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CYP2C19 and CYP2C9: New aspects of pharmacogenetics and transcriptional regulation

Jessica Mwinyi

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From the Department of Physiology and Pharmacology
Section of Pharmacogenetics
Karolinska Institutet, Stockholm, Sweden

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' but 'That's funny ...'

Isaac Asimov

All truths are easy to understand
once they are discovered; the point
is to discover them.

Galileo Galilei

*To
Alama and Papa
and my sisters
Adina and Miriam*

ABSTRACT

Cytochrome P450s (CYPs) are responsible for approximately 75% of the phase I-dependent drug metabolism. Several important polymorphisms in these enzymes are known to significantly affect the individual drug response. CYP2C9 and CYP2C19 are polymorphically expressed CYP family members which are responsible for the metabolism of many different clinically important drugs, e.g. anticoagulants, antidepressants and antiulcer drugs. This thesis focuses on novel aspects with regard to the regulation of CYP2C9 and CYP2C19 gene expression.

The influence of the recently found common allele variant *CYP2C19*17* on CYP2C19 enzyme activity towards two CYP2C19 substrates was investigated in the frame of two pharmacokinetic *in vivo* studies. The studies compared the single-dose pharmacokinetics of omeprazole and the steady-state kinetics of escitalopram in healthy *CYP2C19*17/*17* carriers with the pharmacokinetic outcome obtained for *CYP2C19* wild-type subjects. While no significant differences in the metabolic efficacy were observed for escitalopram, *CYP2C19*17/*17* subjects showed significantly lower plasma levels of omeprazole compared to *CYP2C19* wild type carriers. This observation suggests that *CYP2C19*17/*17* carriers might be at a higher risk for therapy failure during treatment with proton pump inhibitors.

The transcriptional regulation of *CYP2C9* and *CYP2C19* gene expression by GATA transcription factors and by estrogen receptor α (ER α) was investigated *in vitro*. In the proximal promoter regions of both the *CYP2C9* and the *CYP2C19* gene, two adjacent putative GATA-binding sites with an ER-binding half-site in their vicinity were predicted *in silico* and initially studied by luciferase gene reporter assay. HepG2 and Huh-7 hepatoma cells were transfected with *CYP2C9* or *CYP2C19* promoter fragment carrying pGL3basic-constructs along with expression vectors for the transcription factors GATA-2, GATA-4, or ER α . Luciferase activities driven by wild-type *CYP2C19* or *CYP2C9* promoter were highly increased by GATA-4 and GATA-2 in both cell lines, whereas mutations introduced into the GATA binding sites or the co-transfection of the GATA-4 antagonist FOG-2 caused a significant loss of luciferase activity. In contrast, treatment with estradiol derivatives of ER α -transfected cells caused a significant inhibition of *CYP2C19* and *CYP2C9* promoter activity that was antagonized by site-directed mutagenesis of the putative ER α -binding half-sites. Additionally, estradiol derivatives significantly suppressed both *CYP2C9* and *CYP2C19* mRNA expression in human hepatocytes, as measured by real time PCR. Electrophoretic mobility shift assays revealed sequence-specific binding of GATA-4, GATA-6, and ER α to the two adjacent GATA binding sites and to the predicted ER binding half sites, respectively. CHIP assay in the cultured cells furthermore confirmed the association of both GATA-4 and ER α with *CYP2C9* and *CYP2C19* gene promoter. In conclusion, we have established novel mechanisms of *CYP2C9* and *CYP2C19* transcriptional regulation that involve transcription factors from the GATA family and estrogen receptor α . The estrogen mediated regulation may explain the clinically observed inhibitory effects of oral contraceptives on CYP2C19 and CYP2C9 activity.

LIST OF PUBLICATIONS

- I.** Increased omeprazole metabolism in carriers of the CYP2C19*17 allele; a pharmacokinetic study in healthy volunteers.
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- II.** Kinetics of omeprazole and escitalopram in relation to the CYP2C19*17 allele in healthy subjects.
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- III.** The transcription factor GATA-4 regulates cytochrome P450C19 gene expression.
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- V.** Regulation of CYP2C19 expression by estrogen receptor alpha. Implications for estrogen dependent inhibition of drug metabolism.
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* Equal contribution

** Shared last authorship

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
AR	Androgen receptor
AUC	Area under the (plasma) concentration curve
CAR	Constitutive androstane receptor
ChIP	Chromatin immunoprecipitation
CL	Clearance
C _{max}	Maximum plasma concentration
CYP	Cytochrome P450
DHEA	Dehydroepiandrosteron
ds oligonucleotide	Double stranded oligonucleotide
EE	17 β -estradiol
EET	epoxyeicosatrienoic acid
EM	Extensive metabolizer
EMSA	Electromobility shift assay
ER	Estrogen receptor
ETE	17 α -ethinylestradiol
FOG	Friend of GATA
FXR	farnesoid X receptor
GERD	Gastroesophageal reflux disease
GR	Glucocorticoid receptor
HEET	hydroxyeicosatrienoic acid
HNF-4 α	Hepatocyte nuclear factor-4 α
HPLC	High performance liquid chromatography
LXR	Liver X receptor
MAPK	Mitogen-Activated Protein Kinase
MR	Metabolic ratio
MR	Mineralocorticoid receptor
NF- κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NR	Nuclear receptor
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PM	Poor metabolizer
PPAR	Peroxisome proliferator-activated receptor
PPI	Proton pump inhibitor
PR	Progesterone receptor
PXR	Pregnane X receptor
RAR	Retinoic acid receptor
RT PCR	Real time PCR
RXR	Retinoid X receptor

SERM	Selective estrogen receptor modulator
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
SP-1	SP-1 transcription factor
SRF	Serum response factor
SSRI	Selective serotonin re-uptake inhibitor
$t_{1/2}$	Half life
TBP	TATA box-binding protein
T_{max}	Time to maximum plasma concentration
TR	Thyroid hormone receptor
UM	Ultrarapid metabolizer
VDR	Vitamin D receptor

1 INTRODUCTION

1.1 CYTOCHROME P450 ENZYMES AND PHARMACOGENETICS

Cytochrome P450 enzymes (CYPs) belong to a large protein family and are the most important group of xenobiotic metabolizing enzymes in humans. CYPs are particularly abundantly expressed in the liver, where they play a critical role in determining the bioavailability of a wide range of important drugs. It is estimated that CYPs are responsible for approximately 75% of phase I-dependent drug metabolism. Furthermore, CYPs are involved in the transformation of a large amount of dietary constituents and endogenous chemicals (Ingelman-Sundberg, 2004a). While members of the families CYP4 to CYP51 are involved in metabolism of endogenous substrates such as steroids, fatty acids and prostaglandins, the isoenzymes of the families CYP1, CYP2 and CYP3 are collectively responsible for the biotransformation of the majority of therapeutics used in clinics (Ingelman-Sundberg et al., 2007). The human genome comprises 57 *CYP* genes and approximately the same number of pseudogenes, which are grouped into 18 families and 44 subfamilies according to their sequence similarity (Nebert, 1999).

CYPs are characterized by a wide interindividual and intraindividual variability in enzyme activity. Major sources of these differences in enzyme activity are environmental factors, e.g. enzyme inhibition or induction by xenobiotic substances including drugs, biological factors including gender, hormonal state or disease state, and genetic variability. For many *CYP* genes genetic variants have been described caused by single nucleotide polymorphisms (SNPs), insertions/deletions or gene copy number variation (CNVs). The consequences are abolished, decreased, normal or increased rate of drug metabolism. The consequent variation in drug plasma levels can either result in adverse effects or lead to reduced therapeutic efficacy.

The field of 'pharmacogenetics' studies the variability in drug response due to heredity. Genetic variability in drug metabolism as reflected in differences in clearance, half-life, or maximal plasma concentration of a drug can be considered for individual genotype-based dose adjustments. Systematic use of pharmacogenetic information should help to improve the number of therapy responders, decrease the number of patients suffering from adverse drug reactions, thus reducing the risk of drug induced morbidity and mortality (Nebert, 1999; Ingelman-Sundberg et al., 2007; Seeringer and Kirchheiner, 2008).

1.2 CYP2C19 - GENE AND PROTEIN

1.2.1 Polymorphisms in the *CYP2C19* gene

CYP2C19 is mainly expressed in the liver, but has also been detected in the duodenum (Klose et al., 1999; Lapple et al., 2003). The enzyme is involved in the metabolism of approximately 5% of drugs currently used in clinics (Ingelman-Sundberg, 2004b). Important *CYP2C19* substrates include proton pump inhibitors (e.g. omeprazole, lansoprazole, pantoprazole), diazepam, the selective serotonin reuptake inhibitors (SSRI) citalopram and escitalopram, the anti-malaria drug proguanil, and the anti-coagulant clopidogrel (Herrlin et al., 2000; Klotz et al., 2004; Schwab et al., 2004; Ingelman-Sundberg et al., 2007; Mega et al., 2009).

The *CYP2C19* gene, which is localized on chromosome 10, is highly polymorphic. More than 20 different alleles have been described for *CYP2C19*, located in both the 5'-flanking region as well as in coding and noncoding parts of the gene (Sim and Ingelman-Sundberg, 2006). However, only a few of these variants have been characterized with regards to their potential to modulate the metabolic capacity of *CYP2C19*. Individuals can be classified according to their *CYP2C19* genotype and the associated *CYP2C19* enzyme activity. Thus, subjects can be divided into extensive metabolizers (EM; homozygous or heterozygous for the wild type allele), intermediate

metabolizers (IM; one mutant allele responsible for the slow metabolizer phenotype), poor metabolizers (PM; two mutant alleles responsible for the slow metabolizer phenotype), or ultrarapid metabolizers (UM; carriers of the promoter SNP *CYP2C19*17*) (Sim et al., 2006). Compared to EMs, PMs are to a lesser extent able to metabolize CYP2C19 substrates, as shown in several *in vivo* studies using classical CYP2C19 probe drugs such as S-mephenytoin or omeprazole (de Morais et al., 1994; Chang et al., 1995; Masimirembwa et al., 1995; Jurima-Romet et al., 1996; Roh et al., 1996; Furuta et al., 2005).

Several alleles have been described, which are responsible for the occurrence of a PM phenotype, when present in a homozygous manner. The most common defective variants in Caucasians and Asians are the alleles *CYP2C19*2*, which causes a splice defect, and *CYP2C19*3*, which leads to the formation of a premature stop codon (Klotz et al., 2004). Subjects homozygous for *CYP2C19*2* or *CYP2C19*3* do not express any CYP2C19 protein. The interethnic variability in the distribution of the PM genotype is fairly high, ranging from approximately 2.8% in Caucasians to 23% in Asians (Xie et al., 1999; Desta et al., 2002). It has been shown that CYP2C19 PMs exhibit three to thirteen times higher exposure to proton pump inhibitors (PPIs) than EMs. In IMs the bioavailability of PPIs is two to four times higher than in EMs (Klotz et al., 2004). This has important clinical consequences: It was shown in several studies that PMs have significantly higher benefits from therapy with PPIs compared to EMs, as mirrored in e.g. higher *Helicobacter pylori* eradication rates during triple therapy with anti-ulcer drugs (Yang and Lin, 2010). On the other hand, PMs suffer more often from side effects induced by other CYP2C19 substrates such as diazepam or clopidogrel than EMs (Bertilsson, 1995; Mega et al., 2009).

In 2004 two novel SNPs in the *CYP2C19* gene were identified in our laboratory (Sim et al., 2006). These variants are in complete linkage disequilibrium and are localized in the 5'-flanking region of CYP2C19 at position -806 and at position -3402, together constituting the *CYP2C19*17*

allele. The allelic frequency of *CYP2C19*17* was found to be 18% in both a Swedish and an Ethiopian population compared to only 4% in Chinese and 1.3% in Japanese individuals (Sim et al., 2006; Sugimoto et al., 2008).

A retrospectively performed genotyping study of a Caucasian cohort previously phenotyped for omeprazole metabolism revealed a significantly reduced omeprazole AUC of 40% in homozygous *CYP2C19*17* carriers compared to *CYP2C19* wild type carriers, as predicted from the metabolic ratios for omeprazole. Furthermore, *in vivo* hepatic transfections in male CD1 mice (Charles River Laboratories, Uppsala, Sweden) revealed that luciferase plasmids containing the *CYP2C19*17* allele showed enhanced hepatic transcriptional activity compared to the corresponding wild-type promoter (Sim et al., 2006).

