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**CYTOCHROME P450 PHARMACOGENETICS:
IMPLICATIONS FOR ANTICANCER AND
WARFARIN THERAPY**

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Cytochrome P450 Pharmacogenetics: Implications for Anticancer and Warfarin Therapy

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To my family

ABSTRACT

There is a pronounced interindividual variability in the drug disposition, response and toxicity. Pharmacogenetics aims at identifying genetic biomarkers that could help to increase the drug efficacy, reduce adverse drug reactions and contribute to the development of personalized medicine. Polymorphic cytochrome P450 genes encoding heme-containing ER membrane bound monooxygenases that metabolize xenobiotics, drugs and also endogenous compounds, strongly contribute to interindividual variations in drug response. In the present work we have investigated molecular mechanisms of the adverse drug reactions caused by the polymorphic changes in the cytochrome *P450 2C8 (CYP2C8)*, *CYP2C9* and *CYP3A4* genes and, in addition developed a novel enzymatic assay for CYP2W1.

CYP2W1, a P450 enzyme mainly expressed in colon cancer, has an unknown function and no specific substrates were previously identified. Despite the unusual inverse membrane topology of CYP2W1 that allows its glycosylation but prevents interaction with the redox partner, P450 oxidoreductase (POR), we discovered specific CYP2W1-mediated metabolism of indolines, which indicates the presence of a yet unknown electron transport chain in the lumen of ER.

CYP2C9 catalyzes the metabolism of anticoagulant drug warfarin. We characterized the newly discovered rare *CYP2C9*35* allele encoding an enzyme with two amino acid changes including Arg125Leu that was found in a patient with warfarin hypersensitivity. The expression of the variant proteins in the mammalian HEK293 cell system showed abolished activity of the CYP2C9.35 enzyme towards warfarin in NADPH supported reaction, but the enzyme could be activated when NADPH was replaced by hydroperoxides. This indicates that CYP2C9.35 is unable to receive electrons from POR because of the impaired interaction with this redox partner. Indeed, *in silico* modeling confirmed this conclusion showing disrupted salt bridges between CYP2C9.35 and POR due to the mutation of key residues involved in such interaction.

CYP3A4 is one of the key enzymes, which metabolizes the anticancer drug paclitaxel. In a cohort of 236 Spanish patients with a paclitaxel induced neuropathy whole exome sequencing revealed the presence of different rare *CYP3A4* gene variants, *CYP3A4*8*, *CYP3A4*20*, *CYP3A4*25* (p.Pro389Ser) and *CYP3A4*27* (p.Leu475Val), the latter two previously not described. The expression of these two novel gene variants in HEK293 cells revealed that the corresponding enzymes are more unstable than the CYP3A4.1 enzyme and carriers of these rare CYP3A4 variants had much higher risk for neuropathy and a need in paclitaxel treatment modifications. The data indicate enrichment of these rare defect *CYP3A4* alleles in the group of the paclitaxel induced neuropathy patients and suggest that genotyping of *CYP3A4* defective variants may provide a basis for paclitaxel treatment individualization.

Based on previous data indicating a role for the defective *CYP2C8*3* allele for paclitaxel induced neuropathy, we also investigated the influence of this polymorphism on paclitaxel induced neuropathy and neuropathy risk in 148 patients receiving paclitaxel as well as the CYP2C8.3 catalyzed metabolism of paclitaxel in a mammalian expression system. However, in contrast to many other studies we found no significant effect of this allele on paclitaxel induced neuropathy or paclitaxel metabolism *in vitro*.

In conclusion, our data indicate the importance of rare genetic CYP variants for induction of selective drug induced adverse reactions and emphasize the necessity of more extensive genetic analyses, *e.g.* whole exome sequencing, before fully individualized drug therapy can be achieved.

LIST OF SCIENTIFIC PAPERS

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

- I. Gomez A, Nekvindova J, Travica S, **Lee M-Y**, Johansson I, Edler D, Mkrchian S, Ingelman-Sundberg M. (2010) Colorectal cancer specific cytochrome P450 2W1 (CYP2W1): intracellular localization, glycosylation, and catalytic activity. *Molecular Pharmacology*. 78: 1004-1011

- II. **Lee M-Y**, Borgiani P, Johansson I, Oteri F, Mkrchian S, Falconi M[§], Ingelman-Sundberg M[§] (2014). High warfarin sensitivity in carriers of *CYP2C9*35* is determined by the impaired interaction with P450 oxidoreductase. *The Pharmacogenomics Journal*, 2014 Aug;14(4):343-9

- III. Apellániz-Ruiz M[§], **Lee M-Y**[§], Sánchez L, Gutiérrez-Gutiérrez G, Calvo I, García-Estévez L, Sereno M, García-Donás J, Castelo B, Guerra E, Leandro-García L.J, Cascón A, Johansson I, Robledo M, Ingelman-Sundberg M, Rodríguez-Antona C (2014). Whole-exome sequencing reveals defective CYP3A4 variants predictive of paclitaxel dose-limiting neuropathy. *Clinical Cancer Research*, 2014 Nov 14. Epub ahead of print.

- IV. **Lee M-Y**, Apellániz-Ruiz M, Johansson I, Vikingsson S, Bergmann T-K, Green H, Rodríguez-Antona C, Ingelman-Sundberg M (2014). The role of cytochrome P450 2C8*3 (*CYP2C8*3*) in paclitaxel metabolism and paclitaxel-induced neurotoxicity. (*manuscript*)

§ Equal contribution

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LIST OF ABBREVIATIONS

ACN	acetonitrile
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary DNA
CIPN	chemotherapy induced peripheral neuropathy
CL_{int}	intrinsic clearance calculated as $CL_{int} = V_{max}/K_m$
CYPs (or P450s)	cytochrome P450s
DHPLC	denaturing high performance liquid chromatography
DMSO	dimethylsulfoxide
EtOH	ethanol
gDNA	genomic DNA
g	relative centrifugal force
HPLC	high-performance liquid chromatography
HEK293	human embryonic kidney cells
K_m	Michaelis constant- substrate concentration that gives half maximal velocity of an enzymatic reaction
MeOH	methanol
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NSAIDs	Non-steroidal anti-inflammatory drug
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
RCR	polymerase chain reaction
PIPn	paclitaxel induced peripheral neuropathy
POR	P450 (cytochrome) oxidoreductase
RCF	relative centrifugal force
rtPCR	reverse transcription PCR
SDS	Sodium dodecyl sulfate
SNP	single nucleotide polymorphism
UV	ultraviolet

VKORC	vitamin K epoxide reductase complex
V_{\max}	the maximal velocity of a reaction
WES	whole-exome sequencing

1 INTRODUCTION

1.1 Cytochrome P450 Superfamily

Cytochrome P450s (CYP) are heme-containing enzymes embedded in the membrane of the endoplasmic reticulum of eukaryotic cells. The prosthetic group of CYPs, heme is non-covalently bound to the apoprotein and the name 'cytochrome P450' is derived from the fact that the cytochrome exhibits a spectral absorbance maximum at 450 nm when reduced (Fe^{2+} -heme) and complexed with carbon monoxide [1, 2]. In general, cytochrome P450s play important roles in the conversion of xenobiotics or endogenous compounds into the corresponding metabolites, which are further conjugated by phase II enzymes in course of detoxification or biotransformation pathway and then excreted by urine. The human genome encodes 57 cytochrome P450 enzymes, roughly a third of which are involved in the biosynthesis of essential sterols, signaling molecules or regulatory factors. Another third is largely devoted to the metabolism of xenobiotics. The function and substrates of the remaining (orphan) enzymes are still unclear [3, 4].

Nomenclature system

The Cytochrome P450 nomenclature is based on amino acid sequence homology, which allows classifying isoenzymes into families (>40% homology, *e.g.* CYP2), subfamilies (>55%, *e.g.* CYP2C) and individual enzymes (*e.g.* CYP2C9) [5-7]. The polymorphic alleles with nucleotide variations are named according to the guidelines [7], as to which the gene and the allele name are separated by an asterisk followed by Arabic numerals designating the specific allele (*e.g.* CYP2C9*35). The names for the corresponding proteins have a period between the name of the gene product and the allele number (*e.g.* CYP2C9.35). The thesis follows this nomenclature system: *see* the CYP allele nomenclature database (www.cypalleles.ki.se).

P450 mediated drug metabolism

Enzymes that belong to family 1-3 are primarily involved in the metabolism of majority of xenobiotics including clinically used drugs, and therefore considered as major drug metabolizing enzymes. The contribution of individual cytochrome P450 isoforms to the metabolism of several major classes of drugs is presented on Figure 1 [8].

