Interindividual variation in drug metabolism with focus on polymorphic cytochrome P450 2C9

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Stockholm 2005

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ABSTRACT

Cytochromes P450 (CYP) are enzymes, mainly catalysing the oxidation of xenobiotics, in order to facilitate their excretion from the body. CYP2C9 is a polymorphic enzyme, which is responsible for the metabolism of about 10 % of all known drugs, e.g sulphonylureas, anticoagulants, angiotensin II blockers and NSAIDs, which show a considerable interindividual variation in their metabolic clearance. The primary aim of this PhD thesis was to investigate possible factors, which could be responsible for, or contribute to, the variation in CYP2C9 metabolic activity. We also wanted to further elucidate the impact of *CYP2C9* genotypes on the turnover of both exogenous and endogenous substrates.

In healthy Caucasians a three-fold higher metabolic ratio (MR)[*] of the CYP2C9 probe drug losartan was found in volunteers genotyped as *CYP2C9*1/*3* compared to *CYP2C9*1/*1* subjects ($p<0.05$), even though there was considerable variation within each genotype group. The subjects homozygous for the *CYP2C9*3* allele had metabolic ratios that were between 22 and 220 times higher than *CYP2C9*1/*1* subjects. No novel allelic variants of *CYP2C9*, explaining the low metabolic activity in some of the *CYP2C9*1/*1* subjects, could be identified. A two-fold higher metabolic ratio of losartan was evident in women taking oral contraceptives (OCs) compared to women not taking OCs ($p< 0.05$), showing that CYP2C9-dependent drug metabolism is reduced during concomitant intake of OC.

When comparing healthy Swedish and Korean subjects, it was discovered that Swedes had a slower losartan metabolism than Koreans, regardless of genotype. No difference between female subjects of the two populations, having the same genotype, was detected. Swedish men, though, had a higher metabolic ratio (MR=0.83) than Korean men (MR=0.54), $p< 0.001$, but no novel polymorphisms could explain this difference.

The intrinsic clearance of the selective COX-2 inhibitor celecoxib was studied in human liver microsomes of different *CYP2C9* genotypes, as well as in yeast microsomes with recombinantly expressed CYP2C9 variants. The formation rate of OH-celecoxib was significantly reduced in *CYP2C9*3* samples, compared to samples with two functional alleles. The *CYP2C9*2*-samples did not differ from *CYP2C9*1* in any of the systems studied. Alcohol dehydrogenase (ADH1 and ADH2) was identified as being responsible for the further oxidation of OH-celecoxib, *in vitro*.

After a single oral dose of 200 mg celecoxib, the exposure was three times higher in healthy *CYP2C9*3/*3* subjects than in *CYP2C9*1/*1* and *CYP2C9*1/*3* subjects. After one week of repeated celecoxib administration, the celecoxib exposure was more than seven times higher in subjects genotyped as *CYP2C9*3/*3*, compared to individuals in the other two genotype groups. The heterozygous *CYP2C9*3* carriers were almost identical to *CYP2C9*1/*1* subjects. Subjects genotyped as *CYP2C9*3/*3* may be more likely to experience concentration-dependent side-effects after long-term treatment with celecoxib.

In human liver microsomes there was a significantly lower formation rate of epoxyeicosatrienoic acids (EETs) by 34% in samples with the composite genotype of *CYP2C8*3/*3/CYP2C9*2/*2* compared to *CYP2C8*1/*1/CYP2C9*1/*1*. Inhibition experiments confirmed the involvement of both CYP2C8 and CYP2C9 in the oxidation of arachidonic acid to vasoactive metabolites. The results imply that variant alleles of *CYP2C8* and *CYP2C9* might be involved in the pathophysiology of cardiovascular diseases.

[*] The ratio between the parent compound and its metabolite. A high MR reflects a low metabolic activity.

LIST OF PUBLICATIONS

The thesis is based on the following papers:

I **Sandberg M**, Johansson I, Christensen M, Rane A, Eliasson E
The impact of CYP2C9 genetics and oral contraceptives on cytochrome P450 2C9 phenotype, *Drug Metabolism and Disposition* (2004) 32; 484-9.

II **Sandberg Lundblad M**, Roh H-K, Kang J-H, Eliasson E, Bertilsson L
Differences in CYP2C9-dependent drug metabolism between men and women in Sweden and Korea, *manuscript*.

III **Sandberg M**, Yasar Ü, Strömberg P, Höög J-O, Eliasson E
Oxidation of celecoxib by polymorphic cytochrome P450 2C9 and alcohol dehydrogenase, *British Journal of Clinical Pharmacology* (2002) 54; 423-429.

IV **Sandberg Lundblad M**, Ohlsson S, Peter Johanson, Lafolie P, Eliasson E
Accumulation of celecoxib with a seven-fold higher drug exposure in individuals homozygous for CYP2C9*3, *manuscript*.

V **Sandberg Lundblad M**, Stark K, Eliasson E, Oliw E, Rane A
Biosynthesis of epoxyeicosatrienoic acids varies between polymorphic CYP2C enzymes. *Biochemical and Biophysical Research Communications* (2005) 327; 1052-57.

Eigentlich weiß man nur, wenn man wenig weiß. Mit dem Wissen wächst der Zweifel.

(Maximen und Reflexionen, Goethe)

ABBREVIATIONS

ADR	Adverse drug reaction
ANOVA	Analysis of variances
AUC	Area under the curve
BCRP	Breast cancer resistance protein
CAR	Constitutive androstane receptor
Cl	Clearance
COX	Cyclooxygenase
CYP	Cytochrome P-450
DHET	Dihydroxyeicosatrienoic acid
DNA	Deoxyribonucleotide acid
EET	Epoxyeicostrienoic acid
EH	Epoxide hydrolase
EM	Extensive metaboliser
GCP	Good clinical practice
GR	Glucocorticoid receptor
HETE	Hydroxyeicosatetraenoic acid
HNF4α	Hepatic nuclear factor 4 alpha
HPLC	High performance liquid chromatography
ICH	International conference on harmonisation
LC-MS	Liquid chromatography-mass spectrometry
MDR	Multi drug resistant gene
MR	Metabolic ratio
mRNA	Messenger ribonucleic acid
MS	Mass-spectromy
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PM	Poor metaboliser
PXR	Pregnane X receptor
SNP	Single nucleotide polymorphism
SSCP	Single stranded conformational polymorphism
TNFα	Tumour necrosis factor α
TPMT	Thiopurine methyltransferase
UM	Ultra rapid metaboliser

GENERAL INTRODUCTION

People respond differently to the same drug treatment, and this can result in drug toxicities in some patients, while others will experience therapeutic failure. Environmental factors such as diet and pollutants, as well as drug interactions, physiological status and genetic variation within genes coding for drug metabolising enzymes, drug transporters and receptors can all contribute to this interindividual variation.

Cytochromes P450 (CYP) are enzymes mainly catalysing the oxidation of xenobiotics, in order to facilitate their excretion from the body. CYP2C9 is a polymorphic isozyme, which is responsible for the metabolism of about 10 % of all known drugs. Anticoagulants, NSAIDs, and sulphonylureas are metabolised by this enzyme, and they all display a large interindividual variation in their metabolic clearance. Allelic variants of the *CYP2C9* gene are responsible for some of the observed variation, but there is still a large part of the differences in the metabolic clearance of these drugs that remains unexplained. Different aspects of interindividual variation of CYP2C9-dependent metabolism will be extensively discussed throughout this thesis.

First, an overview of the course of events that drugs will be exposed to, on their passage through the body, will be given. Factors, which have an influence on these processes, i.e. absorption, distribution, metabolism, and excretion, will also be discussed.

PHARMACOKINETICS

Pharmacokinetics describe the processes of absorption, distribution, metabolism, and excretion of drugs in the body.

After oral ingestion, drugs enter the body from the gastrointestinal tract via active transporters, filtration or through diffusion. The absorption process mostly utilised by drugs is passive diffusion, requiring no energy. The rate and extent of absorption is dependent on several factors such as the physico-chemical properties of the substance, the drug formulation, concomitant intake of food and other drugs, gastric emptying and motility. Drugs can also enter the body via alternative routes of administration, e.g. through the skin or via the lungs. If the drug is given intravenously, no absorption is required (Speight TM and Holford NHG, 1997).

After absorption, the drug passes via the portal vein into the liver. Already during the absorption from the gastrointestinal tract, and on the passage through the liver, many drugs are being transformed, reducing the amount of drug reaching the systemic circulation. This process is called first-pass metabolism and limits the bioavailability of substances entering the body.

Intravenously administered drugs bypass this first metabolic process and thus have a bioavailability of 100 % (Rowland M and Tozer TN, 1995).

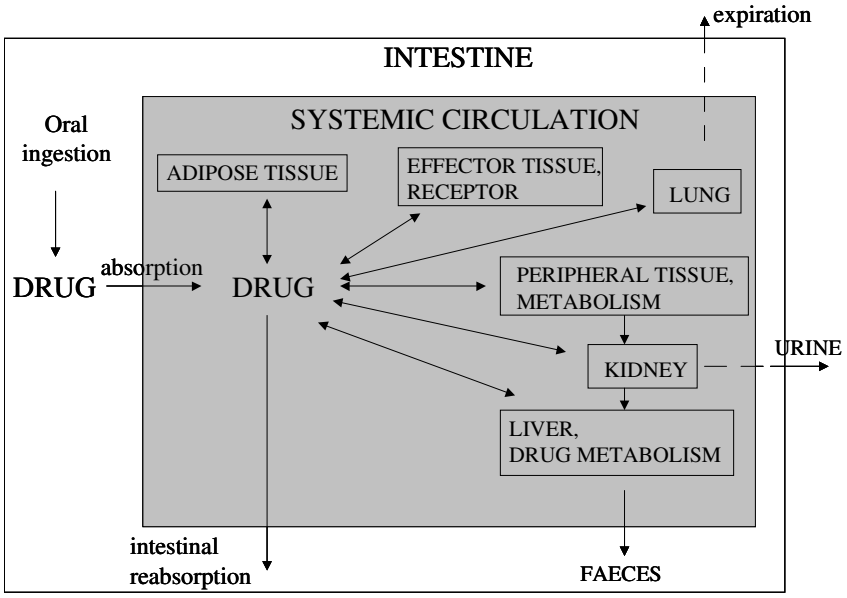


Figure 1. Schematic overview of the absorption, distribution, binding, metabolism and excretion of drugs.

When absorbed, the drug can be distributed throughout the body to the various tissues and organs where the specific targets, e.g. enzymes and receptors, of the drug reside. The distribution is dependent on the lipid solubility of the drug, its concentration, permeability of tissue membranes, blood perfusion, and the binding of the drug to plasma proteins, such as albumin and α_1-acid glycoprotein. The binding is reversible, and it is the free fraction of the drug that exerts the pharmacologic action (Hardman JG et al., 2001).

In order not to be accumulated the drug must be cleared from the body. Since drugs usually are relatively lipophilic, they need to be transformed into more hydrophilic compounds before elimination can occur. This transformation primarily takes place in the liver. Different enzyme systems are involved in the metabolism of both exogenous and endogenous substances. The cytochromes P450 belong to a group of enzymes, abbreviated CYPs, which often are responsible for the first step (phase I) in the metabolic process, resulting in metabolites, through oxidative reactions. After the first transformation, conjugation reactions (phase II) occur, i.e. water-soluble side-chains are linked to the compound through the action of e.g. glutathione S-transferase, and UDP-glucuronosyl transferase, further facilitating the excretion (Hardman JG et al., 2001).

Usually, the phase I and phase II reactions result in metabolites with less pharmacologic effect than the parent compound, but this is not true for all metabolic transformations. In some cases the products are more active than the parent compound, or even toxic.

The drugs and their metabolites are finally *excreted* from the body, via the kidneys into the urine, or via the liver into the bile. After biliary excretion, reabsorption from the intestine can occur, and this is called enterohepatic recirculation. The renal excretion is largely dependent on urinary pH, urinary flow, and active secretion.

VARIATION IN PHARMACOKINETICS

Factors influencing the variation in drug effect

- Food
- Age
- Gender
- Disease
- Environment
- Concomitant drug treatment
- Compliance

Patients are commonly given standardised treatment of a specific drug, independently of other concomitant therapies, age or relevant physiological factors. Many drugs do not exhibit the same effect, and do not obtain the same concentrations in different individuals, despite the same dosage. This effect is called *interindividual* variation, and compared to *intraindividual* variation (variation within the same subject), its importance in drug therapy is relatively large (Meyer UA, 2001). Genetic polymorphisms within genes involved in drug metabolism seem to be one of the major causes to the variable outcomes (Speight TM and Holford NHG, 1997; Ingelman-Sundberg M et al., 1999; Phillips KA et al, 2001; Oscarson M et al., 2002). This thesis will mostly focus on variations due to altered drug metabolising capacity, even if variations within receptor proteins and drug transporters also are important for the final effect of a drug (Guzey C et al., 2004). Although this is not the main focus of this thesis, factors other than genetic, influencing the pharmacokinetics of drugs, are briefly discussed below.

Food

For some drugs the simultaneous intake of food can change the rate and extent of absorption. Food can physically bind the drug, alter the gastric emptying, intestinal motility, and pH, and thereby influence the bioavailability of the drug. In most cases, food delays or reduces the absorption, although enhanced absorption also has been observed. For example, dietary calcium forms chelating complexes with tetracyclines and fluoroquinolones, compromising their absorption. Furthermore, certain dietary components can induce or inhibit the drug metabolising enzymes. Flavonoids in grapefruit juice reduce the intestinal CYP3A4-activity, simultaneously inhibiting P-glycoprotein, resulting in enhanced concentrations of drugs, which are substrates of CYP3A4/P-gp. In contrast, polycyclic aromatic hydrocarbons in chargrilled meat

induce the expression of CYP1A (Toothaker RD et al., 1980; Hathcock JN, 1985; Fontana RJ et al., 1999; Harris RZ et al., 2003).

Age

Newborns have different enzymatic capacities as compared to adults, and both phase I and phase II reactions are usually low in activity during the first months compared to adults, but around six months they might even have a faster clearance of certain drugs. In addition, plasma protein binding, total body fat, plasma volume, total extracellular and intracellular volumes, and rate of gastric emptying differ in infants compared to the grown-up individual. The elimination rates of indomethacin and ibuprofen have been shown to be dependent on postnatal age, and the half-life of ibuprofen was reported to be substantially longer in prematures than in adults. This is important to consider when treating premature infants for patent ductus arteriosus. In contrast, the oxidation of theophylline is much faster in children between 1 and 5 years than in adults (Rane A et al., 1973; Rane A, 1980; Rane A, 1999; Van Overmeire B et al., 2001; Strolin Benedetti M et al., 2003). With increased age, several physiological functions are reduced. Renal function decreases with age, and attention should be paid to elderly patients given drugs that are eliminated mainly via urinary excretion, since there is a risk of reaching supratherapeutic levels, due to drug accumulation. Examples of drugs that are mainly excreted through the kidneys include; digoxin, sotalol, antibiotics and most ACE-inhibitors. Furthermore, increased fat and decreased water content in elderly, influence the distribution volumes of drugs, and thereby the elimination rates (Dorne JL et al., 2004). Liver blood flow also decreases with age, and this will mainly affect orally administered drugs with high hepatic extraction ratios.

Gender

Gender difference is another factor that can influence the pharmacokinetics of drugs, but its relative importance has been poorly evaluated (Giudicelli JF et al., 1977; Fletcher CV et al., 1994; Kashuba AD et al., 1998; Kaiser J, 2005). Even if adjustments are made for body mass, percent body fat, renal clearance, plasma volume, and organ blood flow, unexplained differences still remain between men and women with regard to the exposure of certain drugs (Gandhi M et al., 2004; Anderson GD, 2005). There are reports that women would have a faster clearance of CYP3A4-substrates and also higher CYP3A4 mRNA and protein levels (Wolbold R et al., 2003). The metabolic changes are usually rather moderate, and most studies have a limited number of subjects and a large interindividual variation, why it is difficult to draw any general conclusions about gender differences based on these studies (Harris RZ et al., 1996; Chen ML et al., 2000; Chiou WL et al., 2001). Results from studies using human liver microsomes are incoherent, but in general data point toward similar metabolic activity, although the hepatic content of some drug metabolising enzymes can differ between men and women (Wolbold R et al., 2003; Schmucker DL et al., 1990; Hunt CM et al., 1992; George J et al., 1995). During pregnancy physiological factors will change, e.g. plasma volume, body

fat and body water increases, and the intestinal motility decreases. The clearance of drugs metabolised by CYP2D6, have been shown to be significantly higher during pregnancy as compared to after delivery (Hogstedt S et al., 1985; Wadelius M et al., 1997). Taken together, the pharmacokinetics of most drugs will be altered in pregnant women, although the final outcome of these changes is difficult to predict (Fletcher CV et al., 1994; Kaiser J, 2005; Pennell PB, 2003).