Interestingly, the C>T exchange at position -806 creates a potential consensus DNA-binding motif for the members of the transcription factor family GATA. EMSA analysis revealed that -806T is indeed capable of forming complexes with proteins present in nuclear extracts of human liver. These observations led to the hypothesis that the T variant at position -806 influences the transcriptional rate of the *CYP219* gene (Sim et al., 2006).

1.2.2 Important CYP2C19 substrates

1.2.2.1 Omeprazole

Omeprazole belongs to the group of proton pump inhibitors (PPIs). PPIs are first choice drugs for the treatment of peptic ulcers (PU) and their complications (e.g. bleeding), gastroesophageal reflux disease (GERD), nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal lesions, Zollinger-Ellison syndrome, dyspepsia, and eradication of *Helicobacter pylori* together with antibiotics (Shi and Klotz, 2008). As typical for PPIs, omeprazole binds selectively and irreversibly to the H⁺-K⁺-ATPase in the parietal cells of the stomach and inhibits gastric acid secretion. Omeprazole is administered as racemic mixture and is transformed via two

major metabolic pathways, hydroxylation catalyzed by CYP2C19 and sulfoxidation catalysed by CYP3A4. The metabolism of omeprazole is enantioselective, whereby the *R*-enantiomer is mainly metabolized by CYP2C19 while the *S*-enantiomer is mainly transformed by CYP3A4 (Laine et al., 2003; Schwab et al., 2005).

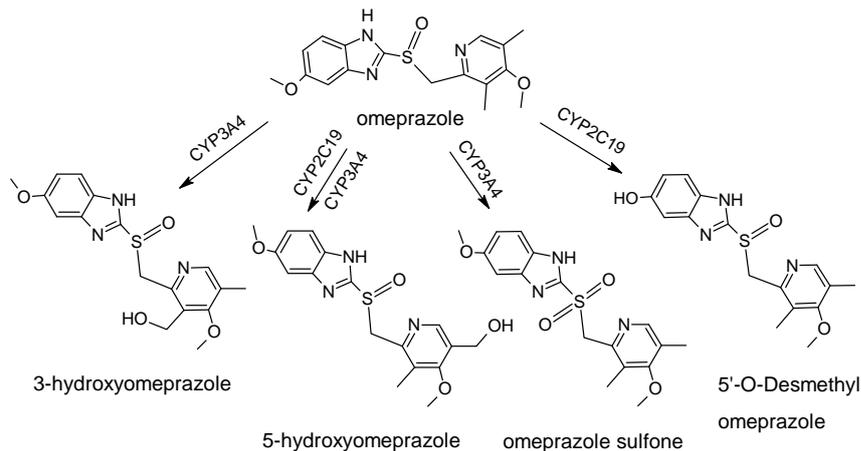


Fig. 1: The metabolic pathways of omeprazole

An effective drug treatment with PPIs, such as omeprazole, is highly dependent on the CYP2C19 genotype (Ingelman-Sundberg, 2004b). CYP2C19 PMs show prolonged half-lives as well as significantly higher omeprazole AUCs compared to EMs (Chang et al., 1995; Sagar et al., 1998). In a study using 20 mg of omeprazole for the treatment of *Helicobacter pylori* infections in peptic ulcers, the observed cure rates were very low in EMs (25%), 50% in IMs, and complete (100%) in PMs (Furuta et al., 1998).

1.2.2.2 Escitalopram

Citalopram acts as a selective serotonin reuptake inhibitor (SSRI) in the central nervous system, and enhances serotonin-dependent neurotransmission by enlarging the serotonin amount in the intersynaptic cleft (Milne and Goa,

1991). Citalopram is marketed as racemate; its *S*-enantiomer is sold as the self-contained drug escitalopram and appears to mediate the major antidepressant effect due to especially high affinity to the serotonin transporter (SERT) (Herrlin et al., 2003).

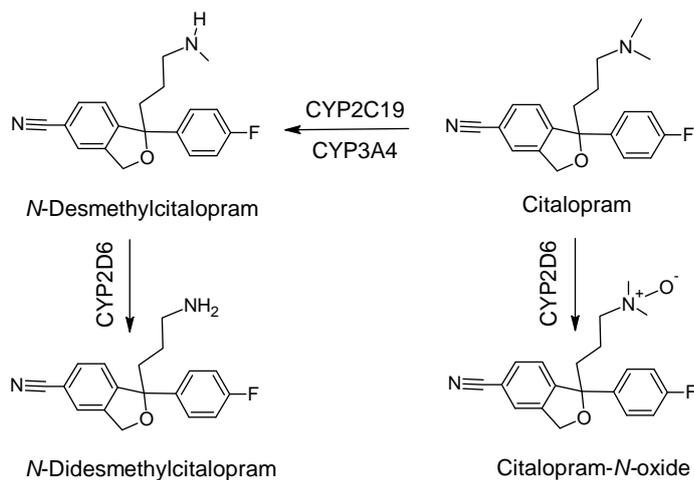


Fig. 2: The metabolic pathways of citalopram

Escitalopram is a well established drug in clinics and is used for the treatment of dysthymias and depression, as well as of anxiety disorders, obsessive compulsive disorder, substance abuse, and dementia-related behavioural disturbances. Escitalopram is mainly eliminated through the liver by *N*-desmethylation to desmethylcitalopram (DCT) and didesmethylcitalopram (DDCT). CYP2C19 is mainly responsible for the first elimination step (Yu et al., 2003). The transformation of DCT to DDCT is catalyzed by CYP2D6. Both metabolites are considered to be inactive (Herrlin et al., 2003). A major problem in clinical use of escitalopram is given by the high interindividual variations in plasma levels, which can lead to a less effective clinical response or to a higher risk for the development of toxic drug effects. The observed differences in escitalopram plasma concentrations are considered to be at least

partly associated with interindividual differences in the activity of the polymorphic expressed CYP2C19 (Yu et al., 2003; Rudberg et al., 2008).

1.2.3 Transcriptional regulation of the CYP2C19 gene

In phenotyping studies a wide range in individual CYP2C19 activities have been observed, which are only partly explained by polymorphic expression or by xenobiotic-induced modulation of CYP2C19 activity. Interestingly, CYP2C19 activity also varies significantly among homozygous extensive metabolizers (*1/*1 carriers) (Chang et al., 1995; Sim et al., 2006). The reasons underlying such variability are still unknown.

Considerable attention has recently been paid to the potential significance of transcriptional regulation of *CYP2C19*. In 2004 Arafeyene *et al.* predicted *in silico* a number of potential binding sites for transcription factors in the *CYP2C19* 5'-flanking region, including the progesterone receptor (PR), Hox proteins, GATA proteins, TATA box-binding protein (TBP), and Pbx-1 (Arafeyene et al., 2003). Later, Kawashima *et al.* identified and experimentally addressed the functional relevance of two neighbouring direct repeat-1 (DR1) elements within the first 200 bp of both the *CYP2C9* and *CYP2C19* promoters (Kawashima et al., 2006). Whereas all these sites were capable of binding the transcription factor hepatocyte nuclear factor-4 α (HNF-4 α), transactivation by HNF-4 α was only shown for the *CYP2C9* promoter. However, silencing experiments in human hepatocytes knocking down HNF-4 α recently showed that HNF-4 α is important for rifampicin-mediated upregulation of *CYP2C19* gene expression (Rana et al., 2010). Furthermore the mRNA expression levels of *CYP2C19* were recently found to be significantly associated with the HNF-4 α content in twenty human liver samples, underlining the important role of HNF-4 α in maintaining the basal CYP2C19 expression (Wortham et al., 2007). Chen *et al.* identified a putative CAR/PXR-responsive element and a putative glucocorticoid receptor (GR) responsive element further upstream within the first 2 kb of the 5'-

Introduction

flanking-region of the *CYP2C19* gene, which were transactivated by CAR/PXR, and GR in HepG2 cells and enhanced the *CYP2C19* promoter activity upon treatment with rifampicin and dexamethasone, respectively (Chen et al., 2003). Bort et al. described three putative HNF-3 binding sites within the first 610 bp of the *CYP2C19* 5'-flanking region and showed that *CYP2C19* mRNA expression is significantly upregulated by HNF-3 γ in HepG2 cells (Bort et al., 2004).

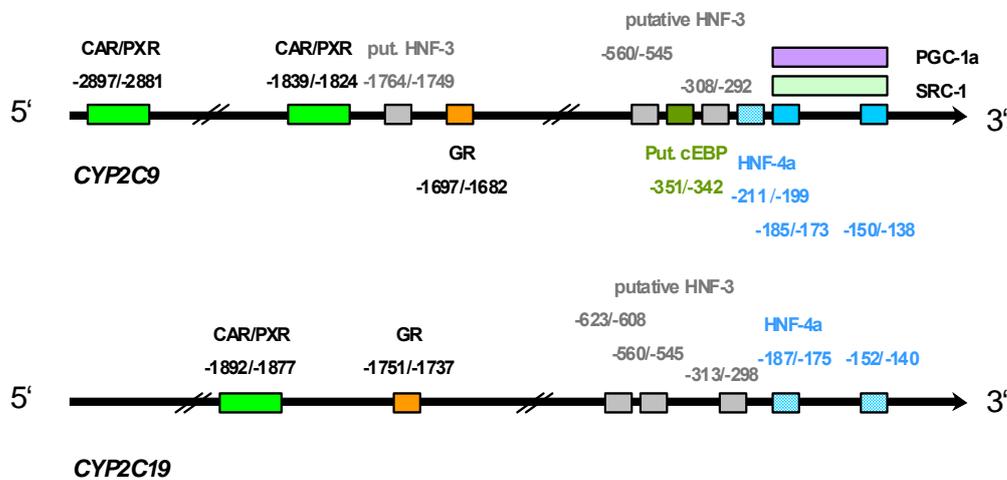


Fig. 3: Functionally confirmed and putative transcription factor binding sites within *CYP2C9* and *CYP2C19* promoters. The checked boxes show HNF-4 α binding sites, which appear not to be functional

It still remains to be elucidated how these transcription factors contribute alone or in concert to the observed interindividual differences in *CYP2C19* expression and activity.

1.3 CYP2C9 - GENE AND PROTEIN

1.3.1 General aspects of CYP2C9

CYP2C9 shows a wide range in interindividual hepatic expression levels and is the second most abundantly expressed CYP enzyme in the human liver (Miners and Birkett, 1998). CYP2C9 is also expressed in several extrahepatic tissues including kidneys, gut, brain, heart, aorta and other vessels, as well as in lungs (Klose et al., 1999; Delozier et al., 2007). It has been estimated that CYP2C9 metabolizes approximately 16% of all clinically prescribed drugs (Chen et al., 2004). This includes many important therapeutics like phenytoin, tolbutamide, warfarin, torasemide, losartan and several NSAIDs. Some of these drugs are characterized by a small therapeutic window (Urquhart et al., 2007). Furthermore, CYP2C9 is, similarly to CYP3A4 and CYP2B6, inducible by exposure to the agents dexamethasone, rifampicin, phenobarbital, and hyperforin (Gerbai-Chaloin et al., 2001).

The involvement of CYP2C9 in warfarin metabolism has particularly important clinical consequences. Warfarin has a narrow therapeutic window: Too low dosing increases the risk of thrombosis, whereas doses too high are associated with a higher incidence of serious bleeding (Urquhart et al., 2007). The inducibility of CYP2C9 and its polymorphic expression explain some of the variation in interindividual responses to warfarin (Peyvandi et al., 2004).

CYP2C9 has also important physiological functions. The enzyme is involved in the endogenous generation of vasoactive molecules such as epoxyeicosatrienoic acids (EETs) and hydroxyeicosatrienoic acids (HEETs) from arachidonic acid in both liver and extrahepatic tissues. EETs play a critical role in vascular homeostasis as endothelial-derived hyperpolarizing factors (EDHF) and are as signalling molecules involved in the promotion of endothelial cell proliferation, migration and angiogenesis (Fleming, 2001; Fleming, 2008).

