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CHARACTERISTICS OF CYTOCHROME P450-CATALYSED DRUG METABOLISM WITH FOCUS ON A BLACK TANZANIAN POPULATION

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To Gunnar and Alice

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ABSTRACT

The cytochrome P450 (CYP) enzymes display interindividual and interethnic variability that may alter the disposition of substances metabolised by these enzymes. Both genetic and environmental factors such as concomitant drug use and concurrent diseases influence the activities of these enzymes. The overall aim of this thesis was to study variability in drug metabolism catalysed by cytochrome P450 enzymes in a Tanzanian population and to assess the consequences of such variability.

In a phenotyping study in Tanzanians, we detected lower metabolic activity of CYP2D6 in Tanzanians compared to Caucasians. The low capacity could not be explained by the partially or fully detrimental *CYP2D6* gene mutations analysed for. Moreover, we found that none of the poor metabolisers of debrisoquine was homozygous for defective *CYP2D6* genes.

A novel *CYP2D6* allele (*CYP2D6*29*) was found at a high allele frequency in the Tanzanian population. The presence of this allele significantly caused a diminished rate of debrisoquine metabolism *in vivo*. Using the CYP2D6 substrate bufuralol, it was shown that CYP2D6.29 had only 26% of the catalytic activity of the wild-type enzyme (CYP2D6.1) in a mammalian cell expression system and also lower activity (63% of CYP2D6.1) when debrisoquine was used as substrate.

We studied the effect of the African-specific *CYP2D6*17* and **29* alleles in healthy Tanzanian subjects on the metabolism of four CYP2D6 probe drugs (codeine, debrisoquine, dextromethorphan, metoprolol). Carriers of *CYP2D6*17* had significantly decreased rate of metabolism for two (debrisoquine and dextromethorphan) of the four probe drugs and *CYP2D6*29* caused a reduced metabolism compared with *CYP2D6*1* and **2* for dextromethorphan and metoprolol but not for codeine and debrisoquine.

The effect of the antimalarial drug amodiaquine on the activities of four P450 enzymes and the recovery of any such effect was studied in healthy Swedish subjects. We found that amodiaquine, its desethylated metabolite, or both inhibited the CYP2D6 (debrisoquine 4-hydroxylation) and CYP2C9 (losartan oxidation) enzymes. This effect was selective affecting neither the CYP2C19 (omeprazole 5-hydroxylation) nor CYP1A2 (caffeine N3-demethylation) enzymes. The inhibitory effect did not persist one week after drug intake.

We validated a novel liquid chromatography-mass spectrometry method, which enabled us to quantify alprazolam, 4-hydroxyalprazolam and α-hydroxyalprazolam in all healthy subjects up to at least 24 hours after drug intake. The plasma concentration ratios (alprazolam/respective metabolite) of samples collected between 1 and 48 hours correlated significantly with the ratios of area under curve values of alprazolam/4-hydroxyalprazolam and alprazolam/α-hydroxyalprazolam. Hence, we suggest that it is possible to estimate alprazolam 4- and α-hydroxylation activities in subjects with a single plasma sample.

In summary, two African-specific *CYP2D6* alleles encode enzymes that exhibit altered substrate specificity compared to the functional enzyme both *in vivo* and *in vitro*. This may preclude extrapolation of results from one specific CYP2D6-drug to another. The antimalarial drug amodiaquine inhibited the metabolism of the CYP2D6 and CYP2C9

enzymes *in vivo*. The 4- and α-hydroxylations of alprazolam can be assessed using a single plasma sample, which makes the method well suited for studies of CYP3A activity in larger populations.

KEY WORDS: Drug metabolism, cytochrome P450, polymorphism, pharmacogenetics, genotyping, phenotyping, CYP2D6, CYP3A, debrisoquine, amodiaquine, alprazolam

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AQ	Amodiaquine
AUC	Area under the plasma concentration versus time curve
Bp	Basepair
CL	Oral clearance
CL_{int}	Intrinsic clearance
C_{max}	Maximum concentration attained in plasma
CPMP	Committee for Proprietary Medicinal Products
CV	Coefficient of variation
CYP	Cytochrome P450
DAQ	N-desethylamodiaquine
DME	Drug metabolising enzyme
DNA	Deoxyribonucleic acid
EM	Extensive metaboliser
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
ICH	International conference on harmonisation
IM	Intermediate metaboliser
Kb	Kilo base
K_m	Michaelis-Mentens constant
LC/MS	Liquid chromatography-mass spectrometry
MDR	Multi drug resistant gene
MR	Metabolic ratio
NADPH	Nicotinamide adenine dinucleotide
OH	Hydroxy
PCR	Polymerase chain reaction
PM	Poor metaboliser
RFLP	Restriction fragment length polymorphism
r_s	Spearman rank correlation coefficient
SNP	Single nucleotide polymorphism
TNFα	Tumour necrosis factor α
UM	Ultrarapid metabolisers
V_{max}	Maximum rate of formation
Å	Ångström (10^{-10} m)

INTRODUCTION

GENERAL INTRODUCTION

Interindividual differences in drug metabolism are explained by genetic and environmental factors such as physiological, pathophysiological, pharmacological and dietary factors. The body excretes lipophilic exogenous as well as endogenous compounds due to enzymes in the liver, converting the substances to more hydrophilic and excretable substances. Genetic polymorphism (allelic variants of the same gene) is common among drug metabolising enzymes (DMEs) and causes variability in drug metabolism between individuals. Allelic variants of genes encoding DMEs have been identified and correlated to plasma levels and adverse effects of certain drugs. Knowledge about the status of metabolic capacity in a population and dose adjustments of drugs to patients carrying certain genotypes will decrease the risk of therapeutic failure or adverse effects. Lately, the knowledge on genetic variability in drug effects has advanced due to the characterisation of the human genome (Lander et al. 2001; Venter et al. 2001).

Variability in drug effects has been investigated since the 1950s (cf. review by (Evans and McLeod 2003)). The first observations were during the Second World War when it was noticed that it was mostly black American soldiers who suffered from hemolysis when using the antimalarial drug primaquine. This could later be explained by glucose-6-phosphate dehydrogenase deficiency (Alving et al. 1956). When isoniazid was introduced as treatment for tuberculosis, it was observed that individuals either had slow or rapid acetylation of the drug (Hughes et al. 1954).

The overall aim of this thesis has been to increase the knowledge of drug metabolism in a black Tanzanian population. I studied the *CYP2D6* gene in the population and found that two African-specific *CYP2D6* alleles coded for enzymes with altered substrate specificity compared to the functional enzyme. Moreover, I studied the inhibitory profile of the antimalarial drug amodiaquine *in vivo* on four different cytochrome P450 enzymes. Finally, I validated a method to study CYP3A activity using a single plasma sample that may be a method suitable for population-based studies.

POPULATION CHARACTERISTICS

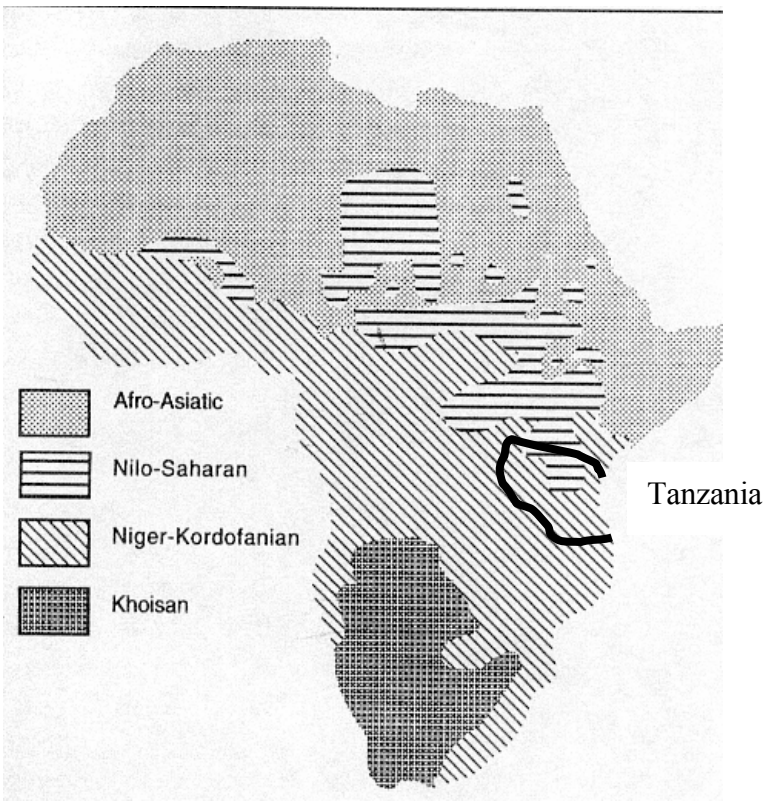
African populations

Africa is the second largest continent in the world after Asia with about 640 million people. The first human species, Homo habilis, originated in Africa about 2.5 million years ago. Later, about 100 000 to 200 000 years ago, it evolved into the modern human species Homo sapiens, which spread to other parts of the world (Cavalli-Sforza 1994; Tishkoff and Verrelli 2003). The dating of the occurrence of Homo sapiens is however controversial (Brooks et al. 1995; Tishkoff and Verrelli 2003).

Human populations can be classified by different approaches, such as by genetic, linguistic, and cultural characteristics. Populations have been classified by means of measuring genetic distances based on the counts of numerous gene frequencies (Cavalli-Sforza 1994). Significant correspondance between linguistic superfamilies and major genetic clusters have been reported (Chen et al. 1995). Linguistic classifications may be useful as an evolutionary tool but only when combined with genetic information. Africa is a continent that is genetically and linguistically diverse (Cavalli-Sforza 1994; Tishkoff and Verrelli 2003). African populations typically have higher levels of genetic diversity and a more complex population substructure compared to non-African populations (Tishkoff and Verrelli 2003). More than 1400 languages are spoken in Africa and these can be divided into four major families (Figure 1): the Afro-Asiatic, Nilo-Saharan, Khoisan and Niger-Kordofanian (Bantu) (Cavalli-Sforza 1994). Linguistic barriers strengthen genetic isolation (minimising genetic mix-up) between groups speaking different languages and hence the four major groups are believed to be genetically different.

Pharmacogenetic data are relatively scarce in Africans compared to European and Asian populations. The existing data on Africans is relatively inconsistent and based on small samples. Several studies in the field have been performed in patients or volunteers from the US, and hence in African-Americans. Noteworthy, is that most African-Americans originated from West Africa. Also, there has been a much higher degree of genetic mixing of the different populations in the US compared to Africa.

Figure 1. **Linguistic families in Africa (modified from Cavalli-Sforza, 1994).** Tanzania is schematically depicted in the figure.



Ethnicity is a multidimensional classification that may encompass shared origins, social background, culture and environment (Xie et al. 2001). The use of ethnic classification in medicine is debated as it risks to undervalue the great diversity within groups, however, it may be useful for generating and exploring hypotheses about environmental and genetic risk factors in complex diseases (Burchard et al. 2003; Phimister 2003)

Tanzanians

Bantu languages are part of the Niger-Kordofanian language family and comprise more than 500 local languages and are spoken by people living from Nigeria and Cameroon across to East Africa and down to South Africa (Cavalli-Sforza 1994). It is believed that about one fourth of all Africans belong to the Bantu linguistic group (Cavalli-Sforza 1994). Throughout history, genetic mixing has occurred within Africa. Besides, along the East African coastline extensive trading with Arabians occured from 900 A.D., which presumably resulted in genetic mixing. Tanzania is one of the East African countries with a long coastline along the Indian Ocean (Figure 1). About 35 million people are Tanzanian nationals and the Bantu people are the major ethnic group in the country.

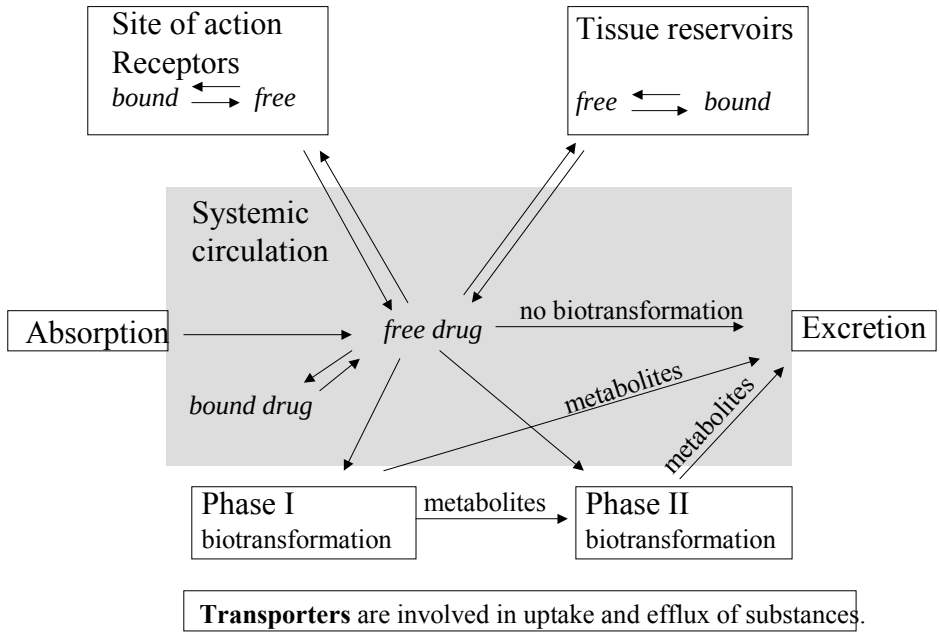
VARIABILITY IN DRUG EFFECTS

The response of a drug depends on multiple factors, whose effects can be synergistic or inhibitory. A substance (drug, endogenous compound, pollutant, dietary constituent) can modify the effect of a drug by influencing its pharmacokinetics (absorption, distribution, metabolism and/or excretion) or its pharmacodynamics (effects on e.g. receptors) or both. The variability of each of these factors can be due to genetic or environmental factors, or both. Existence of profound population differences with small intraindividual variability is consistent with inheritance as a determinant of drug response. Although many environmental factors may influence the effect of a drug, there are several examples in which interindividual differences in drug response are due to variants of genes encoding DMEs, drug transporters or drug targets (cf. review by (Evans and Relling 1999)). Most drug effects have polygenic determinants with interplay of several gene products on the pharmacokinetics and pharmacodynamics of drugs, including inherited differences in drug receptors and drug disposition (e.g. DMEs and transporters). The effect of polymorphisms (i.e. at least two allelic variants with a frequency of at least 1% of the most rare variant) in the DME will be most important when variability in the other pharmacokinetic and pharmacodynamic factors is small.

Pharmacokinetic aspects

The clinically most important interactions are those involving the absorption or metabolism of a drug. Exogenous compounds such as other drugs, or endogenous compounds, can interfere with the pharmacokinetic processes of a specific drug.

Figure 2. Relationship of absorption, distribution, binding, biotransformation and excretion of a drug and the consequences on concentration at the site of action. Adapted from Benet, 1996.



