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Jenni Küblbeck

Use of Xenosensors for Drug Metabolism Studies

Focus on Constitutive Androstane Receptor

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JENNI KÜBLBECK

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ABSTRACT

The inducible expression of different enzymes and transporters involved in the endo- and xenobiotic metabolism and transport is mainly regulated by three xenosensors: Two nuclear receptors (NRs), constitutive androstane receptor (CAR) and pregnane X receptor (PXR), and the bHLH/PAS-family transcription factor aryl hydrocarbon receptor (AhR). These xenosensors share many ligands, target genes and interaction partners, which complicates the regulation of endo- and xenobiotic metabolism. Thus, obtaining clarification on the structure, ligand selectivity and function of the receptors is important for drug development. Species differences in ligand recognition of the xenosensors complicate the use of animal models for metabolism studies. In addition, most of the available assays lack important proteins present in the liver and thus, the results from *in vitro* studies do not necessarily predict the results obtained *in vivo*. Studies on human CAR have been especially limited due to the unique properties of the receptor, such as the high constitutive activity, and the lack of reliable and reproducible assays.

Hepatoma cells are commonly used in drug metabolism studies despite the poor expression of the key metabolic enzymes, cytochrome P450s (CYPs). In this study, chimeric constructs of human CAR and PXR were created by adding the activation domain of NFκB to the full length receptors. These chimeric receptors are constitutively active and exhibit strong target gene activation. The first aim was to study the effect of these chimeric receptors on CYP expression in hepatoma cells. The receptors were shown to up-regulate the expression of CYP2B6, 2C9 and 3A4 in both transiently and stably transfected cells. The generated stable cell lines provide an interesting starting point for further development of hepatoma cell lines to be used in drug metabolism studies.

The second aim was to develop cell-based xenosensor assays which could complement other assays to find novel ligands and to study the properties and function of these receptors. Reporter assays for the three xenosensors were validated and used to screen various xenosensor ligands and CYP inducers. Novel agonists and inverse agonists, selective for human CAR over PXR and AhR, were identified. The compounds discovered were further characterized by different *in vitro* assays as well as with molecular modelling techniques. In particular, the agonist FL81 and inverse agonist S07662 can be used as reference compounds in different assays for human CAR. They could also serve as lead compounds to develop better CAR ligands for research and therapeutic purposes.

National Library of Medicine Classification: QU 120, QV 36, QV 37.5, QV 38

Medical Subject Headings: Receptors, Cytoplasmic and Nuclear; Receptors, Steroid; Xenobiotics; Metabolism; Drug Agonism; Drug Inverse Agonism; Cytochrome P-450 Enzyme System; Ligands; Gene Expression Regulation; Biological Assays; Models, Molecular; Cells, Cultured

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TIIVISTELMÄ

Kaksi tumareseptoria, konstitutiivinen androstaanireseptori (CAR) ja pregnaani X reseptori (PXR), sekä bHLH/PAS-perheeseen kuuluva aryylihiilivetyreseptori (AhR) säätelevät elimistön omien sekä vierasaineiden indusoituvaa metaboliaa ja kuljetusta. Näillä vierasainesensoreilla on yhteisiä ligandeja ja kohdegeenejä sekä useita erilaisia vuorovaikutuksia muiden proteiinien kanssa, minkä vuoksi elimistön omien sekä vierasaineiden metabolian säätely on monimutkaista. Vierasainesensorien rakenteen, ligandiselektiivisyyden ja toiminnan selvittäminen on näin ollen tärkeää lääkekehityksen kannalta. Eläinmallien käyttö metabolian tutkimisessa on haasteellista, sillä vierasainesensoreiden ligandispesifisyydessä on suuria lajienvälisiä eroja. *In vitro* menetelmillä saadut tulokset eivät useinkaan korreloi *in vivo* tulosten kanssa, koska käytetyissä menetelmissä ei ole mukana kaikkia normaalisti maksassa ilmentyviä tärkeitä tekijöitä. Erityisesti ihmisen CAR:n tutkimista ovat rajoittaneet reseptorin ominaisuudet, kuten konstitutiivinen aktiivisuus, ja luotettavien ja toistettavien mittausmenetelmien puute.

Maksan syöpäkudoksesta (hepatooma) eristettyjä soluja käytetään yleisesti lääkeaineenvaihdunnan tutkimisessa, mutta ne ilmentävät huonosti tärkeitä sytokromi P450 (CYP) -entsyymejä. Tässä työssä kehitettiin kimeerisiä ihmisen CAR ja PXR tumareseptoreita lisäämällä niihin NFκB-proteiinin aktivaatioalue. Nämä kimeeriset reseptorit ovat konstitutiivisesti aktiivisia ja aktivoivat voimakkaasti reseptorien kohdegeenejä. Työn tavoitteena oli tutkia näiden kimeeristen reseptorien vaikutusta CYP-entsyymien ilmentymiseen hepatoomasoluissa. Kimeeristen reseptorien osoitettiin lisäävän CYP2B6-, CYP2C9- ja CYP3A4-entsyymien ilmentymistä sekä transientisti että stabiilisti transfektoiduissa soluissa. Kehitettyjä stabiileja solumalleja voidaan käyttää apuna kehitettäessä uusia malleja lääkeainemetabolian tutkimiseen.

Työn toisena tavoitteena oli optimoida solupohjainen aktivaatiomääritys vierasainesensoreille. Näitä uusia määritysmenetelmiä voidaan käyttää yhdessä muiden menetelmien kanssa etsittäessä uusia ligandeja sekä tutkittaessa reseptorien ominaisuuksia ja toimintaa. Validoituja vierasainesensorien aktivaatiomäärityksiä käytettiin seulottaessa erilaisia vierasainesensoreiden ligandeja ja CYP-entsyymien indusoreja. Löydettyjen yhdisteiden ominaisuuksia tutkittiin tarkemmin erilaisilla *in vitro* -menetelmillä sekä molekyylimallituksen keinoin. Uutta selektiivistä ihmisen CAR tumareseptorin agonistia FL81 ja käänteisagonistia S07662 voidaan käyttää referenssiyhdisteinä erilaisissa ihmisen CAR:n tutkimusmenetelmissä. Löydettyjen ligandien avulla voidaan mahdollisesti myös kehittää uusia parempia ihmisen CAR:n ligandeja tutkimukseen ja terapeuttisiin tarkoituksiin.

Luokitus: QU 120, QV 36, QV 37.5, QV 38
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Still round the corner there may wait
A new road or a secret gate

JRR Tolkien

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Kuopio, July 2012

Jenni Küblbeck

List of the original publications

This dissertation is based on the following original publications, referred in the text by Roman numerals **I-IV**.

I Küblbeck J, Jyrkkärinne J, Poso A, Turpeinen M, Sippl W, Honkakoski P, Windshügel B. Discovery of substituted sulfonamides and thiazolidin-4-one derivatives as agonists of human constitutive androstane receptor. *Biochem Pharmacol 76(10): 1288-97, 2008.*

II Küblbeck J, Reinisalo M, Mustonen R, Honkakoski P. Up-regulation of CYP expression in hepatoma cells stably transfected by chimeric nuclear receptors. *Eur J Pharm Sci 40(4): 263-272, 2010.*

III Küblbeck J, Laitinen T, Jyrkkärinne J, Rousu T, Tolonen A, Abel T, Kortelainen T, Uusitalo J, Korjamo T, Honkakoski P, Molnár F. Use of comprehensive screening methods to detect selective human CAR activators. *Biochem Pharmacol 82(12): 1994-2007, 2011.*

IV Küblbeck J, Jyrkkärinne J, Molnár F, Kuningas T, Patel J, Windhügel B, Nevalainen T, Laitinen T, Poso A, Honkakoski P. New in vitro tools to study human constitutive androstane receptor (CAR) biology: discovery and comparison of human CAR inverse agonists. *Mol Pharm 8(6): 2424-2433, 2011.*

The publications were adapted with the permission of the copyright owners. Some unpublished results are also presented.

Contents

Abbreviations

AD	Activation domain
ADME	Absorption, distribution, metabolism and excretion
ADR	Adverse drug reaction
AF-1	Activation function-1
AF-2	Activation function-2
AhR	Aryl hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
ARNT	Aryl hydrocarbon receptor translocator
BCRP	Breast cancer resistance protein
bHLH	Basic helix loop helix
C3A	Hepatoma cell line, clonal derivative of HepG2
CAR	Constitutive androstane receptor
CCRP	CAR cytoplasmic retention protein
C/EBPα	CCAAT/enhancer-binding protein-α
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichloro-benzyl)oxime
CYP	Cytochrome P450
DBD	DNA binding domain
DBP	Albumin D site binding protein
DM	Drug metabolism
DME	Drug metabolizing enzyme
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DT	Drug transporter
DR-n	Direct hexamer repeat (spacer of n nucleotides)
EE2	17α-ethinylestradiol
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ER-n	Everted hexamer repeat (spacer of n nucleotides)
FL81	5-(3,4-dimethoxy-benzyl)-3-phenyl-4,5-dihydro-isoxazole
Fox	Forkhead box protein
GAL4	Yeast transcription activator protein
GRIP	Glucocorticoid receptor interacting protein
GST	Glutathione S-transferase
HLM	Human liver microsome
HNF	Hepatocyte nuclear factor
HPH	Human primary hepatocyte
HTS	High throughput screening
IR-n	Inverted hexamer repeat (spacer of n nucleotides)
LBD	Ligand binding domain
LBP	Ligand binding pocket
LC/MS	Liquid chromatography/mass spectrometry
LUC	Luciferase
MD	Molecular dynamics
MDR	Multidrug resistance protein
MRP	Multidrug resistance associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NAT	N-acetyl transferase
NCoR	Nuclear receptor corepressor
NFκB	Nuclear factor κB
NES	Nuclear export signal
NLS	Nuclear localization signal
NQ	NAD(P)H dehydrogenase, quinone 1
NR	Nuclear receptor
NRIP	Nuclear receptor interaction protein
OME	Omeprazole
OATP	Organic anion transporting polypeptide
PAS	Per-Arnt-Sim
PB	Phenobarbital
PBREM	Phenobarbital response element module
PDB	Protein data bank
PGC	Peroxisome proliferator-activated receptor-γ coactivator
Pgp	P-glycoprotein
PHN	Phenytoin
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
RE	Response element
RIF	Rifampicin
RMSD	Root mean square deviation
RXR	Retinoid X receptor
SMRT	Silencing mediator of thyroid hormone receptor
SNP	Single nucleotide polymorphism
S07662	1-[(2-methylbenzo furan-3-yl)methyl]-3-(thiophen-2-ylmethyl)urea
SRC	Steroid hormone receptor coactivator
SULT	Sulfotransferase
UAS	Upstream activation sequence
UGT	Uridine 5′-diphospho-glucuronosyl-transferase
TAD	Transactivation domain
TF	Transcription factor
VDR	Vitamin D receptor
wt	Wild type
XRE(M)	Xenobiotic response element (module)

1 Introduction

Living organisms are exposed daily to harmful endogenous substances and many xenobiotics, including drugs, carcinogens, environmental chemicals and pollutants. Biotransformation reactions are important in the elimination of these foreign and undesirable compounds from the body. The liver, in addition to its synthetic functions, is the most important xenobiotic metabolizing organ and its multiple functions are carried out by hepatocytes, which contain a variety of different enzymes and transporters. The cytochrome P450 (CYP) superfamily consists of a large and diverse group of enzymes catalyzing the oxidation of endo- and exogenous substances. These are the main enzymes involved in drug metabolism and their inhibition or induction can have drastic effects on the pharmacokinetics of drug molecules, often resulting in therapeutic failures or adverse effects.

The main regulators of xenobiotic metabolism are the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). These three so-called xenosensors are ligand-dependent transcription factors (TFs), which control the inducible expression of their target genes together with coregulator and other accessory proteins. A large and diverse set of endo- and exogenous ligands can bind to one or several of these receptors and result in the enhancement or repression of target gene expression. Certain features of CAR, such as the constitutive activity, distinguish it from other nuclear receptors. Xenosensors are also involved in various physiological functions, such as cell cycle regulation, gluconeogenesis, immune response and the metabolism of fatty acids, cholesterol, bilirubin and bile acids. Many of these functions result from interactions with other TFs, a process called cross-regulation. Due to these various interaction possibilities, the regulation of both xenosensor and target gene function is very complex and remains largely unclear. Studies on the xenosensor function and ligands would be important not only for drug development, but also for therapeutic uses and novel ligands could provide new drug candidates for the treatment of serious metabolic disorders.

Several different methods have been developed to study these processes both to understand the complex network of different proteins and signaling cascades as well as to clarify the metabolic properties of new chemical entities in the early phases of drug development. Since animal studies are unreliable due to species specific differences, the focus has been on the development of *in vitro* methods using human cells or proteins. These methods include cell-based methods using primary cells and continuous cell lines, cell-free metabolic and ligand binding assays as well as computational approaches. An ideal model for xenobiotic metabolism studies would contain all of the functional proteins involved in the metabolic processes. However, all of the currently available assays have problems and limitations and a single assay does not provide information on all the factors and processes involved in the biotransformation of a compound.

2 Review of the literature

2.1 XENOBIOTIC METABOLISM

Xenobiotics, such as drugs, poisons and environmental chemicals, are processed and eliminated in the body via various chemical reactions mediated by different enzymes and transporter proteins. The metabolism of xenobiotics occurs in many tissues (*e.g.* intestine, lung, kidney and skin), but the primary site of these reactions is the liver. The main goal of xenobiotic metabolism is to increase the polarity of the mostly lipophilic xenobiotics and thus, make them more easily excreted. However, these reactions can also activate the xenobiotics into more reactive and toxic metabolites. The metabolic reactions have commonly been divided into three Phases (I-III) but this categorization is slightly artificial because it tells nothing about the order in which these reactions occur. In addition, the term Phase 0 is sometimes used to describe the uptake of compounds into the cell.

2.1.1 Phase I: Functionalization reactions

Phase I reactions introduce or expose certain functional groups on the metabolized compound. The reactions can be roughly divided into three different reaction types: Oxidation, reduction and hydrolysis reactions. Within cells, most Phase I enzymes are located in the endoplasmic reticulum (ER). The hepatic CYP system is the most important oxidation system and approximately 75-80% of all Phase I reactions are carried out by these enzymes (Nebert & Russell 2002, Zuber et al. 2002).

Cytochrome P450 enzymes (CYPs). CYP enzymes are heme-containing mono-oxygenases involved in the oxidative biotransformation of numerous lipid-soluble xenobiotics, such as drugs, solvents, pesticides and plant products, but they also participate in the metabolism and synthesis of various endobiotic substances, such as steroid hormones, prostaglandins as well as bile and fatty acids (Nebert & Russell 2002, Ioannides & Lewis 2004, Denisov et al. 2005, Wienkers & Heath 2005). CYPs are found in all tissues of the body but the highest concentrations are present in the liver and intestine. In humans, the enzymes are divided into different families (18) and subfamilies (44) based on sequence similarity. Altogether 57 CYP genes and 58 pseudogenes have been found (Nelson et al. 2004, Guengerich 2007). Despite the relatively low sequence similarities between different CYP families, they all have a similar and relatively well-conserved structure (CYP-fold), consisting of certain secondary structural elements (12 α-helices (A-L) and 4 antiparallel β-sheets), roughly divided into two domains (***Figure 1A.***) (Guengerich 2001, Raucy & Allen 2001, Johnson & Stout 2005, Sirim et al. 2010). The structure is generally very compact but the precise positioning of the structural elements varies considerably between different CYPs. The most structurally conserved regions are involved in the heme-binding (Cys pocket), whereas the most diverse part is the substrate-binding region. Even minor changes in the amino acid residues in this area have significant effects on substrate binding. The helices F and G, together with F/G and B/C loops, control the access of substrate to the active site, and their position, as well as the volume of the pocket, differs between individual CYPs mirroring the size or dimensions of selective substrates (Williams et al. 2004, Yano et al. 2004, Lewis et al. 2006, Rowland et al. 2006). The heme moiety is essential for the enzyme

activity and the loss of this group, for example due to inhibitor binding, results in the loss of enzymatic activity.

CYPs are primarily membrane-associated proteins, located mainly in the ER but also in the inner membrane of the mitochondria and on the cell surface (Neve & Ingelman-Sundberg 2010). The amino terminus of the protein anchors the enzyme to the membrane and in most cases, the active site is exposed on the cytoplasmic side of the ER (Black et al. 1994). CYPs function as terminal oxidases of an electron transport chain and catalyse a variety of monooxygenase reactions, including epoxidation, N-, O-and S-dealkylation, deamination, dehalogenation, N-, P- and S-oxidation as well as aromatic and aliphatic hydroxylation, depending on the structure of the substrate (Guengerich 2007, Isin & Guengerich 2007). The basic reaction is the addition of one oxygen atom to the substrate, while the other atom is reduced to water (***Figure 1B.***). An essential component for CYP activity is an accessory nicotinamide adenine dinucleotide phosphate (NADPH) -dependent flavoprotein, which forms a temporary complex with CYP and permits the flow of electrons to the heme prosthetic group, enabling the oxidation of the substrate (Laursen et al. 2011).

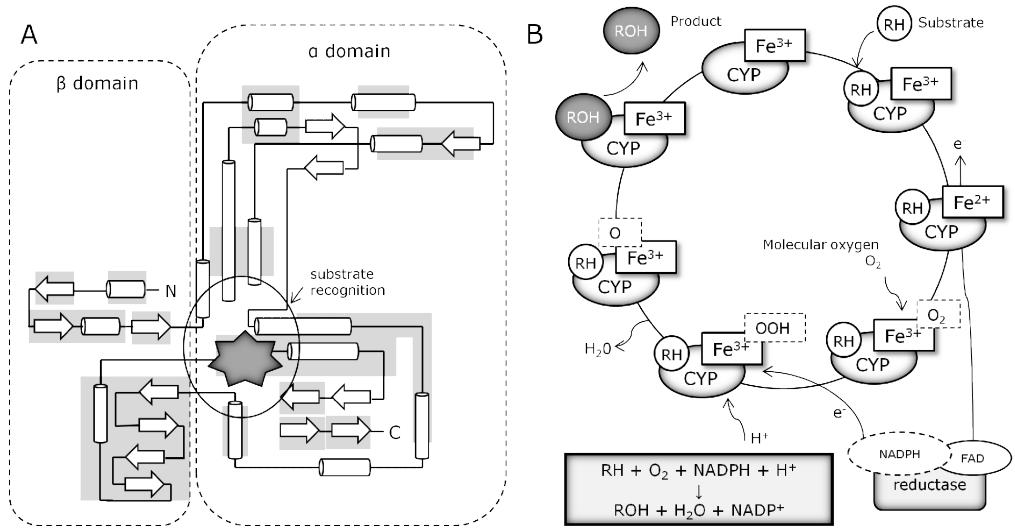

Figure 1. Structure and function of CYP enzymes. **A)** A schematic structure of a CYP enzyme (CYP-fold) (adapted from Raucy & Allen 2001 and Sirim et al. 2010). The tubes represent α-helices, the arrows β-sheets and the grey star heme. The approximate locations of structurally conserved regions are marked by grey squares. The lengths and sizes of the elements are not proportional to their actual sizes. **B)** The catalytic cycle and basic reaction. In short, after the substrate binds the active site of the enzyme and displaces the bound water molecules, an electron is added by an accessory flavoprotein NADPH-P450-reductase and the iron atom is reduced from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) state. The binding of the substrate also induces conformational changes in the active site leading to altered spectral properties. In the following steps, a molecular oxygen binds the enzyme and the adduct is reduced by a second electron transfer. Further, the resulting group is protonated, leading to the release of a water molecule and to a reactive intermediate, which removes the hydrogen atom from the substrate and leads to the generation of the product and its dissociation from the enzyme (adapted from Meunier et al. 2004).

Approximately one fourth of all CYP enzymes, mainly members of families 1-3, are involved in xenobiotic metabolism. Unlike the CYPs responsible for the metabolism of endogenous compounds, these enzymes have broader and overlapping substrate specificities (Guengerich et al. 2005). It is estimated that over 90% of all drugs are

metabolized, at least partially, by different CYPs, the hepatic enzymes being the most important ones (***Table 1.***) (Wienkers & Heath 2005, Pelkonen et al. 2008).

CYP1A2 is mainly located in the liver and is involved in the metabolism of planar aromatic molecules, such as estrogens, caffeine and melatonin, as well as in the bioactivation of polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines, converting them into toxic or carcinogenic metabolites (Wang & Zhou 2009, Zhou et al. 2010). The diverse CYP2 family is the largest single group of CYPs in humans and contains many important drug metabolizing enzymes. In terms of enzyme amount, CYP2A6 is a minor form in the liver. It is best known for the coumarin 7-hydroxylation reaction but is also involved in the metabolism of various small and planar molecules, such as nicotine, and in the activation of several procarcinogens (Honkakoski and Negishi 1997, Pelkonen et al. 2000, Hukkanen et al. 2005, Di et al. 2009). CYP2B6 has been overlooked perhaps due to its highly variable expression between individuals and the lack of good model substrates, inhibitors and antibodies (Wang & Tompkins 2008). It usually metabolizes non-planar, neutral or weakly basic and highly lipophilic molecules but it also both bioactivates and detoxifies some precarcinogens (Mo et al. 2009). The CYP2C family consists of four members (2C8, 2C9, 2C18 and 2C19), together involved in the metabolism of approximately one-fourth of drugs on the market. CYP2C9 is the most well known isoform; it is responsible for the metabolism of relatively small, acidic and lipophilic molecules forming hydrogen bonds, such as various clinically significant drugs, including warfarin and ibuprofen (Rettie & Jones 2005, Zhou et al. 2009a). In addition to liver, the highly polymorphic CYP2D6 enzyme is expressed in many other tissues, and is responsible for the metabolism of numerous CNS and cardiovascular drugs and some endogenous substrates mainly via N-dealkylation reactions (Wang et al. 2009, Zhou et al. 2009b). In contrast to other xenobiotic metabolizing CYPs, CYP2D6 has not been reported to be regulated by any inducers (Ingelman-Sundberg 2005). Despite its high hepatic expression, CYP2E1 does not play a significant part in drug metabolism but has a notable role in toxicology, due to its role in the metabolism of many small molecules (*e.g.* ethanol), the production of reactive oxygen species, the bioactivation of industrial solvents, the activation of chemical carcinogens and acetaminophen-related hepatotoxicity (Caro & Cederbaum 2004, Lu & Cederbaum 2008, Trafalis et al. 2010).

The most well known and clinically important CYP subfamily is the CYP3A family, composing of four functional proteins (3A4, 3A5, 3A7 and 3A43) with overlapping catalytic specificities (deWildt et al. 1999, Burk & Wojnowski 2004, Daly 2006). The CYP3A4 isoform is the main enzyme in the liver. It participates in the metabolism of various drugs and it is also involved in the oxidation of endogenous substrates, such as bile acids, and in the bioactivation of procarcinogens due to its large and flexible active site and a relatively low degree of substrate specificity (Li et al. 1995, Zhou 2008). It is also susceptible to inhibition and is highly inducible by a large number of substances. The other CYP3A family members are minor isoforms in the liver (Daly 2006). The substrate and inhibitor specificities as well as inducibility of CYP3A5 are very similar to those of CYP3A4 but it is only expressed in 20% of livers mainly due to a splice site mutation. CYP3A7 is predominantly a fetal enzyme and it has an important role in normal embryonic development and carcinogenesis (Sim et al. 2005, Leeder et al. 2005). In the adult liver, it is a minor enzyme form and its role in xenobiotic metabolism is unclear. The CYP3A43 is the most recently discovered isoform, which is expressed at low levels in the liver and exhibits a low level of testosterone hydroxylase activity. Its substrate specificity and involvement in xenobiotic metabolism are not known (Westlind et al. 2001, Daly 2006).

The *in vivo* CYP-mediated metabolism is dependent on the relative amount of different CYPs in the liver. The expression of most xenobiotic-metabolizing CYPs differs between individuals and populations due to different genetic and environmental factors. Furthermore, the regulation of the CYP expression by microRNAs (miRNAs) or epigenetic mechanisms (***Section 2.2.***) has been proposed to resolve the so far unexplained interindividual variation (Ingelman-Sundberg et al. 2007). The genetic factors behind the inter-individual variation are mainly due to the different polymorphisms of CYPs, resulting in differences in both expression and function of the enzymes (***Table 1.***) (Zhou et al. 2009c). There can be many reasons for the functional CYP polymorphisms, such as gene duplications, amino acid changes, copy number variation as well as deletions and mutations creating inactive gene products (Ingelman-Sundberg et al. 2007). Clinically significant variations in drug metabolism are mainly caused by the polymorphisms of CYP2C9, 2C19, 2D6 and 3A5. The phenotype differences can sometimes also have beneficial effects for drug therapy. For example, the CYP2C19 phenotype affects the pharmacokinetics of proton pump inhibitors, which can lead to increased responsiveness in poor metabolizers to treatment of different gastrointestinal disorders (Klotz 2006, Kawamura et al. 2007).

Other Phase I enzymes. In addition to CYPs, also other enzymes are involved in Phase I reactions. NAD+ -dependent alcohol and aldehyde dehydrogenases are a group of isozymes mainly catalyzing the oxidation of alcohols usually to more reactive aldehydes or ketones and the oxidation of aldehydes to carboxylic acids, respectively (Vasiliou et al. 2004, Edenberg 2007, Garattini et al. 2008). Flavin monooxygenases are a family of five tissue-specific isozymes with relatively restricted substrate specificities, which catalyze chemical reactions via the bound flavin cofactor (Cashman 2000). They are NADPH and oxygen -dependent and mainly oxidize compounds with nucleophilic N, S or P atoms. Monoamine oxidases are mitochondrial flavoproteins, which catalyze the oxidative deamination of diverse amines (Edmondson et al. 2004). Epoxide hydrolases add water molecules to the epoxides, which are often formed in Phase I metabolic reactions and in the degradation of aromatic compounds, thus converting them to dihydrodiols which can be futher conjugated (Seidegård & Ekström 1997). The majority of xenobiotic epoxides are hydrolyzed by two enzymes, the microsomal epoxide hydrolase and the soluble epoxide hydrolase (Arand et al. 2003). In general, the hydration of epoxides leads to more stable and less reactive products. Finally, the esterases and amidases catalyze the addition of water molecules to esters or amides (Junge & Krisch 1975).

2.1.2 Phase II: Conjugation reactions

In Phase II reactions, hydrophilic moieties are added to the compound by conjugative enzymes. Most of the compounds have already been subjected to Phase I reactions but certain compounds with appropriate groups can be directly conjugated. The most important conjugation enzymes can be roughly divided into uridine 5'-diphospho-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyl-transferases (NATs) and glutathione S-transferases (GSTs) (Zamek-Gliszczynski et al. 2006). The UGTs are localized in the ER and catalyze the conjugation of a glucuronic acid to a substrate molecule (glucuronidation) (Tukey & Strassburg 2000, Guillemette 2003). These enzymes are responsible for the metabolism of at least 35% of drugs metabolized by Phase II enzymes. They are also involved in the metabolism of other xenobiotics as well as many endogenous compounds, *e.g.* bilirubin and steroid hormones. SULTs are cytosolic and weakly inducible enzymes with an overlapping substrate spectrum with UGTs (Gamage et al. 2006, Zamek-Gliszczynski et al. 2006). Typically, the sulfation reactions predominate at low substrate concentrations, whereas glucuronidation takes place at high substrate concentrations.