Disease

Infectious agents have been shown to depress cytochrome P450 dependent drug metabolism, and one of the first reports concerned the decreased elimination of theophylline in patients with upper-respiratory tract infection, caused by influenza A and adenovirus (Chang KC et al., 1978; Renton KW, 2001). Inflammatory processes and infections involve the release of cytokines, such as interferons, interleukins, and tumour necrosis factor (TNF), from macrophages and neutrophils. In animals, these inflammatory mediators have been noted to inhibit the expression of drug metabolising enzymes, resulting in a reduced clearance of drugs, which are substrates of these enzymes. During infections and inflammation, albumin concentrations are decreased, and this will alter the fraction of unbound molecules (i.e. free fraction). The final effect will vary, depending on the intital degree of protein binding, and whether the drug is a high or low extraction compound. The clinical consequences of such alterations in protein binding are generally minor, though. Decreased hepatic blood flow and a lower oxygenation capacity can reduce the metabolic clearance in conditions like cardiac failure (Alvan G, 1986; Renton KW, 2004).

Environment

Compounds in cigarette smoke and pollutants can generate an inflammatory response, and polycyclic aromatic hydrocarbons in smoke induce CYP1A1/1A2 expression. These enzymes are involved in the metabolism of exogenous and endogenous procarcinogens, and will thus generate substantially more toxic and harmful compounds if induced. (Fuhr U, 1998; Churg A, 2003; Raunio H et al., 2005). Exposure to heavy metals influences the cytochrome P450 levels, and lead (Pb) has been shown to decrease drug metabolism in humans. Cadmium (Cd) has been noted to induce CYP1A1 in cultured human cell lines, and increased hepatic CYP2C9 protein levels have been associated with cadmium accumulation in human liver (Moore MR, 2004).

Concomitant drugs

The concomitant administration of two drugs may lead to drug interactions due to altered pharmacokinetics and/or pharmacodynamics, of one or both substances. The pharmacokinetic interactions can occur at the site of absorption, as well as during distribution, metabolism and excretion. Induction is defined as an increase in the amount or activity of the enzyme. Rifam-

picin is a well-known inducer of several drug metabolising enzymes, and the elimination rate of concomitantly administered drugs is usually enhanced, whereby the concentration is reduced and the therapeutic effect is attenuated. Sulphaphenazole and gemfibrozil, on the other hand, are CYP2C9 enzyme inhibitors. Supratherapeutic concentrations of drugs, which are substrates of this enzyme, might be reached if given together with sulphaphenzole or gemfibrozil. Ketoconazole is another inhibitor, increasing the concentrations of concomitantly administered CYP3A4 substrates. Drugs can also interact with each other by forming insoluble complexes in the intestine, compromising absorption, which is the case when aluminium-containing antacids are given concomitantly with fluoroquinolones. Thus, no effect of the fluoroquinolone will be obtained. Probenecid inhibits the tubular secretion of antibiotics, and this action can be used to prolong the effect of antibiotics. Herbal remedies, such as St. John´s Wort (Hypericum perforatum), may also interact with drugs, usually by enhancing their clearance, through induction of CYP3A4 and P-glycoprotein expression (Meisel C et al., 2000; Wen X et al., 2001; Shapiro LE et al., 2002). The effects of inhibition can be rather severe, since unexpectedly high or even toxic concentrations of one or several drugs can be reached. In patients on multi-drug therapy, not only reduced or enhanced concentrations may be obtained, but synergistic or additive pharmacologic effects of two or more drugs can also cause severe side effects.

Compliance

It is common that patients forget to take their medication, or that they do not adhere to the drug prescription. This is likely to cause fluctuations in drug concentrations and could result in toxic or subtherapeutic concentrations. Lack of compliance is considered to be a great problem, but is very difficult to control (Osterberg L and Blaschke T, 2005).

As stated above, the large interindividual variation in drug concentration, and the resulting clinical outcome, is dependent on several factors, one of the most important being the rate of drug metabolism. Different factors that can have an influence on the metabolic clearance of drugs metabolised by CYP2C9 will be discussed throughout this thesis.

CYTOCHROME P450 ENZYMES

In the late 1950s, a membrane-bound carbon monoxide (CO) binding pigment, having an absorbance maximum at 450 nm was isolated and characterised, and named cytochrome P450, due to its physico-chemical characteristics (Klingenberg M, 1958; Omura T et al., 1964). It was later discovered that this pigment was a cluster of several enzymes. Cytochrome P450s are heme-containing proteins, and their catalytic activity is integrated in the membrane of the smooth endoplasmatic reticulum, with a cytosolic orientation (Nebert DW et al., 1987). Cytochrome P450s are monooxygenases, which catalyse oxidative and reductive reactions, where commonly one of the two oxygen atoms of O_2 is incorporated into the substrate.

$$NADPH + H^+ + O_2 + \mathbf{RH} \Longrightarrow NADP^+ + H_2O + \mathbf{R\text{-}OH}$$

The other oxygen is reduced to H_2O (see above). NADPH or NADH function as co-substrates, and are required as electron donors. The electrons are transferred via NADPH-cytochrome-P450-reductase, and cytochrome b_5.

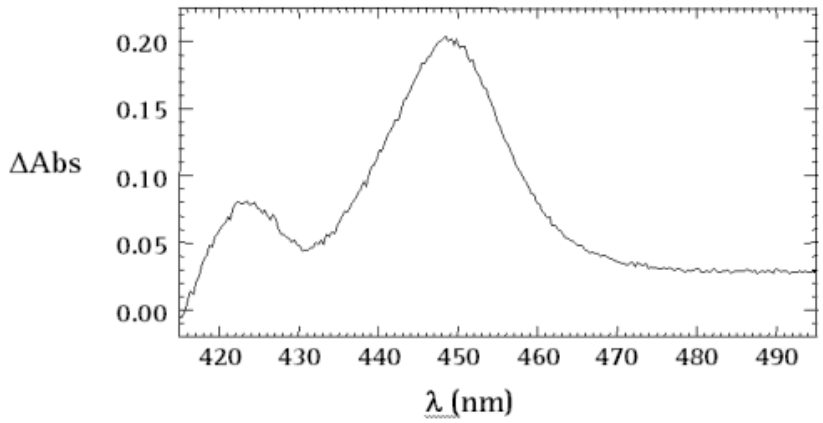
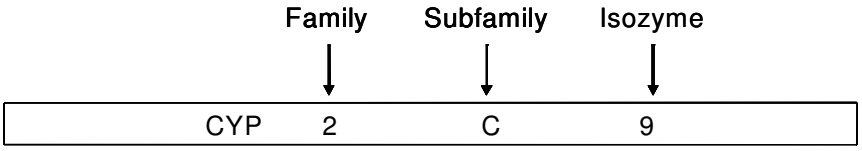


Figure 2. Absorption characteristics of cytochrome P450, where the CO-bound heme is reduced (Fe^{2+}) and bound to the protein via cystein.

A nomenclature for naming the different cytochrome P450s has been adopted (see below), and the enzymes are divided into families, subfamilies and specific enzymes, according to their amino acid homology. CYPs, general abbreviation for cytochrome P450s, belonging to the same family have at least 40 % amino-acid sequence identity, and subfamilies have more than 50 % amino-acid sequence identity. To define different allelic variants, an asterisk followed by a number is used, e.g. *CYP2C9*2* (Werck-Reichhart D et al., 2000).



Cytochrome P450s (CYPs) are a superfamily of enzymes present mainly in the liver, but also in extrahepatic tissues like kidneys, small intestine and lungs. They are responsible for the biotransformation of exogenous substances, but also of endogenous substances, such as arachidonic acid, vitamins, hormones, and bile acids. They are not only present in mammalians, but also in fish, as well as in plants (Nebert DW et al., 2002).

As the concentration of atmospheric oxygen started to increase about 2.5 million years ago, living organisms were required to protect themselves from oxidative stress, and this was partly achieved by ancestral forms of the CYP enzymes. Later, further development of these protective enzyme systems occurred in animals, when they had to detoxify the harmful substances that were present in plants, which they used as an energy source. Throughout evolution, plants and animals successively adapted, and new gene variants emerged, and an association between dietary composition and overall cytochrome P450 activity has been implied. Comparative sequence analyses have revealed a frequent replacement of amino acids within the substrate recognition sites of cytochrome P450 enzymes, showing a successive adaptation to the environment. Accordingly, enzymes with divergent substrate specificities seem to be an evolutionary advantage. In man, 57 functional cytochrome P450 genes have been identified at present (Nebert DW and Gonzalez FJ, 1987; Gotoh O, 1992; Nebert DW et al., 2000; Pirmohamed M et al., 2003; Salisbury BA et al., 2003).

In general, there is no correlation between the hepatic amount of CYPs and their importance in drug metabolism. The most important drug metabolising enzymes are those belonging to the CYP1, CYP2 and CYP3 families. The CYP3A enzymes (CYP3A4, CYP3A5 and CYP3A7) metabolise almost 50 % of all drugs that are being oxidised in the liver. Their abundance is also relatively high, representing about 30 % of the total hepatic CYP content. CYP2D6 is the second most important enzyme, regarding the number of drugs metabolised, approximately 25 %, although its liver content is rather low. The CYP2C family (including CYP2C8, CYP2C9 and CYP2C19) constitutes about 20 % of the total CYP content in the liver, and the different isoenzymes are involved in the metabolism of about 20 % of clinically used drugs (Gibbs MA et al., 2003; Ingelman-Sundberg M, 2004; Wilkinson GR, 2005). Different aspects of CYP2C9 will be further discussed throughout the thesis.

GENETIC POLYMORPHISMS

The most common types of polymorphisms are those called single nucleotide polymorphisms (SNP), and they occur at a frequency of about 1 per 1000 basepairs in the human genome. To be called a polymorphism the nucleotide exchange must be present in more than 1 % of the population, and the more precise definition would be "the occurrence in the same population of two or more alleles at one locus, each with a frequency of at least 1%". A functional polymorphic gene is defined as a stable variant of the gene, with altered enzymatic activity, existing in a population with a known frequency (Tribut O et al., 2002). The first definition of genetic polymorphisms was aimed to distinguish between common and rare phenotypes (Vogel F, 1961; Meyer UA, 1991). Spontaneous mutations occur at a lower frequency and are only distinguished from polymorphisms by definition (Meyer UA, 1991). The frequency of a specific variant allele varies between populations, and while some variants can be rather frequent in one population, it can be totally absent in others (Solus JF et al., 2004).

A recent study indicates that there is a higher genetic diversity among CYP genes than among most other human genes examined. This is believed to have been an evolutionary advantage, since it created enzymes with different substrate specificities. Much of the observed diversity is however due to the occurrence of many variants with low allele frequency, and those are probably of minor importance. The clinical significance of genetic polymorphisms in genes of drug metabolising enzymes, is very much dependent on whether the metabolite is active and/or toxic, the therapeutic index of the drug, the clinical state of the patient, concomitant drug treatment, as well as dose-response relationship (Tucker GT, 2000). Deletions, insertions, and duplications are other examples of mutations, which can be of clinical relevance for drug treatment, when present in genes coding for drug metabolising enzymes (Gotoh O, 1992; Solus J et al., 2004).

The resulting phenotypes of genetic polymorphisms in drug metabolising enzymes are generally called poor and extensive metabolisers, PMs and EMs. The poor metaboliser phenotype is inherited as an autosomal recessive trait, and these subjects are usually homozygous for the defect allele, while the extensive metabolisers are homozygous for the fully functional allele. Heterozygous subjects in general have intermediate metabolic capacity. The presence of duplicated genes is defined as ultrarapid metabolisers (UM) (Kalow W, 1997).

Below, a few clinically important polymorphic drug metabolising enzymes are described:

There are four members of the **CYP3A** family, i.e. CYP3A4**,** CYP3A5, CYP3A7 and CYP3A43, of which CYP3A4 and CYP3A5 are the most important regarding drug metabolism, being responsible for the transformation of about half of all clinically used drugs oxidised in the liver. CYP3A4 accounts for approximately 30 % of the total hepatic CYP content, and in African populations CYP3A5 is suggested to reach almost the same levels. Uncertainty

still exists about the relative importance of CYP3A5 on the overall CYP3A activity, and potential CYP3A5 specific probe drugs are being evaluated for this purpose. CYP3A7 expression is considered to be negligible in adults, while it is the major CYP3A isoform in foetal liver. CYP3A43 is expressed at very low levels and appears to be a pseudogene without functional activity (Ladona MG et al., 1988; Ladona MG et al., 1992; Hustert E et al., 2001; Kuehl P et al., 2001; Westlind-Johnsson A et al., 2003). CYP3A4 has broad substrate specificity, metabolising both endogenous and exogenous substrates, and it shows a large interindividual variability in metabolic activity. There are quite a number of allelic variants of both *CYP3A4* and *CYP3A5*, but most of them are rare. The *CYP3A5*3* and *CYP3A5*6-*8* alleles are all associated with low CYP3A5 protein expression, and their frequencies vary largely among populations (Lamba JK et al., 2002; He P et al., 2005). Many different drugs have been used as probes for CYP3A4/CYP3A5, including midazolam, alprazolam, erythromycin and quinine (Yasui N et al., 1996; Mirghani RA et al., 2003; Wennerholm A et al., 2005).

Although **CYP2D6** is a minor enzyme in quantitative terms, its importance in drug metabolism is relatively large, being responsible for the metabolism of about 25 % of all known drugs. It is the only major drug metabolising enzyme that is not inducible by exogenous compounds, although its activity is clearly higher during pregnancy (Hogstedt S et al., 1983; Wadelius M et al., 1997). It is also the only gene in which gene duplications and amplifications have been discovered (Johansson I et al., 1993). Most antidepressants and neuroleptics are substrates of this enzyme, as well as codeine, ethylmorphine, tolterodine and metoprolol (Lennard MS et al., 1982; Llerena A et al., 1992; Rane A et al., 1992; Sindrup SH et al., 1993; Hamelin BA et al., 1996; Spina E et al., 1997; Brynne N et al., 1998). In the 1970s, debrisoquine and sparteine were recognised as being transformed at different rates in different individuals, and this was later connected to deficient CYP2D6 enzyme activity (Mahgoub A et al., 1977; Tucker GT et al., 1977; Eichelbaum M et al., 1979). About 50 genetic variants of the *CYP2D6* gene have been identified, but only a few of them are important for the actual phenotype (Daly AK, 2003; Bogni A et al., 2005). There is a large interethnic variation in the frequency of PMs and EMs. *CYP2D6*3*, **4*, **5*, **10*, **17* and **41* are the major alleles, and they determine the phenotype. Lack of CYP2D6 activity among Caucasians is mainly due to the presence of the *CYP2D6*4* allele, while decreased enzyme activity in individuals of Asian and Oriental origin is mostly associated to the *CYP2D6*10* allele (Bertilsson L et al., 1992; Wennerholm A et al., 2001; Bapiro TE et al., 2002; Wennerholm A et al., 2002). An increased risk of experiencing side effects has been noted in patients treated with psychoactive drugs carrying defective *CYP2D6* alleles, and there seems to be an overrepresentation of individuals with amplified functional alleles among non-responders of anti-depressive treatment (Topic E et al., 2000; Grasmader K et al., 2004; Rau T et al., 2004; Kawanishi C et al., 2004). In haloperidol treated patients the number of functional *CYP2D6* alleles is significantly correlated to haloperidol clearance, and both ultrarapid and poor metabolisers have an increased risk of experiencing extra-pyramidal symptoms, related either to suboptimal therapy or supratherapeutic concentrations (Brockmoller J et al., 2002). The most striking example of ul-

trarapid metabolisers was found in some subjects with 13 copies of the *CYP2D6* gene, resulting in 25 times lower ratio of nortriptyline over hydroxy-nortriptyline than in subjects with only one functional copy (Dalen P et al., 1998). The metabolism of codeine to morphine is CYP2D6-dependent, and in people who lack a functional *CYP2D6* gene, no or reduced analgesic effect will be obtained. In contrast, individuals who are ultra-rapid metabolisers for CYP2D6 might experience side effects of the rapidly formed morphine (Persson K et al., 1995; Poulsen L et al., 1996; Dalen P et al., 1997). It has also been suggested that CYP2D6-defective individuals are resistant to oral opioid dependence, since no poor metabolisers of debrisoquine were found in a group of opiate dependent subjects (an odds ratio above 7), probably related to insufficient morphine generation in these individuals (Tyndale RF et al., 1997).