Absorption

Xenobiotics can enter the body via e.g. the gastrointestinal tract, lung or skin, where they may be absorbed (Figure 2). The gastrointestinal tract is the most important site of absorption. Several factors affect the gastrointestinal absorption of a drug, such as the physicochemical parameters of the drug, gastrointestinal motility and its concentration at the site of absorption.

Distribution

Distribution describes the reversible transfer of drug from one location to another within the body. The distribution of a drug is influenced by factors such as lipid-solubility, concentration in plasma and in various tissues and binding to plasma proteins, transport proteins and tissues.

Metabolising enzymes and transporters

The so-called first pass metabolism removes drugs when they pass the gastrointestinal membranes and the liver into the systemic circulation. Enzymes catalyse the conversion of lipophilic drugs and endogenous compounds to hydrophilic and thereby excretable metabolites.

Drug disposition can be classified into effects by **phase I** and II enzymes and that of transporters, where the phase I enzyme (e.g. cytochromes P450 (P450), monoaminoxidase and aldehyde dehydrogenases) inserts a functional group (e.g. -OH, -NH_2 or -SH) to the substance (Table 1), the **phase II** enzyme (e.g. UDP-glucuronsyl transferases, glutathione-S-transferases and N-acetyltransferases) is involved in the

conjugation of the intermediate and renders the substances highly water soluble and finally the **transporters** (Siest et al. 2003) are involved in the transport of the metabolite or substance (Figure 2). There is a diversity of reactions catalysed by the P450 enzymes (cf. review by (Mansuy 1998)).

The phase I reaction is generally a detoxifying process, but sometimes the P450-catalysed products can be reactive and cause cell damage by binding to DNA, proteins and membrane components (Nebert et al. 1999). Many of the phase I and II enzymes have been shown to be polymorphic. In some cases the substances may already possess functional groups and hence undergo phase II reactions without first undergoing phase I metabolism (cf. review by (Nebert 2000)).

Table 1. Major drug biotransformation reactions

	Type of reaction
Phase I, oxidation reactions	aliphatic oxidation aromatic oxidation N-dealkylation O-dealkylation N-oxidation S-oxidation deamination
Phase II, conjugation reactions	glucuronidation sulphation acetylation

Transporter proteins are expressed in various tissues with excretory and protective functions such as the intestines, liver and kidney but also in the blood brain barrier. The most thoroughly studied member of the *MDR* (multidrug resistence) gene family is the *MDR1* (*ABCB1*) gene, which encodes P-glycoprotein (P-gp) (Ueda et al. 1987). The *MDR* as well as the *MRP* gene family are ABC (ATP-binding cassette) transporters. P-gp has broad substrate specificity including protease inhibitors for HIV treatment, antiarrhythmics and immunosuppressant drugs. Other drugs are inhibitors such as cyclosporine, verapamil, progesteron and quinidine. In addition, it has been suggested that P-gp and CYP3A4 cooperate in the intestine to form an absorption barrier against xenobiotics (Wacher et al. 1995; Benet et al. 1996). It was suggested that P-gp influences the extent of drug metabolism in the intestine by prolonging the access of drugs to CYP3A4 near the apical membrane and decreasing transport across the cells (Cummins et al. 2002). Polymorphisms have been described for several transporters with 48 SNPs identified in the *MDR1* gene (Kroetz et al. 2003). The *MDR1* C3435T SNP (single nucleotide polymorphism), which cause no amino acid change, is common in most populations studied (Ameyaw et al. 2001). The 3435 C/C genotype has been associated with higher intestinal P-glycoprotein expression and is particularly common in black populations (52 to 83%) compared to white populations (22 to 28%) (Ameyaw et al. 2001; Schaeffeler et al. 2001). There are conflicting reports regarding the clinical

significance of variant *MDR1* alleles (Siegmund et al. 2002; Kroetz et al. 2003; Sakaeda et al. 2003).

MRP2 (ABCC2) is one of the enzymes in the MRP family, and is also implicated in the efflux from the hepatocytes with common drugs such indomethacin and penicillin as substrates (Pauli-Magnus and Meier 2003). There are also other transporter families involved in efflux of substances such as ABCGs and ATP8B, but they are less well studied. There are several classes of hepatocellular transporters involved in the uptake of exogenous and endogenous (e.g. bile constituents) substances into the hepatocyte, such as the OATPs, OATs, OCTs and MRPs transporter families (Pauli-Magnus and Meier 2003). Many common drugs are substrates of these transporters; however, their clinical impact remains to be elucidated.

Excretion

Most drugs leave the body in the urine, either unchanged or as more polar metabolites (Figure 2). Some drugs are secreted in bile via the liver, but may then be reabsorbed from the intestine. Substances with low lipid-solubility such as polar metabolites are excreted more efficiently. Drugs can inhibit the reabsorption or secretion of others by affecting specific transport mechanisms or by affecting urinary pH. No ethnic differences in renal excretion have been documented (Johnson 2000).

Pharmacodynamic aspects

In addition to variants of DMEs, pharmacogenetic variants have been documented in receptors and their activation pathways that can influence drug response (Evans and McLeod 2003). A number of variants of the β2-adrenoreceptor have been identified where the effect of β2-agonists is affected (Evans and McLeod 2003). Also, it was recently shown that improved prediction of response to β-blocker therapy in heart failure might be achieved on the basis of β-adrenergic receptor genotype (Kaye et al. 2003).

CHARACTERISTICS OF CYTOCHROME P450 ENZYMES

Nomenclature

The name P450 was originally a description of a red pigment found in liver microsomes with an absorbance maximum at 450 nm in its reduced carbon-monoxide-bound form (Omura and Sato, 1964). As the number of P450 genes identified and cloned increased, a system for classification of the genes was adopted (Daly et al. 1996; Nelson et al. 1996). It is based on protein sequence identity using a global alignment of the P450 sequences. The enzymes are denoted CYP (Cytochrome P450), followed by a designation of family (number), subfamily (letter) and individual enzyme (number) (http://www.imm.ki.se/CYPalleles). The corresponding gene name is given in *italics*. Enzymes with >40% identity in protein sequence belong to the same family, and enzymes with >55% identity belong to the same subfamily. The names of proteins have a period between the name of the gene product and number. The same nomenclature system covers plants and eukaryotic and prokaryotic species.

Cytochrome P450 genes can be polymorphic, i.e. exist in at least two allelic variants with a frequency of at least 1% of the most rare variant. Such variant alleles can contain one or several SNPs. To be assigned as a unique allele it should contain SNPs or larger genetic rearrangements that have been shown to cause at least one amino acid exchange or to affect transcription, splicing or translation. The wild-type allele was previously used to describe a fully functional allele that occurred at a high frequency in the studied population. Lately, the wild-type definition has been used for the first allele sequenced and refers to a consensus (reference) allele and is designated as **1* (or **1A* and **1B* in case of slightly variant sequences) (http://www.imm.ki.se/CYPalleles) (Ingelman-Sundberg et al. 2000). In many instances, the first genetic material sequenced has been from a Caucasian population, and therefore it has often been the most frequent allele among Caucasians that is named as wild-type.

Evolutionary perspective

The cytochrome P450 enzyme system consists of several different isoenzymes with as many as 2500 P450 genes, identified by the end of 2002. Additional increase in P450 gene number is expected as additional organisms are subjected to whole genome sequencing (Nelson 2003). Sequence comparisons indicate extensive similarity between P450s identified in man and bacteria, and suggest therefore that the P450 superfamily originated from a common ancestral gene about three billion years ago (Nebert and Gonzalez 1987). The P450 genes are found in the genomes of virtually all organisms. Their amino-acid sequences are diverse with levels of identity less than 20% in some cases but their structural fold and general topology seem to have remained the same throughout evolution (Hasemann et al. 1995; Graham and Peterson 1999). When eukaryotic cells evolved and the phylogenetic diversity of organisms developed, mechanisms emerged in order to protect animals and plants against toxic substances in food, pollutants and the like. One of the protective systems that emerged was the P450 system, which is exceptional in that it metabolises such a diversity of substrates and that it is so widespread among species. The P450 enzymes contribute to a series of vital processes such as biosynthesis of hormones, synthesis and degradation of prostaglandins and other unsaturated fatty acids, apart from degradation of xenobiotics (Hasler 1999).

Demographic evolutionary factors (i.e. drift and migration/gene flow) affect all loci similarly, in contrast to loci under selection pressure (i.e. dietary or environmental stress) in the event of shaping genetic variability. Frequency of alleles can be influenced by adaptive selection through dietary stress alternatively through random genetic drift (Tishkoff and Verrelli 2003). In two Amerindian populations where the effect of selection through diet on human CYP2D6 evolution was studied, no disagreements between genotypic and phenotypic data were detected (Jorge et al. 1999). The two tribes diverged 8000 to 10 000 years ago and have low degree of genetic admixture (Torroni et al. 1994). It was therefore concluded that human CYP2D6 among these Amerindians was preferentially affected by random genetic drift and not by adaptive selection. However, local aggregation of subjects with duplicated and multiduplicated *CYP2D6* genes was suggested to be the result of dietary pressure by plant alkaloids (Ingelman-Sundberg et al. 1999). Moreover, the role of population

history and natural selection in shaping genetic diversity in *CYP1A2* was studied (Wooding et al. 2002). It was shown that haplotypes found outside Africa were mostly a subset of those found within Africa and that African populations had the lowest level of linkage disequilibrium of the populations studied.

Cytochrome P450-mediated reactions

Figure 3. The equation for cytochrome P450-dependent reactions

$$NADPH + O_2 + SH + H^+ \quad ========> \quad NADP^+ + SOH + H_2O$$

S = substrate

Cytochrome P450s used to be called mixed function oxidases since many reactions lead to incorporation of an oxygen atom, but P450s can also catalyse reduction reactions (Table 1). The P450s use electrons from NADPH to catalyse activation of molecular oxygen, leading to regio- and stereospecific oxidation of a large number of substrates (Figure 3). In mammalians, P450s and NADPH-P450 reductases are anchored on the cytosolic side of the endoplasmic reticulum (cf. review by (Werck-Reichhart and Feyereisen 2000)). There are also P450s in the inner membrane of the mitochondrias that catalyse steroid synthesis and fatty acid metabolism. These P450s are well conserved between species and have high affinity for their substrates.

The P450s are heme-thiolate proteins. They have a conserved structural fold, but only a few completely conserved amino acids. The highest structural conservation is found in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. The most variable regions are associated with amino-terminal anchoring and substrate binding (Graham and Peterson 1999; Werck-Reichhart and Feyereisen 2000). The latter regions are located near the substrate-access channel and catalytic site and are often referred to as substrate-recognition sites (SRSs; Gotoh et al., 1992).

In mammalian cells, the P450s are membrane bound and have therefore proven difficult to crystallise. The bacterial P450s are non-membrane bound and are possible to solubilise and further crystallise, i.e. P450cam or CYP101 (Poulos et al. 1987). Since, six additional P450 structures (bacterial and fungal) have been described. A molecular model of CYP2D6 was constructed from the bacterial form CYP102 via homology alignment between the CYP2D subfamily and the CYP102 protein sequence (Modi et al. 1996; Lewis et al. 1997).

Later, crystallisation and structural determination of the first eukaryotic P450, CYP2C5 from rabbit, was described (Williams et al., 2000; Cosme et al., 2000). This structure has been important for structural characterisation of human P450s. Using the CYP2C5 coordinates, a homology model for CYP2C9 was suggested (Afzelius et al. 2001). Also, molecular modelling of CYP1B1, 2A6 and 2B6 has been pursued based on homology with the CYP2C5 crystal structure (Lewis et al. 2002; Lewis et al. 2003;

Lewis et al. 2003). Interestingly, the first 3D crystal structure of a human P450 protein was published in July 2003 (Williams et al., 2003). It showed how CYP2C9 interacts with the anti-coagulant drug warfarin and revealed a novel binding pocket. The same group has also announced that it has solved the structure of a second isoform, CYP3A4, although details remain confidential (http://www.astex-technology.co.uk). The crystal structures of human P450s will be of importance for future drug development, and the company behind the first P450 structures has already expressed its intent to crystallise several of the remaining human DMEs (http://www.astex-technology.co.uk).

CYTOCHROME P450-CATALYSED DRUG METABOLISM

Interindividual variability

The capacity to metabolise drugs shows both intra- and interindividual variability. The intraindividual variability is due to environmental factors, whereas the interindividual variability can be due to genetic factors, or environmental factors, or both. The impact from each factor depends on e.g. enzyme, probe drug, ethnic group, concomitant medication. The activity of the CYP3A enzymes has been shown to be influenced by both environmental and genetic factors. On the other hand, in a study comparing between- and within-person variances, it was suggested that the genetic contribution to the variability between subjects was 60 to 90% (Ozdemir et al. 2000). Moreover, using path analysis of nuclear families, it was suggested that genetic factors are of more importance (79%; (Steiner et al. 1985)) than environmental factors for the *in vivo* activity of CYP2D6 (Bock et al. 1994).

Influence of environmental factors is discussed in more detail later.

Genetic polymorphisms

At the gene level, polymorphisms have been shown for most of the important DMEs such as CYP1A2, 1B1, 2A6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 (http://www.imm.ki.se/CYPalleles) (Table 2). The study of the *effects* of the polymorphisms in expressed variant enzymes or *in vivo* is lagging behind; only an average of 49% of the variant P450 enzymes have been studied.

Among the P450 genes, it was the *CYP2D6* gene that was first found to be polymorphic (Mahgoub et al. 1977; Tucker et al. 1977; Eichelbaum et al. 1979). By 1996, 25 alleles had been described (Daly et al. 1996) but today, as many as 80 different alleles are described (http://www.imm.ki.se/CYPalleles). Genetic polymorphisms can result in no enzyme or an inactive enzyme being expressed due to frameshift or splice site mutations. Polymorphisms resulting in amino acid substitutions can also give rise to an unstable enzyme or an enzyme with an altered active site possibly causing a change in substrate specificity. Moreover, duplication or multiduplication of active genes can result in higher levels of mRNA and enzyme and therefore increased metabolic activity (Ingelman-Sundberg et al. 1999).

Table 2. Polymorphic cytochromes P450.

		Number of variants		
CYP	**Year[a]**	**Alleles**	**Studied for enzyme activity[b]**	**Causing altered activity (%)[c]**
1A2	1994	16	5	80
2C9	1994	12	5	80
2C19	1994	19	14	71
2D6	1990	79	43	86
3A4	1998	25	4	50
3A5	1996	23	15	93

Data from CYP homepage (http://www.imm.ki.se/CYPalleles).
[a]Year first polymorphism was identified at the gene level.
[b]*In vivo* or *in vitro*.
[c]Out of studied variants (*in vivo* or *in vitro*).