1.3.2 Polymorphisms in *CYP2C9*

More than 50 SNPs have been described in both the exonic and intronic regions of the *CYP2C9* gene (Sim and Ingelman-Sundberg, 2006). Furthermore, more than 520 variants have been detected in the 5' upstream region of the gene (Zhou et al., 2009). However, only two common coding variants, termed *CYP2C9**2 (the missense mutation 430T>C causing the amino acid substitution R144C (Rettie et al., 1994) and *CYP2C9**3 (the missense mutation 1075A>C on exon 7 leading to the I359L substitution) (Sullivan-Klose et al., 1996), have functional consequences for enzyme activity, having allele frequencies of around 11% (*CYP2C9**2) and 7% (*CYP2C9**3) in white subjects. They occur at much lower frequencies in African and Asian populations (Kirchheiner et al., 2004). Both genetic variants lead to reduced enzyme activity and thereby to a less effective metabolism towards *CYP2C9* substrates, such as warfarin or S-naproxen. *CYP2C9**2 and *CYP2C9**3 have been associated with a higher risk for drug side effects in patients treated with coumarin derivatives, such as warfarin (Kamali and Wynne, 2010). Both polymorphisms lead to significantly lower hydroxylation rate of S-warfarin and have a strong impact on the intrinsic clearance of this drug. Therefore carriers homozygous for *CYP2C9**2 or *CYP2C9**3 require smaller loading and maintenance doses of warfarin and have a four-fold increase in risk of bleeding complications, particularly at the beginning of a warfarin therapy (Gage and Lesko, 2008)

1.3.3 Transcriptional regulation of the *CYP2C9* gene in the liver and in liver-derived cell lines

As for *CYP2C19*, the transcriptional regulation of the *CYP2C9* gene is not completely understood yet. PXR, CAR, and GR have been implicated in regulating *CYP2C9* expression. *Chen et al.* showed that PXR binds to two PXR/CAR-responsive elements within the first 3 kb of the *CYP2C9* 5'-flanking region and mediates the induction of *CYP2C9* by rifampicin,

hyperforin, and phenobarbital (Chen et al., 2004). The more proximally localized PXR/CAR-binding element at position -1839/-1824 is also able to bind vitamin D receptor (VDR) *in vitro* and to mediate a modest upregulation of *CYP2C9* promoter by 1,25-hydroxyvitamin D₃ (Drocourt et al., 2002). Moreover, induction of *CYP2C9* mRNA expression by dexamethasone and phenobarbital is likely to be mediated by several GR and CAR response elements within the *CYP2C9* promoter (Gerbai-Chaloin et al., 2001; Ferguson et al., 2002; Chen et al., 2004). HNF-4 α and HNF-3 γ are important regulators of the constitutive expression of CYP2C enzymes. Both transcription factors have been shown to modulate the basal expression of *CYP2C9* (Jover et al., 2001; Ferguson et al., 2002; Bort et al., 2004; Chen et al., 2005). Kawashima *et al.* could show that an HNF-4 α mediated modulation of *CYP2C9* promoter activity is mediated by two proximally located DR1 binding elements at positions -150/-138 and -185/-173 (Kawashima et al., 2006). Several possible binding sites for HNF-3 γ have been detected within the 5'-flanking region of *CYP2C* genes. HNF-3 γ -dependent activation of *CYP2C9* has been proven both on the mRNA level and in reporter gene studies (Bort et al., 2004). Furthermore, *CYP2C9* expression seems to be modulated by the transcription factor C/EBP α . The C/EBP α effect is mediated by a CCAAT box element within the proximal promoter of *CYP2C9* (Ibeanu and Goldstein, 1995).

In addition to the described isolated effects of various transcription factors on *CYP2C9* promoter activity, regulatory proteins mediate their influence on the expression of *CYP2C9* by interacting with each other or by interaction with further co-factors. For example, HNF-4 α synergistically enhances the promoter activity of *CYP2C9* with CAR and PXR (Chen et al., 2005). HNF-4 α moreover interacts with the co-activators SRC-1 and PGC-1 α , whereby promoter enhancement of *CYP2C9* by especially PGC-1 α is highly dependent on HNF-4 α abundance (Martinez-Jimenez et al., 2006).

two major progestins progesterone and 17α -hydroxyprogesterone are formed in a metabolic side way by transformation of pregnenolone.

1.4.2 The hypothalamic-pituitary-ovarian axis

The hypothalamic-pituitary axis is responsible for the regulation of the estrogen/progesterone production of the ovary and, thus, responsible for the hormone/menstrual cycle of the woman. Neurons of the hypothalamus synthesize and release gonadotropin-releasing hormone (GnRH) in a pulsatile manner. The hormone binds to receptors at the anterior pituitary and stimulates the release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). During the first half of the menstrual cycle elevated FSH levels lead to the maturation of the follicle in the ovary and stimulate aromatase production needed for estradiol synthesis in the granulosa cells of the follicle.

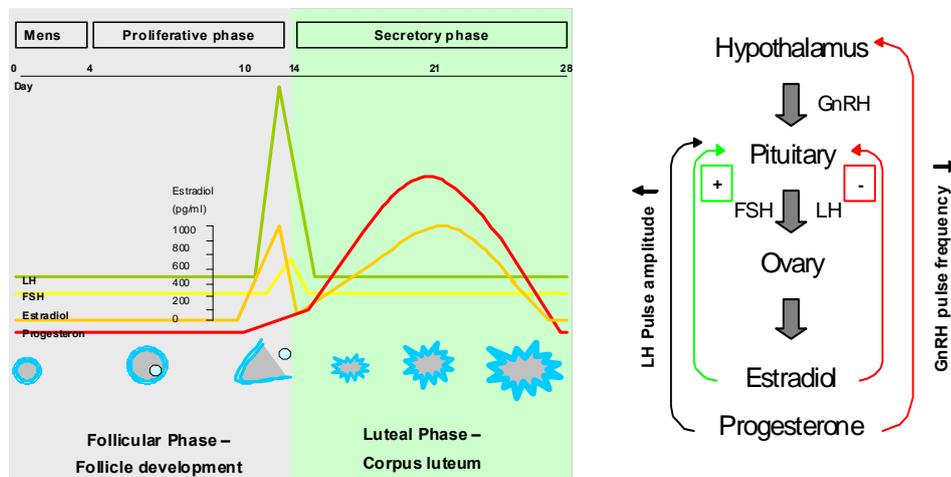


Fig.5 A) The hormonal cycle of a woman. Steep raises of LH and FSH are reached during the preovulatory phase, leading to the rupture of the follicle. **B)** Estrogens are inhibiting the anterior pituitary during early follicle phase. Two days before ovulation high estrogen levels have a positive effect on LH and FSH production. Progestins lower the frequency of LH pulses and higher the amplitude of them. Figures adapted from (Boron, 2009) and (Aktories and Forth, 2009)

The aromatase converts androstendione to estrone, which is itself further converted to estradiol. LH is meanwhile stimulating the androstendione synthesis in the theca cells of the maturing follicle.

The androgens are diffusing into the granulosa cells and are here finally aromatized to estrogens. The peak level of FSH is reached one day before a steep raise of LH which characterises the ovulation with is characterized by the rupture of the graafian follicle. The high LH levels stimulate especially a high production of progesterone by the theca-lutein cells of the corpus luteum during the luteal phase. High levels of progesterone inhibit follicular growth during this phase. Luteal function begins to decrease around 11 days after ovulation and ends with the demission of the corpus luteum.

1.4.3 The Estrogen Receptor

1.4.3.1 The family of nuclear receptors (NRs)

The estrogen receptor family belongs to the large protein group of nuclear/steroid receptors, most of which are ligand-dependent transcription factors. There are around 50 different nuclear receptors known at the moment (selected members shown in Table 1). NRs have several characteristics in common. They are structurally composed of six functional regions (A–F) showing various degrees of sequence conservation. The N-terminal A/B domain is not well conserved among NRs, and harbours the transactivation domain AF-1. The highly conserved C region comprises the DNA-binding domain (DBD) and the conserved E region contains the ligand-binding domain (LBD). The two remaining regions, D, a linker peptide between the DBD and the LBD and F, the C-terminal extension region of the LBD are again of variable size and are not conserved. The LBD binds agonistic or antagonistic ligands (e.g. hormones, vitamins, or toxins). The DBD is composed of two zinc fingers. The typical domain composition of NRs is shown on figure 6.

An often occurring scenario is that NRs homo- or heterodimerize after ligand

binding. The NR dimer travels from the cytoplasm into the nucleus, binds to its specific consensus sequence in the target gene promoters and modulates gene expression. The recognition sites for NRs comprise typically a pair of 5 to 6 bp long DNA sequences (two half sites) which are often separated by a spacer of 1 to 6 bases of length.

Table 1. Selected members of the NR family

Receptor	Ligand	Subtypes	gene symbol
TR	thyroid hormones	α , β	NR1A1, NR1A2
ER	estrogens	α , β	NR3A1, NR3A2
PR	progesterone		NR3C3
AR	testosterone, DHT		NR3C4
MR	mineralocorticoids	α , β	NR3C2
GR	glucocorticoids		NR3C1
VDR	vitamine D		NR1I1
RAR	All-trans retinoic acid	α , β , γ	NR1B1, NR1B2, NR1B3
RXR	9-cis-retinoic acid	α , β , γ	NR2B1, NR2B2, NR2B3
LXR	oxysterols	α , β	NR1H1, NR1H2, NR1H3
FXR	bile acids		NR1H4
PPAR	fatty acids, prostaglandins	α , γ , δ	NR1C1, NR1C2, NR1C3
PXR	xenobiotics		NR1I2
CAR	xenobiotics, androstane		NR1I3
HNF-4	fatty acids	α , γ	NR2A1, NR2A2

1.4.3.2 The estrogen receptors – physiology of action

Estrogen receptors (ERs) mediate estrogenic effects. There are two types of ERs: ER α and ER β . Particularly ER α is responsible for physiological reproductive functions and reproductive behaviour (McDevitt et al., 2008). ER α is ubiquitously expressed and is especially abundant in endometrial tissue, mammary glands, bone tissue, blood vessels, and hypothalamus, in which it mediates estrogen-dependent tissue development, and influences the bone density, the cardiovascular system, and the mating behaviour, and takes

part in the regulation of the hypothalamic-pituitary axis. ER β can be detected in the ovary, prostate, testis, lung, thymus, spleen, heart, and brain. (Hall and McDonnell, 2005). It has become evident that ER β has functions that are distinct from ER α . Studies with knock-out mice have shown that that ER α is the main player in mediating female reproductive functions, whereas ER β is more important in non-classical target tissues. Mice lacking ER β develop hypertension, malignancies in colon and prostate, and show increased anxiety (Swedenborg et al., 2009).

Both ER α and ER β share modest overall sequence identity. The conservation is, however, significantly higher within the DBD and LBD domains (Fig. 6) (Hall and McDonnell, 2005).

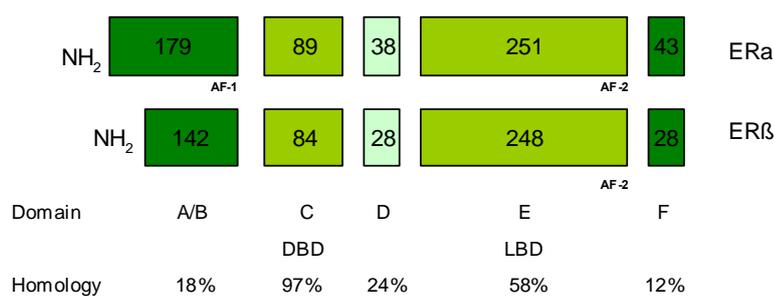


Fig. 6: Domain composition and amino acid similarity of ER α and ER β . Numbers within the boxes represent the number of amino acid residues. The percentages denote the grade of amino acid homology. DBD, DNA-binding domain; LBD, ligand-binding domain. Adapted from (Hall and McDonnell, 2005)

Both ER α and ER β show a similar affinity to 17 β -estradiol, but have got different relative affinities towards several other synthetic or naturally occurring ligands (Barkhem et al., 1998).

In the non-activated state, ERs are located in the cytoplasm and are bound to heat shock proteins (e.g. Hsp90), which block the DNA binding domain. Upon hormone binding the receptor dissociates from its chaperone molecules and undergoes a conformational change, which leads to dimerization and

translocation of the receptor into the nucleus. ER α and ER β can either homo- or heterodimerize, which can lead to the formation of ER α -ER α , ER α -ER β or ER β -ER β complexes.