The treatment of *H. pylori*, peptic ulcer, and reflux diseases has been successful in recent years, through the introduction of proton pump inhibitors. **CYP2C19** is involved in the metabolism of all these drugs, (shown originally for omeprazole) and in PMs of CYP2C19, the AUC of omeprazole has been shown to be about 13 times higher than in EMs (Andersson T et al, 1990). The acid inhibition is also more pronounced in PMs than in EMs, especially after long-term treatment. By taking the actual genotype into consideration, adequate acid inhibition in individuals with EM genotype can be achieved by a more frequent dosing (Furuta T et al., 2004; Klotz U et al., 2004). In contrast, there seems to be no relation to *CYP2C19* polymorphisms and the antimalarial activity of proguanil, which also is a substrate of CYP2C19 (Skjelbo E et al., 1996). Many different variant alleles of CYP2C19 have been detected, several of them (*CYP2C19*2-CYP2C19*8*) being so-called null-alleles with no enzyme activity at all. The most common variants are the *CYP2C19*2* and the *2C19*3* alleles, which show a large interethnic variation. Poor metabolisers of CYP2C19 substrates are more common among Asian populations than among Caucasians, with a frequency of approximately 20 % and 3 %, respectively (Andersson T et al., 1992; Xie HG et al., 1999; Klotz U et al., 2004).

The relevance of ***CYP2C8*** polymorphisms for the clinical outcome of treatment with paclitaxel, rosiglitazone and amodiaquine remains to be established (Dai D et al., 2001; Li XQ et al., 2002; Hichiya H et al., 2005). The occurrence of the *CYP2C8*2* allele is about 18 % among African populations, but very rare in Caucasians, while the opposite is true for the *CYP2C8*3* allele (Cavaco I et al., 2005). The *CYP2C8*3* allele, recombinantly expressed in *E.coli,* was first shown to be defective for the metabolism of arachidonic acid and paclitaxel (Dai D et al., 2001). For repaglinide, a partial CYP2C8 substrate, the total exposure after a single dose was unexpectedly decreased by 45 % in healthy subjects genotyped as *CYP2C8*1/*3* compared to *CYP2C8*1/*1* subjects, although no pharmacodynamic association to blood-glucose levels was observed (Niemi M et al., 2003). The *CYP2C8*3* allele has been shown to be linked to the *CYP2C9*2* allele, and their relative importance for the clearance of arachidonic acid, an endogenous compound, was examined in Study V (Yasar U et al., 2003). The *CYP2C8*4* allele frequency is very low, both among Caucasians and Africans.

In total there are ten different alleles of CYP2C8, five of them *CYP2C8*6-*10*, were only recently discovered in Japanese individuals at a very low frequency (Hichiya et al., 2005).

CYP2C9 is more thoroughly described in other parts of this thesis.

Polymorphism in other enzymes than the cytochrome P450s may also be of importance for drug treatment, and one such example is thiopurine methyltransferase **(TPMT)**. This is a polymorphic enzyme, of which the genotype is of importance to the clinical outcome of mercaptopurine and azathioprine treatment, which have narrow therapeutic indicies. Azathioprine, a prodrug to 6-mercaptopurine, is used to prevent allograft rejections and to treat autoimmune diseases, such as systemic lupus erytematosus, and inflammatory bowel diseases. Mercaptopurine is used for treating acute lymphoblastic leukaemia in children. TPMT metabolises both of these drugs, and a person with deficient TPMT enzyme activity will experience haematological toxicity or bone marrow suppression. In Caucasians, 10 % have intermediate TPMT-activity, and are usually heterozygous for the *TPMT*2* or **3* allele, while 1 out of 300 have a complete enzyme deficiency. Phenotypic tests have been developed to determine the TPMT enzyme activity in red blood cells, before initiating antipurine therapy, in order to avoid lack of effect or supratherapeutic concentrations, which can be potentially fatal (Weinshilboum R, 2001).

Drug transporters act as barriers, preventing exogenous compounds to be absorbed and/or facilitate their excretion. The P-glycoprotein, an ATP-dependent efflux-protein, was the first of these transporters to be discovered, in 1976 (Juliano RL et al., 1976). It now belongs to a big superfamily of **ATP-binding cassette (ABC) proteins**, comprising about 50 members. The function of these transport proteins is mainly to limit the absorption and accumulation of drugs intra-cellularly, through an efflux mechanism that pumps the compounds out of the cell. They are expressed in the blood-brain barrier and placenta, but are mainly localised in the liver and intestines. Polymorphisms within the *MDR1* gene, coding for the P-glycoprotein, are of special interest since they have been shown to be involved in drug resistance of chemotherapy, and affect drug disposition (Chan LM et al., 2004). The effects of polymorphisms within the *MDR1* gene on drug concentrations are unclear, and results are incongruous, i.e. no clear *MDR1* genotype-phenotype association has been established. A specific polymorphism of the *MDRC1* gene has been associated with four-fold higher digoxin levels however (Hoffmeyer S et al., 2000), and there are suggestions that certain *MDR* genotypes would influence the incidence of specific diseases (Lehmann S et al., 2004; Marzolini C et al., 2004). Research within the field of transporters has increased, and the impact of drug transporters along with the CYP enzymes on the clinical effects of drug treatment should definitely be recognised.

GENOTYPING

Genotyping is the method used for determining the allelic variants of a certain gene. The genotype refers to the genetic constitution or make-up of an individual, and is the internally coded, inheritable information. The genotype should preferentially predict the phenotype (see below), and is determined from DNA, which can be extracted from e.g., a blood sample or buccal swab. Several techniques for genotyping exist, and the most common are different types of PCR (Polymerase Chain Reaction) methods, such as RFLP (Restriction Fragment Length Polymorphism) and SSCP (Single Stranded Conformation Polymorphism). With high-throughput methods such as dynamic allele-specific hybridization (DASH), matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF), and TaqMan, many samples can be analysed in one single run. In addition, different sequencing methods are also available. DNA chips and microarray methods, using the principle of hybridisation of complementary nucleotide sequences, also offer the possibility of high throughput screening of polymorphisms (Bray MS et al., 2001; Tribut O et al., 2002; Daly AK, 2004). The method of choice is dependent on many factors, e.g. availability, cost, number of samples to be analysed, and accuracy.

PHENOTYPING

Phenotype can be defined as the visible expression of the genotype, and it can be determined not only by one factor, or one gene, but by several (Tribut O et al., 2002). Phenotyping of a specific cytochrome P450 enzyme *in vivo,* is performed by using a specific probe drug of the enzyme. Usually, a single dose of the drug is ingested and urine or blood samples are collected. Also endogenous compounds can be used as probe substances, an example being cortisol. Phenotyping of TPMT is performed *ex vivo*. By measuring the concentration of the drug and its metabolite, usually by HPLC, LC-MS or GC-MS, the enzyme activity, expressed as the ratio between the substrate and its metabolite(s), can be determined (Daly AK, 2004).

If a so-called cocktail approach is used, the activity of several enzymes can be assessed simultaneously. Different cocktails, using different probe drugs and administration strategies, have been developed in order to phenotype for several cytochrome P450 activities in parallel (Zhu B et al., 2001; Chainuvati S et al., 2003; Christensen M et al., 2003). Therapeutic drug monitoring can also be used for phenotyping purposes (Dahl ML and Sjoqvist F, 2000).

GENOTYPING VS. PHENOTYPING

The limitations using phenotyping is that the probe drug itself can cause side-effects. Usually this is a minor problem, since the doses used are relatively low, and administered only once. However, sometimes concomitant medications must temporarily be withdrawn, in order not to

influence the results by causing drug interactions. If using endogenous substrates the method will be less invasive. Since many of the factors influencing the phenotype, are not constant over time, the test usually has to be repeated. The advantage of phenotyping is the ability to provide information about the overall drug metabolising capacity at a certain time-point, also taking hepatic and renal function, diet, and other environmental factors into account. For enzymes that do not have any polymorphisms of clinical interest, but still exhibit variation in activity, phenotyping is the method of choice for dose prediction (Caraco Y, 1998; Bachmann KA, 2002). Phenotyping tests are usually expensive and sample analysis is time consuming. When using a cocktail approach, a lot of information can be acquired simultaneously. The drawback of a cocktail is the large amount of samples required for analysis, and the potential risk of interactions between the probe drugs (Tanaka E et al., 2003; Zhou H et al., 2004).

Since the genotype is constant over time, and is not affected by other interacting factors, such as concomitant drug treatment, dietary habits, hormonal levels, and diseases, genotyping for a specific gene variant does not have to be repeated. In addition, genotyping results are obtained relatively fast, and the test can be performed without withdrawal of any ongoing drug therapy. Only small amount of sample is required, and the problem with interindividual variation does not exist (Kirchheiner J and Brockmoller J, 2005).

Irrespective of which method is being used, it has to be thoroughly validated. Usually, genotyping methods have proven to be highly accurate (high sensitivity and specificity). As can be concluded from above, the method of choice is dependent on many factors, but despite the many advantages with genotyping, its use in clinical practice has so far been limited (Evans WE et al., 2004). This might be due to the lack of conclusive results proving that the therapeutic outcome would benefit from performing genetic testing, which in turn reflects the difficulties of performing pharmacogenetic and/or pharmacogenomic studies.

PHARMACOGENETICS

The term pharmacogenomics is sometimes used interchangeably with pharmacogenetics, and there seems to be some confusion about the exact interpretation of the two words. Pharmacogenetics is usually defined as the inherited genetic components of variability in individual responses to drugs. Pharmacogenomics, on the other hand, deals with the development of drugs, based on the knowledge of the human genome, and can be defined as the correlation between the genotype of an individual and the response to drug treatment. The field of pharmacogenetics was already recognised by Pythagoras, when he observed that some people, but not others, were at danger when eating fava beans. This was later associated to glucose-6-phosphate dehydrogenase deficiency, resulting in anaemia. The first thorough description of the field was introduced by Vogel in the beginning of the 1960s (Vogel F, 1961; Kalow W, 1965; Kalow W, 1967; Kalow W, 2002).

INDIVIDUALISED DRUG THERAPY (PERSONALISED MEDICINE)

The recommended standard doses of a certain drug are not appropriate for all patients. Due to interindividual variations such as genetic factors, diagnosis, drug interactions, as well as the actual disease and physiological status of the patient, the initial dose must be adjusted in order to achieve optimal effect, without causing any side-effects (Meyer UA, 2001; Tsai YJ et al., 2002; Kalow W, 2002). Pharmacogenetics is thought to be one of several useful tools for optimising drug treatment and developing new therapies. The aim of pharmacogenetic research is to identify genetic differences, which could be responsible for unpleasant or harmful reactions, so-called adverse drug reactions (ADRs), or in the opposite case, lack of therapeutic response (Oscarson M and Ingelman-Sundberg M, 2002; Pirmohamed M and Park BK, 2003). Since approximately 56 % of all known drugs are completely or partly cleared via cytochrome P450 enzymes, and 40 % of those are polymorphic, it is of importance to understand the overall influence of polymorphisms on drug metabolism, in order to avoid unwanted effects. The *CYP2D6* gene, having about 50 genetic variants, demonstrates the complexity of the issue (Ingelman-Sundberg M et al., 2000). Results have been presented, stating that 59 % of the 27 drugs occurring most frequently in ADR reports, were metabolised by at least one enzyme having a variant allele known to cause decreased enzymatic activity. This figure can be compared with 7-22 % of randomly selected drugs that were metabolised by polymorphic enzymes (Phillips KA et al., 2001; Pirmohamed M et al., 2001; Pirmohamed M and Park BK, 2003). Although no exact figure regarding the involvement of polymorphic drug metabolising enzymes in adverse effects can be provided, pharmacogenetics should be recognised as an important research field that can help optimise drug treatment and reduce patient suffering, and hopefully also health care costs (Bates DW et al., 1997; Lazarou J et al., 1998; Anderson GD, 2005). Traditionally, studies have been performed on single genes, trying to determine their association to a given outcome, but lately polymorphic patterns for several different genes have been established. Data derived from the human genome project (HUGO) has been

able to associate polymorphisms in drug receptors, transporters, drug metabolising enzymes and disease genes to drug responses. The are a lot of examples demonstrating the potential benefits of pharmacogenetic research, but still few conclusive results from prospective, randomized, clinical trials have been presented, which are able to prove the advantages, i.e. improved efficacy or reduced number of adverse events, of knowing the genotype of patients before initiating a drug treatment (Evans WE and Relling MV, 2004).

The lack of substantial benefit of knowing the genotype of certain genes before initiating drug therapy is dependent on several factors. First, the genotype does not always reflect the actual phenotype, due to factors such as phenocopy, i.e. different factors giving rise to the same trait or phenotype. Other common problems are unknown affecting environmental factors, the physiological changes caused by the actual disease, drug interactions, and last but not least the fact that most drugs are metabolised by several enzymes. The high frequency of single nucleotide polymorphisms also limits the possibility to establish a phenotype-genotype association. This is in contrast to the general belief that the identification of SNPs will help us predict the phenotype, and thereby the ultimate dose of a specific drug to a certain patient. Furthermore, the expression of genes can be affected by the drug treatment itself, as well as the disease. The aim to find a specific genotype, which will affect the phenotype, has thus been more complex than first anticipated, and it is rather complicated to predict the optimal drug therapy and dose for each patient. Anyway, efforts to establish dose recommendation charts for some drugs, metabolised by polymorphic enzymes, have been done (Meisel C et al., 2000; Kirchheiner J et al., 2001). These are far from complete, and additional factors, like renal and hepatic function, are generally not considered, why they should only be regarded as rough guidelines. In the future there are however hopes that a CYP genotype chart might be available for each patient, helping the physician choosing the right drug and the right starting dose. It must however be remembered that if the frequency of the variant allele is low, a large number of people would have to be genotyped in order to identify one exceptional case at risk of adverse effects, and this would mean substantial additional costs.

Despite the complexity of pharmacogenetic research described above, there are examples proving that genotyping can be a helpful tool in order to optimise drug therapy for the individual patient, and save money, before initiating certain therapies. Studies have shown that poor metabolisers of CYP2C19 have better effect of proton-pump inhibitor treatment than extensive metabolisers, given the recommended doses. In a study, based on patients with duodenal ulcers and *H. pylori* infection, the cost-effectiveness analysis supported the use of CYP2C19 genotyping before initiating drug treatment (Lehmann DF et al., 2003). It has also been implicated that side-effects in patients treated with amitriptyline would be reduced if the *CYP2D6* and *CYP2C19* genotypes were known beforehand (Steimer W et al., 2005).

Therapeutic drug monitoring

Instead of genotyping, traditional therapeutic drug monitoring (TDM) is still the method most widely used in order to determine that adequate drug concentrations have been obtained, and to optimise the dose for each patient. If there is an established dose-concentration correlation, the drug dose can be adequately adjusted according to the TDM result. TDM can be regarded as a phenotyping method, and it is preferred if there is an existing validated method, which is cheap and accessible (Veenstra DL et al., 2000). It is usually used for drugs with a narrow therapeutic window, which have an unpredictable clearance together with a large interindividual variation. The analysis is expected to be accurate, with high sensitivity, specificity and precision. TDM can also be performed when there is suspicion about drug toxicities, non-compliance, drug-interactions, or at therapeutic failure. Immunosuppressants, antiepileptics, antibiotics, antidepressants and antipsychotics are examples of drugs that are routinely being analysed in the clinical practice (Bengtsson F, 2004, Dahl ML and Sjoqvist F, 2000). The problem with TDM is often that samples are drawn without knowing at what time-point the last dose was ingested, which can make an interpretation of the concentration impossible. It is also essential to know for how long time the patient has been taking the drug, in order to determine if steady-state conditions have been obtained or not. Usually plasma samples are used, since the handling is easier, but for some drugs, whole blood or serum is preferred, and it must be remembered that results might differ depending on the method of analysis and blood fraction utilised (Rombo L et al., 1985).