Although the metabolites from these reactions usually are less toxic than their parent compounds, occassionally they also produce active and toxic metabolites (Zamek-Gliszczynski et al. 2006). NATs are responsible for the acetylation of arylamine drugs, such as isoniazid (Cascorbi 2006, Sim et al. 2008). Glutathione conjugation reactions, catalyzed by the membrane-bound isoforms of GSTs, are important in drug biotransformation, especially in the metabolism of electrophiles (Zamek-Gliszczynski et al. 2006). After conjugation, the highly hydrophilic products are usually excreted in the bile or urine by a transporter protein (TP).

2.1.3 Phase III: Other modification reactions and excretion

The term Phase III is commonly used to describe the reactions and proteins involved in the transport of the drug metabolites across different membranes. However, it can also be used to describe the further processing of the conjugates from Phase II reactions (*e.g.* Delaforge et al. 2005).

Although many compounds permeate cell membranes passively, two major groups of TPs, ATP binding cassette (ABC) transporters and solute carriers (SLC), are involved in the disposition of xenobiotic compounds and their conjugates (Giacomini et al. 2010). ABC transporters are the most important efflux pump proteins, which derive energy from ATP hydrolysis in order to transport compounds out of cells independently of the substrate concentration gradient (Chang 2003, Linton & Higgins 2007). The main proteins involved in the transport of xenobiotics are P-glycoprotein or multidrug resistance protein 1 (P-gp, MDR1), multidrug resistance associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) (Chan et al. 2004). The SLCs form a large family of transporters containing proteins (*e.g.* organic anion transporting polypeptides, OATPs) which transport their substrates along the concentration gradient or as secondary active transporters and thus, do not require ATP (Steffansen et al. 2004, Schlessinger et al. 2010). In contrast to the ABC transporters, SLCs are mostly responsible for hepatocellular uptake (Phase 0).

2.1.4 Inhibition, induction and adverse effects

Adverse drug reactions (ADRs) are unexpected responses to a given drug and are often caused by inter-individual differences in enzyme expression and activity, but can also be due to the absorption, distribution, metabolism and excretion (ADME) or chemical properties of the pharmaceuticals. Due to the complex regulation of signaling, drug-drug (or drug-xenobiotic) interactions can take place at any level of the ADME process but the most clinically relevant interactions occur primarily in the absorption or metabolism phase. As CYPs are usually the rate-limiting factors in the metabolic processes, they have an important role in many of these adverse effects. The activity of CYP enzymes can be modulated by external factors via enzyme inhibition, leading to reduced activity, or induction, which can be defined as an adaptive increase in the expression of the enzyme. Due to the impact of CYP inhibition and induction in drug therapy, novel drug candidates are routinely tested for their potential to cause inhibition or induction in the early phases of drug discovery (***Table 1.*** shows the most commonly used probe substrates, inducers and inhibitors).

Inhibition. The most common cause for adverse effects is the inhibition of CYP enzymes, possibly leading to toxic effects or reduced efficacy of a drug (prodrugs) (Bjornsson et al. 2003, Pelkonen et al. 2008). CYP inhibition can occur at various stages of the catalytic cycle and can be roughly divided into two main types, reversible and mechanism-based (irreversible) inhibition (Hollenberg 2002, Ortiz de Montellano & Correia 2005, Pelkonen et al. 2008). Reversible inhibition is more common, for example occurring as direct competition between the substrate and the inhibitor for a binding site. Mechanism-based

inhibition requires biotransformation of the inhibitor and occurs either by a reactive metabolite binding covalently to the enzyme or by formation of metabolite intermediate complexes and is only terminated by re-synthesis of the enzyme (Ortiz de Montellano & Correia 2005). The effects of irreversible inhibition in general are considered to be more serious due to the longer duration as well as the formation of covalent bonds between the enzyme and inhibitor. This may lead to an autoimmune response triggered by hapten formation, *e.g.* the CYP2C9 inhibitor tielinic acid causing fulminant hepatic failure (Lecoeur et al. 1996). The hydrophobic interactions with the enzyme also play a role in the inhibition process as can be seen with ketoconazole which, compared to cimetidine, is a more potent inhibitor of CYP3A4 (Martinez et al. 1999, McGinnity & Riley 2001). Different CYP isoforms show some degree of inhibitor selectivity. In terms of metabolism, CYP3A4 is the most prevalent and important isoform and is thus often involved in many clinically significant ADRs. Examples of CYP3A4 inhibitors include some antifungals (*e.g.* ketoconazole, Greenblatt et al. 2010), macrolide antibiotics (*e.g.* erythromycin, Zhang et al. 2009), protease inhibitors (*e.g.* ritonavir, Sevrioukova & Poulos 2010) and certain dietary compounds (*e.g.* bergamottin from grapefruit juice, Lin et al. 2012). Sometimes the inhibition potential of a compound can be a useful property in drug therapy. One such example is the human immunodeficiency virus (HIV) protease inhibitor ritonavir which enhances the action of other peptidomimetic HIV protease inhibitors by inhibiting their CYP3A4-mediated metabolism (Kempf et al. 1997, Zeldin & Pertruscke 2004). The inhibition of conjugation enzymes (mainly UGTs) and drug transporters (*e.g.* P-gp) can also lead to adverse effects or inadequate pharmalogical activity (Grancharov et al. 2001, Balayssac et al. 2005, Beringer & Slaughter 2005).

Induction. Induction is a protective response against foreign compounds but, in the case of drug metabolism, it can lead to inadequate therapeutic levels or toxic/ carcinogenic effects (prodrugs). For example, induction of CYP2E1 can increase the risk or severity of hepatotoxicity due to the generation of reactive oxygen species and the formation of reactive metabolites of for example ethanol and acetaminophen (Jaeschke et al. 2002, Liu et al. 2005). CYP induction is a slow regulatory process and usually occurs *in vivo* during continuous use of the inducing compound. Most of the human hepatic CYPs are inducible (***Table 1.***) by drugs, dietary compounds and environmental factors. The extent of CYP induction has been shown to vary between individuals, both at the mRNA or protein levels as well as in terms of the levels of activity, in response to inducers (Lin & Lu 2001). Despite this variability in the inductive effect, there appears to be a "threshold" above which the activity cannot be increased (*i.e.* enzyme levels following maximal induction are quantitatively similar) (Graham & Lake 2008). In contrast to enzyme inhibitors, inducers act indirectly and do not require any physical interaction with the induced enzyme (Hollenberg 2002). In most cases, the increase of the expression of an enzyme is the result of an increase in transcription of the gene encoding the enzyme via receptor-mediated mechanisms (***Section 2.2.2***) and further, *de novo* synthesis of the protein, but can also be due to a decrease in the rate of protein degradation. The best characterized example of such an inducer is ethanol, which acts by decreasing the degradation rate of CYP2E1 protein (Roberts et al. 1995).

A typical inducer can induce a wide spectrum of CYPs (Pelkonen et al. 2002). Such well-known inducers include for example phenobarbital (CYP2A, 2B, 2C and 3A), phenytoin (CYP2C and 3A), rifampicin (CYP1A, 2A, 2B, 2C and 3A) and carbamazepine (CYP1A2, 2C19 and 3A4) (Dickins 2004, Hewitt et al. 2007). The best known example of a drug-herbal interaction involves St. John's wort (hyperforin), which induces at least CYP2C19, 2E1 and 3A4 and thus causes decreased plasma concentrations of several drugs (*e.g.* oral

Table 1. Properties and recommended in vitro substrate, inhibitors and inducers of main hepatic CYP enzymes.

	% of total / % of drugs[a]	Preferred *in vitro* probe substrates (S) and inhibitors (Inh)[b]	Preferred *in vitro* inducers[b]	Genetic variants, important alleles and their clinical effects[c]
CYP1A2	13 / 5-20	phenacetin, *theophylline, caffeine* (S) planar, small volume to surface ratio molecules, *e.g.* furafylline (Inh)	omeprazole, lansoprazole	Over 40 different alleles. CYP1A2*1F common (approx. 40% in Caucasians), others relatively rare or frequency is not known. Constitutive expression varies by 15 (mRNA) to 60 (protein) -fold between individuals. Rare effects, typically decreased expression and changes in inducibility (often in smokers)[d].
CYP2A6	1-10 / 2	coumarin, nicotine (S) tranylcypromine, *pilocarpine* (Inh)	*phenobarbital, rifampicin*	Over 80 different alleles. CYP2A6*4 (gene deletion, frequency 11-18%) accounts for the poor metabolizer phenotypes in Asians (15% vs. Caucasians 0.5%). Reduced or abolished activity, may influence smoking habits.
CYP2B6	1-10 / 2-8	buproprion, efavirenz (S) no preferred, *thiotepa, ticlopidine, clopidogrel* (Inh)	phenobarbital	Over 50 different alleles. CYP2B6*6 (frequencies of over 40% in people of African descent, up to 43% in Hispanics, up to 29% in Europeans and 17-44% in Asians) is associated with decreased activity and increased concentrations of anticancer and HIV drugs (*e.g.* efavirenz).
CYP2C8	5-7 / 1-5	paclitaxel, *amodiaquine* (S) quercetin, *rosiglitazone* (Inh)	rifampicin	Less than 20 different alleles. CYP2C8*3 (frequency of 13% in Caucasians and 2% in African-Americans) is associated with defective metabolism of some anticancer drugs (*e.g.* paclitaxel).
CYP2C9	20- 34 / 10-15	diclofenac, S-warfarin, tolbutamide (S) sulfaphenazole (Inh)	rifampicin	Over 40 different alleles. CYP2C9*2 and *3 (8-12% and 3-8% in Caucasians, lower in Asians and Africans) result in decreased metabolic activity (*e.g.* warfarin), although some substrate dependent variation is observed.
CYP2C19	7 / 5-10	S-mephenytoin (S) no preferred, *ticlopidine* (Inh)	rifampicin	Less than 40 different alleles. CYP2C19*2 and *3 account for 50% to 90% of poor metabolizer phenotypes (frequencies 2-5% in Caucasians and 18-23% in Asians).
CYP2D6	2-3 / 20-30	dextromethorphan (S) quinidine (Inh)	none identified[e]	Over 140 different alleles. Various alleles causing 4 different phenotypes: poor metabolizers (PM, 3-11% in Europeans, 1-2% in Asians and higher in Africans), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizer (UM, 39% in Ethiopians or North Africans, also high in Middle Eastern populations, 7% in Caucasians). Changes in the metabolism of a variety of drugs, *e.g.* antidepressants, opioids, antipsychotics and beta-blockers.

	% of total / % of drugs[a]	Preferred *in vitro* probe substrates (S) and inhibitors (Inh)[b]	Preferred *in vitro* inducers [b]	Genetic variants, important alleles and their clinical effects [c]
CYP2E1	7 / 2-4	chlorzocazone (S) no preferred, *clomethiazole* (Inh)	*ethanol, isoniazid*	Less than 15 different alleles. Highly conserved function. Clinical significance is unclear, although the altered function has been associated with increased risk of various cancers.
CYP3A4	28-40 / 40- over 50	testosterone, midazolam (S) ketoconazole (Inh)	rifampicin	Over 40 different alleles. Variants in coding regions occur at < 5% frequency and as heterozygous with the wild type allele. At least CYP3A4*4, *5, *6 and *18 show altered activity, but the effects are minor or moderate and the allelic frequencies are low[f].
CYP3A5 CYP3A7 CYP3A43	Minor isoforms / not known	*testosterone, midazolam (S) ketoconazole (Inh)*	*rifampicin*	Over 20 (CYP3A5) or less than 10 (CYP3A7, CYP3A43) different alleles. The expression (up to 50% of total hepatic CYP3A) and activity of CYP3A5 varies within and between populations mainly due to CYP3A5*1 (functional) and CYP3A5*3 (non-functional) alleles. The *1 allele is more common in Africans than Caucasians or Asians. Some genetic polymorphisms have been described for both CYP3A7 and CYP3A43 but their functional significance is unclear. Different genotypes may affect cancer susceptibility.

[a]The relative amounts of CYPs in liver (% of total) vary highly between individuals and literature sources (Shimada et al. 1994, Pelkonen et al. 2008, Zhou et al. 2009), [b]FDA recommended (USFDA Draft Guidance for Industry 2012, 6.4.2012), other commonly used are marked in *italics*. For a comprehensive review on substrates, inhibitors and inducers, see *e.g.* Pelkonen et al. 2008, [c]Human Cytochrome P450 (CYP) Allele Nomenclature Committee (SNPs with determined haplotypes, 12.2.2012), Lamba et al. 2002 and 2003, Mizutani 2003, Cauffiez et al. 2004, Marks et al. 2004, Scordo et al. 2004, Solus et al. 2004, Xie et al. 2004, Henningsson et al. 2005, Rodriguez-Antona et al. 2005, Roy et al. 2005, Daly 2006, Ohkubo et al. 2006, Ingelman-Sundberg et al. 2007, Kharasch et al. 2007, Nakamoto et al. 2007, Gage & Lesko 2008, Bozina et al. 2009, Zhou et al. 2009, Gao et al. 2010, Lindfelt et al. 2010, Xu et al. 2012, [d]CYP1A2 shows large interindividual variation in activity with no known polymorphic site, possibly due to the exposure to environmental chemicals (Jiang et al. 2006), [e]The increased detoxifying potential of the enzyme is due to the multiple gene copies (polymorphisms), [f]The interindividual variability in activity could be due to environmental or physiological factors and by allelic variations of factors regulating CYP3A4 expression (*e.g.* PXR) (Goodwin et al. 1999, Zhou 2008).

contraceptives and warfarin) metabolized by these enzymes (Delgoda & Westlake 2004, Borelli & Izzo 2009). In addition to the classical view of induction where one compound causes induction affecting the metabolism of another compound, some drugs *e.g.* carbamazepine and artemisinin can induce their own metabolism (autoinduction) by inducing CYP3A4 and CYP2B6, respectively (Bertilsson et al. 1980, Simonsson et al. 2003). Even though the ADRs involving the induction of transporters remain to be demonstrated, some compounds, such as artemisinin (P-gp) and rifampicin (MRP2), have been shown to increase the expression of these proteins (Payen et al. 2002, Burk et al. 2005).

2.2 REGULATION OF XENOBIOTIC METABOLISM AND TRANSPORT

The expression of a gene can be controlled at multiple levels including transcription, mRNA splicing and stability, translation as well as post-translational mechanisms such as protein stability and modifications (Day & Tuite 1998). The most important and most widely studied mechanism for hepatic CYPs is the transcriptional regulation by various TFs, such as the so-called xenosensors (AhR, CAR and PXR, ***Section 2.2.2***).

Liver-specific gene expression in adult cells is controlled by at least six families of liver-enriched, albeit not tissue restricted, TFs (Schrem et al. 2002 and 2004). These factors include several families of hepatocyte nuclear factors (HNFs), the CCAAT/enhancer-binding proteins (C/EBPs) and the albumin D site-binding protein (DBP), controlling the expression of different hepatic proteins. The basal expression of hepatic CYP genes is regulated by at least HNF1α, HNF3γ and HNF4α. HNF4α is known to maintain the constitutive expression of many hepatic genes, such as enzymes in the subfamilies CYP2 and CYP3A4 as well as different TPs and other nuclear receptors (NRs) (Honkakoski and Negishi 2000, Jover et al. 2001, Rodríguez-Antona et al. 2003, Naiki et al. 2004, Kamiyama et al. 2007). C/EBPα is involved in the regulation of at least CYP2 family members (Luc et al. 1996, Jover et al. 1998, Rodríguez-Antona et al. 2003). Together, these and other factors play critical roles in activating the expression of many hepatic genes and maintaining the hepatic phenotype of the cells.

Other mechanisms are also involved in the regulation of CYPs and the interindividual variability in expression can, in addition to different polymorphisms described in ***Section 2.1.1,*** be due to genetic changes at the RNA level or due to epigenetic regulation (Ingelman-Sundberg et al. 2007). Specific RNA-protein interactions in non-coding regions control the gene expression by either activating mRNA degradation or by preventing ribosomal access to the translation start codon. MicroRNAs (miRNAs) are short RNA molecules, acting as post-transcriptional regulators by binding to complementary sequences on target messenger RNAs (mRNAs) (Bartel 2009). This process usually results in translational repression or target degradation and thus, gene silencing. The miRNAs regulate numerous cellular processes such as proliferation, differentiation and apoptosis. The role of miRNAs in CYP regulation has only been demonstrated for CYP1B1 but also other CYPs containing a long 3'-UTR region (*e.g.* CYP1A2, CYP2B6 and CYP3A4) are likely to be regulated by miRNAs (Tsuchiya et al. 2006, Rodríguez-Antona et al. 2010). Epigenetic regulation refers to heritable functionally relevant modifications, including DNA methylation and histone modifications, which change gene expression without altering the nucleotide sequence of the silenced gene (Rodríguez-Antona et al. 2010). Methylation of gene promoters may hinder the binding of some transcription factors to their DNA binding sites or alternatively, the DNA methylation may silence genes by various indirect mechanisms causing changes in chromatin conformation. So far, the DNA methylation processes have been shown to

mainly affect CYP1A1, CYP1A2, CYP2C19 and CYP2D6 genes with potentially functional methylation sites (Rodríguez-Antona et al. 2010). These processes are generally very flexible and the environment can have a role in the expression profile of different CYP genes.

2.2.1 Xenosensors

The inducible expression of several genes involved in xenobiotic metabolism and transport is mainly regulated by three so-called xenosensors AhR, CAR and PXR. However, they are also involved in other regulatory processes in the body, such as homeostasis, cell differentiation and the metabolism of endogenous compounds. Understanding the mechanisms of the translocation and activation of these receptors is of interest and, because of their multiple functions and ligand-dependent activity, they have also become interesting targets for drug design.

2.2.1.1 Constitutive androstane receptor and pregnane X receptor

Human (h)CAR and (h)PXR are two members of the NR superfamily of TFs. The family consists of 48 receptors which can be divided into different groups based on their function, ligands or sequence similarities (Aranda & Pascual 2001, Benoit et al. 2004, Bain et al. 2007). Unlike classical steroid hormone receptors, which bind relatively small and rigid ligands with high (nM) affinity, PXR and CAR are very nonspecific and bind an exceptionally large variety of ligands but with low (μM) affinity (Chawla et al. 2001, Willson & Kliewer 2002, Germain et al. 2006).

Structure. All NRs have a relatively conserved, domain-like structure with different regions each having a specific function, but also interacting with each other to regulate the overall receptor function (***Figure* 2.**) (Renaud & Moras 2000, Aranda & Pascual 2001, Benoit et al. 2004, Germain et al. 2006, Bain et al. 2007). The most variable and structurally disorganized region is the N-terminal domain (A/B), which is able to bind specific coactivators and is responsible for the ligand-independent activation (activation function 1, AF-1). The region also contains multiple phosphorylation sites regulating the transcriptional activity of the receptor (Lavery & McEwan 2005). The DNA-binding domain (DBD, C) is the evolutionary most conserved region formed by three α-helices (H) separated by zinc finger motifs and required for DNA binding and receptor dimerization. The hinge region (D) enables the rotation of the ligand binding domain (LBD) and often contains nuclear localization signals and phosphorylation sites affecting the transcriptional activity of the receptor. The most important region for ligand-dependent function is the LBD (E), which is essential for ligand and coregulator binding as well as for NR dimerization. Commonly, this region consists of 10-13 α-helices (H1, H2 etc.) and 2-5 β-strands, and is highly conserved in structure, but not in sequence. A ligand binding pocket (LBP) is formed in the middle of the domain and sandwiched between two layers of helices. The last C-terminal helix (usually H12, AF-2) is allosterically controlled by the binding of ligands and forms a site on the receptor surface for coregulator (***Section* 2.2.2.3**) binding and modifies the effects of the receptor. The C-terminal part of the receptor (F) is not present in all receptors and its function remains unknown. In contrast to most other NRs, both PXR and CAR lack the N-terminal (A/B) and the C-terminal (F) domains (Timsit & Negishi 2007). In addition, the large species-specific differences in the LBD sequence of CAR and PXR have a substantial impact on the ligand binding and function of the receptors, thus leading to differences in the responses to xenobiotics (*e.g.* Lehmann et al. 1998, Maglich et al. 2003).

The most original feature of hPXR is its large (1280-1544Å^3) and flexible LBP, which is about twice as large as that of hCAR (675Å^3) (Watkins et al. 2003a, Xu et al 2004, Timsit & Negishi

2007). In addition to the seven α-helices present in other NRs, the PXR LBD contains distinct features critical for its specialized function and promiscuity (Watkins et al. 2001, Watkins et al. 2003b, Chrencik et al. 2005, Ngan et al. 2009). Two additional flexible strands (β1′ and β1) enable the expansion of the pocket to accommodate ligands of various sizes and shapes. Like the vitamin D receptor (VDR), PXR contains an approximately 60 amino

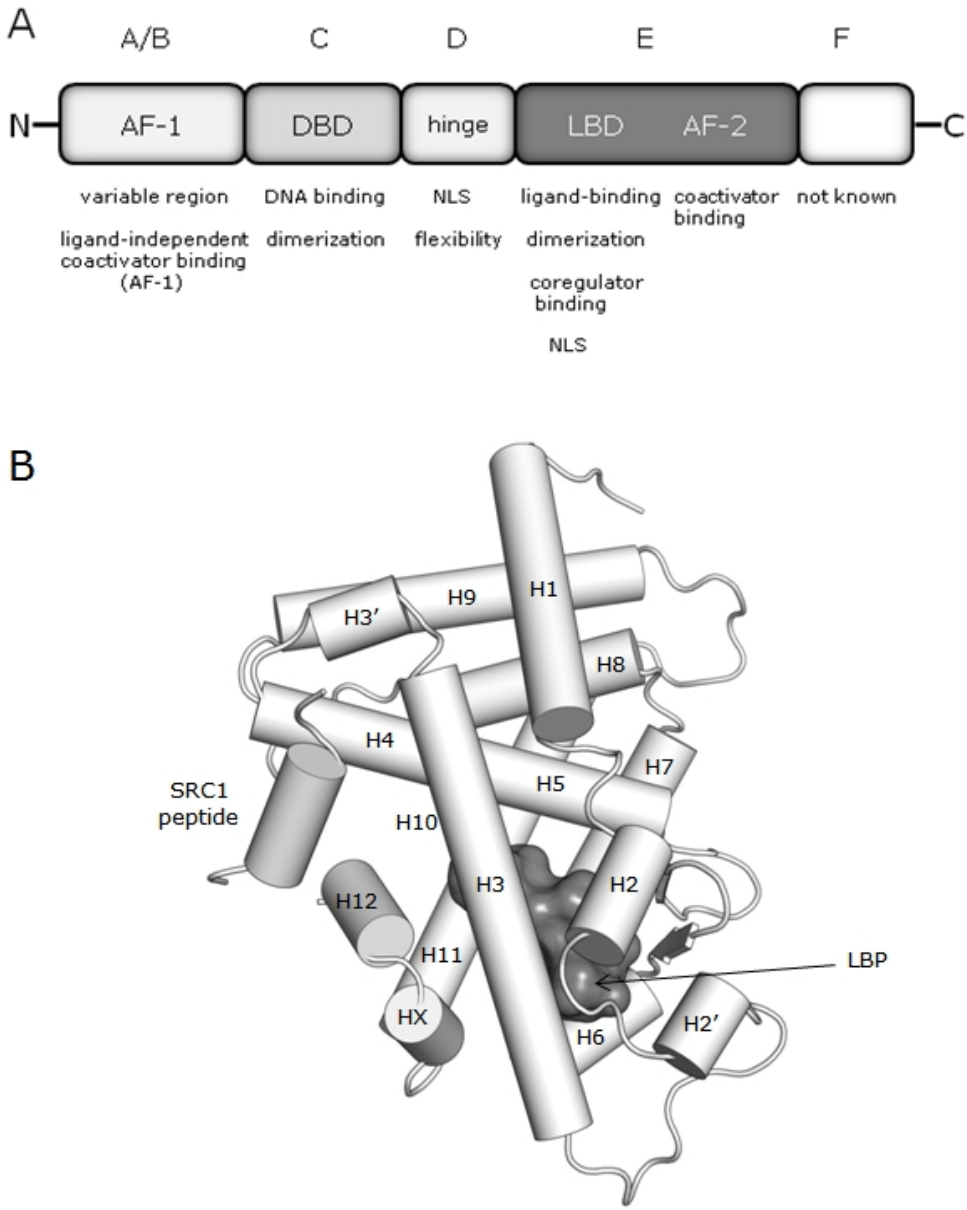

Figure 2. NR structure. **A)** A general schematic structure of nuclear receptors and the main functions of the domains. Both PXR and CAR lack the N- and C-terminal regions (A/B and F). NLS = nuclear localization signal **B)** A 3D representation of human CAR in complex with CITCO and SRC1 peptide (adapted from Xu et al. 2004). The cylinders represent the α-helices H1-H12 (including the 3_{10} helices H2' and H3') and the arrows represent β-strands. The ligand binding pocket (LBP) is shown in dark grey.

acid region between H1 and H3, creating an extended five-stranded antiparallel β-sheet, a stretch adjacent to the LBP and the H6 is replaced by a flexible loop (Watkins et al. 2001, Orans et al. 2005). The hydrophobic LBP is formed by 28 mostly non-polar amino acids with eight polar residues able to form hydrogen bonds with the ligands (Kliewer et al. 2002). The pocket can change both shape and volume and adopt unique conformational structures depending on the binding ligand and thus allow different ligand binding configurations. hCAR LBD is formed by 11 α-helices, two 3_{10} helices and three β-strands (diMasi et al. 2009). The most unique feature of the receptor is the conformationally rigid and short H12, which lacks a C-terminal extension and is responsible for ligand-dependent and independent function of CAR. Furthermore, instead of the extended loop between a shorter H10 and H12, present in most other NRs, active CAR structures contain an additional short helix (HX, residues L336, S337, A338 and M339), which is tightly packed against helices H3 and H10 in the agonist-bound structures and restricts the movement of H12. The LBP is framed by H2, H3, H4, H5, H6, H7 and H10, and two β-strands and consists of 27 mostly hydrophobic residues (diMasi et al. 2009, Windshügel & Poso 2011). The polar and potentially charged residues form two regions which might contribute to the ligand binding, although no direct hydrogen bonding has been observed.