Both ER α and ER β preferentially bind to similar palindromic DNA consensus sequences (EREs) within their target promoters, which are composed of two GGTC A half sites (GGTCANNNTGACC). ER α is also able to bind to imperfect EREs or ERE half sites (van de Stolpe et al., 2004; Welboren et al., 2009). ER binding to DNA leads to a relocation of central α -helices and the formation of a hydrophobic pocket which can bind co-activator or co-repressor proteins.

A large number of co-regulators can interact with ERs via binding the transactivation domains within the NH₂-terminus (AF-1) and next to the LBD (AF-2). In some cells binding to both AFs is required for maximal transcriptional activity (McDonnell and Norris, 2002). Especially the AF-2 domain has been studied concerning its co-regulator recruitment. Here, in particular co-activators of the p160 family are able to recruit histone acetyltransferases, such as p300 and CBP. Histone acetylation leads to the decondensation of the chromatin structure within the promoter regions of the target genes, which facilitates the binding of the general transcription initiation machinery (Kamei et al., 1996). Co-activators such as TRAP220 are able to directly interact with transcription-associated proteins, e.g. RNA-polymerase II (Kang et al., 2002). In contrast, co-repressor proteins, such as RIP140, SMRT or NCoR, recruit proteins with histone deacetylase activity, which induce a more compact chromatin formation within the promoter region, thus impeding transcription. RIP140 can also occlude the access of coactivators to the AF-2 domain (Hall and McDonnell, 2005).

Besides directly binding to their DNA consensus sequences (classical pathway), ERs can act via at least three non-classical pathways: ER activation can occur ligand-independently through second-messenger pathways that

alter the activity of intracellular kinases and phosphatases, resulting in an altered phosphorylation status of ER. Furthermore, ligand-activated ER can interact in an indirect manner with regulatory promoter regions through protein-protein binding. A typical example is the ER interaction with AP1-consensus sequences through a binding to the transcription factors c-Fos and c-Jun. Finally, cell membrane-bound ER can influence cellular signalling pathways, which lead to activation of kinases that phosphorylate other transcription factors (McDevitt et al., 2008).

1.4.4 Selective ER modulators (SERMs)

SERMs are tissue-selective ER modulators, which differ from pure estrogen antagonists, such as ICI 182,780, in their ability to display tissue-selective ER agonist-antagonist activities. Typical examples for this substance group are the therapeutics tamoxifen and raloxifene. The estrogen-antagonistic effect of tamoxifen in the breast makes it a first-line therapy against ER-expressing breast cancer. However, tamoxifen shows in parallel estrogen-agonistic effects in the uterus and is therefore associated with a higher risk for the development of endometrial cancer. Raloxifene is used in therapy of osteoporosis, and does not have a stimulatory effect on the endometrial tissue. The mechanisms by which SERMs express both agonistic and antagonistic effects, are likely to be based on the fact that SERMs induce a spectrum of different AF-2 binding site conformations which define the recruitment of co-activators and/or co-repressors. Dependent on the AF-2 conformational change, on the exact promoter that is targeted, and on the abundance and expression level of co-activators and co-regulators in the cell, tissue-selective ER-dependent modulation of gene expression is achieved.

1.4.5 Oral contraceptives

Ethinylestradiol (ETE), mestranol, and estradiol (EE) are estradiol derivatives mainly used in oral contraceptives (OCs) and hormone replacement therapy.

Whereas ethinylestradiol and mestranol are utilized in OC preparations, estradiol can be found in products used for hormone replacement therapy. In OCs the estradiol derivatives are commonly combined with progesterone derivatives to obtain better cycle control in women with intact uterus (Laine et al., 2003).

The effect of OCs is based on the mechanism that contraceptive steroids exert negative feedback to the hypothalamic-pituitary axis, leading to suppressed secretion of the gonadotropins FSH and LH. Low FSH levels are responsible for insufficient folliculogenesis, whereas low LH levels inhibit ovulation. Progestins help to thicken the cervical mucus and thus inhibit sperm penetration into the uterus. Furthermore, they decrease uterus motility and induce changes in the endometrial tissue, making the endometrium more resistant against embryo implantation.

1.4.6 Estradiol derivatives and their influence on CYP2C9 and CYP2C19 activity

OC formulations are able to influence CYP2C19 dependent metabolism as shown in several *in vivo* studies with different CYP2C19 marker substances. OCs e.g. double the plasma AUC ratio of omeprazole/5-hydroxyomeprazole and the urinary metabolic (S)/(R) ratio of mephenytoin. Interestingly, the inhibitory effect on CYP2C19 activity was only observed during parallel treatment with combined oral contraceptives containing both ethinylestradiol and progestins, but not during a treatment with minipills, containing progestins only (Tamminga et al., 1999; Laine et al., 2000; Hagg et al., 2001; Palovaara et al., 2003).

Furthermore, several studies have shown that estrogen-containing OC formulations are able to affect the pharmacokinetics and metabolism of several other drugs metabolized by CYP2C19 including propranolol, proguanil and selegiline (Walle et al., 1996; Laine et al., 1999; McGready et al., 2003). The observed OC related effects probably cannot be ascribed to an

inhibition of CYP2C19 alone as other CYP enzymes are also involved in the metabolism of the mentioned drugs. Both weak and strong inhibitory effects of estradiol derivatives on CYP2C19 activity have been also observed *in vitro*. Here the measured enzymatic activity of CYP2C19 towards mephenytoin and omeprazole was reduced in liver microsomes upon treatment with EE (Jurima et al., 1985; Laine et al., 2003; Rodrigues and Lu, 2004). A direct inhibitory effect on the CYP2C19 enzyme has been suggested as the mechanism behind the observed effects.

The enzyme CYP2C9 has been found to be significantly inhibited by ethinylestradiol *in vitro* (Laine et al., 2003). Furthermore a genotype-phenotype correlation study, which included individuals treated with the CYP2C9 substrate losartan revealed that high variation in losartan plasma levels observed within the CYP2C9 wild-type group was partly caused by the parallel intake of OCs during the study, as the use of OCs led to slower losartan metabolism (Sandberg et al., 2004).

1.5 THE TRANSCRIPTION FACTOR FAMILY GATA AND ITS CO-REGULATORS

The GATA protein family comprises six members of evolutionarily conserved and widely expressed zinc finger transcription factors, GATA-1 to -6, which all recognize the DNA consensus motif (A/T)GATA(A/G).

Especially the family founding member GATA-1 as well as GATA-2 and GATA-3 have been suggested to play a central role in the regulation of a number of important genes involved in the development and control of haematopoiesis and some ectodermal derivatives (Ohneda and Yamamoto, 2002). GATA-4, -5 and -6 are especially abundantly expressed in cardiac tissue and endodermal derivatives (Peterkin et al., 2005) and have been shown to play a significant role in the transcriptional regulation of several

genes important for cardiac development and cardiomyocyte differentiation (Crispino et al., 2001; Peterkin et al., 2003; Brewer and Pizzey, 2006).

Table 2: Expression profile of GATA proteins and their implication in the development of pathological phenotypes

SUB-GROUP	NAME	EXPRESSION PROFILE IN HUMANS	*	PHENOTYPIC EFFECTS WHEN MUTATED/ KNOCKED OUT**
I	GATA-1	Erythroid, eosinophil and mast cells, megakaryocytic lineages	X	anemia, thrombocytopenia, dyserythropoietic anemia, early death of embryo
	GATA-2	Early erythroid cells, mast cells, megakaryocytic lineages, multipotent progenitor cells, endothelial cells	3	anemia, thrombocytopenia, dyserythropoietic anemia, early death of embryo
	GATA-3	T-lymphocytes, developing parathyroid	10	Hypoparathyroidism, deafness, renal dysplasia
II	GATA-4	heart, gut, ovary, testis, liver, small intestine	8	hypoplastic myocardium, cardia bifida, septal defects early death of embryo
	GATA-5	Heart, small intestine	20	
	GATA-6	pancreas, adrenal cortex, ovary, testis, lung, liver, brain, small intestine	18	

* Chromosome

** Mutations in humans or in animals; knock out in animal experiments

GATA-4 is also abundantly expressed in the liver where it is involved in the regulation of expression of albumin (Bossard and Zaret, 1998), erythropoietin (Dame et al., 2004) and Hex homeodomain proteins (Denson et al., 2000).

All GATA proteins have two highly conserved zinc fingers, which comprise the DNA-binding domain. It seems that especially the C-terminal zinc finger and its adjacent basic domain are required for DNA sequence recognition and DNA-binding. Studies on GATA-1 revealed that the N-terminal zinc finger

does not contact DNA directly during binding to a single consensus motif (Visvader et al., 1995; Morrisey et al., 1997). The N-terminal zinc finger rather seems to stabilize the DNA-binding and to assure binding specificity (Whyatt et al., 1993). However, in case of two adjacent GATA binding sites, which often occur in vertebrate genes, both zinc fingers are required for high affinity DNA-binding. Furthermore both zinc fingers can directly interact with other regulatory proteins (Trainor et al., 2000; Brewer and Pizzey, 2006). GATA genes have been shown to be strongly conserved in mammals. All GATA genes possess at least one non-coding exon and the zinc fingers are encoded on separate exons. Especially the cardiac subgroup GATA-4, GATA-5 and GATA-6 show very high homology in their DNA-binding domains but diverge considerably in their N- and C-terminal regions, which contain the transcription activation modules (Temsah and Nemer, 2005).

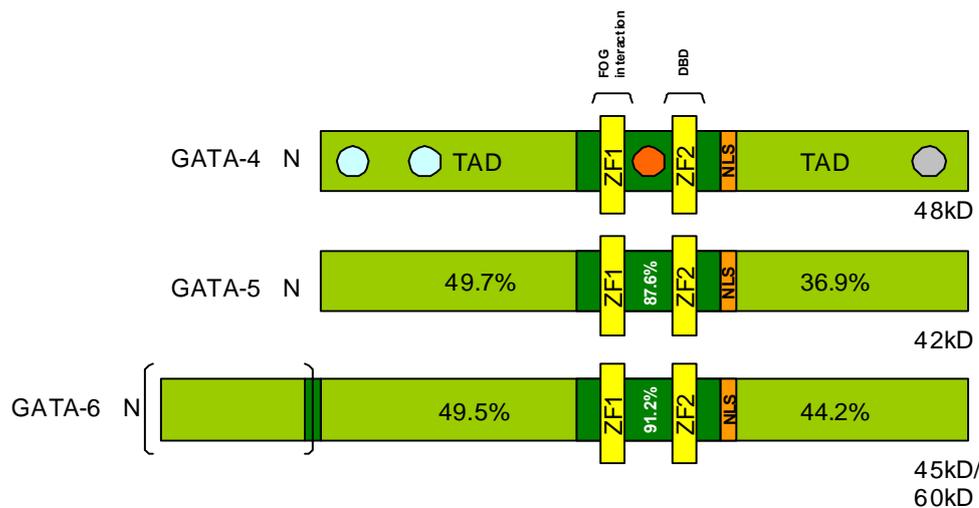


Fig. 7: Domain composition and amino acid similarity of GATA-4, GATA-5 and GATA-6 in vertebrates. Numbers within the boxes represent the percentage of amino acid similarity towards GATA-4. DBD, DNA-binding domain; ZF, zinc finger; NLS nuclear localisation signal; TAD transactivation domain; blue circle: MAPK phosphorylation site; orange circle: PKA phosphorylation site, grey circle: PKC phosphorylation site. In brackets: longer GATA-6 isoform. Figure adapted from (Temsah and Nemer, 2005) and (Brewer and Pizzey, 2006)

GATA factors have been shown to interact with many co-regulatory proteins. Studies have been especially focussed on GATA-4-dependent promoter regulation of cardiac genes. Examples of interactive proteins that enhance a GATA-4-dependent promoter activation include Nkx2-5, MEF-2, SRF, dHAND, SMAD1/4, Tbx5, c-Fos, STAT proteins, YY1, Zfp260, and p300 (Brewer and Pizzey, 2006).