IN VITRO STUDIES OF DRUG METABOLISM

Regulatory authorities require that the metabolic pattern of new drugs have been thoroughly investigated before they are administered to humans, in order to predict drug interactions and influence of polymorphic drug metabolising enzymes. For this, animals are often used, but since there are rather large interspecies differences, other "test systems" are also needed, before the drug is given to humans. Commonly, *in vitro* studies with liver microsomes, hepatocytes, liver slices, or recombinantly expressed enzymes, in combination with specific inhibitors, are used for this purpose (Bertz RJ et al., 1997). From *in vitro* studies, using human liver microsomes, the metabolic pathways for the formation of certain metabolites can be derived, and the interindividual variation in metabolic clearance can be determined. In addition, the intrinsic clearance *in vivo* can be predicted from extrapolations of the *in vitro* results (Rane A et al., 1977; Rane A et al., 1984; Ashforth EI et al., 1995; Zomorodi K et al., 1995). Recombinant expression of different CYPs in bacteria, yeast or insects cells can be used to determine the overall influence of one single enzyme to the overall metabolism of a substrate. Human hepatocytes and liver slices offer the advantage of being more intact systems than liver microsomes, but they require very delicate handling, and quickly loose their metabolic activity. To be able to conclude which enzymes are involved in the metabolism of a certain substrate and to what extent, it is usually recommended to perform studies using at least two of the methods described (Carlile DJ et al., 1999; Houston JB et al., 2000).

CYP2C9

BACKGROUND

The *CYP2C9* gene was first cloned in 1991 (Romkes M et al., 1991; Ohgiya S et al., 1992). It is 55 kb long, including the 9 exons, and is located on chromosome 10, surrounded by the genes of the other members of the subfamily (Figure 3), and it encodes a protein consisting of 490 amino acids (de Morais SM et al., 1993; Goldstein JA and de Morais SM, 1994; Gray IC et al., 1995). The crystal structure of CYP2C9 was only recently resolved (Williams PA et al., 2003), and the understanding of substrate binding has since then improved (Figure 4). CYP2C9 is only one member of the CYP2C subfamily, which contains at least three others, i.e. CYP2C8, CYP2C18 and CYP2C19.

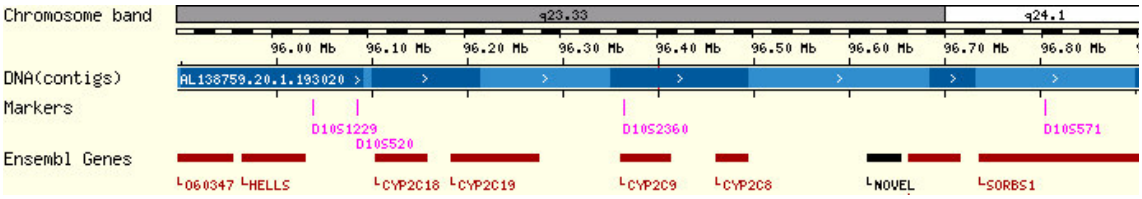


Figure 3. The location of the *CYP2C9* gene, from **www.ensembl.org**.

POLYMORPHISMS

The *CYP2C9* gene contains quite a few variant alleles, both in the exons and the *5´*-flanking region. For the moment there are 24 different alleles identified (*CYP2C9*1-CYP2C9*24*) (www.imm.ki.se/cypalleles). Screening of the major cytochrome P450 genes in samples from different populations revealed that the members of the CYP2 family contain more mutations than for example the CYP1 and CYP3 families (Solus JF et al., 2004). Most *CYP2C9* alleles result in decreased enzyme activity, at least *in vitro*.

The most common and most studied variant alleles, the *CYP2C9*2* and *CYP2C9*3*, are located in exon 3 and 7 respectively, and result in decreased activity of the enzyme. The *CYP2C9*2* variant codes for a cysteine instead of an arginine at position 144, while the *CYP2C9*3* variant results in an exchange of isoleucin to leucin at position 359. The clinical relevance of these polymorphisms is dependent on the substrate. For warfarin, the clearance in *CYP2C9*2/*2* subjects is about 32 % lower compared to *CYP2C9*1/*1* subjects, while the clearance for glibenclamide is only 10 % lower in subjects genotyped as *CYP2C9*2/*2* compared to *CYP2C9*1/*1* subjects. The *CYP2C9*3* variant generally has a greater impact on drug clearance than the *CYP2C9*2* allele, and heterozygous *2C9*3* carriers usually have at

least 50 % decreased clearance of CYP2C9 substrates (Pirmohamed M and Park BK, 2003; Kirchheiner J et al., 2005). Just as the *CYP2C9*3* allele, the *CYP2C9*4* and *CYP2C9*5* variants are located in exon 7. *CYP2C9*4* has so far only been found in Japanese epileptic patients (Imai J et al., 2000), while *CYP2C9*5* only has been detected among African-Americans and Hispanic-Americans (Dickmann LJ, 2001; Yasar U et al., 2002a). The *CYP2C9*6* allele, identified in one African-American subject experiencing phenytoin toxicity, is the result of a deletion of a base-pair at position 818 in exon 5 (Kidd RS et al., 2001). In a panel of samples from different ethnic populations, new mutations in the *CYP2C9* gene were recently discovered, denoted *CYP2C9*7-*12,* and *CYP2C9*14-*20*. Of these, the *CYP2C9*11* variant seemed to be the one with lowest catalytic activity, with regard to tolbutamide metabolism. This mutation is located in exon 7, and the R335W substitution probably alters the secondary structure of the protein. Most of these "new" variants seem to be present at rather low frequencies in all populations, and do not have any major impact on the catalytic activity of the enzyme (Blaisdell J et al., 2004; Zhao F et al., 2004). The *CYP2C9*13* allele is located in exon 2, and was found in a Chinese subject, being a poor metaboliser of lornoxicam as well as tolbutamide. This subject was also heterozygous for the *CYP2C9*3* allele (Si D et al., 2004).

Several of the nucleotide exchanges have been identified in the *5'*-flanking region and the *3´*-UTR region, but they usually occur at very low frequencies. Many of the mutations located in the *5´*-flanking region of the gene are linked to either the *CYP2C9*2* or *CYP2C9*3* alleles, and are part of different haplotypes (Veenstra DL et al., 2005; King BP et al., 2004; Zhao F, et al., 2004). Their influence on gene expression is probably minor, although some reports claim that they may cause reduced enzymatic activity on their own (Shintani M et al., 2001). None of the *5´*-flanking mutations are localized at any transcription factor binding sites, further dismissing their potential influence on enzyme activity (King BP et al., 2004).

Despite the existence of several polymorphisms within the *CYP2C9* gene, there is still a large and unexplained variation of the enzyme activity among individuals genotyped as *CYP2C9*1/*1*. Several investigations have been undertaken, including some of the papers in this thesis (Study I and II), trying to find possible explanations (Shintani M et al., 2001; King B et al., 2004).

EXPRESSION

The CYP2C enzyme expression accounts for approximately 20 % of the total cytochrome P450 content, of which CYP2C9 is the major constituent (Shimada T et al., 1994; Inoue K et al., 1997). While CYP2C9 is the predominant protein of the different CYP2C enzymes in adult liver, CYP2C19 dominates in prenatal livers (Treluyer JM et al., 1997; Koukouritaki SB et al., 2003). CYP2C9 mRNA expression has also been detected in extra-hepatic tissues like

kidney, adrenal gland and endothelial cells (Klose TS et al., 1999; Lapple F et al., 2003; Michaelis UR et al., 2004). In the kidney, CYP2C8 mRNA is expressed at highest quantities among the various CYP2C enzymes, followed by CYP2C9. In testes, ovaries, adrenal gland and prostate, on the other hand, CYP2C9 is predominant. In the small intestine, both CYP2C9 and CYP2C19 expression has been detected (Klose TS et al., 1999). The CYP2C9 protein content is about 10 times lower in the intestine, compared to the liver (Lapple F et al., 2003). mRNA of CYP2C18 has been found to be highly expressed in human epidermis (Zaphiropoulos PG, 1997).

REGULATION

In recent years, several studies have been performed in order to elucidate the mechanisms involved in the regulation of CYP2C9 expression. HNF4α was the first transcription factor, shown to participate in the regulation of hepatic expression of CYP2C9 (Ibeanu GC et al., 1995). In addition, pregnane X receptors (PXR) are involved in the regulation of CYP2C9 transcription, and recent studies have implicated that HNF4α and constitutive androstane receptor (CAR) act synergistically, and that a cross-talk between HPF1 (HNF4α binding site) and CAR-RE (CAR and PXR binding site) occurs (Chen Y et al., 2005). Both distal and proximal CAR responsive elements are important for the constitutive expression of CYP2C9 (Ferguson SS et al., 2002; Chen Y et al., 2004). HNF3 binding sites also exist in the *CYP2C9* gene, and it has been observed that HNF3γ is another important transcription factor for the expression of hepatic *CYP2C* genes in humans (Bort R et al., 2004). There are contradictory reports about the ability of phenobarbital and rifampicin to upregulate CYP2C9 transcription levels, but most of the reports indicate that these two substances, as well as dexamethasone, induce CYP2C9 activity (Gerbal-Chaloin S et al., 2001; Ferguson SS et al., 2002).

The glucocorticoid responsive element (GRE), located between –1684 and –1648 in the *5´*-flanking region of the *CYP2C9* gene, and activated by dexamethasone, controls the expression of PXR, CAR and RXR and is therefore suggested to be indirectly involved in the regulation of CYP2C9 expression (Gerbal-Chaloin S et al., 2001; Ferguson SS et al., 2002; Pascussi JM et al., 2003). A mechanism has been proposed by Pascussi *et al*., suggesting that glucocorticoids activate the glucocorticoid receptor (GR), which in turn induces the PXR, CAR and RXR expression, regulating the transcription of xenobiotic-mediated or glucocorticoid-mediated responses (Pascussi JM et al., 2003).

In human liver foetal samples (between 8 and 24 weeks of gestation), CYP2C9 seems to be expressed in low quantities, with progressively increasing amounts during the remaining part of gestation. The CYP2C9 specific content has been shown to be significantly higher in neonatal samples than in foetal samples, although the variation between samples is extensive (Koukouritaki SB et al., 2003).

SUBSTRATES

Although the different CYP2C subfamily members have about 82 % amino-acid identity, they have quite different substrate selectivities (de Morais SM et al., 1993). For CYP2C9, it is the B-C loop in the protein structure that mostly contributes to the substrate specificity. CYP2C9 metabolises about 20 % of all known drugs, and due to its preference for small acidic substrates, containing electronegative groups, it has been hypothesised that the active site would contain basic residues (Rettie AE et al., 1994; Sullivan-Klose TH et al., 1996; Yasar U et al., 2001a; Williams PA et al. 2003). The recently published crystal structure of CYP2C9 reveals no such entities (Williams PA et al., 2003). Instead, acidic residues were found to be present in the active site of CYP2C9, and the region responsible for selectivity is probably located elsewhere within the protein. A new warfarin-binding site was recently discovered, which previously had not been identified as a substrate-binding site. It was also observed that when *S*-warfarin is present at the binding site, no major conformational change of the protein seems to be induced, indicating the ability of CYP2C9 to bind more than one substrate simultaneously (Williams PA et al., 2003).

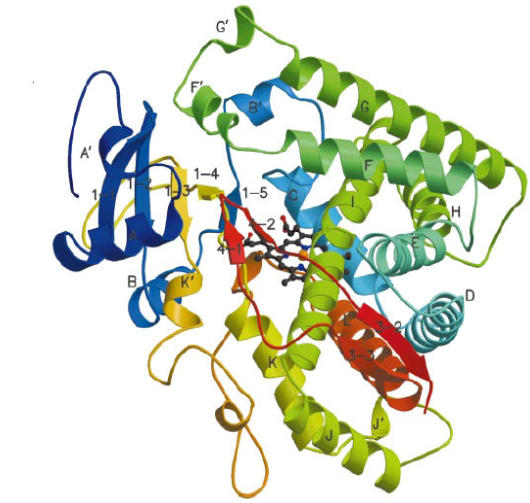
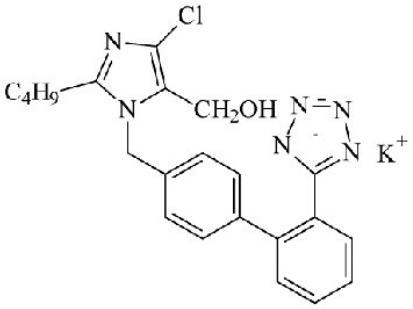


Figure 4. Crystal structure of CYP2C9 (Williams PA et al., 2003).

For many CYP2C9 substrates, the *CYP2C9*2* allele does not seem to have the same impact as the *CYP2C9*3* allele on the metabolic activity, probably due to the location of these two mutations (Kirchheiner J et al., 2003; Vormfelde SV et al., 2004). Since the *CYP2C9*3* mutation is located in the substrate recognition site of the *CYP2C9* gene, it is expected to have a larger effect than the point mutation at amino acid 144 (*CYP2C9*2*), which is not located at any such site or elsewhere in the active site (Gotoh O, 1992).

Some features, which are important for the binding of substrates to the CYP2C enzymes, are the number of hydrogen donors and acceptors, and the acid-base character of the compound (Lewis DF, 2003). Except NSAIDs, also sulfonylureas, angiotensin II receptor antagonists, anticoagulant drugs, as well as phenytoin are examples of drugs that are substrates of CYP2C9 (Sullivan-Klose TH et al., 1996; Klose TS et al., 1998; Yamazaki H et al., 1998; Thijssen HH et al., 2000; Scordo MG et al., 2002).

Losartan



Losartan (Merck Sharp & Dohme) is an angiotensin II receptor antagonist used to treat hypertension and heart failure. Angiotensin II is a potent vasoconstrictor, which activates proinflammatory mediators via NF-KB, and angiotensin II receptor antagonists have been shown to suppress the expression of e.g. free radicals, C-reactive protein (CRP), tumour necrosis factor α (TNF-α), and to reduce inflammation and oxidative stress (Dandona P et al., 2003). Losartan together with its carboxyl metabolite, E-3174, exert the pharmacological effect, but only about 14 % of a given losartan dose is transformed into the carboxyl metabolite. High plasma concentrations of E-3174 are anyway observed, due to a much lower clearance than losartan. E-3174 is more pharmacologically potent than losartan itself (Munafo A et al., 1992). The aldehyde-metabolite, E-3179, which is rapidly converted to the E-3174, was demonstrated to have both anti-inflammatory and anti-thrombotic activity, through COX-inhibition *in vitro*. The clinical significance of this activity has not been clearly established (Kramer C et al., 2002).

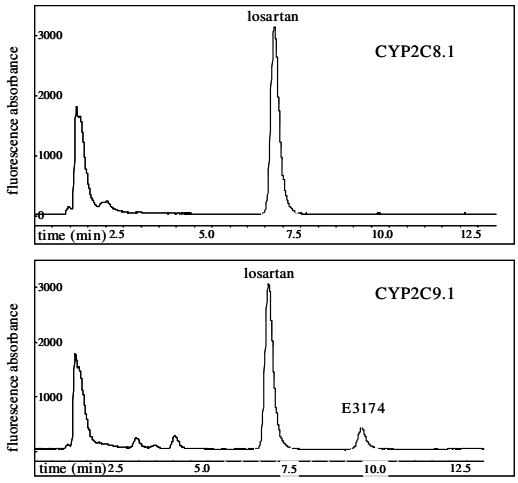
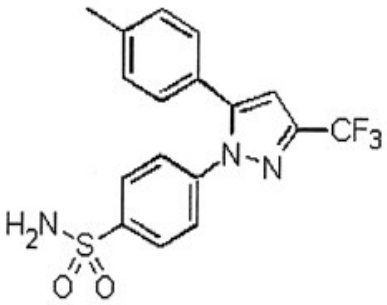


Figure 5: HPLC chromatogram for the analysis of losartan and E-3174 in yeast microsomes expressing CYP2C8.1 or CYP2C9.1

The bioavailability of losartan is relatively low, only 33 %, probably due to first-pass metabolism (Lo MW et al., 1995). The pharmacokinetics for losartan and its active metabolite is linear up to doses of 200 mg. In 1995, it was first reported that losartan was primarily metabolised by CYP3A4 and CYP2C enzymes (Stearns RA et al., 1995). Later it was discovered that the formation of the losartan metabolite, at clinically relevant concentrations, is dependent on *CYP2C9* genotypes (Yasar U et al., 2001a), and it has been proven to be a good and selective

phenotyping probe drug (Yasar U et al., 2002b; Yasar U et al., 2002c). CYP2C8, although sharing high amino acid identity with CYP2C9, is not involved in the transformation of losartan to E-3174, as seen from our experiments with yeast microsomes expressing either CYP2C8.1 or CYP2C9.1 (Figure 5) (Babaoglu MO et al., 2004). Contrary to the large inter-individual variation, the intra-individual variability for losartan is very low (Yasar U et al., 2002b). Losartan was used as a phenotyping probe drug in two of our studies (Study I and II).