Splicing variants and SNPs. Numerous alternatively spliced hCAR and hPXR mRNAs, with varying functions and expression patterns, have been detected in different human tissues (Dotzlaw et al. 1999, Fukuen et al. 2002, Lamba et al. 2004, Lamba et al. 2005, diMasi et al. 2009). The best studied isoforms of hPXR are PXR.1 (wild type), PXR.2 (111 bp deletion in exon 5, LBD), PAR-2 (39 additional amino acids at the N-terminal end) and PXR.3 (123 bp deletion in exon 5, LBD) (Bertilsson et al. 1998, Lamba et al. 2004, Lamba et al. 2005, Zhou et al. 2009). PXR.2 is the second most abundant transcript in human liver (approx. 7% of total PXR mRNA, Lamba et al. 2004). Most likely due to the missing portions of the LBD, the PXR.2 and PXR.3 variants are not able to bind PXR.1 ligands (Lamba et al. 2005). Based on the high prevalence of splice variants with alterations in exon 5, this region has been thought to be the least conserved of the LBD (Lamba et al. 2005).

The three most studied isoforms of hCAR are CAR.1 (wild type), CAR.2 (SVPT insertion in exon 7, in the vicinity of the LBP) and CAR.3 (APYLT insertion between exons 8 and 9, in the ligand binding/heterodimerization domain) (Auerbach et al. 2003, Jinno et al. 2004, Lamba et al. 2005). CAR.2 and .3 have been shown to be prominently expressed in human liver and primary hepatocytes (up to 50% of total CAR) (DeKeyser et al. 2011). While the wild type CAR has high basal activity (see below) in the absence of ligand, both of the splice variants are ligand-activated, although no variant-specific ligands for CAR.3 have been found. Di(2-ethyl-hexyl)phthalate (DEHP) has been shown to be a potent specific activator of CAR.2, while bisphenol A (BPA) is a CAR.1 and CAR.3 agonist but it does not activate CAR.2 (DeKeyser et al. 2009 and 2011). Other hCAR splicing variants have shown to result for example from the deletion of exon 7 or exons 10-12, leading to complete loss of transactivation (Auerbach et al. 2003, Savkur et al. 2003, Arnold et al. 2004, Jinno et al. 2004). The hCAR gene is able to produce many alternative mRNA transcripts and thus, they are difficult to quantify (Lamba et al. 2005). It has been suggested that these variants may also account for many of its functional characteristics, such as cellular localization (Li & Wang 2010).

In addition to splicing variants, numerous SNPs have been identified for hCAR and hPXR (Lamba et al. 2005, Lamba 2008). As with SNPs in general, different polymorphisms are concentrated in different populations (*e.g.* PXR*2 in African-Americans, Zhang et al. 2001) and have different effects (*e.g.* PXR*5, which cannot bind DNA, Koyano et al. 2002). Some of

the SNPs have been shown to affect the basal or induced activity of CYP2B6 and 3A4 *in vitro* and hence, it is possible that some of the hCAR and hPXR allelic variants could affect the expression of their target genes. However, the allelic frequencies of these variants are very low and their impact *in vivo* has not been conclusively proven (Lamba et al. 2005, Thompson et al. 2005, Lamba 2008, Wyen et al. 2011).

Function. The simplified view on NR function is that the inactive forms, residing in the cytoplasm, dissociate from chaperone proteins and translocate into the nucleus after activation by ligands or other stimuli (Aranda & Pascual 2001) (***Figure 4.***). In addition, some receptors are retained in the nucleus bound to unspecific DNA sequences regardless of the ligand-binding status (Germain et al. 2006). Many NRs, including PXR and CAR, shuttle between the cytoplasm and nucleus but the details of the mechanisms involved are still poorly understood. For CAR, it has been suggested that different phosphorylation and dephosphorylation events, affecting the dissociation of CAR from chaperone proteins, or cofactors (*e.g.* glucocorticoid receptor-interacting protein 1, GRIP1) could be involved in the localization of the receptor (Maruvada et al. 2003, Guo et al. 2006, Timsit & Negishi 2007). The dominant mechanism of the activation of PXR is the direct binding of an agonist (Moore et al. 2003). In contrast, activation of CAR can happen either by the direct binding of an agonist or by indirect mechanisms, involving induced nuclear translocation *e.g.* by phenobarbital (PB) which is generally believed not to bind to the CAR LBP (Moore et al. 2000, Swales & Negishi 2004, Qatanani & Moore 2005). In cell-based assays, CAR has a high basal activity, which can be further elevated by agonists or depressed by inverse agonists (*e.g.* Forman et al. 1998, Li et al. 2008). The unique structural features (*e.g.* HX, extended H2, short H12), different hydrogen bonds and other interactions between various amino acid side chains as well as the interaction with RXR, all thought to stabilize the active conformation of the receptor, have been suggested to play a role in the constitutive activity of the receptor (Dussault et al. 2002, Xu et al. 2004, Suino et al. 2004, Windshügel et al. 2007).

In the nucleus, the receptors bind to specific response elements (REs) within their target gene promoters and, together with various coregulators (***Section 2.2.1.3, Figure 4.***), alter the chromatin structure and regulate the expression of these genes. The REs are bipartite elements with two similar hexamer DNA sequences, called half-sites, separated by one or more nucleotides (spacer) (***Table 2.***) (Mangelsdorf & Evans 1995). The sequence and orientation as well as the spacer and 5'-flanking sequences of the half-sites are important for NR recognition (Germain et al. 2006). The elements are usually found clustered in the 5'-flanking promoter region of the NR target gene but also from more distant locations (Aranda & Pascual 2001, Claessens & Gewirth 2004).

Especially the xenobiotic-response enhancer module (XREM) and phenobarbital responsive enhancer module (PBREM), which contain several NR-binding elements and are found in the distal *CYP3A4* promoter and *CYP2B* regulatory regions, respectively, play a major role in *CYP3A4* and *CYP2B* induction by hPXR and hCAR ligands (Honkakoski et al. 1998, Goodwin et al. 1999). Both PXR and CAR bind DNA mainly as nonpermissive heterodimers (*i.e.* partner ligands cannot activate the complex) with retinoid X receptor (RXR, NR1B), although PXR has been shown to function also as a homodimer and CAR to bind to DNA as a monomer (Kliewer et al. 2002, Frank et al. 2003, Noble et al. 2006).

In addition to DMEs and DTs (***Table 2.***), both PXR and CAR participate in the regulation of the metabolism of endogenous substances, such as bilirubin, bile acids and vitamin D (Huang et al. 2003, Xie et al. 2003, Wagner et al. 2005, Hosseinpour et al. 2007). This happens mainly indirectly by regulation of the expression of genes, such as CYP7A1 and

Table 2. Response elements, cofactors and regulated genes of human xenosensors.

	REs[a]	Known cofactors[b]	Regulated genes[c]	*References*
CAR	**DR4**	*Coact.*	CYP1A1/2	*Lee et al. 2007, Yoshinari et al. 2010*
NR1I3	DR5	SRC1	CYP2B6	*Sueyoshi et al. 1999, Wang et al. 2004*
	ER6	SRC2	CYP2C9	*Ferguson et al. 2002*
	ER8	PGC1α	CYP3A4/5/7	*Burk et al. 2002, Burk et al. 2004*
	IR	PBP	ALDH1A4	*Maglich et al. 2003*
		ASC2	UGT1A1/6, 2B1	*Bock & Köhle 2005, Sugatani et al. 2005*
	(AGGTCA)	FoxO1	GSTA1/2	*Assenat et al. 2004*
			SULT1A1/2, 2A1	*Saini et al. 2004, Chen et al. 2007*
		Corepr.	NAT1	*Westerink & Schoonen 2007a*
		NCoR	MDR1	*Burk et al. 2005*
		SMRT	MRP2-4	*Kast et al. 2002, Assem et al. 2004*
PXR	**DR3**	*Coact.*	CYP1A1/2[d]	*Maglich et al. 2002*
NR1I2	DR4	SRC1	CYP2A6	*Itoh et al. 2006*
	DR5	SRC2	CYP2B6	*Wang et al. 2003*
	ER6	SRC3	CYP2C8/9/19	*Gerbal-Chaloin et al. 2002,*
	ER8	PGC1α		*Ferguson et al. 2005,*
	IR0	NRIP1		*Chen & Goldstein 2009*
	PBRE	FoxO1	CYP3A4/5/7	*Lehmann et al. 1998, Pascussi et al. 1999, Burk et al. 2004*
	(AGGTCA)	*Corepr.*	ALDH1A4	*Maglich et al. 2002*
		NCoR	GSTA1/2	*Falkner et al. 2001, Maglich et al. 2002*
		SMRT	SULT1A1, 2A1	*Maglich et al. 2002, Sonoda et al. 2003*
		SHP	UGT1A1/3/4/6/9	*Chen et al. 2003,*
				Gardner-Stephen et al. 2004
			MDR1	*Geick et al. 2001*
			MRP2-5	*Kast et al. 2002, Teng et al. 2003*
			OATPs	*Meyer zu Schwabedissen et al. 2008*
AhR	**XRE**	*Coact.*	CYP1A1/2	*Quattrochi et al. 1994, Mandal 2005*
		ARNT	CYP1B1	*Lin et al. 2003*
	(TNGCGTG)	SRC1	CYP2S1	*Saarikoski et al. 2005*
		SRC2	NQO1	*Yeager et al. 2009*
		CBP/p300	UGT1As	*Zhou et al. 2005,*
		RIP140		*Bock & Bock-Hennig 2010*
			BCRP	*Tan et al. 2010*
		Corepr.	GSTAs	*Yeager et al. 2009*
		AHRR	SULTs	*Yanagiba et al. 2009*
		RIP140		

[a]Orientation of half-sites: head-to-tail (direct, DR-n), head-to-head (inverted, IR-n) or tail-to-tail (everted, ER-n). Core sequences are shown in brackets and the favoured element in bold (Kliewer et al. 2002, Honkakoski et al. 2003, Sonoda et al. 2003, Saini et al. 2004, Song et al. 2004, Beischlag et al. 2008). [b]AHRR=aryl hydrocarbon receptor repressor, ARNT=aryl hydrocarbon receptor nuclear translocator, ASC2=activating signal cointegrator 2, CBP/p300=CREB binding protein/E1A binding protein p300, FoxO1=forkhead transcription receptor 1, NCoR=nuclear receptor corepressor 1, NRIP1=nuclear receptor interaction protein 1, PBP=peroxisome proliferator-activated receptor-binding protein (TRAP220), PGC-1α=peroxisome proliferator-activated receptor gamma coactivator 1α, RIP140=receptor interacting protein 140, SHP=small heterodimer partner, SMRT=silencing mediator for retinoid or thyroid-hormone receptors, SRC1-3=nuclear receptor coactivator 1-3 (Synold et al. 2001, Min et al. 2002, Shiraki et al. 2003, Choi et al. 2005, Jia et al. 2005, Jyrkkärinne et al. 2005 (CAR); Takeshita et al. 2002, Ourlin et al. 2003, Watkins et al. 2003, Johnson et al. 2006, Li & Chiang 2005 (PXR); Kumar et al. 1999, Kumar & Perdew 1999, Matthews et al. 2005, Watt et al. 2005, Madak-Erdogan & Katzenellenbogen 2012 (AhR). [c]Examples of regulated genes involved in xenobiotic metabolism, NQO1 = NADPH dehydrogenase quinone 1, [d]The regulation of CYP1A2 is somewhat questionable (Kojima et al. 2007).

UGT1A1, important for the metabolism or detoxification of these compounds. There are more recent findings indicating that the receptors are also involved in energy metabolism either by direct gene regulation or via crosstalk with other transcriptional regulators, such as the forkhead box proteins O1 (FoxO1) and A2 (FoxA2), involved in gluconeogenesis and fatty acid oxidation (Kakizaki et al. 2008, Moreau et al. 2008, Wada et al. 2009, Gao & Xie 2010).

Ligands. The ligands of hPXR and hCAR are a miscellaneous group consisting both endo- and exogenous compounds with different structures and sizes (diMasi et al. 2009). Examples of compounds binding hPXR include various drugs (*e.g.* rifampicin, metyrapone, statins, anticancer compounds and ketoconazole, Harvey et al. 2000, Moore et al. 2000, Ekins et al. 2007), herbal compounds (*e.g.* hyperforin and certain compounds used in the traditional Chinese medicine, Watkins et al. 2003, Mu et al. 2006, Yu et al. 2011), steroid hormones and metabolites (*e.g.* progesterone, Kliewer et al. 1998), dietary compounds (*e.g.* coumestrol and carotenoids, Rühl et al. 2004, Wang et al. 2008), vitamins (Landes et al. 2003), bile salts and acids (*e.g.* lithocholic acid, Staudinger et al. 2001, Xie et al. 2001) as well as various pesticides (Lemaire et al. 2004). The molecular weight, as well as the three-dimensional shape of a compound, appears to be important if it is to undergo a strong binding to PXR, despite of the exceptional flexibility of the LBP (Xiao et al. 2011). The first identified mouse (m)CAR ligands were endogenous androgen metabolites (androstanol and androstenol), which act as inverse agonists, blocking the constitutive activity of hCAR at high concentrations (Forman et al. 1998). Other published hCAR inverse agonists include 17α-ethinylestradiol (EE2, Mäkinen et al. 2002), clotrimazole (Moore et al. 2002, Auerbach et al. 2003), meclizine (Huang et al. 2004) and the isoquinoline carboxamide PK11195 (Li et al. 2008), although results on clotrimazole and meclizine are controversial (Mäkinen et al. 2002, Toell et al. 2002, Lau et al. 2011). Compounds shown to activate (but not necessarily bind to) hCAR include various drugs (*e.g.* phenobarbital, phenytoin, artemisinin and statins, Sueyoshi et al. 1999, Wang et al. 2004, Burk et al. 2005, Kobayashi et al. 2005), synthetic chemicals (*e.g.* CITCO, Maglich et al. 2003), environmental chemicals (*e.g.* nonylphenol, Hernandez et al. 2009), herbal medicines (*e.g.* Chinese herbal compounds, Huang et al. 2011) and flavonoids (Yao et al. 2010). In addition, hCAR can be activated by high concentrations of bile acids and bilirubin (Huang et al. 2003, Wagner et al. 2005).

2.2.1.2 Aryl hydrocarbon receptor

AhR is a member of the family of the basic-helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factors. The receptor is present in many tissues, such as liver, lung and placenta (Dolwick et al. 1993) and is involved not only in drug metabolism and toxic responses of chemicals, but also in hematopoiesis (Gasiewicz et al. 2010) and the differentiation of the lymphoid system (Kiss et al. 2011, Li et al. 2011, Hooper 2011), T-cells (Quintana et al. 2008), neurons (Akahoshi et al. 2006) and hepatocytes (Walisser et al. 2005).

Structure and variants. Similarly to NRs, AhR has a domain-like structure (***Figure 3.***). The N-terminal bHLH motif, formed by two α-helices connected by a loop, is responsible for DNA binding (basic region, b) and protein-protein interactions (HLH) (Murre et al. 1994, Whitlock 1999). The N-terminal domain also contains the nuclear localization and nuclear export signals (NLS and NES), required for the shuttling between the cytoplasm and the nucleus (Ikuta et al. 2000). The two PAS domains (A and B) have specific interactions with other PAS domain containing proteins, such as aryl hydrocarbon receptor nuclear translocator (ARNT).

These domains are homologous and highly similar to protein domains originally found in the Drosophila genes period (*Per*) and single-minded (*Sim*). The ligand binding site, containing several residues critical for ligand binding, is located in the PAS-B domain, which also defines which dimerization partner can bind the receptor and contributes to the dimer stability. The C-terminal part of the receptor contains the large transactivation domain (TAD), consisting of several subregions, which is involved in coactivator recruitment and transactivation/repression (Reen et al. 2002, Beischlag et al. 2008). The subregions are a glutamine-rich region, important for coactivator interactions, the acidic region and a region rich in Pro/Ser/Thr residues (Kumar & Perdew 1999; Hankinson 2005; Beischlag et al. 2008). The complexity of these regions is responsible for the various interactions and thus, the "flexible" transactivation potential of the receptor. The length of the C-terminus varies between species or even between different strains of laboratory rats resulting from different stop codon usage (Poland & Glover 1987). A single genetic AhR variant seems to have no or little effect on CYP1A inducibility in humans, whereas a combination of AhR variants may have significant effects (Harper et al. 2002). However, such combinations of genotypes are rare and seem to occur primarily in people of African descent (Wong et al. 2001).

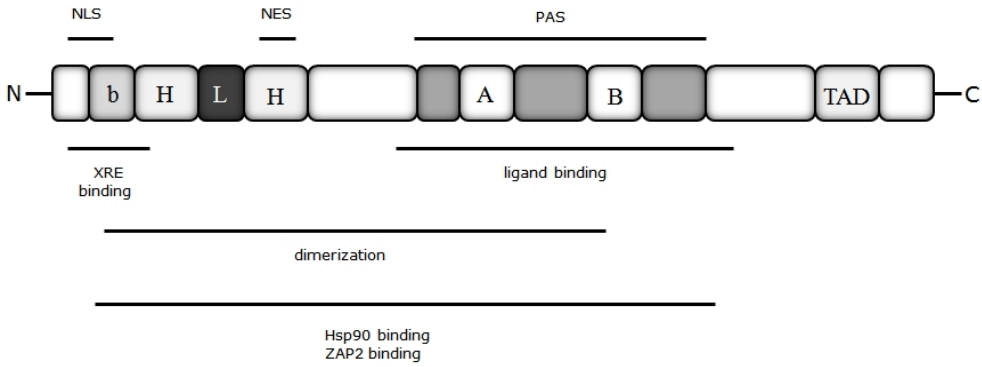

Figure 3. AhR structure. A schematic presentation of AhR structure and the main functions of the domains. NLS = nuclear localization sequence, NES = nuclear export sequence, bHLH = basic helix-loop-helix domain, PAS = Per-ARNT-Sim domain (A and B repeat regions), TAD = transactivation domain.

Function. Despite the different structure and receptor class, the functional properties of AhR closely resemble those of NRs (***Figure 4.***). In the inactive state (no ligand), the receptor resides in the cytosol, bound to two molecules of heat shock protein 90 (Hsp90), the immunophilin like X-associated protein 2 (XAP2) and other proteins, such as p23 (Petrulis and Perdew 2002). Hsp90 and p23 protect the receptor from proteolysis and maintain a conformation suitable for ligand binding (Cox & Miller 2004). XAP2, interacting with Hsp90, binds to the NLS sequence, stabilizes the complex and prevents the receptor from entering the nucleus without a ligand (Petrulis et al. 2000). The ligand binding to the PAS-B domain induces the dissociation of the chaperone proteins and the exposure of NLS (Ikuta et al. 2004). The receptor then translocates to the nucleus where it dimerizes with ARNT, in order to form an active DNA-binding complex, and controls the expression of its target genes by binding to the xenobiotic response element (XRE) in the regulatory region of these genes (Denison and Nagy 2003). Another PAS protein, AhR repressor (AHRR), inhibits both inducible and constitutive AhR activity, by competing with ARNT, or by binding to the XRE (Hahn et al. 2009). Protein kinase C and tyrosine kinase seem to be also involved in the AhR signal transduction but the mechanisms have been a matter of debate (Backlund &

Ingelman-Sundberg 2005). Despite differences in receptor sequence or size between different species, the signalling pathway is highly conserved in all vertebrates as CYP1A induction is seen in all species (Bank et al. 1992). In addition to the members of the CYP1A family, AhR regulates numerous other genes, including Phase II enzymes (***Table 2.***) and is required for the toxic effects of some important environmental compounds, such as dioxins (Céspedes et al. 2010).

Ligands. The AhR binds a wide variety of structurally diverse synthetic and natural ligands. The established ligands include the synthetic halogenated aromatic and polycyclic aromatic hydrocarbons (HAHs and PAHs) as well as natural ligands tetrapyrroles (bilirubin), flavonoids, tryptophan derivatives and dietary carotinoids (Denison & Nagy 2003). The best example is the CYP1A1 inducing agent, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), which produces a wide variety of species- and tissue-specific toxic effects (Poland & Knutson 1982, Mimura & Fujii-Kuriyama 2003). Although various potential candidates, such as heme degradation products, have been suggested, so far no true endogenous ligand for human (h)AhR has been found (Denison et al. 2002). Similarly to the NRs, the ligand-binding affinities of AhR differ between species possibly due to the differences in the amino acid sequences in the LBDs (Ema et al. 1994).

2.2.1.3 Coregulators

As TFs, in general, lack the enzymatic activities required for the modulation of chromatin structure, all three xenosensors either activate or repress target gene expression by binding either coactivators or corepressors, respectively (***Table 2.***). Cofactors are a diverse group of relatively unstable proteins, which lack a distinguishing structural motif possibly due to the number of different transcriptional steps in which they are involved (Bulynko & O'Malley 2011). Coactivators act as histone acetyltransferases (HATs, *e.g.* p160 factors) or methyltransferases or serve as partners for such enzymes and are involved in chromatin relaxation and thus, the recruitment of the basic transcriptional machinery and activation of gene expression (Gronemeyer et al. 2004, Bulynko & O'Malley 2011). In contrast, corepressors recruit histone deacetylases (HDACs) and promote chromatin condensation and repression of gene expression (Privalsky 2004). Some of the coregulators can also have mixed effects, depending on the bound receptor. The activity of the receptors and coregulators can be further controlled by different post-translational modifications, such as methylation or phosphorylation, which can affect all levels of the NR activation process (Bulynko & O'Malley 2010).

The recruitment of a certain type of coregulator depends on the NR conformation, or more specifically, the position of H12, usually determined by the nature of bound ligand (agonist or antagonist) or the lack of ligand (Privalsky 2004). The recruitment of coregulators is mainly mediated through special motifs on the surface of NRs (***Figure 2.***). Interactions with agonists stabilize the LBD to a conformation, which allows the H12 to form a hydrophobic cleft together with helices 3 and 4. The so-called charge clamp, holding the conserved amphiphatic α-helical LxxLL motif (NR box), formed by two highly conserved charged residues (Lys in H3 and Glu in H12) of the LBD, can be found within this cleft (Renaud & Moras 2000). The binding of a coregulator has also been shown to further stabilize the LBD structure and the orientation of the bound ligand. In response to antagonists, the conformation of the LBD and the positioning of the H12 do not allow the binding of coactivators (Privalsky et al. 2004). The H12 can be reoriented from the body of the LBD or bound to another site outside of the AF2, allowing the corepressors with longer LxxI/HIxxxI/L motifs to bind to the cleft (Steinmetz et al. 2001). NRs bind different cofactors depending on their expression levels and the activity of the receptor is dependent on the relative amount of cofactors in the cell (Gronemeyer et al. 2004, Germain et al. 2006).

In analogy to NRs, both AhR and ARNT are able to bind coregulators of various classes (***Table 2.***) but the identity of these factors as well as the mechanisms by which they are recruited are still poorly understood (Beischlag et al. 2008). The AhR can be inhibited or downregulated by corepressors (Rushing & Denison 2002, Fallone et al. 2004), by the binding of AHRR or by degradation (Karchner et al. 2002, Swanson 2002, Mimura & Fujii-Kuriyama 2003).

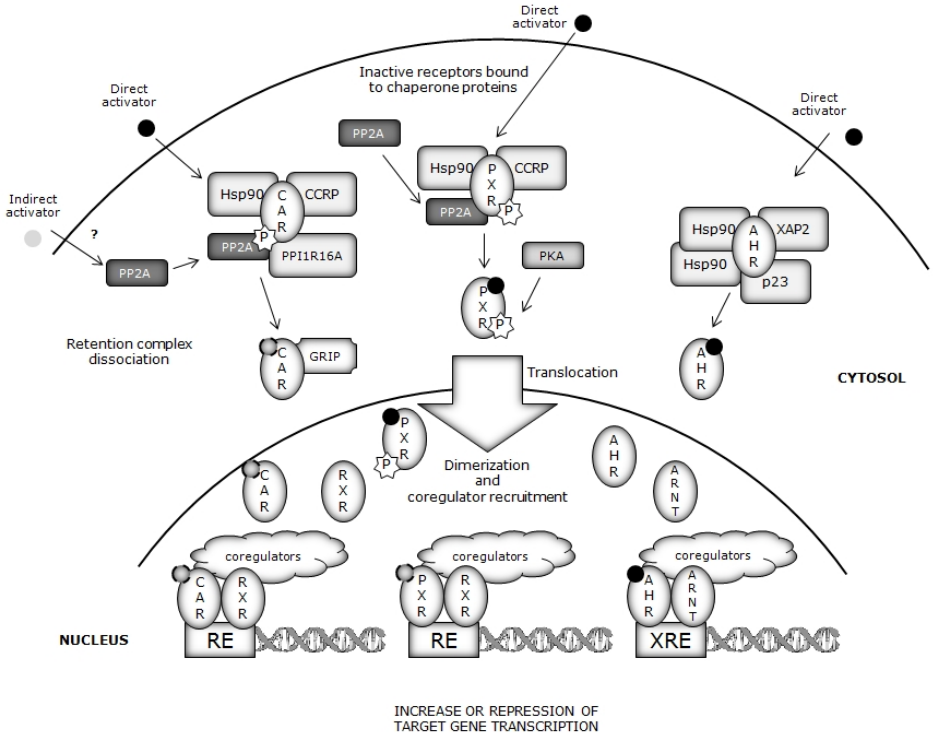

Figure 4. Schematic illustration of xenosensor activation and function. Xenosensors are retained in the cytosol by a multi-protein retention complex, protecting the receptors from proteolysis and preventing nuclear translocation and the binding of coregulators. After activation by binding of the ligand (black circle) and/or different phophorylation events (PP2A=protein phosphatase 2A, PKA=protein kinase A), the receptors are released from the chaperone proteins and translocated to the nucleus. Once in the nucleus, the receptors dimerize with their respective partners, bind the promoter regions of their target genes (REs) and alter the transcription of their target genes together with the coregulator proteins, which affect the chromatin structure. Without ligand, PXR has also been suggested to act as a gene silencer. Modified from diMasi et al. 2009 and Pavek & Dvorak 2008.

2.2.2 Cross-regulation

Cross-regulation refers to the functional interaction between different signalling pathways, involving different steps of these pathways or shared components interacting and affecting both or all of the pathways (Pascussi et al. 2004). NR and AhR signaling pathways are usually described in a simplified fashion and interactions with other pathways are neglected (***Figure 4.***). Cross-regulation can be divided into different levels of action, such as

sharing of ligands, DNA-binding elements or coregulators, receptor-receptor interactions, biotransformation of the ligand/activator and secondary modulation via other pathways (Pascussi et al. 2008).

Due to the xenosensors' ability to bind various types of ligands, different receptors can share ligands, inducing either similar or opposite effects. An example of such a ligand is PK11195, which is an hCAR inverse agonist and hPXR agonist (Li et al. 2008). PXR has also been shown to be activated by liver X receptor (LXR) and farnesoid X receptor (FXR) agonists, such as the synthetic T0901317 and bile acids, respectively (Duniec-Dmuchowski et al. 2007, diMasi et al. 2009).

Another level of cross-regulation is the sharing of DNA response elements between PXR and CAR or between xenosensors and other nuclear receptors, leading to the regulation of the target genes of other receptors by xenosensor ligands. Examples include the DR4 element in the CYP2B6 promoter, shared by CAR and PXR (Mäkinen et al. 2002), as well as REs shared by PXR/CAR and the vitamin D receptor (VDR, Drocourt et al. 2002) or AhR and estrogen receptor (ER, Ohtake et al. 2003). In general, it appears that some receptors are able to bind to and transactivate motifs, which are different from their preferred consensus sequence. However, the binding does not necessarily correlate with the the efficacy of gene transcription. An RE can also be shielded by a receptor (CAR) by its binding to an overlapping or adjacent element of another receptor (HNF4α) (Miao et al. 2006).

