. 2002; Copeland & Anderson 2002).

2.4.2 Induction of CYP enzymes

The amount of metabolising enzymes can increase due to stabilization of the protein or due to de novo synthesis of protein as a result of increased transcription. Most of the human hepatic CYPs, e.g. CYP2C9, CYP2E1, and CYP3A4 appear to be inducible. Unlike the inhibition of CYPs, induction is a slow regulatory process involving nuclear receptors, and clinically relevant induction occurs during multiple dosing of the inducing compound (Lin & Lu 1998, Pelkonen et al. 2002, Xu et al. 2005). From a biological view, induction is a protective response increasing the detoxification activity, but regarding drug metabolism, it leads usually to therapeutic failure as plasma concentrations of the drugs remain at subtherapeutic levels. In the case of prodrugs, induction can also result in the increase of active metabolite.
Classical CYP-inducers include carbamazepine, phenytoin, rifampicin, and St. John’s wort (Dickins 2004). Also, dietary compounds and environmental factors such as smoking, alcohol, and charcoal-grilled meat may act as inducers of drug metabolism. A typical characteristic for inducers is the ability to induce a wide spectrum of CYPs (Pelkonen et al. 1998, Pelkonen et al. 2002). Some agents can lead to auto-induction and thereby increase their own metabolism and elimination as well as potentially any other compounds metabolised by the same enzyme (Chang et al. 1997, Simonsson et al. 2003).

2.5 Applying in vitro results to the in vivo situation

The very first attempts to predict the in vivo behaviour of a drug on the basis of in vitro experiments were presented by Rane and coworkers in the late 70’s (Rane et al. 1977). Followed soon by others, the in vitro-in vivo extrapolation divided into two main areas: prediction of the metabolic clearance and prediction of drug-drug interactions.

2.5.1 Prediction of intrinsic clearance

Metabolic clearance (intrinsic clearance, CL_{int}) is an important determinant for extrapolation purposes. CL_{int} is a direct measure of the efficacy of an enzyme to metabolise a substrate. Knowing that the drug concentrations in in vivo situations are usually far below their K_{m} values, namely under linear velocity conditions, CL_{int} is equal to the ratio of v_{max}/K_{m} (Houston et al. 1994, Iwatsubo et al. 1997, Pelkonen et al. 1998).

When the contribution of multiple enzymes to the metabolism is assumed, the net in vitro CL_{int} in the whole study system can be expressed as the sum of each metabolic pathway (Iwatsubo et al. 1997, Ito 1998):

\[ CL_{\text{int, tot}} = \sum_{i=1}^{n} CL_{\text{int, i}}. \]

Several elements limit the reliability of predicting CL_{int} on the basis of in vitro studies. The most important factor to be taken into account is the nonspecific binding of the substrate to the microsomal protein fraction. Generally, for drugs with high protein binding affinity, underestimations with CL_{int} can occur if protein binding is taken into account. However, inclusion of both microsomal and plasma protein binding usually results in good agreement between extrapolated and actual clearance values (Obach 1999, Obach 2001).

Altered hepatic blood flow may have an impact on the accuracy of the in vitro-in vivo extrapolation. On the basis of their hepatic clearances, drugs can be classified either as low-clearance or high-clearance compounds. In the previous case, the hepatic blood flow has a minor effect on the total clearance of the drug (enzyme-limited clearance), whereas in the latter case, changes in the hepatic blood flow will have a drastic effect on the total clearance of the drug (flow-limited clearance). The clinical outcome, though is, hard to

2.5.2 Prediction of drug-drug interactions

Based on the requirements of the authorities, new drugs need to be tested with respect to their potential to cause drug-drug interactions (European Medicines Agency 1997, U.S. Food and Drug Administration 1997). These estimations rely primarily on projected in vivo concentrations of compounds and on estimates of their inhibitory constants obtained from in vitro studies.

The degree of inhibition depends also on the inhibition pattern when the substrate concentration is high. However, when the \([S] \ll K_m\), the degree of inhibition \((R)\) can be expressed by the following equation independent of the inhibition pattern, except in the case of the uncompetitive inhibition (Tucker et al. 1992, Ito et al. 1998):

\[
R = \frac{V_0}{V_i} = \frac{1}{1 + [I]/K_i}
\]

When \([S] \geq K_m\), the degree of the inhibition can be estimated from the following assuming competitive inhibition (Tucker et al. 1992, Ito et al. 1998):

\[
R = \frac{V_0}{V_i} = \frac{K_m + [S]}{K_m(1 + [I]/K_i) + S}
\]

In the case of competitive inhibition, it is possible to calculate the inhibition constants on the basis of experimentally determined IC\(_{50}\) values using the Tornheim equation (Tornheim 1994):

\[
K_i = \frac{[I]}{(V_0/V_i)(1 + [S]/K_m)}
\]

A recent study by Obach and co-workers (Obach et al. 2006) involving a variety of drugs attempted to estimate the utility of in vitro data for prediction of DDIs in in vivo situations. They concluded that in vitro inhibition data could be reliably used for predictions for at least CYP1A2, CYP2C9, CYP2C19, and CYP2D6, while for CYP3A4, the effects of both hepatic and intestinal metabolism should be also considered. Other factors affecting in vivo – in vitro extrapolation will be discussed below.
2.5.2.1 Factors affecting in vitro – in vivo extrapolation

As presented above, in vivo drug interaction potential can be estimated from in vitro experiments. However, free inhibitor concentrations at the site of action (adjacent to the enzyme) are unknown in in vivo situations. Based on the hypothesis that only an unbound fraction of a drug is capable of diffusing into hepatic tissue, predictions are made assuming that unbound inhibitor concentrations in plasma and hepatic tissue are equal (Ito et al. 1998, von Molkke et al. 1998). For very lipophilic compounds, the previous assumption is known to be false; despite an extensive binding to plasma proteins, their hepatic concentrations are known to be multiple to their plasma values (Chou et al. 1993, von Molkke et al. 1998, Schmider et al. 1999, Cook et al. 2004). A recent analysis by Ito and coworkers (Ito et al. 2004) suggested that total inhibitor concentrations with in vitro Ki values would probably be the most useful approach for the categorization of CYP inhibitors.

The role of transporters, especially P-glycoprotein (P-gp) and human organic anion-transporting polypeptides (OATPs) has been recognised as a major contributor to DDIs (Chandra & Brouwer 2004, Shitara et al. 2005). P-gp is located at the apical surface of the cell membrane in a variety of tissues and serves as an efflux pump transporting substrates against a concentration gradient across the cell membrane (Fromm 2000, Johnstone et al. 2000, Schinkel & Jonker 2003, Hoffmann & Kroemer 2004). Inhibition of P-gp results in increased plasma and organ drug levels (Schinkel et al. 1994, Bauer et al. 2005, DuBuske 2005). P-gp is known to possess a significant substrate overlap with the CYP3A family: drug substrates for both CYP3A4 and P-gp include cyclosporin A, verapamil, quinidine, erythromycin, and HIV-1 protease inhibitors (Wacher et al. 1995, Kim 2002). The role and importance of OATPs has emerged during recent years. In liver, all known OATPs are located at the basolateral membrane and are involved in the uptake of different endogenous agents and drugs (Tirona & Kim 2002, Kim 2003, Mikkaichi et al. 2004, DuBuske 2005). Among the drug substrates of OATPs are fexofenadine (Cvetkovic et al. 1999, Dresser et al. 2002) and pravastatin (Hsiang et al. 1999, Nishizato et al. 2003). As discussed, transporters contribute significantly to the absorption, bioavailability, and disposition of drugs. Nevertheless, estimations concerning the net effect of transporters on DDIs are very uncertain and have been poorly taken into account in in vitro–in vivo extrapolation calculations.

Although the expression of CYPs is centered in the liver, several other barrier tissues such as intestinal mucosa, lungs, and skin contain metabolic enzymes on a smaller scale and contribute to xenobiotic biotransformation (Kapitulnik & Strobel 1999, Ding & Kaminsky 2003). Knowing that the systemic bioavailability depends both on the dose absorbed and the fraction surviving from hepatic and extrahepatic metabolism (see fig. 1), it should be kept in mind that especially intestinal metabolism may affect in vitro–in vivo extrapolation (Wu et al. 1995, Hall et al. 1999, Kivistö et al. 2004). It has been suggested that both the biotransformation by intestinal CYP3A4 and the active efflux of P-gp are responsible for the poor bioavailability observed for many orally administered drugs (Benet et al. 1996, Wacher et al. 2001, Kivistö et al. 2004). However, often the amount of extrahepatic metabolism is not known and consequently, estimations concerning the in vivo situation may be misleading.
Results obtained from *in vitro* test systems are highly dependent on several technical aspects including the microsomal protein amount, incubation time, and initial velocity conditions. The best predictive value is usually obtained when the substrate concentration used is within the linear part of the time and protein concentration dependence curve for the metabolite formation (Mäenpää *et al.* 1998, Lin & Lu 1998, Yu *et al.* 1999). It is supposed, with some restrictions, that the values obtained from inappropriate experimental settings would lead to the greatest extrapolation error with compounds of intermediate inhibitory potency (for a recent review, see Ghanbari *et al.* 2006).

Finally, it should be noted that although the potential for DDIs can be estimated on the basis of *in vitro* potency and specificity studies, the clinical relevance of the interaction is based mainly on two prerequisites: the concentration of the substrate in the *in vivo* situation and the therapeutic index and side-effect profile of the interacting drug (Pelkonen *et al.* 1998).

### 2.6 Approaches to study CYP enzymes, drug metabolism and drug-drug interactions

During the past years, several *in vitro* and *in silico* approaches for CYP screening purposes have been developed and taken into routine use within industry and academia. Most of the interest has focused on developing assays suitable for HTS purposes, but lately an increasing need for validation of these test systems has emerged (Rodrigues & Lin 2001, Yan & Caldwell 2001, Bjornsson *et al.* 2003, Pelkonen *et al.* 2005, Hutzler *et al.* 2005, Coecke *et al.* 2006). All the models presented have certain strengths and weaknesses, and usually an intergrated approach using several different techniques in drug metabolism studies produces the best outcome (Figure 3).