CYP3A4 and 2C9 are two of the most abundant isoforms, as compared with the other P450s metabolizing nearly 50% of the known drugs (Figure 1). Polymorphism of P450 genes, inducibility, and other factors including age, sex, *etc.* may contribute to the variability of drug metabolism.

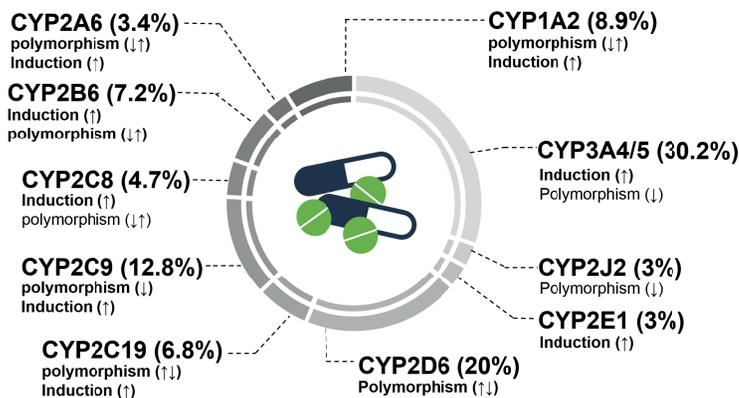


Figure 1 Contribution of known CYPs to the metabolism of 248 drugs (modified from [8]). Arrows indicate increase or decrease in the activities of corresponding CYPs. (%) indicate the fraction of total 248 drugs metabolized by corresponding CYP.

One specific type of CYP mediated metabolism is the activation of prodrugs, which are transformed by P450s into active metabolites, whereas prodrugs themselves display lower or absent pharmacological activity. For instance, CYP2C9 (and CYP3A4) plays an important role in the metabolism of losartan, highly selective angiotensin II receptor type 1 (AT₁) antagonist, and the oxidized metabolite E3174 is 10-40 fold more potent in blocking the target AT₁ receptor compared to losartan itself [9].

Some P450s are expressed in extra-hepatic tissues and often these are orphan enzymes with unknown functions. One of such orphan P450s, CYP2W1 is expressed preferentially in colon tumors [10]. Due to such specific expression profile and the ability to convert duocarmycin prodrugs into cytotoxic metabolites, CYP2W1 was recently suggested as a potential target in the treatment for colorectal cancer [4, 11, 12].

Structure and function of cytochrome P450

A typical monomeric molecular weight of cytochrome P450 proteins is approximately 45,000 – 55,000 Daltons. This hemoprotein contains specific sites for oxygen binding/activation and also substrate (e.g. drugs) binding pockets. Particular regions of CYP proteins are thought to be involved in membrane anchoring, binding to cytochrome b₅ (cyt b₅) or P450 oxidoreductase (POR). A highly conserved region near the carboxy terminus is responsible for heme binding via the invariant cysteine residue. It was shown that mostly in the drug metabolizing CYPs, a fraction of protein molecules (2-3%) can be inversely incorporated into the ER-membrane (with a bulk of the protein facing the ER lumen instead of the cytosol) dependent on the specific features of the N-terminal anchoring sequence [13], however predominantly luminal orientation found in the case of CYP2W1 is unique. (Paper I, [14])

Due to the length of CYP proteins (approximately 490 residues), the X ray crystallography technique is applicable more than the NMR method for the resolution of their three-dimensional structures. During the last decade the technical advances in this area contributed significantly to the elucidation of the structures of many mammalian membrane bound CYPs. A number of crystal structures of mammalian CYPs, in particular CYP2C5, 2C8, 2C9, 3A4, 2D6, 2B4, 2A6 and 1A2 are now available in the PDB bank [15]. This allowed the development of various computational approaches that are currently applied for the quantitative prediction of pharmacokinetics, the study of the nature of interaction between the enzyme and its substrates, and also CYPs' redox partners [15, 16]. In addition, molecular dynamic simulations and subsequent substrate-docking simulations are often applied to study the mechanisms of enzymatic catalysis.

Catalytic cycle of cytochrome P450

The catalytic turnover cycle of cytochrome P450 enzymes requires two electrons per substrate oxidation event as shown in Figure 2. For all of the mammalian drug-metabolizing cytochrome P450 enzymes these electrons are provided by P450 oxidoreductase (POR), although the second electron can sometimes be delivered by cytochrome b_5 [17]. Under certain experimental conditions organic hydroperoxides may also serve as an electron source bypassing the POR and reducing thus the cytochrome P450. This pathway is known as a peroxide shunt (Figure 2).

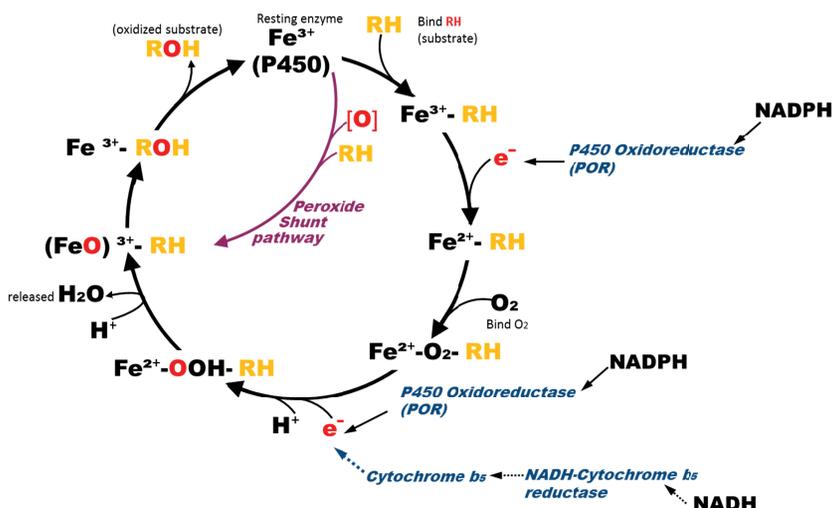


Figure 2 Catalytic cycle of cytochrome P450.

RH represents the drug substrate and ROH the corresponding hydroxylated metabolite. Blue letters indicate proteins donating single electron to P450. Purple arrow, peroxide shunt pathway.

1.2 Pharmacogenetics of P450 and Clinical Implications

What is pharmacogenetics?

Drug metabolizing CYP enzymes are highly polymorphic, which is one of the main factors determining interindividual differences in drug response [18]. Indeed, these variations can lead to significant decrease of efficacy of clinical drugs and/or exacerbate adverse drug reactions (ADR) [19]. Although other factors, such as disease heterogeneity, environmental factors, *etc.* may aggravate ADRs, the genetic factors have been extensively studied due to the relatively simple genotyping methodologies applicable to the individual patients.

Considerable progress in understanding the structure, regulation and function of cytochrome P450s has contributed to elucidation of the details of the mechanisms behind the functional changes associated with its polymorphisms, and as such linking them to the variations in the drug disposition and response. The latter can be manifested by the absence of pharmacological effect in case of the high enzymatic activity of a particular CYP or, in opposite, by the adverse drug reactions (ADR), due to the low activity of a corresponding enzyme. Most cases of ADRs are dose-related, and they often occur when drugs have a narrow therapeutic index (*e.g.*, hemorrhage with oral anticoagulants, *see Introduction 1.2.1*) [20, 21].

1.2.1 CYP2C9 polymorphism and warfarin therapy

CYP2C9 is one of the most abundant hepatic CYP enzymes responsible for approximately 13% of the metabolism of clinically important drugs including warfarin, losartan and diclofenac ([22], Figure 2). The *CYP2C9* gene is highly polymorphic and 58 variant alleles have been listed in the Human Cytochrome P450 Allele Nomenclature Database until November 2014 (*cypalleles.ki.se*). Among them, defective *CYP2C9*2* and *CYP2C9*3* were the most thoroughly investigated. The *CYP2C9*2* and *CYP2C9*3* encode enzymes with Arg144Cys and Ile359Leu substitutions, respectively. In Caucasians, the allele frequencies of the *CYP2C9*2* and *CYP2C9*3* are 17% and 6% respectively, whereas they are much rarer in Asians [22]. Both alleles are associated with significantly decreased enzyme activity toward many drugs, such as diclofenac [23], losartan [24] and others [25, 26] *in vivo* and *in vitro*.

The clinical importance of the CYP2C9 polymorphism is exemplified by the metabolism of the anticoagulation drug warfarin, a vitamin K antagonist. This widely used oral anticoagulant has a narrow therapeutic window meaning that too high doses may cause very serious and possibly lethal bleedings. Warfarin reduces coagulation by inhibiting the vitamin K epoxide reductase complex (VKORC), an enzyme necessary for vitamin K dependent

production of functional coagulation factors. Warfarin is administered as a racemic mixture and the most potent (*S*)-enantiomer is inactivated by CYP2C9. An overview of warfarin and Vitamin K mechanisms is presented on Figure 3.

