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Novel Extrahepatic P450 Enzymes with Emphasis on
the Tumor Specific CYP2W1

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ABSTRACT

Cytochrome P450 is a superfamily of enzymes involved in the metabolism of endogenous and exogenous compounds. The P450 enzymes are expressed at high levels in the liver, but are also found in extrahepatic tissues and in transformed tissues. The expression of P450s in tumors has drawn interest because their presence might influence cancer therapy and since the enzymes may be utilized as drug targets in new cancer therapy strategies. In the current study two novel extrahepatic P450s, CYP2U1 and CYP2W1, were identified and characterized. The expression of CYP2W1 in transformed tissues was investigated, resulting in the proposal for CYP2W1 as a novel potential drug target for colon cancer therapy.

CYP2U1 was cloned and identified as a highly conserved P450, having an ortholog in Fugu fish (Takifugu rubripes). The gene contained only 5 exons and the deduced amino acid sequence harbored two unique NH2-terminal stretches, with one of them being proposed to be important for efficient folding. The CYP2U1 cDNA was expressed in HEK293 cells yielding a properly folded enzyme. Antibodies were developed against a human COOH-terminal sequence, and analysis showed that the CYP2U1 mRNA and/or protein from human and rat were found to be expressed especially in brain, in particular in the limbic structures and cortex, and in thymus. It is suggested that the enzyme can be used as a target for drugs in brain.

CYP2W1, initially found as a partial clone in HepG2 cells, was cloned and expressed in HEK293 cells yielding an enzyme with a proper folding capable of metabolizing arachidonic acid. Antibodies were developed and showed the presence of three different immunoreactive bands in Western blotting, probably representing different post-translational modifications. In adult human tissues, CYP2W1 mRNA was found to be expressed at no or very low levels. In rat, high mRNA expression was seen in fetal colon, with expression levels increasing by fetal age and then decreasing again after birth, but CYP2W1 was not seen in other adult tissues investigated. CYP2W1 mRNA was expressed in human tumors originating from the adrenals and colon. High CYP2W1 mRNA and protein expression were especially seen in Caco-2TC7 cells and colon tumors. The CYP2W1 gene was found to contain CpG islands, and demethylation of a CpG island located in the exon1-intron 1 region appeared to be required for expression in tumor samples. It is concluded that CYP2W1 most probably is a colonic enzyme expressed in fetal life and overexpressed in about 50% of human colon tumors, which suggest the use of the enzyme as drug target in cancer therapy. However, more studies are needed in order to fully understand its role in tumor biology and its potential as drug target.
LIST OF PUBLICATIONS


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*these authors contributed equally to this work
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>arnt</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>AzaC</td>
<td>5-Aza-2’-deoxycytidine</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DHET</td>
<td>Dihydroxyeicosatrienoic acid and</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EM</td>
<td>Extensive metabolizer</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin-containing monooxygenase</td>
</tr>
<tr>
<td>GDEPT</td>
<td>Gene directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HAH</td>
<td>Halogenated aromatic hydrocarbons</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non-polyposis colorectal cancer</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IM</td>
<td>Intermediate metabolizer</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>NB</td>
<td>Northern blotting</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PM</td>
<td>Poor metabolizer</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>rtq-PCR</td>
<td>Real-rime quantitative PCR</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>sqRT-PCR</td>
<td>Semi quantitative reverse transcriptase PCR</td>
</tr>
<tr>
<td>TDEC</td>
<td>Tumor derived endothelial cells</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP glucuronosyltransferase</td>
</tr>
<tr>
<td>UM</td>
<td>Ultrarapid metabolizer</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic response element</td>
</tr>
<tr>
<td>Δ2W1</td>
<td>Truncated CYP2W1</td>
</tr>
</tbody>
</table>
1 BACKGROUND

1.1 BIOTRANSFORMATION

Humans, as well as all other organisms, are exposed to a large number of foreign compounds, also known as xenobiotics. These substances are absorbed across the skin, by inhalation or more commonly by ingestion, like food components or drugs. Many xenobiotics are lipophilic which make them difficult to excrete. Consequently, they have to be metabolized into more hydrophilic compounds before they can be eliminated from the body. This metabolism, or biotransformation, occurs mainly in the liver and is usually carried out sequentially in two steps called phase I and phase II reactions (Figure 1).

The phase I reactions include oxidation, reduction and hydrolysis and, by insertion of -OH, -NH₂, -SH or -COOH groups, the compound/drug becomes more chemically reactive, often toxic or carcinogenic, sometimes more than the parent compound. This first modification is mainly carried out by cytochrome P450 enzymes, but also other systems like flavin-containing monooxygenases (FMOs), cyclooxygenases (COXs) and monoamine oxidases (MAOs) etc. are involved in phase I reactions (for a list of phase I and phase II enzymes see [1]).

In the phase II reaction the parent drug or the phase I metabolite undergoes a conjugation reaction like glucuronidation, sulfation etc. The phase II reactions are carried out by for example UDP glucuronosyltransferases (UGTs), N-acetyltransferases (NATs) glutathione S-transferases (GSTs) and methyltransferases. Phase II conjugations often increase the water solubility of the compounds making them more easily excreted [1-3].

![Figure 1](image.png)

Figure 1. The two phases of biotransformation of foreign compounds including drugs. For abbreviations see the text.
1.2 CYTOCHROME P450s

1.2.1 History, evolution and nomenclature

The first report about cytochrome P450 enzymes is from 1958 by Martin Klingenberg who found that rat liver microsomes contained a carbon monoxide-binding pigment with an absorbance maximum at 450 nm [4]. A few years later, Ryo Sato and Tsuneo Omura reported that this pigment was a heme protein [5] and proposed the name cytochrome P450. Estabrook, Cooper and Rosenthal later showed that cytochrome P450 was involved in hydroxylation reactions and was oxidating several drugs [6, 7]. (For a review regarding P450 history see [8, 9])

Today as many as 57 cytochrome P450 genes encoding active enzymes as well as 58 pseudogenes have been identified in the human genome [10]. Originally all P450 genes have diverged from a common ancestral gene. The initial branching of the P450 phylogenetic tree is thought to have occurred 2100-1400 million years ago with the emergence of eukaryotic cells [11]. During evolution, genetic events like mutations, gene duplications, gene conversions etc. have lead to a whole P450 superfamily.

The P450 superfamily is divided into families and subfamilies based on amino acid sequence similarity according to a nomenclature system. According to the guidelines sequences that are more than 40% identical belong to the same family, whereas sequences that are more that 55% identical belong to the same subfamily [12]. The abbreviation for cytochrome P450, CYP, is followed by an arabic number designating the family, a capital letter (or letters) designating subfamily, and finally an arabic number defining the individual enzyme. For example, CYP2W1 is a family 2 member, a member of subfamily W and within this subfamily enzyme number 1. Gene names are written in italics (CYP2W1), whereas mRNA and protein are written in regular letters (CYP2W1).

1.2.2 P450 function

The cytochrome P450s are heme-containing enzymes called monooxygenases or mixed function oxidases since they incorporate one atom of molecular oxygen into the substrate whereas the other oxygen atom is reduced to water. The activities of P450s require, besides molecular oxygen, also a reducing agent, like NADPH. The electron transfer is mediated mainly by cytochrome P450 reductase, but also cytochrome b₅ participates in several reactions. The catalytic reaction can be summarized as follows:

\[
\text{RH} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O}
\]

In mammals the P450s are membrane bound and found mainly in the ER but also in mitochondria. The N-terminal part of mammalian P450 enzymes consists of a
hydrophobic $\alpha$-helix which spans the membrane and serves as an anchor with the P450 enzyme facing the cytoplasmic side.

The P450 enzymes can be roughly divided into those involved in the metabolism of xenobiotics, and those involved in the biosynthesis or metabolism of endogenous compounds. The P450 families mainly responsible for xenobiotic metabolism are the enzymes belonging to family 1-3. However, also some of the family 4 enzymes have been reported to be involved in xenobiotic metabolism [13]. Besides being of major importance for drug metabolism, some of the CYP1-3 enzymes also have endogenous functions, like for example vitamin D metabolism or metabolism of arachidonic acid (AA). Although P450s are of importance for drug metabolism the majority of P450 families have endogenous substrates and important endogenous functions, like in the biosynthesis or metabolism of cholesterol, steroids, AA, regulation of blood homeostasis etc. Most of the human CYP5-51 families have orthologs in fugu fish (*Takifugu rubripes*) [14], emphasizing the importance of these conserved P450s in endogenous pathways. For an overview of the main function of human P450 families see table 1.

<table>
<thead>
<tr>
<th>P450 family</th>
<th>Number of subfamilies</th>
<th>Number of genes coding for functional enzymes</th>
<th>Main function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1</td>
<td>2</td>
<td>3</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>CYP2</td>
<td>13</td>
<td>16</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>CYP3</td>
<td>1</td>
<td>4</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>CYP4</td>
<td>6</td>
<td>12</td>
<td>Fatty acid, aracidonic and xenobiotic metabolism</td>
</tr>
<tr>
<td>CYP5</td>
<td>1</td>
<td>1</td>
<td>Thromboxane synthase</td>
</tr>
<tr>
<td>CYP7</td>
<td>2</td>
<td>2</td>
<td>Cholesterol, bile acid synthesis</td>
</tr>
<tr>
<td>CYP8</td>
<td>2</td>
<td>2</td>
<td>Prostacyclin synthase, bile acid synthesis</td>
</tr>
<tr>
<td>CYP11</td>
<td>2</td>
<td>3</td>
<td>Steroidogenesis</td>
</tr>
<tr>
<td>CYP17</td>
<td>1</td>
<td>1</td>
<td>Steroid 17-hydroxylase, 17/20-lyase</td>
</tr>
<tr>
<td>CYP19</td>
<td>1</td>
<td>1</td>
<td>Estrogen aromatase</td>
</tr>
<tr>
<td>CYP20</td>
<td>1</td>
<td>1</td>
<td>Unknown function*</td>
</tr>
<tr>
<td>CYP21</td>
<td>1</td>
<td>1</td>
<td>Steroid 21-hydroxylase</td>
</tr>
<tr>
<td>CYP24</td>
<td>1</td>
<td>1</td>
<td>Vitamin D 24-hydroxylase</td>
</tr>
<tr>
<td>CYP26</td>
<td>3</td>
<td>3</td>
<td>Retinoic acid hydroxylases</td>
</tr>
<tr>
<td>CYP27</td>
<td>3</td>
<td>3</td>
<td>Bile acid biosynthesis, vitamin D hydroxylations</td>
</tr>
<tr>
<td>CYP39</td>
<td>1</td>
<td>1</td>
<td>Oxysterol 7$\alpha$-hydroxylase</td>
</tr>
<tr>
<td>CYP46</td>
<td>1</td>
<td>1</td>
<td>Cholesterol 24-hydroxylase</td>
</tr>
<tr>
<td>CYP51</td>
<td>1</td>
<td>1</td>
<td>Lanosterol 14$\alpha$-demethylase</td>
</tr>
</tbody>
</table>

*For CYP20 no function has been described so far. The mouse Cyp20 mRNA is however found in adult heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis and in whole mouse embryo at four development stages [16].
1.2.3 The CYP2 family

The CYP2 family is a large enzyme family mainly involved in the metabolism of drugs and other xenobiotics. However, several of the CYP2 enzymes are also involved in the metabolism of endogenous substrates, like the metabolism of AA, with the main AA epoxygenases belonging to the CYP2 family. The ω-hydroxylation, however, is mainly performed by CYP4 family members [17]. For an overview of the AA metabolism pathway see figure 2. Despite involvement in endogenous metabolism, the CYP2 enzymes are generally expressed in tissues involved in xenobiotic metabolism, above all the liver, but also the respiratory tract etc. However, as evident e.g. from the current thesis, the individual enzymes can have different expression profiles.