*In silico* screens based on predictions of $K_i$

*Primary in vitro* screens with rCYPs and fluorescence-based assays (determine $IC_{50}$)

*Secondary in vitro* screens with HLMs and LC/MS-based assays (determine $K_i$)

*In vivo* studies

*In vivo* human studies

Fig. 3. Representative steps of a drug-interaction screening process of NCE (modified from Rodrigues & Lin 2001).

#### 2.6.1 Humanized *in vivo* models

Genetically modified mice lines expressing various human CYP forms (Gonzalez 2003, Gonzalez & Kimura 2003, Gonzalez & Yu 2006) and chimeric mice with humanized liver (Katoh *et al.* 2004, Tateno *et al.* 2004) with the aim of creating more predictive models
for human response have been developed during past few years. The use of these strains is still very limited and requires further validation and characterisation.

### 2.6.2 Human-derived in vitro techniques

#### 2.6.2.1 Primary hepatocytes

Hepatocytes are known to contain the full complement of both phase I and phase II DMEs. Therefore, they are a valuable tool for multiple approaches including metabolite identification, transportation studies, and elucidation of possible toxic effects of the NCE. Good \textit{in vitro} – \textit{in vivo} correlations in the metabolic activity of a number of drugs have been demonstrated and consequently, currently cultured human hepatocytes are the most recommended tools to study CYP-mediated metabolism and induction (Guillouzo \textit{et al.} 1993, Li \textit{et al.} 1997, LeCluyse 2001, Worboys & Carlile 2001, Gomez-Lechon \textit{et al.} 2003, Gomez-Lechon \textit{et al.} 2004).

Widespread use of primary hepatocytes is limited by the restricted availability of liver tissue. Hepatocyte cultures can be prepared from whole livers and surgical wedge biopsies. The maintenance of normal cellular physiology and intercellular contacts in hepatocytes requires special matrix configurations and demanding technical abilities. Although several cryopreservation applications have been developed, the time for efficient use of a single hepatocyte batch is still quite short (Cross & Bayliss 2000, Ekins \textit{et al.} 2000, Hengstler \textit{et al.} 2000, Bjornsson \textit{et al.} 2003).

#### 2.6.2.2 Immortalised cell lines

Permanent cell lines expressing one or a few DMEs have been used for several years. The most common ones for metabolism studies are probably the liver-derived HepG2 and BC2 cell lines and the lung-derived line A549 (Hukkanen \textit{et al.} 2000, Yoshitomi \textit{et al.} 2001, Gomez-Lechon \textit{et al.} 2001). In order to increase the expression patterns and levels of DMEs, a number of genetically engineered cell lines and co-culture systems have been developed. However, most of these approaches have failed or been shown to possess a very restricted metabolic capacity (Coecke \textit{et al.} 2006).

Very recently a human hepatoma-derived cell line HepaRG was introduced (Gripon \textit{et al.} 2002) and demonstrated to possess several liver-specific functions and morphological resemblance to normal human hepatocytes (Parent \textit{et al.} 2004, Le Vee \textit{et al.} 2006). The levels of major drug-metabolising CYPs are relatively high in the HepaRG line, making it a very promising tool for drug metabolism and toxicity studies in the future (Aninat \textit{et al.} 2006, Turpeinen \textit{et al.} unpublished).
2.6.2.3 Liver slices

Precision-cut liver slices retain a wide range of enzymatic activities and closely resemble the organ level of the liver. Since the transport systems are present in liver slices, they are suitable for studying the transport of an NCE through cell membranes. Liver slices are also suitable for induction studies (Edwards et al. 2003). If a whole cell metabolism needs to be studied for short periods, liver slices represent a valuable tool (Ekins et al. 2000).

Maintenance of liver slices is demanding. For proper oxygen and nutrient transportation the thickness of the slice needs to be as thin as possible, and therefore special slicers are needed (Vickers et al. 1995). The time for use and prepare of a single slice is limited; however, cryopreservation methods have overcome some of these problems (Ekins et al. 1996, Glöckner et al. 1998, Ekins et al. 2000). Many studies have shown lower cellular uptake, clearance, and metabolic capacity in liver slices compared to hepatocytes (Worboys et al. 1996, Worboys et al. 1997, Ekins et al. 2000), which has probably influenced their popularity.

2.6.2.4 Subcellular fractions

Liver samples can be used directly as homogenates or subcellular fractions, including microsomes, can be prepared. Liver homogenate contains all phase I and II enzymes. Microsomes are derived from endoplasmic reticulum after homogenisation and differential ultracentrifugation and contain CYPs and UGTs. Together with liver homogenates, microsomes are the most widely used in vitro system for drug metabolism studies. Liver homogenates can be applied to metabolic stability and metabolite identification studies when the biotransformation pathways of an NCE are unknown. Microsomes are utilised widely, especially in CYP-inhibition studies (Ekins et al. 1999b, Pelkonen et al. 2005).

Subcellular fractions are very easy to prepare, reproduce, use, and store. Their disadvantages are the loss of some enzyme activity during preparation, which can be satisfactorily recreated by the addition of appropriate cofactors. Liver homogenate fortified with appropriate cofactors (e.g. NADPH, UDPGA, GSH and PAPS) exhibits the same enzyme activities that are present in intact tissue (Pelkonen et al. 2005, Ekins et al. 1999b, Ekins et al. 2000, Coecke et al. 2006). If the fresh tissue is correctly handled and frozed immediately after excision, the enzymatic activities are shown to be stable even during prolonged storage (Pearce et al. 1996, Yamazaki et al. 1997). Supposedly the validation and harmonisation of the handling of liver tissue between laboratories would diminish the inter-laboratory variation between assays employing subcellular fractions (Boobis et al. 1998, Kremers 1999).
2.6.2.5 \textit{cDNA-expressed CYPs}

Isolated heterogeneous human CYP enzymes, expressed as single enzymes at a time from cDNA in bacterial, yeast, and mammalian cells have been commercially available for several years. Recombinant CYPs have been adopted as frontline tools in early drug development. These systems are suitable for HTS purposes and can be utilised to ascertain whether an NCE is a substrate for a particular CYP form and what metabolite is generated by that specific enzyme. Moreover, recombinant enzymes can be used as small-scale bioreactors to generate usable amounts of metabolic product (Friedberg \textit{et al.} 1999, Moody \textit{et al.} 1999, McGinnity & Riley 2001).

2.6.2.6 \textit{Novel cell-based technologies}

Future applications for drug metabolism studies include bioartificial liver systems (Iwahori \textit{et al.} 2003, Park & Lee 2005) and stem cell-derived cultures and assays (Ogawa \textit{et al.} 2005, Tsutsui \textit{et al.} 2006). Preliminary studies concerning the expression, inhibition, and regulation of CYPs in these settings have already been published, making these technologies a quite interesting possibility for future drug metabolism studies.

2.6.3 \textit{Computational in silico methods}

The use of computer-based methods in the prediction of ADME properties of NCEs has expanded rapidly in drug discovery and has become increasingly accepted in both industry and academia. \textit{In silico} models provide high throughput and are applied in the very early stages of the drug development process. These approaches can be roughly classified as ligand-based (pharmacophore and QSAR), protein-based (crystallographic protein and homology models), and ligand-protein interaction models (ligand-protein docking) (de Groot & Ekins 2002, de Graaf \textit{et al.} 2005).

Several expert systems utilising rule- and knowledge-based databanks have been introduced. These systems are capable of predicting the potential sites of metabolism in the drug molecule and to further provide metabolic trees and pathways with estimations about the likelihood for the production of each metabolite (Greene \textit{et al.} 1999, Button \textit{et al.} 2003, Langowski & Long 2002, Coecke \textit{et al.} 2006).

A good drug is likely to be a compromise between multiple characteristics (lead optimisation). A possible pitfall of \textit{in silico} based models is the possibility of losing good compounds during early screens. Clear advantages include economic efficiency, the possibility to screen an unlimited amount of compounds, and the possibility to explore the effects of minor structural changes on selected targets.
### Table 2. Comparison of in vitro enzyme sources used in preclinical research (modified from Pelkonen et al. 2005 and Coecke et al. 2006).

<table>
<thead>
<tr>
<th>Enzyme sources</th>
<th>Availability</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver homogenates</td>
<td>Relatively good.</td>
<td>Contains basically all hepatic enzymes</td>
<td>Liver architecture lost. Cofactor addition necessary</td>
</tr>
<tr>
<td></td>
<td>Commercially available.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>Relatively good.</td>
<td>Contains most important rate-limiting enzymes. Relatively inexpensive. Easy storage.</td>
<td>Contains only Phase I enzymes and UGTs. Requires strictly specific substrates or antibodies for individual DMEs. Cofactor addition necessary</td>
</tr>
<tr>
<td></td>
<td>Transplantations or commercial sources.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA-expressed individual CYP enzymes</td>
<td>Commercially available.</td>
<td>Can be utilised with HTS substrates. The role of individual CYPs in the metabolism can be easily studied.</td>
<td>The effect of only one enzyme at a time can be studied.</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>Difficult to obtain.</td>
<td>Contains the whole complement of DMEs cellurally integrated. The induction effect of an NCE can be studied. Cryopreservation possible.</td>
<td>Requires specific techniques and well established procedures. The levels of many DMEs decrease rapidly during cultivation</td>
</tr>
<tr>
<td></td>
<td>Relatively healthy tissue needed.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commercially available.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver slices</td>
<td>Difficult to obtain.</td>
<td>Contains the whole complement of DMEs and cell-cell connections. The induction effect of an NCE can be studied. Cryopreservation possible.</td>
<td>Requires specific techniques and well established procedures. Limited viability.</td>
</tr>
<tr>
<td></td>
<td>Fresh tissue needed.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immortalised Cell lines</td>
<td>Available at request.</td>
<td>Non-limited source of enzymes.</td>
<td>The expression of most DMEs is poor. Genotype/phenotype instability.</td>
</tr>
<tr>
<td></td>
<td>Only few adequately characterised cell lines exist.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*Kremers et al. (1999); Rodrigues et al. (1999); Guillouzo et al. (1995), Gomez-Lechon et al. (2004); Ferrero & Brendel (1997); Allen et al. (2005)

### 2.7 Individual drugs studied

#### 2.7.1 Bupropion

Bupropion (amfebutamone) is a monocyclic aminoketone approved as a non-nicotine medical aid for smoking cessation and as an antidepressant. The exact mechanism of bupropion in smoking withdrawal is not known, but it has been thought to produce its therapeutic effects via inhibition of the neuronal uptake of noradrenaline and/or dopamine.