Besides co-activators, several co-repressors of GATA proteins have been described. Amongst them, a family of nuclear zinc finger proteins, which comprises the members FOG-1 and FOG-2 (Friend of GATA), plays an important role (Hirai et al., 2004). While FOG-1 has been shown to be co-expressed together with GATA-1, -2, and -3 and to be involved in the differentiation of hematopoietic cell lineages (Tsang et al., 1997), FOG-2 is co-expressed with GATA-4, -5, and -6 in the heart throughout its development and in the adult (Lu et al., 1999; Svensson et al., 1999). In animal experiments it has been shown that FOG proteins are essential for the development of both the hematopoietic and cardiovascular system (Tsang et al., 1997; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999). Highest expression levels of FOG-2 are observed in the heart, brain, and testis. However, it has been also found, though at lower levels, in human liver (Lu et al., 1999; Svensson et al., 1999). FOG-2 interacts via its C-terminal zinc fingers 5 to 8 with the N-terminal zinc finger of GATA-4, -5 and -6, thus repressing GATA-mediated transcriptional activation of target promoters (Lu et al., 1999). Engineered mice in which the GATA-4/FOG-2 interaction is disrupted display cardiac malformations similar to those observed in FOG-2 deficient embryos, suggesting that most of the functions by FOG-2 in the heart are mediated via its interaction with GATA-4 (Crispino et al., 2001). Recently, Roche *et al.* showed that the metastasis-associated proteins (MTA) -1, -2 and -3, as well as the retinoblastoma proteins RbAp46 and RbAp48, interact with FOG-2 and are essential for the repressive effect of FOG-2 on GATA-4 activity (Roche et al., 2008).

In addition to its involvement in the expression of many genes important for cardiac and endocrine function (reviewed in (Brewer and Pizzey, 2006) and (Viger et al., 2008)), GATA-4 has also been shown to regulate the expression of drug-metabolizing enzymes and liver transporters, such as epoxide hydroxylase (Zhu et al., 2004) and the ATP-binding cassette transporters ABCG5 and ABCG8 (Sumi et al., 2007). Furthermore the expression of CYP19, one of the key genes in estrogen synthesis, is regulated by GATA-4 (Cai et al., 2007). These data suggest that GATA-4 is also important for the expression of genes implicated in metabolism and transport of both endogenous and exogenous compounds.

2 AIMS

The following *in vitro* and *in vivo* studies were performed to investigate how a polymorphic promoter site in *CYP2C19* and specific transcription factors contribute to *CYP2C9* and *CYP2C19* expression and thereby to the observed interindividual variation in drug metabolism efficacy.

Specifically, the following projects were included in my thesis:

1. An *in vivo* study investigating the effect of *CYP2C19*17* on omeprazole pharmacokinetics
2. An *in vivo* study investigating the effect of *CYP2C19*17* on escitalopram pharmacokinetics
3. Investigation of transcription factor GATA-dependent regulation of *CYP2C9* gene expression
4. Investigation of transcription factor GATA-dependent regulation of *CYP2C19* gene expression
5. An *in vitro* study to investigate the influence of female sex steroids on *CYP2C19* gene expression
6. An *in vitro* study to investigate the influence of female sex steroids on *CYP2C9* gene expression

3 COMMENTS ON METHODOLOGY

3.1 BIOINFORMATICS

Nucleotide sequences were obtained from the online database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Sequencing results for cloned promoter constructs were compared using the pairwise Basic Local Alignment (BLAST) algorithm of NCBI. Any identified mutations in promoter constructs, generated during PCR reactions, were corrected with the GeneTailor mutagenesis kit from Invitrogen (GeneTailor™ Site-directed mutagenesis, Invitrogen™). The corrected plasmids were resequenced and reblasted for control. Transcription factor binding sites were predicted using search engines, such as TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and MatInspector (<http://www.genomatix.de/>).

3.2 CELL LINES AND CELL CULTURE

In the *in vitro* studies described in this thesis the human hepatoma cell lines HepG2 and Huh7 as well as primary human hepatocytes were used. HepG2 and Huh7 cells were used in transient cell transfection experiments as well as for the production of nuclear extracts used in EMSA experiments. These two cell lines were chosen as an *in vitro* model for the hepatic *in vivo* situation with the aim to mimic the hepatic transcription factor composition as well as possible. Furthermore, both cell lines are well transfectable with Lipofectamine™ 2000. A disadvantage of both cell lines is that CYP2C9 and CYP2C19 are very poorly expressed, so that e.g. measurements of RNA expression levels upon treatments with transcription factor ligands are not possible. Therefore, primary human hepatocytes were used to determine the changes in the endogenous expression level of CYP2C19 in Paper V.

For stable transfections of CYP2C19 cDNA, Flp-In™- 293 cells purchased from Invitrogen™ were used. This system allows integration of foreign cDNA into the cell genome at a specific Flp site at a transcriptionally active genomic locus within the cells.

3.3 IN VITRO TRANSFECTIONS

3.3.1 Transient transfections

Transient transfections were performed using Lipofectamine™ 2000 (Invitrogen™). With this method nucleic acids are introduced into cells with the help of cationic lipids. The lipids enclose the genetic material and deliver it to the inside of the cell by fusing with the outer cell membrane. As a result, the transfected nucleic acids are transiently expressed in one generation of a transfected cell line.

Other transfection methods, which are not utilized in this thesis, differ mainly by the nature of the transfection reagent, which can also be based on e.g. calcium-phosphate, cationic polymers, or highly branched organic compounds.

3.3.2 Establishment of a cell line stably expressing CYP2C19

Stable transfections can be achieved by introducing the transfected DNA fragments into the cell genome, so that the foreign DNA can be passed to the next cell generations. In Paper V a stable cell line expressing CYP2C19 was constructed. For that purpose the Flp-In™ system comprising Flp-In™-293 cells from Invitrogen™ were used. This specific cell line expresses the pFRT/*lacZeocin* fusion gene, which contains a single integrated Flp Recombination Target (FRT) site.

In Paper V the cell line stably expressing CYP2C19 was established to measure enzyme activity in dependency of estradiol treatment. For that purpose CYP2C19 cDNA was inserted into the pcDNA5/FRT plasmid. This

construct was co-transfected into the Flp-InTM-293 cells together with the Flp recombinase expression plasmid pOG44 using Lipofectamine 2000. The expressed Flp recombinase mediates insertion of the expression construct, which carries the *CYP2C19* cDNA into the cell genome at the FRT site through site-specific DNA recombination. Afterwards, single colonies resistant to Hygromycin B (InvitrogenTM) were selected and subcultured. The selection of positive clones was performed by detection of *CYP2C19* mRNA and measurement of enzyme activity as described in the next sections. Mock cells were transfected with pcDNA5/FRT empty plasmid and pOG44 and prepared as described above.

3.4 GENE REPORTER ASSAYS

Gene reporter assays are working with plasmid vectors that carry a reporter gene, which encode a well quantifiable protein. The reporter construct used in this thesis is the vector pGL3basic, which carries the reporter gene coding for the protein luciferase. The luciferase protein is able to catalyze the cleavage of a marker reagent which is added to the cell lysate. The metabolites emit fluorescent light. The amount of light is easily detected and measured and is proportional to the amount of expressed luciferase. The 5'-flanking promoter regions of genes are cloned in front of the luciferase gene, and regulate and modulate its expression. Thereby it is possible to measure the effect of a specific transcription factor on the subcloned gene promoter, e.g. by co-transfecting and overexpressing the regulatory proteins of interest or by site-directed mutagenesis of putative transcription factor binding sites within the subcloned promoter.

In paper III and IV gene reporter assays were performed co-transfecting GATA-2-pCMV and GATA-4-pcDNA1.1 constructs together with pGL3basic plasmids carrying around 2 kb long 5'-flanking regions of *CYP2C9* or *CYP2C19* into HepG2 and Huh7 cells. In paper V and VI the construct

ER α -pcDNA3.1 was co-transfected together with *CYP2C19* promoter or *CYP2C9* promoter carrying pGL3basic plasmid into Huh7-cells. Furthermore, site-directed mutagenesis (GeneTailor™ Site-directed mutagenesis, Invitrogen™) was utilized, to confirm any regulatory influence of putative GATA- and ER-binding sites within the *CYP2C9* and *CYP2C19* promoters.

3.5 DETECTION OF CYP2C19 ENZYME ACTIVITY BY P450-GLO™ ASSAY.

In paper V the CYP2C19 enzyme activity dependent on ER α ligand treatment was quantified by measuring the catalytic activity towards a specific substrate in cells stably transfected with CYP2C19. For that purpose Flp-In™-293/CYP2C19 cells were seeded into 96-well plates and incubated with increasing amounts of the CYP2C19 substrate Luciferin-H-EGE (Promega). Luciferin H-EGE is a luminogenic substrate which is converted to luciferin specifically by CYP2C19. The CYP2C19 activity was stopped by adding inhibitors and the amount of produced luciferin was detected by adding Ultra-Glo™ Luciferase enzyme (provided in the P450 Glo™ Assay, Promega) that generates a luminescent signal. The produced luminescence, which is proportional to CYP2C19 activity, was measured in the cell medium using a TD-20/20 Luminometer (Turner Designs).

3.6 ELECTROMOBILITY SHIFT ASSAY (EMSA)

To investigate possible binding of the GATA transcription factors and ER α and ER β to *CYP2C9* and *CYP2C19* promoter elements, EMSAs were carried out in papers III, IV, V and IV.

The EMSA method uses oligonucleotides of 30-40bp in length, which comprise the *in silico* predicted transcription factor (TF) binding sites of interest. Reverse complementary oligonucleotides are hybridized to double

stranded fragments, which are radioactively labelled with ^{32}P . Radiolabelled oligonucleotides are then incubated with nuclear proteins extracted from various cell lines or with *in vitro* translated proteins. Subsequently, the electrophoretic mobilities of the formed TF-oligonucleotide complexes are tested on a polyacrylamide gel. Supershift reactions using antibodies specifically interacting with the transcription factors of interest, reactions using binding site-specific mutated oligonucleotides and competition reactions with unlabelled oligonucleotides (cold probe) are executed to further specify the type of protein which binds to the DNA fragment and to confirm site-specific binding.

In papers III to VI nuclear extracts from HepG2 and Huh7 cells were used in EMSA experiments. Furthermore, *in vitro* translated ER α protein was generated and utilized in EMSAs performed in paper V to further confirm ER-specific binding to the predicted ERE half-site.

3.7 CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) assays investigate the protein-DNA interactions in living cells. In paper III to VI ChIP assays were carried out using HepG2 and Huh7 cells. In the ChIP assay, DNA-bound proteins are crosslinked with formaldehyde, DNA is sheared into fragments by sonication or by enzyme digestion, and protein-DNA complexes of interest are pulled down with the help of antibodies which specifically react with the transcription factor of interest. After reversing the crosslinks, the binding of the protein is finally confirmed by PCR reactions with specific primers for the DNA fragment, which the protein has been initially crosslinked to.

In papers III and IV ChIP assays have been performed to investigate a possible interaction of GATA-4 with the proximal promoter of *CYP2C9* and *CYP2C19* and in paper V and VI to answer the question whether ER α binds to regulatory elements within *CYP2C19* and *CYP2C9* promoter.

3.8 QUANTITATIVE REAL TIME PCR

To measure a putative increase or decrease in mRNA amount due to promoter induction or inhibition quantitative real time PCR methods using either SYBR Green (paper V) or the TaqMan® technology (paper VI) were employed. In paper V, a primer pair specific for the CYP2C19 mRNA was designed and used in the PCR reaction. In the first step reverse transcription was performed to obtain the cDNA template for the PCR reaction. Afterwards SYBR Green, a fluorescent marker, was added to the PCR reaction. SYBR Green intercalates between DNA strands. The amount of emitted fluorescence is therefore directly proportional to the amount of DNA produced in the PCR reaction. In paper VI *CYP2C9* specific TaqMan® oligos were used. The Taqman® probes are characterized by a covalently bound fluorophore at the 5'-end and a covalently bound quencher at the 3'-end of the primer. During PCR reactions the utilized polymerase unfolds a 5' to 3' exonuclease activity which destroys the probe that has annealed to the DNA template. As a consequence, the fluorophore is released and emits a fluorescent signal which is computational detected. The signal is directly proportional to the amount of released fluorophore and therefore directly proportional to the amount of cDNA template (reverse transcribed mRNA) in the PCR reaction. *CYP2C19* and *CYP2C9* mRNA measurements were normalized to the mRNA amount of the reference gene human TATA box binding protein (TBP). In paper V and VI, the technique of quantitative real time PCR was used to measure the amount of produced *CYP2C19* or *CYP2C9* mRNA in primary human hepatocytes in dependency of a cell treatment with different ER α ligands.