![Figure 2. Overview of the arachidonic acid metabolism pathway. EET=epoxyeicosatrienoic acid, DHET=dihydroxyeicosatrienoic acid and HETE=hydroxyeicosatetraenoic acid. Adapted from [17].](image)

In humans, 16 CYP2 genes encoding active enzyme, as well as 16 pseudogenes, divided into 13 subfamilies have been described [10]. The enzymes within family 2 responsible for the metabolism of the majority of drugs are the CYP2C subfamily as well as CYP2D6 [18]. The CYP2C and CYP2D6 enzymes are also highly polymorphic which can affect the enzyme activity. In the CYP2 family alleles causing defective, qualitatively altered, diminished or enhanced rates of metabolism have been identified [19]. This can lead to an extensive inter-individual variability in drug response, usually resulting in three or four major phenotype groups; poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs) and ultrarapid metabolizers (UMs) [20].
1.2.4 Regulation of P450s

1.2.4.1 Transcriptional regulation of gene expression
Induction or inhibition by exogenous or endogenous factors is affecting the catalytic activity of many P450 enzymes, either by acting on the transcriptional activation or by altering the catalytic properties of the enzyme (post-transcriptional regulation). The transcriptional regulation of P450 gene expression involves several pathways and nuclear receptors, like the pregnane X receptor (PXR), the constitutive androstane receptor (CAR) and the vitamin D receptor (VDR) [21]. Another example of P450 induction at the transcriptional level is the induction of CYP1A1 by dioxin mediated via the aryl hydrocarbon receptor (AhR) signalling pathway [22].

The AhR is a ligand-dependent transcription factor that has a large number of ligands, both endogenous and exogenous. The high affinity ligands for the AhR however include environmental contaminants like halogenated aromatic hydrocarbons (HAHs) e.g. dioxins, polycyclic aromatic hydrocarbons (PAHs) etc. In short, upon binding of a ligand to the receptor, AhR is dissociated from the cytosolic receptor complex and translocated to the nucleus. In the nucleus the AhR dimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) and binds to multiple xenobiotic response elements (XREs) in the promoter region leading to gene induction [22-24]. The P450s traditionally thought to be transcriptionally regulated via the AhR signalling pathway is the CYP1 family including CYP1A1, CYP1A2 and CYP1B1. However, recently also CYP2S1 was shown to be upregulated by TCDD via AhR [25].

1.2.4.2 Epigenetic mechanisms - DNA methylation
Besides transcriptional and post-transcriptional regulation there are also epigenetic mechanisms that control the transcription of genes. Epigenetic mechanisms are heritable although they do not depend on changes in the genome sequence. Examples of epigenetic regulations are histone modification and DNA methylation. In mammalian cells DNA methylation occurs at the 5’-position of cytosine within a CpG dinucleotides. Clusters of CpG dinucleotides or CpG island are on average from 200bp to 1kb long and are often found within or near the promoter or first exons of genes [26, 27]. DNA methylation is a form of cell memory which is stably inherited and transmitted through cell lineages, and is believed to ensure a highly condensed chromatin configuration leading to a transcriptionally inactive gene.

The methylation status is not constant during development. In the early embryo genomic DNA is highly methylated, but later at the morula - early blastula stages a genome wide demethylation occurs followed even later by a de novo methylation giving highly methylated adult somatic cells (Figure 3) [28]. This is only true for the genome as a whole; the methylation pattern during development for an individual gene
can be different as exemplified below for the *CYP2E1* gene. Methylation is essential in various genomic events such as imprinting of the genome, inactivation of the X chromosome etc. [27]. The best known human diseases caused by a malfunction of the imprinting mechanism are the Prader-Willi and Angelman syndromes involving genes on chromosome 15 [28]. However, hypo- and hypermethylation events are commonly involved in cancer development. As evident from figure 3, in general the genomes of tumor cells are hypomethylated as compared to normal cells, but hypermethylation of individual genes is also common. These changes of methylation status can lead to activation of oncogenes and silencing of tumor suppressor genes and thereby contribute to tumor development [28-33].

![Diagram showing developmental stages and methylation levels](image)

**Figure 3.** Changes in the overall DNA methylation levels of genomic DNA during development. Genome-wide methylation changes occur in the pre-implanted and post-implanted embryo, followed by high methylation levels in the adult somatic cells. As shown to the right, in general, tumor cells are characterized by hypomethylated genomic DNA. Adopted from [28, 34].

### 1.2.4.3 P450s and DNA methylation

The cytochrome P450 enzyme *CYP1B1* is, as mentioned below, overexpressed in many tumors. Recently it was found that hypomethylation of the *CYP1B1* promoter results in overexpression of *CYP1B1* in prostate cancer [35]. The data suggested that the hypomethylation of the *CYP1B1* promoter was an event that occurred early during tumor development, and since CYP1B1 activates several procarcinogens this might be important for the initiation of tumor formation. Also CYP24, involved in vitamin D metabolism, have altered methylation levels in tumor derived endothelial cells (TDEC) compared to non-tumor endothelial cells [36]. Two CpG islands in the upstream region of the *CYP24* gene were found to be hypermethylated in the TDEC. CYP24 metabolizes calcitriol, one form of vitamin D, which has a growth inhibitory effect.
CYP24 is upregulated via the VDR and hypermethylation of the CYP24 promoter is hypothesized to silence the CYP24 expression in TDEC and hence also be responsible for the increased sensitivity of these cells to calcitriol [36].

Besides being involved in the different expression patterns of P450s in tumors compared to normal tissue, methylation has also been shown to regulate the tissue-specific and age-specific expression of other P450 genes [37-40]. One example of methylation being involved in regulation of age-specific expression is methylation of the rat and human CYP2E1 gene [39, 40]. CYP2E1 is not expressed in fetal livers; however in newborns the CYP2E1 protein is detected. This difference of expression during development is hypothesized to depend on demethylation of the CYP2E1 gene after birth, since demethylation could be correlated with the onset of CYP2E1 RNA accumulation [39, 40].

1.2.5 Novel P450 isoforms
Because of the completion of the sequencing of the human genome and initiatives related to this project, there has been a progress in the identification and characterization of novel P450 genes over the last years. Many of these P450s are, unlike the previously well characterized hepatic enzymes, mainly expressed in extrahepatic tissues. Some of these novel P450s are CYP2R1, CYP2S1, CYP2U1, CYP2W1 and CYP3A43 as well as several CYP4 enzymes. By now most of these enzymes have been at least initially characterized and some substrates have been found [41]. However, further studies are needed to fully understand the roles/functions of these new extrahepatic enzymes.

1.2.6 Extrahepatic xenobiotic metabolizing P450s
Many of the P450s in extrahepatic tissues are expressed at higher levels in the liver; however there are also P450s that are preferentially present in extrahepatic tissues. Extrahepatic tissues having high P450 expression levels are the respiratory and gastrointestinal tract, being exposed to foreign compounds entering the body. The importance of extrahepatic P450s for drug clearance is relatively small compared to the liver, but they can be important for the tissue specific or cell specific metabolic activation or inactivation of xenobiotic compounds and can therefore influence e.g. the tissue exposure of carcinogens or effectiveness of therapeutic agents. For a review about extrahepatic P450s, with emphasis on the respiratory and gastrointestinal tract see [42].

1.2.6.1 Respiratory tract
The respiratory tract, consisting of nasal mucosa, trachea and lung, is exposed to xenobiotics both via the blood and from inspired air. Unlike the liver, the lung contains
more than 40 different cell types. Many of these cells, including Clara cells and bronchial epithelial cells etc., are capable of metabolizing xenobiotics [42, 43]. Several P450s are expressed preferentially in the respiratory tract for example CYP2A13, CYP2F1 and CYP2S1 all belonging to the CYP2 gene cluster on chromosome 19 [42].

CYP2S1 is a recently identified P450 enzyme [44]. Besides the respiratory tract, the CYP2S1 mRNA is found in tissues from the gastrointestinal tract, like stomach and small intestine [44, 45]. The CYP2S1 protein has been detected by Western blotting in human lung [44]. In another study the authors showed expression of CYP2S1 in human skin, and could also show that CYP2S1 is inducible by UV-light, coal tar and all-trans retinoic acid, which is also a substrate for CYP2S1 [46]. Regarding regulation CYP2S1 possesses a characteristic mainly associated with the CYP1 enzymes, namely being inducible by dioxin and PAHs via the AhR [25]. Like many other P450, the CYP2S1 gene has been found to exert genetic polymorphism [47].

1.2.6.2 Gastrointestinal tract
The gastrointestinal tract is exposed to orally ingested xenobiotics, and P450s expressed in the gastrointestinal tract is, together with the hepatic P450s, the major enzymes involved in first-pass metabolism. Especially the expression of P450s and the metabolism of xenobiotics by the small intestine is well documented [42, 48]. The most abundant P450s in the small intestine are the CYP3A enzymes with CYP3A4 being the predominant enzyme [49]. Other P450s that have been shown to be expressed in small intestine at the protein level include CYP1A1, CYP2C9, CYP2C19, CYP2J2 and CYP2D6 [42, 49, 50]. However, the P450 content seems to vary between different parts of the small intestine [50, 51].