Bupropion is extensively metabolised in human liver, resulting in only low levels of the parent compound in plasma. The major metabolite, hydroxybupropion, is formed selectively by CYP2B6 and is pharmacologically active (Laizure et al. 1985, Posner et al. 1985, Hesse et al. 2000, Faucette et al. 2001). The other two metabolites, stereoisoforms threo- and erythrohydrobupropion, are suggested to form via the ketone reduction pathway (Borges et al. 2004, Jefferson et al. 2005).

A moderate inhibitory potency of CYP2D6 by bupropion in vitro has been reported (Hesse et al. 2000), but the clinical importance of this inhibition could be noteworthy (Kotlyar et al. 2005, Jefferson et al. 2005). Although not formally evaluated in clinical interaction studies, the potent in vitro inhibition of bupropion metabolism by a number of newer antidepressants such as paroxetine, fluvoxamine, and sertraline suggests the potential for clinical DDI’s (Hesse et al. 2000).

Bupropion is generally a well tolerated drug, the main side-effects being insomnia and dry mouth (Settle et al. 1999, Holm & Spencer 2000). However, a small risk of severe side-effects, e.g. seizures, has been associated with high concentrations of bupropion in plasma (Dunner et al. 1998, Johnston et al. 2001), and this may be of clinical importance when bupropion is used with other drugs affecting its metabolism.

### 2.7.2 Ticlopidine and clopidogrel

Thienopyridine derivatives clopidogrel and ticlopidine are prodrugs that selectively inhibit adenosine diphosphate mediated platelet aggregation and have been for several years in clinical use for the prevention of atherothrombotic events (Kam et al. 2003). A combination of aspirin and clopidogrel has become a standard treatment to prevent thrombosis after intravascular stenting procedures and in the management of atherothrombosis in high-risk patients with recurrent transient ischaemic attack or ischaemic stroke (Hacke 2002).

Only a few studies concerning DDIs related to clopidogrel have been published. Recent studies demonstrating the ability of atorvastatin to make clopidogrel ineffective (Clarke et al. 2003, Lau et al. 2003) have been refuted by several others (Saw et al. 2003, Mitsios et al. 2004 & 2005, Mukherjee et al. 2005). Ticlopidine has been previously characterized to be a potent mechanism-based inhibitor of CYP2C19 (Ko et al. 2000, Ha-Duong et al. 2001).

Compared to aspirin, the risk of gastrointestinal bleeding was decreased and the risk of diarrhoea and skin rash was increased with both clopidogrel and ticlopidine (Hankey et al. 2000). Despite the high efficacy of ticlopidine, its widespread use has been limited by frequent adverse effects including life-threatening neutropenia and thrombotic thrombocytopenic purpura (Bennett et al. 1998, Lubbe & Berger 2002).
Fig. 4. Chemical structures of study compounds and original articles concerned.
3 Aims of the present study

Since the importance of CYP enzymes for drug metabolism is beyond dispute, the goal of present thesis was to explore the possibilities to study drug metabolism and drug-drug interactions utilising in vivo, in vitro, and in silico techniques using CYP2B6 as the target enzyme. In addition, we aimed to development and validation of in vitro screening assay for commercial purposes. The specific aims were:

1. To find a potent and selective chemical inhibitor for CYP2B6 enzyme using conventional in vitro techniques.
2. To evaluate the use of CYP specific model substrates and inhibitors with known in vivo properties in a novel in vitro test system and to further develop a robust screening assay for the screening of the CYP inhibition potential of NCEs.
3. To compare the performance and utility of three distinct in vitro assay settings in CYP inhibition screening.
4. To further evaluate the effects of known in vitro inhibitors of CYP2B6 in vivo.
5. To create a comprehensive in silico model for CYP2B6 inhibition screening and to evaluate the CYP2B6 inhibition potencies of structurally diverse compounds.
6. To investigate the effect of renal impairment on the eactivity of CYP2B6 in vivo.
4 Materials and methods

4.1 In vitro and in silico studies (I-III, V)

4.1.1 Chemicals

Bupropion and hydroxybupropion were kind donations from Glaxo SmithKline, Inc. (Research Triangle, NC); omeprazole, 5-hydroxyomeprazole and omeprazole sulphone form Astra Zeneca (Mölndal, Sweden); midazolam and α-hydroxymidazolam from F. Hoffman-La Roche (Basel, Switzerland) and rac-mephenytoin from Sandoz Pharma (Basel, Switzerland). 4-benzylpyridine (BP) and 4-(4-chlorobenzylpyridine) (CBP) were obtained from Aldrich-Chemie (Heidenheim, Germany) and 4-(4-nitrobenzylpyridine) (NBP) from Acros Organics (Morris Plains, NJ). The metabolite standards hydroxytolbutamide, 4’-hydroxymephenytoin, 6-hydroxychlorzoxazone, 6β-hydroxytestosterone and dextrophan were purchased from Ultrafine Chemical Company (Manchester, U.K.). Montelukast was obtained from Sequoia Research Products (Panbourn, UK). HPLC-grade solvents were from Rathburn (Walkerburn, U.K.). The laboratory water was purified trough a Milli-Q system (Millipore S.A., Molsheim, France). Other chemicals were obtained mainly from Sigma Aldrich (St. Louis, MO) and Boehringer (Ingelheim, Germany) and were of the highest purity available.

4.1.2 cDNA expressed CYPs

Baculovirus-insect cell expressed human CYPs were purchased from BD Biosciences Discovery Labware (Bedford, MA) and were used according to manufacturer’s instructions.
4.1.3 Human liver samples

Human liver samples were obtained from the University Hospital of Oulu as surplus from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All liver samples were of Caucasian race. Characteristics of the samples are presented in Table 3.

The livers were transferred to ice immediately after the surgical excision, cut into approximately 1 cm³ pieces, 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 in CYP enzyme activities took place (data not shown, for details see e.g. Pearce et al. 1996 and Yamazaki et al. 1997).

A weight-balanced microsomal pool of seven (I, II) or ten (III, V) liver microsomal preparations which have been extensively characterized to be used for primary screening (sufficient model activities, no known polymorphisms, expected effects of model inhibitors, quantitation of CYPs by Western blotting) was employed. The final microsomal pellet was suspended in 100 mM phosphate buffer pH 7.4. Protein content was determined by the method of Bradford (Bradford 1976).

Table 3. Characteristics of the human liver samples.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Age</th>
<th>Sex</th>
<th>Cause of death</th>
<th>Drug history</th>
<th>Liver pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL20</td>
<td>54</td>
<td>M</td>
<td>ICH</td>
<td>Diazepam⁴</td>
<td>None</td>
</tr>
<tr>
<td>HL21</td>
<td>44</td>
<td>M</td>
<td>ICH</td>
<td>Phenytoin⁴, alcohol abuse</td>
<td>Cirrhotic</td>
</tr>
<tr>
<td>HL22</td>
<td>40</td>
<td>F</td>
<td>ICH</td>
<td>Dexamethasone⁴, nizatidine⁴, phenytoin⁴</td>
<td>None</td>
</tr>
<tr>
<td>HL23</td>
<td>43</td>
<td>M</td>
<td>ICH</td>
<td>Diazepam⁴, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL24</td>
<td>47</td>
<td>M</td>
<td>ICH</td>
<td>No medication, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL28</td>
<td>21</td>
<td>M</td>
<td>Stroke</td>
<td>Dexamethasone⁴, smoker</td>
<td>None</td>
</tr>
<tr>
<td>HL29</td>
<td>39</td>
<td>F</td>
<td>ICH, SAH</td>
<td>Dexamethasone⁴</td>
<td>None</td>
</tr>
<tr>
<td>HL30</td>
<td>53</td>
<td>F</td>
<td>ICH, SAH</td>
<td>No medication</td>
<td>Steatosis</td>
</tr>
<tr>
<td>HL31</td>
<td>44</td>
<td>F</td>
<td>ICH, SAH</td>
<td>No medication</td>
<td>Steatosis</td>
</tr>
<tr>
<td>HL32</td>
<td>62</td>
<td>M</td>
<td>ICH, SDH</td>
<td>Metformin, alcohol abuse, smoker</td>
<td>None</td>
</tr>
</tbody>
</table>

* Drugs were administrated only during the last 24 hours before death; M, male; F, female; ICH, intracerebral haemorrhage; SAH, subarachnoidal haemorrhage; SDH, subdural haematoma. NA, not available.

4.1.4 Enzyme assays and analytical methods

4.1.4.1 N-in-one and single substrate assays

The incubation and analysis conditions used in single substrate assays are summarised in Table 4 and described in detail in the original publications (I, II, IV and VI). The metabolites of ethoxyresorufin and coumarin were determined using Hitachi F-4010 fluorescence spectrophotometer. For analysis of amodiaquine metabolism a Waters Alliance 2695 HPLC system with Waters X Terra RP18 column (2.1×50 mm, 3.5 µm...
particle size, Waters Corp, Milford, MA) and Phenomenex Luna C18 precolumn, (4.0×2.0 mm, 3.0 µm, Phenomenex, Torrance, CA) was used. For other metabolites a Shimadzu VP series HPLC system with Waters Symmetry C_{18} column (3.9×150 mm, 5 µm, Waters Corp, Milford, MA) and Lichrospher 100 RP-18 guard column (4.0×4.0 mm, E.Merck, Darmstadt, Germany) was employed.