Human genomes contain approximately one SNP per 1250 bp of DNA sequence (Venter et al. 2001). This means that most genes have several polymorphic sites distributed throughout their coding and regulatory regions, suggesting that there are in fact no wild-type sequences in nature, but rather that all phenotypes are quantitative traits (Hartman et al. 2001). An individual's haplotype (combination of SNPs in and around the gene, as arranged on each parental chromosome) offers a greater predictive ability than an SNP (Judson et al. 2000). When a genetic study shows a new association between an SNP and effect, it must be recognised that the variant in question may be in linkage disequilibrium with another variant in the gene or in a nearby gene, which is the true pharmacogenetic locus (Liggett 2001). The background to genetic variability in drug effect can be complex because a number of factors may have to be considered depending on the drug in question:

- Genotype of phase I enzyme
- Genotype of phase II enzyme
- Genotype of transporter
- Genotype of drug targets, e.g. receptors
- Unknown SNPs in genes in question

Genotype versus phenotype approach

In **phenotyping**, a probe drug is used that reflects the activity of a specific P450 enzyme. The activity is correlated to a pharmacokinetic parameter such as clearance, area under the plasma concentration versus time curve (AUC) or to a molar ratio (MR) of parent compound to the metabolite in e.g. plasma or urine. A significant correlation of the chosen parameter, such as MR, to clearance needs to be shown. A complete validation of *in vivo* probe drugs for assessment of activity of a specific P450 isoform includes; correlations to other probe drugs of the enzyme, inhibition by specific inhibitors, correlation to genotype, stability of test and lack of interferences in the analytical method. Other important features of a probe drug are safety at the selected dose and preferably a wide therapeutic index. The phenotypic measure should cover a

wide range in activity. It is further also advantageous if the phenotyping test is relatively non-invasive, i.e. low dose and few samples.

Phenotyping reveals the activity of the P450 enzyme at a particular time point and indicates the actual metabolic capacity of the patient. Concomitant medication and concurrent diseases may influence the phenotype measurement and the result may not be reproducible over time, and hence significant intraindividual variations may result.

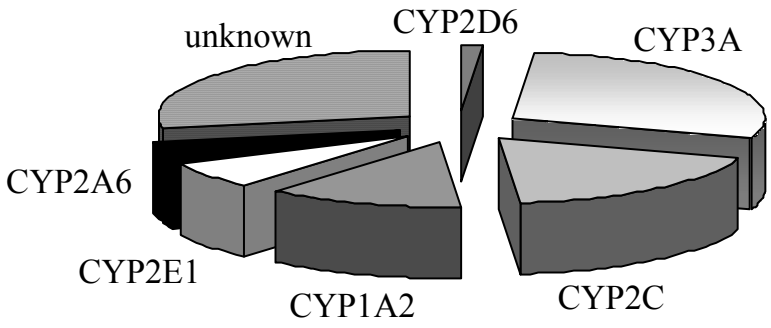
Predictive **genotyping** strategies exist for some enzymes, i.e. genotyping for the 6 to 7 most common defective *CYP2D6* alleles in Caucasians will predict the phenotype with 99% certainty (Sachse et al. 1997; Griese et al. 1998). Genotyping has major advantages in that it is done once in a lifetime and also can be done in patients who are unable to take a phenotyping probe due to risk for interactions. If genotyping is to replace phenotyping and be used for dose selection, it should not only allow for identification of poor metabolisers (PMs), but also give some prediction of metabolic clearance among extensive metabolisers (EMs). When the predictive power of genotyping of CYP2D6 was studied it was concluded that genotyping correctly identified PMs, but quantitative prediction of drug metabolism among EMs was not possible (Griese et al. 1998). When the utility of *CYP2D6* genotyping as an alternative to traditional phenotyping as a predictor of PM status was assessed for use in clinical trials, the genotyping methodology successfully predicted all but 1 of the 46 PMs (McElroy et al. 2000).

SPECIFIC CYTOCHROME P450 ENZYMES IN MAN

In September 2003, 57 P450 genes had been identified in humans (http://drnelson.utmem.edu/human.P450.table.html). It is believed that this number will not increase much as the whole human genome has been sequenced. Mainly enzymes from the CYP1, CYP2 and CYP3 families metabolise xenobiotics in humans. The CYP1 family includes 3 subfamilies (3 genes and 1 pseudogene); CYP2 family, 13 subfamilies (16 genes and 16 pseudogenes) and CYP3 family, 1 subfamily (4 genes and 2 pseudogenes) (http://drnelson.utmem.edu/P450lect.html). Most P450s are hepatic and few are expressed exclusively extrahepatically. Also, the most important site for metabolism of exogenous compounds in man is the liver. About 70% of liver P450s (Figure 4) are accounted for by the CYP1A2, CYP2A6, CYP2C, CYP2D6, CYP2E1 and CYP3A enzymes (Shimada et al. 1994). These P450 enzymes are responsible for the metabolism of 90% of all drugs (Bertz and Granneman 1997; Rendic and Di Carlo 1997).

The cytochrome P450 enzymes that are relevant for this thesis work will be discussed in the section below.

Figure 4. The relative abundance of the different P450 isoforms in the liver
Contents of total P450 determined spectrally and individual isoforms determined immunochemically (percentage of total P450) in human liver microsomes (Shimada et al. 1994). CYP3A consists of CYP3A4, 3A5 and 3A7; and CYP2C of CYP2C8, 2C9, 2C18 and 2C19.



CYP2D6

CYP2D6 belongs to the *CYP2* gene family, which is the largest P450 gene family in humans with 16 members spread on 7 different chromosomes (http://drnelson.utmem.edu/CytochromeP450.html). The *CYP2D6* gene is normally found in a cluster with the two pseudogenes, *CYP2D7P* and *CYP2D8P,* on chromosome 22 (Heim & Meyer, 1992). The enzyme is mainly expressed in liver (Zanger et al. 2001), but also in tissues such as heart (Thum and Borlak 2000) and brain (Siegle et al. 2001).

In the late 1970s, it was found that between 5 and 10% of European population were unable to metabolise the drugs debrisoquine (Mahgoub et al. 1977; Tucker et al. 1977) and sparteine (Eichelbaum et al. 1979), respectively. Since, the large range in activity of the CYP2D6 enzyme has been explained by significant heterogeneity in the *CYP2D* gene cluster. Deficiency of the CYP2D6 enzyme is inherited as an autosomal recessive trait and subjects without functional enzyme are classified as poor metabolisers. Gene conversion events in which inactivating sequences were transferred across genes and accumulation of point mutations have generated many known *CYP2D6* alleles (http://www.imm.ki.se/CYPalleles) (Heim and Meyer 1991; Heim and Meyer 1992). Distribution of the frequencies of the *CYP2D6* alleles show marked interethnic variability (Table 3). Several groups have undertaken systematic analyses of the *CYP2D6* gene in Caucasian populations. Marez et al. (Marez et al. 1997) analysed 672 Europeans and revealed 48 mutations and 53 different alleles, while Sachse et al.

(Sachse et al. 1997) analysed the mutational profile of 589 Caucasian subjects. Furthermore, the genetics of the *CYP2D* locus has been reviewed and genotype linked to protein expression and function (Griese et al. 1998; Zanger et al. 2001).

CYP2D6 is the only major P450 enzyme that is not inducible. In contrast, this gene is the only one where gene duplications and amplifications have been shown, resulting in higher enzymatic activity (Johansson et al. 1993). It has been suggested that in certain geographical areas (e.g. Ethiopia) there has been a dietary pressure to increase detoxification by the CYP2D6 enzyme, which was made possible through recombination events creating haplotypes with duplicated and multiduplicated *CYP2D6* genes (Ingelman-Sundberg 1999).

In order to study genetic reasons for Caucasian IMs, Raimundo et al. (Raimundo et al. 2000) sequenced the 5'-flanking region of the *CYP2D6* gene and were able to show that the *CYP2D6*2* allele consisted of at least two different alleles in a German population because the mutation C-1584G mutation specified a subgroup of the *CYP2D6*2* allele showing a higher activity (about 4-fold; *in vivo*) than the wild-type (numbering of nucleotides is consistent with the CYP allele homepage but not with the original article; there is a discrepancy between the two systems of 88 nucleotides). This increased rate in CYP2D6-dependent metabolism explained about 60% of intermediate Caucasian metabolisers. The C-1584 allele with decreased activity is now named *CYP2D6*41*, whereas the G-1584 variant is named *CYP2D6*2* (http://www.imm.ki.se/CYPalleles). It is believed that the most common defect and partly defect *CYP2D6* alleles among Caucasians have been identified (Griese et al. 1998), however the molecular basis for partly defect CYP2D6-dependent metabolism in other populations is not fully known.

In contrast to the thorough investigations in Caucasians, few consistent sequencing studies had been undertaken in any African population. Masimirembwa et al. (Masimirembwa et al. 1996) sequenced Zimbabwean subjects and identified the *CYP2D6*17* allele, which was shown to have a reduced function both *in vivo* (Masimirembwa et al. 1996) and *in vitro* (Oscarson et al. 1997). It is probable that alleles coding for nonfunctional enzymes in black populations have been identified, but the molecular reasons of intermediate metabolisers in Africans is not fully known (Griese et al. 1999; Wennerholm et al. 1999). Indeed, we compared Tanzanian subjects with EM phenotypes (i.e. including only subjects with the *CYP2D6*1* or **2* alleles) to Caucasian EMs. We showed that 41% of Tanzanian EMs had a debrisoquine MR > 1, whereas only 7% of the Caucasian EMs did. *Possible genetic reasons for the decreased CYP2D6 activity in Africans are reviewed in the Discussion part.*

CYP2D6 phenotypes can be divided into ultrarapid (UM; debrisoquine metabolic ratio (MR) < 0.1), extensive (EM), intermediate (IM; debrisoquine MR 1-12.6) and poor metabolisers (PM; debrisoquine MR > 12.6) (Dahl et al. 1995; Meyer and Zanger 1997). In a phenotyping study in Germans, the metabolic clearance of sparteine varied 80-fold within the EM group (Griese et al. 1998). A close relationship existed between the MR of sparteine and its metabolic clearance, which varied over approximately the same range (Griese et al. 1998). The PM phenotype is based on the MR of a probe drug (i.e. debrisoquine MR >12.6) and Caucasian subjects with such phenotype would not express any functional enzyme. Nevertheless, in African populations this definition

Table 3. Important *CYP2D6* alleles in African populations. Frequencies (%); n = number of subjects. Data from other populations included.

				Africans			Caucasians	Asians
Allele	**Mutations**	**Consequence**	**Activity (*in vivo*)**	**Tanzanians** n = 106[a]	**Ethiopians** n = 122[b]	**West Africans (Ghana)** n = 193[c]	**Europeans** n = 672[d]	**Chinese** n= 113[e]; n = 119[f]
**1*	-	-	normal	28	22[k]	44	33	27[e]
**2*	C-1584G; G1661C, C2850T, G4180C[g]	R296C, S486T	normal	16[l]	14	11	33	13[e]
**2 x N*	G1661C, C2850T, G4180C, **gene duplication**	R296C, S486T, **2 or more functional genes**	increased	3.3[h]	16	1.6	1.5[j]	1.3[e]
**3*	**A2549 deletion**	**Frame shift**	none	0	0	0	1.8	0[f]
**4*	C100T, C974A, A984G, C997G, G1661C; **G1846A;** G4180C	P34S, L91M, H94R, S486T, **splicing defect**	none	0.9	4.1	7.0	19	0.8[f]
**4 x N*	As **4*, but with gene duplication	-	none	0.9	NA	NA	NA	NA
**5*	**deletion**	No protein	none	6.1	3.3	6.0	7.3	5.7[e]
**6*	**T1707 deletion**	**Frameshift**	none	0	NA	0	1.4	NA
**10*	**C100T**, G1661C, G4180C	**P34S**, S486T	decreased	3.8	8.6	3.1	1.4	51[e]
**17*	**C1023T**, G1638C, C2850T, G4180C	**T107I,** R296C, S486T	decreased	17	11	28	0.1	NA
**29*	**G1659A**, **G1661C**, C2850T, **G3183A**, G4180C	**V136I**, R296C, **V338M**, S486T	decreased	20	NA	NA	0.1	NA
**41*	**C-1584**, A-1235G, C-740T, G-678A, G1661C; C2850T, G4180C[g]	R296C, S486T	decreased	4.0[i]	22	NA	NA	NA

NA, not assessed. The functional mutation is indicated in bold. However, effect of variant enzyme may be dependent on a combination of SNPs and substrate (Oscarson, 1996; Wennerholm, 2001). Most of the above alleles represent a number of allelic variants, e.g. *CYP2D6*2A* to *2K* (http://www.imm.ki.se/CYPalleles). In the genotyping studies referred to, the exact allele has not always been elucidated but instead it is the functional mutation that was genotyped for. [a]Wennerholm et al., 2001; [b]Aklillu et al., 1996 and 2002 (Ethiopians living in Ethiopia); [c]Griese et al., 1999; [d]Marez et al., 1997; [e]Johansson et al., 1994 and [f]Wang et al., 1993; [g]frequently with a *CYP2D7* gene conversion in intron 1; [h]*CYP2D6*2 x 2* and/or *CYP2D6*1 x 2*; [i]Wennerholm, unpublished data; [j]Griese et al., 1998; [k]Calculated from figures below; [l]Figure for *CYP2D6*41* deducted.

may not be valid; e.g. among Tanzanian subjects phenotyped with debrisoquine, none of the PMs was homozygous for defective *CYP2D6* alleles (Wennerholm et al. 1999). *The lack of correlation of CYP2D6 geno- and phenotype data in African populations will be reviewed in the Discussion part.*

Poor correlations of phenotyping results using different CYP2D6 probe drugs in African populations were observed early on (Woolhouse et al. 1985; Lennard et al. 1992; Simooya et al. 1993). Additionally, it was reported that the African-specific *CYP2D6*17* allele encoded an enzyme with changed substrate specificity compared to CYP2D6.1 and CYP2D6.2 *in vitro* (Oscarson et al. 1997). *The possibility of CYP2D6 alleles coding enzymes with altered substrate specificity in vivo will be reviewed in the Discussion part.*

CYP2D6 is one of the most important phase I enzymes involved in the metabolism of drugs (Table 4). Within the P450 family, this enzyme is second to CYP3A4 in the number of drugs it is known to metabolise (cf. review by (McCarthy et al. 2002)). One of many estimates is that it metabolises, at least partly, about 25% of currently known drugs (Bertz and Granneman 1997; Rendic and Di Carlo 1997). CYP2D6 preferably metabolises lipophilic drugs with a basic protonable nitrogen atom between 5 to 7 Å from the position of oxidation (Eichelbaum and Gross 1990). The protonated amine seems to interact with the carboxylate anion of Asp301 in the enzyme active site (Ellis et al. 1995). There are, however, also high affinity substrates lacking a basic nitrogen, which challenges accepted models of the CYP2D6 active site (Guengerich et al. 2002).

CYP2C subfamily

The *CYP2C* subfamily is a cluster with four genes (*CYP2C8, 2C9, 2C18* and *2C19*) found on chromosome 10 (http://drnelson.utmem.edu/CytochromeP450.html). These enzymes are expressed mainly in liver, but CYP2C18 only at low levels. The number of described alleles of the *CYP2C* genes is continually increasing as more populations are sequenced (http://www.imm.ki.se/CYPalleles). Among Caucasians, most PMs of CYP2C9 and CYP2C19 are identified by screening for the *CYP2C9*2*, **3* and *CYP2C19*2* alleles (de Morais et al. 1994; de Morais et al. 1994; Scordo et al. 2001). Also, among black PM subjects, the same alleles are the most common (Herrlin et al. 1998; Scordo et al. 2001).