1.2.6.2.1 Colon
Cytochrome P450 expression in colon has drawn interest since xenobiotic metabolism, like the activation of procarcinogens, or the lack of expression of certain P450s, have been proposed to be a contributing factor to colon cancer. However, compared to liver and small intestine, few studies of P450 expression in colon have been reported. Furthermore the P450 expression levels in human colon seem to be lower than both the liver and the small intestine [52, 53], although the limited data available have to be considered. The P450s detected either at the mRNA or protein level in normal human colon is shown in table 2. Most studies are found regarding CYP3A enzymes, however many of these studies also emphasize that the CYP3A expression levels in colon are lower than the ones seen in the small intestine. Beside the CYP1-3 enzymes also
Table 2. Human P450s in family 1-3 expressed in normal human colon and the methods used for detection. Several of the enzymes detected using immunohistochemistry (IHC) by Kumarakulasingham et al. are however expressed at low frequency and showing weak immunoreactivity [54]. Data regarding expression in colon derived cell lines etc. have been excluded. For abbreviations see the abbreviation list.

<table>
<thead>
<tr>
<th>P450</th>
<th>Protein Localization</th>
<th>Method</th>
<th>Localization</th>
<th>Method</th>
<th>Localization</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1*</td>
<td>IHC</td>
<td>RT-PCR</td>
<td>[55]</td>
<td>WB</td>
<td>[54]</td>
<td>[56]</td>
</tr>
<tr>
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<td>IHC</td>
<td>RT-PCR</td>
<td>[55]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1B1</td>
<td>IHC° epithelial cells, blood vessel walls, muscularis smooth muscle cells</td>
<td></td>
<td>[57]</td>
<td>IHC</td>
<td>[54]</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WB</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>CYP2A/2B</td>
<td>IHC</td>
<td></td>
<td>[54]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C</td>
<td>IHC</td>
<td>RT-PCR</td>
<td>high: ascending, low: descending &amp; sigmoid</td>
<td>[54]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C8</td>
<td>WB</td>
<td>sigmoid, low: descending, ascending not analyzed</td>
<td>[58]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>WB</td>
<td></td>
<td>[56]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>WB</td>
<td></td>
<td>[56]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>IHC</td>
<td></td>
<td>[54]</td>
<td>WB</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>WB</td>
<td>high: descending, low: sigmoid, ascending not analyzed</td>
<td>[58]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IHC</td>
<td></td>
<td></td>
<td>RT-PCR</td>
<td>descending &amp; sigmoid</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>rtq-PCR</td>
<td>[60]</td>
<td></td>
</tr>
<tr>
<td>CYP2J2</td>
<td>WB, IHC high: epithelium, autonomic ganglion cells, low: muscularis smooth muscle cells, goblet cells lining the crypts of Lieberkühn, vascular endothelium</td>
<td>NB, ISH</td>
<td>high: epithelium, autonomic ganglion, low: muscularis smooth muscle cells, blood vessel endothelium</td>
<td>[61]</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CYP2R1</td>
<td>IHC</td>
<td></td>
<td>[54]</td>
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<td></td>
</tr>
<tr>
<td>CYP2S1</td>
<td>IHC</td>
<td></td>
<td>[44]</td>
<td>IHC</td>
<td>[45]</td>
<td>[54]</td>
</tr>
<tr>
<td>CYP2U1</td>
<td>IHC</td>
<td></td>
<td>[54]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A</td>
<td>WB</td>
<td>ISH</td>
<td>epithelial cells of all colon segments</td>
<td>[62]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rtq-PCR</td>
<td>[63]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4**</td>
<td>WB</td>
<td>descending &amp; sigmoid, ascending not analyzed</td>
<td>[62]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB</td>
<td></td>
<td>[58]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IHC</td>
<td></td>
<td>[56]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A5</td>
<td>WB</td>
<td>descending &amp; sigmoid, ascending not analyzed</td>
<td>[62]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IHC</td>
<td></td>
<td>[58]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*There are also several studies showing no expression of CYP1A mRNA or protein in human colon.
**In some papers indicated as CYP3A3 or CYP3A3/3A4.
°Normal tissues obtained from colon cancer patients.
CYP4B1 [65], CYP4F12 [66, 67], CYP24A1 [54, 68], CYP27B1 [69-71] CYP4F11, CYP4V2, CYP4X1, CYP26A1, CYP39 and CYP51 [54], have been detected in human colon, with most of them being involved mainly in endogenous metabolism.

Similar to the small intestine, there is a variation in P450 expression levels, as seen for some enzymes, in different parts of the colon [58]. The cells/tissues in colon showing highest P450 expression are the epithelial cells but also the vascular endothelium, which are also the cells being exposed to most foreign compounds. Besides these cell/tissue types also the muscularis smooth muscle cells as well as autonomic ganglions have been reported to express P450 mRNAs or proteins. For expression of the individual enzymes and references see table 2.

Several of the P450s listed in table 2, although expressed in normal colon, show higher expression levels in colon cancers. That includes CYP1B1, which is one of the major P450s expressed in tumors (see below) [57] as well as CYP2J2, CYP2S1 and CYP3A5 [54, 72]. In contrast most studies regarding CYP3A4 expression show no difference in expression levels between normal colon and colon tumors.

1.2.6.2.2 Caco-2
A common model for intestinal biotransformation is Caco-2 cells. This human cell line is derived from a colon carcinoma and the cells are able to spontaneously differentiate in culture [73]. When the cells reach confluency they start to polarize, develop tight junctions and microvilli on the apical side as well as develop enzymatic activities typical to the small intestine. Several clones have been derived from Caco-2 cells. One of them, Caco-2TC7, expresses higher levels of enterocytic differentiation markers compared to the parent Caco-2 cells. For the TC7 clone the expression of CYP3A4 is higher that in Caco-2 cells, but in both Caco-2 and Caco-2TC7 cells the CYP3A4 expression levels are increased after confluency. In contrast, the expression of CYP1A1 is decreased after confluency in both cell types [74].

1.2.6.3 Brain
The brain is less available to blood-borne drugs than many other tissues due to the blood-brain-barrier (BBB), which makes the brain not considered as a tissue capable of drug metabolism. Unlike the liver, the brain differs dramatically in cell density and function between different regions. Overall the P450 content in brain has been reported to range between 0.5-5% of the hepatic levels [75-77], but when looking at specific regions or cells P450 levels equivalent to the levels in hepatocytes have been seen [75]. The expression of P450s in brain is highest in the cerebellum [78], but expression of different P450 isoforms have been observed in almost all brain regions [75]. Members of many P450 families have been identified in brains from humans as well as animals,
e.g. CYP2D6, CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C, CYP2E1, CYP3A5, CYP4F, CYP7B, CYP26, CYP46 etc. (see reviews by Hedlund et al. and Miksys et al. [75, 77]).

1.2.6.4 Thymus

Just like the brain, the thymus is not considered as a major drug metabolizing organ. Instead the thymus is important in early life in the development of the immune system. Although not involved in drug metabolism, in adult rats the P450 content in thymus has been reported to be approximately 8% of the content in liver [79]. Several P450s have been reported to be expressed in adult and fetal thymus from different species, although most of them involved in endogenous metabolism and hence not belonging to the CYP1-3 families. The main family 1-3 members found in thymus are CYP1A1, CYP1B1, CYP2A1/2, CYP2B1/2, CYP2D25, CYP2E1, CYP2J3 and CYP3A7 [79-85]. Of them the only human P450s are CYP1B1 [83] and CYP3A7 [85], both found in human fetal thymus. Besides the xenobiotic metabolizing P450s also CYP27B1, CYP17, CYP11B1, CYP19 and CYP21 are reported to be expressed in human thymus [86, 87].

1.2.7 P450s and cancer

The expression pattern of P450 enzymes in tumors is in many cases different from P450 expression patterns in the corresponding normal tissues. One reason for this can be the stronger DNA instability in tumor cells resulting in genetic and epigenetic changes which can alter the P450 expression profile. It is however difficult to get an overall picture of the P450 expression in tumors due to different quantification techniques etc. For an attempt to give an overview of P450 expression or activity in tumors compared to the normal tissue see Oyama et al. [88].

1.2.7.1 Tumor-specific P450s

CYP1B1 is one P450 enzyme that has been shown to be highly expressed in several tumor types, including tumors originating from bladder, connective tissue, kidney, stomach, uterus, lung, esophagus, skin, brain, breast, colon, testis, lymph node as well as in primary and metastatic ovarian cancers, [54, 89-92]. Besides expression in tumors, there are also several publications that have shown CYP1B1 mRNA in normal and fetal human tissues [93-99] and the corresponding protein has been found in a few normal tissues including colon, although at lower levels as compared to colon tumors [57]. Other normal tissues showing CYP1B1 protein expression include brain, kidney, testis, breast, prostate, bladder, lung, ovary etc. [100-104]. In normal liver the CYP1B1 expression levels is low or absent [100, 105].
Although no protein has been observed, the CYP1B1 mRNA has been shown to be abundantly expressed in ocular tissues [106] and mutations in the CYP1B1 gene was shown to be linked to primary congenital glaucoma [98, 106]. This finding suggests that CYP1B1 has an important role in eye development.

The expression of CYP1B1 in cancer tissue is specifically localized to the tumor cells. Interestingly, also an increased CYP1B1 specific estradiol 4-hydroxylase activity has been observed in breast cancer and in uterine leiomyomas but not in the corresponding normal tissues [107, 108]. Besides estradiol, CYP1B1 has other substrates including procarcinogens and anticancer drugs, which raised the question if CYP1B1 can promote tumorigenesis. On the other hand, CYP1B1 is also utilized in several cancer therapy approaches that are under development (see below).

Besides CYP1B1, also other human P450s are overexpressed in tumors compared to the normal tissues, including CYP4Z1 (breast carcinoma) and CYP2J2 (tumors originating from breast, stomach, esophagus, liver, colon and lung) [72, 109]. Also CYP2J2 has, just like CYP1B1, been suggested to be beneficial for tumor development. As previously mentioned, also CYP2S1, CYP2U1 and CYP3A5 have been reported to be overexpressed in colon cancer compared to normal colon [54]. In addition there is a study showing overexpression of the following ten P450s in primary and metastatic ovarian cancer: CYP2F1, CYP2R1, CYP2S1, CYP2U1, CYP3A5, CY3A7, CYP3A43, CYP4Z1, CYP26A1 and CYP51 [92]. It can be noted, however, that CYP3A43 is considered to be a non functional P450 [110].