Celecoxib



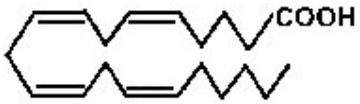
Celecoxib (Searle/Pharmacia&Upjohn/Pfizer) is a selective COX-2 inhibitor, which was first introduced in 1999. It is used to relieve the symptoms in patients with reumathoid arthritis and osteoarthritis, and it exhibits anti-inflammatory, analgesic and antipyretic effect. Studies with celecoxib for the treatment of familial adenomatous polyposis (FAP), which could lead to colon cancer, have been completed, and there has also been interest in the possibility of celecoxib to prevent Alzheimer (Aisen PS, 2002; Breitner JC, 2003; Sinicrope FA et al., 2004). At inflammatory sites, COX-2 is overexpressed in comparison to COX-1, and selective COX-2 inhibitors were mainly developed to replace the traditional NSAIDs, which inhibit both COX-1 and COX-2. They were marketed as having the same analgesic effect as NSAIDs, but without the common and well-known gastro-intestinal side effects. *In vitro* studies suggest that celecoxib has a more than 350-fold selectivity to COX-2 over COX-1 (Davies NM et al., 2000). Celecoxib has no significant effect on platelet aggregation or thromboxane levels, not even at supratherapeutic doses, and celecoxib does not affect the prophylactic activity of low-dose aspirin (Wilner KD et al., 2002). The use of COX-2 inhibitors have been under debate and recently rofecoxib, a much more potent COX-2 inhibitor than celecoxib, was withdrawn due to a 2-fold higher risk of heart attacks after long-term treatment, compared to placebo (Lenzer J, 2005). Similar results were implicated from a study on celecoxib for the treatment of colon polyposis, showing a two to three-fold increase in deaths from cardiovascular events, in patients treated with celecoxib (400 or 800 mg daily), compared to placebo (Solomon SD et al., 2005). Restrictions for prescribing celecoxib have thereafter been introduced. For both rofecoxib and celecoxib the side-effects seem to be dose-dependent, with a higher incidence at higher doses.

Celecoxib itself exerts the pharmacological effect, while none of the main metabolites have any effect, neither on COX-1 nor COX-2. The primary route of elimination is via metabolism, and less than 3 % of the total dose is excreted unchanged in the faeces. Celecoxib is first oxidised to a primary alcohol, mainly by CYP2C9, and then further oxidation occurs whereby a carboxylic acid is formed (Tang C et al., 2000). The carboxylic acid is the major metabolite

excreted in urine and faeces (Paulson SK et al., 2000). Celecoxib is an inhibitor of CYP2D6 (Werner U et al., 2003).

In single-dose studies the oral clearance of celecoxib has been shown to be extensively reduced in subjects homozygous for the *CYP2C9*3* allele, compared to *CYP2C9*1/*1* subjects (Tang C et al., 2001; Kirchheiner et al., 2003), while a steady-state study of celecoxib could not demonstrate any association between *CYP2C9* genotypes and celecoxib exposure (Brenner SS et al., 2003). Thus, the metabolism and pharmacokinetics of celecoxib in relation to *CYP2C9* genotypes between single-dose studies and studies with repeated dosing were incoherent, why we decided to perform an additional study *in vivo*, but also *in vitro* (Study III and Study IV).

Arachidonic acid



Arachidonic acid is an endogenous compound, which is esterified to phospholipids and released by phospholipase A_2 during cell stimulation. It is the precursor of many physiologically active compounds, referred to as eicosanoids (Dennis EA, 2000). Cyclooxygenases and lipoxygenase are the enzymes responsible for the formation of prostaglandins, thromboxanes and leukotrienes, while the epoxyeicosatrienoic acids (EETs), and hydroxyeicosatetranoic acids (HETEs), are the major metabolites generated by cytochrome P450s (Capdevila J et al., 1981; Oliw EH et al., 1981; Rifkind AB et al., 1995; Zeldin DC et al., 1996; Kroetz DL et al., 2002, Fleming I, 2004).

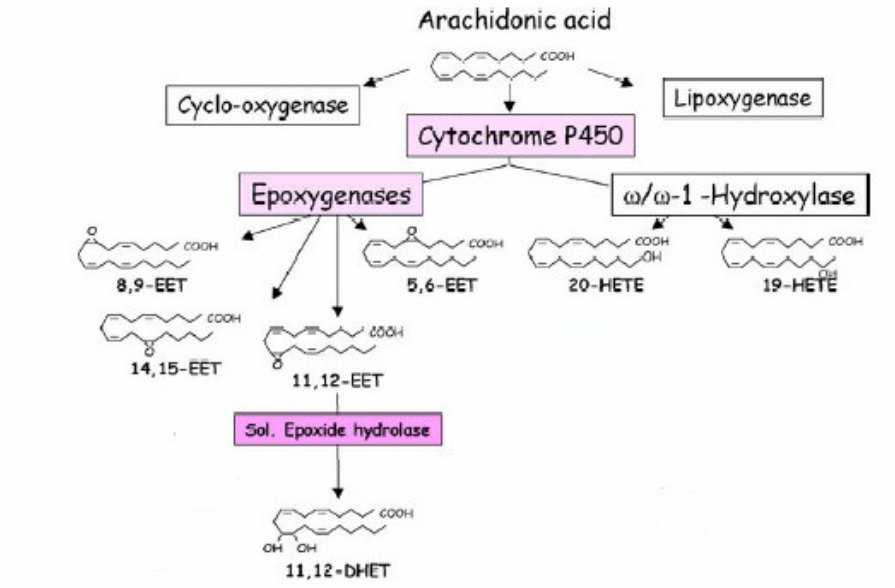


Figure 6. Schematic overview of the metabolism of arachidonic acid (adapted from Fleming I, 2004).

EETs have been shown to have important vasodilating function, through activation of K_{Ca+} channels as well as Na-K-ATPase, thereby inducing hyperpolarisation (Fleming I et al., 2001; Gauthier KM et al., 2002). They also possess anti-inflammatory and fibrinolytic properties (Capdevila JH et al., 2001; Roman RJ, 2002; Michaelis UR et al., 2003).

CYP2C9 and CYP2C8 are involved in the biosynthesis of the vasoactive metabolites (EETs) from arachidonic acid. It has been speculated that altered enzymatic activity of the *CYP2C8* and *CYP2C9* allelic variants could be important for the pathogenesis of cardiovascular diseases, since one of the EET-metabolites, 11,12-EET, is identical to endothelium derived hyperpolarizing factor (EDHF) (Fisslthaler B et al., 1999; Fleming I, 2004). Indeed, the risk of acute myocardial infarction tended to be higher in female patients with rare allelic variants of *CYP2C8* and *CYP2C9*, i.e. *2C8*3*, *2C9*2* and *2C9*3*, than in women genotyped as *CYP2C9*1/*1* or *CYP2C8*1/*1* (Yasar U et al., 2003). In a larger case-control study, with 403 patients with myocardial infarction and 431 control individuals (without myocardial infarction) the results were inconsistent with those of Yasar *et al.*, as the *CYP2C9*2* or *CYP2C9*3* allelic variants were more frequent among male controls than in male subjects with myocardial infarction. In females no such differences were observed (Funk M et al., 2004). Similar results were recently published in relation to hypertension and *CYP2C9* genotypes, showing that Chinese healthy controls had a higher *CYP2C9*3* frequency than hypertensive patients of the same ethnic origin (Yu BN et al., 2004). These conflicting results might be explained by the dual activity of EET, being able not only to cause vasodilation, but also vasoconstriction in certain vascular beds. Hence, the role of EETs in the pathogenesis of hypertension is certainly quite complex, and further studies are warranted (King LM et al., 2005). The importance of CYP2C enzymes for the metabolism of arachidonic acid was investigated in Study V.

Other important CYP2C9 substrates

Warfarin is used as anticoagulation therapy in many European countries, as well as the US. It is a racemic mixture with *S*-warfarin being the more potent enantiomer. Warfarin exhibits a large interindividual variation in its anticoagulant effect. *S*-warfarin is metabolised by CYP2C9 to 7-OH-warfarin, while CYP3A4, CYP1A2 and CYP2C19 are responsible for the metabolism of *R*-warfarin (Scordo MG et al., 2004). Despite dose adjustments according to standardised anticoagulation measures, such as INR (international normalised ratio), bleeding complication is still a major cause of death in warfarin treated patients. The risk for serious bleedings is approximately 1.3-4.2 % per year of exposure, and has been shown to be related to *CYP2C9* polymorphisms (Taube J et al., 2000). Patients heterozygous for the *CYP2C9*3* allele have about 60 % decreased warfarin clearance, while the reduction is 90 % in homozygous *CYP2C9*3* patients (Takahashi H et al., 1998). Accordingly, the warfarin dose is usually significantly lower in subjects carrying *CYP2C9*2* or *CYP2C9*3* alleles, compared to *CYP2C9*1*. Patients genotyped as *CYP2C9*1/*1* have a relatively large variation in warfarin

clearance, demonstrating that the *CYP2C9* genotype is not the only dose predicting factor. Maximally 20 to 30 % of the variation observed can be accounted for by the *CYP2C9* genotype, and other factors like age and body weight have been shown to be additional predictors of warfarin dose. Recently, a variant allele within the gene coding for microsomal epoxide hydrolase (mEH), involved in the reduction of vitamin K epoxide, was demonstrated to be of importance for warfarin dose requirements (Loebstein R et al., 2005). A newly identified polymorphism within the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene was shown to be responsible for another 13 % of the interindividual variability in warfarin dosage (D'Andrea G et al., 2005). This still leaves us with a relatively large part of the variation which can not be explained, although efforts trying to identify mutations in important genes, as well as other factors of interest, are being performed (Rost S et al., 2004; Scordo MG et al., 2004; Herman D et al., 2005; Veenstra DL et al., 2005). Phenprocoumon and acenocoumarol, are two other used anticoagulants, which also are metabolised by CYP2C9 (Thijssen HH and Ritzen B, 2003; Ufer M et al., 2004).

Phenytoin is used for treatment of seizure disorders and it displays nonlinear pharmacokinetics, with a narrow therapeutic range, which results in dose-adjustment problems when trying to achieve optimal concentrations. Both CYP2C9 and CYP2C19 are involved in the metabolism of phenytoin, and it is also a substrate for P-gp. The drug exhibits large interpatient variation in clearance, and even though the major polymorphisms of *CYP2C9* are predictive of its pharmacokinetics, a substantial part of the variation still remains to be explained by other factors (Taguchi M et al., 2005). A polymorphism within the *SCN1A* gene, encoding for a subunit of the voltage-sensitive sodium channels, was recently shown to be present in patients with high dose requirements of phenytoin (Kerb R et al., 2001; Kidd RS et al., 2001; Hung CC et al., 2004; Tate SK et al., 2005). Phenytoin has been used as a phenotyping probe drug for CYP2C9, using the metabolic ratio of phenytoin and its phenylhydantoin metabolite (Caraco Y et al., 2001).

Many of the non-selective **NSAIDs** are substrates of CYP2C9. The effect of the common *CYP2C9* polymorphisms on the elimination of these drugs varies, due to the different selectivities of the substances to the enzyme. The metabolism of the pharmacological active *S*-ibuprofen is quite extensively reduced in carriers of the *CYP2C9*3* allele, while carriers of the *CYP2C8*3* allele have a reduced metabolism of the *R*-isomer (Martinez C et al., 2005). *In vitro*, there are clear differences between the *CYP2C9* genotypes regarding the metabolism of diclofenac, while *CYP2C9* polymorphisms are not predictors of diclofenac transformation, *in vivo* (Yasar Ü et al., 2001b; Kirchheiner J et al., 2002; Kirchheiner J et al., 2003; Brenner SS et al., 2003). Flurbiprofen is also metabolised by CYP2C9, showing a decreased clearance in subjects carrying one or two *CYP2C9*3* alleles, and it has been shown to be a good probe drug for CYP2C9 (Lee CR et al., 2003a; Lee CR et al., 2003b). Other anti-inflammatory drugs, such as tenoxicam and lornoxicam, also exhibit *CYP2C9* genotype–dependent metabolism (Vianna-Jorge R et al., 2004; Zhang Y et al., 2005).

Sulfonylureas, including tolbutamide, glibenclamide, glipizide and glimepiride, are all metabolised by CYP2C9, through which glibenclamide forms an active metabolite. Tolbutamide was demonstrated to be a good probe drug for CYP2C9 (Lee CR et al., 2003b). The pharmacokinetics of these substrates have been shown to be significantly altered in subjects with one or two *CYP2C9*3* alleles, compared to *CYP2C9*1/*1* subjects. Even though no significant changes in blood glucose responses after single doses of glibenclamide or glimepiride have been noted in enzyme deficient healthy volunteers (Niemi M et al., 2002), there were substantially more diabetic patients with low CYP2C9 activity experiencing hypoglycaemia than in controls treated with glimepiride or glibenclamide (Holstein A et al., 2005). Altered pharmacokinetics of these drugs might influence the duration of the insulin release refractory period.

INTERETHNIC VARIATION

Molecular genetic variation among different populations was first demonstrated to exist for the ABO blood groups in 1919, and it was soon discovered that genetic variations of proteins among different populations was a rather common phenomenon. Natural selection and genetic drift are two factors, which are responsible for the difference in allele frequency among different populations, but migration also plays an important role. The variation between populations seems to be less than the variation within populations, and there are several reports confirming this. Interethnic variation only accounts for 5 to 15 % of the variance, the rest being variations within populations. By studying these interethnic variations, it may be possible to reconstruct the evolutionary history of populations (Cavalli-Sforza LL et al., 2003). Within populations, the African-American contains much more diversities than the Asian population, having the least variability. Not only genetic variation can account for the interethnic variations, but diet, life-style, and general health are also contributing factors that can have either an inhibitory or inducing effect on protein expression (Salisbury BA et al., 2003).

Figure 7 demonstrates a common situation, when comparing drug clearances between populations. As illustrated, the mean metabolic clearances between the two populations are one standard deviation (SD) apart, but the statistical difference is non-significant, due to substantial overlap between the two curves.

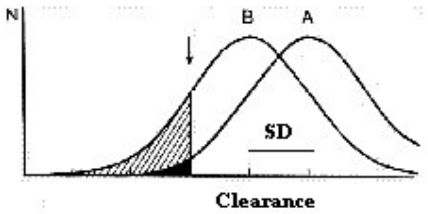


Figure 7. Variation in metabolic clearance between different populations, adapted from Kalow W and Bertilsson L, 1994.

The arrow indicates the clearance below which there is a risk of developing side effects. For population A, about 2 % of the individuals will fall into this risk group, while as much as 16 % of population B is at risk. This example clearly highlights the difficulties when interpreting results, only comparing mean metabolic activities (Kalow W and Bertilsson L, 1994). In clinical practice the genetic background of patients are generally not recognised, and as demonstrated by this example, the effect in single patients is difficult to predict from large phenotyping studies where the overall metabolic clearance between populations are similar.

The two most common *CYP2C9* polymorphisms with functional effect, the *CYP2C9*2* and *CYP2C9*3* variants, occur at a frequency of about 11 and 7 %, respectively, in Caucasian populations. In African subjects, the frequency of the *2C9*2* variant is much lower, while it is completely absent among Asians. The *CYP2C9*3* allele has a lower frequency in both Asians and Africans, compared to Caucasians. Other alleles, like the *CYP2C9*5, CYP2C9*6, CYP2C9*8* and *CYP2C9*11*, have only been detected among Africans (Kidd RS et al., 2001; Yasar U et al., 2002a; Allabi AC et al., 2004; Blaisdell J et al., 2004). When the *CYP2C9* gene in different ethnic populations was characterised, it was found that Pygmies carried the highest percentage of mutated alleles, which is in accordance with the high genetic diversity observed in African populations (Blaisdell J et al., 2004).

Results from a warfarin study showed that Japanese patients genotyped as *CYP2C9*1/*1* had higher clearance of *S*-warfarin than Caucasians with the same genotype, even after adjustment for body weight, and irrespective of mutations in the *5´*-flanking region of the *CYP2C9* gene (Takahashi H et al., 2003; Takahashi H et al., 2004). However, some of the patients (not stated whom) were treated with other drugs known to interact with the metabolism of warfarin, and the time point for blood sampling was not clearly defined (although stated not to be of importance). In addition, genotyping was performed by RFLP, which can cause erroneous results (Yasar U et al., 1999; Okuda R et al., 2004). In contrast, another study indicated that Chinese patients required lower maintenance doses of warfarin than Caucasians, in order to obtain the same anticoagulant effect as Caucasians, but here age, body weight and other concomitant diseases influenced the results (Yu HC et al., 1996). No differences between Caucasians and African-Americans regarding warfarin doses or INR-values were

noted, in a study with totally 153 patients (Tabrizi AR et al., 2002). In warfarin-treated Korean patients, it was concluded that no association between *CYP2C9* genotypes and warfarin dose or therapeutic effect, existed, but relatively few patients were evaluated and none of the patients carried any of the *CYP2C9*2* or *CYP2C9*3* allelic variants (Lee S et al., 2003).