The biological function of the xenosensors depends on the availability of cofactors and the recruitment of cofactors to another signalling pathway may decrease their availability. The competition for interaction partners between the three xenosensors (***Table 2***.) or between receptors in general might lead to concurrent activation and inhibition of different pathways (Beischlag et al. 2002, Gurevich et al. 2007). For example, CAR inhibits ER signalling by reducing the levels of available GRIP1 leading to reduced activity of estrogen receptor (squelching) (Min et al. 2002). Similarly, the AhR dimerization partner, ARNT, has been shown to act as a coactivator for the ER, leading to competition between AhR and ER (Brunnberg et al. 2003). Other TFs can also act as coregulators for xenosensors. For example, the forkhead transcription factor FoxO1, important for gluconeogenesis, can act as a coactivator for CAR and PXR (Kodama et al. 2004, Kachaylo et al. 2011). Conversely, CAR and PXR can act as corepressors of FoxO1 and downregulate the FoxO1-mediated transcription in the presence of their activators.

The xenosensors can be also regulated by each other or by other TFs or can themselves be a part of a signalling cascade regulating the expression or function of another receptor. For example, CAR can be regulated by AhR (Patel et al. 2007). Other examples include the regulation of CAR by HNF4α together with PGC1α via binding to an RE in *CAR* promoter (Ding et al. 2006). Similarly, both PXR and CAR expression is glucocorticoid-dependent in primary hepatocytes and thus, the activity of the glucocorticoid receptor (GR) is assumed to influence the expression PXR/CAR target genes (Pascussi et al. 2000, Pascussi et al. 2003). In addition, cross-regulation may result from the target gene products (enzymes) affecting the ligands/activators of a receptor controlling another pathway. For example, CAR participates in the thyroid hormone metabolism by inducing the expression of UGTs and SULTs, which in turn inactivate thyroid receptor (TR) ligands T3 and T4 (Qatanani et al. 2005). The AhR antagonist, omeprazole-sulfide, is also converted to the AhR activator, omeprazole, by the PXR target gene CYP3A4 (Gerbal-Chaloin et al. 2006).

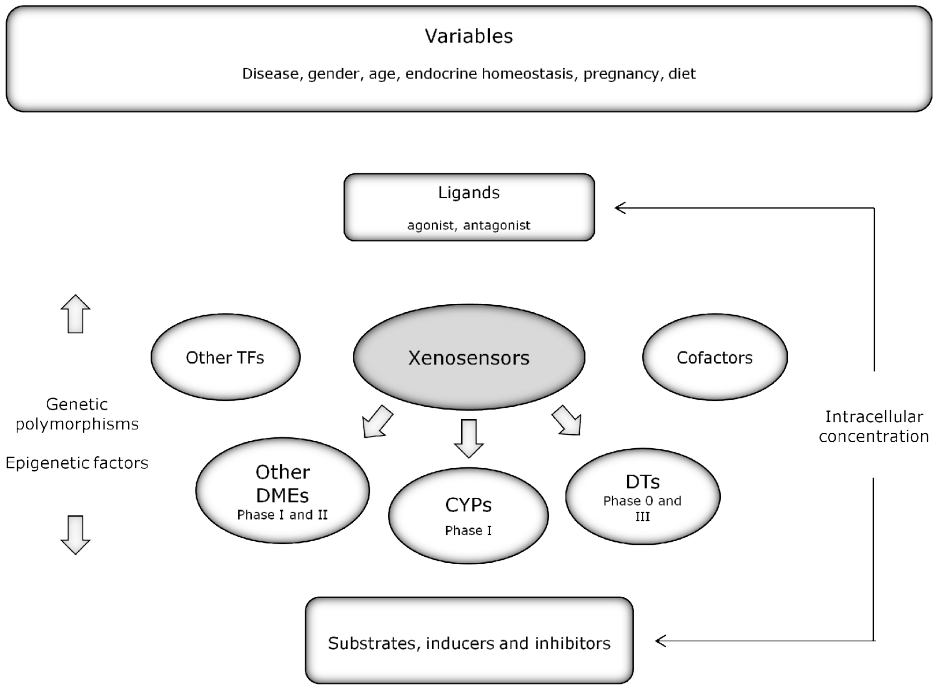

Figure 5. Major factors and variables contributing to interindividual differences in xenobiotic metabolism. The metabolism of or the induction caused by a compound is affected on different levels of the xenobiotic metabolism by different physiological and environmental variables, genetic variations of xenosensors, DMEs and DTs as well as the concentration of ligands, inducers/inhibitors and substrates. Modified from Tang et al. 2005.

These are only a few examples highlighting the complexity of the networks involved in gene regulation by xenosensors. In general, a single xenobiotic compound can initiate a very complicated process involving numerous regulatory pathways controlling homeostasis of the body and its overall effects will depend on the specific effects of the compound to various receptors, cofactors and enzymes (***Figure 5.***).

2.3 EXPERIMENTAL TOOLS TO STUDY XENOBIOTIC METABOLISM (INDUCTION) AND XENOSENSOR FUNCTION

An ideal biological model for xenobiotic metabolism studies should express all the receptors, cofactors, enzymes and transporters important in the metabolic process, in the correct proportions. An *in vivo* system containing all these components would be the most favourable choice but human studies are considered unethical and animal models are very often not suitable due to the species specific differences in the responses to xenobiotics. For example the European Union is tightening the rules to reduce the number of animals being used in scientific research. Thus, *in vitro* and *in silico* studies are increasingly recommended to study xenobiotic metabolism and to screen novel drug candidates. All of these models or methods have their own different strengths and weaknesses but in general they are cheaper, faster and less complex compared to *in vivo* models. They are also easier to use and enable the use of human cellular preparations and cell lines. Often, the main aim in the development of these assays has been to generate systems suitable for high throughput

screening (HTS) and less emphasis has been placed on the proper validation of the assays or on the assessment of their limitations (Pelkonen & Turpeinen 2007).

2.3.1 *In silico* methods

Owing to the increasing knowledge about the structures of xenosensors and CYPs and their ligands, several computational (*in silico*) methods have been developed to complement biological assays, especially for use in the early phases of drug discovery process (Schuster et al 2006, Ai et al 2009). These methods are intended to reduce the number of molecules needing to be tested in biological assays but also to study the molecular properties and interactions of xenosensors or enzymes. However, they have been often unable to provide accurate predictions on the effects of a compound *in vivo*, due to the flexibility and promiscuity of the proteins, the diverse molecular properties of the compounds and the complexity of ligand-protein interactions (Ekins et al. 2007a,b).

A pharmacophore defines the essential molecular features of a ligand needed for optimal interactions with a receptor, which then cause the desired biological response (Wermuth et al. 1998). It can be designed based on the binding site of the target and the active conformation of the ligands (direct) or by a set of active compounds (indirect), when the structure of the protein is not known. In both cases, the ligands have to be superimposed (aligned) (Ekins et al. 2007b, Wolber et al 2008). The features, which are then defined for the superimposed ligands, are usually various electrostatic and steric properties (hydrophilic or hydrophobic areas, cationic or anionic groups and hydrogen bond donors/acceptors) necessary for an optimal interaction between a ligand and a target molecule (van Drie 2003, Wolber et al. 2008). When the distances between these features are defined, they can be used to screen different chemical libraries for similar compounds, assumed to exhibit similar biological activity. This assumption or principle is called the structure-activity relationship (SAR). Quantitative SARs (QSARs) link the chemical structures to biological activity with a mathematical expression, which can be used to predict the biological activity of other chemicals (Wermuth et al. 1998). The quality of a QSAR model depends mainly on the accuracy of the biological data, the alignment of the compounds used to build the model and the selection of descriptors, statistical tools and validation of the developed model. 3D QSAR models are based on the calculation of the force fields around the aligned ligands and the correlation of the ligands to the field properties (Verma et al. 2010). Various pharmacophore and 3D-QSAR models have been used to study the active sites of CYP enzymes and to classify molecules into CYP substrates and inhibitors (Ekins et al. 2001, Mankowski & Ekins 2003, Li et al. 2008). Even though CYPs have been shown to have many common structural features, the interactions with substrates and inhibitors differ significantly. CYP pharmacophore or QSAR models would be useful in drug discovery (finding of lead structures) and in predicting of ADME properties but, due to different or inadequate datasets or the different methodologies being used, the predictive power of these approaches may be questionable (Kurogi & Güner 2001, Shen et al. 2004, Lill 2007). However, other methodologies based on the ligand's chemical structure, have been shown to predict the site of metabolism with up to 80% accuracy (Afzelius et al. 2007). Xenosensor pharmacophores and QSAR-models have been used to study the binding of ligands as well as the properties of ligands required for specific binding and activation of the receptors. Several predictive ligand-based AhR QSAR models have been devised to study the hAhR ligand binding affinity and the possible mechanism of toxicity of various ligands (*e.g.* Waller & McKinney 1992, Mekenyan et al. 1996, Lo Piparo et al. 2006, Li et al. 2011, Gu et al. 2012). Due to the huge numbers and structural diversity of the ligands as well as the size and flexibility of the LBP, there are only a few pharmacophore or QSAR-based studies on hPXR (*e.g.* Ekins & Erickson 2002, Schuster & Langer 2005, Dring et al. 2010). In the case of

hCAR, the lack of known specific agonists has perhaps hindered the use of ligand-based approaches (*e.g.* Jyrkkärinne et al. 2003 and 2008, Dring et al. 2010).

In order to obtain a crystal structure of a protein, it has to be produced either in bacterial or eukaryotic cells, purified and crystallized. The atomic structure is obtained using X-ray diffraction and the final model is built based on an interpretation of the electron density data (Gouding & Perry 2003, Rupp & Wang 2004, Chayen & Saridakis 2008, Wlodawer et al. 2008). In general, certain structural modifications are needed to improve the water solubility of CYPs and to enable the crystallization process (*e.g.* Cosme & Johnson 2000). Thus, the proteins are subjected to highly unphysiological conditions in the crystallization procedure. Multiple crystal structures (with or without mutations), with inhibitors or substrates, exist for human CYPs. The first published human CYP crystal structure was that of CYP2C9, which became available in 2003 (Williams et al. 2003), followed by structures of all main hepatic CYPs (Sansen et al. 2007 (CYP1A2), *e.g.* Yano et al. 2005, DeVore et al. 2011 (CYP2A6), *e.g.* Gay et al. 2010 (CYP2B6), *e.g.* Schoch et al. 2004 and 2008 (CYP2C8), Rowland 2005 (CYP2D6), *e.g.* Porubsky et al. 2008, Scott & Porubsky 2010 (CYP2E1), *e.g.* Yano et al. 2004, Sevrioukova & Poulos 2010 (CYP3A4)). Due to the size and flexibility of the NRs, the purification and crystallization of an entire receptor is extremely challenging and thus, only two crystal structures (PPARγ and VDR) containing the entire receptor exist so far (Chandra et al. 2008, Orlov et al. 2011). Otherwise, either LBD crystals, containing a bound ligand and/or the partner RXR LBD and a cofactor peptide, or DBD structures, attached to the response element and the partner RXR DBD, exist for these receptors. Published crystal structures for hCAR and hPXR and their properties are listed in ***Table 3.*** At present, no published crystal structure for AhR is available and the amino acids important for ligand binding have mainly been studied with the help of the established homology models as well as through mutation studies (***Table 3.***, Ema et al. 1994, Goryo et al. 2007). The main problem with the crystal structures is that they are snapshots of a protein conformation in an unnatural surrounding, and thus, do not necessarily provide sufficiently detailed information about all of the properties of the protein.

Although the Protein Data Bank (PDB) provides numerous 3D structures for different proteins, many structures remain uncharacterized (*e.g.* AhR). In homology modelling, a model of the desired protein (target) is created based on sufficient sequence homology (at least 30-40% identity) of proteins with known structures (templates) (Ginalski 2006, Liu et al. 2011). Sequence similarity searches are used to find proteins with known high-quality 3D structures and often, multiple template structures are used to build a single model of the target. In general, flexible structures, such as loops, or insertions and deletions are the most difficult to predict. The model is built based on the sequence alignment between template and target. Steric clashes within the model are eliminated by energy minimization and the model is validated by comparing with the template and by calculating stereochemical factors (Hillisch et al. 2004, Xiang 2006, Liu et al. 2011). Many homology models, based on bacterial CYP isoforms and later the first crystallized mammalian enzyme (rabbit CYP2C5), were created for human CYPs when no crystal structure was available (Kirton et al. 2002a,b, de Groot et al. 2004). These models have been used to explain and predict the likely sites of metabolism in a variety of CYP substrates (Hillisch et al. 2004, Afzelius et al. 2007). Some examples of xenosensor homology models and their applications are presented in ***Table 3***. The quality and applicability of homology models depend on the similarity between the template and target. The evaluation or comparison of the homology models has been difficult due to the lack of information available or due to different template structures used (Windshügel et al. 2007). The advantage of homology models is

that their generation is relatively quick and easy. Nonetheless, homology models tend to become rather obsolete when a validated crystal structure is available.

Table 3. Crystal structures and examples of homology models of human xenosensors.

Protein	Crystal structures[a] and homology models	*References*
hCAR	***Crystal structures*** (2): LBD (chains B and D) with RXRα heterodimer (chains A and C), SRC1 peptide (chains E-H) and agonists 5βP (1XV9) or CITCO (1XVP). Used to determine the binding modes of the two ligands as well as amino acid side chains important for ligand binding.	*Xu et al. 2004*
	Homology models: Five models based on PXR or ERα alone or both PXR and VDR. Consist of mCAR or hCAR LBD alone or hCAR LBD with a cofactor (SRC1). Used to study the ligand binding and binding modes (docking), the key residues in the LBP, influence of coactivator binding and the mechanisms of the constitutive activity of CAR.	*Dussault et al. 2002* *Xiao et al. 2002* *Jacobs et al. 2003* *Jyrkkärinne et al. 2005* *Windshügel et al. 2005 & 2007*
hPXR	***Crystal structures*** (8): LBD (chain A) Apo (1ILG) or with SR12813 (1ILH), hyperforin (1M13), rifampicin (1SKX), colupulone (2QNV) and PNU-1412721 (3R8D) or the LBD (chains A and B) with SRC1 peptide (chains C and D) and SR12813 (1NRL) or with SRC1 peptide (chains C and D, res. 625-639) and T0901317 (2O9I)	*Watkins et al. 2001* *Watkins et al. 2003a and b* *Chrencik et al. 2005* *Xue et al. 2007a and b* *Teotico et al. 2008* *Cheng & Redinbo 2011*
	Homology models: Different models based on ERα, PXR crystal structure (apo) (with two SMRT interacting domains from PPAR/SMRT structure). Used to study the key residues for ligand and cofactor binding.	*Jacobs et al. 2003* *Wang et al. 2006*
hAhR	***Crystal structures*** (0): No published crystal structures.	
	Homology models*:* Different models based on the hypoxia-inducible factor 2α (HIF-2α) (apo or holo), ARNT or other PAS domains as well as ERα. Used to study and compare the ligand binding properties of AhR from multiple species (e.g. human, mouse and rat) and the properties of its high-affinity ligands.	*Jacobs et al. 2003* *Pandini et al. 2007* *Bisson et al. 2009* *Motto et al. 2011* *Salzano et al. 2011*

[a]The number of published crystal structures and their PDB codes are in brackets (PDB 15.12.2011)

Knowledge of the structural features of CYP active sites or the xenosensor LBPs is crucial in predicting the interactions of a drug and a protein. Both crystal structures and homology models of human CYPs have produced useful estimates of substrate binding affinities and in predicting the sites of metabolism in the substrate (Lewis 2002). The crystal structures of NR LBDs have mainly been used to study the changes in receptor conformation in response to different ligands and to identify crucial residues or structural elements for ligand specificity or receptor function (***Table 3.***). However, the ligand-induced changes in receptor structure extend outside of the LBD via allosteric interactions between the LBD and DBD. Thus, the crystal structures of the full length receptors would provide a more accurate view on the receptor function. Furthermore, the lack of an apo structure has partly hindered studies on the constitutive activity of CAR (Windshügel & Poso 2011, Jyrkkärinne et al. 2012).

Docking methods predict the energetically most favourable binding orientation of a ligand and thus, can be used to predict the affinity and activity of the ligand (Taylor et al. 2002).

The best pose of the ligand is usually selected based on a scoring function (empirical, knowledge-based or physics-based molecular mechanics force fields), even though their predictive value is questionable (Huang et al. 2010). Most docking methods suffer from the fact that the flexibility, a crucial feature for NR function, is omitted or too limited. Different docking programs (*e.g.* AutoDock, GOLD) and scoring functions can be used as a part of virtual screening approaches and to examine the binding modes and features of different compounds as well as to obtain information on the protein binding site (Huang et al. 2010, Meng et al. 2011). Docking methods are often used to predict the binding orientation and affinity of drug candidates for their target receptors. Docking of a compound into the LBP or active site usually does not capture the whole picture of transcriptional or other activation (*e.g.* allosteric) events. However, docking has been used to visualize the PXR antagonist ketoconazole binding to the AF-2 regions and not to the LBP (Wang et al. 2007). These methods have also been used to study the binding of substrates into the CYP active sites in order to understand the structural basis of enzyme selectivity as well as the specific interactions between the substrate and different amino acid residues affecting the orientation of the substrate and thus, their metabolism (Lewis et al. 2006). The sites of metabolism have also been predicted by using docking methods (*e.g.* CYP2D6, de Graaf et al. 2006, Afzelius et al. 2007).

Molecular dynamics (MD) simulations calculate the time-dependent behaviour of a solvated receptor and ligand system and are able to provide detailed information on the fluctuations and conformational changes occurring in the protein (Carloni et al. 2002). The simulations are often used to refine structural models obtained by other methods since they are especially suited for studying flexible structures, such as loops, within a protein (*e.g.* Nurisso et al. 2012). For example, using the MD simulations, it has been shown that the flexibility of a CYP correlates with their substrate specificity, *i.e.* 1A2 and 2A6 were the most rigid and 3A4 most malleable of the enzymes studied (Skopalík et al. 2008, Hendrychová et al. 2011). The constitutive activity of CAR, the effects of long-range motions within the PXR LBD as well as AhR ligand interactions have been studied with MD simulations (Windshügel et al. 2005, Teotico et al. 2008, Jogalekar et al. 2010).

2.3.2 Cell-free assays

Cell-free assays are mainly based on either *in vitro* produced peptides or proteins, or proteins isolated from the liver tissue, which can be used to study protein-protein as well as protein-ligand interactions. These *in vitro* studies are generally accepted to be used as supportive and mainly qualitative studies (Brandon et al. 2003).

Metabolic assays. Specifically expressed human recombinant metabolic enzymes or their genetic variants are commercially available (Supersomes™, Becton Dickinson) and have been used extensively in early drug development (Bjornsson et al. 2003). In addition to CYPs, these cDNA-derived enzymes also include UGTs, FMOs and NATs (Pelkonen & Turpeinen 2007). NAPDH-regenerating system or NADPH alone, as well as uridine 5'-diphospho-glucuronic acid (UDPGA) or 3′-phosphoadenosine-5′-phosphosulfate (PAPS), are required in these assays as cofactors of CYPs and UGTs or SULTs, respectively. They are suitable for screening purposes and can be used to study substrate specificities as well as to generate metabolic products on a small scale (McGinnity & Riley 2001). They can also be used to study drug-interactions as demonstrated with fluvoxamine-theophylline interaction (CYP1A2) (Yao et al. 2001).

Liver homogenates, isolated from (pre-treated) animals or humans contain all Phase I and II enzymes (Brandon et al. 2003). The cytosolic fraction contains soluble Phase II enzymes (*e.g.*

NATs, GSTs, SULTs that require exogenous cofactors for catalytic activity) and can be obtained from a liver homogenate by differential centrifugation. The S9-fraction is a mixture of human liver microsomes (HLMs) and cytosol separated from the liver homogenate by low-speed centrifugation and it requires NADPH and other cofactors for functionality. The S9 fraction has been mainly used in metabolism studies, especially when primary hepatocytes have not been available, but also in conjunction with the Ames test, to evaluate the mutagenic potential of compounds and their metabolites (*e.g.* Hakura et al. 1999). In general, it may provide a more complete metabolic profile but their enzyme activity is lower than those of HLMs or cytosol. HLMs are derived from the S9 fraction by differential ultracentrifugation. They are the most widely used *in vitro* system for drug metabolism, especially in CYP inhibition studies, and they contain CYPs and UGTs but no cytosolic enzymes (Pelkonen et al. 2005). Thus, they do not fully resemble the physiological environment. The inhibition studies are usually carried out by analysing the inhibition of substrate metabolism using specific probe substrates and different analysis techniques, such as fluorometry or liquid chromatography/mass spectrometry (LC/MS) (Fowler & Zhang 2008). The activity of HLMs can vary substantially between individuals and thus, pooled microsomes are often used in experiments (Brandon et al. 2003). Furthermore, it has to be kept in mind that the results obtained from HLMs are not quantitative estimates of *in vivo* biotransformation due to the enrichment of CYPs and UGTs in the microsomes (Sidelmann et al. 1996). The absence of other enzymes may leave some of the potential *in vivo* metabolites unnoticed (Crommentuyn et al. 1998).

In general, subcellular fractions are easy to prepare, use and store. However, they do have their disadvantages, such as the possible loss of enzyme activity during preparation and the need for supplemental cofactors. Since the different enzymes and transporters involved in the sequential steps of drug metabolism and transport also reside in different subcellular fractions, the entire metabolic process cannot be studied with these systems.

Receptor assays. Different simple and high-throughput *in vitro* assays, based on the displacement of a radio-/fluorescently labeled high-affinity ligand by a test compound, can be used to study the binding of a ligand to a receptor (Raucy & Lasker 2010). In the scintillation proximity assay (SPA), the isolated receptors are bound to a bead and incubated with a test compound and a high-affinity radiolabeled ligand (***Figure 6A.***). The binding of nonradioactive ligands can be measured by their ability to compete with the radioligand (Wu & Liu 2005, Glickman et al. 2008). The assay is generally very flexible due to the ease of the adjustment of all the components and does not require separation of the bound from the free radioligand. A less direct approach is the coactivator receptor ligand assay (CARLA), in which the binding of an agonist results in a conformational change in the receptor, permitting an interaction with a radiolabeled coactivator protein, and the interaction can be measured by co-precipitation of the coactivator with the receptor of interest (pull-down assay, ***Figure 6B.***) (Krey et al. 1997, Kliewer et al. 2002). Compared to SPA, CARLA is more labour-intensive but has the advantage of not requiring a high-affinity radiolabeled ligand. Fluorescence polarization (FP) employs a fluorescently labeled molecule such as a coregulator peptide (***Figure 6C.***) (Parker et al. 2000). The technique can be used to analyze the interaction of two proteins in solution. Time-resolved fluorescence resonance energy transfer (TR-FRET) is a nonradioactive, homogenous proximity assay, which uses the transfer of energy between two fluorescent probes as well as the phenomenon of time-resolved fluorescence (***Figure 6D.***) (D'Souza et al. 2008, van Royen et al. 2009). The method has been used to study the interaction of coregulators and NRs labeled with different fluorophores. The Amplified Luminescent Proximity Homogenous Assay, or AlphaScreen, is a relatively new, sensitive and easy-to-use technology which has been applied in NR coregulator recruitment assays (***Figure 6E.***) (Rouleau et al. 2003). The

NR and coregulator are coupled to donor and acceptor beads. Upon excitation, the donor bead releases a singlet oxygen which will cause a luminescent signal to be released from the acceptor bead. Compared to TR-FRET, AlphaScreen has better sensitivity and a broader dynamic range (Glickman et al. 2002). In addition, it has been shown to be a very sensitive, versatile and robust to efficiently screen NR modulators (Rouleau et al. 2003).

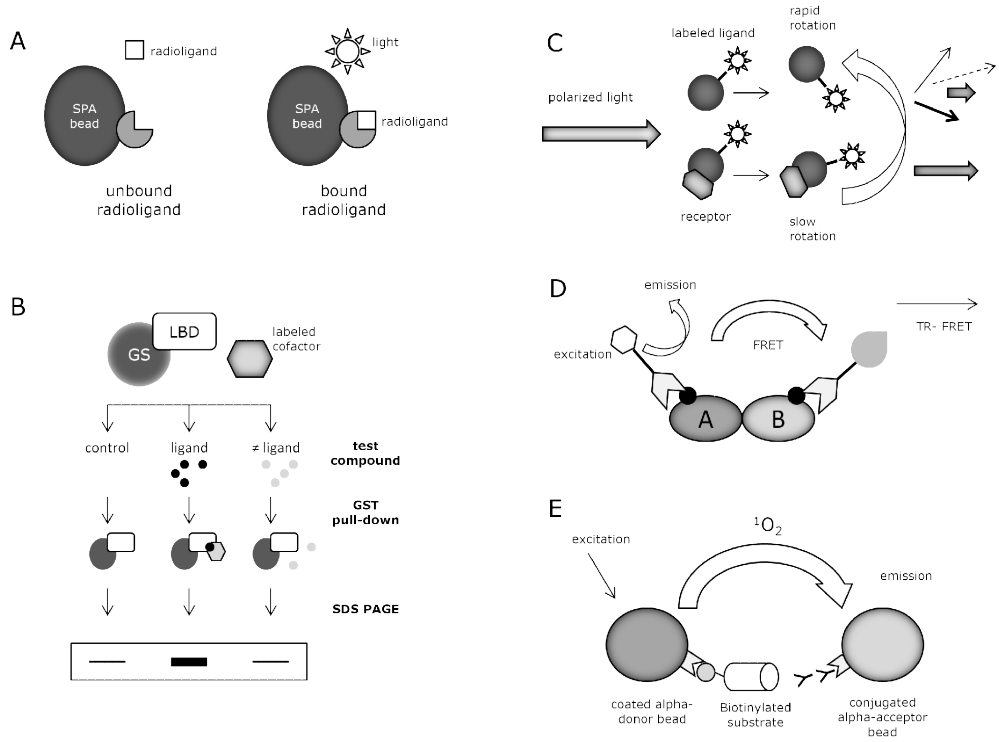

Figure 6. Principles of ligand-binding assays. ***A) SPA***: The LBD of a receptor (light grey) is affixed to a scintillant-containing bead and incubated with a radioligand (square). A bound radioligand is in close enough promixity to the bead and thus stimulates the scintillant in the bead to emit light, whereas the unbound radioligand or bound non-radioactive test ligand does not. ***B) CARLA***: A GST fusion protein of the receptor is bound to glutathione beads and the labeled coregulator is added together with the test compound. The protein-protein interaction is analyzed by SDS-PAGE. ***C) FP***: Protein bound fluorescent molecules, excited with a plane-polarized light, emit light into a fixed plane (polarized light) whereas unbound molecules emit light into a different plane due to faster rotation during excitation (depolarized light). The interaction between the protein and ligand can be evaluated by monitoring the change in polarization value. ***D) TR-FRET***: The coregulator peptide and NR are labeled with donor and acceptor fluorophores, respectively. Energy transfer to the acceptor fluorophore occurs upon excitation of the donor fluorophore, when they are in sufficient proximity (bound) and the emission from the acceptor fluorophore can be detected in a time-resolved manner. The level of light emitted is proportional to the degree of the donor-acceptor complex formation. ***E) Alpha Screen***: The hydrogel coated donor and acceptor beads are brought into close proximity by the interaction between two attached molecules. A singlet oxygen, converted by the donor bead upon excitation, reacts with the chemiluminescer in the acceptor bead, resulting in an activation of fluorophores in the same bead.