The CYP2C enzymes are estimated to metabolise about 20% of currently known drugs (Bertz and Granneman 1997; Rendic and Di Carlo 1997). So far, it has been primarily the CYP2C9 and CYP2C19 enzymes that have been shown to be important for drug metabolism among the CYP2C enzymes (Table 4). More recently, information on the importance of the CYP2C8 enzyme is emerging. It has been shown that CYP2C8 metabolises paclitaxel (Rahman et al. 1994) and lately, it was shown that CYP2C8 is an important DME for the metabolism of the antimalarial drugs amodiaquine and chloroquine *in vitro* (Li et al. 2002; Projean et al. 2003).

Table 4. Examples of substrates or drug classes (in bold), inhibitors and inducers of five of the major DMEs. Commonly used *in vivo* probe drugs are indicated in *italics*.

Enzyme	Substrates/ Drug classes		Inhibitors	Inducers
CYP1A2	*caffeine,* amitriptyline, clomipramine, clozapine, estradiol, fluvoxamine,	imipramine, melatonin, theophylline, paracetamol, propranolol, verapamil	fluvoxamine, oral contraceptives, quinolone antibiotics	charbroiled meat, fluvoxamine, omeprazole, PAH[a], verapamil
CYP2C9	*diclofenac, losartan,* celecoxib, fluvastatin,	ibuprofen, indomethacine, phenytoin, S-warfarin	amiodarone, fluconazole, fluvoxamine, paroxetine	carbamazepine, rifampicin
CYP2C19	*S-mephenytoin, omeprazole,* amitriptyline, carisoprodol, S-citalopram, clomipramine	diazepam, imipramine, lansoprazole, moclobemide, proguanil, R-warfarin	cimetidine, fluoxetine, fluvoxamine, indomethacine, oral contraceptives	rifampicin, artemisinin
CYP2D6	*debrisoquine, dextromethorphan, metoprolol, sparteine,* chlorpheniramine, chlorpromazine, codeine,	ethylmorphine, **antiarrythmics, antidepressants, β-adrenoceptor blockers, neuroleptics**	chlorpheniramine, chlorpromazine, fluoxetine, ritonavir, sertraline, quinidine	none[b]
CYP3A	*erythromycin*, *midazolam*, alprazolam, carbamazepine, clarithromycin, cyclosporine, indinavir, nifedipine, quinidine, quinine,	ritonavir, saquinavir, tacrolimus, tamoxifen, terfenadine, **antifungals, calcium channel blockers, steroid hormons**	erythromycin, grapefruit juice (bergamottin), indinavir, itraconazole, ketoconazole, ritonavir, saquinavir	carbamazepine, dexamethasone, phenytoin, rifampicin, ritonavir, St John's wort (hyperforin)

Adapted from Flockhart (http://medicine.iupui.edu/flockhart/index.html) (Baciewicz 1986; Rane et al. 1992; Flexner 1998; Svensson et al. 1998; Laine et al. 2000; Facciola et al. 2001). A drug has been included in the list if there is published evidence that it is metabolised, at least in part, via that isoform. [a]Polycyclic Aromatic Hydrocarbons. [b]CYP2D6 activity may be induced in pregnant women (Högstedt et al. 1983; Wadelius et al. 1997).

CYP1A2

The *CYP1A1* and *CYP1A2* genes are found on chromosome 15 (http://drnelson.utmem.edu/CytochromeP450.html). The CYP1A2 enzyme is expressed mainly in the liver (Sesardic et al. 1990; Windmill et al. 1997). About 20 clinically important drugs are predominantly or partly metabolised by CYP1A2 (Table 4). Some studies have shown that CYP1A2 activity is polymorphically distributed (Butler et al. 1992), whereas other studies have not been able to confirm this (Welfare et al. 1999). A number of allelic variants of the *CYP1A2* gene have been identified (http://www.imm.ki.se/CYPalleles), however their impact on activity needs to be shown. Recently, a novel haplotype affecting the inducibility was described (Aklillu et al. 2003). CYP1A2 is affected by environmental factors such as smoking to a great extent.

CYP3A subfamily

The CYP3A subfamily is found in a cluster on chromosome 7 (http://drnelson.utmem.edu/CytochromeP450.html). The *CYP3A4*, *3A5, 3A7, 3A43* genes and the two pseudogenes (*CYP3A5P1* and *3A5P2*) have high sequence homology (Gellner et al. 2001). CYP3A4 is primarily expressed in the liver and small intestine but less so in other tissues (Watkins et al. 1987; Lown et al. 1994; Shimada et al. 1994). CYP3A5 is polymorphically expressed in a range of tissues including the liver, stomach and kidney (Wrighton et al. 1989; Wrighton et al. 1990; Kolars et al. 1994). CYP3A7 is a foetal form and rarely expressed in adults (Schuetz et al. 1994). The *CYP3A43* gene is the most recently cloned *CYP3A* gene (Domanski et al. 2001; Westlind et al. 2001) but it has not yet been shown to be functional.

The rate of metabolism of drugs that are substrates for CYP3A vary more than 10-fold *in vivo* (Kleinbloesem et al. 1984; Wilkinson 1996; Schmidt et al. 2001). Several screening studies have been performed to elucidate the genetic reasons of variability in CYP3A activity. So far 25 allelic *CYP3A4* variants have been identified (http://www.imm.ki.se/CYPalleles). Generally, variants in the coding region of CYP3A4 occur at allele frequencies < 5% and so far, no common allele with a significant impact on activity has been identified. This is also in agreement with the reported unimodal distribution of CYP3A4 expression (Koch et al. 2002). The most common allelic variant in African-Americans together with *CYP3A4*1,* is the *CYP3A4*1B* allele, which has an A-392G exchange in the 5'-flanking region, and an allele frequency of 55% (Ball et al. 1999). In white Americans the frequency was only 4% (Ball et al. 1999). Studies were not able to link the *CYP3A4*1B* allele to alterations in the metabolism of the CYP3A4 substrates erythromycin and nifedipine (Ball et al. 1999).

CYP3A5 was shown to be polymorphically expressed in both Caucasian and black populations, but at a much higher frequency in black populations (Hustert et al. 2001; Kuehl et al. 2001) (Table 5). The major defect alleles in black populations are *CYP3A5*3* (splicing defect), **6* (splicing defect), **7* (frameshift and premature stop) and **8* (amino acid substitution). The haplotypes of the *CYP3A5*3, *6* and **7* have not yet been fully elucidated (see Table 5). By analysing a black Tanzanian population, we

found that 77% had at least one functional *CYP3A5*1* allele assuming that G6986, G14690A and G27131-32insT are located on separate alleles (Wennerholm, unpublished data). Recently, it was suggested that the contribution of CYP3A5 to hepatic drug CYP3A metabolism is insignificant in Caucasians (Westlind-Johnsson et al. 2003).

Table 5. Selected *CYP3A5* allele frequencies (%) in black populations and Caucasians. Alleles with A6986 are classified as *CYP3A5*1* alleles as haplotypes of *CYP3A5*3, *6* and **7* have not been elucidated; therefore, the total allele frequency >100%.

Allele (SNP)	**Activity**	**Tanzanians**[a] n = 112	**Africans**[b] n = 24	**African-Americans**[c] n = 45	**German/Swiss Caucasians**[c] n = 183	**British Caucasians**[d] n = 100
**1* (A6986)	wild-type	83	65	73	4.9	6.5
**3* (G6986)[e]	none	17	35	27	95	94
**6* (G14690A)[e]	none	19	17	13	0	NA
**7* (G27131-32insT)[e]	none	10	8	10	0	NA
**8* (C3699T)	decreased	NA	4	NA	0	NA

NA; not assessed.

[a]Wennerholm, unpublished data.

[b]DNA from Coriell Institute, samples of African-Americans and African Pygmies (Lee et al. 2003).

[c](Hustert et al. 2001); fewer subjects were analysed for the positions of *CYP3A5*6, *7* and **8.*

[d](King et al. 2003).

[e]G6986A was found in some subjects with G14690A and G27131-32insT, respectively, however haplotypes have not been elucidated.

The CYP3A enzymes metabolise about half of clinically used drugs (Li et al. 1995; Bertz and Granneman 1997). The CYP3A enzymes have broad substrate specificity and the binding site can accommodate both small and large molecules with a range in charge and lipophilicity (Bertz and Granneman 1997). Several groups have attempted to correlate the activities of various *in vivo* probes of CYP3A4 activity (Watkins et al., 1994; Thummel et al., 1998) but it is common that the activity of one *in vivo* probe fails to accurately predict the metabolism of other CYP3A substrates (Hunt et al., 1992; Watkins et al., 1992; Kinirons et al., 1993, Stein et al., 1996). Different routes of administration, presence of more than one binding site in the CYP3A4 active site (Khan, 2002; Khan 2003) and impact of intestinal metabolism could be reasons for a low degree of correlation between CYP3A probe drugs. Influence of P-gp could as well be one contributing factor as the CYP3A enzymes and P-gp have overlap in their substrate specificities (Wacher et al. 1995).

It is still unclear what proportion of the CYP3A drugs that are substrates for both CYP3A4 and CYP3A5. Some CYP3A drugs have been suggested to be differentially metabolised by CYP3A4 and CYP3A5, i.e. CYP3A4 was shown *in vitro* to be more efficient than CYP3A5 in metabolising quinidine and erythromycin (Wrighton et al. 1990; Nielsen et al. 1999) and testosterone (Williams et al. 2002). The published data are sometimes conflicting however, as in the case of erythromycin, where Gillam et al. (Gillam et al. 1995) showed comparable rates *in vitro* of erythromycin demethylation by CYP3A4 and CYP3A5. It has also been shown *in vitro* that the 1'-hydroxymidazolam/4'-hydroxymidazolam ratio was higher for CYP3A5 than CYP3A4 (Gorski et al. 1994; Kuehl et al. 2001; Williams et al. 2002). In analogy, a preference for formation of 4-hydroxyalprazolam by CYP3A4 and that of α-hydroxyalprazolam by CYP3A5 was shown *in vitro* (Gorski et al. 1999; Hirota et al. 2001). It is suggested though that *in vitro* data on catalytic activity of the CYP3A enzymes should be interpreted with caution because their function appears to be highly sensitive to reconsitution environment (Gillam et al. 1995; Lamba et al. 2002).

Both midazolam and alprazolam have been used in studies in healthy subjects for the analysis of impact of CYP3A5 expression on CYP3A activity (Shih and Huang 2002; Floyd et al. 2003; Gashaw et al. 2003). *The pharmacokinetics of alprazolam and its possible use for assessing CYP3A activity will be reviewed in the Discussion part.*

ENVIRONMENTAL FACTORS OF IMPORTANCE FOR DRUG METABOLISM

Interindividual variability in drug metabolism can be caused by environmental factors such as pharmacological, hormonal, dietary, pathophysiological and physiological factors.

Metabolic drug interactions may lead to adverse drug reactions due to increased concentrations of active substance. Induction may also result in lower drug concentrations, which may be critical in certain cases. Cyclosporine has a narrow therapeutic index and if its concentration falls below the maintenance dose due to enzyme induction there may be risk for transplant rejection (cf. review by (Campana et al. 1996)). Several antimalarial drugs have been shown to inhibit P450 enzymes (Steiner et al. 1988; Simooya et al. 1998). Amodiaquine (AQ) and its major metabolite N-desethylamodiaquine (DAQ) have been shown to inhibit primarily CYP2D6 in a system of recombinant enzymes (Bapiro et al. 2001). Also, in patients, a single 600-mg amodiaquine dose given one hour prior to regular chlorpromazine intake caused a 2.5-fold increase in chlorpromazine levels compared to baseline levels (Makanjuola et al. 1988). *Interference of antimalarial drugs on the disposition of drugs metabolised by polymorphic P450 enzymes will be reviewed in the Discussion part.*

There are several herbal drugs that have been shown to have significant impact on drug metabolism (Zhou et al. 2003). Hyperforin, a constituent in St Johns wort, has been shown to induce CYP3A4 supposedly via activation of PXR (Pregnane X receptor) (Wentworth et al. 2000). This herb also contains ingredients that inhibit CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (Obach 2000). Use of oral contraceptives is

common and these substances have been shown to inhibit CYP1A2 and CYP2C19 activity, but not CYP2D6 activity (Abernethy and Todd 1985; Balogh et al. 1995; Laine et al. 2000; Hagg et al. 2001).

CYP2D6 activity was indicated to be induced in pregnant women (Högstedt et al. 1983; Wadelius et al. 1997). No difference in CYP2D6 activity, as measured by dextromethorphan phenotyping, between the mid-follicular and mid-luteal phases of the menstrual cycle was observed when studied (Kashuba et al. 1998).

Intake of grapefruit juice was shown to increase the bioavailability of the CYP3A substrates felodipine and nifedipine (Bailey et al. 1991), whereas red wine was shown to decrease the bioavailability of cyclosporine (Tsunoda et al. 2001). Peppers are common food ingredients used worldwide, and one of the constituents in pepper, piperine was shown to inhibit CYP3A4 activity (Tsukamoto et al. 2002). Charcoal-grilled meat and cruciferous vegetables were shown to induce CYP1A2 activity (Parsons and Neims 1978; Vistisen et al. 1992). It has also been shown that tobacco smoke (polycyclic aromatic hydrocarbons) induces CYP1A2 (Landi et al. 1999).

In hepatic cirrhosis of cholestatic or hepatocellular origin, levels of especially CYP1A2, but also CYP3A, CYP2C and CYP2E1 proteins were decreased by 20 to 80% (Farrell 1999). Significant decreases in P450 levels in human liver are primarily limited to cases of severe liver failure and decompensated cirrhosis (Farrell 1999). Indeed, disposition of the CYP2D6 probe drug debrisoquine was unaffected by liver disease, even in patients with cirrhosis of moderate severity (Adedoyin et al. 1998). However, plasma clearance of a CYP2C19 probe drug (mephenytoin) decreased 79% in patients with liver disease, and the reduction was related to the severity of the disease (Adedoyin et al. 1998).

Infections have also been shown to down-regulate the expression of a number of P450s, supposedly with cytokines (e.g. interleukins, TNFα and interferons) as mediators (Cheng and Morgan 2001). However, few studies have been done *in vivo*. In man, CYP1A2 activity has been shown to be decreased in influenza, adenoviral infectious and malaria (*Plasmodium falciparum*) (Morgan 1997; Akinyinka et al. 2000). Data on most P450 enzymes are scattered and mainly from *in vitro* studies (Cheng and Morgan 2001). However, quinine (a CYP3A4 substrate) concentrations in plasma were elevated in malaria-infected patients as a result of reduced clearance (White et al. 1982). Changes may occur in HIV-positive patients such that their CYP2D6 activity approaches that of PMs, despite having an EM genotype (O'Neil et al. 2000). Also, disease progression in HIV infection and AIDS may alter expression of the phase II enzyme N-acetyltransferase 2 (O'Neil et al. 1997).