3.9 IN VIVO PHENOTYPING OF CYP2C19*17 CARRIERS

To evaluate the influence of the *CYP2C19**17 allele on the enzymatic activity of CYP2C19 *in vivo*, two phenotyping studies with specific probe drugs for

CYP2C19 were designed and performed as described in papers I and II. In both studies 17 healthy volunteers, 12 genotyped as *CYP2C19*1/*1* and five as *CYP2C19*17/*17* carriers, were included. Genotype determination had been performed in advance with a nested PCR approach. Both studies were conducted as open-label, one phase trials.

In paper I, 17 individuals were treated with a single dose of 40 mg omeprazole after a fasting period of at least 8 hours. After drug administration, 10 ml venous blood were drawn at time points 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10 hours after treatment. Omeprazole and the two metabolites 5-hydroxyomeprazole and omeprazole sulfone were quantified using a reversed-phase high-performance liquid chromatography (R-HPLC) method. Main kinetic parameters that were determined and compared in both genotype groups included the extrapolated AUC_{∞} (area under the curve), the total body clearance (CL), T_{max} , C_{max} and $t_{1/2}$ for omeprazole and its metabolites.

In paper II, the 17 participants earlier included in the omeprazole study were treated as outpatients with 5 mg of the probe drug escitalopram over 1 week. Participants were only allowed to take part after a wash out period of at least one week for omeprazole. At the last day of treatment steady state pharmacokinetics of escitalopram was determined by drawing blood samples at the time points 1.5, 3, 4, 5, 8, 10 and 12 h after the morning dose. Pharmacokinetic data were evaluated by determining the pharmacokinetic main parameter AUC_{1-12h} for escitalopram and its main metabolite desmethylescitalopram. Homozygous *CYP2C19*1* and *CYP2C19*17* carriers were compared concerning their pharmacokinetic characteristics for escitalopram. In a last step a correlation analysis between escitalopram kinetics at steady state (AUC_{1-12h}) and omeprazole single-dose kinetics (AUC_{∞}) was performed to directly compare the *in vivo* behaviour of both drugs with respect to the *CYP2C19* genotype.

3.10 STATISTICAL ANALYSIS

Comparisons of pharmacokinetic data (AUC, CL, T_{max} , C_{max} , $t_{1/2}$) in paper I and II were performed using the unpaired two-tailed t-test (paper I) or the Mann-Whitney-U test and the one-sided t-test (paper II). P-values < 0.05 were considered as significant. The AUC_{1-12} and AUC_{∞} (Paper I and II) were calculated using the linear trapezoid rule. The extrapolation to infinity was performed with help of the log-linear terminal elimination phase (λ_z). The Spearman correlation coefficient was determined to correlate the AUC_{1-12} of escitalopram steady state kinetics determined in paper II with the AUC_{∞} of omeprazole single dose kinetics determined in paper I.

In paper III and IV comparisons of luciferase activities obtained upon co-transfections with GATA transcription factors mediating *CYP2C19* and *CYP2C9* promoter activity were performed using the one-way ANOVA followed by a Tukey's post hoc test. P-values < 0.05 were considered as significant.

In paper V and VI statistical comparisons of *CYP2C19* and *CYP2C9* promoter driven luciferase activities obtained upon co-transfections with ER α and ER α ligand treatment as well as real time PCR based mRNA level measurements after cell treatments with ER α ligands were performed using the one-sample t-test. Also here, p-values < 0.05 was considered to be significant.

4 RESULTS

4.1 PAPER I – INFLUENCE OF *CYP2C19*17/*17* ON OMEPRAZOLE PHARMACOKINETICS *IN VIVO*

To investigate, whether *CYP2C19*17* has a significant impact on omeprazole metabolism *in vivo*, we performed a pilot study with 16 healthy individuals, which were either homozygous carriers of *CYP2C19*17* (5 participants) or homozygous of *CYP2C19*1* (11 individuals). The *CYP2C19* genotype of all participants was determined by PCR/RFLP analysis before inclusion into the study. Sample size calculations for the study were based on the prior estimate of a 40% difference in omeprazole AUC between *CYP2C19*1/*1* and *CYP2C19*17/*17* groups, which was considered to be significant (Sim et al., 2006). The subjects were treated with a single dose of 40 mg omeprazole and the main pharmacokinetic parameters of omeprazole obtained in both genotype subgroups were determined and compared.

We found that the homozygous carriers of *CYP2C19*17* exhibit significantly higher metabolic activity towards omeprazole, when compared to the wild-type carriers ($p=0.04$). This observation is especially reflected in a twice as high AUC_{∞} of omeprazole and a twofold higher AUC_{∞} ratio of omeprazole/5-hydroxy omeprazole in *CYP2C19* wild-type carriers compared to the ratio calculated for *CYP2C19*17* carriers. Furthermore, *CYP2C19*17* carriers showed 3-fold lower levels of the metabolite omeprazole sulfone. This observation can be explained with the fact that less omeprazole was available to enter the CYP3A4-dependent metabolic pathway in subjects carrying the *CYP2C19* variant. As a result, a very strong correlation of ($r^2=0.95$) occurred between the AUCs measured for omeprazole and the sulfonated metabolite. No significant differences were observed in the AUCs of the 5-hydroxy metabolite between the *CYP2C19* genotype groups.

Interestingly, a large interindividual variation in CYP2C19 activity was observed within the *CYP2C19* wild-type group in contrast to the relatively similar low AUC values determined in the *CYP2C19*17* group.

4.2 PAPER II – INFLUENCE OF *CYP2C19*17*17* ON ESCITALOPRAM PHARMACOKINETICS *IN VIVO*

In a second approach we investigated how the genotype *CYP2C19*17/17* influences the metabolism of the CYP2C19 probe drug escitalopram compared to *CYP2C19*1*1*. A sample size of six *CYP2C19*17*17* subjects and twelve *CYP2C19*1*1* subjects was estimated to have 80% power to show a true difference in the AUC of 40% between the groups (as predicted from (Sim et al., 2006)). Eventually we were able to include 11 wt carriers and 5 homozygous *CYP2C19*17* carriers into the study and treat them with 5 mg of escitalopram over one week. Steady state kinetics of the drug was analyzed on day 7 of treatment.

The analysis focused on the determination of the pharmacokinetic characteristics of escitalopram and its main metabolite desmethylescitalopram. A 21% lower mean escitalopram AUC₀₋₁₂ was observed in homozygous *CYP2C19*17* carriers compared to the mean AUCs measured in *CYP2C19* wild-type carriers. This observation was in agreement with the finding of faster metabolism towards the CYP2C19 probe drug omeprazole in *CYP2C19*17*17* carriers presented in paper I. The results obtained for escitalopram were, however, not statistically significant. Furthermore, no differences in the concentration-time curves of desmethylescitalopram were observed between the genotype groups.

Interestingly, a low variability in the measured AUC_{S1-12} of escitalopram was observed within the *CYP2C19*17*17* group, compared to considerable higher interindividual differences within the *CYP2C19* wild-type group. Subjects homozygous for *CYP2C19*17/17* were thereby found within a low

escitalopram AUC value range. Similar observations were made for the omeprazole AUCs in paper I.

Both observations are reflected in a relatively strong correlation (coefficient 0.67) between escitalopram AUC₁₋₁₂ values and the determined omeprazole AUCs_∞.

4.3 PAPER III AND IV – THE ROLE OF GATA-4 IN *CYP2C19* AND *CYP2C9* GENE EXPRESSION

The initial hypothesis that GATA transcription factors could be possibly the responsible regulatory elements behind the modulating effect of the promoter variant *CYP2C19*17* on *CYP2C19* activity (which by (Sim et al., 2006) and in paper III was proven not to be the case) prompted us to the idea to further investigate the role of GATA proteins in the general transcriptional regulation of *CYP2C19* and the sequence wise near relative *CYP2C9* in paper III and IV. In luciferase assays we could show that several members of the GATA family are able to stimulate *CYP2C9* and *CYP2C19* promoter activities. In cotransfection experiments, the highest degrees of upregulation were observed for GATA-2 and GATA-4 (paper III, figures 2A/B and 3 A/B, paper IV, figures 2A and 2B).

Bioinformatic analysis predicted two adjacent potential GATA-binding sites at position -165/-156 in the *CYP2C19* promoter which is conserved in the *CYP2C9* promoter at position -163/-154. Co-transfection experiments in HepG2 and Huh7 cells applying GATA constructs and several deletion constructs of different lengths derived from the *CYP2C19* and *CYP2C9* 5'-flanking regions narrowed the binding region of GATA down to the predicted loci (shown in paper III for the *CYP2C19* promoter, figures 2A/B and 3A/B). To prove the functional importance of the predicted GATA binding sites for both genes we artificially changed the putative binding sites by site-directed mutagenesis and co-transfected the modulated *CYP2C19* and *CYP2C9*

promoter constructs together with GATA-2 and GATA-4 expression plasmids into Huh7 and HepG2 cells. As shown in paper III, figure 2A/B and 3A/B, and paper IV figure 2A/B, the introduction of mutations resulted in a significant loss of GATA-dependent promoter stimulation in the case of both *CYP2C19* and *CYP2C9*. This confirmed the significant role of the predicted response elements for a GATA-dependent transcriptional regulation.

EMSAs using nuclear extracts from HepG2 and Huh7 cells revealed specific binding by GATA-4 and partly by GATA-6 to the oligonucleotides that carried the GATA binding sites of the *CYP2C19* and *CYP2C9* promoters. Specificity of binding was proven by successful supershifts with GATA-4- and GATA-6-specific antibodies (paper III figure 4, paper IV figure 3).

To test whether GATA-proteins are associated with the predicted binding sites in the *CYP2C9* and *CYP2C19* promoters in intact cells we performed ChIP assays using genomic DNA from HepG2 cells and an antibody specific for the liver-enriched GATA family member GATA-4. Promoter fragments were amplified using one primer pair (paper IV) or two primer pairs (paper III) encompassing the putative GATA binding sites in question. As shown in paper III (figure 5), and in paper IV (figure 4), PCR products could be successfully generated matching the size of the promoter fragments, which carry the GATA binding sites when GATA-4 antibody was utilized. Furthermore, the control reactions with unspecific IgG antibodies led to a negative PCR output, thus proving the specificity of the experiment.

GATA proteins are known to interact with the co-regulatory acting transcription factor family FOG (Friend of GATA), which contains two FOG proteins, FOG-1 and FOG-2. Especially FOG-2 has been proven to co-regulate GATA-4 and to influence the transcription of its target genes. To test, whether FOG-2 influences a GATA-4-dependent regulation of *CYP2C19* and *CYP2C9* gene expression, we performed luciferase gene reporter experiments where GATA-4 and FOG-2 were co-transfected and *CYP2C9* or

CYP2C19 promoter activity was detected in Huh7 cells. As shown in paper III on figure 6, and in paper IV on figure 5, GATA-4 alone upregulated both *CYP2C19* and *CYP2C9* promoter-driven luciferase expression as expected. Increasing amounts of co-transfected FOG-2 construct inhibited the observed GATA-4 dependent upregulation, proving the antagonistic effect of FOG-2 on GATA-4 activity on both promoters. The co-transfection of an expression plasmid encoding a mutated FOG-2 variant, lacking the fragment coding for the GATA-interacting zinc finger domain, showed a significantly reduced inhibitory effect on GATA-4-dependent promoter stimulation.

4.4 PAPER V AND VI – THE INFLUENCE OF ESTROGEN RECEPTOR ALPHA ON *CYP2C19* AND *CYP2C9* GENE EXPRESSION

Both *CYP2C9* and *CYP2C19* activity are known to be inhibited by female sex steroids. The mechanisms how estradiol derivatives influence the enzyme activities are not yet fully understood. In Paper V and VI we tested whether ER α and ER β and their ligands are putative modulators of *CYP2C19* and *CYP2C9* gene expression.