PXR assays are probably the most commonly and successfully used *in vitro* receptor-ligand binding assays mainly due to the simplicity of the ligand-based activation of the receptor

(*e.g.* Jones et al. 2000, Moore et al. 2000, Shukla et al. 2009). In contrast, due to the unique activation of CAR, ligand-binding assays are of only limited value in identifying CAR activators and, although sometimes used, the compounds identified by these methods have often proved to be poor inducers of CYP2B6 expression in human primary hepatocytes (HPHs) (Moore et al. 2000, Maglich et al. 2003, Li & Wang 2010). Some studies on AhR ligand affinities and mutations have been conducted using these assays (Delescluse et al. 2000, Backlund & Ingelman-Sundberg 2004). The problem with competition assays using TCDD is that weak ligands may not be able to compete for the binding to the receptor. While highly usable, these cell-free methods can produce false positive or negative results depending on the type of chemical tested. For example, compounds requiring active transport may never reach the receptor *in vivo* and thus, receptor-ligand binding assays can lead to false positives. A similar effect may be seen if the ligand binding does not evoke appropriate recruitment of coregulators and thus, a comparable response *in vivo* (*e.g.* paclitaxel and docetaxel with PXR) (Zhu et al. 2004, Harmsen et al. 2007).

2.3.3 Xenosensor transactivation assays

Different transactivation assays, such as cell-based reporter assays, are routinely used to study the receptor-mediated effects on gene transcription to screen NR and AhR ligands and to predict the CYP induction resulting from the activation of the receptor (Moore & Kliewer 2000, Kliewer et al. 2002, Zhu et al. 2004). Most often, the receptor cDNA, the NR-responsive reporter gene and a transfection control (*e.g.* β-galactosidase) are co-transfected transiently into a chosen cell line after which the cells are treated with selected test chemicals (Sinz et al. 2008, Raucy & Lasker, 2010). Due to the increased expression of the reporter in response to the ligand-receptor interaction, the extent of the receptor activation can be measured as an increased reporter activity (***Figure 7***.). A good reporter gene should be sensitive and easily measured and it should not affect the normal function of the cells. Commonly used reporter genes include luciferase (LUC), chloramphenicol acetyl-transferase (CAT) or secreted placental alkaline phosphatase (SEAP) (Schenborn & Groskreutz, 1999).

The receptor construct can include either a full-length or so-called chimeric receptor, in which the DBD of the NR has been replaced *e.g.* by yeast GAL4-DBD (1-hybrid assay) (Moore & Kliewer 2000, Kliewer et al. 2002). The main benefit of chimeric receptors is the common GAL4-driven reporter, which can be used for all receptors without prior knowledge of the DNA-binding properties or optimal response elements for each receptor. For full-length receptors, reporter constructs with natural *CYP3A4* and *CYP2B6* promoters have been used (Moore et al. 2000). Even though most continuous cell lines do not express significant levels of CAR and PXR, the effects of endogenous NRs on the expression levels of the reporter with natural promoter cannot be completely ruled out (Goodwin et al. 1999, El-Sankary et al. 2001). The full-length receptor requires either endogenous or a co-transfected dimerization partner (RXR) for normal function. Both assay types require endogenous coregulators to initiate transcription. The development of hCAR-based assays has been more difficult due to the high basal activity of the receptor, its spontaneous translocation to the nucleus in continuous cell lines and the suggested dual mechanism of activation (Li et al. 2009). Thus, transactivation assays are often complemented with nuclear translocation assays. Assays utilizing the newly discovered hCAR variant (CAR.3) have been found to be able to identify potential hCAR activators more easily due to the lower basal activity of the receptor (Auerbach et al. 2005, Faucette et al. 2007). AhR is endogenously expressed in most hepatic cell lines and AhR-activating chemicals have been mainly studied with the very sensitive chemically-activated luciferase expression (CALUX) assay (Murk et al. 1996, He et al. 2011). The system is based on stable transfection of a luciferase reporter, with TCDD-responsive CYP1A1 elements, into a hepatoma cell line

(Yueh et al. 2005). Examples of different xenosensor reporter assays are shown in ***Table 10.*** ***(Section 5.2.1***).

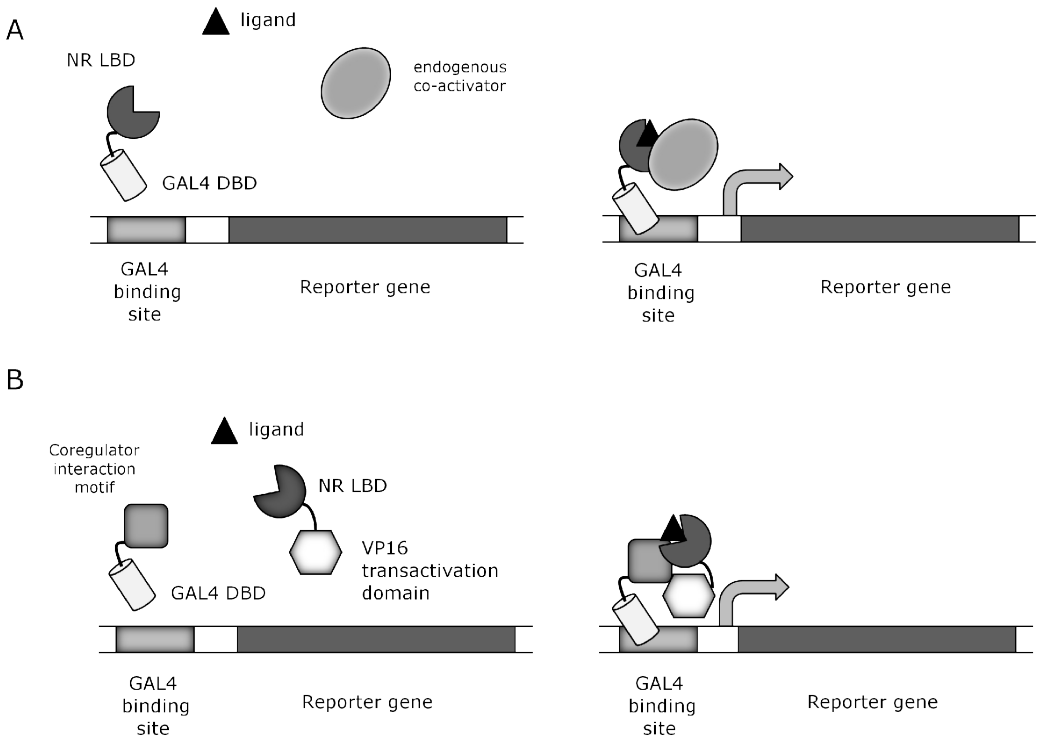

Figure 7. Principles of different NR transactivation assays. ***A) M1H***: The produced two-domain (GAL4-NRLBD) fusion protein controls the expression of the reporter gene in response to test compounds. ***B) M2H***: Two produced fusion proteins (GAL4-cofactor and NRLBD-AD) interact with each other in response to the binding of the ligand to NR LBD and control the expression the reporter gene.

Mammalian or yeast 2-hybrid assays (M2H or Y2H) have been used to study the interactions between coregulators and NRs (Fields & Song 1989, Gietz et al. 1997). The use of yeast cells is perhaps more straightforward due to the lack of endogenous NRs and coregulators, avoiding any competing interactions, but suffers from restricted compound permeability (Khazak et al. 2005). M2H assays use a coregulator, containing the NR interaction motifs, fused with yeast GAL4 DBD and the NR LBD fused with an activation domain (AD) of a strong transcriptional activator. These two constructs are co-transfected into the chosen mammalian cell and assayed to evaluate the interaction with a reporter construct. The binding of an agonist induces a conformational change in the NR LBD, resulting in the recruitment of the interacting cofactor peptide. Since no transcription of the reporter gene occurs unless the receptor and the coregulator protein interact, this assay allows detailed studies on ligand-dependent interactions between the receptor-coregulator pair.

The main problem with transient transfections is the variation in the transfection efficiency and thus, in the activation response. Stable lines, such as DPX2 for hPXR, have been developed to overcome these problems (Trubetskoy et al. 2005, Lemaire et al. 2007). Another option to streamline the procedure is to freeze a batch of transiently transfected

cells, which have been shown to produce similar results as the unfrozen cells (Zhu et al. 2007). The choice of the host cell line and culture conditions are generally important since it has an impact on the magnitude of the induction response (Goodwin et al. 1999). For example, differences in culture conditions are known to give different responses even with the established PXR ligands (over 10-fold difference in activation with rifampicin) (Stanley et al. 2006). In general, transactivation assays are useful for rapid screening of potential xenosensor activators. However, the overexpression of NRs and high ligand concentrations used create unphysiological conditions and the impact of the compounds on CYP activity *in vivo* cannot be directly predicted. Nonetheless, at least the PXR transactivation has been shown to correlate well with CYP3A4 mRNA levels and enzyme activity (Luo et al. 2002, Luo et al. 2004). Similarly to ligand binding assays, these techniques only assess the activation of one receptor at a time and the effects of crosstalk are not assessed (Wang & LeCluyse 2003). However, they are preferred over the *in vitro* ligand-binding assays to screen new chemical entities due to the better correlation with *in vivo* data and fewer false positives.

2.3.4 Hepatic cell models

The liver, together with pancreas are crucial in controlling the metabolism and structural modification of many compounds *e.g.* digested nutrients, hormones and exogenous compounds (Zaret & Grompe 2008). Structurally, the liver is formed by lobules, which are hexagonal structures consisting of the central vein surrounded by sheets of parenchymal hepatocytes and have a complicated blood circulation system (Turner et al. 2011). The lobuli are responsible for the majority of liver-specific functions. As the liver is the main site of the biotransformation of xenobiotics, hepatic *in vitro* models are of considerable interest. Hepatocytes are highly differentiated and polarized cells and their functional properties are at least partly dependent on their location within the lobules. The normal function of hepatocytes is greatly affected by the liver architecture and the communication between hepatocytes and non-parenchymal cells. The liver can regenerate after many insults or injuries by activating the mitosis of mature hepatocytes, meaning that these cells possess a stem-cell like regenerative capacity (Zaret & Grompe 2008). Despite this extensive regenerative capacity, it has proven difficult to grow and expand hepatocytes or to maintain their differentiation state in culture. The common problem with all hepatic cell lines is the need for cell-cell or cell-matrix contacts or the requirement for special growth factors and other media components for their differentiated function.

2.3.4.1 Human primary hepatocytes

Isolated human primary hepatocytes (HPHs) are used extensively as an *in vitro* model not only in metabolism studies but also to study the physiology and metabolism in the liver. Due to their resemblance to hepatocytes *in vivo* and their well-characterized properties, they are considered to be the golden standard and are an FDA and EMA -approved method for drug metabolism and interaction studies (LeCluyse et al. 2005, Gómez-Lechón et al. 2007, Hewitt et al. 2007, EMA Guideline on the Investigation of Drug Interactions 2010 – 29.6.2012, USFDA Draft Guidance for Industry 2012 – 6.4.2012).

The isolation of viable HPHs is a slow and difficult process, which often causes cellular damage (Li 2007). Mixtures of hepatocytes from different donors have been used to overcome the large variability in the CYP expression and inducibility between different cell lots. This variability can result from genetic variations or simply from the age, gender and prior chemical exposure or illness of the donor as well as the source of the cells (surplus tissues) (Gómez-Lechón et al. 2007, ***Figure 5.***). After isolation, the cells can be maintained and used in suspension for a few hours or as monolayer cultures for up to several weeks (Elaut et al. 2006). However, cultured hepatocytes do not proliferate and show a loss of

liver-specific functions, such as a rapid and uneven decline of CYP expression and activity, over time (Gómez-Lechón et al. 2007). Supporting the cell-cell interactions, along with adequate culture media and appropriate 3D structure of the culture, are important for prolonging cell survival and for maintaining their differentiation state (***Section 2.3.6***).

Due to the limited supply of fresh hepatocytes, different cryopreservation techniques have been developed for the long-term storage of these cells. The established techniques have provided viable hepatocytes with inducible CYP enzymes, which can produce qualitatively similar results as freshly isolated hepatocytes (Schehrer et al. 2000, Roymans et al. 2005, Chu et al. 2009). The advantages of cryopreservation are that experiments can be planned in advance and repeated experiments can be conducted with cells from the same donor in order to minimize the variation between responses. Cryopreserved cells are also approved for metabolism and induction studies by the FDA and EMA. Fetal hepatocytes proliferate in culture but the regulation of CYPs and the general CYP profiles differ from adult hepatocytes. For example, these cells show only marginal PXR-dependent induction and express high amounts of CYP3A7 (Pelkonen et al. 2008).

2.3.4.2 Continuous hepatic cell lines

Various continuous hepatic cell lines have been used in the early ADME studies due to their better availability and ease of culturing compared to HPHs. However, even though some liver-specific functions are retained, the unstable genome and strongly altered phenotype of hepatic cell lines limit their use in drug metabolism studies.

Tumour-derived cells. Hepatomas, also known as hepatocellular carcinomas (HCC), and hepatoblastomas are two types of primary liver cancers. Cells derived from both tumour types are commonly referred to as hepatoma cell lines, regardless of their origin (Donato et al. 2008). Most hepatoma cell lines have maintained certain properties of normal hepatocytes, such as the ability to secrete albumin. For drug metabolism studies, the main problem has been the low or non-existing expression and inducibility of CYPs or other DMEs and DTs. This is thought to be mainly due to a decrease in transcription rather than impaired CYP enzymatic function (Donato et al. 2008). In addition, the former property has been thought to be due to the lower expression or activity of the key regulators, such as HNFs, C/EBPs, CAR and PXR or their coregulators (Martinez-Jimenez et al. 2006, Benet et al. 2010).

The xenosensor and CYP expression profiles of the most commonly used hepatoma cell lines are shown in ***Table 4.*** The HepG2 cell line is the most widely used hepatoma cell line even though the expression and activity of the major CYPs, as well as UGTs, are lower (0.1-10% mRNA expression) than in HPHs (Rodríguez-Antona et al. 2002, Westerink & Schoonen 2007a,b, Hariparsad et al. 2008). The relatively new cell line, HepaRG, has notably higher CYP expression and activity levels than the other hepatoma cell lines, but requires a relatively long differentiation period (Aninat et al. 2006, Guillouzo et al. 2007). For many of these cell lines, the level of CYP activity depends on the origin of the cells, culture conditions, growth status and possibly also on clonal selection in different laboratories (Hewitt & Hewitt 2004). Another problem is the heterogeneity and/or different subtypes present in the culture (Rencurel et al. 2005, Kanebratt & Andersson 2008a,b). For example, subtypes of HepG2 have been enriched and several cell lines appear to exhibit higher CYP induction potentials (*e.g.* WGA, Rencurel et al. 2005). HepaRG also tends to present two cell types at confluency: Cholangiocytes and hepatocytes. After culturing with 2% DMSO and 50 μM hydrocortisone hemisuccinate, the hepatocytes make up approximately 50% of the total cell population and form structures resembling bile

canaliculi (Cerec et al. 2007). Due to this heterogeneity, the extrapolations to *in vivo* hepatic induction are not clear because the contributions from the other cell subtypes in the hepatoma culture is not known.

Despite these problems and weaknesses, hepatoma cell lines are being used in DM studies as well as in some clinical applications (Donato et al. 2008). With the exception of the HepaRG line, they are not suitable for direct *in vivo* comparisons. However, they can provide useful information on the metabolism and possible toxicity of new chemical entities in the early drug discovery process. Most of these cell lines express AhR and ARNT endogenously and thus can be used for assessing ethoxyresorufin-O-deethylase (EROD) activity or quantitative RT-PCR assays of CYP1A1 but not the predominant CYP1A isoform, CYP1A2 (Hewitt & Hewitt 2004, Westerink & Schoonen 2007b).

Immortalized cells. Replicative senescence is a phenomenon by which cells lose their ability to divide and thus remain in the G1 phase of the cell cycle (Cascio 2001, O'Hare et al. 2001). Shortening of telomeres (the repetitive end sequences of eukaryotic chromosomes) in humans induces senescence and is thought to prevent genomic instability (Cascio 2001). In most eukaryotic organisms, telomerases (ribonucleo-proteins maintaining telomere ends) are active only in stem cells, germ cells and in certain white blood cells (Hahn & Meyerson 2001).

The choice of the immortalization method depends on the properties of the cells, such as the intrinsic telomerase activity (O'Hare et al. 2001). However, several secondary factors, such as factors preventing proliferation or oxidative stress, can also affect the immortalization results. Most immortalization methods used for hepatic cells have involved the use of viral genes, most commonly the Simian virus 40 T antigen (SV40T) (Ali & DeCaprio 2001). SV40T inactivates tumor suppressor genes (*e.g.* p53, pRb), but may also induce telomerase activity in the infected cells. Viral oncogenes appear to disrupt many cellular pathways and evoke undesirable changes, such as loss of differentiated properties (Yeager & Reddel 1999). Most of the SV40T-immortalized adult hepatocyte lines express several CYP enzymes (e.g. 1A2, 2C9 and 3A4) both at the mRNA and enzyme activity levels (Pfeifer 1993, Akiyama et al. 2004, Mills et al. 2004, Ripp et al. 2006, Hariparsad et al. 2008). The Fa2N-4 line, grown on collagen in a proprietary medium, has been used for the assessment of CYP induction (Ripp et al. 2006). Cells immortalized by the overexpression of telomerase (TERT), such as TTNT-1, have been shown to maintain a stable genotype and retain critical phenotypic markers (Hahn & Meyerson 2001, Wege et al. 2003). hTERT transfection has also been used in the differentiation of mesenchymal stem cells into hepatocyte-like cells (Liang et al. 2012).

Reversible or conditional immortalization can be achieved by the Cre-loxP system or temperature sensitive SV40T (SVtsA58), which can be inactivated by increasing the temperature (Cascio 2001, Daniele et al. 2002, Kobayashi et al. 2003). The immortalizing gene is introduced into the cell in a vector between two loxP sites. After expanding the immortalized cells in culture, the reversion can be achieved by transduction of Cre recombinase and thus, removal of the inserted gene. The main drawback of these strategies is the possible residual activity of the immortalising agent even after reversion (Hoekstra & Chamuleau 2002). A reversibly immortalized adult hepatocyte line, NKNT-3, is morphologically similar to hepatocytes and is functional *in vitro*, but the expression levels or activities for CYP enzymes are somewhat limited (Kobayashi et al. 2000, Hoekstra et al. 2006). In addition, the cell line has been shown to be heterogenous and genetically unstable.

The immortalization of primary cells was thought to be one way to provide functional and differentiated cells in large quantities in order to compensate for the lack of homogenous populations of hepatocytes. However, they do not function as normal cells due to the mutations and karyotypic changes caused by the immortalization process and they have an increased sensitivity towards neoplastic transformation (Drubin & Clawson 2004). Similarly to tumour-derived cells, the expression of CAR and several hepatic uptake transporters are also significantly lower than in HPHs (Hariparsad et al. 2008).

Stem cell -derived cell lines. In order to be defined as a stem cell, a cell must possess two properties: 1) The ability to divide continuously, while maintaining the undifferentiated state (self-renewal) and 2) The capacity to differentiate into specialized cell types (Weissman et al. 2001). In adult tissues, somatic stem cells participate in the renewal or regeneration of damaged tissues and are usually multipotent (Alison & Islam 2009). Embryonic stem cells (ESCs) are pluripotent cells found in the inner cell mass of the blastocyst (Hadjantonakis & Papaioannou 2001). Induced pluripotent stem (iPS) cells can be artificially derived from adult somatic cells by inducing expression of stem-cell expressed transcription factors (*e.g.* Oct4, Sox2, Klf4 and c-Myc) and have quite similar properties as ESCs (Takahashi et al. 2007).

The differentiation of cell types during embryogenesis is mainly based on the presence of critical growth factors, which induce cell type -specific expression of genes. *In vivo*, this process consists of fine-tuned signalling, which is difficult to imitate in *in vitro* culture conditions. The described phases of hepatic differentiation vary between groups and the effects of individual factors have not been systematically studied. The first step is the differentiation of the endoderm from the epiblast, a process regulated by TGF β-related Nodal and Wnt-signaling -proteins (D'amour et al. 2005). The differentiation of hepatoblasts from the endoderm requires the presence of proteins from the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families (Lemaigre 2009). Further differentiation also involves the hepatocyte growth factors (HGF), epidermal growth factor (EGF) and oncostatin M (OSM). Transcription factors, such as Oct4, Nanog, FoxA2, Sox17, HNF and c/EBP, are expressed in the different phases of the differentiation process and can be monitored during the *in vitro* differentiation (Keller 2005, Lemaigre 2009, Takayama 2012). Normally, an adult hepatocyte should not express alpha-fetoprotein (AFP) and the expression of CYP3A7 should decrease while the expression of CYP3A4 increases (Daly 2006, Meier et al. 2006). The *in vitro* differentiation approaches used with human ESCs often rely on providing the established growth factors with minor adjustments, such as the replacement of Nodal proteins with Activin A. The differentiation into endoderm prior to growth factor addition significantly enhances the differentiation into hepatocyte-like cells (Hay et al. 2008). In addition to growth factors, the differentiation protocols sometimes involve the use of other cell types or matrices (Basma et al. 2009, Pei et al. 2009). Hepatocyte differentiation continues during embryonic development and birth; thus a short *in vitro* differentiation protocol may be impossible to develop (Lemaigre 2009).

Adult stem cells with stem-cell like properties have also been used to create hepatocyte-like cells. The liver contains cells which, unlike primary hepatocytes, can be cultured and possess some differentiation capacity but their exact function is unknown (Herrera et al. 2006, Alison 2009, Fonsato et al. 2010). When these cells are grown with HGF and FGF, they start to differentiate into hepatocyte-like cells but this process requires the use of a special bioreactor. Mesenchymal stem cells of bone marrow or adipose tissues and blood monocytes have been coaxed by the above-mentioned growth factors to differentiate into hepatocyte-like cells (*e.g.* Ruhnke et al. 2005, Sato et al. 2005, Ong et al. 2006, Zheng et al.

2008, Saulnier et al. 2009, Snykers et al. 2011). The iPS cells have also been used to generate hepatocyte-like cells using similar protocols as with the ESCs (Si-Tayeb et al. 2010, Sullivan et al. 2010). The presence of iPS cells has also enabled the generation of hepatocyte-like cells with different genotypes, which could be used to study the interindividual differences in drug metabolism (Sullivan et al. 2010).

Adult cells do not suffer from similar ethical problems as ESCs and would be more readily available. If successfully differentiated, these cells could provide a good source for *in vitro* DM testing (Pouton & Haynes 2005). The cells could also be used in bioartificial liver devices and for hepatocyte transplantation to treat metabolic liver diseases (Nussler et al. 2006). One problem is that the cells may later transdifferentiate into possibly cancerous cells in the body. Some of the differentiated hepatocyte-like cells and their properties are presented in ***Table 5.*** Often, the cells have not been adequately characterized especially for their DM properties and their suitability for DM studies is difficult to assess.

2.3.4.3 Modifications on hepatic cell lines

A differentiated cell in any tissue has a limited ability to proliferate. Conversely, an increase in the proliferation rate effectively means a decrease in differentiation. All cell types also adapt to the *in vitro* environment through physiological or morphological changes. The common cell culture plate and growth media can keep the cells alive but may not maintain their *in vivo* differentiated properties. Thus, different modifications have been applied to increase the expression and functionality of DMEs in both HPHs and continuous cell lines.

The most direct way to increase protein expression is to transfect the desired gene into a cell line. For example, the HepG2 cell line has been transfected with various CYP cDNAs, which are expressed for long periods of time (Yoshitomi et al. 2001, Tolosa et al. 2011). However, since *in vivo*, multiple enzymes are responsible for the metabolism of xenobiotics, a cell line overexpressing a single CYP gene can only be used to study the metabolism of a compound by that particular enzyme. Controlled expression of transfected genes can be achieved in several ways, *e.g.* by a doxycycline-inducible gene regulation or by adenoviral vectors (Goldring et al. 2006, Aoyama et al. 2009). The controlled expression of CYPs could allow tuning of the protein levels to resemble those observed *in vivo*. In addition, the lack of transcription factors could be one reason for the low CYP expression in hepatoma cell lines (Aninat et al. 2006, Ding et al. 2006, Hariparsad et al. 2008, Pelkonen et al. 2008). A stable transfection of CAR and PXR into the small intestine model Caco-2 has increased the expression of several CYP mRNAs (Korjamo et al. 2005 and 2006) and, the transfection of C/EPBα into HepG2 has also increased the expression of CYP2 family members but the CYP3A4 expression requires cotransfection of HNF3γ. Similarly, the expression and activity levels of CYP2B6 approach those of HPHs after transfection of C/EPBα, HNF4α and CAR to HepG2 (Jover et al. 1998, Rodríguez-Antona 2003, Benet et al. 2010). Due to the complexity of the regulation of xenobiotic metabolism, especially the number of factors involved, the restoration of a hepatocyte-like phenotype in hepatoma cells by gene transfers may be impossible.

Multiple chemicals or media formulations increase the expression or activity of CYP enzymes and other factors in HPHs and hepatoma cell lines (***Table 6.***). This indicates that these agents are able to enhance the maintenance of hepatic functions or to promote the re-differentiation in the cells. It should be kept in mind that some of the compounds may also act as xenosensor ligands (*e.g.* retinoic acid and CAR) and thus, the increased expression of CYPs would be due to induction and not differentiation (Chen et al. 2010b). The medium formulation is especially crucial for HPHs if they are to maintain their specific functions.

Table 4. Properties and applications of the most commonly used human hepatoma cell lines.