In original publication II, a Waters Alliance 2695 chromatographic system with Waters X Terra MS C_{18} (2.1×50 mm, 3.5 µm particle size, Waters Corp, Milford, MA) and Phenomenex Luna C_{18} precolumn, (4.0×2.0 mm, 3.0 µm, Phenomenex, Torrance, CA) were employed for n-in-one assay.

The IC_{50} and K_{i} values were determined graphically using Microcal Origin version 6.0 (Microcal Software Inc., Northampton, MA).

### 4.1.4.2 Fluorescence based assay

Incubations for fluorescence based assays were performed in 96-well plates and analysed with a Victor^{2} plate counter (Perkin Elmer Life Sciences Wallac, Turku, Finland). The experimental conditions are summarised in Table 5. and described in detail in original publications IV and VI. The IC_{50} values were calculated using non-linear regression analysis with Prism 4.0TM software (San Diego, CA, USA).
Table 4. Summary of the CYP selective in vitro assay conditions used in single substrate assays (I, II, III and V).

<table>
<thead>
<tr>
<th>CYP</th>
<th>Activity</th>
<th>[S]/K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Protein / incubat.</th>
<th>Incubat. time</th>
<th>Incubat. volume</th>
<th>Assay method and principal reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple</td>
<td>Ethoxycoumarin O-de-ethylation</td>
<td>0.3</td>
<td>0.1 mg</td>
<td>10 min</td>
<td>500 µl</td>
<td>Fluorometric (365/454 nm) Greenlee and Poland 1977</td>
</tr>
<tr>
<td>1A2</td>
<td>Ethoxyresorufin O-de-ethylation</td>
<td>2.5</td>
<td>0.2 mg</td>
<td>5 min</td>
<td>1000 µl</td>
<td>Fluorometric (530/585 nm) Burke et al. 1978</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>10</td>
<td>0.1 mg</td>
<td>10 min</td>
<td>500 µl</td>
<td>Fluorometric (365/454 nm) Aitio et al. 1978 Raunio et al. 1988</td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion hydroxylation</td>
<td>0.6</td>
<td>0.4 mg</td>
<td>15 min</td>
<td>200 µl</td>
<td>UV-HPLC (204/214 nm) Hesse et al. 2000 Faucette et al. 2000</td>
</tr>
<tr>
<td>2C8</td>
<td>Amodiaquine de-ethylation</td>
<td>12</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (342 nm) Li et al. 2002</td>
</tr>
<tr>
<td>2C9</td>
<td>Tolbutamide hydroxylation</td>
<td>0.5</td>
<td>0.15 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (236 nm) Knodell et al. 1987 Sullivan-Klose et al. 1996</td>
</tr>
<tr>
<td>2C19</td>
<td>Mephenytoin 4′-hydroxylation</td>
<td>1.7</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (204/240 nm) Wrighton et al. 1993</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole 5-hydroxylation</td>
<td>2</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (204/304 nm) Abéló et al. 2000</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>2</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (204/280 nm) Park, et al. 1984 Kronbach et al. 1987</td>
</tr>
<tr>
<td>2E1</td>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>2.5</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>UV-HPLC (282 nm) Peter et al. 1990</td>
</tr>
<tr>
<td>3A4</td>
<td>Midazolam α-hydroxylation</td>
<td>2</td>
<td>0.1 mg</td>
<td>5 min</td>
<td>200 µl</td>
<td>HPLC (245 nm) Kronbach et al. 1989</td>
</tr>
<tr>
<td>3A4</td>
<td>Omeprazole sulphotidation</td>
<td>0.4</td>
<td>0.1 mg</td>
<td>20 min</td>
<td>200 µl</td>
<td>HPLC (204/304 nm) Abéló et al. 2000</td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>2</td>
<td>0.4 mg</td>
<td>20 min</td>
<td>500 µl</td>
<td>TLC; autoradiography Waxman et al. 1983</td>
</tr>
</tbody>
</table>
Table 5. Summary of the CYP selective in vitro assay conditions used in fluorescence based assays (III and V).

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Metabolite</th>
<th>[S]</th>
<th>Amount of enzyme</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>7-ethoxyresorufin</td>
<td>Resorufin</td>
<td>1 μM</td>
<td>0.5 pmol</td>
<td>20 min</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin</td>
<td>7-hydroxycoumarin</td>
<td>3 μM</td>
<td>0.75 pmol</td>
<td>15 min</td>
</tr>
<tr>
<td>2B6</td>
<td>EFC</td>
<td>HFC</td>
<td>2.5 μM</td>
<td>0.75 pmol</td>
<td>30 min</td>
</tr>
<tr>
<td>2C8</td>
<td>DBF</td>
<td>Fluorescein</td>
<td>1 μM</td>
<td>3 pmol</td>
<td>30 min</td>
</tr>
<tr>
<td>2C9</td>
<td>MFC</td>
<td>HFC</td>
<td>75 μM</td>
<td>0.75 pmol</td>
<td>45 min</td>
</tr>
<tr>
<td>2C19</td>
<td>BFC</td>
<td>HFC</td>
<td>25 μM</td>
<td>2.5 pmol</td>
<td>45 min</td>
</tr>
<tr>
<td>2D6</td>
<td>MAMC</td>
<td>HAMC</td>
<td>7.5 μM</td>
<td>1.5 pmol</td>
<td>60 min</td>
</tr>
<tr>
<td>2E1</td>
<td>MFC</td>
<td>HFC</td>
<td>100 μM</td>
<td>1.5 pmol</td>
<td>45 min</td>
</tr>
<tr>
<td>3A4</td>
<td>BFC</td>
<td>HFC</td>
<td>50 μM</td>
<td>0.75 pmol</td>
<td>30 min</td>
</tr>
</tbody>
</table>

EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; DBF, dibenzyl fluorescein; MFC, 7-methoxy-4-(trifluoromethyl)coumarin; BFC, 7-benzyloxy-4-(trifluoromethyl)coumarin; MAMC, 7-methoxy-4-(aminomethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; HAMC, 7-hydroxy-4-(aminomethyl)coumarin

4.1.5 Comparative molecular field analysis

Comparative molecular field analysis (CoMFA) modelling was performed using Sybyl molecular modeling software version 6.9 (TRIPOS Associates Inc., St. Louis, MO). Atomic point charges were calculated using the MMFF94 method. Standard CoMFA analysis included steric and electrostatic fields which were calculated using the sp3 carbon probe atom with a +1 charge and a 2 Å grid spacing.

4.2 In vivo studies (IV, VI)

4.2.1 Subjects and ethics

Healthy, non-smoking volunteers participated this study series (IV and VI) after having given their written informed consent. The subjects were ascertained to be in good health by medical history, a full clinical examination and standard haematological and blood chemistry laboratory tests (i.e. blood haemoglobin, serum creatinine, serum alanine transferase, plasma glucose) before enrolment. Pregnancy was excluded by urine hCG test in female subjects. None of the subjects were using continuous medications or natural products.

In addition, ten patients with impaired kidney function (VI) had a stable, long-term renal impairment with elevated serum creatinine value and decreased glomerular filtration rate (GFR). Their liver function test results were in the normal range and none had have a treatment with peritoneal dialysis or haemodialysis. The medications taken by
the patients and causes of renal failure are collected in Table 7. Other characteristics are described in detail in the original publication (VI). Smoking or the use of natural products was not allowed during the study. The estimated GFR (eGFR) was calculated according to Cockcroft-Gault equation (Perrone et al. 1992, Lesley et al. 2006):

\[
eGFR = \frac{(140 - \text{age}) \times \text{body weight} \times 0.85 \text{ (for women)}}{\text{plasma creatinine} \times 72}
\]

The trials were designed in accordance with Good Clinical Practice and the Declaration of Helsinki. The study protocols were approved by the Ethics Committee of the Varsinais-Suomi healthcare district, Finland and by the Finnish National Agency for Medicines.

**Table 6. Demographics of the volunteers in studies IV and VI (range).**

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects (m/f)</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>12 (12/0)</td>
<td>22-27</td>
<td>67-95</td>
<td>21-26</td>
</tr>
<tr>
<td>V</td>
<td>Patients: 10 (5/5)</td>
<td>21-50</td>
<td>69-83</td>
<td>22-27</td>
</tr>
<tr>
<td></td>
<td>Controls: 17 (12/5)</td>
<td>32-43</td>
<td>57-96</td>
<td>21-27</td>
</tr>
</tbody>
</table>

**Table 7. Causes of compromised kidney functions medications of the renal-impaired patients (VI).**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cause of renal failure</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diabetes</td>
<td>Bisoprolol, calcium, darpoietin α, ezetimibe, felodipine, fluvastatin, furosemide, insulin, moxonidinone, ramipril, sodium polystyrene sulphonate, sodium bicarbonate</td>
</tr>
<tr>
<td>2</td>
<td>Congenital renal dysplasia</td>
<td>Allopurinol, atorvastatin, calcium, calcium carbonate, ramipril</td>
</tr>
<tr>
<td>3</td>
<td>Diabetes</td>
<td>Acetylsalicylic acid, furosemide, labetalol, ramipril, simvastatin</td>
</tr>
<tr>
<td>4</td>
<td>Diabetes</td>
<td>Alpha-calcidol, acetylsalicylic acid, calcium, enalapril, epoietin β, folic acid, furosemide, insulin, iron, metoprolol, thyroxin, sodium bicarbonate</td>
</tr>
<tr>
<td>5</td>
<td>Diabetes</td>
<td>Alpha-calcidol, calcium, carvedilol, felodipine, furosemide, insulin, iron, moxonidinone, pratsosine, ramipril, sodium polystyrene sulphonate</td>
</tr>
<tr>
<td>6</td>
<td>Diabetes</td>
<td>Atorvastatin, calcium, felodipine, hydrochlorothiazide, insulin, ramipril</td>
</tr>
<tr>
<td>7</td>
<td>Diabetes</td>
<td>Amlodipine, acetylsalicylic acid, darpoietin α, dipyridamole, furosemide, insulin, iron, ramipril, simvastatin, sodium bicarbonate</td>
</tr>
<tr>
<td>8</td>
<td>Diabetes</td>
<td>Bisoprolol, calcium, captopril, felodipine, furosemide, insulin moxonidinone, sodium polystyrene sulphonate</td>
</tr>
<tr>
<td>9</td>
<td>Polycystic renal disease</td>
<td>Allopurinol, calcium, felodipine, metoprolol, ramipril</td>
</tr>
<tr>
<td>10</td>
<td>IgA nephropathy</td>
<td>Calcium, furosemide, sodium bicarbonate, ramipril</td>
</tr>
</tbody>
</table>
4.2.2 Study designs