There are also differences between subpopulations of the same origin, and it was revealed that Chinese and Malay subjects required lower warfarin doses than Indians (Zhao F et al., 2004).

Phenytoin is another CYP2C9 substrate that shows interethnic variation. To evaluate results from clinical studies on phenytoin is complicated, since it exhibits non-linear kinetics, and the hepatic metabolism may be saturated already at therapeutic doses (Lund L et al., 1971; Idestrom CM et al., 1972). Still, comparisons have been performed, and a study comparing African and Caucasian subjects showed that the half-life of phenytoin was significantly longer in Africans than in Caucasians, and a further study revealed that the excretion of one of the metabolites of phenytoin, *p*-HPPH, was higher in Caucasians than in people from Ghana, despite similar phenytoin doses (Edeki TI and Brase DA, 1995).

No interethnic differences have been observed for tolbutamide, having similar pharmacokinetics in Chinese and Caucasian healthy volunteers (Gross AS et al., 1999).

Interethnic variations were further studied in Study II.

INTERINDIVIDUAL VARIATION

Interindividual variation in the metabolic capacity of CYP2C9 is an important issue to consider, when trying to predict dose-effect relationship. Several attempts have been made trying to find an explanation to the large variation observed. There are reports that the content of CYP2C enzymes in relation to the total cytochrome P450 varied between 7 and 35 % in human liver microsomes (Shimada T et al., 1994). In another study with human liver microsomes, it was shown that the CYP2C9 activity varied almost 12-fold, while the CYP2C9 content varied about 6-fold (Yasar U et al., 2001a; Shu Y et al., 2001). No plausible explanations to these observations yet exist, although attempts have been made trying to identify relevant features. For example, interferons and other cytokines, expressed during inflammation, have been shown to suppress the CYP expression in both human cell lines and rodents (Morgan ET et al., 1994). In rats, the CYP2C11 transcription was depressed after administration of bacterial endotoxin (Morgan ET et al., 2002). Since many diseases, like fever, diabetes, cancer and reumathoid arthritis have inflammatory components, the clearance of drugs might be altered in individuals suffering from such illnesses, and cancer has been associated with reduced hepatic drug metabolism (Slaviero KA et al., 2003). The impact of diseases on CYP2C9 activity was further demonstrated in a study where the plasma warfarin S/R ratio was increased by 50

% in patients with end-stage renal disease (ESRD), compared to healthy control subjects (Dreisbach AW et al., 2003).

When looking at gender differences, it has been found that drugs, which are substrates of CYP2C19, might be metabolised faster by men than by women (Harris RZ et al., 1995; Anthony M et al., 2002). It has also been demonstrated that CYP2C expression is up-regulated in pregnant women (Zhou Y et al., 2005), and the phase of the menstrual cycle can influence pharmacokinetics of certain drugs (Gerdin E and Rane A, 1992). Very little is however known about the influence of gender on therapeutic outcome (Harris RZ et al., 1995).

Various receptors, like CAR and PXR, regulate the expression of CYPs. In general, these receptors have to be translocated from the cytoplasm to the nucleus, where they form heterodimers and bind to different response elements. For CYP2C enzymes, the influence of these receptors and their ligands on the interindividual response is not exactly known (Pascussi JM et al., 2005). The stability of mRNA and protein can also differ, and might influence the total enzymatic activity (Salisbury BA et al., 2003).

Interindividual differences are further discussed in the studies included in this thesis, especially in Study I.

AIMS

The primary aim of this PhD work was to investigate possible factors, which could be responsible for, or contribute to, the variation in CYP2C9 metabolic activity, especially within the *CYP2C9*1/*1* group. We also wanted to further elucidate the impact of *CYP2C9* genotypes on the turnover of both exogenous and endogenous substrates, and on the pharmacokinetics of drugs metabolised by CYP2C9. The specific aims for each study were the following:

Study I: To characterise the variation in CYP2C9 phenotype in relation to genotype, in a healthy Swedish population, with further analysis of the *CYP2C9* gene in metabolic outliers, i.e. subjects with slow metabolism of losartan, not carrying any *2C9*3* allele.

Study II: To study the CYP2C9 dependent metabolic clearance of losartan in healthy Korean subjects, in relation to their *CYP2C9* genotype, as well as to compare the outcome with the results from Study I.

Study III: To compare the rate of celecoxib hydroxylation by different genetic variants of *CYP2C9* in human liver microsomes as well as in yeast expressed enzymes, and to identify the enzyme(s) involved in the oxidation of hydroxy-celecoxib.

Study IV: To investigate the influence of *CYP2C9* genotypes on single-dose, and steady state pharmacokinetics, of celecoxib in healthy Caucasian subjects.

Study V: To assess if the three main genotypes of *CYP2C9* would influence the biosynthesis of epoxyeicosatrienoic acids (EETs) from arachidonic acid, and to determine the relative contribution of the different CYP2C isoenzymes to this process.

METHODS

SUBJECTS

Healthy Swedish and Korean subjects were recruited in Study I and II, for participation in a so-called cocktail study, where they were given five different probe drugs of the cytochrome P450 enzymes, including a single oral dose of 25 mg of losartan, as well as quinine, caffeine, omeprazole and debrisoquine. The protocol had been designed in order to avoid potential drug interactions (Christensen et al., 2003). No medications, except oral contraceptives, were allowed throughout the study period. Only one Korean woman stated the use of oral contraceptives, but she was excluded from all analyses. Among Swedish women, a substantial number were users of oral contraceptives. These females were excluded when compared with the Korean population. All subjects had to abstain from caffeine containing food and beverages at least 48 hours before study start. In Study I, 126 Swedish individuals were included in the analyses of losartan and *CYP2C9* genotypes, and in Study II there were 147 eligible Korean subjects. Another 25 Swedish participants were included in the analyses in Study II.

Genotype	Swedes (n)[a]	Koreans (n)[b]
*CYP2C9*1/*1*	96	137
*CYP2C9*1/*2*	26	0
*CYP2C9*2/*2*	2	0
*CYP2C9*2/*3*	2	0
*CYP2C9*1/*3*	22	8
*CYP2C9*3/*3*	3	0

a: Total number of subjects after inclusion of another 25 subjects. Women using OCs are included.
b: The single woman using OCs is excluded.

Table 1. Total number of subjects phenotyped with losartan.

Healthy Caucasian subjects, with previously known *CYP2C9* genotypes, were selected for participation in Study IV. Only subjects with *CYP2C9*1/*1* (n=7), *CYP2C9*1/*3* (n=3) and *CYP2C9*3/*3* (n=3) genotypes were included. Individuals genotyped as *CYP2C9*1/*2* or *CYP2C9*2/*2* were excluded, since individuals with *CYP2C9*2* alleles did not differ from *CYP2C9*1/*1* subjects with regard to celecoxib metabolism (Kirchheiner J et al., 2003). In order to minimise the risk for potential cardiovascular side-effects, which have been reported after long-term use with celecoxib, all subjects had to be below 55 years of age, with no previously known history of angina or hypertension ($\geq$140/90 mm Hg), and with a BMI of maximally 30. In addition, they had to have normal ECG recordings, and not take any other medication, including oral contraceptives and herbal remedies, and they were not allowed to be smokers. Blood pressure was checked regularly throughout the study. The subjects were in fasting condition both before the first and last drug intake, since food can influence the absorption of celecoxib, and thereby the maximum plasma concentration.

PHENOTYPING WITH LOSARTAN

The CYP2C9-phenotyping method, using 25 mg of losartan, was developed in our lab and has been previously validated in healthy volunteers (Yasar U et al., 2002c). Both sampling and analysis procedures are relatively simple, and no extraction processes are needed. In addition, the intra-individual variability seems to be low (Yasar U et al., 2002b). Losartan is given to subjects in the morning, where after urine is collected for 8 hours. Aliquots were stored at –20˚ C, until analysis. The ratio of the peak area of losartan and its CYP2C9 generated carboxy-metabolite (molar concentrations) is calculated as an index of the CYP2C9 activity. A high metabolic ratio thus indicates a slow metabolism, and vice versa.

DRUG ANALYSES

Quantification of losartan and its active metabolite, E-3174, was performed in urine (8-hour sampling) by an established RP-HPLC-method, using fluoroscent detection (Ritter MA et al., 1997) (Study I and II).

Celecoxib and its hydroxy- and carboxy-metabolites were also detected and quantified by RP-HPLC, using UV-detection (Study III and IV). In Study III the supernatant of the microsomal incubation was directly injected onto the column, without any previous extraction procedures. In Study IV, modifications of a published HPLC-method were performed, which resulted in a simplified work-up procedure of the plasma samples, and a shorter run-time (Stormer E et al., 2003).

In Study V, samples were separated on a C_{18}-column, and then subjected to electrospray ionization (ESI), according to a previously established method (Bylund J et al., 1998). The efflu-

ent was connected to an ion trap mass spectrometer, and detection was performed on the molecular ions in a negative ion mode. Single ion monitoring was performed on the epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DHETs), and hydroxyeicosatetraenoic acids (HETEs).

MOLECULAR BIOLOGY METHODS

Genotyping

Genotyping of study participants, as well as of human livers, was performed by extracting DNA, either from leukocytes or from human liver tissue. All genotyping methods were based on the polymerase chain reaction (PCR) technique. Mostly, allelic discrimination using a TaqMan assay was used, but also allele-specific PCR, for identifying known polymorphisms of the *CYP2C8* and *CYP2C9* genes. For the allele-specific reactions we designed our own specific primers, and for the TaqMan assay we either used pre-developed reaction kits, or designed our own specific primers and probes (Yasar U et al., 2003; Halling J et al., 2005).

DNA sequencing analyses

For characterising gene fragments and identifying new mutations, sequencing of purified DNA was performed. This method is also based on the PCR technique, and ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits with fluorescent dideoxynucleotides were used, with reagent concentrations recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). We designed our own specific primers for sequencing 3.5 kb of the *5'*-flanking region of the *CYP2C9* gene, as well as the nine exons and exon-intron junctions. The fragments were analysed by ABI 377 DNA sequencer.

Reporter gene assay

Experiments to determine the influence of different mutations in the promotor region of the *CYP2C9* gene were performed. This was done by cloning of the *5´*-flanking region of the *CYP2C9* gene, and ligating it into the promotorless pGL3 basic firefly luciferase reporter vector (Promega, Madison, WI). The construct was further transformed into super competent XL-1 Blue *E. Coli* bacteria and plasmid DNA was purified. The purified plasmid was co-transfected into HeLa cells with reporter plasmid pRL-SV40 (containing the *Renilla* luciferase gene) as control. Firefly and *Renilla* luciferase activities were measured on a luminometer.

Recombinant enzyme expression

Heterologously expressed enzymes are used for studying the importance of one enzyme, without the interference of others. These systems are also useful for studying the impact of different allelic variants on the metabolic activity. In Study III and V we used *Saccharomyces cerevisae* (baker´s yeast), which overexpresses NADPH cytochrome P450 reductase, but in itself has low endogenous cytochrome P450 activity. cDNA of interest was cloned into the yeast expression vector pYeDP60 and expressed in *Saccharomyces cerevisae*, and microsomal fractions were prepared.

In Study III, human alcohol dehydrogenases ADH1C2, ADH2 and ADH3, were expressed in *E.coli* in order to determine their involvement in the oxidation of hydroxy-celecoxib.

HUMAN LIVER BANK

A human liver bank has previously been established at the department, and was enlarged with livers from the transplantation surgery unit at Karolinska University Hospital, Huddinge (von Bahr C et al., 1980; Tybring G et al., manuscript). Five of the livers were from patients suffering from familiar amyloidotic polynephropathy (FAP). Three of these were of Japanese origin, two of which were monozygous twins. FAP livers had higher amounts of CYP2C9 protein content than non-FAP livers, according to Western blot results. Three of the livers were genotyped as *CYP2C9*1/*1*, one as *CYP2C9*1/*2*, and one as *CYP2C9*2/*3*. Some of the FAP-livers were included in Study III and V, but these livers were shown not to significantly influence our results (Tybring G et al., manuscript).

Consent was obtained from the donors, or their relatives, to use the liver tissue for scientific purposes. Most donors were given dopamine before surgery, and almost all livers were transfused with 5-10 L Belzer UW Cold Storage Solution (ViaSpan, Du Pont Pharmaceuticals, USA). Prior to transfusion, bensyl penicillin (0.12 mg), human insulin (1.4 mg) and dexamethasone (16 mg) solutions were added. The livers were handled within the ischemic time of about 16 hours, cut into small pieces, frozen in liquid nitrogen and stored at – 80 °C (Tybring G et al., manuscript).

MICROSOME AND CYTOSOL PREPARATION

Microsomes from human livers, belonging to the liver bank described above, were prepared in order to study the kinetics and metabolic pathways of drugs, as well as the formation rates of their metabolites *in vitro*. They are well characterized with respect to metabolic capacity and genotype. Until preparation of cytosol and microsomes, the livers were stored at –80 °C. Pieces of liver were homogenized in buffer (10 mM Na/K phosphate buffer, pH 7.4, containing 1.14 % (w/v) KCl) and then centrifuged (14 000 × *g* at 4 °C for 15 min). The resulting supernatant was further exposed to centrifugation, whereby a microsomal pellet and a cytoso-

lic fraction were obtained. The pellet was homogenized and mixed with buffer (50 mM potassium phosphate buffer, pH 7.4), and the cytosolic fraction was mixed with dithiothreitol, EDTA, sucrose and glycerol, before storage at –80 °C. The total protein content was determined by the Lowry method, and the total cytochrome P450 content was quantified spectrophotometrically (Lowry OH et al., 1951; Omura T and Sato R, 1964). Western blot was used for determining the specific protein content.

MICROSOMAL INCUBATIONS

Microsomes and cytosol from human livers as well as yeast were used in Study III and V. Incubations were performed in the linear range, regarding time and protein concentration. NADPH or NAD^+ were used as co-enzymes. The reactions were allowed to proceed at +37 ºC, in a shaking water bath, and were terminated with either acetonitrile or ethanol. Samples were then immediately put on ice, before further work-up procedures.

For all methods, duplicate samples, standard curves and quality controls were run. The *in vitro* incubations were performed in the linear range, with regard to protein concentration, and time.

ENZYME KINETICS

The enzyme kinetics of celecoxib hydroxylation and arachidonic acid oxidation in different allelic variants of *CYP2C9*, expressed in yeast, was determined by applying the non-linear Michaelis-Menten regression model. From the metabolite formation vs. substrate concentration curve, K_m could be determined as the concentration where half V_{max} was obtained. Also yeast microsomes expressing *CYP2C8* and *CYP2C19,* were used for determining the kinetic parameters of arachidonic acid oxidation (Hidestrand M et al., 2001; Li XQ et al., 2002).

BIOINFORMATICS

The nucleotide sequence of the *5´*-flanking region, as well as of the exons and introns of the *CYP2C9* gene were obtained from the National Center for Biotechnology Information Locus-Link database, a public database. In order to compare the results from the sequencing analyses with the published “wild-type” sequence, sequence alignments were performed using ClustalW, which identifies the best match within multiple sequences, with the help of algorithms.

Primer specificity was checked in BLASTN at NCBI, which compares a query sequence against a nucleotide sequence database.

In study I, a search for potential transcription factor binding sites was performed by MatInspector V2.2 based on Transfac 4.0, which can locate transcription factor binding sites in sequences of unlimited length.

PHARMACOKINETIC CALCULATIONS

Noncompartmental analysis (WinNonlin, version 4.1, Pharsight Inc., Mountain View, CA) was used for calculating the pharmacokinetic parameters of celecoxib and its metabolites in Study IV. C_{max} (maximum plasma concentrations) and t_{max} (time to achieve C_{max}) were determined from the observed concentration versus time profiles. The half-life ($t_{1/2}$) was obtained from the slope of the terminal phase ($t_{1/2}=\ln 2/\lambda$). $AUC_{0\text{-}24h}$ (area-under-time curve-vs-drug concentration, for the zero-to-24h time interval after drug intake) was calculated using the trapezoidal rule. The remaining area ($AUC_{24h\text{-}\infty}$) was extrapolated from the last dose to infinity by the software, by dividing the concentration at the last sample time (C_t) by the rate constant of the terminal elimination phase (λ). Apparent clearance (Cl/F) was calculated as dose/$AUC_{0h\text{-}\infty}$ after the first dose, while it was calculated from dose/$AUC0_{\text{-}24h}$ on day 7, i.e. considering only the dosing interval at steady state.