Cell line	mRNA expression			Properties and applications	*References*
	Receptor	**CYP**	**Other**		
HepG2/C3A (male, 15y)	AhR PXR (low) HNF4α	1A1/2* 2A6 2B* 2C9/19 2D6 2E1 3A4*/5/7	UGTs* GSTs*/** SULTs*/** NATs** EPHX1*/**	**HepG2:** Adherent and epithelial-like. Secretes plasma proteins (*e.g.* albumin). Shows significant changes in gene expression during culture and the source of the cells as well as passage number or different culture conditions may lead to varying results. Widely used in drug metabolism, induction and toxicity assays regardless of the lack of adequate CYP expression or inducibility. **C3A:** Clonal derivative of HepG2. Exhibits strong contact inhibition, secretes plasma proteins (*e.g.* albumin) and can be grown in glucose-free medium. Mainly used in bioartificial liver (BAL) devices on different culture platforms or conditions due to the production of albumin and clotting factors and expression of normal metabolic pathways (urea- and gluconeogenesis) as well as some P450 activity.	*Aden et al. 1979* *Rodriguez-Antona et al. 2002* *Wilkening & Bader 2003* *Hewitt & Hewitt 2004* *Medina-Diaz & Elizondo 2005 Westerink & Schoonen 2007 a & b* *Wang et al. 1998* *Filippi et al. 2004* *Elkayam et al. 2006* *Mavri-Damelin et al. 2008*
Hep3B (male, 8y)		1A1/2 2B, 2E1 3A4	GST NAT1 SULT 1A1/3	Adherent and epithelial-like. Secrete plasma proteins (*e.g.* albumin). Poor phase I enzyme activity (except for CYP1A1). Higher phase II enzyme activity than in HepG2. Mainly used to study apoptosis and toxicity.	*Knowles et al. 1980* *Fukuda et al. 1992* *Brandon et al. 2003* *Majer et al. 2004* *Zhu et al. 2006*
BC2 (male, 61y)		1A1/2* 2A6, 2B6(*), 2C9, 2D6, 2E1 3A4	GST UGT	Differentiates at confluency and remains differentiated for several weeks. Displays a more appropriate metabolic profile than HepG2. Mainly used in (repeated) toxicity studies, to generate metabolites as well as in some induction studies. A subpopulation (ADV-1) has higher drug-metabolizing enzyme activity than the parent cell line.	*Glaise et al. 1998* *Ingster et al. 1996* *Gomez-Lechon et al. 2001* *Fabre et al. 2003* *O'Connor et al. 2005*

Cell line	mRNA expression			Properties and applications	*References*
	Receptor	**CYP**	**Other**		
Huh7 (male, 57y)	PXR CAR HNF4α	2C19 2D6 3A4* 3A5	UGTs	Secretes plasma proteins (*e.g.* AFP, albumin). Acquires a more differentiated (polarized) phenotype when cultured in 3D or at confluency. Contains a mutation associated with hereditary hemochromatosis. Used in various studies related to hepatitis virus C infection, hepatotoxicity and gene regulation.	*Nakabayashi et al. 1982* *Sainz et al. 2009* *Sivertsson et al. 2010* *Vecchi et al. 2010* *Meex et al. 2011*
HepaRG (female, ?)	AhR** PXR** CAR PPARα**	1A1/2*/** 2A6*** 2B6* 2C8/9/19* *2D6**** *2E1**** 3A4*	OCT-1 OATP-B**/C/8 BSEP* MRP2*/3** NTCP MDR1*/** GSTA1/2/4** GSTM1 UGT1A1	Differentiated (requires DMSO and hydrocortisone) cells derived from liver progenitor cell line. Hepatic morphology. Higher phase I and phase II enzyme expression and activity than in other hepatoma cell lines but CYP induction potential is dependent on culture conditions. Long-term culture stability enables long *in vitro* toxicity studies. Has been used for studies on metabolism, uptake as well as induction screening.	*Gripon et al. 2002* *Aninat et al. 2006* *LeVee et al. 2006* *Guillouzo et al. 2007* *Kanebratt & Andersson 2008a,b* *Josse et al. 2008 and 2011* *Turpeinen et al. 2009* *Anthérieu et al. 2010* *Hart et al. 2010* *Laurent et al. 2010* *Gerets et al. 2012*

*Inducible by classical inducers (HepaRG cultured without DMSO after differentiation), **In general, the expression levels of genes involved in xenobiotic metabolism are significantly lower than in primary hepatocytes but the expression levels of the marked gene transcripts have been shown to be similar or higher than those of primary hepatocytes, ***Expression levels and activities of CYP2A6 and 2E1 are low even in the differentiated HepaRG cells and become undetectable after 3 days in DMSO-free medium. Also the CYP2D6 expression and activity are lower than other CYPs, suggesting a poor metabolizer phenotype of the donor. However, the formation of the CYP2D6 probe substrate metabolite is clearly detectable in HepaRG cultures.

Table 5. Examples of hepatocyte-like cells differentiated from various human (stem) cell types.

Origin	**Gene expression (differentiated cells)[a]**			**Other properties (differentiated cells)[a]**	***References***
	TFs CoFs	**CYPs**	**Other**		
ESC	AhR CAR C/EBPα GATA4 GR HNF1α/β HNF4α PGC1α PXR SRC1	1A2 2D6 1A1 *(up to ~adult liver, inducible)* 2B6 *(<adult liver, inducible)* 2C9/19 *(<adult liver, inducible)* 3A4* *(<adult liver, inducible)* 3A7 *(<fetal liver, >adult liver)*	AAT AFP ALB CD26/54 CK7/8/9 CK18/19 UGT1A1 MRP2 OATP2	The differentiated cells have a polygonal shape and (partly) double nuclei. The cells exhibit some hepatocyte-like functions, such as albumin and urea synthesis and secretion, LDL uptake and glycogen storage. The cells are able to integrate into the liver of a CCl4-treated adult mouse and display characteristics of differentiated hepatocytes. *HNF4α transduction produces cells in which the induction of CYP3A4 is similar to primary hepatocytes.	*Cai et al. 2007* *Ek et al. 2007* *Agarwal et al. 2008* *Hay et al. 2008* *Basma et al. 2009* *Brolén et al. 2010* *Synnergren et al. 2010* *Touboul et al. 2010* *Funakoshi et al. 2011* *Takayama et al. 2011** *Yu et al. 2012*
Liver progenitor	C/EBPα HNF1α HNF4α	1A1 2B6 2C9 2D6 2E1 3A5	AFP ALB CK8/18	The differentiated cells exhibit hepatocyte-like functions such as glycogen synthesis and storage and albumin production. The expression of drug metabolizing CYPs is lower than in primary hepatocytes but at least CYP7A1 is expressed at similar levels. The cells are able to integrate to the liver of a mouse.	*Chen et al. 2010a* *Fonsato et al. 2010*
Adipose tissue	C/EBPβ HNF4α	1A1 *(functional)* 2C9 2E1 3A4	AAT AFP ALB CD26 CK18/19 TDO2	The differentiated cells have round epithelial morphology, tight cell to cell interactions and visible bile canaliculi. Exhibit hepatocyte-like functions, such as urea and albumin synthesis (approx 50% of primary hepatocytes), LDL uptake and glycogen storage. Incorporate into the liver of a CCl4-treated adult mouse.	*Seo et al. 2005* *Timper et al. 2006* *Banas et al. 2007* *Taléns-Visconti et al. 2007* *Aurich et al. 2009* *Zemel et al. 2009* *Lue et al. 2010* *Goradeghini et al. 2010* *Puglisi et al. 2011*

Origin	Gene expression (differentiated cells)[a]			Other properties (differentiated cells)[a]	*References*
	TFs CoFs	**CYPs**	**Other**		
Bone marrow	C/EBPα GATA4 HNF1α HNF4α	1A1/2 2B6 *(inducible)*	AFP ALB CD26 CK18/19	The differentiated cells have a polygonal shape, double nuclei and a small percentage of them are polarized. Exhibit hepatocyte-like functions, such as albumin and urea secretion, LDL uptake and glycogen storage.	*Schwartz et al. 2002* *Snykers et al. 2006* *Ishii et al. 2008* *Chivu et al. 2009* *Lin et al. 2010* *Ayatollahi et al. 2011*
iPS	C/EBPβ GATA4 HNF4α HNF6	CYP2A6 CYP3A4	AFP ALB CK8 CK18/19 PEPCK TDO2	Morphologically similar to hepatocytes. Exhibit some hepatocyte-like functions, such as LDL uptake, urea synthesis, albumin secretion and glycogen storage.	*Song et al. 2009* *Si-Tayeb et al. 2010* *Takata et al. 2011* *Ishikawa et al. 2012*

Comparisons of expression levels or functionality have often made between differentiated cells from different origins, between differentiated cells and hepatoma cell lines or between differentiation protocols, not between differentiated cells and hepatocytes/liver. Comparisons with primary hepatocytes/liver are presented, when available in the original publications. [a]Either mRNA or protein levels. CYPs important to drug metabolism are shown. In addition, most of the cell lines express CYP7A1. AAT=alpha-1-antitrypsin, AFP=alpha-fetoprotein, ALB=albumin, CD26=dipeptidyl-peptidase IV, CD54=intercellular adhesion molecule-1 CK=cytokeratin, GATA4=GATA binding protein 4, GR=glucocorticoid receptor, PEPCK=phosphoenolpyruvate carboxykinase, TDO2= tryptophan 2,3-dioxygenase.

Table 6. Examples of modifications on hepatoma and HPH cultures.

Modification	Hepatoma cell lines	Primary hepatocytes	*References*
Chemicals			
Insulin	Modest (2-3-fold) increase in hepatic marker and albumin expression.	Enhances cell response to growth factors *in vivo*. CYP2E1 activity increased / CYP3A activity decreased *in vitro* (*rat*).	*Kang-Park et al. 1995 Woodcroft & Novak 1997, 1999*
DEX[a]	Increases CYP3A4 expression together with rifampicin (HepG2).	Stabilization of morphology and function (long-term cultures). < 2-fold increase in CYP mRNA expression and activity (2B and 3A).	*Luo et al. 2002 Faucette et al. 2004 Maruyama et al. 2007*
DMSO	Increase in NR and CYP expression and CYP activity. Decreased induction by classical inducers (HepaRG).	Max. 3-fold increase in the CYP1, CYP2 and CYP3A mRNA expression.	*Nishimura et al. 2003 Aninat et al. 2006*
TSA[a]	Increase in the expression of hepatic transcription factors (HepG2). Changes in morphology and growth.	Promotes functional and morphological differentiation and prevents apoptosis. Positively affects the CYP-mediated biotransformation (*rat*).	*Yamashita et al. 2003 Henkens et al. 2007*
Vitamin E	2-fold increase in CYP3A activity	3-15 -fold increase in CYP3A and CYP2 mRNA expression.	*Landes et al. 2003 Brigelius-Flohe 2005*
Culture conditions			
Confluency	Increased CYP activity, albumin and transcription factor expression and hepatocyte-like morphology (BC2, Huh7).[b]	90% confluence recommended to avoid detaching. Cell-cell contacts are important for maintaining differentiated properties.	*Gómez-Lechon et al. 2001 Hamilton et al. 2001a Sivertsson et al. 2010*
Co-culture	Increase in CYP expression (HepG2 + bovine endothelial cells).	Longer functional lifespan (fibroblasts/biliary epithelial cells, *rat*)	*Hamilton et al. 2001a,b Ohno et al. 2008*
Matrigel	No effect (HepG2). Increased CYP1A1/2 activity (C3A).	Improves morphology, increase in expression of genes involved in drug metabolism and decrease in the expression of some proteins, *e.g.* cytokeratins.	*Page et al. 2007 Sung et al. 2009*

[a] DEX = dexamethasone, TSA=Trichostatin A (antifungal antibiotic and mammalian HDAC inhibitor) [b] Polygonal shape and two nuclei

The use of serum is not recommended due to the changes in hepatic morphology, such as the inhibition of bile canaliculi formation (Runge et al. 2000, Tuschl et al. 2009).

The different matrix components of the epithelial cells of bile ducts and blood vessels form the extracellular matrix (ECM) scaffolds to which hepatocytes attach *in vivo*. They provide mechanical integrity and act as dynamic modulators of various cellular processes. This essential environment has been simulated by different culture matrices, *e.g.* the ECM isolated from liver tissue or co-culture of epithelial or mesenchymal cells (Castell & Gómez-Lechón 2009). In comparison to conventional monolayer culturs on collagen I, often used to culture HPHs, a sandwich culture can enhance the expression of many liver specific proteins, such as albumin, and to maintain these specialized functions for a longer period of time (LeCluyse 2001, LeCluyse et al. 2005, Meng 2010). The sandwich culture provides a more liver-like morphology with functional bile canaliculi and correct localization of efflux transporters and thus, allows studies on hepatic metabolism, transport-mediated uptake and biliary excretion of substrates for example to determine mechanisms of hepatotoxicity (Sahi 2006, Ogimura et al. 2011, Schaefer et al. 2012).

Different matrices have also been used for hepatoma cell lines. HepG2 and C3A cell lines form cell clusters on 3D polystyrene matrices or natural hydrogels, such as Matrigel and alginate, which can promote differentiation as shown by the increase of albumin or CYP expression (Elkayam et al. 2006, Bokhari et al. 2007). When culturing continuous cell lines, the cells are normally split either before or at confluency (Gómez-Lechón et al. 2001), which prevents any long-lasting contacts with neighbouring cells. For example, the BC2, Huh7 and HepaRG cell lines require a period of confluency for differentiation (***Table 4.***, Gómez-Lechón et al. 2001, Aninat et al. 2006). In contrast, growing HepG2 cells in confluent cultures does not seem to alter CYP expression (Ohno et al. 2008).

2.3.5 Liver slices and perfused liver

Precision-cut liver slices are physiologically relevant models for the metabolism of xenobiotics, mainly because they retain an intact cellular tissue architecture, a wide range of inducible Phase I and II enzymes and cofactors as well as transport proteins (Ekins et al. 2000, Edwards et al. 2003, Jia & Liu 2007, Olinga et al. 2008). Due to the poor circulation, poor permeability or slow transport through the cell layers, the metabolism is generally slower in liver slices than in HPHs. Thus, the thickness of the slice is crucial both for the cellular and architectural integrity and for adequate diffusion. A perfused liver would be the best model of the *in vivo* situation, but so far, human livers have not been used and experiments on animal livers have been performed only a very small scale (Brandon et al. 2003). The main problems associated with both models are their suitability only for short-term studies, poor reproducibility of the experiments and species specific differences when using animal-derived tissues (Brandon et al. 2003). Both methods are also labour-intensive and require special instrumentation. Even though the xenosensor pathways and thus, the induction of DMEs and DTs, are functional, these methods have been used almost exclusively in metabolism and toxicity studies (Vermeir et al. 2005, Olinga et al. 2008, Elferink et al. 2011).

2.3.6 *In vivo* models

In vivo models, *i.e.* laboratory animals and human clinical trials, incorporate the parallel dynamic ADME processes missing from static *in vitro* assays. Although some induction studies of CYP3A4 with known inducers have been conducted to compare the activity between healthy volunteers and isolated HPHs, human clinical studies are mainly used in the late development phases of new drug molecules (*e.g.* McCune et al. 2000). The use of

animal models is associated with the problem that the data cannot be extrapolated to humans due to species differences in the isoforms, catalytic activities and expression levels of CYPs (Martignoni et al. 2006, Pelkonen et al. 2008, Puccinelli et al. 2011). As discussed earlier, also xenosensors display similar variation.

The mouse has a shorter lifespan, faster breeding, larger litter sizes and lower maintenance costs compared to other rodents or larger animal models and is thus the most widely used animal model (Cheung & Gonzalez 2008). In attempts to overcome the problems arising from species differences, various knock-out (loss of function), transgenic (gain of function) and humanized mouse models have been developed for the xenosensors and many CYPs (Lin 2008). Both PXR and CAR null mice have a normal phenotype, are viable and fertile, but the induction of *Cyp3a11* and *Cyp2b10*, respectively, is lost. These animals do not metabolize endo- or exogenous toxicants effectively and are thus prone to serious liver damage (Staudinger et al. 2001, Zhang et al. 2002). The CAR null mouse has been important in the discovery of the role of CAR in bilirubin and bile acid metabolism (Huang et al. 2003). The AhR knockout mice exhibit decreased liver size, decreased expression of *CYP1A2* as well as resistance to TCDD-elicited CYP1A1 induction (Gonzalez & Fernandez-Salguero 1998, Mimura & Fujii-Kuriyama 2003). Most of the CYP null mice have a normal phenotype and the loss of enzymes in general does not result in any significant changes in development or cause any abnormal physiology (Gonzalez 2003).

A transgenic or humanized mouse can be generated by various techniques (Cheung & Gonzalez 2008). Since most transcription factors are conserved in mammals, it is assumed that the replacement of the mouse TF by the human counterpart will maintain regulation of the target gene in the transgenic animal. The simplest approach is to introduce a human xenosensor- or CYP-coding cDNA, under a tissue-specific promoter controlling the expression of the gene, or a whole human gene, containing all of the regulatory elements, into an animal by pronuclei injection or by using genomic clones, respectively. Since the resulting animals still harbour the corresponding murine genes, they may exhibit overlapping functions and this may interfere with the expression or function of the human transgene. Thus, a better approach is to introduce the human transgene into a knockout animal either by direct incorporation (knock-in) of the human gene (cDNA) to the site of the endogenous mouse gene or by breeding the hNR transgene into the mNR knockout background. The xenosensor or CYP-humanized mice could be used to predict potential toxicological and metabolic problems and to overcome the species differences in the *in vivo* drug metabolism studies.

Different transgenic PXR mice have been created by fusing the hPXR cDNA to albumin or fatty acid binding protein (FABP)-promoters with or without the (constitutively active) VP16 coactivator and breeding the transgene onto the murine *Pxr*-null background (Xie et al. 2000, Zhou et al. 2006). In these mice, the endogenous *Cyp3a11* gene is induced by known human PXR inducers, such as rifampicin, but only minimally by the mouse PXR ligand PCN. Similarly, CAR-humanized mice have been produced with the albumin promoter-driven hCAR cDNA (Zhang et al. 2002). The AhR-humanized mouse, resistant to target gene induction by TCDD, has been generated by knocking in the human AhR cDNA into the mouse AhR gene promoter (Moriguchi et al. 2003). Various transgenic CYP mice (*e.g.* CYP1A1/2, 2A6, 2C18/19, and 3A4), which exhibit metabolic activity similar to humans *in vivo*, have been generated (Gonzalez & Yu 2006, Cheung & Gonzalez 2008, Uno et al. 2009, Hasegawa et al. 2012). These models have been used to study the differences in CYP-mediated metabolism between the two species, to determine the mechanisms of regulation of the human gene and to search for potential endogenous substrates for the enzymes.

Another approach is to transplant human hepatocytes into an immunodeficient mouse and thus, to generate humanized livers ("chimeric mouse", Tateno et al. 2004). CYP enzymes as well as PXR and CAR have been shown to be expressed at near normal (human) levels in these mice and the inducibility of at least CYP1A2 and CYP3A4 was shown to be similar to hepatocytes (Emoto et al. 2008, Katoh et al. 2008). Further, double humanized mice (*e.g.* PXR/CYP3A4) could provide even better models for biotransformation studies or drug development (Felmlee et al. 2008, Cheng et al. 2011).

2.3.7 Summary

Obtaining valid information on the different factors involved in drug metabolism is critical for drug discovery and development, but also for the understanding of the molecular mechanisms behind the function of the xenosensors and CYP enzymes. For drug development, the main aim is to increase safety and efficacy of new drugs as well as to lower the costs of the drug development process. As described in the previous sections, multiple *in vitro* and *in vivo* methods, involving numerous different technologies and instrumentation, exist with which to study xenosensor and CYP function and xenobiotic metabolism. Each of these systems has unique properties and all of them can provide some useful information. But how should one choose the most appropriate method for the phenomenon under study?

In most cases, the different *in vitro* or *in silico* methods can serve as an initial screen to rule out metabolic pathways or to predict xenosensor activation or CYP induction. They can also produce results which can be further used to plan or interpret toxicological and clinical studies. The appropriate design of an experiment as well as proper validation and understanding of the method, and the mechanisms by which all the biological components function, is essential for the correct evaluation of the properties (*e.g.* induction potential) of different compounds. In addition to the appropriate experimental conditions, the choice of positive and negative controls, incubation times/endpoints as well as relevant concentrations of both study compounds and controls are critical for making effective and correct interpretations or conducting *in vivo* extrapolations of the obtained data.

In essence, all of the assays described in the previous sections have their own advantages but also certain disadvantages or limitations (***Table 7.***) and thus at the moment, the use of several different methods (in sequence) to study the different aspects of xenobiotic metabolism produces the best results (Gómez-Lechón et al. 2007, Pelkonen et al. 2008). As described previously, the models are also constantly being developed to be better suited for studies in drug discovery and development.

Table 7. Summary of the different methods used to study xenosensor function and xenobiotic metabolism (induction)

Method	Advantages	Disadvantages	Availability	Applications
In silico assays	Generally fast and cheap. Enable virtual screening for thousands of chemicals. Relatively good predictive power at least for ligands within the same chemical class.	Require adequate hardware and software. The use of different software types or versions may make comparing the results difficult. No AhR crystal structure available. The predictive power may be questionable. The relationship between binding and activity is not clear for CAR and predictions are hard to make.	+++ *(commercial)*	Used to study the structures and ligand/substrate -binding properties of xenosensors and CYPs as well as for virtual screening to find potential ligands, substrates, inducers and inhibitors (lead structures).
Cell-free assays	High capacity (HTS). Inexpensive. One isozyme present, also different genotypes (*supersomes*). CYP enzymes and other essential proteins present in physiological proportions in (*microsomes* and *liver homogenates)*. Use of mechanism-based assays to enhance specificity.	Lack of validation. *Recombinant CYPs*: Lack of other enzymes and transporters or excessive CYP concentrations relative to *in vivo*. *Microsomes*: Contain only Phase I enzymes and UGTs. Requires specific substrates and inhibitors or antibodies for individual enzymes. Problems with extrapolation. *Receptor assays*: False positives with several NR binding assays.	+++ *(commercial)*	*Enzyme assays:* Used to study inhibition, the metabolic stability of chemicals and in the identification of the isozyme responsible for the metabolism of a compound as well as CYP phenotyping. *Receptor assays:* Used to study the ligand/coregulator binding to a xenosensor.
Reporter assays	Reproducible, high capacity (HTS) assays. Receptor-mediated activation is a relevant endpoint. Stable lines available.	Partly cell line -specific responses. Activation of a single receptor does not reflect the complete induction process *in vivo*. No information of the extent of target gene activation. Transcriptional activation may not relate to changes in CYP activity.	+++ *(partly commercial)*	Both transient and stable assays used for ligand screening, prediction of induction and xenosensor activation (and inactivation) as well as the binding of cofactors (2-hybrid methods). Studies on species specific differences.

Method	Advantages	Disadvantages	Availability	Applications
Continuous cell lines	*Hepatoma cells:* Unlimited source of enzymes, CYP1A family induction readily detected (endogenous AhR).	*Stem cells:* Ethical issues (mainly ESCs). *All:* Incomplete differentiation, possible genotype instability, lack of character-ization, poor CYP expression and activity profiles. CAR is not present in most of the cell lines.	- - - *(stem cell derived)* + *(immortalized)* +++ *(hepatoma cells, commercial)*	*Hepatoma cells:*Various lines used to study metabolism, induction and toxicity of different compounds. Also used in bioartificial livers (C3A). *Stem cells and immortalized cells:*So far no validated applications for research or therapeutic purposes.
Primary hepatocytes*	Full spectrum of DMEs, receptors and coregulators. Good *in vitro - in vivo* correlation.	Interindividual and batch-to-batch variations. New preparation required for each experiment (fresh). Need for healthy tissue. Poor availability and quality.	+ *(fresh, commercial)* + + + *(cryo, commercial)*	The golden standard method for different drug metabolism, induction, inhibition and toxicity studies.
Liver slices and perfused liver	Highly preserved 3D cytoarchitecture, best representation of the *in vivo* situation, morphological studies possible. Direct measurement of metabolism.	Fresh tissue needed. *Slices:* Difficult to handle, limited experimental period, inadequate permeation of drugs, issues with heterogeneity, poor reproducibility and no validated data. *Livers:* Inadequate penetration of medium, damaged cells, limited viable period, noninducible and expensive.	- - - / + + *(human/animal liver slices)* + + *(animal livers)*	Used (rarely) in drug biotransformation studies, to assess metabolic routes and profiles within or between species and to study target gene activation.
In vivo (animals and clinical trials)	Humanized animal models: (human) *in vivo*-like information, Human: relevant (human) *in vivo* data	Animals: Poor correlations, expensive, ethical issues, retain animal enzymes. Humans: Expensive, ethical issues, interindividual differences, results depend on the probe drug used.	++ *(animals)* - - - *(volunteers)*	Animals used for toxicity or metabolism studies. Different genetically modified mice (*e.g.* disease models) available for specific experiments. Human trials focused only on CYP3A4.

* Preferred model.

3 Aims of the study

The main aim of this study was to develop different cell-based models and to complement them with other *in vitro* and *in silico* methods for studies on human xenosensors.

The specific aims were as follows:

1. To compare the impact of different natural and chimeric transcription factors on CYP expression in hepatoma cells and to develop novel genetically modified hepatoma cell lines expressing chimeric hCAR and hPXR receptors.

2. To develop optimized and validated screening assays for human xenosensor activation.

3. To find novel selective agonists and inverse agonists for human CAR by using virtual screening techniques and different *in vitro* assays.

4. To use these methods to study the essential ligand characteristics to achieve specific activation of hCAR and the induction of CYP enzymes.

4 Materials and methods

4.1 CHEMICALS

The commercial chemicals were of at least analytical grade and their suppliers, as well as dilution media, have been specified in the original publications (**I-IV**). Chemicals identified by virtual screening were ordered from Tripos Inc. (St.Louis, MO, USA) (**I, III**) or from Maybridge (Trevillet, UK) (**IV**). S07662, flexible diaryl (FL) compounds, TPP and TMPP were synthesized and purified as described in the original publication **IV**, Pulkkinen et al. 2008 and Honkakoski et al. 2004, respectively. Meclizine was a kind gift from Drs. Hongbing Wang (University of Maryland, MD) and Tatsuya Sueyoshi (NIEHS, NC).

4.2 PLASMIDS

The constructs were created by standard DNA cloning protocols (Sambrook and Russell 2001) and verified by dideoxy sequencing. The plasmids were amplified in DH5α and XL10 strains of *E. coli* and purified with Qiagen columns (Hilden, Germany).

NR expression vectors. The CMX-GAL4-LBD constructs of hCAR (residues 108-348) and hPXR (107-434) have been described in Mäkinen et al. 2002 and the mutated hCAR LBD constructs in Jyrkkärinne et al. 2005 (**I**, **III**, **IV**). The full-length, wild-type hCAR (residues 1-348) and hPXR (residues 1-434) expression vectors have been described in Mäkinen et al. 2002 and Korjamo et al. 2005. For chimeric constructs (***Figure 8.***), the full length NRs were fused either N- or C-terminally with the fragment containing the nuclear localization signal and the activation domain (AD) of the human NFκβ subunit (residues 284-551; designated as "p65"), as described in detail in the original publication **II**.

Reporter plasmids. The hPBREM-tk-luciferase, mutated hPBREM-tk-luciferase, hCYP2B6-luciferase and $(ER6)_3$-tk-luciferase have been previously described in Sueyoshi et al. 1999, Honkakoski et al. 1998 and Mäkinen et al. 2002. CYP3A4-XREM-luciferase (Goodwin et al., 1999) was donated by Dr. Chris Liddle (University of Sydney, Westmead, Australia), GAL4-responsive UAS_4-tk-luciferase by Prof. R.M. Evans (Salk Institute, La Jolla, CA) and the CYP1A1 promoter-driven luciferase by Dr. Stephan Safe (Texas A&M Health Science Center, TX).