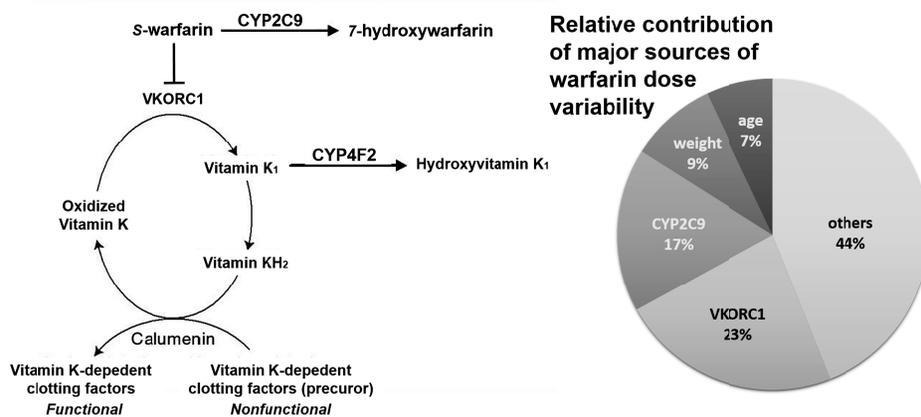


Figure 3. Schematic warfarin and vitamin K metabolism pathways and the relative contribution of major sources of warfarin dose variability. Left panel, *S*-warfarin is metabolized predominantly to 7- hydroxyl metabolites via CYP2C9. Warfarin exerts its anticoagulant effect through inhibition of its molecular target, VKORC1, which in turn limits availability of reduced vitamin K, leading to decreased formation of functionally active clotting factors. The metabolism of reduced vitamin K to hydroxyvitamin K1 is catalyzed by CYP4F2, which removes vitamin K from the vitamin K cycle [27, 28]. Right panel, pie chart shows major sources of warfarin dose variability (adapted from [29]).

Polymorphisms in the *CYP2C9* gene (responsible for the metabolism of warfarin) and *VKORC1* gene (target for the warfarin) are relatively common. The genotyping of both genes in combination with other environmental factors (*i.e.* age, weight, diet, *etc.*) can predict the factual dose in up to 60% of cases [20]. Carriers of certain *CYP2C9* SNPs, coding for enzymes with reduced enzymatic activity require a lower average warfarin dose to achieve the desired therapeutic effect [20, 28, 30, 31]. Such are, for instance, *CYP2C9.2* (~70% of the *CYP2C9.1* activity) and *CYP2C9.3* (~10%). Moreover, a higher risk of bleeding and lower warfarin dose requirements have been reported in carriers of the *CYP2C9*2* and *CYP2C9*3* alleles. The molecular and structural basis for the altered catalytic properties of *CYP2C9* enzyme variants are poorly investigated. However, two recent reports attempted to explain the reduced catalytic activity of *CYP2C9*-Arg144Cys by the altered interaction between *CYP2C9* and POR [26] or by the differences in the spin state of *CYP2C9* [32].

In addition to *CYP2C9* polymorphisms, the warfarin dosage should be also modified (lowered) in case of two common SNPs located in the promoter region of *VKORC1* gene that decrease the gene expression [20]. Finally, *CYP4F2* has also some minor contribution in defining the warfarin dosage [28, 33].

Due to the large interindividual variation in the dose needed to reach adequate levels of anticoagulation, the anticoagulant effect needs to be closely monitored, especially during the initiation of therapy. Accordingly, the US FDA agency recommends the optimal ranges of warfarin dose (mg/day) according to CYP2C9 and VKORC1 genotypes of patients [28, 34, 35].

1.2.2 Polymorphisms in CYP3A4 and CYP2C8, clinical implications for drug induced neuropathy

CYP3A4 polymorphisms

The CYP3A locus is found on chromosome 7 and includes genes encoding for CYP3A4, 3A5, 3A7 and 3A43 isoforms. CYP3A4 is the major isoform of CYP3A subfamily. It is the most abundant CYPs in human liver (~50%) [36] being also expressed in small intestine. CYP3A4 metabolizes ~ 30 % of clinically used drugs. (Figure 2, [8]). The ability to convert such broad range of drugs is possible due to the flexibility of CYP3A4 substrate binding pocket [15]. CYP3A4 is considered to be susceptible to induction by environmental chemicals, drugs, *etc.* (Figure 1, [37])

The bioavailability and systemic clearance of the CYP3A4 metabolized drugs is usually subject to interindividual variations [38], albeit without clear distinction between the groups of slow and rapid metabolizers [39].

Unlike such highly polymorphic enzyme as CYP2C9, only a few SNPs are known to influence CYP3A4 expression or function. Among all alleles currently considered in the Human Cytochrome P450 Allele Nomenclature Database, the group of reference *CYP3A4*1* alleles contains 19 subtypes, including 18 SNPs in non-coding regions not affecting the mRNA expression. Only one CYP3A4 allele, *CYP3A4*22*, carrying an intronic mutation in intron 6 has been shown to be associated with decreased CYP3A4 activity towards cyclosporine A. The allele frequency of this variant is 0.08 in Caucasians and 0.04 in Asian and Africa population [40]. In addition, at least 24 exonic SNPs in *CYP3A4* with the changes of amino acid sequence have been described including rare *CYP3A4*20* and *CYP3A4*26* that cause the loss-of-function phenotype due to the completely absent protein expression [39]. Still, the proteins expressed from the most of identified allelic variants are often functional, which cannot therefore explain the large interindividual variability observed in CYP3A expression and *in vivo* activity.

CYP2C8 polymorphisms

CYP2C8 accounts for about 7 % of total human hepatic CYPs [41, 42] and it mediates the metabolism of 4.7% of the commonly used drugs (Figure 2). The X-ray crystal structure of CYP2C8 [43] showed a characteristic large active site cavity, which could accommodate reasonably large substrates [44], such as anticancer [45, 46], antiepileptic drugs [47], and HMG-CoA reductase inhibitors [48]. In addition, the CYP2C8 active site is similar in size, but different in shape to that of CYP3A4 [43, 49, 50], which might explain the overlapping substrate specificity, albeit with different metabolite profiles for the same substrate [44].

CYP2C8 is located on a chromosome 10 in a CYP2C genes cluster (centromere-CYP2C18-CYP2C19-CYP2C9-CYP2C8-telomere). Given the close proximity of CYP2C8 and CYP2C9, some linkage disequilibrium exists between these genes [51]. There is substantial interindividual variability of the CYP2C8 protein levels and catalytic activity [46, 52, 53], which can be partly explained by genetic polymorphisms. Fourteen (14) CYP2C8 SNPs have been identified to date and some of these SNPs are located in the coding region and are connected with the variability in CYP2C8-mediated metabolism and altered drug disposition and response. In general, polymorphic CYP2C8 alleles have not been assigned any phenotypic classification (*e.g.* poor metabolizers). This is primarily due to the relatively limited amount and controversial *in vitro* data and discrepancies between the *in vitro* and *in vivo* findings [50] (Table 1, 2). CYP2C8.3 have been reported to have both decreased and increased or even unaltered activity toward paclitaxel as compared to the reference CYP2C8.1 enzyme [50], whereas some other substrates such as pioglitazone, repaglinide, and rosiglitazone were shown to be metabolized with higher rate by CYP2C8*3. It has also been demonstrated that in reconstituted systems the increase in the molar share of redox partners, such as POR and cyt b₅ results in the higher activity of CYP2C8.3 compared to CYP2C8.1 [54].

CYP2C8*3 has an allele frequency of 10-13% in the Caucasians. The encoded variant enzyme (CYP2C8.3) contains two amino acid substitutions, Arg139Lys and Lys399Arg [55]. However, the allelic frequency of CYP2C8*3 reported in African population is relatively low (2%) and this allele is completely absent in Japanese population. CYP2C8*3 is in partial linkage disequilibrium with CYP2C9*2 [51].

CYP2C8 and CYP3A4 mediated paclitaxel metabolism; clinical implications

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the dose limiting side effects of many older, commonly used chemotherapeutic agents, including taxanes. The incidence of CIPN can be variable, but often ranges from 30 to 40% of patients receiving chemotherapy [56]. Common chemotherapeutic taxane agents including paclitaxel originate from the taxol isolated from the natural product of the bark of the Pacific yew tree. Paclitaxel is widely used as an effective chemotherapy for cancers of ovary, breast and lung. It inhibits the disassembly of microtubules, and consequently causes the cell death [21, 57-60].