The clinical studies were conducted in a single center (University of Turku, Dept. of Pharmacology and Clinical Pharmacology) and volunteers were under direct medical supervision during the study days. Bupropion was administered by study personnel in both studies. In all phases the volunteers fasted for 8 hours before and 4 hours after administration of bupropion. Identical meals were provided on the study days in each study (IV, VI). Volunteers were also required to refrain from strenuous physical exercise, alcohol- or caffeine-containing drinks, smoking, grapefruit juice and other medications for two days before and after the study drugs.

The study IV was a three-phased, open study with randomised and balanced cross-over design. First phase was the control phase where each subject received a single oral dose of bupropion (Zyban sustained release, 150 mg, GlaxoSmithKline, Uxbridge, UK) without pretreatment. In phases 2 and 3, a four day long oral antiplatelet agent pretreatment either with clopidogrel (Plavix, 75 mg once daily, Sanofi Synthelabo, Guildford, UK) or ticlopidine (Ticlid 250 mg twice daily, Sanofi Synthelabo, Guildford, UK) was received. On phases 2 and 3, the administration of clopidogrel and ticlopidine was done by the volunteers themselves on days 1 to 3 and by the research personnel on day 4. Between phases 1 and 2 there was a one week long washout period and between phases 2 and 3 there a washout period of two weeks was carried out.

The study VI was conducted with an open parallel design. A single oral dose of bupropion (150 mg) was ingested by the healthy subjects as well as the renal-impaired patients.

In both studies, venous blood samples (10 ml each) for determination of bupropion and the metabolites hydroxybupropion and hydrobuprobion, were taken prior to administration of bupropion and 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, and 72 hours later. All blood samples were collected into lithium-heparin tubes and centrifuged for 10 min at 1600* g. Plasma was separated and stored at -70 °C until analysed.

4.2.3 Bioanalytical methods

The plasma samples were filtrated through Sirocco 96-well protein precipitation plates (Waters Corp., Milford, MA) and plasma concentrations for bupropion and hydroxybupropion were determined using HPLC-MS/MS. Mass spectrometric detection with multiple reaction monitoring (MRM) mode was performed with Micromass Quattro II triple quadrupole mass spectrometer (Micromass Corp., Altrincham, UK) with positive ion mode electrospray ionization. The fragmentation reactions monitored (MRM) were from m/z 256 to m/z 238 for hydroxybupropion and from m/z 240 to m/z 184 for bupropion. The lower limits of quantitation were 0.4 ng/ml for both bupropion and hydroxybupropion. Interday and intraday coefficients of variation were less than 15% for both compounds through the linear quantitation range 0.4-400 ng/ml.
4.2.4 Genotyping

The subjects and patients were genotyped with respect to the CYP2B6 gene SNPs 1459 C>T (CYP2B6*5) and 516 G>T (CYP2B6*6) using 5' nuclease assay assessed by an ABI PRISM 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK). The identification of CYP2B6 516G>T mutation was done by using primers cttctctagggcctca and ggtagtgggaactttcaagac and the MGB (minor groove binder) dark quencher probes 6-FAM-cttcctctt ccaGtcca and VIC cctcttccaTtccatt (Applied Biosystems, Warrington, Cheshire, UK). The CYP2B6 1459 C>T assay was performed with the primers ggecccaagacatcgat and cttccctcagccccttcag and 6-FAM-cagatcTgcttcctg and VIC-cagatcCgcttcct MGB dark quencher probes. Both of these assays were described previously in detail in Palovaara et al. (2003). Genotyping of SNP 785 A>G (CYP2B6*4) was performed by amplifying the DNA-sequence with primers gtctctctgtacgtctga (forward) and cctcttcctttcccttct (reverse), and sequencing the amplified product by using the same primers.

4.2.5 Pharmacokinetic calculations

The pharmacokinetic analyses were carried out with WinNonlin Pro 5.0.1 (Pharsight Corp, CA). Pharmacokinetic parameters for bupropion and hydroxybupropion were calculated by standard noncompartmental methods. The area under the plasma concentration–time curve (AUC) was determined by use of the linear trapezoidal rule up to the last measurable concentration and thereafter by extrapolation of the terminal elimination phase to infinity. The maximum plasma concentration (C max) and the time to C max (t max) for each subject were derived directly from the plasma concentration data. The half-life (t 1/2) was calculated by least-squares regression analysis of the terminal linear part of the log concentration–time curve. The estimate of the weight-corrected apparent oral clearance (CL/F/Wt) was calculated by dividing the bupropion dose by the AUC of bupropion and the subject’s weight.

4.2.6 Statistical analysis

Results were expressed as mean values ± SD in the text and tables and as mean ± SEM in figures. For t max, median with a range was given. In study IV, the percentage differences between the treatments were calculated within subjects, and 95% CIs were given. In both studies (IV, VI), the minimum statistical significance level was set at P < .05.

In the study IV, for the statistical testing of the absolute changes in the pharmacokinetic parameters, an ANOVA model for repeated measures and Student’s t-test for post hoc analysis were used. Logarithmic transformation was used for the non–normally distributed data before analysis. The Friedman test and Wilcoxon signed rank test for pairwise comparisons were used for t max. The Bonferroni adjustment for repeated significance testing was used for the P values.
In the study VI, a Mann-Whitney U-test for differences in medians was used. The difference in means between the study groups was tested with analysis of variance, with gender as confounding variable. Pearson’s correlation coefficients (r) were calculated to correlate eGFR and metabolic ratios.
5 Results

5.1 In vitro approaches for CYP inhibition screening purposes (II, III)

5.1.1 Development of n-in-one assay

The development of the n-in-one assay began with selection of probe substrates, optimisation of the analytical method, and compatibility studies with the substrates. A number of chemicals were tested to find suitable probe substrates for important drug-metabolising CYPs, as well as structurally diverse probe substrates for CYP3A4. Clinical significance was an important prerequisite in the selection of the probe substrates: the substrates themselves had to be drugs which had clinical data available, and for which both in vitro and in vivo interaction studies demonstrating their potential interactions could be obtained. The assay also had to cover all major drug metabolising CYP enzymes. The robustness of the assay was based on the employment of a LC/TOF-MS.

Several combinations of the selected probe substrates were made and compared to single substrate incubations, and no evidence of any interaction or interference between these substrates was observed. The final cocktail of incubation mixture contained substrates for nine CYP enzymes and included the ten probe compounds presented in Table 8. For CYP3A4, three structurally unrelated probes (midazolam, testosterone and omeprazole) were employed.
Table 8. Substrates, concentrations and CYP-specific model reactions used in the n-in-one assay.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Activity</th>
<th>[S] (μM)</th>
<th>K_m (μM)</th>
<th>Extracted ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Melatonin 6-hydroxylation</td>
<td>4</td>
<td>6</td>
<td>271+287</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>2</td>
<td>2</td>
<td>163</td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion hydroxylation</td>
<td>1</td>
<td>90</td>
<td>238+256</td>
</tr>
<tr>
<td>2C8</td>
<td>Armodiaquine de-ethylation</td>
<td>2</td>
<td>2.4</td>
<td>328</td>
</tr>
<tr>
<td>2C9</td>
<td>Tolbutamide methylhydroxylation</td>
<td>4</td>
<td>200</td>
<td>287+309</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole 5-hydroxylation</td>
<td>2</td>
<td>10</td>
<td>362</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole demethylation</td>
<td>-</td>
<td></td>
<td>332</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>0.2</td>
<td></td>
<td>258</td>
</tr>
<tr>
<td>2E1</td>
<td>Chloroxazone 6-hydroxylation</td>
<td>6</td>
<td></td>
<td>186</td>
</tr>
<tr>
<td>3A4</td>
<td>Midazolam α-hydroxylation</td>
<td>0.4</td>
<td>5</td>
<td>342</td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>1</td>
<td>40</td>
<td>305+327</td>
</tr>
<tr>
<td>3A4</td>
<td>Omeprazole sulfoxidation</td>
<td>-</td>
<td>4</td>
<td>362</td>
</tr>
<tr>
<td>3A4</td>
<td>Omeprazole 3-hydroxylation</td>
<td>-</td>
<td>47</td>
<td>362</td>
</tr>
</tbody>
</table>

As the [S] were below K_m (Table 8), the contribution of single CYPs to the model reaction was also observed using recombinant CYPs. CYP1A2, CYP2A6, CYP2B6 and CYP2E1 were the only enzymes involved in their probe substrate metabolism. Other metabolites were also formed mainly by the proposed recombinant CYP isoform. The CYP3A4 model reactions midazolam α-hydroxylation and omeprazole sulphoxidation were catalysed mainly by CYP3A5 instead of CYP3A4. The combined contribution of CYP3A family members was > 80% for midazolam α-hydroxylation and 100% for omeprazole sulphoxidation.