1.2.7.2 Activation of procarcinogens – role of P450s in tumor development

The major P450 enzymes involved in the metabolism of procarcinogens are the CYP1 family, consisting of CYP1A1, CYP1A2 and CYP1B1, as well as CYP2A6, CYP2B6, CYP2E1 and the CYP3 family [111, 112]. The procarcinogen metabolism by P450s has been extensively studied especially in vitro. However, in vivo a lot of additional factors have to be accounted for including the exposure to carcinogens, the tissue specific concentration of P450 and the effect of further metabolism/inactivation of the carcinogen by other enzymes. There is many studies regarding P450 polymorphisms and if genetic variants of the procarcinogen activating P450s can predispose to certain cancer types. However, the P450s being highly polymorphic are mainly the enzymes involved in drug metabolism and not the enzymes involved in the activation of procarcinogens [112].

Besides activation of procarcinogens, P450s can have other roles in tumor development. As mentioned earlier CYP2J2 is an enzyme recently shown to be overexpressed in tumors. CYP2J2 is metabolizing AA into epoxyeicosatrienoic acids.
(EETs) and experiments show that EETs, endogenous via CYP2J2 metabolism or added exogenous, promotes cell viability and stimulates proliferation [72, 113]. Xenograft models have shown that CYP2J2 enhance tumor formation [72] and CYP2J2 expression also predicts the disease prognosis for lung and esophageal cancers in humans with a lower survival for patients with CYP2J2-expressing tumors (Lecture by D. Zeldin at the MDO 2006 meeting, [114]).

1.2.7.3 Activation/inactivation of anti-cancer drugs
Activation or inactivation of anti-cancer drugs by P450s takes place mainly in the liver. Several of the drug metabolizing P450 enzymes are highly polymorphic which can severely affect the outcome of cancer therapy for the individual patient. One example is the enzyme CYP2D6. In Caucasians approximately 7% of the population is defined as PMs for CYP2D6, which means that they are lacking active CYP2D6 enzyme. CYP2D6 is one of the enzymes involved in the metabolism of tamoxifen, an agent widely used in treatment of estrogen receptor positive breast cancer. Tamoxifen is metabolized by CYP2D6 into the metabolites 4-hydroxytamoxifen and endoxifen, with endoxifen being the most abundant and also being much more potent than the parent compound [115]. Recently several studies have shown that the patients genotype is affecting the outcome of tamoxifen treatment, with individuals lacking CYP2D6 enzyme having significantly lower plasma concentrations of endoxifen [115-117]. The same effect on endoxifen concentration is seen in patients concomitantly treated with tamoxifen and CYP2D6 inhibitors [115].

Besides hepatic metabolism, P450 activity in the tumor cells can be of crucial importance for the success of cancer therapy. As mentioned earlier, CYP1B1 is overexpressed in many tumors, e.g. ovarian tumors [91]. In vitro studies have shown that CYP1B1 expressing cells are more resistant to docetaxel, a cytotoxic drug being one alternative in the treatment of ovarian cancers [118]. Although not significantly shown, a CYP1B1 increased resistance may affect treatment outcome and overall survival for the cancer patients [91]. Considering these results, P450 dependent inactivation of anti-cancer drugs within the tumor can be one of the mechanisms responsible for drug resistance.

In table 3 an overview of some of the anti-cancer drugs metabolized by P450s is shown. Note that even if many P450s are listed for one drug it necessarily does not mean that all enzymes have equally clinical significance since some P450s only represent minor pathways of metabolism.
### Table 3. Some anticancer agents metabolized by cytochrome P450 enzymes. P450s shown in bold represent major pathways. Data adapted from [112, 119-122].

<table>
<thead>
<tr>
<th>Drug/Prodrug</th>
<th>P450s involved in metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altretamine</td>
<td>2B</td>
</tr>
<tr>
<td>Bexarotene</td>
<td>2C9, 3A4</td>
</tr>
<tr>
<td>Busulfan</td>
<td>3A4</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2E1, 3A4</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>2B6, 2C19, 3A4, 2A6, 2C8, 2C9</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>3A4</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>3A4, 3A5, 1B1, 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>3A4, 2D6</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>3A4, 1A1, 1A2, 1B1, 2C9</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1A1, 1A2, 3A4</td>
</tr>
<tr>
<td>Etoposide</td>
<td>3A4, 3A5, 2E1, 1A2</td>
</tr>
<tr>
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<td>3A4</td>
</tr>
<tr>
<td>Flutamide</td>
<td>1A2, 3A4, 3A5</td>
</tr>
<tr>
<td>Fulvestrant</td>
<td>3A4</td>
</tr>
<tr>
<td>Gefitinib/Iressa</td>
<td>3A4, 3A5, 2D6</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>2D6, 2C9</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>3A4, 2B6, 2A6, 2C8, 2C9, 2C9, 2C19, 3A5</td>
</tr>
<tr>
<td>Imatinib</td>
<td>3A4, 3A5, 2C9, 2D6, 2C19, 1A2</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>3A4, 3A5</td>
</tr>
<tr>
<td>Letrozole</td>
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<tr>
<td>Mitoxantrone</td>
<td>1B1,3A4</td>
</tr>
<tr>
<td>Paclitaxel/Taxol</td>
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<tr>
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</tr>
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<td>Tegafur/Ftorafur</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td>3A</td>
</tr>
<tr>
<td>Vincristine</td>
<td>3A</td>
</tr>
<tr>
<td>Vindesine</td>
<td>3A</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>3A4</td>
</tr>
</tbody>
</table>

### 1.2.7.4 Cancer treatment approaches based on P450s

Besides the conventional cytostatic drugs metabolized by P450s several new anticancer therapy approaches utilizing P450 enzymes are under development. These approaches involve both gene directed enzyme prodrug therapy (GDEPT), tumor specific P450s like CYP1B1 and enhancement of already existing therapies by inhibition of P450s.

In the P450 GDEPT approach the gene for an exogenous P450 is introduced into tumor cells followed by treatment of a prodrug activated by that P450 enzyme. The result is a local activation of the prodrug within the tumor leading to an increased exposure of the
tumor cells to the cytotoxic metabolite and an enhanced tumor cell killing effect, while minimizing the toxicity for normal tissues. This approach has been shown to be successful both in vitro, using several human cancer cell lines, and in vivo, using tumor xenograft models. This work has mainly been focused on the use of CYP2B enzymes, with the canine CYP2B11 being the most successful [123], and the oxazaphosphorine prodrugs cyclophosphamide (CPA) and ifosfamide [124, 125]. The advantage with the P450 GDEPT approach is the use of mammalian enzymes, which reduce the likelihood of immune responses, and prodrugs already used in conventional cancer treatment. Another advantage of using e.g. CPA as a prodrug is the ability of the metabolite 4-OH-CPA to cross the cell membrane and thereby also affect tumor cells not expressing the P450 gene. This bystander effect is important for an efficient response. Several strategies have been described to improve P450 GDEPT and increase tumor cell death, including local prodrug delivery [126], modified administration schedule [127, 128], co-expression with P450 reductase [129], inhibition of the hepatic activation of prodrug [130, 131] as well as increased bystander effect by co-expression of antiapoptotic factors thereby delaying tumor cell death [132]. For a review regarding this gene therapy approach see [125].

The P450 GDEPT approach has so far been successful also in the clinical setting, both in a Phase I study in breast and melanoma cancer patients and in a Phase I/II study in pancreatic cancer patients. In the Phase I study, metastatic tumors were injected with MetXia-P450, a retroviral vector expressing CYP2B6, followed by treatment with CPA. The study showed that the MetXia approach was well tolerated and that 5 out of 12 patients got a partial response or stable disease. Besides that, also an antitumor immune response as well as a reduction in breast cancer marker levels was observed [133]. In the Phase I/II study 14 patients having inoperable pancreatic cancer got encapsulated cells modified to overexpress CYP2B1 by angiographic placement flowed by treatment with ifosfamide. Also in this study the treatment was beneficial with two patients showing partial response, two showing minor response and eight patients showing stable disease. In total, this increased the median survival with 20 weeks compared to the control group as well as increasing the one-year-survival from 11% to 36% [134]. For an overview of ongoing and completed clinical trials see [135].

There are several cancer therapy approaches being developed involving the CYP1B1 enzyme. One is an immunotherapy based approach where a DNA vaccine, ZYC300, has been designed to promote the immune system to react towards the tumor cells expressing CYP1B1 [136, 137]. A Phase I clinical trial has already been completed using this vaccine [138]. The outcome was positive with 6 out of 17 patients developing an immune response to CYP1B1. Of these patients five required disease stabilization with one of them remaining stable without need for further therapy.
However, all others who responded to the CYP1B1 vaccination had a significant response to the next treatment regimen, suggesting that a CYP1B1 immune response is of advantage for the patients.

The evaluation of ZYC300 is continuing. Right now a Phase I study is being performed aiming to determine the feasibility, safety and tolerability of repeated ZYC300 administrations following CPA treatment. ZYC300 has not been co-administered with CPA before and the hope is that CPA will enhance the effect of ZYC300 by inhibition of the regulatory T cells. This study is expected to involve 20 patients and to be completed until April 2007 [139].

Also other approaches like CYP1B1 prodrug activation, which would work in a similar way like the GDPET concept, and CYP1B1 inhibition, to overcome anti-cancer drug resistance, are being developed (for a review see [140]). Some of the CYP1B1 protein inhibitors being investigated are stilbene derivatives (patent application WO03018013) and α-naphtoflavone (patent application WO0158444, [141]). However, also other enzymes, not specifically expressed in tumors, can be targets for inhibition to improve cancer treatment. One example is inhibition of CYP3A4 using CYP3A4 antisense inhibitors (patent application WO0187286, [142]). Inhibition of CYP3A4 expression would then reduce the drug metabolism, extend drug half life and thereby offer a more effective treatment with CYP3A4 inactivated drugs e.g. paclitaxel.

1.3 COLON CANCER

Colorectal cancer is one of the most common cancer forms of today. Worldwide the incidence for colorectal cancers is more than 1 million new cases with a mortality of about half a million deaths as for year 2002 [143]. There is a large variation in cancer incidence between countries, with developed countries having high incident rates as compared to developing countries. In Sweden, as for year 2005, almost 4000 new colon cancer cases were reported making it the fourth (men) or second (female) most common form of cancer [144].

Both genetic and environmental factors contribute to the development of colon cancer, with diet being one of the factors believed to be of importance, with individuals on a typical Western diet, i.e. high levels of saturated fat and low fiber intake, being of high risk [145]. This is shown for example in the increased incidence in countries recently adapting to western life style and the rapid increase in incidence for populations moving from low-risk to high-risk areas [143, 146].