The factors that influence the incidences of paclitaxel induced peripheral neuropathy (PIPNe) are the same as in patients receiving other types of neurotoxic chemotherapy and include patient age, dose intensity, cumulative dose, therapy duration, co-administration of other neurotoxic chemotherapy agents, and pre-existing conditions such as diabetes and alcohol abuse [56]. PIPNe can be painful and disabling, causing significant loss of functional abilities especially in hands and feet, and consequently decreasing quality of life. Although the exact pathophysiology of paclitaxel induced peripheral neuropathy is not fully elucidated, different underlying mechanisms have been suggested for different classes of anticancer drugs. Neurotoxicity caused by taxanes may arise due to disruption of microtubule structure of neurons leading to the impairment of axoplasmic transport and dying back neuropathy [21] and/or toxic effect on mitochondria in primary afferent neurons leading to a deficit in axonal energy supply and thus chronic sensory neuropathy [61].

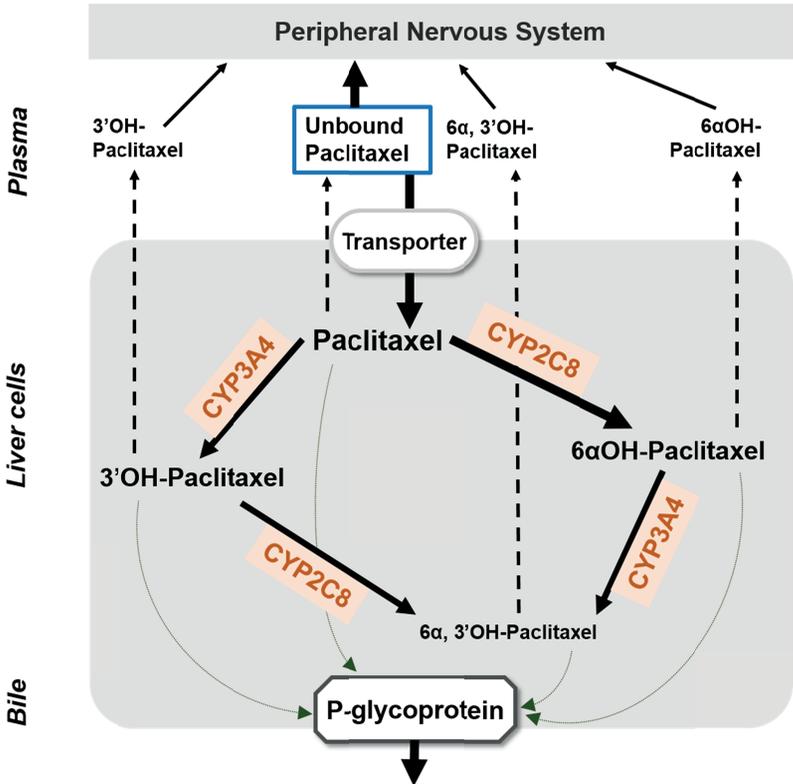


Figure 4. Paclitaxel metabolism. Paclitaxel enters hepatocytes via transporters (e.g. OATP 1B1/3 [62, 63]). In hepatocytes paclitaxel is metabolized by CYP2C8 to 6α-hydroxypaclitaxel, which is the major metabolite, and by CYP3A4 to C3'-hydroxypaclitaxel. Paclitaxel and its metabolites either diffuse to plasma or are excreted to the biliary canaliculi by P-glycoprotein. (modified from [64])

Figure 4 demonstrates the paclitaxel metabolism pathways mediated by both CYP3A4 and CYP2C8 enzymes in the liver and its elimination mediated by biliary excretion. It has been observed in *in vitro* study that paclitaxel 6 α -hydroxylation by CYP2C8 enzyme is more dominant than 3'-paclitaxel hydroxylation by CYP3A4 [65]. The two primary metabolites of paclitaxel are hydroxylated again by the same CYP2C8 and CYP3A4 enzymes. Excessively produced less potent secondary metabolites or potent unbound paclitaxel in plasma will be exposed to peripheral nervous system leading to PIPN.

Genome wide association studies as well as studies with selected target genes have identified many single nucleotide polymorphisms (SNP's) in paclitaxel metabolizing and transporter genes that were suggested to be responsible for interindividual differences in the toxicity and drug response. Some of them were found to be associated with the risk of developing neurotoxicity [66].

Study Type	Paclitaxel dose Patient number	Genotype distributions	Association results for carriers of CYP2C8*3	Ref.
Genotype vs CL	80-225 mg/m ² n=97	CYP2C8*1/*1, n=78 CYP2C8*1/*3, n=11 CYP2C8*3/*3, n=3	No association between CYP2C8 genotype and mean paclitaxel CL	[67]
	575-775 mg/m ² n=93	CYP2C8*1/*1, n=72 CYP2C8*1/*3, n=11 CYP2C8*3/*3, n=1	No association between CYP2C8 genotype and paclitaxel CL	[68]
	135-175 mg/m ² n=38	CYP2C8*1/*1, n=27 CYP2C8*1/*3, n=6	Lower CL in patients with the CYP2C8*1/*3 and ABCB1 2677G/T vs CYP2C8*1 homozygotes and ABCB1 2677G/T	[69]
	175 mg/m ² n=93	CYP2C8*1/*1, n=74 CYP2C8*1/*3, n=19	11% lower paclitaxel CL in patients carrying CYP2C8*3.	[70]
	175 mg/m ² n=119	CYP2C8*1/*1, n=95 CYP2C8*1/*3, n=21 CYP2C8*3/*3, n=1	No association between neuropathy and CYP2C8*3 Log-Rank test (p=0.98), overall survival HR=1.18 (95% CI: 0.66-1.92)	[71]
	175 mg/m ² n=92	CYP2C8*1/*1, n=74 CYP2C8*1/*3, n=18	No association between sensory neuropathy, Log-Rank test (p=0.46), and CYP2C8*3	[72]
	dose of paclitaxel not available n=36	allele frequency CYP2C8*3=0.14	No correlation between CYP2C8*3 and paclitaxel induced neuropathy. (statistic data not provided)	[73]
	80-175 mg/m ² n=118	allele frequency CYP2C8*1B=0.15 allele frequency CYP2C8*3=0.18	HR=1.72 (95% CI: 1.05-2.82, p=0.032) multivariable cox regression, cumulative paclitaxel dose test	[64]
	80-170 mg/m ² n=111	CYP2C8*1/*1, n=81 CYP2C8*1/*3, n=22	No significant of the effect of CYP2C8*3 in neurotoxicity OR=3.13 (95% CI: 0.89-11.01, p=0.075)	[74]
	50 mg/m ² n=261	CYP2C8*1/*1, n=92 (female);n=105 (male) CYP2C8*1/*3+*3/*3, n=30 (female);n=27 (male)	No association between neurotoxicity (National Cancer Institute's Common Terminology Criteria for Adverse Event version 2-4, grade 0-3) and CYP2C8*1/*3 + CYP2C8*3/*3 (p=1.0)	[75]
80-170 mg/m ² European-American (n=209) African-American (n=107)	CYP2C8*1/*1, n=155 CYP2C8*1/*3, n=51 CYP2C8*3/*3, n=3 in European-American, n=209	HR (per allele) =1.93 (95% CI: 1.05-3.55, p=0.006) in CYP2C8*3 status This paper also showed that African-American had higher neuropathy risk than European of similar genotype (p=0.03)	[76]	
80-170 mg/m ² n=412	Low metabolizer group (CYP2C8*2,*3,*4), n=142 Reference group (CYP2C8*1), n=270	HR = 1.722 (95% CI 1.10-2.70, p = 0.018) in low metabolizer group (CYP2C8*2,*3 & *4) for paclitaxel induced neuropathy	[77]	
175 mg/m ² n=1040	allele frequency CYP2C8*3 (R139K)=0.1	No significant association COX HR 1.21, p=0.23	[78]	
175 mg/m ² n=1303	allele frequency CYP2C8*3 (R139K) is not available	No significant association with the risk of neuropathy OR=1.22 (95% CI 0.93-1.59, p=0.14)	[79]	
paclitaxel-platinum 175mg/m ² n = 456	CYP2C8*1/*1 =0.87 (799/905) CYP2C8*1/*3 (R139K)=104/905 CYP2C8*3/*3 (R139K) =2/905	No association in both χ^2 analysis (Pearson's χ^2 test) and Log-Rank (progression-free survival) analysis	[80]	
docetaxel-carboplatin 75 mg/m ² n=458	CYP2C8*1/*1 =0.88 (650/841) CYP2C8*1/*3 (K399R) = 174 /841 CYP2C8*3/*3 (K399R) =17/841			

Table I. Summary of *in vivo* studies of CYP2C8*3 association with neuropathy.