Constructs for the mammalian 2-hybrid assay (I, III and IV). The NR interaction domains of co-repressor NCoR (residues 1972-2290) and co-activator SRC1 (residues 547-789) were cloned in-frame downstream of the GAL4-DNA-binding domains between the EcoRI and BamHI sites of the pM vector (Matchmaker kit, BD Clontech) and the NdeI and BamHI sites of the CMX-GAL4-vector, respectively. The human CAR LBD (residues 108–348) was fused with the VP16 transactivation domain present in the pVP16 plasmid. The luciferase reporter pG5-luc was purchased from Promega (Madison, WI, USA).

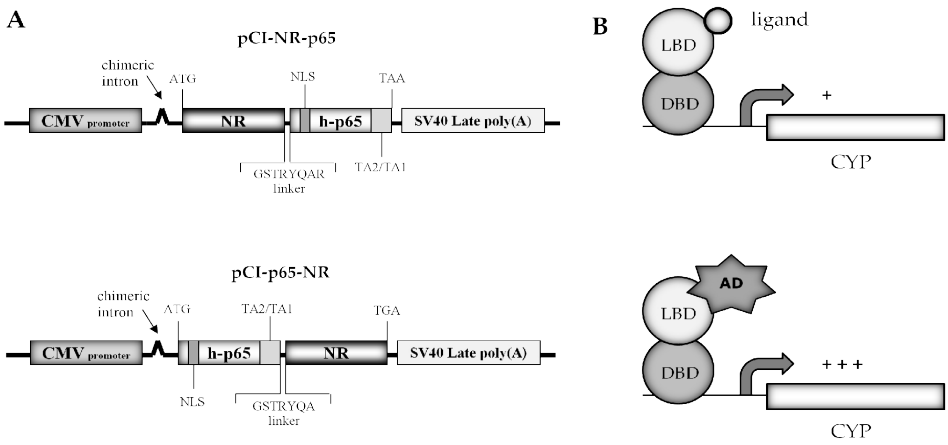

Figure 8. Schematic presentation of the chimeric NR constructs and their function. **A)** All constructs contain amino acids 284-551 of p65 activation domain. CMV prom = cytomegalovirus early promoter, TA2/TA1 = transactivation domain 1 and 2, NLS = nuclear localization signal, SV40 Late poly(A) = simian virus 40 polyadenylation signal. **B)** The chimeric receptors with the added activation domain (AD) exhibit higher *trans*-activation potential compared to the wild type receptor without ligand.

4.3 CELL CULTURE

The continuous cell lines were maintained as described in the original publications and passages between 3 and 30 were used in the experiments (**I-IV**). The human hepatoma cell line C3A (ATCC CRL-10741) was used in most of the assays. Different cell lines (ARPE-19, HEK293, HepG2, C3A) were tested in pilot studies for their NR responses, ease of culturing and transfection efficiency. The C3A cells are easily transfected, easy to culture and show clear and reproducible responses in NR reporter assays, perhaps reflecting a suitable cofactor environment for NR function. They also express the AhR endogenously. In study **III**, the more commonly used HepG2 hepatoma cell line was used to compare the expression of the selected genes in these two related cell lines.

Human primary hepatocytes from donors with no known exposure to CYP inducers, were obtained from BD Gentest/BD Discovery Labware (Woburn, MA) (**I**) or Biopredic International (**III, IV**). Upon receipt, the medium provided by the supplier was replaced and the cells were allowed to stabilize for 24-48 hours (Abadie-Viollon et al. 2010) before chemical exposures, according to the supplier's instructions. The cells were exposed to DMSO or H_2O (0.1%, v/v), selected test chemicals and reference compounds for 24 hours for mRNA studies and for 48-72 hours for CYP induction studies.

Modified C3A cell lines expressing chimeric hPXR and hCAR were generated from wild-type C3A cells by transfections of expression vectors for chimeric NR cDNAs described in ***Section 4.2*** and selected with G418 (Honkakoski et al. 2001). Proliferating G418-resistant colonies were selected first based on hPXR/hCAR- responsive reporter gene activity and subsequently with quantitative RT-PCR for CYP mRNAs (**II**).

4.4 REPORTER GENE ASSAYS

The principles of the methods are described in ***Section 2.3.3*** as well as in the original publications (**I-IV**). For the xenosensor activity assays and cofactor binding assays (hCAR, M2H) (**I, III, IV**), C3A cells were seeded on 48-well plates and transfected with the calcium phosphate method (Chen & Okayama 1987) and constructs described in the original publications. The assays were optimized and validated by using control chemicals chosen based on initial screening and NIH guidelines (modified for 48-well plates) as well as the assay performance measures recommended in Iversen et al. 2006. The empty CMX-GAL4-vector was used as a negative control for M1H assays and the SRC1 assay and the empty pM vector for the NCoR assay. For the reporter assays with full length and chimeric hCAR and hPXR constructs, HEK293 and C3A cells were seeded onto 48-well plates and transfected with selected reporter genes with natural or mutated NR binding sites and NR constructs **(II)**. The empty pCI/neo vector was used as a negative control.

Stably transfected colonies (**II**) were expanded and tested for the chimeric NR expression and functionality by a transient transfection assay. Replicate cell aliquots were seeded on 48-well plates and transfected either with the NR-responsive luciferase reporter or the non-responsive control. Positive and negative controls were prepared by co-transfection of reporters and the appropriate chimeric construct or the empty pCI/neo vector, respectively. Colonies yielding responses higher than the transient negative controls underwent further testing.

After transfection, the medium was replaced with fresh medium including either a vehicle control (DMSO or H_2O, 0.1% v/v), reference compounds or test chemicals as described in the original publications and incubated for 24 hours. After chemical treatment, the cells were lysed and stored at -80ºC at least overnight to completely lyse the cell membranes and to maintain the functionality of the enzymes. Luciferase and β-galactosidase activities were measured from the cell lysate (Honkakoski et al. 2001) using the Victor multiplate reader (PerkinElmer, Turku, Finland) and the luciferase activity was normalized to the control β-galactosidase activity of each respective sample.

4.5 YEAST 2-HYBRID ASSAY

The yeast 2-hybrid assay is essentially based on the same principle as its mammalian counterpart described in ***Section 2.3.3***. However, the NR and coregulator constructs differ in that the NR LBD is fused with the yeast GAL4 DBD and the coregulator with the GAL4-AD. The NR/DBD construct binds to the yeast genomic LacZ gene and the coregulator/AD construct binds the activated NR, thus enabling the expression of the β-galactosidase gene. The pGBKT7 and pGADT7 plasmids (Matchmaker GAL4 Two-Hybrid System 3, Clontech, CA) encoding the human CAR LBD and the human NCoR interaction domain have been described (Jyrkkärinne et al. 2005) Yeast colonies expressing both CAR LBD and the interacting partner (NCoR) were grown, treated with DMSO vehicle (0.1 % v/v) or selected test chemicals and assayed for β-galactosidase activity, cell density and turbidity as described in Mäkinen et al. 2002. The β-galactosidase activities were normalized to cell density.

4.6 GENE EXPRESSION STUDIES

Effects of unmodified and chimeric NRs and NR ligands on CYP expression in wild type C3A cells (II). In order to determine the effects of NR agonists and/or transiently transfected natural and chimeric NRs on CYP expression (**II**), wild type C3A and HepG2 cells were seeded onto 6-well plates one day before transfection (C3A) or three (C3A) and five (HepG2) days before exposing to NR agonists. C3A cells were transfected with chimeric and natural NR expression vectors by polyethyleneimine (PEI) 25 or calcium phosphate precipitation as described in the original publication. Standard growth medium in the absence or presence of NR agonists was added to selected wells and the cells were incubated for 48 h before measurement of CYP mRNAs. The empty vector (pCI/neo) was used as a negative control.

Effects of FoxA2 and HNF4α on CYP expression in wild type C3A cells (unpublished). Wild type C3A cells were grown and transfected with human HNF4α or FoxA2 expression vectors (SD123863 and SC327867, Origene, MD) by calcium phosphate precipitation as described above. After 24 hours, the medium was replaced with standard growth medium. The mRNA expression of CYP2B6 and CYP3A4 was measured after 48 hours and 7 days of culture and the expression levels were compared to non-transfected cells.

Nucleic acid isolation. Total RNA from 6-well plates was isolated with TRI Reagent (Sigma) and any contaminating DNA was removed using the DNase Free kit (Ambion, Austin, TX). The RNA was quantified with RiboGreen dye (Molecular Probes, Netherlands) and 2 μg of RNA was reverse transcribed to cDNA with M-MuLV reverse transcriptase (Fermentas, Hanover, MD) using random priming. The RNA from smaller culture plates was isolated and reverse transcribed using the TaqMan Gene Expression Cells to Ct kit (Ambion, Austin, TX) according to the manufacturer's instructions. The genomic DNA was isolated from selected cell lines (**II**) with standard proteinase K treatment, phenol-chloroform extraction and ethanol precipitation and treated with RNase to remove any residual RNA contamination.

Quantitative RT-PCR. All studies were done using TaqMan chemistry, which uses a fluorogenic probe for the detection of a specific product as it accumulates during PCR cycles, and an ABI Prism 7500 instrument (Applied Biosystems, UK). Forty ng of the cDNA obtained from 6-well plates or samples with an unknown cDNA amount obtained with the TaqMan Gene Expression Cells to Ct kit were used in the PCR reaction. The fluorescence data were processed in the QGene program (Müller et al. 2002) and the results were calculated according to comparative Ct-method assuming equal amplification efficiencies of 2. The measured CYP levels were normalized either to total RNA or to selected control gene expression (Bustin 2000 and 2002). The primers and probes and the liver sample used as a positive control (**II**) are presented in the original publications (**I-IV**). For copy number determination, primer/probe sets for hPXR and hCAR were used for detection of the integrated chimeric NR cDNAs. The used probes are able to span an exon/exon junction and can detect the transgenic cDNA with no intronic sequences but cannot detect genomic CAR or PXR gene sequences. Dilutions of corresponding chimeric NR plasmids were used as standards.

4.7 METABOLIC EXPERIMENTS

Fluorometric assays (II, III, unpublished). The metabolic activity of CYP3A4 (**II,** unpublished results) was measured with a fluorometric assay from a lysate obtained from wild type and modified C3A cells, with or without chemical treatment, grown on 6-well plates. The lysate was incubated in buffer containing the substrate (BFC) with or without the CYP3A4 specific inhibitor, ketoconazole. BFC is dealkylated into fluorescent HFC by CYP3A4, CYP1A2 and to a lesser extent by CYP2B6 (Donato et al. 2004). Ketoconazole, at the chosen concentration (5 μM), is sufficient to inhibit all CYP3A4 activity (Turpeinen et al. 2005), thus making it possible to differentiate the BFC metabolism catalyzed by CYP3A4 from other CYP enzymes. Microsomes obtained from the human liver sample HL24 were used as a positive control. The CYP3A4 and CYP2B6 inhibition potential of selected chemicals (**III**) was studied with cDNA expressed recombinant enzymes (BD Gentest, Franklin Lakes, NJ) in 96-wells as described by Salminen et al. 2011. The incubation mixtures, containing the recombinant CYP enzymes, probe substrates (BFC for 3A4 and EFC for 2B6) and test chemicals, have been described in detail in the original publication. The reference inhibitors were ketoconazole for CYP3A4 and ticlopidine for CYP2B6. All dilutions of substrates and test chemicals were prepared so that the final concentration of the solvent (ACN or DMSO) did not exceed 0.1% v/v. The plates were pre-incubated in +37ºC for 10 min and the NADPH-regenerating system was added to start the reaction. The plates were then incubated in 37ºC for 6 hours (activity, **II**) or 20 min (inhibition, **III**). A standard curve (0–1000 nM) was constructed from HFC. The fluorescence was measured at 405/535 nm with VICTOR^2 multiplate reader.

LC-MS/MS (III). The compounds in the sample are first separated by liquid chromatography (LC) and then introduced into the mass spectrometer (MS), which measures the mass-to-charge ratio of charged particles (Korfmacher 2005). Hepatocytes on 48-well plates were treated as described in ***Section 4.3***. Salicylamide was used to saturate the conjugation enzymes and the samples were incubated with CYP specific substrates (testosterone for CYP3A4 and buproprion for CYP2B6). The samples were then analyzed for the amounts of CYP isoform specific metabolites as described by Tolonen et al. 2007.

4.8 RECOMBINANT HUMAN CAR-LBD PROTEIN PRODUCTION AND LIMITED PROTEASE DIGESTION ASSAY (III, IV)

The limited protease digestion (LPD) assay is based on the principle that a conformational change of the LBD may limit access to a cutting site for a protease and thus, create a protease-resistant receptor fragment. These assays have been used to analyze NR ligand binding, ligand-induced conformational changes to the LBD and to characterize the functional conformations of the receptors (Reichman et al. 1984, Benkoussa et al. 1997, Nayeri & Carlberg 1997). The detailed protocols have been described in the original publications (**III, IV**). Briefly, the N-terminal His6-hCAR-LBD (residues 103-348) fusion protein was produced in *E. Coli* BL21 (DE3) cells and purified. The resulted recombinant His6-hCAR-LBD was preincubated with DMSO or selected hCAR ligands and digested with subtilisin. The obtained proteolytic fragments were denatured and analyzed with SDS-PAGE.

4.9 MOLECULAR MODELING

Two-step virtual screening procedures, based on a pharmacophore searches and molecular docking studies, were used to screen large databases for novel hCAR ligands. The principles of the methods have been described in ***Section 2.3.1***.

In study **I**, the Tripos LeadQuest database and in study **IV**, the Maybridge database, were used as starting points for the pharmacophore searches. Selected ligands were docked into hCAR LBD (Protein Data Bank (PDB), crystal structure 1XVP, chain D) using GOLD docking suite (version 2.2, CCDC, Cambridge, UK) and the docking site was defined either within a sphere around atom CD1 of Leu206 (**I**) or using the ligand molecule (CITCO) extracted from the crystal structure (**IV**). Compounds to be tested in biological assays were selected according to their GoldScore and visual inspection. Possibilities for interaction between ligands and the LBP were predicted by molecular interactions fields calculations (**I**). The detailed features of the pharmacophores as well as docking protocols have been explained in the respective original publications.

Selected ligands (**III, IV**) were also docked to the 1XVP with a new version of the GOLD docking suite (4.0), which allowed selected amino acid side chains to move freely ("flexible docking"). The docking site was defined using the ligand molecule (CITCO) and the side chains of amino acids F161 and Y224 were allowed to move freely, based on the data obtained from previous MD simulations (Windshügel et al. 2005 and 2007). The rescoring of the docking poses was done by calculating the theoretical binding energies of ligands and the contact preference maps of ambiguous docking poses were inspected visually. The best pose for each ligand was selected based on both the binding energy and the adequacy of the interaction fields. Shorter (1.0 ns, **III**) or longer (10 ns, **IV**) MD simulations were performed for the best poses obtained from flexible docking to study the structural behaviour of selected ligand/LBP complexes. The trajectories were analyzed for root mean square deviations (RMSD), atomic positional fluctuation (APF) and protein secondary structures and visually examined. The volume of the LBP and ligands were calculated in order to evaluate the possible differences in the filling degree of the pocket by different ligands, which was thought to influence the activity of the receptor. The detailed methods are presented in the respective original publications and in Jyrkkärinne et al. 2012.

4.10 STATISTICAL ANALYSIS

The results are presented as mean ± SEM of at least 3 replicates. Student's paired *t*-test with Bonferroni or Šidák correction was used to assess the statistical significance of the results.

5 Main results and discussion

5.1 STUDIES ON CYP EXPRESSION IN HEPATOMA CELLS

The applicability of human hepatoma cell lines for drug metabolism and toxicity studies has been questioned due to the downregulation of CYP enzymes in cultured cells. The suggested mechanisms behind this phenomenon and different approaches to increase the CYP expression have been discussed in ***Section 2.3.4***. Since the gene expression levels in hepatoma cell lines vary depending on the culture conditions, the basal mRNA levels of selected CYPs, NRs and NR cofactors were determined in C3A and HepG2 cell lines in comparison with a single human liver sample giving a rough estimate of the transcript levels *in vivo* (**II**). In general, the two hepatoma cell lines showed a similar expression profile and the expression levels of the genes were clearly lower (≤ 1%) than in the liver sample as described previously (Rodriguez-Antona et al. 2002, Westerink & Schoonen 2007a,b). However, the transcript levels of NCoR, PGC1, PXR and HNF4α were higher than in the liver sample in both cell lines, whereas the levels of CYPs, CAR and other coregulators were very low. The high expression of HNF4α has been previously described for HepG2 whereas, in contrast to the present finding, the PGC1α has been claimed to be underexpressed in these cells (Martínez-Jiménez et al. 2006). The largest differences between the two cell lines were in the expression levels of CYP2B6, PXR, TIF2 and NCoR, with higher expression levels (260-fold in the case of CYP2B6) in the C3A cell line. C3A was chosen for further studies mainly due to the higher expression of CYP2B6, the ease of culturing, better transfection efficiency and reproducibility (unpublished observations) as compared to HepG2.

5.1.1 Transient transfections and NR activators (II, unpublished)

One possible reason for the downregulation of CYPs in hepatoma cells is the lack of the different TFs which control their expression. Both PXR and CAR regulate the inducible expression of many DME genes and the expression of these NRs in general correlates with the expression of CYP mRNAs in liver samples (Pascussi et al. 2001, Chang et al. 2003, Vyhlidal et al. 2006, Wortham et al. 2007). Different modifications related to these receptors, such as treating the cells with their agonists or transfecting the cells with the receptor expression vectors, have been applied to increase or enhance the expression and activity of CYPs in hepatoma cells (***Section 2.3.4.3***). The usefulness of the former technique depends on the endogenous expression of PXR and CAR, whereas the latter approach can upregulate numerous CYP and DT mRNAs, albeit modestly (Korjamo et al. 2006). In this work, the effects of human PXR and CAR activators as well as the overexpression of different TFs to the CYP mRNA levels or reporter activities were studied (***Table 8.***).

NRs and NR agonists. As expected, based on the NR mRNA levels of unmodified C3A cells, the cells displayed only a modest response to a PXR but not to a CAR activator, which is consistent with the low expression of CAR in hepatoma cells (Zelko & Negishi 2000). The overexpression of hPXR or hCAR increased the expression of CYP3A4 and CYP2B6 mRNAs only slightly and further treatment with NR agonists could only increase the expression of CYP3A4. Even though PXR is expressed in the C3A cells at the mRNA level, the protein might be non-functional due to the unfavourable expression levels of cofactors

in these cells (low SRC1, high NCoR) or due to the expression of splice variants (Donato et al. 2008, Lamba 2008, Zhang et al. 2008). Ligand-free PXR is also known to associate with corepressors decreasing its activity (Johnson et al. 2006). However, due to the over 2-fold increase in CYP3A4 expression by RIF, the wild type C3A contains at least a small amount of functional PXR, as reported by others for HepG2 (Fery et al. 2010).

Chimeric NRs. The modular structure of NRs generally allows the modification of the receptor by addition of other transcription factor domains to modulate their function (Germain et al. 2006). Thus, a strong activation domain (AD) of the p65 subunit of the nuclear factor κB was fused to both hPXR and hCAR either at the N- or the C-terminus in order to create receptors with constitutively enhanced activity. Similar approaches using herpes simplex viral protein 16 (VP16-AD) have been used to generate exdysone-inducible regulator and in mice, the NR-VP16 fusion proteins have been shown to increase the expression of NR target genes (No et al. 1996, Rosenfeld et al. 2003, Saini et al. 2004). The functionality of the constructs was tested with reporter assays: Whereas the wild type receptors had a modest (less than 5-fold) effect on the reporter gene expression even with added agonists, the chimeric receptors displayed a strong constitutive activation of both *CYP3A4* (20- to 28-fold) and *CYP2B6* (9- to 16-fold) promoter driven reporter constructs (***Table 8.***). Furthermore, all chimeric constructs notably increased the CYP3A4 expression (hCAR: 6- to 30-fold, hPXR: 8- to 10-fold) (***Table 8.***).

Interestingly, the hCAR constructs, especially the version with the C-terminal AD, exhibited higher activation of CYP3A4/XREM- and ER6-reporters and higher upregulation of CYP3A4 mRNA than either of the hPXR constructs. The natural CYP2B6 driven and heterologous hPBREM reporters exhibited highest activation by the hCAR with N-terminal AD, while the hPXR mediated activation was modest or even decreased by over 50% compared to empty vector. The hPXR construct with the N-terminal AD was slightly better than the C-terminal AD in most assays. These differences could be due to the stronger activation potential of the chimeric hCAR constructs as such, which is also seen in HEK293 cells, or the impaired function of hPXR constructs in this particular cell line, since in HEK293 cells the differences in reporter activity between different constructs were not as pronounced. In summary, the activation profile of the CYP3A4 reporters was in general very similar to the expression profiles of CYP3A4 mRNA.

HNF4*α* and FoxA2 (HNF3β) (unpublished). As explained in ***Section 2.2***, HNF4*α* regulates many hepatic genes involved in the differentiated function of hepatocytes and is responsible for the basal expression of liver specific genes. The receptor also regulates the hepatic expression of different CYPs and reduction of HNF4*α* protein significantly decreases at least the 3A4 mRNA levels in HepG2 cells (Akiyama et al. 2004, Matsumura et al. 2004). It is also required for the expression of PXR and thus, may also affect the CYP3A4 expression via this pathway (Li et al. 2000). The forkhead box transcription factor A2 (FoxA2, previously HNF3β) is involved in the energy metabolism, bile duct development and bile acid homeostasis (Wolfrum et al. 2004, Li et al. 2009). It is also involved in the endoderm differentiation via binding to the sequences required for hepatocyte-specific expression of genes and activated during the differentiation of definitive endoderm (Kaestner 2000). The deletion of FoxA2 in mice results in a significant decrease in the levels of certain proteins, such as PXR and Cyp3a11 (Bochkis et al. 2008). In addition, a significant correlation between FoxA2, PXR and CYP3A4 levels in human hepatic samples has been shown (Lamba et al. 2010).

Table 8. CYP2B6 and 3A4 reporter gene activity and mRNA expression in C3A cell lines

	CYP3A4		CYP2B6	
	RGA	**mRNA**	**RGA**	**mRNA**
NR agonists	N.D.	↑(CITCO) ↑ (RIF)	N.D.	↑ (CITCO) ↑ (RIF)
Transient transfections				
- *wild type NR*	↑-↑↑	↑-↑↑	↓/↓	↑↑
- *p65-NR*	↑↑↑↑	↑↑↑	↓-↑↑ / ↓-↑↑↑↑	N.D.
- *NR-p65*	↑↑↑↑	↑↑↑ - ↑↑↑↑	↓-↑ / ↑-↑↑↑	N.D.
- *HNF4**	N.D.	↓(48h) ↑↑ (7d)	N.D.	↓ (48h) ↑↑ (7d)
- *FoxA2**	N.D.	↑ (48h) ↑↑↑ (7d)	N.D.	↓ (48h) ↑↑ (7d)
Stable transfections				
- *p65-NR*	↑↑-↑↑↑↑	↓-↑↑↑↑	N.D.	↓-↑↑↑↑
- *NR-p65*	↑↑↑↑	↑↑-↑↑↑↑	N.D.	↓-↑↑↑↑

The results are expressed as fold-increase in activity or expression compared to untreated or wild type C3A cells. N.D. = not done, ↑ = 1-2.5 -fold, ↑↑ = 2.5-5 -fold, ↑↑↑ = 5-10 -fold, ↑↑↑↑ = over 10-fold, ↓ = less than 1-fold, RGA=Reporter gene activity: CYP3A4.XREM.distal.luc/ (ER6)3.tk.luc for CYP3A4; hPBREM.tk.luc/ CYP2B6.luc for CYP2B6, CYP3A4.XREM.distal.luc for stable transfectants, *unpublished results

A moderate increase of the expression of both CYP2B6 (2 to 3 -fold) and CYP3A4 (4 to 6-fold) was observed only after 7 days of culture (***Table 8.***) after transient transfection of either HNF4α or FoxA2. The somewhat delayed effect might be due to indirect or secondary (via other TFs) effects on CYP expression. Interestingly, even though the C3A shows high levels of HNF4α (see above), transfection of the gene increased the CYP mRNA expression, suggesting that the endogenously expressed HNF4α is not fully functional. In particular, PGC1α is considered to be a key coactivator for sustaining the expression of HNF4α-dependent genes in hepatoma cells and thus, the lack of functionality has previously been suggested to be due to the observed low levels of SRC1 and PGC1α (Martínez-Jiménez et al. 2006). As the present results revealed that the PGC1α was expressed at higher levels than in the liver sample, the lack of (endogenous) HNF4α functionality could be due to the levels of other cofactors (such as the high levels of corepressor NCoR). However, these results show that the CYP mRNA levels can be upregulated also by the overexpression of HNF4α and FoxA2.

5.1.2 Generation of stable cell lines expressing chimeric PXR and CAR (II)

Previously, the upregulation of CYP expression HepG2 and Caco-2 cells has been achieved by stable transfections of TFs, such as c/EBPα, HNF4α, CAR and PXR (Jover et al. 1998, Sueyoshi et al. 1999, Naiki et al. 2004, Korjamo et al. 2005, 2006, Trubetskoy et al. 2005). Due to their promising transactivation properties, chimeric NRs were used to develop stable C3A cell lines. The use of chimeric receptors to modulate CYP levels in hepatoma cells has not been previously published. The majority of the obtained positive colonies (80%), chosen based on reporter gene activities, were based on hCAR-chimeras, which also showed a higher transactivation potential in transient transfections (***Section 5.1.1***). This could be due

to the lower expression of endogenous CAR in C3A cells or simply to a random integration of the transgene. It was also recently shown that the PXR-mediated upregulation of the p38 MAPK signaling resulted in morphological changes and migration of HepG2, while cell growth itself not affected (Kodama & Negishi 2011). No significant morphological changes were observed in the hPXR sublines by microscopy but no detailed studies were done on the effects of the overexpression of the constitutively active receptor construct on cellular functions. Furthermore, the CYP3A4 reporter activities used to select the colonies for further testing did not correlate with the later measured CYP3A4 mRNA levels and thus, the use of reporter based method is not necessarily the best choice for the selection of positive colonies.

The mRNA levels of CYP2B6, 2C9 and 3A4 were increased somewhat differently in the sublines. All hCAR sublines showed increased levels of all three CYPs, whereas the hPXR sublines showed a decrease in CYP2B6 expression, compared to the unmodified cells. The increase in expression was most impressive with CYP2C9 but nonetheless the expression levels still remained below those of the liver sample. In contrast, CYP2B6 expression levels were close to or exceeded the levels of the liver sample. The mRNA levels of at least CYP2B6 and CYP3A4 have been shown to be stable throughout continuous passaging for several months and between different stocks of individual sublines (unpublished observation). Most of the modified cell lines also showed slightly elevated CYP3A4 function compared to the wild type C3A cells. The subline hCAR28 was considered to be the most promising cell line due to the overall enhancement of CYP expression and CYP3A4 activity and its functionality has since been confirmed in experiments examining bioactivation and metabolism of cytotoxic drugs (Ma et al. 2012).