Age-related effects that may have an impact on drug metabolism are altered renal function, increased gastric pH and decreased gastric motility (Tam 1993). Drug disposition has also been shown to be affected in many different ways in malnourished children (Krishnaswamy 1989).

CLINICAL RELEVANCE OF CYTOCHROME P450 DRUG METABOLISM

Effects of variability in drug metabolism

Cytochrome P450-catalysed metabolic reactions show marked interindividual variability leading to large differences in plasma concentrations; e.g. a 30-fold variability in steady-state plasma concentration of nortriptyline was seen in patients with the same dose (Hammer and Sjöqvist 1967). It was also shown that subjects with three functional *CYP2D6* genes needed three times the normal dose of nortriptylin, i.e. 300-500 mg daily (Bertilsson et al. 1985; Bertilsson et al. 1993). Schizophrenic patients phenotyped as PMs of CYP2D6 were shown to be at risk of adverse drug reactions upon treatment with haloperidol (Brockmoller et al. 2002). In contrast, patients with extra *CYP2D6* gene copies were at risk of low therapeutic efficacy when treated with haloperidol (Brockmoller et al. 2002). Also, the CYP2D6 substrate codeine, which is a pro-drug of morphine, have a lower analgesic effect in PMs (Eichelbaum and Evert 1996; Poulsen et al. 1996), whereas it was reported that an ultrarapid metaboliser got severe abdominal pain after codeine intake (cf. case report (Dalen et al. 1997)).

Polymorphisms in DMEs may have clinical importance when **1)** a substantial amount of the drug is metabolised by the enzyme with no or minor alternative pathways available, **2)** the resulting variability in elimination rate has clinical impact (i.e. drugs with narrow therapeutic ranges), **3)** the drug is a prodrug, **4)** there is low variability in other pharmacokinetic processes, and **5)** there is low pharmacodynamic variability.

In PMs, the potential consequences of polymorphic drug metabolism are extended pharmacological effect, adverse drug reactions from the substrate, lack of prodrug activation, metabolism by alternative, deleterious pathways or drug-drug interactions. UMs may have an increased effective dose and may have adverse drug reactions from formed metabolites. Adverse effects are a serious clinical issue and it has been estimated that about 100 000 American patients die each year and 2.2 million are injured by adverse drug reactions to prescribed drugs (Lazarou et al. 1998). This data have been criticised for overestimation of figures though (Fremont-Smith 1998). In Sweden, 10 to 15% of patients acutely admitted to a clinic of internal medicine were admitted due to an adverse drug reaction (Sarlöv et al. 2001; Mjörndal et al. 2002). Known risk factors for adverse drug reactions include polytherapy, old age, liver and renal disease. Moreover, it has been estimated that more than half of the drugs in reports on adverse drug reactions are metabolised by polymorphic enzymes (Phillips et al. 2001). Even though drug-drug interactions may cause adverse drug reactions, it has been difficult to improve pharmacogenetic awareness in the clinic. It is believed that to do so, prospective studies may have to be performed, where measurable differences in clinical outcome that patients and physicians care about are considered (Meisel et al. 2000; Meisel et al. 2003).

Clinical relevance in African populations

Parasitic and infectious diseases are major threats to the health of most populations in Africa. It is recognised that pronounced differences in drug metabolism exist between as well as within ethnic groups (Bertilsson 1995; Masimirembwa and Hasler 1997). New drugs have seldom been studied for use in developing countries where unique

factors influence the effect of a given dose, e.g. polymorphic DMEs, nutritional status, interfering diseases. It is now accepted in principle that differences in genetic inheritance and environment preclude extrapolation of results of drug studies from non-Africans to Africans. Therefore, it is important to do specific molecular genetic and clinical studies in African populations to understand the mechanisms behind differences that may require population-specific dosage schedule of important drugs.

Besides, combination therapy is particularly common in tropical medicine. It was first used for treatment of tuberculosis (TB) and leprosy, but with time this strategy has also been used to treat HIV and malaria. Due to the limited number of locally available antiparasitic drugs it has become necessary to prolong the efficacy of these drugs (Bloland and Ettling 1999). This has lead to the use of combination therapy which may help to avoid resistance development (White 1999). The high incidence of parasite diseases as well as HIV and its associated infections means increased exposure to multiple drugs, thus increasing the risk of drug-drug interactions. Drugs in a combination therapy can interact in several different ways, either by competing for the same DME or by inhibiting the enzyme metabolising one of the other drugs. The DMEs involved may also exist in allelic variants affecting the drugs differently. It is important to study whether such polymorphisms influence suitable dosage of these drugs in African populations. The CYP3A enzymes are of major importance in Africa, since they metabolise so many drugs including certain protease inhibitors used in HIV treatment (Barry et al. 1997). Some protease inhibitors are also inhibitors of the CYP3A4 enzyme (van Heeswijk et al. 2001) thus requiring studies of their inhibitory potential in African populations, which might differ from that observed in Caucasians due to differences in genetic variants between the populations.

Knowledge of the CYP3A enzymes is also important for future treatment of TB, since one of the drugs in TB treatment, rifampicin, is a potent inducer of CYP3A4 (Hebert et al. 1992). In a study in healthy volunteers, dosing of rifampicin (600 mg per day) for 7 days increased the oral clearance of midazolam 22-fold (Gorski et al. 2003). Rifampicin being a potent P-gp inducer complicates the issue further (Greiner et al. 1999). The complexity of handling combination treatments for HIV patients with active or latent tuberculosis is recognised (CDC 2000). In future, anti-HIV and TB treatments will become more readily available to risk groups of patients also in Africa. It is hence important to know the effects of differences in drug metabolism to make informed choices on drug dosages. This emphasises the importance of phenotyping methods that are possible to use in larger population studies.

Quinoline drugs (e.g. chloroquine and quinine) have been among the most widely used drugs for treatment of *Plasmodium falciparum*. However, resistance development has decreased their utility. It was shown that chloroquine resistance could be reversed *in vitro* by verapamil, an inhibitor of multi-drug resistance in cancer cell lines (Martin et al. 1987; Martiney et al. 1995). Chloroquine resistance has been associated to a P-glycoprotein homologue present in the parasite (Foote et al. 1989), and resistance of the malaria parasite may therefore in some aspects be compared to P-gp-mediated multidrug resistance to chemotherapy in patients. A chloroquine resistance reversing agent, chlorpheniramine, together with chloroquine was successfully used in the treatment of malaria in children in an area where chloroquine-resistance reaches 35%

(Sowunmi et al. 2000). Some malarial drugs (e.g. mefloquine, quinine and halofantrine) show cross-resistance in their malaria activity, which also indicates a multi-drug resistance mechanism (Price et al. 1999).

Recently, the World Trade Organisation (WTO) (August 2003) decided that developing countries would be allowed to import cheaper copies of drugs protected by patents for the treatment of HIV and other serious infections. This is a continuation of the decision taken at a WTO meeting in 2001, where it was decided that developing countries had the right to produce generics of certain drugs protected by patents. A condition for the agreement was that the drugs would only be used for treatment of diseases threatening the general health of the population (Frankish 2001).

Population-specific drug dosages

Oriental populations have been shown to require lower doses of antipsychotics compared to Caucasians (Poolsup et al. 2000). Also, slower metabolism in Asians due to the *CYP2D6*10* allele has resulted in lower dosing recommendations (Lou 1990; Lin et al. 1991) and Japanese federal requirements that pharmacokinetic studies should be performed in Japanese subjects (Shah 1993). Moreover, major steps towards subpopulation (extensive, intermediate and poor metabolisers) specific drug dosages relevant for Caucasian populations have been taken for psychiatric drugs (Kirchheiner et al. 2001). In these, dose reductions around 50% were generally recommended for tricyclic antidepressants in PMs of substrates of CYP2D6 or CYP2C19, whereas differences were smaller for the selective serotonin re-uptake inhibitors.

It is important to study whether genetic diversity in Africans translates into clinically important variations in responses to drugs. It was reported that African-Americans showed significantly higher plasma concentrations of nortriptyline than Caucasians after administration of the same drug dose (Ziegler and Biggs 1977). It has also been shown that depressed Tanzanian patients required lower doses of clomipramine compared to those recommended for Caucasians (Kilonzo et al. 1994). Population-specific drug dosages may also be important in the light of drug interactions caused by polymorphic P450 enzymes. Several clinically used drugs are known inhibitors of these enzymes, which affect possible drug combinations as well as drug dosages. In the CPMP/ICH guideline "Note for Guidance on Ethnic Factors in the Acceptability of Foreign Clinical Data" (CPMP/ICH 1998), issued by the European Agency for the Evaluation of Medicinal Products, recommendations are given for evaluation of the impact of ethnic factors. They divide drugs into those that are less likely to be sensitive to ethnic factors and those that are more likely to be sensitive to ethnic factors. Factors rendering a drug sensitive to ethnic factors are, e.g. steep pharmacodynamic curve for efficacy and safety, extensively metabolised, metabolism by polymorphic enzymes, low bioavailability (more susceptible to dietary absorption effects) and high likelihood of use in a setting of multiple co-medications.

AIMS

The overall aim of this thesis was to study variability in drug metabolism catalysed by cytochrome P450 enzymes in a Tanzanian population and to assess the consequences of such variability.

Specific aims of the studies were:

- To investigate CYP2D6 geno- and phenotypes in a black Tanzanian population.
- To identify genetic reasons for the generally lower capacity of the CYP2D6 enzyme in a Tanzanian population.
- To characterise the metabolic capacity of CYP2D6 in Tanzanian subjects with selected *CYP2D6* genotypes using four different substrates to explore the discrepancies in phenotypic measurements.
- To investigate any inhibition of CYP2D6, 2C9, 2C19 and 1A2 activities *in vivo* by the antimalarial drug amodiaquine that is commonly used in Tanzania.
- To study the elimination of alprazolam *in vivo* and develop a suitable phenotyping method and procedure to be used to assess CYP3A4 and 3A5 activities in population-based studies.

STUDY DESIGN, SUBJECTS AND METHODS

STUDY DESIGN

Study I

A *geno- and phenotyping study* of CYP2D6 performed in healthy Tanzanians (n=106) to characterise the metabolic capacity of this population.

Study II

A *screening study* to identify novel SNPs present in a Tanzanian population to understand the *mechanism* behind the generally lower capacity of the CYP2D6 enzyme. Subjects from study I with only functional *CYP2D6* alleles (*CYP2D6*1* and/or *CYP2D6*2*) were analysed by DNA sequencing. The identified allele was expressed in two different expression systems and the enzyme variant was assessed using two different substrates.

Study III

A *phenotyping study* where each volunteer was given four different CYP2D6 substrates on four different occasions. The subjects were selected based on *CYP2D6* genotype. The study included 35 healthy Tanzanians and a control group of 23 healthy Swedes.

Study IV

An *inhibition study* with the antimalarial drug amodiaquine performed in healthy Swedish subjects (n = 12). The subjects obtained a phenotyping cocktail (four out of five drugs from the Karolinska cocktail; cf. Discussion) to define the baseline level of their drug metabolising capacities. Thereafter, the subjects received a single dose of amodiaquine followed 2 hours later by the phenotyping cocktail. One week after intake of amodiaquine the cocktail was administered a third time to assess recovery from possible inhibition.

Study V

A *validation study* of a liquid chromatography-mass spectrometry (LC/MS) method to quantify alprazolam, 4-hydroxyalprazolam and α-hydroxyalprazolam in plasma. Moreover, a *pharmacokinetic study* with 12 healthy Swedes was performed and the alprazolam/metabolite ratios were correlated to the AUC-ratios of alprazolam/metabolite to determine a suitable time for a single plasma sample to assess CYP3A activity.

SUBJECTS

Healthy adults (Tanzanian and Swedish) participated. Each study had specific inclusion- and exclusion criteria relevant for the population and study in question. In **Study III**, we wanted to compare the results obtained in Tanzania to the Swedish situation, and therefore used a Swedish control group.

SCREENING OF SUBJECTS & LOGISTICS

For details on screening methodology used in this work see papers I-V.

To avoid confounding factors thorough screening procedures were undertaken in the studies. Screening was done by interview, physical examination and by laboratory tests to ensure that the volunteers were healthy and not taking concomitant medications. In **Study III-V**, the subjects also had dietary restrictions. This was done to study only the effect of the variant DME or potential inhibitor, and to avoid additive effects by confounders. Avoiding confounders makes it easier to draw conclusions and to compare to similar studies; however, it makes it more difficult to extrapolate the findings to the general population.

Emphasis was put on securing the validity of data, i.e. system for avoiding mix-up of samples, identification control of subjects, verification of quality control measures at local laboratories used in Tanzania, verification of correct storage and transport temperature of samples.

ETHICAL CONSIDERATIONS

The studies involving healthy volunteers (**study I, III-V**) were approved by the Ethics Committee at Huddinge University Hospital, Karolinska Institutet, Sweden. The studies involving Tanzanian healthy volunteers (**study I and III**) were approved as well by the Human Research Ethics Committee at Muhimbili University College of Health Sciences, Tanzania. The studies were performed according to the 'Declaration of Helsinki' and all subjects gave their written informed consent, on the basis of verbal and written information in their local language, Swahili or Swedish.

MOLECULAR BIOLOGY METHODS

Genotyping analyses

DNA isolated from peripheral leucocytes by a guanidinium isothiocyanate method or a QIAamp DNA Blood Midi kit was used for genotyping (**study I-V**) and genomic DNA sequencing (**study II**).

In the studies we used several methods for genotyping that were based on Polymerase Chain Reaction (PCR) and Southern blot techniques such as:

- Allele-specific PCR for identification of specific mutations.
- Long PCR to amplify large genomic sequences in order to identify alleles with larger rearrangements.
- Restriction Fragment Length Polymorphisms (RFLP), which is a Southern blot hybridisation method. This method was used in **study I** to identify CYP2D6 *Eco*RI and *Xba*I haplotypes.
- PCR-RFLP, for identification of specific mutations.
- Allelic discrimination using Taqman, for identification of specific mutations.

Genomic DNA sequencing

The design of specific primers to PCR-amplify only the sequence in question, and avoiding e.g. similar genes and pseudogenes, is crucial for sequencing. In **Study II**, the exons and the intron–exon boundries of the *CYP2D6* genes were sequenced using Sequenase T7 DNA polymerase with α-^{35}S dATP labelling. In later studies, PCR-based methods with fluorescent dideoxynucleotides such as the ABI PRISM Big Dye terminator cycle sequencing kit were used. The amplified DNA fragments were then analysed on a gel with an ABI Prism 377 DNA sequencer.

Heterologous expression

Because there are large interspecies differences in the DMEs, it is not relevant to use animal models for studies of enzyme activity. Instead, techniques for heterologous expression of human DMEs have been developed. In contrast to human liver microsomes, these systems are convenient in that one enzyme at a time is expressed; it is possible to express allelic variants after site-directed mutagenesis and relatively large quantities could be produced. Often, though, it is valuable to have a more complete structure, as found in human liver microsomes, with many of the phase I and some phase II enzymes available with the right co-enzymes.