The assessment of the n-in-one assay for interaction screening purposes was performed using diagnostic CYP selective inhibitors. The inhibitory effects of these chemicals were also characterised with single substrate incubation studies. In general, the lowest IC_{50} value for each enzyme activity was seen with the reference inhibitor for the given enzyme, and the results were in good accordance with the literature values obtained from more conventional assays (Table 9). Some inhibitors proved to be highly selective for, and potent against, the selected model reaction, while others also inhibited a number of other enzymes, too.

Table 9. Performance of the n-in-one test with CYP selective chemical inhibitors.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Inhibitor</th>
<th>IC_{50} value in cocktail</th>
<th>IC_{50} value in single substrate assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Fluvoxamine</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>2A6</td>
<td>Tranylcypromine</td>
<td>1.00</td>
<td>0.38</td>
</tr>
<tr>
<td>2B6</td>
<td>Ticlopidine</td>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td>2C8</td>
<td>Quercetin</td>
<td>57.8</td>
<td>28.2</td>
</tr>
<tr>
<td>2C9</td>
<td>Sulphaphenazole</td>
<td>0.2</td>
<td>0.35</td>
</tr>
<tr>
<td>2C19</td>
<td>Fluconazole</td>
<td>6.4</td>
<td>23.8</td>
</tr>
<tr>
<td>2D6</td>
<td>Quinidine</td>
<td>0.035</td>
<td>0.08</td>
</tr>
<tr>
<td>2E1</td>
<td>Pyridine</td>
<td>36.6</td>
<td>2.75</td>
</tr>
<tr>
<td>3A4</td>
<td>Ketoconazole</td>
<td>0.07-1.8</td>
<td>0.065–0.3</td>
</tr>
</tbody>
</table>
5.1.2 Comparison and evaluation of single substrate, fluorescent probe, and n-in one assays

Validation of the n-in-one assay was carried out by comparing the performance of traditional single substrate assays and the n-in one assay utilising human liver microsomes (HLMs) as an enzyme source, and the fluorescent probe method with recombinant human CYPs. To cross-validate the assays, the inhibitory capacities of six structurally diverse drugs with known CYP inhibition profiles were selected from three classes of therapeutic agent (β-adrenergic receptor antagonists, selective serotonin reuptake inhibitors, and benzodiazepines). These study compounds were known to display distinct inhibition profiles compared to other compounds in the same class. The study compounds were: sotalol, propranolol, citalopram, fluoxetine, oxazepam, and diazepam (Figure 4).

All three assay types yielded remarkably similar results with the majority of tested CYP forms. With sotalol, an IC₅₀ value of > 100 µM was seen with all assay types and all CYPs studied. The known potent inhibitory effects of propranolol and fluoxetine against CYP2D6 were observed in all three test settings. The n-in-one technique suggested moderate inhibition of CYP2B6 by propranolol and diazepam, which was not observed in the other two assay types. The fluorescent probe assay predicted inhibition of CYP2C8 by fluoxetine, which was not confirmed by the other two tests. A 100-fold difference in the IC₅₀ value for fluoxetine with CYP2C19 was also observed between the fluorescent probe assay and the other two assay types.

5.2 Inhibitory effects and potential interactions (I, IV and V)

5.2.1 Inhibition of CYP2B6 in vitro

At the beginning of these studies, knowledge about potential CYP2B6 inhibitors was scarce. In study I, more than 30 different chemicals were screened for CYP2B6 inhibition. The most potent compounds were selected for more detailed investigation.

Ticlopidine and thioTEPA were found to be highly effective and potent inhibitors of CYP2B6. The mode of inhibition of bupropion hydroxylation by ticlopidine in HLMs and recombinant CYP2B6 was found to be of mixed type with a Kᵢ value of 0.2 µM (Figure 5 a-d). ThioTEPA inhibited bupropion hydroxylation competitively with a Kᵢ value of 2.8 µM in both HLMs and recombinant CYPs.
The selectivity of thioTEPA proved to be excellent, with IC$_{50}$ values of > 100 µM for all other CYPs studied. Besides CYP2B6, ticlopidine was also found to inhibit CYP1A2, CYP2C19, and CYP2D6 associated model activities, though with > 10-fold higher IC$_{50}$ values compared to CYP2B6. At a concentration level of 1 µM, where CYP2B6-activity was completely suppressed, ticlopidine inhibited other CYPs by less than 15% (Figure 6).
The CYP2B6 inhibition potential of three benzylpyridine derivatives, CBP, NBP, and BP was studied using two distinct \textit{in vitro} methods: the bupropion hydroxylation assay with HLMs and the 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) hydroxylation assay using recombinant CYP2B6 as an enzymatic source (V). With bupropion and HLMs potent inhibition of CYP2B6 was seen with IC\textsubscript{50} values of 0.02, 0.03 and 0.03 µM for CPB, NBP, and BP, respectively. With cDNA–expressed CYP2B6 and 7-EFC, the IC\textsubscript{50} values for CPB, NBP, and BP were 0.3, 0.4 and 0.4 µM, respectively.

At a 0.1 µM concentration of CBP, complete inhibition of CYP2B6 activity was observed. By comparison, no inhibition at a concentration level of 0.1 µM of CBP with other CYP model reactions was found, and only minimal inhibition of other CYPs at 1 µM of CBP was seen. Both NBP and BP were more unselective, especially regarding CYP1A2 (Figure 7).
5.2.2 Inhibition of CYP2B6 in silico

41 chemicals were analysed using the 3D-QSAR method. The CoMFA model created was of high quality. The sterically favoured area was located around a substitution at carbon 4 of the benzyl ring of benzylpyridine in the CoMFA model. A broad partial negative charge near to a nitrogen atom and around the benzyl group was observed to increase the inhibition potency (Figure 8).

The structural features of ticlopidine and CBP were compared in order to explore the CYP selectivity (Figure 9). CBP was found to be approximately 190 times more potent towards CYP2B6 than the next most sensitive form, CYP1A2. The chloro substituent on the benzyl ring in CBP includes a partial negative region near the chlorine atom, which was observed to affect the CYP selectivity of CBP. The nitrogen and sulphur atoms in the structure of ticlopidine also showed partial negative charges on the colour contour map.
Fig. 8. Stereofigure of colour contour maps of CYP2B6 CoMFA. The reference structure is CBP. Areas where negative partial charge and bulkier groups increase the inhibition potency are indicated in red and green, respectively. Areas where negative partial charge and bulkier groups decrease the potency, are indicated in blue and yellow, respectively.

Fig. 9. Electrostatic fields of CBP (on the left) and ticlopidine (on the right). The red color represents a region with a partial negative charge and the blue color a region with a partial positive charge.
The predictive power of the CoMFA model was evaluated by estimating pIC\textsubscript{50} values for an external test set of 11 compounds. The predictions were in good agreement with the measured values. For bupropion, the pIC\textsubscript{50} value predicted by CoMFA model was 4.55 and the pIC\textsubscript{50} value measured with the fluorescent probe system was 4.23. For thioTEPA, the measured and CoMFA-predicted pIC\textsubscript{50} values were 5.00 and 4.3, respectively.

### 5.2.3 Inhibition of CYP2B6 in vivo

Due to their wide clinical use and relevance, the antiplatelet agents clopidogrel and ticlopidine were selected for further in vivo interaction studies with healthy volunteers. A significant reduction in the AUC of hydroxybupropion was seen after both clopidogrel (-52%, \( P = .001 \)) and ticlopidine (-84%, \( P < .0001 \)) treatments. Clopidogrel increased the AUC of bupropion by 60% (\( P = .02 \)) and ticlopidine by 85% (\( P < .0001 \)). The AUC ratio of metabolite to parent compound was reduced by 68% (\( P = .002 \)) and 90% (\( P = .001 \)) with clopidogrel and ticlopidine, respectively (Figure 10).

![Fig. 10. Effect of clopidogrel and ticlopidine on hydroxybupropion and bupropion AUC and metabolic ratio.](image)

After clopidogrel and ticlopidine treatments, the CL/F of bupropion was decreased by 26% (\( P = .006 \)) and by 36% (\( P = .01 \)), respectively. The \( C_{\text{max}} \) of bupropion of was increased 40% (\( P = .01 \)) and 38% (\( P = .01 \)) by clopidogrel and ticlopidine, respectively. Consequently, the \( C_{\text{max}} \) of hydroxybupropion was decreased 50% (\( P < .0001 \)) by clopidogrel and 78% (\( P < .0001 \)) by ticlopidine treatment.
5.3 CYP2B6 activity and bupropion pharmacokinetics in healthy subjects and patients with impaired kidney function (VI)

In patients with kidney disease, the bupropion AUC was 126% higher (P < 0.0001), C_{max} 86% higher (P = 0.001), and t_{1/2} 140% longer (P = 0.001) compared to healthy controls. The CL/F of bupropion was 64% lower (P < 0.0001) in the patients with kidney disease. Only minor changes were detected in the concentrations and pharmacokinetic parameters of the metabolites hydroxybupropion and hydrobupropion. In patients with impaired kidney function, the metabolic ratio of hydroxybupropion/bupropion was 66% (P ≤ 0.0001) lower and the hydrobupropion/bupropion AUC ratio 69% (P = .001) lower compared to the controls (Figure 11).

Fig. 11. Effect of kidney function on bupropion PK. Individual and mean values for bupropion AUC (a), hydroxybupropion AUC (b) and hydroxybupropion/bupropion AUC ratio (c) in 17 healthy controls (open circles) and 10 renal-impaired patients (solid squares) after a single 150-mg dose of bupropion.