CL: clearance, HR: hazard ratio, OR: odds ratio

*CYP2C8*3 and the paclitaxel induced neuropathy*

Possible association of *CYP2C8*3* expression with paclitaxel induced neuropathy has been investigated in many studies, which however produced a multitude of contradictory results (summarized in table 1). *Green et al.* and *Bergmann et al.* showed lower clearance of paclitaxel in patients being heterozygotes for *CYP2C8*3* (n = 23, n=93 respectively) as compared to patients homozygous for *CYP2C8*1* [69, 70] indicating reduced metabolism of paclitaxel *in vivo* by *CYP2C8*3*. Recently *Leskelä et al (2011)* and *Hertz et al (2013, 2014)* have reported a link between *CYP2C8*3* and paclitaxel induced neuropathy using the accumulated dose analyses showing the hazard ratio as 1.72 (p=0.032) in 118 patients, 1.93 (p=0.006) in 209 patients and 1.77 (p=0.018) in 412 patients [64, 76, 77], respectively. However, there are many studies showing lack of correlation between the polymorphism of *CYP2C8* and taxane induced neuropathy (e.g. in *Marsh S et al* done in 800 patients [80] or in *Abraham et al.* in more than 800 patients, (OR=1.22; 95% CI, 0.93-1.59; P= 0.14)) [79] or in a genome-wide association study in 855 breast cancer patients (COX HR= 1.21, p= 0.23) [78]. A summary of studies of *CYP2C8*3* association with neuropathy is presented in Table 1.

A number of *in vitro* studies have been carried out to characterize the catalytic activity of *CYP2C8.3* enzyme variant by using heterologous expression systems. Similar to the *in vivo* studies these data are very controversial (Table 2.)

Table 2. *In vitro* kinetic parameters for paclitaxel metabolism mediated by *CYP2C8.1* and *CYP2C8.3*.

Expression system	CYP2C8.1				CYP2C8.3				Ref.
	Km (μM)	Vmax	CL/Int (Vmax/Km)	Turnover (Kcat) nmol/min/nmol P450	Km (μM)	Vmax	CL/Int (Vmax/Km)	Turnover (Kcat) nmol/min/nmol P450	
<i>E.coli</i>	15±7	0.8±0.11 (nmol/min/nmol P450)	63±14.5	0.26±0.1	ND	ND	ND	0.04±0.04*	[55]
	3.7 ± 0.6	-	0.11	0.29 ± 0.01	4.7 ± 0.7	-	0.16	0.76 ± 0.06**	[54]
	20.9 ± 2.13	2.5 ± 0.07 (nmol/min/nmol P450)	119.6 (μl/min/nmol P450)	-	22.6 ± 2.3	0.31 ± 0.008 (nmol/min/nmol P450)	13.7 (μl/min/nmol P450)	-	[81]
<i>Yeast</i>	4.17 ± 1.33	2.4 ± 0.14 (pmol/min/pmol CYP2C8)	0.57 ± 0.12	-	8.87 ± 1.84**	2.41 ± 0.87 (pmol/min/pmol CYP2C8)	0.27 ± 0.03*	-	[82]
<i>Insect cells</i>	8.65	69.89 (pmol/min/pmol protein)	8.09 (μl/min/pmol protein)	-	16.25±1.45**	84.15 ± 3.47 (pmol/min/pmol protein)	5.25 ± 0.25** (μl/min/pmol protein)	-	[83]
<i>HepG2</i>	16.2	102 (pmol/min/mg protein) 29.8 (pmol/min/nmol CYP2C8)	6.33 (μl/min/mg protein) 1.88 (μl/min/pmol CYP2C8)	-	15.1	71.1 (pmol/min/mg protein) 19.6 (pmol/min/nmol CYP2C8)	4.75 (μl/min/mg protein) 1.34 (μl/min/pmol CYP2C8)	-	[84]

ND, kinetics of *CYP2C8*3* could not be determined because of its low activity.

*p<0.05, **p<0.01 compared with *CYP2C8.1*

1.3 Heterologous expression systems used to characterize enzymatic properties of cytochrome P450s

General aspects

Traditionally, the expression and enzymatic properties of CYPs were studied using the endoplasmic reticulum (microsomes) isolated from the tissues or cultured cells. Alternatively, the enzymes were purified from microsomes and reconstituted in the lipid vesicles together with P450 oxidoreductase in order to determine the substrate and inhibitor specificity of the enzymes. Such approach has advantages but also obvious shortcomings, such as non-physiological conditions of the assay, laborious isolation and purification of the large amounts of proteins and often poor availability of human tissues.

In vitro transfection systems using recombinant technology allowed to overcome many of these hinders. Several expression systems, including bacteria, yeast, baculovirus, or mammalian cells, have been used to produce catalytically active CYPs. Characterization of enzymatic properties of different CYP allelic variants, such as substrate specificity, K_m and V_{max} values for determining the intrinsic clearance of the drug can help in predicting the appropriate drug dose and clearance for the carriers of the corresponding variants of CYPs. In addition, the data on the expression levels of the variant proteins and their interaction with POR in such model systems may also be extrapolated to the situation *in vivo* [85].

The criteria for choosing the type of heterologous system should include the convenience of handling, protein yield, functional activity, proper folding and posttranslational modifications of the expressed protein. In addition, it is desirable to have a clean background, i.e. low or absent expression of the enzymes of interest. As CYPs are membrane-bound enzymes the suitable environment mimicking the ER membranes is important. The redox partners, P450 oxidoreductase and in some cases cytochrome b_5 should be co-expressed in the system, if not already present. This section describes the commonly used systems for heterologous expression of drug metabolizing enzymes and discusses some advantages and disadvantages for each of the systems.

Bacterial system

Due to the several benefits such as low maintenance costs and high protein yield compared to other expression systems, many polymorphic P450 variants have been characterized using recombinant enzymes expressed in *E.coli*. Barends et al. first expressed a bovine CYP17A1 in *E.coli*. No immunoreactive CYP17A1 protein was produced when the native cDNA was introduced into an expression vector, however modification of the N-terminus of CYP17A1 led to the expression of the protein and incorporation into the *E.coli* membrane [85]. This method was used further to modify the NH_2 regions of other mammalian CYPs in order to enhance the protein expression levels. Further purification of

the expressed enzymes and incorporation them into the liposomal reconstituted system including other components of electron transport chain, POR and cytochrome b₅ demonstrated sufficient levels of enzymatic activity. However, using different P450: b₅: POR ratios in such systems led to some conflicting results in the evaluation of different P450 variant enzymes, in particular for the metabolism of paclitaxel, amodiaquine and rosiglitazone by CYP2C8 variants [54]. The lack of mammalian posttranslational machinery that might be important for certain CYPs and the absence of native redox partners were found to be additional confounding factors limiting the use of *E.coli* for characterization of mammalian CYPs.

Yeast system

Saccharomyces cerevisiae was the first eukaryotic heterologous expression system [86] used for characterization of CYPs and most of the human enzymes have been expressed in yeast. Advantages of using yeast system overlap with those of the bacteria, *e.g.* cost, ease to manipulation and high protein yield. These are combined with the advantages of eukaryotic cells, *i.e.* presence of endoplasmic reticulum and mitochondria similar to human cells [85]. Yeast has low background levels of its own P450s, namely lanosterol-C14-demethylase, which are totally inactive toward xenobiotics. Unlike *E. coli*, no N-terminal modification of CYPs is needed [87]. The specific yeast NADPH-P450 reductase gene is constitutively expressed, but it cannot couple efficiently to the human P450s, especially to CYP3A4 [88] and yeast cytochrome b₅ is an unsuitable donor of electrons to the human enzymes.

Insect cell system

The insect Sf9 cells is a eukaryotic system, which yields very high expression levels of many human enzymes upon baculovirus mediated transfection. However, these cells are devoid of all electron-transport chain components needed for P450 activity so similar to other above mentioned systems these have to be added (co-expressed) to reproduce a fully active electron transport chain [89]. Another problem is the insufficient level of heme synthesis, which should be boosted by the addition of hemin to the culture medium. This addition however proved to be toxic for insect cells in long term cultures [90].

Mammalian cell system

Currently this system is considered as the most relevant for the expression and activity studies of the human proteins including CYPs. COS-1 or COS-7 cells (derived from African green monkey kidney) and HEK293 cells (derived from the human embryonic kidney) are the most widely used because of the simple handling, adequate physiological environment including folding and posttranslational machinery and no need in modification of cDNA for

the expression of membrane bound proteins. Additionally, all of these cells express enough amounts of the redox partners for CYP enzymes and have absent or low CYP background.