In **Study II**, we used a *Saccharomyces cerevisae* (bakers yeast) strain that has been genetically engineered to overexpress the yeast reductase (Truan et al. 1993). Because yeast is eukaryotic, it possesses endoplasmic reticulum and mitochondria, characteristics that are important for the expression of human P450s. The yeast is incorporated into the endoplasmic reticulum and the normal heme levels in yeast are sufficient for the heterologous P450s (Waterman 1994). Yeast has low background levels of endogenous P450s that, furthermore, seem to be inactive towards drugs (Renaud et al. 1990). For expression, a galactose inducible promoter is used that enables separation of cell growth and P450 production (Pompon et al. 1996). Also, in our experiments we used constructs with a triplicate of adenine bases just prior to the initiating ATG codon, which has been shown to increase the amount of expressed protein (Krynetski et al. 1995).

We also used mammalian COS-1 cells in **Study II** to investigate functional properties and enzyme stability. This cell line was derived from the African green monkey kidney CV-1 cell line and it contains P450s involved in vitamin D metabolism. Enzyme stability can be studied because COS-1 cells contain the protein synthetic machinery and subcellular compartments of animal cells (Waterman 1994). Transfection efficiency is low, which results in low expression levels. Hence the obtained enzyme quantities are not enough for spectral analysis of P450 levels but enough for determination of apoprotein levels by Western blot.

Enzyme kinetics analyses

Kinetic analysis of CYP2D6 activity was performed in yeast microsomes with different concentrations of bufuralol, and the samples were analysed by reversed-phase high-performance liquid chromatography (HPLC). Moreover, the supernatant from COS-1 cells was incubated with bufuralol and the samples were analysed in the same manner.

Subsequently, the supernatant from COS-1 cells was incubated with debrisoquine and the samples were worked up prior to analyses by ion-pairing HPLC. K_m and V_{max} values were determined by nonlinear regression analysis and compared to the Lineweaver-Burke plots.

PHENOTYPING

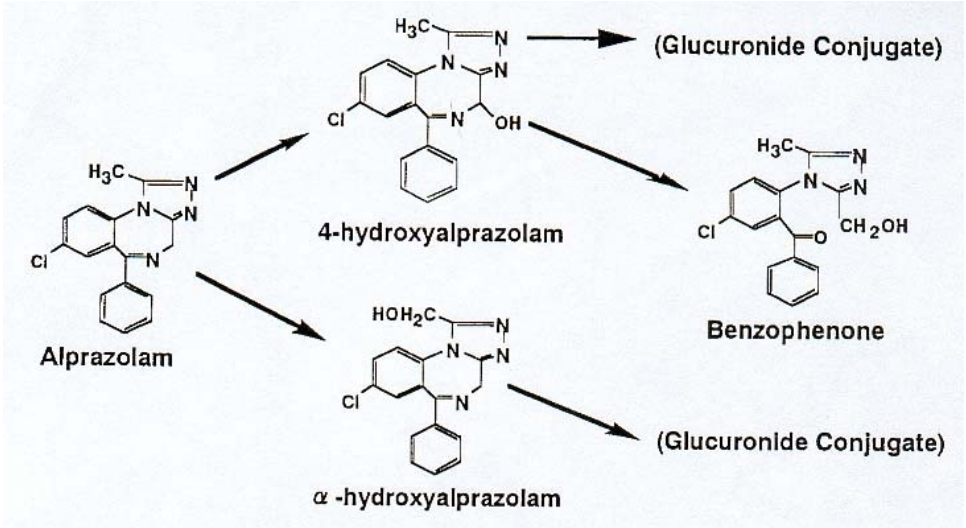
Debrisoquine is one of the most commonly used probe drugs for CYP2D6 phenotyping (Idle and Smith, 1979). It is an adrenergic blocking agent that was used as an antihypertensive drug during a short period in few countries. In **Study I** and **III**, a single oral 10-mg dose was given. Debrisoquine is metabolised via two primary pathways; oxidation of the alicyclic and aromatic structures or by ring cleavage (Allen, 1975; Angelo, 1976). *In vitro* data indicated that debrisoquine may be a substrate for P-gp (Kim et al. 1999). However, the effect of the affinity to P-gp on CYP2D6 phenotype has not yet been studied.

Codeine is an analgesic and antitussive drug. In clinical practice, the recommended daily dose is between 25 to 150 mg. In **Study III**, a single oral 25-mg dose was given. It has an oral bioavailability of 50% and a mean half-life of 3 hours. It is O-demethylated (via CYP2D6), N-demetylated (via CYP3A4) and glucuronidated.

Dextromethorphan is an antitussive agent with a recommended dose of 120 mg/day. In **Study III**, a single oral 15-mg dose was given. Dextromethorphan has a low bioavailability in CYP2D6 extensive metabolisers and a half-life of about 2 hours. It is O-demethylated via CYP2D6 to dextrorphan and N-demetylated via CYP3A4 to 3-methoxymorphinan.

Metoprolol is a β-blocker used for treatment of hypertension. Recommended daily dose is 100 to 200 mg. In **Study III**, a single oral 100-mg dose was given. Oral bioavailability is about 45% and its elimination half-life about 4 hours. Metoprolol has two CYP2D6-derived metabolites: α-hydroxymetoprolol and O-desmethylmetoprolol (partly via CYP2D6).

Figure 4. The metabolic pathways of alprazolam. From Hirota et al., 2001.



Alprazolam is a benzodiazepine with anxiolytic activity. Recommended daily dose is 0.5 to 3 mg. In **Study V**, a single oral 1-mg dose was administered. It has an oral bioavailability of about 90% and an elimination half-life of 12 hours. The main metabolic pathways of alprazolam in humans are 4- and α-hydroxylation, and both hydroxy-metabolites are subsequently glucuronidated (Figure 4). Alprazolam is mainly hydroxylated by CYP3A4 and CYP3A5 (Gorski et al. 1999).

Amodiaquine is a 4-aminoquinoline used as an antimalarial drug in Africa. For some years, sulfadoxine-pyrimethamine has been the recommended first line antimalarial in several African countries, and commonly amodiaquine is the second line alternative (Kitua 1999). Amodiaquine in combination with artesunate is also an option recommended by the WHO for use in malaria control programmes (WHO 2001). For treatment of malaria, the recommended dose schedule is 10 mg/kg day 1, 10 mg/kg day 2 and 5 mg/kg day 3, i.e. in total 1750 mg to a 70-kg person. In Europe, amodiaquine was replaced by other antimalarials in the 1980s, due to reported agranulocytosis and hepatotoxicity during long-term prophylactic use (Olliaro et al. 1996). In **Study IV**, the subjects were given a single oral dose of 600 mg amodiaquine hydrochloride. Moreover, amodiaquine has been suggested for use as probe drug based on *in vitro* data showing selectivity for the CYP2C8 enzyme (Li et al. 2002).

Cocktail approach

The cocktail approach permits the simultaneous assessment of the activities of multiple DMEs in a single experimental session. The activities can be studied by fewer sampling occasions compared to assessing each enzyme separately. This is to advantage for the subject, and it also means that possible interfering factors such as concurrent drugs or diseases are the same for all enzymes studied. Any risk of pharmacokinetic or pharmacodynamic interactions between the probe drugs, or analytical interferences, is assessed during the validation of the cocktail. A number of phenotyping cocktails have been described using different drug combinations (Setiabudy et al. 1994; Frye et al. 1997; Streetman et al. 2000). In the Karolinska cocktail, the following probe drugs are administered: caffeine (CYP1A2), debrisoquine (CYP2D6), omeprazole (CYP2C19), losartan (CYP2C9) and quinine (CYP3A4) (Christensen et al. 2003). The cocktail approach also enables investigators to study the selectivity in induction and inhibition of a concomitant drug on the activity of different isoenzymes (Breimer and Schellens 1990).

DRUG ANALYSES

For details on drug analyses performed in this work see papers I-V.

Phenotyping was done with debrisoquine (**Study I, III and IV**), codeine (**Study III**), metoprolol (**Study III**), dextromethorphan (**Study III**), caffeine (**Study IV**), omeprazole (**Study IV**) and losartan (**Study IV**). Chloroquine was analysed from filter paper (**Study III).** Furthermore, the pharmacokinetics of amodiaquine (**Study IV**) and alprazolam (**Study V**) were studied. Assays of debrisoquine and bufurolol from incubates were done (**Study II**). During the studies, analyses were made from urine,

plasma, dried blood and incubates. The analyses included parent compounds and metabolites.

LC/MS was used for analyses of codeine (**Study III**) and alprazolam (**Study V**) and their metabolites. We developed and validated the alprazolam method within this thesis work. The other analyses were done on HPLC. All analyses in the thesis work were performed at Huddinge University Hospital with the exception of the metoprolol analysis that was done at AstraZeneca R&D.

PHARMACOKINETIC CALCULATIONS

*See Material and Methods sections in S**tudy IV and V**.*

STATISTICAL ANALYSES

Nonparametric methods were used in **Study I, II and III**. In **Study IV and V**, parametric methods including linear correlation analysis were used. Moreover, in **Study IV** the volunteers served as their own controls and intra-individual comparisons were made between phenotyping results using repeated measures analysis of variance (ANOVA). For statistical analysis the software StatMost version 2.01 for windows was used in **Study I**. Furthermore, Statistica for windows was used, version 5.0 for **Study II and III** and version 6.0 for **Study IV and V**. In all studies, a *P*-value < 0.05 was considered statistically significant.

RESULTS (STUDY I-V)

Details of the results are found in paper I-V.

STUDY I

We demonstrated that Tanzanians of Bantu origin have a generally decreased capacity to metabolize the CYP2D6 substrate debrisoquine, compared to Caucasian populations. The lower metabolic capacity in Tanzanians could not be explained by the partially or fully detrimental *CYP2D6* gene mutations analysed for. We detected heterogenous molecular genetic characteristics of the *CYP2D6* gene and found that none of the poor metabolisers of debrisoquine was homozygous for defective *CYP2D6* genes. The mutational profile indicated a closer association of the Tanzanian *CYP2D* locus to that of Zimbabweans rather than to that of Ethiopians.

STUDY II

A novel *CYP2D6* allele (*CYP2D6*29*) was found at a high allele frequency in the Tanzanian population. The corresponding enzyme was characterized *in vitro* after transfection of cDNA variants into COS-1 cells and yeast. The presence of this allele significantly caused a diminished rate of debrisoquine metabolism *in vivo*. Indeed, the *CYP2D6*29* allele, together with *CYP2D6*2*, was found to be the second most common *CYP2D6* allele with a frequency of 20%. Using the CYP2D6 substrate bufuralol, it was shown that CYP2D6.29 had only approximately 26% of the catalytic activity of the wild-type enzyme (CYP2D6.1) in the mammalian cell expression system and also lower activity (63% of CYP2D6.1) when debrisoquine was used as substrate. The data thus indicate altered substrate specificity of the CYP2D6.29 enzyme compared to the wild-type form when expressed in COS-1 cells.

STUDY III

Lack of correlation of different CYP2D6 phenotyping drugs had been reported from black populations (Woolhouse et al. 1985; Lennard et al. 1992). Additionally, it had been reported that the *CYP2D6*17* allele encoded an enzyme with changed substrate specificity compared to CYP2D6.1 and .2 *in vitro* (Oscarson et al. 1997). We wanted to study the role of this finding *in vivo* and therefore investigated the effect of the African-specific *CYP2D6*17* and *29 alleles in healthy Tanzanian subjects on the metabolism of four CYP2D6 probe drugs (codeine, debrisoquine, dextromethorphan, metoprolol) with control for interfering factors. Swedish subjects were used as a control group. We found that carriers of *CYP2D6*17* had significantly decreased rate of metabolism for two (debrisoquine and dextromethorphan) of the four probe drugs and that *CYP2D6*29* caused a reduced metabolism compared with *CYP2D6*1* and *2 for dextromethorphan and metoprolol but not for codeine and debrisoquine. There are thus difficulties in translating the effect of a certain mutation from one drug to another.

STUDY IV

It had previously been shown that the antimalarial drug chloroquine inhibited the CYP2D6 enzyme *in vivo* (Adedoyin, 1998). We investigated whether the structurally similar antimalarial drug amodiaquine also inhibited the drug metabolising enzymes *in*

vivo. We studied the effect of a single oral 600 mg dose of amodiaquine hydrochloride on the activities of four P450 enzymes as well as recovery from any such effects. We found that amodiaquine, its desethylated metabolite, or both inhibited the CYP2D6 (debrisoquine 4-hydroxylation; $P = 0.005$; ANOVA) and CYP2C9 (losartan oxidation; $P = 0.017$; ANOVA) enzymes and that this effect was selective, not affecting the CYP2C19 (omeprazole 5-hydroxylation) or CYP1A2 (caffeine N3-demethylation) enzymes. The inhibitory effect did not persist one week after drug intake. The decreases in CYP2D6 and CYP2C9 metabolic capacities were seen in 10 and 9 out of 12 subjects, respectively.

STUDY V

Some genetic *CYP3A5* variants have been reported to exist at much higher frequencies in black populations compared to Caucasians (Kuehl, 2001; Hustert, 2001). CYP3A5 might therefore contribute significantly to the metabolism of CYP3A substrates in Africa. Previous *in vitro* data indicated that alprazolam might be possible to use in order to distinguish between CYP3A4 and CYP3A5 activity *in vivo* (Hirota et al. 2001). Therefore, we validated a novel liquid chromatography-mass spectrometry method, which enabled us to quantify alprazolam, 4-hydroxyalprazolam and α-hydroxyalprazolam in all healthy subjects up to at least 24 hours after drug intake. The plasma concentration ratios (alprazolam/respective metabolite) of samples collected between 1 and 48 hours correlated significantly with all ratios of area under curve values of alprazolam/4-hydroxyalprazolam and alprazolam/α-hydroxyalprazolam. Hence, we suggest that it is possible to estimate alprazolam 4- and α-hydroxylation activities in subjects with a single plasma sample.

DISCUSSION

RELATIONSHIP BETWEEN CYP2D6 GENO- AND PHENOTYPES *(PAPER I, III)*

When **Study I** was performed, the only studies in which *CYP2D6* genotype was correlated to phenotype in black Africans, were one in Zimbabwe (Masimirembwa et al. 1993) and one in Ethiopia (Aklillu et al. 1996). Two studies from the west African countries Ghana and Gabon and one study from Tanzania were published in parallell to our study (Bathum et al. 1999; Griese et al. 1999; Panserat et al. 1999). Since, poor correspondance between CYP2D6 genotype and phenotype in Africans has continued to be reported, in contrast to Caucasian and Oriental populations where there has generally been good correspondance (Eichelbaum et al. 1982; Roh et al. 1996; Sachse et al. 1997; Gaedigk et al. 2003). In **Study I**, we were able to show that when using the antimode for debrisoquine, established in Caucasians, none of the PMs had two defective *CYP2D6* alleles. Moreover, in **Study III** we confirmed that using debrisoquine as phenotyping probe resulted in poor correspondance of genotype and phenotype because only one out of five Tanzanian PMs had two defect *CYP2D6* alleles. The seven Swedish PMs identified in the same study, all had two defect *CYP2D6* alleles. The finding is important because debrisoquine, together with sparteine and dextromethorphan, is one of the most commonly used probe drugs for CYP2D6. The subjects in **Study III** had undergone a screening procedure where sources of possible confounding factors for inhibition of P450 activity would be excluded, including concurrent infections and concomitant medications. Probably, the lack of concordance is partly due to higher frequency of partly detrimental alleles in black populations compared to Caucasians.