Even if most cases of colon cancer are non-hereditary a number of cases can be linked to hereditary conditions. Most common are the high-risk syndromes familial
adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), caused by mutation in the APC tumor suppressor gene or genes involved in DNA replication error repair, respectively [28]. Together FAP and HNPCC account for 3-5% of the colorectal cancer cases but other still unknown genetic/hereditary causes are estimated to account for up to one third of the colorectal cancer cases [147].

Colon cancer cases are classified according to the primary tumor, lymph node metastasis as well as distant metastasis and then grouped into different stages [148], which give valuable information for prognosis of the patient, and hence also for the choice of treatment.

1.3.1 Treatment
The standard treatment for colorectal cancer patients is surgery but pre- or postoperative radiation treatment (mainly for rectal cancers) and chemotherapy are also used. Chemotherapy is recommended for colon cancer classified as stage III, whereas it is not standard for patients with stage II colon cancer. The treatment regimes used are commonly involving the drugs fluorouracil (5-FU) and leucovorin (folinic acid) [146, 149, 150]. Recently also two other drugs, irinotecan or oxaliplatin, have been used for chemotherapeutic treatment. Studies have shown that addition of oxaliplatin to the treatment regime improves disease free survival, and concomitant treatment with 5-FU, leucovorin and oxaliplatin are therefore also considered for stage III patients [149].

1.3.2 New therapy approaches
Several new colon cancer therapy approaches are being developed. The targets for these novel agents are growth factor receptors, signaling transducers, matrix metalloproteinases etc. [151]. The most promising of these agents are the monoclonal antibodies cetuximab and bevacizumab, both with successful phase II/III clinical trials. Bevacizumab is directed towards the vascular endothelial growth factor receptor (VEGFR), whereas cetuximab is directed towards the epidermal growth factor receptor (EGFR) [148, 151]. Besides these two agents, also many other approaches, including e.g. immunotherapy but also small-molecular inhibitors, signaling kinase inhibitors etc., are being developed (for a review see [151, 152]).

Also P450s have received attention with respect to colon cancer and cancer prevention or treatment. One example is the publication by Chang et al. were the objective was to evaluated the possibility of using CYP1B1 as a biomarker for early stages of colon cancer [153]. IHC analysis showed that CYP1B1 expression was induced also at early stages indicating that CYP1B1 in combination with β-catenin or COX-2 could be potential biomarkers in screening for colorectal adenoma or carcinoma. Other P450 enzymes getting attention are CYP24A1 and CYP27B1, both expressed in colon and
involved in vitamin D metabolism. The vitamin D metabolite calcitriol has growth inhibitory effects, and it is hypothesized that an increased intratumoral calcitriol concentration, obtained by affecting the expression of the involved P450s by adjusting diet calcium etc., may be one way to inhibit tumor progression or prevent tumor formation, as reviewed by Cross et al. [154].
2 AIMS

As mentioned in the background, several cytochrome P450 genes have been identified over the last years because of the initiatives related to the sequencing of the human genome. For most of the corresponding enzymes only limited knowledge are available at present. This study aims to bring further insight into this field by cloning and characterizing novel P450 genes.
3 COMMENTS ON METHODOLOGY

3.1 mRNA EXPRESSION
In this study different approaches have been used to investigate mRNA distributions. The main methods used are mRNA blot analysis and real-time PCR, with real-time PCR being the most sensitive. Both commercially available blots and cDNA panels (Clonetech, Palo Alto, CA) as well as RNAs/cDNAs made directly from tissues/cells have been used. The commercially available RNAs/cDNAs are pooled from several individuals, mainly Caucasians. The expression levels detected by using these samples are therefore the mean expression in a number of individuals.

When analyzing the results from real-time PCR two different methods for calculation have been used. In paper III standard curves were constructed for each gene product analyzed with the use of serial 10-fold dilutions ranging from $10^{-2}$ fmol/µl to $10^{-9}$ fmol/µl of full-length or partial cDNA fragments. The obtained CYP2W1 concentration was then normalized with the concentrations obtained for the housekeeping gene. In paper V a relative quantification method, called the $2^{-\Delta\Delta C_T}$ method, have been used. Here, HepG2 cells have been used as the calibrator [155], i.e. the other samples analyzed were compared to HepG2 to determine the relative change in CYP2W1 expression. In paper V, besides $\beta$-actin, two additional housekeeping genes have been used. This is to make sure a housekeeping gene is used that is unaffected by experimental/patient treatment.

3.2 SUBCELLULAR FRACTIONATION
In this study both cells and tissue samples have been fractionated by differential centrifugation into cytosolic, mitochondrial and microsomal fractions, later used for e.g. western blotting, incubations in search of substrate etc.. These fractions can not be regarded as pure mitochondrial/microsomal isolations, instead they should be regarded as enrichments.

In addition to differential centrifugations subcellular fractionation using sucrose gradients have also been performed for some samples (although not included in any publication). Using this method the post-nuclear supernatant is loaded on top of a discontinuous sucrose gradient, and after centrifugation fractions are collected from the bottom of the tube. However also using this method some overlapping is seen for the mitochondria and microsomes.
3.3 TUMOR SAMPLES
Several tumor tissue samples, as well as some corresponding normal samples, were analyzed for CYP2W1 expression or methylation of the *CYP2W1* gene. Through collaboration these tissue samples were obtained from the Hospital Clínico Universitario “Lozano Blesa”, Zaragoza, Spain, and from the Karolinska University Hospital. The different samples have most likely been handled differently and, although examined by pathologist at the hospitals, have varying levels of heterogeneity, which have to be considered when interpreting the results.

For the preparation of mRNA, DNA or subcellular fractions small pieces of the tissue samples were used. Hence, in the presence of liquid nitrogen the tissue samples were divided into smaller pieces and different pieces were used for the different preparations. The fact that different tumor pieces have been used can also contribute to the sometimes varying results obtained for the same sample regarding mRNA expression, protein expression and/or methylation.

3.4 EXPRESSION SYSTEMS
The two enzymes studied, CYP2U1 and CYP2W1, have both been cloned and expressed using different expression systems. Best results were obtained when using a human embryonic kidney cell line, HEK293, and these are the results included in our publications. Besides mammalian cells also yeast (*Saccharomyces cerevisiae*) overexpressing the yeast or human P450 reductase as well as the drosophila expression system Schneider 2 cells have been used. An advantage with using a mammalian cell line like HEK293 cells as expression system is that it is more likely that all necessary factors are present and that the enzyme will be correctly folded. However, a disadvantage with HEK293 cells is, since it is a human cell line, the risk for background, regarding both expression of human P450s and P450 metabolism. The HEK293 cells are not considered to express P450s, but as seen in figure 5B, paper I, mock-transfected HEK293 microsomes are expressing CYP2U1.

3.5 METHYLATION OF THE CYP2W1 GENE
For analysis of CYP2W1 gene methylation two different methods were used. In paper III we analyzed CYP2W1 gene methylation by methylation sensitive restriction digestion. There cells were treated with the demethylated agent 5-Aza-2’-deoxycytidine (AzaC) and genomic DNA from treated and untreated cells were digested using the isoschizomers *Msp*I and *Hpa*II. Both these enzymes recognize the CCGG restriction site, however *Hpa*II is methylation sensitive and will not cut if the DNA is methylated at this site. Digestion was followed by amplification by PCR to determine the methylation status.
In paper V the sodium bisulfite sequencing method was used to determine methylation levels. When using this method the genomic DNA was denatured followed by incubation with sodium bisulfite. This will change the unmethylated cytosines into uracils. By PCR amplification and sequencing the methylation status of each individual CpG dinucleotide can then be determined (Figure 4). This is in contrast to the previously used method which can only detect presence or absence of methylation.

**Figure 4.** Chemical conversion of cytosine to uracil (A). Sodium bisulfite sequencing method for DNA methylation analysis (B). Unmethylated cytosines are converted to uracil while methylated cytosines remain unchanged by the bisulfite treatment. PCR and subsequent sequencing will show the unmethylated cytosines as thymines while methylated cytosines remain as cytosines. Adapted from [156].
4 RESULTS

The results obtained and presented in this study are the following:

- Cloning and characterization of CYP2U1, with regard to gene structure, distribution and activity.
- Cloning and characterization of CYP2W1, with regard to gene structure, distribution, activity, substrate specificity and regulation.

4.1 CHARACTERIZATION OF CYP2U1 (PAPER I)

4.1.1 Identification and initial characterization

Using bioinformatics approaches sequences were identified that correspond to the P450 CYP2U1. The open reading frame (ORF) was verified by PCR amplification and sequencing. The CYP2U1 gene is located on chromosome 4 and unlike other P450s belonging to family 2 CYP2U1 has only five exons in comparison to other cases nine. The first two exons are rather long (~500-600 bp), whereas the last three exons are much shorter (<200 bp). Later on, when amplifying the CYP2U1 cDNA from various human tissues an additional splice variant was seen that after sequencing turned out to lack the second exon (Figure 5).

![Figure 5. Detection of CYP2U1 cDNA, wild type and splice variant, in different human tissues as assessed by gene specific PCR amplification. L=1 kb plus ladder, N=negative control, 1=heart, 2=lung, 3=colon, 4=prostate and 5=spleen.]

When comparing the deduced CYP2U1 amino acid sequence with other human P450s the enzyme with highest identity to CYP2U1 is CYP2R1, also having only five exons. Alignment of the CYP2U1 amino acid sequence with other CYP2 sequences, as well as with CYP2U1 orthologs from fugu fish and mouse, revealed that CYP2U1 has two segments of additional amino acids in its N-terminal region compared to the other CYP2 enzymes (Figure 6A). The comparison with mouse and fugu CYP2U1 also indicated that the region in the very N-terminal have evolved later in time compared to
the other additional amino acid region. In paper I we speculate if these extra regions can affect i.e. membrane incorporation or structure of the CYP2U1 enzyme.

Figure 6. (A) Alignment of the N-terminals of human CYP2U1 and CYP2R1. The amino acids removed or mutated in constructs 1, 2 and 3 are shown in bold. (B) Schematic figure of the CYP2U1 expression constructs made in order to see how the additional N-terminal amino acid regions are affecting the CYP2U1 enzyme. Striped or checked boxes represent the mutated or deleted amino acids shown in bold in figure A. wt denotes wild type.

4.1.2 mRNA and protein distribution
RNA blot analysis revealed that CYP2U1 is highly expressed in both fetal and adult human thymus. Lower expression levels were seen in several tissues although the most pronounced were heart and brain. CYP2U1 mRNA expression was also examined in RNA from rat liver, thymus and brain, showing a slightly higher expression in brain compared to thymus and no expression in liver. Similar results were obtained when determining the CYP2U1 protein expression pattern in rat tissues, although here the expression in brain was much higher than in thymus. The brain regions showing highest CYP2U1 expression were the limbic structures and the cortex.