STATISTICAL ANALYSES

One-way analysis of variance (ANOVA), using different *post-hoc* tests if significant differences ($p < 0.05$) were observed, was mostly used. The conditions for performing ANOVA, like normal distribution, homogenous variances etc., were checked and when needed, log transformations were performed in order to fulfil the criteria. ANOVA is used for studying the effects of two factors separately and/or their interaction effect.

In Study IV, the non-parametric Kruskal-Wallis test was used. This test can be applied when three or more groups are compared. There are no restrictions for normal distribution, but instead the test is less powerful as compared with parametric tests.

All statistical calculations were performed using Statistica version 6.0 or 6.1.

RESULTS

STUDY I

The allele frequency of *CYP2C9*2* and *CYP2C9*3* were 9.9 and 10.3%, respectively, among the 126 healthy Caucasian subjects analysed. For the *CYP2C9*3* allele this is somewhat higher than previously reported. As expected, the metabolic ratio of losartan differed significantly between subjects genotyped as *CYP2C9*1/*1* and *CYP2C9*1/*3*. The variation within the *CYP2C9*1/*1* group was large, the difference being 30-fold between the highest and lowest MR value. The highest ratios were obtained in the two subjects homozygous for the *CYP2C9*3* allele. Several single nucleotide polymorphisms (SNPs) were identified in the *5´*-flanking region of the *CYP2C9* gene, but none which could explain the large interindividual variation within the *CYP2C9*1/*1* subjects (Table 2). Most of the SNPs were heterozygous and linked to the *CYP2C9*2* or *CYP2C9*3* alleles. The occurrence of several SNPs makes the haplotype prediction difficult, since it must be known whether the polymorphisms reside on the same allele or not. Preliminary results (unpublished), where we examined the importance of one of the mutations, at –1188 with reporter gene assay in HeLa cells, did not show any difference in luciferase activity between the –1188T and –1188C constructs. This mutation was chosen since it was relatively frequent among our samples. Although no genetic explanation for the interindividual variation could be identified, it was observed that women genotyped as *CYP2C9*1/*1*, taking oral contraceptives, had two-fold higher metabolic ratio of losartan, compared to women of the same genotype, taking no oral contraceptives. Men genotyped as *CYP2C9*1/*1* had a metabolic ratio in-between women taking oral contraceptives and those not taking oral contraceptives, but the differences did not reach statistical significance.

SNP location	Frequency
-3579 G>A	1 (4%)
-3455 T>G	6 (22%)
-3360 T>C	12 (44%)
-2958 A>T	2 (7%)
-2902 A>T	1 (4%)
-1911 T>C	10 (37%)
-1885 C>G	10 (37%)
-1537 G>A	6 (22%)
-1188 T>C	8 (30%)
-1096 A>G	4 (15%)
-1091 G>A	1 (4%)
-981 G>A	8 (30%)
-620 G>T	2 (7%)
-485 T>A	7 (26%)
-484 C>A	7 (26%)
-77 C>A	1 (4%)

Table 2. SNPs identified in the *5'*-flanking region of the *CYP2C9* gene of 27 healthy volunteers. Frequency data presented as the number of subjects carrying each SNP, and corresponding percentage within parenthesis.

STUDY II

Interethnic comparisons between Swedes and Koreans demonstrated that Swedish subjects had a higher metabolic ratio of losartan, irrespective of genotype. Women genotyped as *CYP2C9*1/*1* had similar metabolic ratios in the two populations, while Caucasian men of

the same genotype had more than 1.5 times higher metabolic ratio of losartan than Korean men ($p < 0.05$). No genetic explanation within the *CYP2C9* gene could be identified to be responsible for this discrepancy. The involvement of other enzymes, such as CYP3A4 and UDP glucuronosyltransferase, could also be dismissed. In addition to the ethnic differences between men, a gender difference was evident in Korean men and women.

As for the Caucasian population, there was a statistically significant difference in the metabolic ratio of losartan between *CYP2C9*1/*1* and *CYP2C9*1/*3* subjects in Asians. No homozygous *CYP2C9*3* carriers or any individuals carrying *CYP2C9*2* alleles were identified. The variation in metabolic ratio among individuals genotyped as *CYP2C9*1/*1* was wide (10-fold), but not as large as among the Swedish subjects.

STUDY III

From experiments with allelic variants of *CYP2C9* expressed in yeast, it could be shown that the intrinsic clearance of celecoxib in the CYP2C9.3 variant was more than 3 times lower than for the CYP2C9.1 variant. No differences between CYP2C9.1 and CYP2C9.2 samples were apparent. In human liver microsomes of different *CYP2C9* genotypes, the *CYP2C9*1/*1* samples (n=14) had a more than 5-fold higher formation rate of OH-celecoxib than the single *CYP2C9*3/*3* sample. The heterozygous *CYP2C9*3* microsomes (n=8) exhibited a formation rate of OH-celecoxib that was 2.2. times lower, than samples genotyped as *CYP2C9*1/*1*, while samples carrying *CYP2C9*2* alleles were similar to the *CYP2C9*1/*1* samples. Inhibition experiments with sulphaphenazole and triacetyloleandomycin, indicated CYP2C9 to be the major catalyst of celecoxib to OH-celecoxib. Furthermore, it could be proved for the first time that alcohol dehydrogenases (ADH), especially ADH1 and ADH2, are involved in the oxidation of OH-celecoxib. ADH3 showed no activity.

STUDY IV

After a single-dose of 200 mg celecoxib, the $AUC_{0\text{-}24h}$ of celecoxib was more than 3 times higher in individuals genotyped as *CYP2C9*3/*3* compared to those genotyped as *CYP2C9*1/*1* ($p < 0.05$), but there were no significant differences in the maximum plasma concentrations of celecoxib (C_{max}), calculated as median values, between the three genotype groups of *CYP2C9*. At steady-state conditions, the peak concentration of celecoxib in homozygous *CYP2C9*3* carriers was almost 4 times higher than in subjects belonging to the two other genotype groups, and celecoxib exposure was 7-fold higher. Subjects belonging to the *CYP2C9*1/*1* and *CYP2C9*1/*3* groups were almost identical, both at single-dose and steady-state conditions. Compared to single-dose data, the clearance of celecoxib seemed to be slightly decreased after repeated administration in subjects genotyped as *CYP2C9*3/*3.*

STUDY V

Among microsomes from 25 different human livers of various *CYP2C8* and *CYP2C9* genotypes, it could be concluded that samples homozygous for the *CYP2C8*3* and *CYP2C9*2* alleles (n=4) had a 34 % decreased synthesis of EET and DHET metabolites compared to *CYP2C9*1/*1/CYP2C8*1/*1* (n=17) samples ($p < 0.05$). No significant differences were observed between other *CYP2C8/CYP2C9* haplotypes. Both sulphaphenazole (specific CYP2C9 inhibitor) and quercetine (non-specific CYP2C8 inhibitor), significantly decreased the metabolite synthesis. In addition, experiments with inhibitory monoclonal antibodies and recombinantly expressed enzymes, proved both CYP2C8 and CYP2C9 to be about equally important for the synthesis of vasoactive metabolites from arachidonic acid, *in vitro*.

DISCUSSION

INTERINDIVIDUAL DIFFERENCES, LOSARTAN

Different probe drugs for CYP2C9 phenotyping have been suggested. A recent study showed that the clearances for both tolbutamide and flurbiprofen were better correlated to the *CYP2C9* genotype than losartan (Yasar Ü et al., 2000; Lee CR et al., 2003b). In Study I, *CYP2C9* genotypes were only able to explain about 30 % of the variability in subjects genotyped as *CYP2C9*1/*1*. This made us believe that losartan would be able to distinguish between yet unknown genotypes within the *CYP2C9*1/*1* group. By selecting metabolic outliers, i.e. individuals having high MR of losartan and not carrying any *CYP2C9*3* alleles, and perform sequencing analyses of the *CYP2C9* gene, we hoped to identify new polymorphisms of importance to the enzyme activity. Although quite a few new polymorphisms were detected, especially within the *5´*-flanking region, none of them seemed to be of any importance for the enzyme activity or the metabolic variation. Most of the base-pair exchanges were linked to either the *CYP2C9*2* or the *CYP2C9*3* allele, and none of them were located at any binding site for transcription factors. Instead, some of them now constitute predicted haplotypes (www.imm.ki.se/cypalleles). About 3.5 kb of the *5´*-flanking region was characterised, which is only a short part of the entire regulatory region. Although more recent studies have sequenced about 10 kb of the same region only finding rare mutations, there is still a large part that remains to be examined, and there might be not yet identified mutations located at sites, which could be of importance for gene transcription (Veenstra DL et al., 2005). If finding such mutations and consider them to be relevant for the phenotype, they have to be relatively frequent. They should preferably also be evaluated in functional studies. Quite a few new mutations have recently been found, but all of them seem to be present at low frequency, irrespective of the ethnic origin of the subjects (Takahashi H et al., 2003; Zhao F et al., 2004; Allabi AC et al., 2004; Blaisdell J et al., 2004). Not only variations within the *CYP2C9* gene itself, but also variations in other genes coding for drug receptors and transcription factors are important to consider.

Since previous studies have indicated CYP3A4 to be partly involved in the metabolism of losartan (Stearns RA et al., 1995), the influence of this enzyme on the overall metabolism was studied, by correlating quinine data with losartan data from the same subjects. No correlation with the CYP3A4-dependent metabolism of quinine could be observed, indicating no substantial influence of this enzyme on the metabolism of losartan.

INTERACTION, LOSARTAN

Although no novel polymorphisms of importance to the CYP2C9 activity were identified, it was discovered that oral contraceptives had an inhibitory effect on the metabolism of losartan. Both *in vitro* and *in vivo* studies have shown that ethinylestradiol as well as other steroid hor-

mones definitely have the potential to interact with concomitant drugs, e.g. omeprazole (Laine K et al., 2000; Laine K et al., 2003; Palovaara S et al., 2003; Reimers A, 2004). Also phenytoin has been shown to reach higher concentrations in users of oral contraceptives, irrespective of other potential interfering factors (De Leacy EA et al., 1979). In addition, the *S*-mephenytoin activity was reduced by 60 % in women taking oral contraceptives, compared to women not taking oral contraceptives (Hagg S et al., 2001). In many clinical trials the use of oral contraceptives has not been an issue for exclusion, and this may be important to consider when evaluating the results. None of the steroid hormones are known to be substrates of CYP2C9, but most of the substances contained in oral contraceptives are metabolised by CYP1A2 and CYP3A4, of which the latter has regulatory mechanisms in common with CYP2C9. An interference is therefore not unlikely (Harris RZ et al., 1995). In study II, only one Korean woman stated the use of oral contraceptives, and a comparison among users and non-users in Korean subjects could therefore not be performed. A comparable interaction would however be expected. In conclusion, women taking oral contraceptives have a similar decrease in activity as heterozygous *CYP2C9*3* subjects. They might need increased doses of losartan in order to achieve sufficient therapeutic effect. Although the mechanism of interaction is not always clear, it is important that the influence of oral contraceptives on drug elimination is not dismissed.

GENDER DIFFERENCES, LOSARTAN

Results from Study I indicated a gender difference, although the analysis did not reach statistical significance. When another 25 subjects were included there was a statistically significant difference ($p < 0.05$), for women not taking oral contraceptives having a 40 % lower metabolic ratio of losartan than men. In the Korean population men had somewhat faster losartan elimination than women ($p=0.08$). Gender differences with regard to pharmacokinetics of drugs have been noted before, but are usually attributable to gender-related physiological factors such as weight, muscle mass, total body water, etc. (Overholser BR et al., 2004). There is no evidence of sex related differences for CYP2C9 substrates such as warfarin, phenytoin or tolbutamide (Anderson GD, 2005). A few studies have indicated that drugs, which are substrates of CYP3A4, have a faster elimination in women than in men, and there seems to be a significant sex related difference in the extent of CYP3A4-induction by rifampicin (Wolbold R et al., 2003). Since CYP2C9 is also induced by rifampicin, a similar response could not be excluded. It has been speculated that CAR, which is involved in the regulation of CYP2C9 expression, might be an essential factor contributing to sex-related differences, but there are contradictory results regarding CAR expression between male and females (Lamba V et al., 2002; Wei P et al., 2003; Wolbold R et al., 2003). With regard to inhibition though, there are no gender differences reported for any of the CYP enzymes in humans (Chen ML et al., 2000; Gorski JC et al., 2003; Pascussi JM et al., 2003). Overall, the clinical significance of the gender differences seems to be minor, and probably no dose-adjustments for losartan are needed, based on gender.

INTERETHNIC DIFFERENCES, LOSARTAN

In Study II, we revealed differences in the metabolism of losartan between Swedish and Korean populations, with Swedish men showing a lower CYP2C9-dependent losartan oxidation than Korean men, while Swedish and Korean females had similar metabolic activity. There was no obvious reason to the differences between males in the two populations, and metabolic outliers of Swedish origin, i.e. subjects carrying no *CYP2C9*3* alleles, had identical DNA sequences of the *CYP2C9* gene as Korean subjects, genotyped as *CYP2C9*1/*1*, with low, intermediate and high metabolic ratio of losartan. Differences in metabolic capacity between populations have been recognised for many drugs, mostly due to different frequencies of variant alleles. In our case however, the discrepancies were present among subjects with the same *CYP2C9* genotype and the same gender. For phenytoin, another CYP2C9 substrate, there was a higher K_m in Caucasians than in Orientals (Edeki TI and Brase DA, 1995). In another study, Chinese patients required lower warfarin doses than Caucasian patients, despite a much lower frequency of the *CYP2C9*3* allele in Asian populations, but here age seemed to be the most important underlying factor determining the actual dose (Yu HC et al., 1996). In our study, the mean age of the two populations was similar, and did not affect the results.

It was recently discovered that the lower urinary testosterone glucuronide levels in Asian men compared to Caucasian men were associated with a deletion of the *UGT2B17* (UDP glucuronosyltransferase) gene, responsible for the glucuronidation of testosterone (Jakobsson J et al, in manuscript). We therefore treated selected urine samples, displaying different losartan activities, with β-glucuronidase. If there was any conjugated losartan present, β-glucuronidase would have hydrolysed the conjugated compound and the concentration of losartan (non-conjugated) would have increased. The losartan metabolic ratios remained the same in all samples, both those with the gene deletion and those having an intact gene. Furthermore, no correlation between testosterone levels and metabolic ratio of losartan could be established, and altered glucuronidation could be dismissed as a factor influencing the interethnic variation.

The difference in mean metabolic ratio between men of the two populations was less than the difference observed between subjects genotyped as *CYP2C9*1/*1* and *CYP2C9*1/*3*. The example by Kalow and Bertilsson (Figure 7) must be remembered, though. As a consequence, Swedish men are more likely to experience inadequate blood pressure lowering effects compared to Korean men, receiving standard losartan doses. In contrast, more Asian men will experience pronounced hypotensive effects than Swedish men (Kalow W and Bertilsson L, 1994). The opposite would be true if losartan had been more potent than its major metabolite (Munafo A et al., 1992). Differences in blood pressure between genotype groups have been observed in normotensive Japanese individuals, and subjects genotyped as *CYP2C9*1/*1* had a greater hypotensive effect than individuals in the *CYP2C9*1/*3* group taking losartan

(Sekino K et al., 2003). These results could not be confirmed in another study in healthy individuals of Caucasian origin (Lee CR et al., 2003c).

Despite efforts, described elsewhere as well as in this thesis, trying to find explanations to the observed differences in CYP2C9 catalytic effect between gender and ethnic populations, as well as between individuals of the same genotype, no reasonable answer can yet be offered, and there are still areas, which remain to be explored. Environmental factors like diet, physicological status, and/or yet unidentified mutations further upstream in the *5´*-flanking region might have influenced the result, and remains to be studied. Transcription factors, such as CAR, are also of potential interest.

CELECOXIB *IN VITRO*

As previously reported, we could confirm that celecoxib was mainly metabolised by CYP2C9 and that human liver microsomes genotyped as *CYP2C9*1/*3* or *CYP2C9*3/*3* had clearly reduced capacity of forming OH-celecoxib (Study III). The influence of *CYP2C9*2* alleles was negligible, in contrast to results by Tang *et al*. who showed an intermediate activity in two heterozygous *CYP2C9*2* samples (Tang C et al., 2001). Experiments with recombinantly expressed enzymes supported our results from human liver microsomes, but they were not appropriate to compare with data from Tang *et al.*, since different expression systems were used. In addition to CYP2C9, CYP3A4 was shown to be involved in the metabolism of celecoxib, although to a limited extent.