The mode of transfection (stable or transient) of vectors carrying cDNA of choice into the mammalian cells is chosen depending on the experimental design and objective. Stably transfected genes integrate into the host genome and sustain transgene expression even after host cells replicate [91]. Therefore it is more suitable for the production of the large amounts of recombinant proteins with proper folding and posttranslational modifications. As the development of stable cell lines can be laborious, the use of transient expression when appropriate is beneficial. Transiently transfected genes are only expressed for a limited period of time and are not integrated into the genome [91], however the protein folding, posttranslational modifications and functionality are identical with the stably expressed proteins.

2 AIMS

The overall goal of this thesis was to investigate the contribution of genetic components to the interindividual variations in the response to the drugs metabolized by the cytochrome P450s from the CYP2 family and CYP3A4.

More specifically, the thesis is focused on:

1. Development of the novel enzymatic assay for the orphan CYP2W1 using various indoline derivatives.
2. Enzymatic and structural validation of the hypothesis that the altered interaction of *CYP2C9*35* with POR determines the low activity of this allelic variant toward warfarin.
3. Identification of CYP3A4 polymorphisms associated with the paclitaxel induced peripheral neurotoxicity and their functional characterization.
4. Evaluation of the role of *CYP2C8*3* in paclitaxel induced neuropathy and characterization of CYP2C8.3 enzymatic properties.

3 RESULTS AND DISCUSSION

3.1 Development of the novel enzymatic assay for the orphan CYP2W1 using various indoline derivatives. (Paper I)

Overexpression of CYP2W1 in HEK293 cells results in the appearance of two immunoreactive bands of approximately 52 and 54 kDa. The latter has been suggested to be a product of posttranslational modification of the protein [14].

In silico analysis suggested possible N-glycosylation site at the Asn177 residue and indeed, treatment of the cell extracts with deglycosylating enzymes PNGase or Endo H ablated the band with a higher molecular weight confirming its glycosylation origin. Similar results were shown upon the treatment of samples from colon tumors with high expression levels of CYP2W1. These findings were further supported by the expression and analysis of the Asn171Ala CYP2W1 mutant.

Protease protection assay demonstrated unusual inverted ER membrane topology of CYP2W1, which is consistent though with its modification by the luminal glycosylation machinery. Such topology raises the question of the enzymatic function of CYP2W1 as its main redox partners are localized on the other side of ER membrane.

There were no specific assays described for CYP2W1 activity determination, however it was shown that CYP2W1 may participate in the metabolism of indole [92]. Therefore we decided to test different indole and indoline derivatives incubating them with the CYP2W1 expressing HEK293 cells. The cells were then extracted and metabolites separated using reverse-phase HPLC system. Using this method we showed specific CYP2W1 dependent metabolism of 5-bromoindoline and 2-methyl-5-nitroindoline. The indoline metabolizing capacity of CYP2W1 was later used in our laboratory in collaboration with Bradford University to develop a novel class of chloromethylindoline based suicidal prodrugs for the treatment of colon cancer [11, 12].

3.2 Molecular mechanisms of impaired interaction between CYP2C9*35 and P450 oxidoreductase (Paper II)

In paper II, we investigated the molecular mechanisms that are responsible for the warfarin hypersensitivity phenotype associated with the CYP2C9*35 allelic variant. This CYP2C9 polymorphism was identified in an Italian patient [93] who needed extremely low doses of warfarin even compared with carriers of the other known defective alleles (CYP2C9*2 and *3). This variant, designated CYP2C9*35 (www.cypalleles.ki.se), expresses an enzyme, which has Arg144Cys (*cf.* CYP2C9*2) and additional Arg125Leu amino acid replacements. Previous *in silico* analysis suggested that Arg125Leu substitution might lead to the reduction of the positive potential of CYP2C9-POR interaction surface preventing the POR recognition, and thus negatively affecting the CYP2C9 activity toward warfarin [93]. In this paper we aimed to validate experimentally this hypothesis using the *in vitro* human cell transfection system, as well as the extended structural bioinformatics analysis.

In order to evaluate in detail the combined and individual contribution of the amino acid changes to the activity of the enzyme, we constructed FlpIn™-HEK293 cell lines that stably express CYP2C9.1, CYP2C9.35, CYP2C9-R125L and CYP2C9-R144C (CYP2C9.2) at the same protein levels.

Further analyses demonstrated that *S*-warfarin metabolism as mediated by CYP2C9.1 and CYP2C9-R144C followed the Michaelis-Menten kinetics, however the activity of CYP2C9-R144C (CL_{int} 0.463 ± 0.038) was lower than the rate of *S*-warfarin metabolism by CYP2C9.1 (CL_{int} 0.839 ± 0.081), which is in line with previously reported data [23, 25, 26]. However, the CYP2C9 variants containing Arg125Leu and Arg125Leu/Arg144Cys (CYP2C9.35) substitutions were found to be completely catalytically silent. The activity was recovered when instead of NADPH-POR as an electron donor we used different hydroperoxides (peroxide shunt system), confirming the impaired CYP2C9.35-POR interaction. Thus, the absence of activity toward warfarin was not due to the defects in the enzyme active site (Figure 5). Similar data with yet another CYP2C9 substrate diclofenac confirmed these conclusions.

The conserved Arginine 125 in CYP2C9 is one of the key residues that has been discussed as one of the functional binding sites of P450 enzymes with their redox partners, such as cytochrome b_5 (cyt b_5) and POR. This was in particular shown by the analysis of the CYP-POR/cyt b_5 interactions of the Arg125Ala mutant in wild type rabbit CYP2B4 or mouse CYP2A5, the proteins with the high degree of homology with CYP2C9 [94].

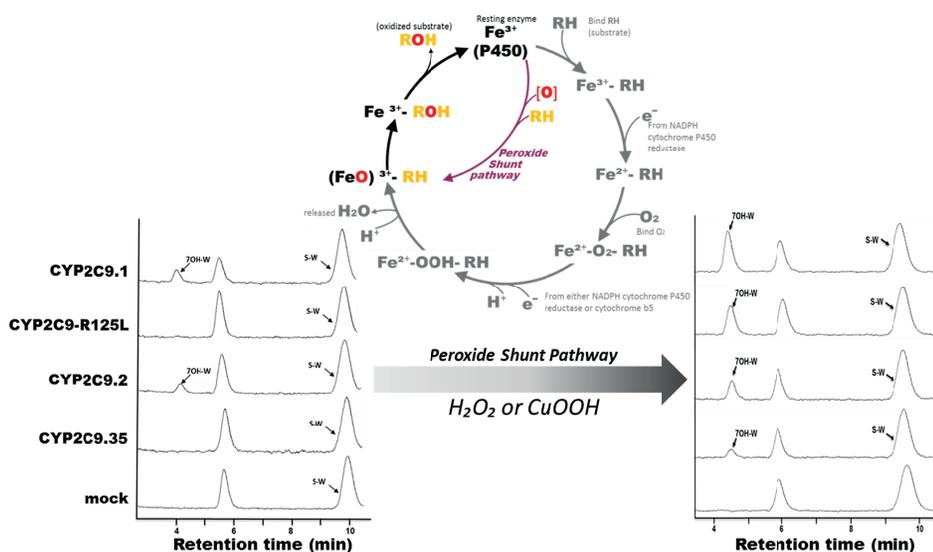


Figure 5. S-warfarin metabolism using peroxide shunt in the cells expressing CYP2C9 variants.

7OH-W: 7hydroxy warfarin, S-W: S-warfarin

Our structural analysis confirmed and extended this hypothesis. The Arg125Leu amino acid change strongly contributes to the decrease of electrostatic potential of CYP2C9 proximal surface that is complementary to the corresponding negatively charged surface of FMN-binding domain of POR. As shown in Figure. 6, such loss of electrostatic interaction results in the disruption of important salt bridges between two proteins (Glu56-Arg125-Glu32). This may lead to destabilization of CYP-POR complex and hinder the electron transport. This mutation however, had no impact on the substrate binding loci of CYP2C9, which suggests that elimination of the positively charged Arg125 cannot perturb the substrate-enzyme interaction. In line with this assumption we found that CuOOH (or H₂O₂) was able to restore the activity of CYP2C9-R125L that was silent in the NADPH-POR driven enzymatic system (Figure 5).

In addition to Arg125Leu, the CYP2C9.35 variant protein includes also an Arg144Cys change. The activity of the variant with Arg144Cys only, was not abolished completely as in case of the double mutant. Using the CuOOH peroxide shunt system its activity was recovered to nearly the level of the wild type enzyme (Figure 5). In contrast to Arg125, this residue is not surface exposed and therefore cannot participate in the CYP/POR interaction. Consequently, it is not clear why the Arg144Cys mutation affects the enzyme activity. It can be speculated that in the CYP2C9-Arg144Cys mutant reactive cysteine might be involved in the fortuitous interactions with POR and/or with other molecular partner, reducing thus the probability of the formation of correct CYP2C9-POR binding domain binary complex.