GENETIC REASONS FOR DECREASED CYP2D6 METABOLISM AMONG TANZANIANS (*PAPER I, II AND III*)

Several groups have undertaken systematic analyses of the *CYP2D6* gene in Caucasian populations (Marez et al. 1997; Sachse et al. 1997). Consequently, the *CYP2D* locus in Caucasians has been extensively characterised and a subset of alleles covering 99% certainty of prediction of phenotype has been identified (Sachse et al. 1997; Griese et al. 1998). Nonetheless, few consistent sequencing studies have been undertaken in black African populations. Masimirembwa et al (Masimirembwa et al. 1996) sequenced Zimbabwean subjects and identified the *CYP2D6*17* allele, which was shown to have a reduced function *in vivo* (Masimirembwa et al. 1996).

Probably, the alleles coding for nonfunctional enzymes in black populations have been identified, but the molecular reasons of IMs in Africans is not fully known (Griese et al. 1999; Wennerholm et al. 2002). In **Study I**, we compared Tanzanian subjects with EM phenotypes (i.e. including only subjects with the *CYP2D6*1* or **2* alleles) to Caucasian EMs. We showed that 41% of Tanzanian EMs had a debrisoquine MR > 1, whereas only 7% of the Caucasian EMs did. The same result was found in Ghanaians (Griese et

al. 1999); median MRs of *CYP2D6*1/*1* EMs were 0.50 in Ghanaians versus 0.28 in Caucasians, which translated into a 40% lower metabolic clearance (CL_{met}) in Ghanaians. These findings prompted us to screen for novel mutations in Tanzanian EM subjects (*CYP2D6*1/*1* or *CYP2D6*2/*2*). In **Study II**, two functional mutations, G1659 to A (causing V136I) and G3183 to A (causing V338M) were identified in a *CYP2D6*2/*2* sample (numbering of nucleotides is consistent with the CYP allele homepage but not with the original article; there is a discrepancy between the two systems of 88 nucleotides). The allele identified (*CYP2D6*29*) was common with an allele frequency of 20% in Tanzanians. Analysis of the distribution of *CYP2D6*29* in subjects phenotyped for debrisoquine revealed that this allele significantly reduced the the rate of debrisoquine hydroxylation *in vivo* compared to wild-type.

Do we now have the full explanation of the lower activity in Tanzanian intermediate metabolisers?
1) *CYP2D6*17* allele
2) *CYP2D6*41* allele
3) *CYP2D6*29* allele
4) Unknown mutations
5) Novel nonfunctional alleles *(CYP2D6*40* and **42)*

1) An allele frequency of 17% of the *CYP2D6*17* allele could not alone explain the general decrease in metabolic activity in Tanzanians as compared to Caucasian populations. In **Study III**, we compared debrisoquine MRs of subjects with (*CYP2D6*1* or **2*)/(*CYP2D6*1* or **2*) genotypes (i.e. having excluded subjects with *CYP2D6*17* and **29* alleles as well as those consistent with PM phenotype), and showed that Tanzanians had higher median MRs compared to Caucasians. All of these subjects had undergone a thorough screening procedure and should be devoid of apparent confounders. However, differences in diet or environmental toxins or other factors cannot be excluded. Interestingly, phenotyping with dextromethorphan, metoprolol and codeine revealed no such interethnic difference among subjects with (*CYP2D6*1* or **2*)/(*CYP2D6*1* or **2*) genotypes.

2) In addition, we studied whether a higher frequency of the lower activity allele, *CYP2D6*41*, in black populations compared to Caucasians may partly explain the lower metabolic capacity in African subjects. The frequency of the mutation in different alleles containing mutations characteristic of the *CYP2D6*2* allele (i.e. also the *CYP2D6*17* and **29* alleles; the possibility of the G-1584 variant in the African-specific *CYP2D6*17* and **29* alleles had not been assessed in the German population) was studied in the Tanzanian population (Table 6). We showed that 4% of the Tanzanians had the G-1584 mutation indicative of the *CYP2D6*2* allele (Wennerholm, unpublished data). In Tanzanians, the G-1584 variant was consistently detected in subjects with at least one *CYP2D6*2* allele. The *CYP2D6*2* allele, as identified in **Study I,** consists in fact of three different alleles in Tanzanians, *CYP2D6*2, *29* and **41*, and its frequency should be 4% instead of 40%. The low frequency of the *CYP2D6*2* allele is in contrast to the level in another African population, Ethiopians, where an allele frequency of 14% was identified, i.e. close to the level in Caucasians (Aklillu et al. 2002). Therefore, the lower frequency of the "true" *CYP2D6*2* allele (the allele with normal activity) in Tanzanians may contribute to the IM phenotype; i.e. it

would in fact not be Tanzanian EMs that are slow but Caucasian populations that are faster than previously thought.

Table 6. Presence of C-1584G mutation in African populations. Allele frequency of total alleles (%). Data from other population is shown.

Allele (SNP)	**Activity**	**Tanzanians**[a] n = 96	**Ethiopians**[b] n =122	**Germans**[c] n = 195
*CYP2D6*2* (G-1584)	normal[f]	4	14	22
*CYP2D6*41* (C-1584)	lower[f]	16	25	10
*CYP2D6*29*	lower[f]	20	ND[d]	ND[e]

[a]Wennerholm 2001, unpublished data.
[b]Data from Aklillu et al. 2002 (Ethiopians living in Ethiopia).
[c]Data from Raimundo et al. 2000 and Griese et al. 1998.
[d]Possibly includes *CYP2D6*29* allele as this was not screened for.
[e]In study by Marez et al. 1997, *CYP2D6*29* was only detected in < 0.1% of subjects and hence is not expected to be identified in Caucasians.
[f]Compared to wild-type.

3) The *CYP2D6*29* allele may contribute to the lower activity (Wennerholm et al. 2001; Gaedigk et al. 2002). Subjects with this allele were overrepresented among IMs compared to EMs in a study in African-Americans (Gaedigk et al. 2002).

4) Besides, to investigate whether other mutations in the 5'-flanking region of the *CYP2D6* gene contribute to the lower activity among EMs, four of the Tanzanian samples were analysed for the six SNPs in the 5'-flanking region identified by Raimundo et al. (Raimundo et al. 2000). The subjects had (*CYP2D6*1* or **2)/(CYP2D6*1* or **2)* genotypes, but with a debrisoquine MR > 1. However, none of the samples had any of the described mutations (Wennerholm, unpublished data).

5) *CYP2D6*40* and *CYP2D6*42* are novel nonfunctional alleles that seem to be African-specific (Gaedigk et al. 2002; Gaedigk et al. 2003). However, the respective frequencies in African-Americans were only 0.6 and 0.3%, and would hence not be of importance for the general shift in activity.

In addition, possible impact of environmental factors cannot be excluded. Comparing Ethiopians living in Ethiopia with those living in Sweden, Aklillu et al. showed that differences between the groups were not due to genetic factors and suggested that the slower debrisoquine hydroxylation activity in Ethiopians in Ethiopia was due to environmental factors (Aklillu et al. 2002). No such comparative study has been done in Tanzanians.

We conclude that the intermediate metaboliser phenotype among Tanzanians probably has a multifactorial background including presence of intermediate activity alleles such as *CYP2D6*17, *29* and **41* and impact of environmental factors. This work is of

principal interest because it illustrates to what extent detailed characterisation of the presence of mutated alleles can help to predict metabolic activity of various genetic variants of *CYP2D6*. In recent years, it has been found that for each detected mutated allele the predicted value of a specific genotype increases.

CYP2D6 SUBSTRATE SPECIFICITY (*PAPER III)*

There is poor phenotype-to-phenotype correlation between phenotypes determined with different CYP2D6 probe drugs in Africans (Masimirembwa and Hasler 1997) whereas in Caucasian and Oriental populations the metabolism of different CYP2D6 probe drugs has been shown to cosegregate (Eichelbaum et al. 1982; Horai et al. 1990). In black populations, PMs for CYP2D6 have varied from 0 to 9% (Masimirembwa and Hasler 1997), whereas in Caucasians they have been 5 to 10% and in Asians 0 to 1% (Bertilsson 1995). The variability in frequency of PMs in Africans is in line with the reported genetic heterogeneity of African populations (Tishkoff and Verrelli 2003). Also, phenotyping in different African populations with various probe drugs has shown either a unimodal distribution or relatively low frequencies of PM subjects when using antimodes established in Caucasian populations. The *CYP2D6*17* allele has been shown to contribute to the IM phenotype in black populations with frequencies ranging from 9 to 34%. The activity of the *CYP2D6*17* allele was studied *in vitro* using codeine and bufuralol, and it was shown that the CYP2D6.17 enzyme with the amino acid substitution T107I, R296C and S486T had a much higher K_m for bufuralol and codeine (Oscarson et al. 1997). The variant in which only the T107I amino acid change of CYP2D6.17 was introduced affected the K_m for codeine but not for bufurolol. The data indicated changed substrate specificity of CYP2D6.17 compared to CYP2D6.1 and CYP2D6.2. The finding also emphasise the importance of studying haplotypes and not single mutations.

We evaluated the *in vivo* effect of the CYP2D6.17 enzyme in **Study III**, and were able to show that carriers of *CYP2D6*17* were slower metabolisers when using debrisoquine or dextromethorphan as probe drugs than when codeine or metoprolol was used. A difference in substrate specificity was also evident from the study when debrisoquine was probe drug; 5 Tanzanian PMs were identified, but with the other three substrates there was only 1 subject identified as PM. A similar discrepancy had been found in a study in Nigerians, where there were 6 PMs with debrisoquine but none with metoprolol (Lennard et al. 1992) (Table 7). Also, in a study in Zambians, there were 2 PMs with debrisoquine but none of these was a PM with metoprolol (Simooya et al. 1993).

In a study with heterologously expressed enzymes, it was found that the CYP2D6.17 enzyme generally had reduced clearance (Bapiro et al. 2002). In contrast, the magnitude of the reduction depended on substrate (Table 8). The largest difference in CL_{int} was seen with dextromethorphan, which is in accordance with our results in **Study III** where we saw 17-fold reduction in subjects homozygous for *CYP2D6*17* compared to Tanzanians with *CYP2D6*1* and **2* alleles. Also, in a comparative study of three probe drugs performed in three ethnic groups, dextromethorphan was identified as the most suitable probe drug to identify outlying metabolic activity

Table 7. Frequency of CYP2D6 PMs in black African populations phenotyped with more than one probe drug (studies in chronological order)

Population (n, ethnicity)	Probe drugs: % PMs	Same PMs	Genotyping? (n)	r_s value (P-value)	2D6*17 (%)
Ghana (141)[a]	deb: 7, spa: 0	-	no	r_s 0.47	-
Nigeria (138, Yoruba)[b]	deb: 0, met: 0	-	no	r_s 0.54; n=102	
South Africa (98, Venda)[c]	deb: 4, met: 7	none	no	ND	-
Nigeria (165)[d]	deb: 4, met: 0, spa: 2	one the same[l]	no	r_s (met/spa) 0.51; P < 0.001 r_s (deb/met) 0.16 NS r_s (deb/spa) 0.31 NS; n=82	-
Zambia (102, different)[e]	deb: 2, met: 1	none	no	r_s 0.60; P < 0.001; n=88	-
Zimbabwe (94, Shona)[f]	deb: 2, met: 5	yes	yes (80)	r_s 0.67; P <0.001; n=89 EMs	34
Ghana-American (21, Canadian nationality)[g]	deb: 0, dex: 0, spa: 0	-	yes (21)	r_s (deb/spa) 0.72; P < 0.002 r_s (deb/dex) 0.52; NS r_s (dex/spa) 0.38; NS	15
Ghana (201)[h]	deb: 3, spa: 4	4 were the same	yes (193)	r_s 0.63; P < 0.001	28
Tanzania (35, Bantu)[i,m]	deb (5 subjects), met (1 subject), dex (1 subject), cod[k]	yes	yes (35)	r_s 0.72 - 0.89; P < 0.001[m]	17
Caucasians (38)[j]	deb, spa	-	no	r_s 0.91; P < 0.001	-

deb=debrisoquine, spa=sparteine; met=metoprolol; dex=dextromethorphan, cod=codeine. r_s = spearman rank correlation coefficient; ND = not done; NS = not statistically significant. [a-j](Eichelbaum et al. 1982; Woolhouse et al. 1985; Iyun et al. 1986; Sommers et al. 1989; Lennard et al. 1992; Simooya et al. 1993; Masimirembwa et al. 1996; Droll et al. 1998; Griese et al. 1999; Wennerholm et al. 2002); [k]No antimode established; [l]Two were not tested with all drugs; [m]Subjects were selected based on genotype.

(Droll et al. 1998). In **Study III**, we showed that when debrisoquine was probe drug there was a 10-fold difference between subjects homozygous for *CYP2D6*17* and those with *CYP2D6*1* and **2* alleles. This is in contrast to the results by Bapiro et al. (Bapiro et al. 2002), where debrisoquine was the least sensitive probe drug as determined *in vitro*. However, our results are in accordance with those from another recent *in vitro* study (Marcucci et al. 2002), where the CL_{int} of debrisoquine with CYP2D6.17 was 22% of that of CYP2D6.1 (Table 8). Our results are also supported by a study in healthy volunteers (n = 359) where subjects homozygous for *CYP2D6*17* had a 6-fold difference in median debrisoquine MR when compared to subjects with *CYP2D6*1* and **2* alleles (Leathart et al. 1998). Comparison of percent reduction in clearance of 10 CYP2D6 substrates, including some antidepressants, of the CYP2D6.17 enzyme revealed a range in effect from 13 to 81% reduction compared to CYP2D6.1, which is consistent with the observed substrate specificity (Bapiro et al. 2002).

Table 8. Substrate specificity as observed with the CYP2D6.17 enzyme compared to CYP2D6.1 *in vitro*

Substrates	System	Activity compared to CYP2D6.1	Reference
Codeine, bufuralol	Yeast	2D6.17 had 5-fold higher K_m (codeine; bufuralol)	(Oscarson et al. 1997)[a]
Codeine, bufuralol	COS-1 cells	2D6.17 had 20% of 2D6.1 activity (bufuralol)	(Oscarson et al. 1997)[a]
Bufuralol, debrisoquine, metoprolol, dextromethorphan	Yeast	CL_{int} 25% of 2D6.1 (bufuralol); CL_{int} 15% of 2D6.1 (dextromethorphan); CL_{int} 70% of 2D6.1 (debrisoquine); CL_{int} 33% of 2D6.1 (metoprolol);	(Bapiro et al. 2002)
Dextromethorphan, bufuralol, debrisoquine	Baculovirus expression system	CL_{int} 18% of 2D6.1 (dextromethorphan); CL_{int} 22% of 2D6.1 (bufuralol); CL_{int} 22% of 2D6.1 (debrisoquine)	(Marcucci et al. 2002)
Dextromethorphan, bufuralol, debrisoquine	COS-7 cells	CL_{int} 25% of 2D6.1 (dextromethorphan); CL_{int} 37% of 2D6.1 (bufuralol)	(Marcucci et al. 2002)

CL_{int} calculated as V_{max}/K_m.