Thirteen individuals were heterozygous (six in the control and seven in the patient group, respectively) and none was homozygous with respect to CYP2B6 SNP 785 A>G. Three of the subjects were homozygous (two in the control and one in the patient group, respectively) and five were heterozygous (three in the control and two in the patient group, respectively) for the 1459 C>T. None of the 27 subjects were homozygous, but 11 (five in the control and six in the patient group, respectively) were found to be
heterozygous for the 516 G>T. None of the SNPs studied was found in seven of the study subjects (five in the control and two kidney impaired patient group, respectively). No statistically significant differences were apparent in the pharmacokinetics of bupropion or its metabolites between the different genotype groups.
6 Discussion

6.1 Methodological aspects

6.1.1 In vitro studies

Although certain metabolic transformations of chemical’s structures have been shown to be mediated by specific CYPs (see e.g. Walsky & Obach 2004), the use of probe substrates and metabolic reactions as an indirect measure of an enzyme activity is not without pitfalls. In the present studies this was seen especially, but not only, with CYP3A4, where the effect of inhibitors was seen to be substantially substrate dependent. The variability in the response to an inhibitor tends also to be higher with flatter IC\textsubscript{50} curves. In the present studies this effect was seen (III), when no inhibition of CYP2D6 with oxazepam was observed at a concentration level of 100 µM in two assays, whereas one test setting displayed an IC\textsubscript{50} value of approximately 92 µM. Thus, the use of multiple probe substrates and close inspection of the inhibition curves is essential when evaluating results obtained from inhibition screens. The utilisation of these model activities also requires that the enzyme kinetics of the model reactions are characterised, and the incubation conditions are within the linear velocity range with respect to metabolite formation.

The dissolution of probe substrates is crucial, because most organic solvents are known to suppress CYP activities significantly. If solubility considerations are excluded, methanol and acetonitrile appear to be the most suitable solvents and DMSO the most harmful for the maintenance of CYP activities. In general, it is recommendable to keep the amount of the solvent below 1% of the total volume of a sample (Chauvet et al. 1998, Hickman et al. 1998, Busby et al. 1999, Easterbrook et al. 2001). In our studies, the stock solutions of chemicals were tested to find the most appropriate solvent to dissolve them in in the following order: water, acetonitrile, methanol, ethanol, and DMSO; and further subsequently diluted in the reaction buffer. The final amount of organic solvent in incubations was 1% or less.
Both human liver microsomes and recombinantly expressed CYPs were used in this study as an enzymatic source. Microsomes are the golden standard for metabolism studies, and depending on the donor, contain the whole pattern of CYPs. The effect of genotype variation is usually minimised by pooling multiple liver samples together or by genotyping the samples with respect to major SNPs. In our studies, a weight-balanced pool of seven or ten livers was employed. cDNA-expressed CYPs are mostly used for the identification of enzymes metabolising the NCE, but also increasingly as an alternative method of predicting hepatic clearance (Pelkonen et al. 2005). In the present studies, recombinant enzymes were used when certain CYPs needed to be studied closer in an isolated and purified form or when HTS-kits were used. The use of HLMs or purified enzymes thus depends on the goals of the study in question.

Usually higher IC₅₀ values in HLMs compared to recombinant enzymes suggests the contribution of multiple CYPs in the metabolic reaction. In study I, the inhibitory values of ticlopidine and thioTEPA were exactly the same when bupropion hydroxylation was measured both in recombinant CYPs and HLMs. This further supports the evidence that bupropion hydroxylation is a highly selective CYP2B6 model reaction.

### 6.1.2 In vivo studies

Both clinical studies were conducted with an open design and clinically used doses of bupropion, ticlopidine, and clopidogrel were administrated. Since only the pharmacokinetics were studied, the use of a closed study setting or placebo-controls were not considered necessary. Study VI was conducted with parallel design and study IV with three-phased, randomised, and balanced cross-over design. The latter study setting, where each subject serves as his own control and the changes in the pharmacokinetic parameters are calculated within each subject, allows minimisation of the effect of inter-individual variation, and thus improves the quality of the results. In study VI, a parallel group study was applied, and all the subjects were genotyped with major CYP2B6 SNPs to ensure that the relevant SNPs were not selected by chance in either group.

The possible effect of food or beverages on the pharmacokinetics of the study drugs was minimised by a fasting period before ingestion of drugs and by serving standardised meals on all study days. In study IV, wash-out periods from one week to two weeks between the study phases were conducted in order to minimise the possible carry-over effects. The compliance was ascertained by requiring short message service (SMS) verification by subjects that they had taken their medication. With this setting we cannot prove that drugs were actually ingested, but could control that subjects did not forget to take the medication.

In study IV, the subjects did not have any concomitant medication. Patients with renal impairment (VI) refrained from their prescribed medication on the first study day when bupropion was administrated, but otherwise they took their medication as usual. The concomitant medication in the patient group did not include any known inhibitors (e.g. oral contraceptives or hormone replacement therapy) or inducers of CYP2B6.

Differences in the bupropion pharmacokinetics (PK) with respect to gender have been suggested by certain studies (Findlay et al. 1981, Stewart et al. 2001), but refuted by
others (Hsuy et al. 1997, Daviss et al. 2005). The effect of gender on bupropion PK was not assessed in the present study due to the low number of subjects. The female subjects and patients were at various phases of menstrual cycle, but the menstrual cycle seems to have only minor effects on CYPs (Kashuba & Nafziger 1998) compared with the magnitude of changes in bupropion pharmacokinetics in studies IV and VI.

In adolescent patients, a more rapid conversion of bupropion to the active metabolites compared to adults has been demonstrated (Daviss et al. 2005). A lower bupropion apparent clearance and a minor increase in bupropion $C_{\text{max}}$ in elderly patients over 65 years compared to subjects aged less than 40 years has also been reported previously (Sweet et al. 1995, Jefferson et al. 2005). Consequently, as all the present in vivo studies were conducted with adult patients aged from 21 to 50 years old, one should avoid extrapolating results to the elderly or adolescents.

The majority of the patients in study VI had the renal impairment due to diabetes. Studies with experimental animals and cell lines have demonstrated both up- and down-regulation of CYP-activities with hyperinsulinemia and hyperglycemia (Pass et al. 2002, Becker et al. 2004, Carmiel-Haggai et al. 2005). The effect of diabetes and/or hyperglycemia itself on CYP2B6 activity or bupropion pharmacokinetics in man is not known.

6.2 Screening multiple CYPs in a single test system

Knowing that metabolic studies are often a bottleneck in drug development, the rationale for developing the n-in-one assay was to create a cost-effective and robust screening technique which would serve in ranking hit/lead candidates in the early phases of drug discovery. To be useful, the assay developed should provide background information for further decisions about the fate of a compound in terms of development and usability. It should also indicate, which CYPs are potentially inhibited by a compound, and to give tentative IC$_{50}$ values or the magnitude of potential inhibition.

A couple of assays employing the same n-in-one -idea for the simultaneous analysis of CYPs in vitro have also been published before (Bu et al. 2001, Dierks et al. 2001, Testino & Patonay 2003). These assays have certain limitations as not all relevant drug-metabolising CYPs can be detected (for example CYP2B6), selection of unselective probe substrates can occur, and in some cases, the probe substrate concentrations may not be in the appropriate range as determined by enzyme kinetics. Compared to previously published assays, our test system managed to keep the substrate concentration below the $K_m$ values for each reaction and comprised all relevant drug-metabolising CYPs (Table 10).
Table 10. Published n-in-one in vitro assays.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Dierks et al. 2001</th>
<th>Bu et al. 2001</th>
<th>Testino &amp; Patonay 2003</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Ethoxyresorufin</td>
<td>Phenacetin</td>
<td>Melatonin</td>
<td></td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin</td>
<td>Coumarin</td>
<td>Coumarin</td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>-</td>
<td>-</td>
<td>Bupropion</td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>Paclitaxel</td>
<td>-</td>
<td>Amodiaquine</td>
<td></td>
</tr>
<tr>
<td>2C9</td>
<td>Diclofenac</td>
<td>Tolbutamide</td>
<td>Tolbutamide</td>
<td></td>
</tr>
<tr>
<td>2C19</td>
<td>S-mephenytoin</td>
<td>-</td>
<td>Omeprazole</td>
<td></td>
</tr>
<tr>
<td>2D6</td>
<td>Bufuralol</td>
<td>Dextromethorphan</td>
<td>Bufuralol</td>
<td></td>
</tr>
<tr>
<td>2E1</td>
<td>-</td>
<td>Chlorozoxazone</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3A4</td>
<td>Midazolam</td>
<td>Midazolam</td>
<td>Midazolam</td>
<td>Testosterone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Omeprazole</td>
</tr>
</tbody>
</table>

A potential problem with the n-in-one approach is that probes may interfere with the analytical detection of other substrates or interact with each others kinetically or chemically. In our study there was no evidence of interaction between probe substrates or analytical difficulties as a result of suppression or co-elution.

A few n-in-one settings for in vivo phenotyping purposes have also been developed and published. The most well known of these are probably the Pittsburgh cocktail (Frye et al. 1997), the Cooperstown cocktail (Streetman et al. 2000), and the Karolinska cocktail (Christensen et al. 2003), all of which comprise five probe substrates. So far, there is no general consensus concerning the applicability of n-in-one assays in preclinical and clinical research. It has been suggested that the use of these approaches may facilitate the rapid delineation of the NCE’s interaction potential and that they offer the potential of providing guidance on conducting clinical trials and limiting co-medication restrictions to only those likely to be clinically relevant (Zhou et al. 2004).

The n-in-assay developed as a part of this thesis has proven to be suitable for fast and reliable in vitro screening of the interaction potential and characteristics of NCEs and has been taken into commercial use. Furthermore, the assay has been applied to studies of CYP expression and function in different biological preparations.

### 6.3 Inhibition of CYP2B6 in silico, in vitro and in vivo

It is interesting – though not surprising – that very small changes in the structure and chemical formula of a compound results in significantly variable inhibition profiles. This was observed especially with three benzylpyridine derivatives studied in original publication IV. Using the relatively simple CoMFA-model, we were able to demonstrate that certain modifications within the molecule tended to increase the CYP2B6 inhibition potency and selectivity. Thus, substrate-structure relationships can provide additional information about the likelihood of an interaction and give warnings about structural or general features regarding problems with specific molecular structures. For CYP2B6, several different in silico models have been applied in order to model the active site of the enzyme (Turpeinen et al. 2006). Although the crystal structure of CYP2B6 is not
known, modelling the 3D-structure provides important insight into the function of CYP2B6.