4.1.3 Subcellular localization and folding of recombinant CYP2U1 variants
Using a CYP2U1-pCMV4 construct we were able to express correctly folded CYP2U1 protein in HEK293 cells as revealed from the P450 spectra. CYP2U1 was found in both microsomal and mitochondrial fractions from the transfected cell, whereas constitutively expressed CYP2U1 in HEK293 cells is located mainly in the microsomes. Expression constructs lacking the extra regions in the N-terminal as previously described were also made using the pCMV4 vector (Figure 6B). A few pilot experiments in HEK293 cells using the wild type construct as well as construct 1 and 2
indicate that the second region located after the membrane spanning region is important for folding since no P450 spectra could be obtained from the HEK293 microsomes transfected with construct 2 (C. Cauffiez and M. Karlgren, unpublished observation). However, more information is needed before any conclusions can be made.

4.2 CHARACTERIZATION OF CYP2W1

4.2.1 Identification and initial characterization (Paper II and III)

In the dbEST database and the Celera sequence database sequences were identified that correspond to the partial CYP2W1 sequence previously found in a HepG2 cDNA library. The CYP2W1 gene is located on chromosome 7 and the CYP2W1 ORF was verified by amplification and sequencing. Unlike CYP2U1, CYP2W1 has a typical family 2 gene structure with nine exons. The CYP2W1 sequence confirmed by sequencing was identical to a reported sequence (GenBank Accession No. NP_060251). Also another CYP2W1 transcript has been reported (GenBank Accession Nos. AK000366, EAW87186) having only eight exons with the eight exon being longer than in the nine-exon-transcript. Compared to other P450s the eight exon variant is however lacking e.g. the conserved cysteine [157], which is the fifth heme ligand. Therefore only the CYP2W1-nine exon transcript has been further analyzed and

![Figure 7. Comparison between the CYP2W1-nine and -eight exon variants. The additional part of the eighth exon in the CYP2W1-eight exon transcript is shown in italics. The conserved cysteine is shown in bold.](image-url)
included in paper II, III and V. For a comparison of the two CYP2W1 transcripts see figure 7.

4.2.2 Subcellular localization and folding of the recombinant CYP2W1 protein (Paper III)
The CYP2W1 cDNA was cloned into the pCMV4 vector and expressed in HEK293 cells. Using microsomes from the transfected cells, a P450 spectrum was obtained indicating that the CYP2W1 enzyme was properly folded. When analyzing microsomes and mitochondria from the transfected cells using Western blotting three bands corresponding to CYP2W1 were found in both microsomes and mitochondria. The expression of CYP2W1 in both microsomes and mitochondria is also seen when performing subcellular fractionation using a sucrose gradient (Figure 8). The multiple bands observed for CYP2W1 is probably the result of post-translational modifications, as indicated by a few pilot experiments (A. Gomez, unpublished observation).

Figure 8. Intracellular localization of human CYP2W1 heterologously expressed in HEK293 cells as determined by discontinuous sucrose density gradient and Western blotting. Fractions were collected from bottom to top and are numbered 1-11. A fraction of the post-nuclear supernatant loaded on the sucrose gradient was also analyzed by Western blotting and is loaded in the last lane. ERp29 is a marker for the microsomes, Hsp70 for the mitochondria and Hsp90 for the cytosol. The band seen in fraction 9-11 (and the upper band in the post-nuclear supernatant) is the result of unspecific binding (u.s.), whereas the bands in fraction 1-7 (and the lower bands in the post-nuclear supernatant) correspond to the CYP2W1 protein (2W1) as indicated with arrows to the right. The middle band seen in Figure 4B in paper III is not visible in this figure.

4.2.3 CYP2W1 substrate specificity (Paper II and III)
To analyze the catalytic activity of CYP2W1, microsomes from the transfected HEK293 cells were incubated with AA. Using liquid chromatography-mass spectrometry (LC-MS) analysis of the EET, hydroxyeicosatetraenoic acid (HETE) and dihydroxyeicosatrienoic acid (DHET) metabolites we could show that CYP2W1 is metabolizing AA at a small rate. The main metabolites seen were 14,15-, 11,12- and 8,9-DHET.
4.2.4 Regulation of the CYP2W1 gene (Paper III, IV and V)

Using bioinformatic approaches two CpG island were identified in the CYP2W1 gene. One island is located in the promoter region and one is located in the exon 1-intron 1 region. The methylation status of the second CpG island was analyzed in HepG2, B16A2 and HEK293 cells using treatment with the demethylating agent AzaC and methylation sensitive restriction digestion. The results showed that the CYP2W1 gene is methylated in the B16A2 and HEK293 cell lines whereas the gene is unmethylated in HepG2 cells. Treatment with AzaC also resulted in activation of the CYP2W1 gene causing significant expression in the previously non-expressing cell lines B16A2 and HEK293.

Using the sodium bisulfite sequencing method the methylation status of a part of this CpG island was examined in colon tumors, normal colon, a few human livers and in Caco-2TC7 cells as well as in the HepG2 and B16A2 cell lines previously examined. Also here HepG2 cells were found to have a hypomethylated CpG island (8.3% methylation) whereas the CYP2W1 gene in B16A2 cells was highly methylated (100% methylation). The Caco-2TC7 cells were just like HepG2 hypomethylated (8.3-16.7% methylation) whereas the liver samples were hypermethylated (mean 93.8%). When looking at tissue samples from colon higher methylation levels were seen in the normal samples (mean 76.6%) compared to the tumor samples (mean 55.1%).

4.2.5 CYP2W1 mRNA and protein distribution (Paper II, III, IV and V)

The CYP2W1 mRNA tissue distribution was analyzed using RNA blot as well as real-time PCR with both commercially available and own made cDNA/RNA as templates. Using RNA blots no expression could be observed in normal adult or fetal human tissue or in any cell line except for HepG2. When analyzing human adult and fetal cDNA panels by real-time PCR some expression could be found in a few tissues although at much lower levels than in HepG2 cells used as reference. Interestingly, slightly higher expression was seen in fetal kidney, liver and lung compared to the corresponding adult samples.

To further look into the fetal expression of CYP2W1, the rat CYP2W1 mRNA expression was examined by real-time PCR in fetal tissues at three different ages. Rat CYP2W1 mRNA was detected and especially in fetal colon high CYP2W1 mRNA levels were seen. In colon the expression increased by fetal age and then decreased again in adult rat colon.

The CYP2W1 expression was also analyzed in human tumor samples originating from various tissues. Both real-time PCR and Western blotting showed that high levels of
CYP2W1 were expressed in colon tumors. Moderate mRNA levels were seen in adrenal gland tumors and one lung tumor sample expressed low levels of the CYP2W1 protein. As mentioned in paper IV and shown more extensively in paper V, we recently also found high expression of CYP2W1 mRNA and protein in a colon carcinoma cell line, Caco-2TC7, with increasing CYP2W1 levels when cells were allowed to differentiate for 3 weeks after reaching confluence.

The study of CYP2W1 expression in colon tumors was extended in paper V where an additional numbers of colon tumors were analyzed as well as some corresponding normal colon samples. Also here high expression of CYP2W1 expression was observed in the tumor samples, although lower expression levels were detected also in some normal colon samples. The results indicate that the tumor samples/patients can be divided into two different groups regarding CYP2W1 expression with one group having similar CYP2W1 expression levels in normal and tumor tissues and one group having highly induced CYP2W1 expression in the tumor samples.

When analyzing tumors samples using Western blotting, CYP2W1 expression was seen in both the mitochondrial and microsomal fractions obtained by differential centrifugation. In paper III only one band was seen in the tumor samples corresponding to the lowest band in the CYP2W1 HEK293 microsomes used as positive control, although in paper V additional bands were seen.
5 DISCUSSION

5.1 CYP2U1 – A HIGHLY CONSERVED P450 ENZYME

CYP2U1 is, as mentioned in Paper I and II, highly conserved across species, being one out of two CYP2 subfamilies having orthologs in fugu fish [14]. This high conservation strongly suggests that CYP2U1 has an important endogenous function. This is also supported by the fact that so far only one polymorphism have been found in the coding region of the human CYP2U1 gene, and that the mutation found is a rare missense mutation, Arg53Lys, involving two fairly similar amino acids (C. Cauffiez, personal communication). Besides the Arg53Lys mutation, many single nucleotide polymorphisms (SNPs) have been found in non-coding regions [158-160]; including 32 SNPs found in the HapMap database and 75 SNPs in the NCBI database. This is in contrast to many of the other CYP2 family members that are highly polymorphic, including polymorphisms in the coding regions. Even though polymorphisms are not frequently found in the CYP2U1 gene, at least one splice variants have been observed (see Results section). So far no reports have been published regarding e.g. tissue distribution or potential functionality of CYP2U1 splice variants.

The main difference between the deduced amino acid sequences of CYP2U1 from fugu fish, mouse and human is the additional amino acid stretches found in the N-terminal of human CYP2U1. As mentioned in the result section constructs were made lacking these regions. One idea was that these additional amino acids could affect the structure or membrane incorporation and thereby also the function of the CYP2U1 enzyme. No comprehensive results are available, but a few pilot experiments, including spectral analysis and western blotting, suggest that the sequence in the very N-terminal (deleted in construct 1) is of no importance for folding and expression of the CYP2U1 enzyme. However, the sequence inserted after the membrane spanning region (deleted in construct 2) is crucial for folding, since no P450 spectra could be observed in microsomes from these samples (C. Cauffiez and M. Karlsgren, unpublished observation).

5.2 ROLE OF CYP2U1

Although the CYP2U1 enzyme is expressed in tissues generally not thought of as major xenobiotic metabolizing organs, it can not be excluded that CYP2U1 is involved in the metabolism of foreign compounds. However, as mentioned the tissue distribution and especially the species conservation strongly suggest endogenous substrates and functions. A crucial role in normal development and physiology would explain the strong selection pressure resulting in the highly conserved CYP2U1 sequence. Unfortunately, sufficient amount of recombinantly expressed CYP2U1 were not obtained using our expression systems, HEK293 or yeast, to allow for substrate...
screening. However, another group could, using recombinant CYP2U1 enzyme from insect cells, show that CYP2U1 is metabolizing AA and other long chain fatty acids [161]. Although the closest human relative to CYP2U1 is CYP2R1, CYP2U1 also has high identity to the CYP2N enzymes, also active in AA metabolism and with especially CYP2N2 being expressed in teleost brain [162].