Bioinformatic analysis of the *CPY2C19* and *CYP2C9* gene promoter revealed four putative ER DNA-binding half-sites at positions (-151/-147), (-1117/-1113), (-1515/-1511), (-1611/-1607) and at positions (-1829/-1825), (-1557/-1553), (-149/-145) and (-104/-100), respectively. To investigate whether these loci are targeted by ER α and/or ER β EMSA analysis was performed. For each promoter region four different oligonucleotides, which comprised the putative ERE half sites and nuclear extracts from Huh7 and HepG2 cells were utilized. We could prove that ER α was capable of binding to ERE half sites (-151/-147) and (-149/-145) within *CYP2C19* promoter and *CYP2C9* promoter, respectively, as confirmed by a positive supershift or a successful competition reaction of the generated binding complex with a specific antibody against

ER α (Paper V and VI, figure 2). ER β did not bind to any of the putative ERE half sites within the 5' flanking region of *CYP2C19* in EMSA experiments.

To further confirm an interaction between ER α and *CYP2C9* and *CYP2C19* gene promoter and to further elucidate the influence of ER α on *CYP2C9* and *CYP2C19* gene expression, we performed luciferase gene reporter assays. Cell experiments co-transfecting a plasmid carrying 1.6 kb or 0.5kb long fragments of *CYP2C19* and *CYP2C9* gene promoter, respectively, and an ER α expression vector revealed a dose-dependent inhibition of reporter luciferase activity upon cell treatment with 17 β -estradiol in the case of *CYP2C19* promoter and with 17 α -ethinylestradiol in the case of both *CYP2C19* and *CYP2C9* promoter. The SERMs 4-hydroxytamoxifen and raloxifene did not influence *CYP2C19* promoter activity but were able to slightly transactivate the *CYP2C9* promoter. Using the approach of site-directed mutagenesis we could confirm, that ERE half site (-151/-147) and (-149/-145) are partly responsible for the ER-mediated modulatory effects on *CYP2C19* and *CYP2C9* gene expression observed.

The inhibitory effect of estrogens on *CYP2C19* and *CYP2C9* gene expression was also confirmed in human hepatocytes. Here, the amount of detected *CYP2C19* mRNA was significantly lower in cells treated with 17 β -estradiol, when compared to cells treated with vehicle substance only (Paper V, figure 5). In the case of *CYP2C9* the inhibitory effect of ETE on *CYP2C9* expression was significant and much more pronounced compared to the inhibitory effect of EE (paper VI, figure 6).

ChIP experiments using Huh7 cells and an antibody specific for ER α confirmed *in vivo* binding of ER α to promoter fragments that comprise the identified functionally relevant ERE half-sites within both *CYP2C19* and *CYP2C9* promoter.

As described in paper V, ETE and EE are also able to directly inhibit *CYP2C19* enzyme activity. Assays using a stably transfected *CYP2C19* HEK-293 cell line and *CYP2C19* enzyme activity measurements in

Results

microsomes of insect cells overexpressing CYP2C19 showed, however, that the dosages of hormone needed for an effective direct inhibition were of 1 to 2 magnitudes higher than the dosages required for an inhibitory effect on the gene promoter in luciferase gene reporter assays (Paper V, figure 7).

5 DISCUSSION

5.1 THE INFLUENCE OF *CYP2C19*17* ON DRUG PHARMACOKINETICS *IN VIVO*

5.1.1 Omeprazole and Escitalopram

Interindividual variability in the activity of drug metabolizing enzymes is a major cause for differences in drug exposure and, hence, a major contributor to variation in drug response (Rudberg et al., 2008).

In case of the enzyme CYP2C19 especially the variants *CYP2C19*2* and *CYP2C19*3* have been over a long time considered to be sufficient for prediction of the CYP2C19 phenotype. The discovery of the variant *CYP2C19*17* and the postulation of a linkage to a lower systemic exposure towards the probe substrate omeprazole opened the possibility for a further explanation of the wide interindividual variability observed for CYP2C19 probe drugs. Since then several studies have been published investigating the influence of *CYP2C19*17* on the pharmacokinetics of several different CYP2C19 substrates (see also chapter 5.1.2)

PPIs represent good probe drugs for CYP2C19 activity, as the drug group is of clinical importance and their pharmacokinetic characteristics, e.g. the area under the plasma concentration time curve (AUC), correlate well with the drug response which is well expressed in the measured levels of the intragastric pH, healing efficacy of peptic ulcer or severity of gastroesophageal reflux disease (Klotz, 2006).

In our pilot study presented in paper I we could show that homozygously expressed *CYP2C19*17* has a significant impact on omeprazole pharmacokinetics. The study was the first systematic approach investigating the impact on *CYP2C19*17* on the *in vivo* pharmacokinetics of a CYP2C19 probe drug. Since then several small studies addressing the same topic have

been published. One study included and compared CYP2C19 wild-type carriers and individuals heterozygous for *CYP2C19*17* concerning the mean AUC₀₋₂₄ levels for omeprazole and pantoprazole after both single dosing and multiple dosing. Despite a trend towards lower AUCs for heterozygous *CYP2C19*17* carriers no significant differences between the genotype groups were observed (Hunfeld et al., 2008). Another study including a cohort of children came to similar results. Homozygous *CYP2C19*17* carriers were, however, not investigated in either of these studies. These findings together with our findings support the hypothesis that a homozygous expression of *CYP2C19*17* seems to bear greater impact on the omeprazole pharmacokinetics than a heterozygous expression. However, additional studies, ideally including larger populations, have to be performed to further elucidate the relevance of *CYP2C19*17*. Moreover, it would be desirable to investigate the influence of *CYP2C19*17/*17* on the clinical outcome with PPIs by correlating the *CYP2C19*17/*17* genotype with e.g. intragastric pH levels or the healing efficacy of peptic ulcer, such as already performed for the *CYP2C19* PM genotype in the past (Kang et al., 2008; Saitoh et al., 2009).

The influence of *CYP2C19*17/*17* on pharmacokinetics of citalopram or escitalopram is not comprehensively investigated yet. We did not observe a significant difference between escitalopram and N-desmethylescitalopram levels when comparing homozygous *CYP2C19*1* and homozygous *CYP2C19*17* carriers. Only one other study so far has addressed this question and has retrospectively investigated the influence of a heterozygous or homozygous expression of *CYP2C19*17* on dose-adjusted escitalopram plasma levels in a pool of 166 depressive patients (Rudberg et al., 2008). This study detected 42% lower escitalopram mean plasma levels in *CYP2C19*17/*17* carriers compared to wild-type carriers. While a positive aspect of the study of Rudberg *et al.* is that a high number of patients have taken the drug over a relatively long time frame, a weak point of this

investigation might be, however that the concentration measurements were made over a wide sampling-time window of 10-30h after the last dose. Moreover, the differences in escitalopram plasma concentrations were much more pronounced between homozygous PMs and *CYP2C19* wild type carriers.

In conclusion, further studies have to be performed in the future – ideally including more participants or patients than we were able in our pilot study - to clarify the importance of *CYP2C19*17* in escitalopram metabolism.

5.1.2 *CYP2C19*17* and other *CYP2C19* substrates

Especially the PM allele *CYP2C19*2* has been associated with reduced effectiveness of the antiplatelet prodrug clopidogrel, which is metabolically activated by both *CYP2C19* and *CYP3A4* (Hulot et al., 2006; Kim et al., 2008; Shuldiner et al., 2009). The poorer response of PMs to clopidogrel is due to less extensive prodrug activation. Two studies, which investigated the role of *CYP2C19*17* in platelet response upon clopidogrel treatment, could show that the *CYP2C19*17* allele has a significant impact on the antiplatelet effect of clopidogrel that is connected to a higher risk for bleeding events due to increased prodrug activation, as shown in a cohort of patients on clopidogrel-treatment for prevention of stent thrombosis (Sibbing et al., 2010a; Sibbing et al., 2010b).

The estrogen antagonist tamoxifen is mainly metabolized by *CYP2D6*, although other CYP enzymes, such as *CYP2C19*, are also involved in the formation of the two main metabolites 2- and 4-hydroxytamoxifen. Especially 4-hydroxytamoxifen is an active metabolite of tamoxifen with a significantly higher therapeutic effect than the maternal substance. Interestingly, *CYP2C19*17* has been associated with better tamoxifen response, as demonstrated by a lower risk of relapse for *CYP2C19*17* carrying breast cancer patients compared to EM or PM phenotype expressing patients (Schroth et al., 2007).

The antifungal drug voriconazole is extensively metabolized by CYP2C19 and to a lesser extent by CYP3A4 and CYP2C9 (Theuretzbacher et al., 2006; Wang et al., 2009). In addition to strong and significant differences in pharmacokinetic key parameters between homozygous PMs carrying *CYP2C19*2/*2* and *CYP2C19*1/*1* carrying individuals, Wang et al. observed significant differences in the AUC₀₋₂₄ of voriconazole in heterozygous *CYP2C19*17* carriers compared to wild-type individuals. Homozygous expression of *CYP2C19*17* was not investigated due to the fact that homozygous carriers of *CYP2C19*17* were not detected in the included Chinese population.

*CYP2C19*17* has been also associated with a fast CYP2C19 dependent metabolism of the antidepressant imipramine and the antimalarial drug chlorproguanil. About 50% higher AUC₀₋₂₄ values were observed for the chlorproguanil metabolite chlorcycloguanil in the *CYP2C19*17* carrier group compared to wild type carrying individuals in a study, which included 43 Gambians treated for malaria (Janha et al., 2009). In addition, about 30% lower imipramine steady state plasma concentration levels were measured in homozygous *CYP2C19*17* allele carriers compared to wild type carrying individuals in a study that investigated 178 patients treated for depression (Schenk et al., 2010).

It remains to be further elucidated in future studies to which extend the three latter findings are of clinical relevance.

5.2 THE ROLE OF GATA PROTEINS IN THE EXPRESSION OF DRUG METABOLIZING ENZYMES

In paper III and IV we have identified a functionally relevant double GATA binding site in the proximal area of both *CYP2C19* and *CYP2C9* gene promoters. We could show that this element can be transactivated by members of the GATA family and binds mainly the liver-abundant protein GATA-4 in liver-derived cell lines HepG2 or Huh7. A broad binding

potential of different GATA proteins to the same element is well in line with the fact that GATA proteins share high amino acid similarity within their double zinc finger domains, responsible for DNA interaction.

In former times GATA family members have been mainly associated with their important regulatory role in the expression of genes either involved in the development of different blood cell lines (GATA-1 to GATA-3) or in the development of the heart and other endodermal derivatives (GATA-4 to GATA-6). An increasing number of publications have shown that GATA factors are also taking part in the regulation of cytochrome P450 enzymes and other xenobiotic metabolizing enzymes as well as drug transporters. CYP17, which is expressed in the adrenal cortex and which is involved in the cortisol biosynthesis, has been shown to be upregulated by GATA-6 in concert with the transcription factor specificity protein 1 (Sp1). Interestingly the stimulatory actions of GATA-6 on CYP17 promoter are based on a protein-protein interaction with Sp1 rather than on a direct DNA binding. The expression of CYP19, an enzyme which is involved in estrogen biosynthesis, is influenced by GATA-4. *Shadley et al.* identified a far upstream enhancer in CYP2E1 5'-flanking region which is stimulated by GATA-4 (*Shadley et al., 2007*). This aspect together with our findings supports the hypothesis that the spectrum of genes regulated by GATA factors is certainly greater than initially thought and investigated. It becomes more and more clear that GATA proteins play a role in the regulation of genes involved in the metabolism of endogenous and exogenous compounds.

Many other transcription factors have been determined, which act in concert with GATA proteins. This feature has been especially well investigated for GATA-1 (*Morceau et al., 2006*) and GATA 4 (*McBride et al., 2003; Temsah and Nemer, 2005*). For the regulation of *CYP2C19* and *CYP2C9* promoter it can also be presumed that GATA-4 cooperates with other transcription

factors. *Kawashima et al.* experimentally proved the functional relevance of two DR-1 elements, which are able to bind the important liver enriched transcription factor HNF-4 α and to transactivate *CYP2C9* (Kawashima et al., 2006). These sites are located in direct neighbourhood to our GATA binding sites identified in this thesis (figure 7). Furthermore, the functionally relevant ERE half site detected in *CYP2C19* promoter (paper V) is also located directly next to the GATA binding sites. The hypothesis of a direct interaction between HNF-4a, ER α and GATA-4 has, however, to be experimentally proven.