For the first time we could show that alcohol dehydrogenase (ADH1 and ADH2) catalysed the further oxidation of OH-celecoxib. Both ADH1 and ADH2 are polymorphic, why this conversion might occur at different rates in samples of different *ADH* genotypes. Since OH-celecoxib has no pharmacologic effect, this metabolic transformation is not considered to be clinically significant. ADH2 was shown to be more active than ADH1, but their relative importance *in vivo* is difficult to evaluate since ADH2 is expressed at lower levels than ADH1 in the liver (Edenberg HJ et al., 1997).

CELECOXIB *IN VIVO*

After a single dose of celecoxib, all pharmacokinetic parameters pointed toward a slower metabolism of celecoxib in subjects genotyped as *CYP2C9*3/*3,* while heterozygous *CYP2C9*3* individuals were almost identical to *CYP2C9*1/*1* subjects. The sampling was only performed for 24 hours, and complete elimination in all subjects had not been obtained during this time period. The clearance estimations might therefore not be optimal, but the values for the *CYP2C9*1/*1* subjects were similar to those obtained by Kirchheiner *et al.* (Kirchheiner J et al., 2003). After one week of celecoxib administration the differences were even more pronounced between *CYP2C9*1/*1* and *CYP2C9*3/*3* subjects, and the AUC was 7-fold larger in individuals genotyped as *CYP2C9*3/*3* compared to *CYP2C9*1/*1* individuals. There are

indications of drug accumulation in homozygous *CYP2C9*3* carriers, as seen from the apparent reduction in clearance rates after repeated administration compared to results from single-dose studies (Kirchheiner J et al., 2003). This would mean that saturation kinetics may occur after long-term treatment in individuals with two deficient *CYP2C9* alleles.

Heterozygous carriers of the *CYP2C9*3* allele were still comparable to the *CYP2C9*1/*1* subjects at steady-state conditions. When considering the metabolic ratio of celecoxib however, i.e. $AUC_{celecoxib}/AUC_{OH\text{-}celecoxib+COOH\text{-}celecoxib}$, it was about 40 % higher in individuals genotyped as *CYP2C9*1/*3* compared to those genotyped as *CYP2C9*1/*1,* at steady-state. This indicates a reduced CYP2C9 metabolic transformation also in heterozygous *CYP2C9*3* samples, which is not obvious when only evaluating the primary pharmacokinetic parameters of celecoxib. Since the metabolites of celecoxib do not posses any pharmacological activity, their impaired formation rate is not of clinical importance. If the metabolites had been active though, the sum of the substrate and metabolite, i.e. the total active moiety, would have been of interest. There are examples of drugs where the total active moiety remains constant in individuals of different genotypes, but there are also substrates where the total amount changes. Thus, for the latter group, the genotype is important to consider prior to drug administration (Brynne N et al., 1998; Lindh JD et al., 2003). The total exposure of the metabolite is also dependent on the further elimination, and to what extent polymorphic enzymes are involved in this step.

A greater difference between heterozygous *CYP2C9*3* subjects and *CYP2C9*1/*1* subjects would have been anticipated from Study III. Since the clearance in subjects with two deficient alleles was so much lower, a single functional allele would not be expected to be sufficient to balance out one *CYP2C9*3* allele. Furthermore, a larger influence of CYP3A4 would have been expected from earlier *in vitro* studies, but also from the fact that CYP3A4 is a high-capacity, low-affinity enzyme. This demonstrates the difficulty of extrapolating *in vitro* results to the *in vivo* situation, the body being a much more complex system. Therefore, it is always necessary to complement *in vitro* experiments with clinical studies.

In summary, we could conclude that the rare individuals genotyped as *CYP2C9*3/*3* will experience dramatically increased and prolonged celecoxib exposure, and thus be at higher risk for experiencing adverse effects.

ARACHIDONIC ACID

The endogenous epoxyeicosatrienoic acids (EETs) derived from arachidonic are known for inducing hyperpolarisation and relaxation of vascular smooth muscle, and to cause angiogenesis and endothelial cell proliferation (Fleming I, 2001; Michaelis UR et al., 2004; Michaelis UR et al., 2003). We were able to show that both CYP2C9 and CYP2C8 are involved in

the biosynthesis of these compounds, and that human liver microsomes genotyped as *CYP2C8*3/*3*/*CYP2C9*2/*2* had lower formation rate of EETs (Study IV). Hence, variant alleles of *CYP2C8* and *CYP2C9* are supposed to play an essential role in the development of cardiovascular events. To really confirm this result, experiments using vascular endothelial tissue should be used, since the local expression of various enzymes may differ. As pointed out above, there are contradictory results about the association between *CYP2C* polymorphisms and the frequency of myocardial infarction and hypertension (Funk M et al., 2004; Yasar U et al., 2003; Yu BN et al., 2004), and the linkage between disease and gene mutations needs to be further studied.

It was discovered relatively recently that an allelic variant in the *5´*-flanking region of the *CYP2J2* gene was significantly more frequent in Caucasian subjects with coronary artery disease than in healthy control subjects, 17.3 and 10.6 %, respectively. This variant was also associated with a decreased 14,15-DHET concentration in plasma, and functional studies revealed that constructs containing this mutation had a 48 % decreased enzyme activity (King LM et al., 2002; Spiecker M et al., 2004; Spiecker M et al., 2005). Our samples were not analysed for this mutation, but this may be carried out retrospectively, provided the enzyme is expressed in human liver.

INFLAMMATION, COX AND ARACHCIDONIC ACID

Already early findings indicated that individuals taking high doses of rofecoxib had an excessive risk of cardiovascular events, but these findings were dismissed (Bombardier C et al., 2000; Ray WA et al., 2002a; Drazen JM, 2005), despite results showing prostacyclin receptor knock-out mice to be more susceptible to thrombotic stimuli than wild-type mice (Cheng Y et al., 2002). Due to increased risk of experiencing myocardial infarction, rofecoxib as well as valdecxoib were withdrawn from the market, and restrictions were introduced for prescribing celecoxib (Bresalier RS et al., 2005; Nussmeier NA et al., 2005; Solomon SD et al., 2005). There are now also concerns if non-selective NSAIDs would elevate the risk of cardiovascular events, and the hypothesis that naproxen would be cardioprotective has been challenged (Ray WA et al., 2002b; Couzin J, 2005; Hippisley-Cox J and Coupland C, 2005; Topol EJ, 2005). In addition, the risk of developing gastro-intestinal side-effects have not been shown to be substantially lower for coxibs, and their costs are much higher compared to conventional NSAIDs. Hence, their use has been questioned.

The increase in cardiovascular side-effects in patients treated with COX-2 inhibitors is not surprising in view of their action. A selective inhibition of the COX-2 enzyme will create an imbalance in the vascular homeostasis, favouring platelet aggregation and vasoconstriction, because of an excessive production of thromboxane A_2 (prothrombotic) in relation to prostacyclin (antithrombotic) (Vane JR, 2002; Psaty BM et al., 2005), and higher doses are more

likely to cause a more pronounced imbalance. Other explanations to these results have also been suggested, and one epidemiological study indicates that patients receiving COX-2 inhibitors have an elevated underlying disease burden, with a higher prevalence of diabetes mellitus and cardiovascular disease compared to patients receiving conventional NSAIDs (Rawson NS et al., 2005). In other words, there might be a chance for so called confounding by indication. The results from recent meta-analyses on the risks of developing adverse effects during anti-inflammatory treatment might also be misleading since there seems to be publication bias. Even in large, prospective, randomised clinical trials, predisposing factors might sometimes also be difficult to detect (Hippisley-Cox J and Coupland C, 2005; Hudson M et al., 2005; Juni P et al., 2005).

The regulation of blood pressure and vascular homeostasis is complex, and both CYP2C9 and 11,12-EET seem to be able to induce the COX-2 expression in endothelial cells. COX-2 expression is also elevated in endothelial cells exposed to shear or stress, a situation similar to atherosclerosis (Michaelis UR et al., 2005). In the kidney, COX-2 is involved in the renal homeostasis, thereby indirectly controlling the blood pressure (Catella-Lawson F et al., 1999; Rossat J et al., 1999; Brater DC et al., 2001; Zhou Y et al., 2005). Furthermore, CYP2C9 seems to be involved in the synthesis of reactive oxygen species (ROS), which causes cardiac damage. Proinflammatory cytokines, such as TNF-α and IL-1β, upregulate the release of arachidonic acid (Fichtlscherer S et al., 2003; Funk M et al., 2004), and at the same time, these inflammatory mediators have been shown to down-regulate both cytochrome P450 expression and activity (Fleming I et al., 2001; Frye RF et al., 2002; Miyoshi M et al., 2005; Richardson TA et al., 2005).

As stated above, it is a complicated network of different proteins interacting with each other, and it is difficult to predict which of these factors that will determine the interindividual variation and be of greatest importance for the pharmacological effect when treating patients with e.g. losartan or celecoxib. Not only the drug treatment, but also the disease itself, as well as local variations of enzyme expression, and allelic variants of drug receptors and metabolising enzymes are probably important for the final pharmacokinetic and pharmacodynamic outcome (Enayetallah AE et al., 2004; Zhou Y et al., 2005). In addition, drug interactions and the metabolism of endogenous substances, such as arachidonic acid, are also of importance.

PERSPECTIVES AND FUTURE STUDIES

There is an ongoing debate whether or not genotyping should be performed prior to initiation of certain drugs with a narrow therapeutic range, but results presented so far do not show any overall convincing advantages. With the help of new techniques there might be a potential to further develop the area of individual drug therapy. For example, transcriptomics and proteomics are approaches, which explore the gene transcripts and the proteins encoded by the genome, trying to predict the prognosis of certain diseases and the response to specific therapies. Metabonomics is another research area that can determine the metabolic profile from urine samples with the help of sensitive analytical techniques, such as different kind of MS and NMR techniques (Lindon JC et al., 2004; Robertson DG, 2005). Due to natural variation, and the enormous amount of data generated, the results are however difficult to interpret, and there is still a long way before these methods can be used in clinical practice. Although many new techniques are available and the knowledge within pharmacogenetics/pharmacogenomics has evolved during the last years, the information is not always used appropriately, since the clinical significance may be uncertain and many of the biomarkers have not been validated. The FDA has therefore recently launched guidelines on the submission of pharmacogenomic data, and there is certainly a need of evaluating the influence of a certain genotype on the drug response as early as possible (www.fda.gov). This will hopefully improve the drug development process and could already from the beginning result in restrictions for prescribing drugs to individuals with certain genotypes, avoiding a market withdrawal later on. Thus, if the patient´s genotype was known beforehand, it would help individualise drug therapy (personalised medicine), at least for certain drugs. Furthermore, individuals carrying defective alleles of drug metabolising enzymes could be excluded from treatment with drugs, which have severe dose/concentration-dependent side effects, like celecoxib.

For warfarin, the question whether genotyping should be introduced in the clinical management remains to be answered, due to contradictory results (Sanderson S et al., 2005). According to a study performed by Scordo *et al.*, the positive predictive value (PPV), i.e. the likelihood that a patient genotyped as a slow CYP2C9 metaboliser actually needs a low warfarin dose, is 88 %. The negative predictive value (NPV), i.e. the likelihood that a patient not genotyped as a slow CYP2C9 metaboliser needs a normal or high warfarin dose, was 65 % in the same study (Scordo MG et al., 2002). The corresponding values in another study were 24 and 76 % (Peyvandi F et al., 2004). Calculations made on assumptions that the bleeding event rate is 8 %, per year and that patients with a variant *CYP2C9* allele have a 2.34 times higher risk of bleedings compared to patients with no variant alleles, arrived at a number of 44 persons who need to be genotyped for CYP2C9 in order to prevent one single bleeding (Higashi MK et al., 2003). The costs for genotyping these individuals must be considered and related to the health care costs for the patients experiencing bleeding complications. Thus, for warfarin, genotyping of patients on already stable warfarin doses seems to be unnecessary, since it would not provide any additional benefits compared to routine monitoring of INR. On the

other hand, it has been clearly shown that patients with *CYP2C9*3* alleles generally require a lower warfarin dose, and these rare individuals might benefit from knowing their genotype prior to drug initiation. Results from the Swedish WARG-study will further contribute to the knowledge about genetic risk factors in anticoagulant drug treatment (Lindh JD et al., 2004).

There are no published trials evaluating the influence of CYP2C9 genotypes on blood pressure lowering effects of losartan in hypertensive patients, why it is difficult to give any advices on losartan dosing. In addition, polymorphisms within the angiotensin II receptor gene, which is the target for losartan, will probably also be of importance (Spiering W et al., 2005). The LIFE-study is a double-blind, randomised, prospective trial, comparing the effects of losartan with atenolol, on cardiovascular morbidity and mortality in approximately 9000 patients with essential hypertension or left ventricular hypertrophy (Okin PM et al., 2000). A subanalysis of the LIFE-study, where the *CYP2C9* genotype has been determined by us for about 4000 of the participants, will hopefully help to conclude if there is a relationship between *CYP2C9* genotype and blood pressure, losartan dose, cardiovascular death, myocardial infarction, stroke, or other adverse events, and whether or not genotyping of *CYP2C9* is of clinical interest before initiating losartan. The results might also be helpful for further interpretation of Study I and Study II.

Another area of interest will be to study the influence of cytokines on CYP activity in general, and CYP2C9 activity specifically. In an ongoing collaboration between our group and the Rheumatology Division at Karolinska University Hospital Huddinge, the metabolic clearance of ibuprofen will be measured before, and three months after, starting treatment with etanercept (a TNF-α inhibitor), in order to determine if the metabolic rate has been changed. If inflammation in itself, and especially TNF-α, has any impact on the CYP2C9 activity, dose adjustments for the NSAIDs, commonly used by these patients, may be warranted.

Despite controversies regarding the predictive value of genotyping, there are examples showing real benefits of genotyping patients before initiating drug treatment. Genotyping or phenotyping of TPMT is routine praxis before starting treatment with mercaptopurine and azathioprine, and this has had a substantial impact on preventing drug toxicities and therapeutic failure (Evans WE et al., 2001). There is also accumulating evidence that ultrapid metabolisers of CYP2D6 are resistant to treatment with antidepressants, and these subjects may thus benefit from being genotyped prior to drug initiation (Kawanishi C et al., 2002, Kawanishi C et al., 2004).

In conclusion, there still remains a lot of research to be done within the field of pharmacogenetics/pharmacogenomics of CYP2C9 in order to tailor the drug treatment of each patient. Genotyping of drug metabolising enzymes is definitely an important tool for assessing the

right dose of certain drugs. Together with the constant development of new techniques and the knowledge about diseases, as well as the influence of drugs and endogenous substances, individualised drug therapy will hopefully increase in the future.

CONCLUSIONS

- No genetic explanation for the large interindividual variation in the metabolism of CYP2C9 substrates in subjects genotyped as *CYP2C9*1/*1*, could be identified.
- Oral contraceptives have an inhibitory effect on losartan metabolism, and the influence of oral contraceptives on drug metabolism should be recognised. Women using oral contraceptives will exhibit a decreased elimination of losartan, which is comparable to subjects genotyped as *CYP2C9*1/*3.*
- Swedish men have a 50 % higher metabolic ratio of losartan compared to Korean men, while Swedish and Korean females are identical with regard to losartan metabolism.
- The clearance of losartan appears to be gender-dependent, although the underlying mechanism of this is unknown.
- No general dose adjustments for losartan can be recommended with regard to ethnicity or gender since the differences are rather moderate.
- The metabolism of celecoxib *in vitro* is significantly lower in human liver microsomes and in yeast microsomes, carrying *CYP2C9*3* alleles, while there is no effect of the *CYP2C9*2* allele.
- Subjects genotyped as *CYP2C9*3/*3* have a 7-fold higher exposure of celecoxib after repeated administrations (200 mg daily) than individuals genotyped as *CYP2C9*1/*1*, while individuals genotyped as *CYP2C9*1/*3* have a similar exposure as *CYP2C9*1/*1* subjects.
- Subjects homozygous for the *CYP2C9*3* allele are likely to have a higher risk of experiencing cardiovascular side-effects after long-term treatment with celecoxib.
- CYP2C9 and CYP2C8 are involved in the synthesis of vasoactive EET-metabolites from arachidonic acid, and human liver microsomes homozygous for the *CYP2C8*3* and the *CYP2C9*2* alleles exhibit a significantly reduced EET synthesis by 34 %.
- Variant alleles of *CYP2C8* and *CYP2C9* might be involved in the pathophysiology of cardiovascular diseases.

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