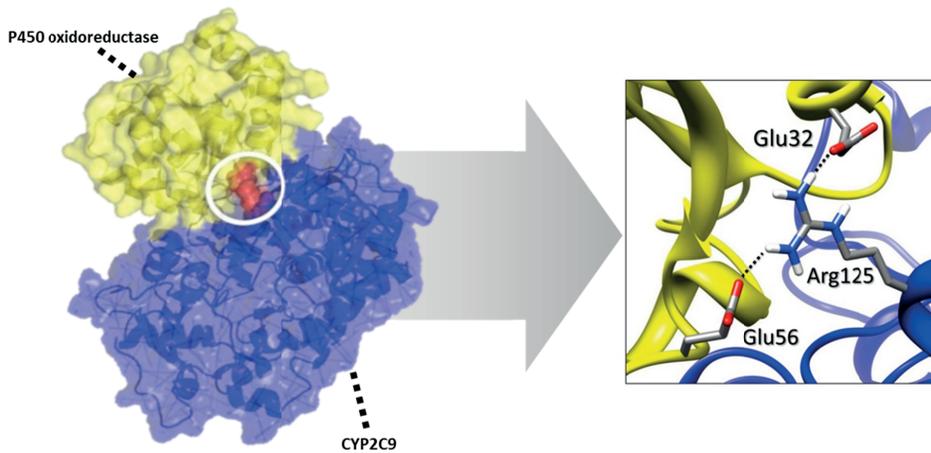


Figure 6. *In silico* analysis of electrostatic interaction between P450 oxidoreductase and CYP2C9

Collectively, paper II demonstrates that the Arg125Leu change in CYP2C9.35 strongly augments the modest inhibitory effect of the Arg144Cys mutation found in the carriers of CYP2C9*2 allelic variant. This suggests that thorough genotyping for the presence of both polymorphic changes might facilitate more accurate warfarin dosing using this allele as a specific genetic biomarker.

3.3 Paclitaxel induced peripheral neuropathy. (Papers III and IV)

Paclitaxel is metabolized by CYP3A4 and CYP2C8 and the polymorphisms of these P450s have been considered as a risk factor for chemotherapy induced neuropathy.

- Paper III: We have identified two novel alleles of CYP3A4, established their association with the neuropathy risk in the patients treated with paclitaxel and characterized these CYP3A4 variant proteins *in vitro* (see 3.3.1)
- Paper IV: We have evaluated the probability of the causative link between the expression of *CYP2C8*3* gene and paclitaxel-induced neuropathy and characterized the catalytic properties of overexpressed recombinant CYP2C8.3 protein. (see 3.3.2)

3.3.1 Novel defective polymorphisms of CYP3A4 associated with neuropathy induced by paclitaxel. (Paper III)

The molecular mechanisms of the paclitaxel induced neuropathy are hitherto not well understood. The discovery of novel CYP3A4 variants associated with this pathology is of importance as it suggests a novel pharmacogenetic biomarker for the peripheral neurotoxicity risk. In paper III, we demonstrated such association in patients and characterized the enzyme kinetics of the variant proteins overexpressed in HEK293 cells.

We have screened 236 Spanish neuropathic patients treated with paclitaxel by using the whole exome sequencing and dHPLC, and focusing on the critical genes involved in paclitaxel metabolism and transport (*CYP3A4*, *CYP2C8*, *ABCB1* and *SLCO1B3*). In this cohort seven patients were found to carry *CYP3A4* variant alleles as shown in Table 3. This finding was confirmed by Sanger-sequencing. The defective *CYP3A4* alleles identified in these patients include *CYP3A4*20* (n=4), *CYP3A4*8* (n=1), two novel missense variants CYP3A4-Pro389Ser and CYP3A4-Leu475Val, later designated as *CYP3A4*25* and *CYP3A4*27* respectively (Table 3).

The *CYP3A4*20* allele is characterized by a frame shift and premature stop codon, which produces truncated and inactive CYP3A4.20 protein in both yeast and HEK293 cells [95]. The protein expressed by *CYP3A4*8* (c.389G>A, p.Arg130Gln) has been shown to display reduced activity due to the not detectable holoprotein levels in *E.coli* [96]. Two novel alleles with missense mutations, *CYP3A4*25* (c.1165C>T, p.Pro389Ser) and *CYP3A4*27* (c.1423C>G, p.Leu475Val) produce proteins with mutated residues located in the highly conserved CYP β -helix 4 and in the C-terminus correspondingly.

When compared with *CYP3A4*1* reference gene, the *CYP3A4*20* was found associated with the significantly higher risk of neuropathy in this cohort study (2 fold higher, p=0.042).

In case of non-synonymous variants (*CYP3A4**8, *25 and *27) the risk of neuropathy grade 3 was increased 1.3 fold. (Table 3). All of these variants were associated with significantly higher neuropathy grade ($p < 0.05$).

Table 3. Characteristics of patients with *CYP3A4* defective variants.

CYP3A4 genotype ^s	Patient number (n)	Neuropathy grade ^a	CYP3A4 variant	CYP3A4 variant (<i>in vitro</i>)	Risk of paclitaxel-induced neuropathy ^b
*1/*20	4	3	<i>CYP3A4</i> *20 c.1461_1462insA, p.P488Frameshift	Due to premature stop codon, truncated protein produced in yeast and HEK293 cells No activity (loss of function) [95]	2 fold increased risk of grade 3 neuropathy (loss of function vs. reference)
*1/*25	1	3	<i>CYP3A4</i> *25 c.1165C>T, p.Pro389Ser	Novel allele, decreased activities	1.3 fold increased risk of grade 3 neuropathy (missense vs. reference)
*1/*27	1	3	<i>CYP3A4</i> *27 c.1423C>G, p.Leu475Val	Novel allele, decreased activities	
*1/*8	1	1	<i>CYP3A4</i> *8 c.389G>A, p. Arg130Gln	no detectable P450 holoprotein, decreased activity [96]	

a Maximum sensory neuropathy grade during paclitaxel treatment (NCI-CTC v4).

b When compared with *CYP3A4**1 homozygotes, the risk of 3 grade neuropathy was higher.

Characterization of *CYP3A4.25* and *CYP3A4.27*

Recombinant *CYP3A4.25* protein was found to have reduced level of expression (40% of *CYP3A4.1*) in HEK293 expression system, and *CYP3A4.27* was also expressed in substantially lower amounts (10% of *CYP3A4.1*) (Figure 7).

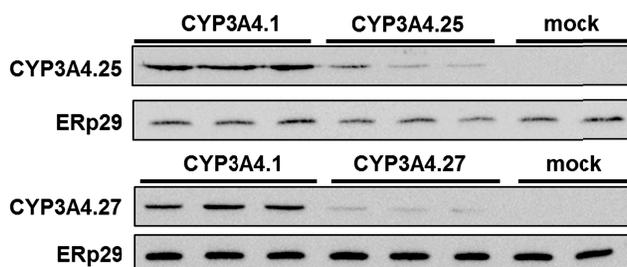


Figure 7. Immunoblotting of *CYP3A4.25* and *CYP3A4.27*

Treatment of the cells with the inhibitor of protein synthesis, cycloheximide suggested that the Pro389Ser substitution in CYP3A4.25 leads to the increased rate of degradation (Figure 8).

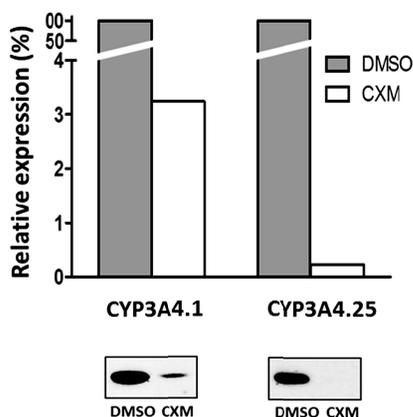


Figure 8. Immunoblots of CYP3A4.1 and CYP3A4.25 expressed in HEK293 cells after cycloheximide treatment. Upper panel, densitometric analysis of the protein bands from the lower panel.

In order to determine the catalytic activity of these CYP3A4 variants, we overexpressed them in HEK293 cells and measured the activities by using dibenzylfluorescein as a specific substrate. The activity was normalized by the expression levels of apoprotein as quantified by the densitometry of immunoblots. The evaluation of enzyme kinetics revealed similar K_m values for CYP3A4.27 and CYP3A4.1, whereas the K_m value for CYP3A4.25 was higher. However, the true V_{max} was difficult to determine because of the low expression of the variant proteins.