[a]The enzyme used had a methionin in position 374 instead of valine, a SNP that has since been shown to affect activity (Ellis et al. 1996).

DRUG-DRUG INTERACTIONS INVOLVING ANTIMALARIALS (*PAPER IV*)

Relatively few studies are pursued on metabolic pathways and inhibitory potential of antimalarial drugs (Table 9). These drugs are common in Africa and combination treatment of antimalarials is becoming more common (White 1999) (http://www.who.int/tdr/research/finalreps/pdf/fr44.pdf). Therefore, any inhibition of P450 enzymes may have clinical impact. Amodiaquine (AQ) and its major metabolite N-desethylamodiaquine (DAQ) have been shown to inhibit primarily CYP2D6 in a system of recombinant enzymes (Bapiro et al. 2001). Also, in patients, a single 600-mg amodiaquine dose given one hour prior to regular chlorpromazine intake caused a 2.5-fold increase in chlorpromazine levels compared to baseline levels (Makanjuola et al. 1988). Because chlorpromazine is primarily metabolised by the CYP2D6 enzyme (Muralidharan et al. 1996) this indicated that the increased drug levels were likely to be caused by the inhibitory effects of AQ on the CYP2D6 enzyme. Nonetheless, this study was small and needed confirmation. In our study in healthy volunteers, we were able to show that a 600-mg amodiaquine dose, i.e. one third of that used during treatment, significantly inhibited debrisoquine (CYP2D6) and losartan (CYP2C9) metabolism and that the effects were reversible. The effect was selective and did not inhibit CYP1A2 and CYP2C19 activities as measured by caffeine and omeprazole metabolism, respectively.

In the study we did not assess inhibition of CYP3A4 activity. There was no prior indication that AQ would inhibit CYP3A4. Baune et al. showed that in human hepatic microsomes, it was only quinine and quinidine out of 11 antimalarials (AQ being one of them) that inhibited the metabolism of the CYP3A4 substrate halofantrine (Baune et al. 1999). Also, Bapiro et al. who used testosteron metabolism to assess CYP3A4 activity *in vitro* detected no inhibition of CYP3A4 activity by AQ or DAQ (Bapiro et al. 2001). Moreover, AQ was replaced by other antimalarials in Europe in the 1980s, due to reported agranulocytosis and hepatotoxicity during long-term prophylactic use (Olliaro et al. 1996). We studied the risk profile of AQ prior to the study and made extensive consultations regarding its safety. Thereafter we decided to perform the study but not to include quinine (the CYP3A4 probe drug in the Karolinska cocktail), which is structurally alike AQ, as both these drugs have been associated with adverse drug reactions.

The 4-aminoquinoline, amodiaquine, was the first antimalarial drug found to be metabolised by CYP2C8 *in vitro* (Li et al. 2002). Other antimalarial drugs such as quinine, quinidine, primaquine and artemisinin had previously been identified to be metabolised by CYP3A4 (Table 9). However, recently it was shown that also another 4-aminoquinoline, chloroquine, is metabolised *in vitro* by CYP2C8 and CYP3A4, and to a minor extent by CYP2D6 (Projean et al. 2003). We studied the pharmacokinetics of AQ and DAQ, and it was apparent that DAQ had multiexponential elimination by visual inspection. Few reports on interindividual variability of AQ kinetics are available (Pussard et al. 1987; Winstanley et al. 1987). The slow elimination of DAQ (half-life of 12 days in our study) is similar to that of chloroquine and desethylchloroquine which in a triexponential modelling gave even longer estimated elimination half-lives (Gustafsson et al. 1987).

Table 9. Antimalarial drugs that are substrates of cytochrome P450 enzymes or that inhibit or induce cytochrome P450 enzymes

Drug	Metabolised by (*in vivo*)	Metabolised by (*in vitro*)	Reference	Inhibits	Induces	Reference
amodiaquine	-	CYP2C8, other minor	(Li et al. 2002)	CYP2D6[a], 2C9[a]	-	Wennerholm, in manuscript
artesunate	-	CYP2A6	(Li et al. 2003)	-	-	-
artemisinin	-	CYP2B6, 3A4	(Svensson and Ashton 1999)	CYP1A2[b]	CYP2C19[a]	(Svensson et al. 1998; Bapiro et al. 2001)
atovaquone	not metabolised	-	(Giao and de Vries 2001)		CYP1A2[b]	(Bapiro et al. 2001)
chloroquine	-	CYP3A4, 2C8, 2D6	(Projean et al. 2003)	CYP2D6[a, b]	-	(Masimirembwa et al. 1995; Simooya et al. 1998)
halofantrine	CYP3A	CYP3A4, 3A5, to a minor extent 2C8	(Halliday et al. 1995; Baune et al. 1999; Charbit et al. 2002)	CYP2D6[a], 3A4[b]	-	(Halliday et al. 1995; Simooya et al. 1998)
primaquine	-	CYP1A2, 3A4	(Li et al. 2003)	CYP2D6[b]	CYP1A1[b], 1A2[b]	(Masimirembwa et al. 1995; Bapiro et al. 2002)
proguanil	CYP2C19	-	(Birkett et al. 1994; Wright et al. 1995)	-	-	-
quinidine	CYP3A4	-	(Min et al. 1996)	CYP2D6, 3A4[b]	-	(Muralidharan et al. 1991)
quinine	CYP3A4	CYP3A4, other minor	(Zhao et al. 1996; Mirghani et al. 1999)	CYP2D6[a], 2C8[b], 3A4[b]	CYP1A1[b], 1A2[b]	(Steiner et al. 1988; Baune et al. 1999; Ong et al. 2000; Bapiro et al. 2002)

A drug has been included in the list if there is published evidence that it is metabolised, at least in part, via that isoform. [a]Tested in healthy subjects. [b]Tested *in vitro*

Interactions may occur when using combination treatment for malaria (cf. Table 9). Besides, because malaria treatment is common in Africa, there are also risks of interactions with other commonly used drugs, e.g. potential interactions between AQ and drugs for psychiatric disorders as such drugs are commonly CYP2D6 substrates (http://www.who.int/medicines/organization/par/edl/infedl11group.html) (NEDLIT 1991). Another antimalarial drug that has strong CYP2D6 inhibitory effect is quinidine. It is one of the more potent CYP2D6 inhibitors and with doses used in malaria treatment (15 mg/kg) there are risks for drug-drug interactions. In a study by Steiner et al. (Steiner et al. 1988) 800 mg quinidine for 2 days decreased the excretion of the CYP2D6-dependent metabolite 2-hydroxydesipramine by 96% in healthy subjects.

There are data available on possible interactions with antimalarials based on *in vitro* data (Bapiro et al. 2001; Bapiro et al. 2002; Li et al. 2003), where also predictions of *in vivo* drug hepatic clearance are made. Now, it would be important to evaluate the clinical relevance of the potential drug interactions in malaria patients. Impact of malaria on pharmacokinetic parameters has been shown for quinine (increase in half-life by 50% (Krishna and White 1996)) and chloroquine (C_{max} two times higher (Na-Bangchang et al. 1994)). In addition, the concentration of chloroquine has been shown to be 100 times higher in liver than in plasma (Mackenzie 1983) indicating risk for interactions in the liver. Based on our results, we can conclude that AQ has potential to cause drug-drug interactions and should be further investigated in malarial patients treated with drug combinations containing amodiaquine.

ALPRAZOLAM AS A POSSIBLE PROBE DRUG FOR MEASURING CYP3A ACTIVITY (*PAPER V*)

In contrast to Caucasian populations, CYP3A5 is commonly expressed in African populations (Hustert et al. 2001; Kuehl et al. 2001). The impact of CYP3A5 expression on the drug metabolism of CYP3A drugs is not known. Therefore, a tool for assessment of both CYP3A4 and CYP3A5 activities using a single probe drug would be of value. *In vitro* data have shown a preference for formation of 4-hydroxyalprazolam by CYP3A4 and that of α-hydroxyalprazolam by CYP3A5 (Gorski et al. 1999; Hirota et al. 2001). The lack of a suitable method for quantification of alprazolam, 4-hydroxyalprazolam and α-hydroxyalprazolam prompted us to develop and validate a novel method. Upon studying the pharmacokinetics of the three substances, we were able to show that the two metabolites had formation-dependent kinetics and hence that it would be possible to assess the 4-hydroxylation and α-hydroxylation activities using a single plasma sample. This was confirmed as both the $AUC_{(0-\infty)}$ ratio of alprazolam/4-hydroxyalprazolam and the $AUC_{(0-\infty)}$ ratio of alprazolam/α-hydroxyalprazolam, and the corresponding clearances, correlated significantly with all plasma concentration ratios collected between 1 and 48 hours. The single plasma sampling makes the method well suited for studies in larger populations.

The 12 subjects in the study were selected so that they represented variable quinine metabolic ratios (quinine/3-hydroxyquinine). Indeed, we observed a significant correlation (r = 0.64; P = 0.045) between the quinine ratio and the AUC-ratio of alprazolam/both metabolites when including the subjects expressing only CYP3A4 (n=10). This is in accordance with quinine being metabolised primarily via CYP3A4 (Mirghani, unpublished data).

The importance of CYP3A5 in total CYP3A activity is debated. Kuehl et al. (Kuehl et al. 2001) showed that CYP3A5 protein constituted more than 50% of total hepatic CYP3A content in one-third of Caucasian livers and over one-half of African-American livers. However, these results were contradicted by Westlind Johnsson et al. (Westlind-Johnsson et al. 2003), who were able to show that in Caucasians the proportion of CYP3A5 of total hepatic CYP3A would only be 17% in the 11% of subjects who had quantifiable levels.

Kuehl et al. (Kuehl et al. 2001) were also showed that the mean reaction velocities using midazolam (total rate of hydroxylation) were 2-fold higher for microsomes prepared from livers from African-Americans with at least one *CYP3A5*1* allele, compared with livers of people homozygous for *CYP3A5*3*. Midazolam (Goh et al. 2002; Shih and Huang 2002; Floyd et al. 2003) and alprazolam (Gashaw et al. 2003) have been used to study the effect of CYP3A5 expression on CYP3A activity also in healthy subjects. However, no significant differences in AUC or clearance of midazolam were observed between subjects expressing CYP3A5 and those not (Goh et al. 2002; Shih and Huang 2002; Floyd et al. 2003). In these three studies only levels of midazolam and 1'-hydroxymidazolam, not 4'-hydroxyalprazolam, were analysed. In the study by Gashaw et al. (Gashaw et al. 2003) no difference in the 10-hour alprazolam concentration was seen between subjects heterozygous for *CYP3A5*1/*3* genotype (n = 6) compared to all subjects (n = 96). It is difficult to draw conclusions from this study because the levels of the two hydroxylated metabolites were not investigated and the number of subjects expressing CYP3A5 was low.

Midazolam is in some aspects an inconvenient probe drug for population-based studies, due to the necessity of multiple blood samples and its adverse drug reaction profile. In addition, alprazolam may be a more suitable probe to assess liver CYP3A activity since it does not undergo extensive first pass metabolism and has an oral bioavailability of 90% (Greenblatt and Wright 1993). It is still unclear whether alprazolam is a substrate or not for P-gp, but it has been suggested that P-gp would not play a clinically significant role in the disposition of alprazolam (Yasui et al. 2000). To evaluate CYP3A metabolic capacity it would be an advantage to use a probe drug without affinity for P-gp to avoid adding variability in uptake onto the metabolic variation.

Expression of CYP3A5 was shown to be important for the disposition of the immunosuppressive drug tacrolimus. Patients expressing CYP3A5 and on treatment with tacrolimus were shown to need higher doses than patients homozygous for the *CYP3A5*3* allele to achieve the same blood concentrations (Hesselink et al. 2003; Thervet et al. 2003; Zheng et al. 2003). Tacrolimus is also a P-gp substrate and a correlation between tacrolimus blood levels and *MDR1* G2677T and C3435T was also seen in two studies (Macphee et al. 2002; Zheng et al. 2003) but not in a third study (Hesselink et al. 2003).

The metabolic ratios of alprazolam to respective metabolite in a single plasma sample are suggested to reflect the alprazolam 4- and α-hydroxylation activities. In future, it is important to study these activities in populations where CYP3A5 is expressed at high frequency (e.g. in Tanzania, 77% carried at least one functional *CYP3A5*1* allele) in order to find out the relative importance of the two enzymatic pathways to the *in vivo* clearance of alprazolam. This knowledge can form the basis for extrapolation of metabolic patterns of other drugs metabolized by both CYP3A4 and CYP3A5, which can be of importance for predictions of suitable drug dosages and risk of drug interactions.

CONCLUSIONS

- Tanzanians of Bantu origin had a generally decreased capacity to metabolise the CYP2D6 substrate debrisoquine with 59% of Tanzanian EMs having debrisoquine MR > 1 compared to 20% of Caucasian EMs.

- The lower metabolic capacity in Tanzanians could not be explained by the partially or fully detrimental *CYP2D6* mutations analysed for. Furthermore, none of the PMs of debrisoquine was homozygous for defective *CYP2D6* genes in contrast to the situation in Caucasians.

- A *CYP2D6* allele, *CYP2D6*29*, identified in the Tanzanian population caused reduced catalytic activity when expressed in COS-1 cells. The allele was common with an allele frequency of 20%.

- The original *CYP2D6*2* allele consists in fact of three different alleles in Tanzanians; *CYP2D6*2, *29* and **41*, and its frequency is therefore 4% instead of 40% as reported earlier.

- The IM phenotype among Tanzanians probably has a multifactorial background including presence of intermediate activity alleles such as *CYP2D6*17, *29* and **41* and impact of environmental factors. When assessing metabolic capacity of a population, it is important to not only study PM frequencies, but also frequency of subjects with IM phenotype.

- Carriers of *CYP2D6*17* were slower metabolisers when using debrisoquine and dextromethorphan as probe drugs than when codeine or metoprolol was used. The substrate specificity of the CYP2D6.17 enzyme seems to explain part of the discrepancies from earlier phenotyping studies in black populations. In Tanzanians, there are difficulties in translating the results of phenotyping from one drug to another.

- A single 600-mg dose of the antimalarial drug amodiaquine hydrochloride, i.e. one third of that used during treatment, significantly inhibited debrisoquine (CYP2D6) and losartan (CYP2C9) metabolism. Due to the generally lower capacity of the CYP2D6 enzyme in Tanzanians, potential inhibition by antimalarials may have to be considered during co-medication with drugs that are substrates for CYP2D6.

- In healthy Swedish subjects, the plasma concentration ratios (alprazolam/respective metabolite) between 1 and 48 hours correlated significantly with all ratios of area under curve values of alprazolam/4-hydroxyalprazolam and alprazolam/α-hydroxyalprazolam. Hence it was possible to assess the 4-hydroxylation and α-hydroxylation activities using a single plasma sample. The single plasma sampling makes the method well suited for studies of CYP3A4 and 3A5 activities in larger populations.

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