The functional relevance of GATA-4-dependent regulation of the *CYP2C9* and *CYP2C19* promoters remains to be further investigated. It would be of

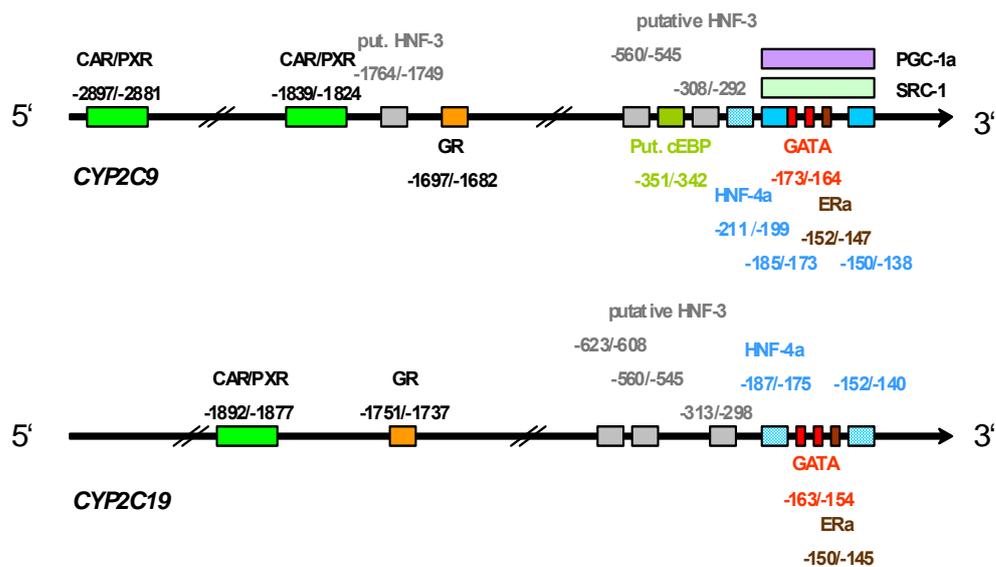


Fig. 8: Functionally confirmed and putative transcription factor binding sites within *CYP2C9* and *CYP2C19* promoters. The checked boxes show HNF-4 binding sites, which are, however, not functionally active. *In red:* newly identified and functionally active double GATA binding site in *CYP2C9* and *CYP2C19*. *In brown:* Functionally confirmed ER α binding site in *CYP2C19* which is conserved in *CYP2C9*.

interest, whether endogenous and exogenous substances including drugs influence the expression or binding capacity of GATA to both promoters. GATA factors are known to be targets for a range of signalling cascades (Temsah and Nemer, 2005). Further understanding of variation in the expression of chief regulators of *CYP2C9* and *CYP2C19*, such as GATA proteins, may shed more light on the unsolved problem, why the enzymes *CYP2C9* and *CYP2C19* show such a wide interindividual range in gene expression, a fact that can only partly be explained by functionally relevant polymorphisms.

It should also be taken into account that GATA transcription factors themselves are polymorphically expressed. Several variants seem to be of functional relevance as they have been associated with various pathological phenotypes (see table 2). Therefore, it can not be excluded that polymorphic expression of especially GATA-4 has an impact on gene expression of *CYP2C9* or *CYP2C19*.

5.3 THE ROLE OF ER ALPHA IN THE EXPRESSION OF DRUG METABOLIZING ENZYMES

A significant inhibitory effect of oral contraceptives (most often comprising ethinylestradiol and levonogestrel) have been observed in several *in vivo* studies with various *CYP2C19* substrates including omeprazole (Palovaara et al., 2003), proguanil, cycloguanil (McGready et al., 2003), and carisoprodol (Bramness et al., 2005). The inhibitory effect is probably only mediated by the estradiol derivative as shown in an *in vivo* study using the *CYP2C19* probe drug omeprazole performed by Palovaara et al. (Palovaara et al., 2003). For *CYP2C9* not as many *in vivo* studies investigating the influence of estradiol derivatives on enzyme activity have been performed yet. Significant inhibitory effects of female sex steroids were, however, not only observed on

the metabolism of the CYP2C9 probe drug losartan. Estradiol derivatives have been shown to also significantly higher the anticoagulative effect of the CYP2C9 substrate warfarin *in vivo* (Sandberg et al., 2004; Zingone et al., 2009). Especially the latter observation is of clinical importance as small alterations in warfarin concentrations can easily lead to bleeding complications.

In our *in vitro* studies presented in paper V and VI we could show that ER α is able to bind to an ERE half-site located in the proximal part of the *CYP2C19* and *CYP2C9* 5'-flanking region and to suppress *CYP2C19* and *CYP2C9* expression in a dose-dependent manner. This study provides evidence that inhibitory effects on *CYP2C19* and *CYP2C9* activity earlier observed with estradiol derivatives can be at least partly explained by this promoter-dependent regulatory pathway. This hypothesis holds probably especially true for *CYP2C19*. The estrogen concentrations needed for the inhibition of *CYP2C19* gene expression were 1 to 2 magnitudes lower than the concentrations needed for direct protein inhibition. Based on these results it is likely that estrogen-dependent inhibitory effects on *CYP2C19* activity are rather transcription factor-mediated than induced by direct competitive inhibition at the active site of *CYP2C19*.

The ERE half-sites detected at positions (-151/-147) and (-149/-145) in *CYP2C19* and *CYP2C9* promoter match exactly one half of the palindromic ERE consensus sequence. Several *in vitro* studies could show that a binding of ER α or ER β to a half site of the ERE consensus sequence is sometimes sufficient to mediate an ER-dependent response of the target promoter (Chen et al., 2008; Kimura et al., 2008). An important observation is, however, that mutations introduced into the functionally relevant ER-binding element led to only a partial reconstitution especially of *CYP2C19* promoter activity. Therefore, it can not be excluded that other ER-dependent pathways are involved in the regulation of the *CYP2C19* gene expression. It is, for example,

known that ER α often mediates its effects via protein-protein interactions. Furthermore, ER α influences the expression of other transcription factors, such as AP-1, SP-1 or NF- κ B, by stabilizing their DNA-binding and/or recruiting co-activators to the binding complex (Higashi et al., 2007).

An ER α -dependent regulation of *CYP2C19* and *CYP2C9* gene expression is well in line with the fact that ER α and both CYP enzymes are abundantly expressed in liver (Rodriguez-Antona and Ingelman-Sundberg, 2006; Higashi et al., 2007). A comparative sequence analysis of the proximal *CYP2C9* and *CYP2C19* gene promoter revealed that the detected functionally relevant ERE half sites are located within a conserved region which is very similarly composed in *CYP2C9* and *CYP2C19*. In addition to *CYP2C9* and *CYP2C19*, genes encoding other CYP enzymes, which are expressed in estrogen related tissues such as mammary, uterus and ovary, have been shown to be regulated by ER α . This includes *CYP2A6* (Higashi et al., 2007) and *CYP1B1* (Tsuchiya et al., 2004). In these cases ER α -dependent regulation is as well mediated by the classical pathway recruiting (complete) ERE consensus sequences.

Interestingly, only the *CYP2C9* promoter but not the *CYP2C19* promoter is (weakly) transactivated by SERMS. It is, however, questionable how important the observed promoter mediated induction is, as one study showed that tamoxifen significantly inhibits CYP2C9 activity *in vivo* (Boruban et al., 2006). Therefore it can not be excluded that tamoxifen exhibits in parallel an inhibitory effect by other not yet investigated mechanisms, which has a stronger impact on enzyme activity than the observed promoter mediated effects.

Especially CYP2C19 expression is ER α and estradiol dependently regulated. What could be the (patho)physiological importance for this observation? Similarly to CYP1B1, which is one of the key enzymes in estrogen metabolism, CYP2C19 is also playing a role in the transformation of estrogens. Thus, it can be assumed that both enzymes play a role in general estrogen homeostasis. It can not be excluded that variability in estrogen

Discussion

dependent regulation of estrogen metabolizing enzymes like CYP1B1 or CYP2C19 influence hormone dependent events such as estrogen-dependent carcinogenesis.

6 THESIS SUMMARY AND CONCLUSIONS

6.1 CYP2C19*17 AND ITS INFLUENCE ON CYP2C19 DEPENDENT METABOLISM *IN VIVO*

The main results obtained in the *in vivo* studies investigating the influence of the promoter SNP *CYP2C19*17* on omeprazole and escitalopram metabolism and the main conclusions to be drawn can be summarized as follows:

- *CYP2C19*17/*17* has a significant influence on omeprazole pharmacokinetics *in vivo*
- *CYP2C19*17/*17* has to be further investigated concerning its impact on therapy efficacy with omeprazole, ideally in large panel studies considering a measurement of therapy outcome parameters
- The impact of *CYP2C19*17/*17* on escitalopram metabolism is not as strong as on omeprazole pharmacokinetics and seems not to be significant
- In both pilot studies a trend towards lower AUCs for both omeprazole and escitalopram were observed. The AUC values were condensed within small value intervals. This leads to the hypothesis that regulatory elements, which bind to the SNP locus in *CYP2C19* promoter seem to have a strong impact towards a high and uniform expression of *CYP2C19*. It still remains to be investigated which transcription factor(s) are responsible for this observed phenomenon

6.2 THE IMPACT OF THE GATA TRANSCRIPTION FACTOR FAMILY GATA ON CYP2C19 AND CYP2C9 EXPRESSION

Conclusions to be drawn and future perspectives arising from the studies investigating the influence of GATA factors on transcriptional regulation of *CYP2C9* and *CYP2C19* can be summarized as follows:

- Both *CYP2C19* and *CYP2C9* promoters are significantly transactivated by GATA factors through a conserved double GATA binding site.
- The strength of inducibility of both promoters by GATA factors leads to the hypothesis that GATA seems to play an important role in the endogenous constitutive expression of *CYP2C9* and *CYP2C19*.
- The binding occurs within promoter areas of *CYP2C9* and *CYP2C19*, which are densely targeted by other transcription factors.
- It remains to be investigated how GATA factors are interacting with other transcription factors on the transcriptional regulation of *CYP2C* genes that bind especially in the neighborhood of the detected GATA binding sites. Putative cooperators include especially the regulatory proteins HNF4 α , ER α , cEBP and HNF-3 γ .
- It would be worth to further investigate, whether GATA expression itself is influenced by xenobiotic agents and endogenous substrates via e.g. nuclear factors like PXR or CAR, thus being able to mediate for example drug dependent effects on *CYP2C9* and *CYP2C19* mRNA and protein expression. This would perhaps also further explain the large interindividual differences observed for *CYP2C19* and *CYP2C9* activity.

6.3 THE IMPACT OF ER ALPHA ON CYP2C19 AND CYP2C9 EXPRESSION

The following conclusions can be drawn from the investigation:

- ER α is able to mediate an estradiol derivative dependent inhibition on both *CYP2C9* and *CYP2C19* gene expression via a conserved ERE half site within *CYP2C9* and *CYP2C19* gene promoter.
- The promoter mediated inhibition of *CYP2C19* by estrogens takes place at significant lower estrogen concentrations compared to the estrogen concentrations needed for a direct enzyme inhibition. This finding leads to

the conclusion that the known inhibitory effect of estrogens on CYP2C19 *in vivo* is likely to be promoter mediated rather than through direct inhibition of the active center of CYP2C19.

- The newly detected ERE half site is likely to be not the only binding site of ER α . It can be assumed that other (non classical) pathways are also taking part in the promoter mediated regulation of especially CYP2C19 expression. This has however to be experimentally proven in the future.
- Whereas both *CYP2C9* and *CYP2C19* gene expression appears to be significantly inhibited by especially α -ethinylestradiol, only *CYP2C9* promoter turns out to be weakly transactivated by SERMS, such as 4-hydroxy-tamoxifen. This observed effect is, however, likely to be overwhelmed by in parallel occurring inhibitory effects of SERMS on CYP2C9 activity, which may be mediated by other mechanisms.

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