Based on these data we suggest that the aberrantly high degradation rates of both CYP3A4.25 and CYP3A4.27 may substantially reduce the paclitaxel metabolism rate in the carriers of these alleles resulting in insufficient paclitaxel clearance and corresponding toxic consequences. Therefore, the patients carrying CYP3A4 defective variants or with preexisting conditions associated with neuropathy, would require treatment modification as paclitaxel drug therapy endpoint.

3.3.2 CYP2C8*3 possible association with a neuropathy risk and *in vitro* validation. (Paper IV)

CYP2C8 is the main paclitaxel metabolizing enzyme (*see Introduction*). Therefore over the last decade much attention (Table 1) was paid to the possible role of genetic variations, in particular of *CYP2C8*3* in the paclitaxel induced neuropathy. However, due to the high degree of controversy between the *in vivo* and *in vitro* data this issue still remains unresolved. (Table 1-2, *Introduction 1.2.2*). In paper IV we have analyzed a possible link between the expression of *CYP2C8*3* and the neuropathy risk in 148 patients receiving paclitaxel, however no significant association (HR =1.70, p=0.078) was found.

In order to evaluate the effects of CYP2C8.3 on the metabolism of paclitaxel we have characterized the catalytic properties of CYP2C8.3 overexpressed in HEK293 cells. The enzyme kinetic parameters for both allelic variants (CYP2C8.1 and 8.3) were found to be practically identical. These data is consistent with some previous *in vitro* studies [84], however other investigations indicate altered paclitaxel metabolism by CYP2C8.3 (*see table 2*).

The reason for such controversy might be associated with the choice of the expression system. For instance, CYP2D6.17 identified in a patient with a defective drug metabolism had full catalytic activity toward bufuralol when it was expressed in the yeast cells but much less activity when expressed in the mammalian expression system [97]. Thus, lower intrinsic clearance of paclitaxel was observed when CYP2C8.3 was expressed in *E.coli*, yeast or insect cells [54, 55, 81-83], whereas when paclitaxel hydroxylation was evaluated in the transfected human hepatoma cells, HepG2 [84] the kinetic parameters of paclitaxel metabolism with CYP2C8.3 and CYP2C8.1 were similar, as also seen in our study using HEK293 cells.

The enzymatic activity of CYP2C8.1 and CYP2C8.3 proteins was also tested using amodiaquine and rosiglitazone. For amodiaquine, we did not observe any changes in kinetic parameters. Conflicting results regarding the amodiaquine have been previously reported for CYP2C8.3, which to a certain extent may again be explained by the different expression systems used [54, 98]. Clearance of yet another substrate, rosiglitazone, was higher in the CYP2C8.3 expressing cells, which is in line with another *in vitro* study [54].

The CYP2C8.3 variant enzyme contains two conserved amino acid changes, Arg139Lys and Lys399Arg. Therefore, it is not surprising that an *in silico* structure analysis using two online tools PolyPhen2 and SIFT predicts unaltered structural properties of CYP2C8.3 as compared with CYP2C8.1. Such predictions are in good agreement with the experimental data showing no difference in catalytic activities of CYP2C8.3 towards paclitaxel and amodiaquine. Interestingly, an *in silico* study based on the crystal structure of CYP2C8 complexed with troglitazone, a drug similar to rosiglitazone, predicts a missing salt bridge in Arg139Lys mutant whereas the salt bridge in Lys399Arg should remain intact. The authors conclude that this may affect the substrate specificity of Arg139Lys protein [98]. These

predictions might be a possible explanation for the different rosiglitazone conversion rates found in our study. Similarly different enzyme kinetics with two different substrates (codeine and bufuralol) was reported for the CYP2D6.17 variant when compared with the reference CYP2D6.1 enzyme [97].

Collectively, in the paper IV, we reported the absence of association of *CYP2C8*3* expression with paclitaxel induced neuropathy and also no changes in *in vitro* activity of CYP2C8.3 toward paclitaxel, yet finding somewhat different substrate specificity with rosiglitazone.

4 SUMMARY

The main focus of current study was the investigation of the role of genetic factors underlying the variability of enzyme activities in CYP2C and CYP3A subfamilies and possible implications for the drug adverse reactions.

- Using the human cell expression system, we have shown the inversed ER membrane topology of the orphan enzyme CYP2W1 favoring its N-glycosylation at residue Asn177. Despite such non-canonical localization that would hinder its interaction with the redox partners, the HEK293 expressed CYPW1 was able to specifically metabolize two indoline based substrates. This raises an interesting question of the hypothetical ER luminal redox partners, the issue that is currently under intensive scrutiny in our laboratory.
- Using similar experimental setup we have characterized expression and enzymatic properties of the novel *CYP2C9*35* allele with two amino acid changes, Arg125Leu and Arg144Cys. Arg125Leu was found to have a strong negative impact on the catalytic activity towards warfarin, which apparently leads to the warfarin hypersensitivity in the carriers of this allele. Bypassing traditional P450 catalytic cycle via peroxidase shunt pathway restored *CYP2C9*35* activity indicating intact enzymatic properties of the enzyme. *In silico* structural analysis suggested that the altered electrostatic interaction between NADPH-P450 oxidoreductase and CYP2C9.35 may be responsible for the abolished activity of this genetic variant of CYP2C9.
- Polymorphic variants of the CYP3A4 gene, *CYP3A4*8*, **20* and two novel alleles *CYP3A4*25*, **27* were found to be associated with the paclitaxel mediated neuropathy in the seven out of 236 paclitaxel treated cancer patients. *In vitro* characterization of CYP3A4.25 and CYP3A4.27 harboring two amino acid changes, Pro389Ser and Leu475Val, respectively revealed higher rates of degradation as compared with the reference enzyme suggesting reduced paclitaxel metabolism, which may be the reason for neurotoxicity in the affected patients.
- Despite the multitude of controversial *in vivo* and *in vitro* data, part of which suggested the connection of *CYP2C8*3* with the increased neuropathy risk upon treatment of cancer patients with paclitaxel, our data derived from the study of a large cohort of such patients could not support that notion. Moreover, our *in vitro* results were consistent with this finding, showing no difference in kinetic parameters of CYP2C8.3 catalyzed metabolism of paclitaxel and amodiaquine with somewhat

higher rosiglitazone conversion rate suggesting different substrate specificity of this variant enzyme.

- In general, our data support the application of HEK293 cell expression model as a robust and versatile host system for the expression of human CYP enzymes.
- This work should also contribute to further mapping of the polymorphic markers of the investigated cytochrome P450s and improve thus the personalized prescription of the drugs metabolized by these enzymes.

5 FUTURE PERSPECTIVES

The modern time and cost effective next generation sequencing technologies provide us with the growing information on a myriads of different genetic variations of all human genes and the drug metabolizing enzymes in particular. To date the databases containing CYP SNPs now contain close to 400 different allelic variants and most of them were found to be associated with the specific clinical phenotypes [19].

The rapidly developing personalized medicine approach requires comprehensive description of the patient including the clinical characterization, family anamnesis, full chemistry of biological fluids and genomic information. The latter is especially important for the pharmacogenetic part of personalized medicine as the drug response is tightly connected with the genetically defined expression levels and activity of the drug metabolizing enzymes.

Although still insufficient, the growing knowledge of pharmacogenetics based on interindividual variances of drug responses and molecular information, is entering the clinical practice. This, for instance is supported by the increasing number of pharmacogenomic labels for drug lists as recommended by FDA and EMA. In particular, special attention is paid to pharmacogenomic labels in cancer treatment and anticoagulant therapy, where the dosing must be precise enough to have efficacy without causing toxicity. Among several targets most attention have been paid to variations in the genes encoding drug metabolizing enzymes, including CYPs, drug transporters, drug targets and their receptors, signal transduction molecules.

However, there are still remaining problems that hinder full implementation of personalized medicine into the clinical practice. One of them is the need in more sophisticated bioinformatic tools and better statistical apparatus for the assessment of ADR risks. For instance, the precise evaluation of the role of *CYP2C8*3* in paclitaxel induced neuropathy obviously requires larger statistical power and more sophisticated methods of its treatment. However, even with the information that we possess at a moment we can substantially improve our knowledge by the deep literature and data mining and comprehensive meta-analysis of the data.

Another problem is the lack of knowledge of the molecular characteristics of the target genes. The approach that we have applied in current study is of course very useful and provides with enough information to judge about the catalytic or other properties of the genes of interest. However, the accumulation of this information is definitely lagging behind the technical progress in deciphering the genetic information (genome sequencing) and requires more efficient high throughput technologies.

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