As mentioned in the introduction, AA is a promiscuous substrate and several CYP2 enzymes are involved in the production of EETs. The AA metabolites produced by CYP2U1 were however 19- and 20-HETE. Several CYP4 isoforms are involved in the formation of 19- and 20-HETE [17], and some of the CYP4 enzymes have also been shown to be expressed in brain [17, 77, 163]. Besides CYP4, also mouse CYP2J9, a brain selective P450, has been shown to metabolize AA into the main metabolite 19-HETE [164]. 19-HETE inhibits voltage-gated Ca\(^{2+}\) channels, and may therefore affect the release of neurotransmitters [164], whereas 20-HETE serves as an endogenous vasoconstrictor in brain by inhibiting vascular smooth muscle cell large conductance Ca\(^{2+}\) activated K\(^+\) channels [17].

Although no immunohistochemical data exist for CYP2U1 in brain and thymus and we hence do not get any clue for the function of CYP2U1 by knowing in which cells CYP2U1 is expressed, the expression pattern as well as the activity reported suggest that CYP2U1 may play a role in the regulation of blood flow and vascular tone in brain and maybe also in thymus, heart and other extrahepatic tissues. Considering the high conservation, as mentioned earlier, it is however unlikely that CYP2U1 plays a major role in xenobiotic metabolism even if it can not be excluded that metabolism by CYP2U1 could be significant for specific drugs in specific tissues, e.g. thymus and brain.

Two reports from 2005 show that the CYP2U1 protein is expressed in normal colon and ovary, but more interestingly that the expression of CYP2U1 is significantly induced in primary tumors from these tissues [54, 92]. For colon tumors there was also a higher CYP2U1 expression in lymph node metastasis compared to normal colon [54]. These results imply that CYP2U1 and a hypothetical exogenous CYP2U1 activity can be important also for cancer therapy.

### 5.3 CYP2W1 – INITIAL CHARACTERIZATION

Similar to CYP2U1, CYP2W1 have orthologs in other species, although CYP2W1 does not seem to be conserved to the same extent as CYP2U1. As mentioned in paper IV the CYP2W1 gene is one of three genes representing deep branches in the CYP2
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CEU: CEPH (Utah residents with ancestry from northern and western Europe)
CHB: Han Chinese in Beijing, China
JPT: Japanese in Tokyo, Japan
YRI: Yoruba in Ibadan, Nigeria

**Figure 9.** Overview of SNPs in the *CYP2W1* gene, with gene exons indicated as black boxes (A). The SNPs with reported allele frequencies are indicated with black arrows, and other SNPs located in the coding region of CYP2W1 are indicated with grey arrows. For the non-synonymous SNPs amino acid exchanges are indicated. Allele frequencies corresponding to SNPs shown with black arrows can be found below (B). For explanation regarding populations see the lower left corner. Allele frequencies are obtained from the HapMap homepage [158].

gene family, which means that it either is evolutionary older or has mutated more rapidly that other *CYP2* genes [165]. Just like for CYP2U1, some polymorphisms have been reported for the *CYP2W1* gene ([158-160], M. Hiratsuka, personal communication). Only six of these polymorphisms are found in the coding region of CYP2W1 and five of these are non-synonymous causing amino acid exchange. As discussed in
paper IV these amino acids are not located in the predicted substrate recognition sites when comparing with CYP2D6 [166], but it is still possible that they can affect substrate specificity by e.g. affecting the folding of the enzyme. Besides SNPs in coding regions also the SNPs located in introns etc. can affect splicing, stability of the CYP2W1 mRNA etc. and can thereby also affect the amount of enzyme being expressed. It remains to see the importance of all these SNPs. For an overview of the SNPs with reported allele frequencies see figure 9.

When examining the CYP2W1 tissue distribution in human adult and fetal tissues mRNA blots as well as cDNA panels were used. Although both we and other groups have found some expression of CYP2W1 in human tissues using these cDNA panels (paper III, [167-169]), the sensitivity when using PCR together with the lack of expression observed when using mRNA blot indicates that the expression of CYP2W1 in normal fetal and human tissues indeed is very low (as discussed in paper IV). However the high fetal expression of CYP2W1 in whole mouse embryo [167] and rat fetal colon (paper III) indicates that CYP2W1 might be involved in development and/or differentiation of the gastrointestinal tract and especially colon. As to be pointed out, the commercial cDNA panels used in the four publications previously cited do not contain human fetal colon. Therefore it is difficult to make any assumptions regarding CYP2W1 function in human development.

Besides expression in fetal tissues, there is also one group who has shown CYP2W1 expression in human keratinocytes, with higher expression in TCDD treated cells [170-172]. Therefore, it is possible that CYP2W1 has exogenous functions in skin. However, so far only CYP2W1 mRNA have been identified and no protein making it difficult to estimate the CYP2W1 expression level in keratinocytes compared to expression levels in HepG2 cells or in tumors. In other respects the expression in skin as well as the induction by TCDD are examples of similarities between CYP2W1 and its relative CYP2S1 [25, 45, 46].

5.4 FUNCTION AND SUBSTRATE SPECIFICITY OF CYP2W1

In paper III we could show that CYP2W1 metabolizes AA, however only at a small rate, indicating that the influence by CYP2W1 on the metabolism of AA is negligible and that AA is not the main substrate for CYP2W1. In a recent publication the authors showed that recombinant CYP2W1 expressed in E. coli metabolizes benzphetamine, an anti-obesity drug closely related to amphetamine [173]. The same authors could also show that recombinant CYP2W1 activates several procarcinogens, including some PAHs. They also registered, together with a second publication, blue colored cultures when expressing CYP2W1 in E. coli indicating indole 3-hydroxylation [169, 173]. In the publication by Yoshioka et al. they showed, using HPLC analysis, that the bacterial
cultures expressing recombinant truncated CYP2W1 (Δ2W1) and P450 reductase contained isatin, oxindole, indigo and indirubin. Using membrane fractions from those cultures they also showed that Δ2W1 metabolises indole to oxindole and isatin [169]. Also several other enzymes in the CYP2 family can catalyze the formation of indoxyl (isomer to oxindole) and isatin from indole [174, 175], and the following dimerization products indigo and indirubin have been shown to be potent AhR ligands [114].

Altogether these results show that CYP2W1 indeed is an active P450 enzyme with quite broad substrate specificity. Considering the activation of procarcinogens by CYP2W1 as well as the suggested regulation via the AhR (see below), CYP2W1 seems more similar to the CYP1 enzymes. However considering the metabolism of benzphetamine and indole, mainly performed by CYP2 and CYP3 enzymes, the CYP2W1 substrate specificity seems less restricted.

Since CYP2W1 is an extrahepatic P450s, expressed both in tissues associated and not associated with xenobiotic metabolism, it is likely that CYP2W1 has endogenous functions. However the expression of CYP2W1 mRNA in adult human tissues is, as discussed previously, very low. Despite the low expression it can not be excluded that CYP2W1 has important endogenous or exogenous functions in specific tissues or cells types, like e.g. keratinocytes. In contrast to the low expression in adult tissues the relatively high mRNA expression in fetal rat colon (paper III) as well as the high expression of CYP2W1 mRNA in mouse embryo [167] suggests that CYP2W1 may have important functions during development.

With respect to substrate as well as the high expression in certain tumors (as discussed below) an interesting point is of course the activation of procarcinogens by CYP2W1 but also the possibility that CYP2W1 is involved in the metabolism of anti-cancer drugs. There are a few indolic compounds produced by plants that are interesting in the context of anticancer drugs [176, 177]. However, so far no drugs used in cancer therapy have been reported to be identified as substrates for CYP2W1, but that is an interesting area for further research.

5.5 MECHANISMS INVOLVED IN THE REGULATION OF THE CYP2W1 GENE

Demethylation of the CYP2W1 gene seems to be a prerequisite for expression of the CYP2W1 gene products, since no samples are found having a methylated CYP2W1 gene together with high expression of CYP2W1 mRNA or protein (as discussed in paper V). However, there is no clear correlation between CYP2W1 gene methylation levels and CYP2W1 expression. This indicates that additional factors are involved in the regulation of CYP2W1 expression. As previously mentioned CYP2S1, which is one
of the human P450s with highest identity to CYP2W1, has XRE elements in the gene promoter and is transcriptionally regulated via the AhR [25]. Also CYP2W1 has some XRE elements and a pilot study in our lab indicates that CYP2W1 expression is induced by TCDD (A. Gomez, unpublished results). Hence, the CYP2W1 gene might be regulated via the AhR. Besides TCDD and other HAHs and PAHs, the AhR also has dietary ligands like dietary indole-3-carbinol etc. [24]. Interestingly, Wu et al. showed that CYP2W1 metabolizes several procarcinogens also being ligands for the AhR [173]. Considering the well characterized CYP1 enzymes, it is common that the compound causing induction of the expression of the P450 gene also is a substrate for the corresponding protein. However, more results are needed to validate if the CYP2W1 gene is the second CYP2 enzyme being transcriptionally regulated via AhR, and in order to fully understand the regulation of CYP2W1 expression.

Interestingly, a recent paper reported that besides dioxin the expression of both mouse and human CYP2S1 is induced by hypoxia [178]. The induction is dependent on the Hypoxia Inducible Factor-1 and hypoxia response elements (HREs) located within the XREs in the promoter region. As mentioned in the background, CYP2S1 is expressed above all in the respiratory and gastrointestinal tract. However, the CYP2S1 protein is also highly expressed in some tumors, including colorectal tumors [45, 54]. Many solid tumors are hypoxic, which can be one explanation for the high expression levels of CYP2S1. It is not possible to extrapolate these results to the CYP2W1 situation; however it is an interesting topic which may be worth investigation.

### 5.6 CYP2W1 – A TUMOR SPECIFIC P450 ENZYME

Even if the CYP2W1 mRNA is expressed at very low levels in human adult and fetal tissues it is highly expressed in some tumors. First indications for CYP2W1 being highly expressed in tumors came from the high expression in HepG2 cells, a human hepatoma cell line. HepG2 cDNA is also the source for the first found partial CYP2W1 sequence and hence, during this study, have been our reference for CYP2W1 expression. Besides HepG2 we also found a slightly higher expression in 2 out of 8 tumor samples, one lung and one colon, from a commercial cDNA panel (Human Tumor MTC panel, Clontech, Palo Alto, CA) (unpublished observation). When further analyzing the CYP2W1 expression in a variety of human tumors we could detect CYP2W1 mRNA or protein in adrenal gland tumors, colon tumors and lung tumors (paper III and V); with considerable higher expression in colon tumors. However, in paper V, we also identified CYP2W1 expression in some normal colon tissues, indicating that the expression of CYP2W1 is not as exclusively restricted to tumors, as first thought. As evident from both the mRNA and protein expression in normal/tumors pairs from colon (paper V) tumors/patients can be divided into two subgroups
regarding CYP2W1 expression, with about 50% of the subjects overexpressing CYP2W1 in tumors. The reason for this discrepancy is not yet known.