Ticlopidine, thioTEPA, and CBP were characterised as potent inhibitors of CYP2B6. The most effective CYP2B6-inhibitors, ticlopidine and CBP, were observed to be rather unselective, but at a concentration level of 1 µM for ticlopidine and 0.1 µM for CBP, the inhibitory effect towards other CYPs was negligible. Consistent with previous studies, ThioTEPA was found to be a highly selective inhibitor of CYP2B6 (Rae et al. 2002, Harleton et al. 2004, Richter et al. 2005). The clinical relevance of this interaction has been reported in least one study, where administration of thioTEPA resulted in a significant reduction in the plasma levels of the active metabolite of cyclophosphamide (Huitema et al. 2000).

One of the key findings of this study was the strong inactivation of CYP2B6 in vivo by clopidogrel, and especially, by ticlopidine. CYP2B6 is known to be expressed in the liver, but not in the intestinal mucosa (Ding & Kaminsky 2003), and consequently, the interaction should primarily take place at the level of hepatic enzymes. The t½ of bupropion was unaffected, Cmax of bupropion was increased and the Cmax of hydroxybupropion decreased after clopidogrel and ticlodipine treatments. These findings suggest that the interaction takes place in the first-pass phase rather than in the elimination phase.

Our studies were carried out using bupropion hydroxylation as a CYP2B6-specific model reaction. It is highly likely that the clinical effect observed can be applied to the other drugs metabolised mainly by CYP2B6 - cyclophosphamide, ifosfamide, ketamine, propofol, efavirenz, and pethidine (Turpeinen et al. 2006), but this is not directly proven by our study design. Besides CYP2B6, ticlopidine was shown to be a potent inhibitor of CYP1A2, CYP2C19, and CYP2D6 in vitro. The inhibitory effect of ticlopidine on CYP2C19 mediated metabolism has been demonstrated previously in a few clinical studies (Donahue et al. 1997, Tateishi et al. 1999, Ieiri et al. 2005). Clopidogrel has been shown to inhibit CYP1A2, CYP2C9, and CYP2C19 in addition to CYP2B6 in vitro (Richter et al. 2004). However, clinical CYP-interaction studies concerning clopidogrel are basically non-existent. Metabolism via the CYP3A4 enzyme could serve as an optional elimination route for propofol, ketamine, and nevirapine. Consequently, the inhibition of CYP2B6 is assumed to have a more minor effect on the clearance of these drugs.

In conclusion, ticlopidine and clopidogrel considerably decreased the plasma concentrations of hydroxybupropion. Concomitant use of bupropion with these drugs may lead to a reduced therapeutic effect by bupropion in smoking cessation and furthermore, increase the risk of concentration dependent adverse effects associated with bupropion.

This study yielded novel information about CYP2B6. In accordance with previous in vitro studies (Faucette et al. 2000, Hesse et al. 2000), bupropion hydroxylation was proven to be a selective and useful in vivo model reaction for CYP2B6. Several potent and selective inhibitors for CYP2B6 were discovered and characterised in distinct test settings. With validated substrates and inhibitors, it is expected that the pharmacological and clinical significance of CYP2B6 will be delineated more fully in the near future.
6.4 Impaired kidney function, CYP2B6, and bupropion PK

Clinical studies exploring bupropion PK in non-smoking renal-impaired subjects without dialysis treatment had not been carried out before. In the present study, a significant increase in the AUC, C_{max} and t_{1/2} of bupropion in patients with renal impairment, but normal liver function, was seen. The apparent oral clearance of bupropion was significantly lower in patients with renal impairment. These findings are interesting and surprising since hepatic clearance is known to contribute almost solely to the total clearance of bupropion (Holm & Spencer 2000, Jefferson et al. 2005). A plausible explanation for this is the suppressed CYP2B6 activity, although this could not be proven beyond question by our study setting.

The pharmacokinetics of the bupropion metabolites were not found to differ between the study groups. In a recent study with a small group of smoking patients with end-stage renal failure that were undergoing hemodialysis, an accumulation of the bupropion metabolites after a single oral dose of bupropion was observed, but no impact was evident on the parent compound pharmacokinetics (Worrall et al. 2004). This difference is interesting and further studies on the cause of this discrepancy are warranted.

Extensive details, nomograms, and guidelines on adjusting pharmacotherapy in patients with renal insufficiency are available and have been adopted for routine use in clinical decision making. Despite these strategies, ADRs are more frequent with renal-impaired patients compared to those with normal kidney function (Bates et al. 1999, Zaidenstein et al. 2002). Usually the recommended adjustments do not consider extra-renalily eliminated drugs. Knowing that the importance of non-renal elimination processes increases when renal function declines, it is quite surprising how few studies dealing with the effect of renal impairment on hepatic drug metabolism have been published. Previously, renal impairment has been shown to have an impact on metabolic reactions mediated by CYP2C9 (Dreisbach et al. 2003), CYP2D6 (Rostami-Hodjegan et al. 1999), CYP2E1 (Nolin et al. 2003a) and CYP3A4 (Dowling et al. 2003). The expression and regulation of drug transporters and Phase II enzymes can also be altered in chronic kidney disease (for a review see Nolin et al. 2003b). Recently, a study by Anttila and coworkers (Anttila et al. 2005) demonstrated a clear effect on the elimination of selegiline, a drug solely eliminated by hepatic biotransformation processes, with subjects with end-stage renal disease.

In conclusion, it appears that impaired kidney function leads to wider effects in pharmacokinetic processes than just changes in renal clearance, and this should be taken into account even if the drug is known to be non-renally eliminated.

6.5 Can in vivo effects be predicted by in silico and in vitro studies?

Inhibition of P450 enzymes is frequently the underlying mechanism of drug-drug interactions. Results obtained from in vitro and in silico assays are intended to guide the decision making in further studies. In our studies, the inhibition of CYP2B6 with ticlopidine was clearly observed both in vitro and in vivo and, furthermore, attributed to certain chemical structures in silico. However, relying and focusing only on inhibition parameters may be misleading with respect to clinical findings. Thus, CYP-inhibition
data should be interpreted together with the other ADME endpoints such as solubility, plasma protein binding affinity, metabolic stability, and other factors discussed in more detail in chapter 2.5.2.1.

Examples of the discrepancy between in vitro and in vivo inhibitory potencies are numerous. For example, montelukast and zafirlukast are known to be highly potent inhibitors of CYP2C8 in vitro (Walsky et al. 2005a, Walsky et al. 2005b), but their effect on the CYP2C8 substrate pioglitazone has been shown to be negligible in vivo (Jaakkola et al. 2006). Similarly, ritonavir has been demonstrated to be a potent in vitro inhibitor of CYP2B6 (Hesse et al. 2001), but seems to have only a minimal impact on CYP2B6 activity in vivo (Hesse et al. 2006). Isradipine was found to be a moderate inhibitor of CYP3A4 in vitro (Wang et al. 1999), but in a clinical setting this effect was not observed (Backman et al. 1999).

In our present study, the strong CYP2B6 inhibition potential of ticlopidine was observed both in vivo and in vitro. Ticlopidine was known to be a slightly stronger inhibitor of CYP2B6 than clopidogrel, with \( K_i \) values of 0.2 and 0.5 \( \mu \text{M} \), respectively (Richter et al. 2004, Turpeinen et al. 2006). Accordingly, in the present study, a more intense in vivo inhibition was seen with ticlopidine compared to clopidogrel with 84% and 52% decreases in the hydroxybupropion AUCs, respectively.

Although the use of in vitro and in silico approaches has become routine during drug development, it is crucial to keep in mind the pros and cons of each method applied and to use the collected data in context, with all the other pieces of information obtained from other applications. Understanding the differences between, and applicability of simulations, the order of magnitude of predictions and the scientific background behind them, allows better utilisation of the results obtained (Smith & van de Waterbeemd 1999, Lin 2000, Rostami-Hodjegan & Tucker 2004).
7 Conclusions

7. Ticlopidine and ThioTEPA were characterised as highly effective and potent inhibitors of CYP2B6. The inhibition type of ticlopidine was found to be mixed type with a component of mechanism-based inhibition, while thioTEPA inhibited CYP2B6 in a competitive manner.

1. A comprehensive in vitro test utilising n-in-one approach was developed and taken successfully into commercial use as a medium-troughput assay for the evaluation of CYP-inhibition potential of NCEs.

2. Compared to conventional single substrate assay, the n-in-one technique, and the fluorescent probe assay were found to be useful and suitable for measuring the inhibition of major drug-metabolising CYP activities.

3. Both clopidogrel and ticlopidine significantly inhibited the CYP2B6-catalysed bupropion hydroxylation. This was observed as average decreases of 68% and 90% in the AUC ratio of hydroxybupropion over bupropion by clopidogrel and ticlopidine, respectively. Patients receiving either clopidogrel or ticlodipine are likely to need dose adjustments when treated with drugs primarily metabolised by CYP2B6.

4. The developed CoMFA model yielded novel structural information about CYP2B6 inhibitors and accurately predicted the inhibition potencies of several structurally unrelated compounds. The three benzylpyridines, CBP, NBP, and BP, were found to be highly potent inhibitors of CYP2B6.

5. Bupropion clearance and metabolic ratios were found to be significantly reduced and peak plasma concentrations increased in patients with renal impairment. Patients with renal impairment may need lower doses when treated with bupropion. A plausible explanation for this is the suppressed hepatic CYP2B6 activity, although this could not be unquestionably proven by present study setting.
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Miia Turpeinen

CYTOCHROME P450 ENZYMES—IN VITRO, IN VIVO, AND IN SILICO STUDIES