The mRNA and protein expression seen in tumor samples do not correlate for all of the samples. This is especially seen when comparing the mRNA and protein expression of CYP2W1 in paper III. In that paper we discuss that this can be due to sample quality, i.e. contribution by untransformed tissue or degradation of CYP2W1 gene products. This is most likely the explanation, however now an additional alternative can be added. Two of the non-synonymous SNPs found for CYP2W1 are located in the very C-terminal, and the antibody used for examining the CYP2W1 protein expression is raised using a C-terminal 15 amino acid peptide. For these two CYP2W1 mutants, less protein will be detected by the peptide antibody due to the changes in epitope. For one of the SNPs, the populations genotyped, and shown on the HapMap homepage [158], are all homozygous for the reference allele, indicating that this may be a minor problem. In contrast, others have got different results with the mutated allele having an allele frequency of approximately 35% in some populations (A. Gomez, unpublished observation, M. Hiratsuka, personal communication).

5.6.1 Involvement in tumor formation and development

As suggested for most P450s expressed in tumors, there is a possibility that CYP2W1 expression is beneficial for the tumor and that CYP2W1 is promoting tumor formation or development. If so, CYP2W1 would be considered as an oncogene. The expression of CYP2W1 protein in normal tissue is however not yet verified, making any assumptions regarding CYP2W1 involvement in tumor formation too vague. However, considering the expression if CYP2W1 in rat fetal tissues and the hypothetical role in fetal development, it is possible that the high expression of CYP2W1 actually seen in tumors is of advantage for the tumor and hence of disadvantage for the patient. This remains however to be shown. A similar characteristic have been suggested for the relative CYP2S1, with one study showing overexpression of the CYP2S1 protein in colorectal tumors, and correlation between strong CYP2S1 immunoreactivity and poorer survival [54].

5.7 IMPORTANCE OF CYP2U1 AND CYP2W1 IN DRUG METABOLISM

The knowledge so far regarding CYP2U1 and CYP2W1 is limited and their role in drug metabolism is therefore not clear. Both enzymes are likely to have endogenous functions, as exemplified by their tissue distribution and expression in fetal tissues. Nevertheless, they still might be important for the tissue specific drug metabolism or drug metabolism at different developmental stages. Of these two enzymes CYP2W1 seems to be the enzyme most likely to have an influence on drug metabolism, based on the less conservation across species compared to CYP2U1 and that CYP2W1 is similar
to CYP2S1, which is induced by and metabolizes exogenous compounds (for a review see [179]).

5.8 CYP2U1 AND CYP2W1 AS DRUG TARGETS

As extensively discussed in paper IV the tumor specific expression of CYP2W1 makes the enzyme interesting as a potential drug target. Depending on what role CYP2W1 play in tumor development or in tumor progression several approaches can be applicable like using CYP2W1 as a prodrug activator, using CYP2W1 as tumor antigen in immunotherapy or, if CYP2W1 expression is beneficial for tumor progression, inhibition of CYP2W1 expression or CYP2W1 activity.

In our lab we are now developing new antibodies hopefully suitable for immunohistochemical detection of CYP2W1. The information that could be obtained using this approach would be valuable both for screening larger number of tumors, but also for giving information regarding what cells that express the enzyme and to what extent. However, more importantly, if considering a cancer therapy approach it is important to identify and select the target group. Immunohistochemical detection of CYP2W1 may then be one way to find those individuals that could benefit from a potential CYP2W1-based cancer therapy.

Also CYP2U1 could be interesting in the aspect of drug targeting, e.g. drug targeting to the brain. The drug supply to the brain is, as commonly known, restricted due to the BBB. If compounds/drugs being activated by CYP2U1 can be found and activation directly in the brain could be advantageous, this might be an opportunity. This requires, however, that CYP2U1 is expressed in the brain structures or cells of interest. Another opportunity for CYP2U1 as drug target would be in ovarian or colorectal tumors were CYP2U1 was shown to be overexpressed in primary tumors [54, 92]. Interestingly, the expression level of CYP2U1 was higher, although not significant, also in metastatic colorectal tumors [54], a characteristic that can be of advantage in a cancer therapy approach. However, just like for CYP2W1 it remains to elucidate what role CYP2U1 plays in tumors and how this could be utilized in the best way.
6 CONCLUSIONS

The results of the current thesis can be summarized as follows:

6.1 CYP2U1

The full length CYP2U1 ORF was identified and verified by sequencing. In addition, also a CYP2U1 splice variant lacking the second exon was found.

Comparison with other P450s showed that the CYP2U1 amino acid sequenced had stretches of additional amino acids in the N-terminal region.

The CYP2U1 mRNA was shown to be highly expressed in fetal and adult human thymus, with other extrahepatic tissues showing lower levels of expression.

The CYP2U1 protein from rat was shown to be highly expressed especially in brain but also in thymus. The brain region having highest expression levels of CYP2U1 were the limbic structures and cortex.

The human CYP2U1 was expressed in HEK293 cells and a reduced CO difference spectrum could be obtained from the microsomal fractions indicating a properly folded and functional CYP2U1 enzyme.

6.2 CYP2W1

The full length ORF for CYP2W1 were identified and verified by sequencing. Also a second presumably non-functional CYP2W1 transcript was found, lacking the fifth heme iron ligand.

CYP2W1 expressed in HEK293 cells was properly folded, as determined using reduced CO difference spectra, and was catalytically active towards AA.

Analysis of the CYP2W1 mRNA expression pattern showed that CYP2W1 is only expressed at low levels in normal fetal and adult tissues. Higher expression levels, with expression increasing by fetal age, were however seen in rat fetal colon.

CYP2W1 mRNA and/or protein were shown to be highly expressed in human tumors originating from the adrenals, colon and lung, with especially colon tumors showing high CYP2W1 levels.
Colon tumors/colon cancer patients was shown to be diverged in the respect of CYP2W1 overexpression, with approximately 50% showing much higher CYP2W1 expression levels in tumors compared to normal tissue.

Two CpG island was found in the proximal part of the CYP2W1 gene, and demethylation of one of these CpG islands was found to be a prerequisite for CYP2W1 expression, both in cell lines and in tissue samples.
7 FURTHER PERSPECTIVES

As mentioned in the introduction colon cancer is one of the most common forms of cancer today, with approximately 1 million new cases and half a million death annually worldwide [143]. The future prospects are not encouraging with more and more people adapting to the western lifestyle and thereby also being exposed to a higher risk of developing colon cancer. The chemotherapy approaches commonly used today are limited and even if new treatments are under development there is still a potential for new approaches, like a CYP2W1-based cancer therapy.

Besides colon cancer there is also a possibility that a CYP2W1-based cancer therapy can be applied also for other gastrointestinal cancers, since CYP2W1 has been show to be overexpressed in gastric cancer [168], and maybe also for e.g. lung and adrenal gland tumors. If expression in rat fetal tissues can be extrapolated to human cancers there is also a possibility for CYP2W1 being expressed in brain tumors, since a transient expression was observed in fetal rat brain.

However, the knowledge of e.g. CYP2W1 expression, regulation and substrate specificity has to be extended before the full potential of a CYP2W1 cancer therapy can be examined. As evident in paper V, tumors/patients can be divided in two subgroups regarding CYP2W1 expression. For estimation of the potential of a CYP2W1 cancer therapy it is important to know at what frequency CYP2W1 is overexpressed in tumors, but based on the small numbers of colon tumors so far analyzed a CYP2W1 therapy approach might be useful in about half of the cases. In addition to colon cancer also gastric cancers, adrenal cancers etc. may further increase the potential. Further insight into CYP2W1 gene regulation might also offer the ability to upregulate the CYP2W1 expression in tumors further in both subgroups, which might be an advantage based on what type of therapy approach that will be suitable.

One of the possibilities for a CYP2W1 based cancer therapy approach is tumor specific activation of a CYP2W1-specific prodrug. The most obvious limitation for such an approach so far is the limited knowledge regarding CYP2W1 substrate specificity and the activity of the constitutively expressed enzyme. For a prodrug approach to be successful the tumor-expressed enzyme has to be active, be expressed at sufficient amount and in sufficient numbers of cells (even if a bystander effect can be possible depending on the nature of the prodrug/active metabolite). In addition also a suitable chemical structure has to be found as substrate/prodrug. Besides the prodrug approach also other strategies can be considered, as evident when comparing to the strategies being developed for CYP1B1, like inhibition of expression/activity or immunotherapy.
At this early time point this is of course merely speculations, however, it will be interesting to see, as more results regarding CYP2W1 will be obtained, what the future will tell regarding the potential of CYP2W1 in cancer therapy.
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9 REFERENCES

8. Estabrook RW. A passion for P450s (rememberances of the early history of research on cytochrome P450). Drug Metab Dispos 2003;31:1461-1473
19. Ingelman-Sundberg M. Genetic susceptibility to adverse effects of drugs and environmental toxicants. The role of the CYP family of enzymes. Mutat Res 2001;482:11-19

44. Rylander T, Neve EP, Ingelman-Sundberg M, and Oscarson M. Identification and tissue distribution of the novel human cytochrome P450 2S1 (CYP2S1). *Biochem Biophys Res Commun* 2001;281:529-535


52. Roediger WE, and Babidge W. Human colonocyte detoxification. *Gut* 1997;41:731-734


77. Hedlund E, Gustafsson JA, and Warner M. Cytochrome P450 in the brain; a review. *Curr Drug Metab* 2001;2:245-263


81. Shimada T, Sugie A, Shindo M, Nakajima T, Azuma E, Hashimoto M, and Inoue K. Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered

82. Lai ZW, Hundeiker C, Gleichmann E, and Esser C. Cytokine gene expression during ontogeny in murine thymus on activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol Pharmacol* 1997;52:30-37


98. Stoilov I, Akarsu AN, and Sarfarazi M. Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum Mol Genet* 1997;6:641-647


121. van Schaik RH. Cancer treatment and pharmacogenetics of cytochrome P450 enzymes. *Invest New Drugs* 2005;23:513-522


159. Japanese SNP Database. [http://snp.ims.u-tokyo.ac.jp](http://snp.ims.u-tokyo.ac.jp)


165. Nelson DR, Zeldin DC, Hoffman SMG, Maltais LJ, Wain HM, and Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* 2004;14:1-18