5.1.3 Further studies on the stable C3A-NR cell lines (unpublished)

The expression and activity of different CYPs in hepatoma cell lines have been shown to depend also on culture conditions, such as media formulation or different cell culture matrices. The impact of few selected chemicals on CYP3A4 activity in wild type and modified C3A cells was briefly studied. Dexamethasone (DEX) is an anti-inflammatory steroidal drug, which has been shown to increase the CYP3A4 activity, via the induction of glucocorticoid receptor (GR) and PXR, up to 5-fold in the BC2 cell line (Gómez-Lechón et al. 2001). Vitamin D3 (1,23-$(OH)_2$-D_3, VD3) is involved in the growth and differentiation of cells in several tissues and it has been shown to increase the CYP activity mainly in intestinal cell lines (*e.g.* Caco-2) but also in HepG2 cells (Drocourt et al. 2002, Elizondo & Medina-Díaz 2003, Fan et al. 2009). Serum is commonly used in cell culture as a source of different hormones and growth factors needed for proliferation.

Table 9. The effects of different media modifications on CYP3A4 activity in wild type and modified C3A cell lines.

Cell line	Media modification[b]			
	Control[a]	100nM DEX	100nM VD3	5% serum
C3A wt	3,5	↑	↑	↑
hCAR28	0,3	↑	↑	↑↑↑
hCAR1.8	2,1	ND	↑↑↑	↑↑
hPXR7	3,7	↓	↑	↓
hPXR15	0,4	↑↑	↑↑↑	↑↑↑

[a]CYP3A4 activity (pmol HFC/h/mg prot), [b]↑ = 1-2.5 -fold, ↑↑ = 2.5-5 -fold, ↑↑↑ = over 5 -fold, ↓ = less than 1-fold, culture time 7 days. DEX=dexamethasone, VD3=vitamin D3 (1,23-(OH)2-D3), ND=not done.

Large variation was observed between cell lines but in general, the addition of DEX or VD3 or the reduced serum concentration caused a notable increase in CYP3A4 activity in those cell lines with lower basal activity (***Table 9.***). However, the overall effects on the tested media formulations were modest in this study and the control values for CYP3A4 activity were low compared to the previous results obtained from these cells (**II**). Further tests using different concentrations or combinations of added chemicals as well as the use of another analytical method (*e.g.* LC-MS) might provide more consistent and accurate results.

5.2 XENOSENSOR ASSAYS AND HUMAN CAR LIGANDS

The development of functional cell-based and other *in vitro* and *in silico* assays to study the biology and function of hCAR and to find novel specific hCAR ligands have been subjects of several studies. The main problems and limitations of the previously used assays as well as the properties of the receptor have been discussed in the previous sections.

5.2.1 Optimization and validation of the xenosensor assays (III)

Various reporter assays employing different cell lines and receptor constructs have been used to study the activation of xenosensors but often they have not been validated or otherwise assessed for predictivity or reproducibility. The Z′ factor (Z′) and signal window (SW) parameters adjust the assay signal to the assay variability and can be used to evaluate the overall quality or performance of an assay, and thus, in assay optimization and validation (Iversen et al. 2006). Validation of assays is crucial; one needs to have both reliable and reproducible prediction of the ability of a compound to activate a xenosensor and thus, cause induction of CYP and other DMEs. However, the validation of the assay does not guarantee an acceptable performance over time, so ongoing monitoring is important.

The developed screening assays in the C3A cell line (**III**) fulfilled the acceptance criteria parameters in repeated experiments, although the Z′ value for the AhR assay was slightly under the acceptance level for an excellent assay (***Table 10.***). Compared to the assays used by others, our hCAR (isoform 1) assay meets the acceptance criteria of an excellent assay (both Z' and SW). The use of assays which do not fulfill these acceptance criteria, suggest that at least some studies on hCAR activators have been performed in sub-optimal conditions and thus, may have provided inaccurate predictions. The most important aspect for the developed hCAR assay is that the basal activity of the receptor in C3A cells is relatively low and the assay is able to identify also weak agonists without artificial suppression of the activity by the use of inhibitors (*e.g.* EE2, Jyrkkärinne et al. 2005), artificial mutations on the LBD or the use of splicing variants (DeKeyser et al. 2009, Chen et al. 2010c). Thus, our assay provides reliable information on the hCAR ligand binding specificity and affinity. The low basal activity of hCAR in this cell line could be due to the cofactor content (low SRC1/TIF2 and high PGC1, ***Section 5.1***) (Liu et al. 2002). In assays which use an inhibitor to lower the basal activity, competition between the inverse agonist and agonist may lead to misclassification of weak affinity ligands or partial agonists. The hCAR.3 isoform has been proposed to be a suitable tool for prediction of hCAR.1 ligands due to their similar ligand-binding properties and the low basal activity of hCAR.3 (Auerbach et al. 2005, Faucette et al. 2007). The hCAR.3 assays in general fulfill the requirements of an excellent assay, while this is often not the case for the hCAR.1 assays (***Table 10.***). However, the variants or mutations may affect the structure of the LBD and ligand specificity of the receptor.

Table 10. Examples of xenosensor reporter assays and their performance parameters.

Receptor[a]	**Reference compound**[b]	**Cell line**	**Performance parameters**[c]		***References***
	(µM)		**Z'**	**SW**	
hCAR (LBD)	FL81(10)	C3A	**0.70**	**7.8**	***III***
hCAR (FL)	CITCO(0.25)	HepG2	0.10	< 1	*Yao et al. 2011*
hCAR (FL)	CLOTR(10)	Huh7	<0	<1	*Anderson et al. 2011*
hCAR.3 (FL)	CLOTR(10)	Huh7	**0.86**	**21**	*Anderson et al. 2011*
hCAR (FL)	CITCO(1)	HepG2	< 0	< 1	*Li et al. 2010*
hCAR.3 (FL)	CITCO(1)	HepG2	**0.55**	**6.0**	*Li et al. 2010*
hCAR (FL)	CITCO(5)	COS-1[d]	< 0	< 1	*DeKeyser et al. 2009*
hCAR.2 (FL)	CITCO(5)	COS-1	< 0	< 1	*DeKeyser et al. 2009*
hCAR.3 (FL)	CITCO(5)	COS-1	**0.60**	**4.5**	*DeKeyser et al. 2009*
hCAR (LBD)	CLOTR(2)	HEK293	**0.79**	<1	*Jyrkkärinne et al. 2005*
hCAR (LBD)	TMPP(10)	HEK293	0.40	**4.4**	*Jyrkkärinne et al. 2005*
hPXR (LBD)	RIF(10)	C3A	**0.59**	**4.7**	***III***
hPXR (FL)	RIF(25)	Huh7	0.40	**3.0**	*Anderson et al. 2011*
hPXR (FL)	RIF(10)	HepG2	**0.56**	**4.2**	*Li et al. 2010*
hPXR (FL)	RIF(10)	DPX2[d]	**0.77**	**10**	*Trubetskoy et al. 2005*
hPXR (FL)	RIF(10)	HepG2	**0.87**	**28**	*Lemaire et al. 2004*
hPXR (FL)	RIF(10)	HepG2/hPXR[d]	**0.52**	**3.4**	*Lemaire et al. 2004*
hPXR (FL)	RIF(10)	C3A	**0.70**	**7.3**	*Luo et al. 2002*
hAhR (EG)	OME(10)	C3A	0.48	**4.3**	***III***
hAhR (EG)	TCDD(5)*	AZ-AHR[d]	**0.82**	**9.6**	*Novotna et al. 2011*
hAhR (EG)	OME(100)	HepG2-A10[d]	0.20	< 1	*Sekimoto et al. 2007*
hAhR (EG)	3MC(1)	HepG2-A10[d]	**0.68**	**6.6**	*Sekimoto et al. 2007*
hAhR (EG)	TCDD(10)*	DRE1A2[d]	**0.97**	**160**	*Yueh et al. 2005*
hAhR (EG)	3MC(2)	DRE1A2[d]	**0.87**	**23**	*Yueh et al. 2005*
hAhR (EG)	TCDD(10)*	TV101L-CALUX[d]	0.45	1.9	*Long et al. 2003*

Acceptance criteria
Iversen et al. 2006

Ideal	*Z′ = 1*	Recommended	*SW > 2*
Excellent	*Z′ > 0.5*	Acceptable	*SW > 1*
Do-able	*0 < Z′ <0.5*	Unacceptable	*SW < 1*
Yes/No	*Z′ = 0*		
Unacceptable	*Z′ < 0*		

The Z′ and SW values fulfilling the acceptance criteria for an excellent or recommended assay are marked in bold. [a]LBD=ligand binding domain (fused with yeast GAL4-DNA binding domain), FL=full length receptor, EG=endogenously expressed, [b]CLOTR=clotrimazole, 3MC=3-methylcholanthrene ,*nM concentration [c]Performance parameters are calculated based on the activation data presented in the respective publications. [d]DPX2=HepG2 derivate harboring hPXR and CYP3A4-promoter driven luciferase reporter (PuraCyp, Carlsbad, CA), AZ-AHR=HepG2 based cell line containing a luciferase reporter with several upstream AhR binding sites, CALUX=chemical activated luciferase expression assay (Murk et al. 1996), DRE1A2=human hepatoma cells harboring a DRE driven luciferase reporter (PuraCyp, Carlsbad, CA).

In general, the hPXR and hAhR assays mostly fulfill, whereas most of the hCAR assays do not, the acceptance criteria as judged by the calculated performance parameters (***Table 10.***). In the present experiments, the hPXR and hAhR assays are also more reproducible in repeated experiments than the hCAR assay, despite their lower Z' and SW values (unpublished observation). The hCAR assay is in general more prone to variation; this may be due to transfection efficiency or perhaps to the condition of the cells. Most of the hPXR assays use a full-length receptor in comparison to the present assay which uses only the receptor LBD. The pros and cons of the use of a full length receptor and the LBD have been discussed in ***Section 2.3.3***.

The hAhR assays are usually based on the use of endogenously expressed AhR and a stably transfected luciferase reporter. In contrast, in the present assay, the reporter is transiently transfected, which might affect the reproducibility of the results. The use of omeprazole as a positive control has also been questioned due to its hPXR activation and CYP3A4 induction potential in comparison to beta-naphthoflavone (BNF) (Faucette et al. 2006, Abadie-Viollon et al. 2010). In the present assay, BNF produced similar or higher reporter activities than omeprazole but the results were highly variable between experiments possibly due to compound instability (unpublished observation). In addition, since TCDD is extremely toxic and thus difficult to use, omeprazole was chosen as a positive control for the hAhR assay.

The validated assays can be used to assess the receptor transactivation by various ligands and thus, predict the possible CYP induction caused, but as such, they cannot necessarily predict the overall effects caused by the chemical *in vivo*. A transient transfection assay is in general laborious and also prone to errors, so to be used as high thoughput screening methods for large amounts of ligands, the developed assays should be scaled to at least 96-well format *e.g.* with the possibility to use a reverse transfection method (Reinisalo et al. 2006).

5.2.2 The search for novel hCAR ligands (I, III, IV)

Possibly due to the promiscuous nature and the ligand independent or indirect activation of hCAR as well as due to the lack of reliable and predictive assays, only a few selective ligands for the receptor are known. Most of the studies used to find and study hCAR activators have relied on different reporter assays; in particular, the selectivity of the ligands over other xenosensors has not been studied (Poso & Honkakoski 2006). For example, perhaps the reason why ambiguous results have been reported for clotrimazole and meclizine is due to the different assays used in the studies (see below). Many studies have also focused mainly on the basal activity or the activation mechanisms of hCAR and not on identifying novel ligands. One aim of this work was to discover novel hCAR agonists and inverse agonists and to study their selectivity, by using the above-mentioned xenosensor assays together with other *in vitro* and *in silico* methods. A total of nearly 400 xenosensor activators or CYP inducers, selected based on virtual screening procedures (**I, IV**) and literature (**III, IV**), were studied.

Agonists. Based on the M1H assay, CITCO, FL81 and permethrin (pyrethroid) (**III**) are strong hCAR activators, while *o,p'*-DDT (organochlorine, minor isomer in technical grade DDT) (**III**) and the substituted sulfonamides (3 and 6) (**I**) and thiazolidin-4-one derivatives (9 and 14) are moderate hCAR activators (**I**) (***Table 11., Figure 9.***). The strong activators are not specific for hCAR since they show at least moderate activation of hPXR (FL81) or hAhR (CITCO and permethrin). Except for *o,p*-DDT, which is specific activator of hCAR at concentrations below 3 μM, the moderate activators are also strong activators of hPXR (all) and hAhR (9) (***Table 11.***). TMPP and TPP have been used previously as positive controls in hCAR assays (Jyrkkärinne et al. 2005, 2008) and they produce fairly modest, although

reproducible, activation of hCAR. However, TPP is a strong hPXR activator and, both compounds are moderate activators of hAhR (***Table 11.***). All agonists evoked a robust recruitment of SRC1, although a decrease in reporter activity was seen with high concentrations (30μM) of CITCO, permethrin and *o,p'*-DDT, which was not due to toxicity (***Table 11.***). None of the agonists showed any significant NCoR recruitment. In the LPD assays, the unliganded hCAR LBD was degraded by subtilisin A, while the binding of different agonists provided protection to various degrees from proteolysis, supporting direct association of the established agonists (CITCO, *o,p'*-DDT, permethrin and FL81) with the hCAR LBD and further hinting at different binding orientations of the ligands within the LBP.

While CITCO showed a strong induction of both CYP2B6 mRNA and activity, the increase of CYP2B6 mRNA expression by other strong activators was very modest, although selective for CYP2B6 over CYP3A4. However, permethrin showed a moderate induction of CYP2B6 activity. Of the moderate activators, compound 14 (**I**) evoked a significant induction of CYP2B6 mRNA and was selective over CYP3A4, while *o,p'*-DDT (**III**) was the only chemical found to increase both CYP2B6 and CYP3A4 as well as CYP1A2 mRNA expression, even though it did not activate hAhR in the M1H assay (***Table 11.***). This is probably due to the PXR and CAR-mediated regulation of CYP1A2 expression (Maglich et al. 2002).

Although the established hCAR agonist CITCO (Maglich et al. 2003) has been used as a positive control for hCAR activation in many reporter gene assays (***Table 10, I***), its activation potential varies significantly between and within studies, possibly due to its poor stability (Jyrkkärinne et al. 2008, Dring et al. 2010). CITCO also fails to show a dose-dependent increase in hCAR activity, cofactor recruitment and CYP induction, at least with over 100nM concentrations. Many pesticides of various chemical classes have been shown to induce CYP expression and recently the developed hCAR assay (***Section 5.2.1***) was used to study different pesticides for their hCAR activation (Abass et al. 2012). Together with permethrin and *o,p'*-DDT, these newly found chemicals, in addition to FL81 and compounds 3, 6, 9 and 14, represent novel chemical types of hCAR ligands.

Inverse agonists. All of the established inverse agonists evoked a decrease in hCAR activity (10-77 %) in the M1H assay (***Table 11., Figure 9.***). Both PK11195 and S07662 were strong hPXR and weak hAhR activators. In contrast, neither EE2 nor androstenol activated hPXR and they even decreased the activity of AhR (50-80%, unpublished results). The SRC1 recruitment was low for all other inverse agonists, except for PK11195, suggesting that the compound might in fact be a partial agonist of hCAR. A strong NCoR recruitment was observed only with S07662 and PK11195, while the results were very modest for both EE2 and androstenol. However, in the Y2H assay, the hCAR/NCoR interaction was strongly induced by all of the inverse agonists, as well as by another steroidal compound, etiocholanolone (data not shown). The interactions with cofactors could be blocked via competition with agonists in both SRC1 and NCoR interaction assays (**IV**, unpublished results). Similarly to the situation with the agonists, the strong inverse agonists protected the LBD from digestion better than the weaker inverse agonist EE2 in the LPD assay. Furthermore, all inverse agonists suppressed the induction of CYP2B6 mRNA by CITCO and phenytoin. In the absence of agonists, CYP2B6 mRNA was induced moderately by the three other inverse agonists; in contrast, S07662 caused only a very modest induction. CYP3A4 mRNA was induced moderately (up to 7-fold) by both S07662 and PK11195, whereas the steroidal inverse agonists showed no effect. Despite some drawbacks, such as

the lesser degree of suppression of CITCO-mediated CYP2B6 induction and hPXR activation, S07662 represents a novel useful tool for studies of hCAR biology.

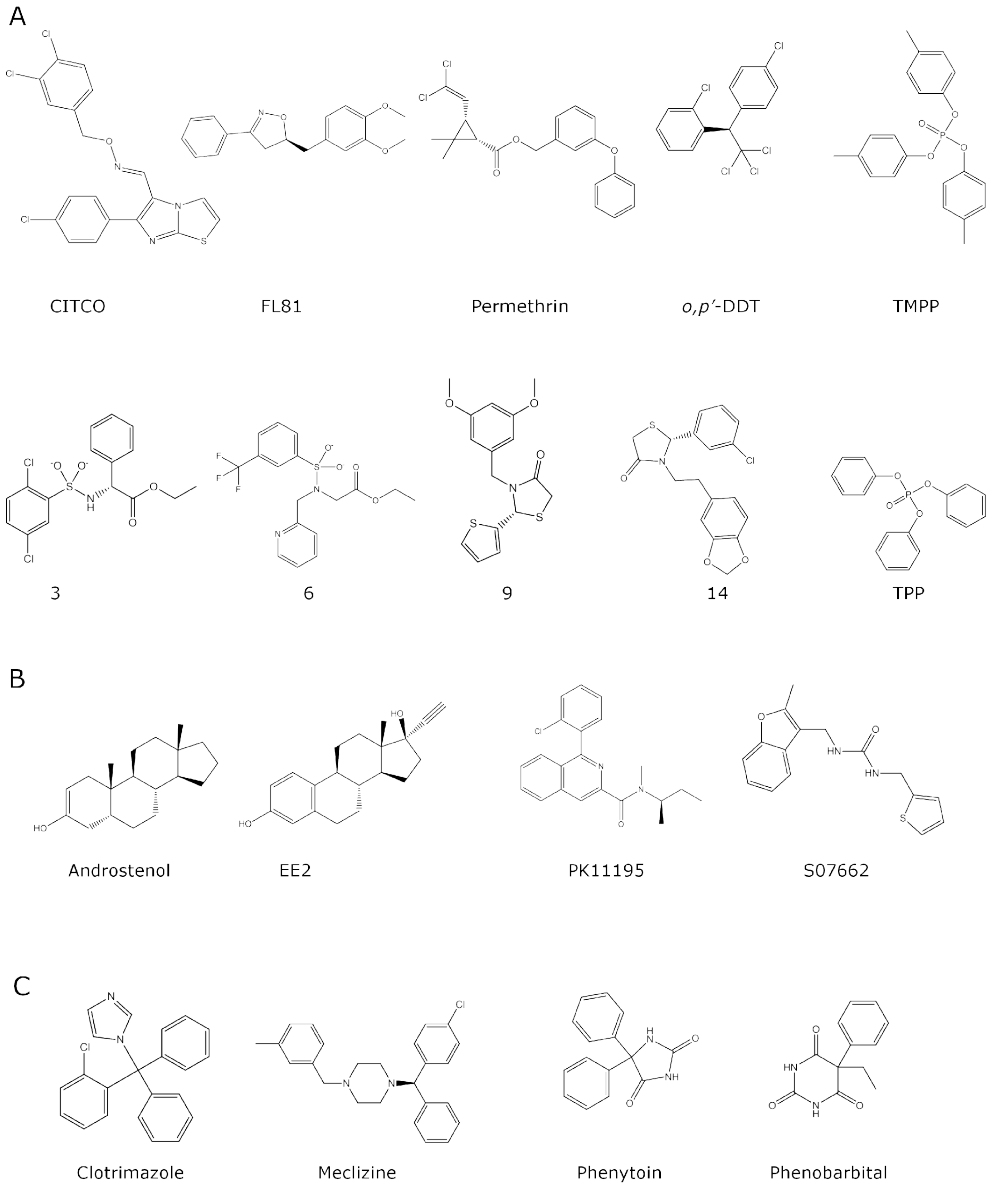

Figure 9. Chemical structures of A) agonists, B) inverse agonists and C) the problem children.

Problem children. The antifungal clotrimazole has been reported to act both as a modest agonist (Jyrkkärinne et al. 2005, Dring et al. 2010) and inverse agonist (Moore et al. 2000 and 2002, Auerbach et al. 2003) for hCAR. In this study, clotrimazole displayed a modest activation of hCAR (M1H), relatively high SRC1 and low NCoR recruitment at the sub-toxic concentrations (***Table 11., Figure 9.***). Meclizine is a histamine H1 receptor antagonist,

reported to act as an inverse agonist at human CAR, based on the decrease in the hCAR/SRC1-interaction at 20μM as well as the decrease of PB-induced CYP expression (Huang et al. 2004). In this study, meclizine exhibited a modest hCAR activation (M1H) as well as SRC1 and NCoR recruitment (M2H) (***Table 11., Figure 9.***); similar observations have also been reported by others (Lau et al. 2011). These results suggest that meclizine is not an inverse agonist of human CAR.

Two anticonvulsant and antiepileptic drugs, phenobarbital (PB) and phenytoin (PHN), are both established CYP2B6 inducers via activation of CAR and PXR (***Figure 9.,*** Kawamoto et al. 1999, Rushmore & Kong 2002, Wang et al. 2004). Both of the compounds were inactive in the hCAR reporter assay but PB activated hPXR as reported earlier (***Table 11.,*** Luo et al. 2002). However, PHN, and to a lesser extent also PB, were able to recruit SRC1 in the M2H assay dose-dependently, to protect the LBD from degradation and the degree of protection correlated with the extent of SRC1 recruitment. This finding suggests that the alleged indirect activation mechanism of hCAR by different phosphorylation events leading to nuclear translocation could in fact be a consequence of the binding of a weak ligand for which the hCAR activation cannot be shown in conventional reporter assays. As expected, both compounds induced CYP2B6 and CYP3A4 mRNAs but had different selectivities, PB favoring CYP3A4 (probably due to hPXR activation), while PHN was clearly more selective for CYP2B6.

Comparison of ligand binding modes. The size of the hCAR LBP allows ligand binding in different orientations and even though the established ligands possess moieties capable of hydrogen bond formation, the hydrophobicity of the pocket encourages binding mainly via van der Waals interactions. Since no clear interaction points (*e.g.* H bond donors or acceptors) can be found in the LBP, it is difficult to define any specific chemical groups or structures that an agonist or inverse agonist should possess in order to bind and/or alter the activation of the receptor. Thus, the identification of interaction areas or amino acid side-chains important for ligand binding would be beneficial for determining the required properties of a ligand. Previous docking and mutation studies have found different coefficient regions (positive I-III and negative IV) and subpockets (S1 and S2), important for ligand binding and hCAR activation (Jyrkkärinne et al. 2008, ***Figure 10.***). The subsite S1, overlapping with coefficient region I, is targeted by compounds that activate hCAR efficaciously. The channel C1, with a negative coefficient region IV at the end, connects the LBP with H12 (Jyrkkärinne et al. 2008, ***Figure 10.***). Occupation of this region may result in steric clashes and displace the H12 from its active conformation, thus preventing coactivator interaction, or conversely, it may promote interactions with corepressors. The amino acid residues F161 (H3), N165 (H3), F234 (H6/H7 loop) and Y326 (H11) form a so-called LBD/H12 interface, creating a barrier between the H12 and the LBP, and this prohibits any direct contacts of ligands with H12 (Xu et al. 2004, Jyrkkärinne et al. 2008, ***Figure 10.***).

In this study, selected compounds were docked into the hCAR crystal structure (PDB entry 1XVP9, chain D) using two versions of the GOLD docking suite. In studies **III** and **IV**, the side chains F161 and Y224 were allowed to move freely ("flexible docking"), whereas in study **I,** all the side chains were "rigid". In study **I**, two different binding modes were observed. Compounds 3 and 9 were shown to bind deep in the LBP in a compact conformation and interact with the small hydrophobic subsites (S1 and S2, ***Figure 10.***) within the LBP. In contrast, compounds 6 and 14 adopted a more stretched L-shaped conformation and were bound to the channel which connects the LBP with H12 (C1, ***Figure 10.***). However, as the earlier studies, as well as study **I**, have been based on a limited

Table 11. Properties of selected hCAR ligands.

		Reporter gene activity						*References*
	µM	**CAR**	**PXR**	**AhR**	**cofactor interaction (SRC/NCoR)[a]**	**CYP mRNA (3A4/2B6)**	**CYP activity (3A4/2B6)**	
Agonists								
CITCO	1	••••	•	••	+ / •	- / ••••	- / •••	*Maglich et al. 2003,* ***III***
FL81	10	•••	••	•	••••• / •	- / •	- / •	*Pulkkinen et al. 2008, Jyrkkärinne et al. 2012,*
o,p'-DDT	10	•••	•	•	•••• / •	•• / ••	• / •	***III***
Permethrin	10	••••	•	••	••••• / •	- / -	• / ••	*Medina-Diaz et al. 2007****, III,***
3 (1554-08332)	10	•••	••••*	•*	••••• / •*	N.D. / •	N.D.	*Abass et al. 2012,* ***III***
6 (1557-02159)	10	••	•••*	-*	••• / •*	N.D. / •	N.D.	***I***
9 (1539-03130)	10	••	••••*	••••*	•••• / ••*	N.D. / •	N.D.	***I***
14 (1539-30112)	10	•••	••••*	••*	••••• / ••*	- * / •••	N.D.	***I***
TPP	10	••	••••	••	•••• / •• *	N.D.	N.D.	***I***
TMPP	10	••	••	••	•••• /•	N.D.	N.D.	*Jyrkkärinne et al. 2005,* ***III***
								Jyrkkärinne et al. 2005, ***I****,* ***III***
Inverse agonists								
	10	- [c]	•	-	•• / •	- / •••	N.D.	*Forman et al. 1998, Jyrkkärinne et al. 2003,* ***IV***
Androstenol	10	- [c]	-	-	• / •	• / ••	N.D.	*Mäkinen et al. 2002,* ***IV***
EE2	10	- [c]	••••	••	•••• / ••••	•• / •• [b]	N.D.	*Li et al. 2008,* ***IV***
PK11195	10	- [c]	•••	••	• / ••••	••• / • [b]	N.D.	***IV***
S07662								
Problem children								
	4	••	••••	••••	•••• / ••	N.D.	N.D.	*Moore et al. 2000 & 2002, Dring 2010,* ***III****,* ***IV***
Clotrimazole	10	•	••	•	• / -	N.D.	N.D.	*Huang et al. 2004, Lau et al. 2011,* ***IV***
Meclizine	50	-	•	•	••• / •	• /••••	• / •••	*Wang et al. 2004* ***III***
Phenytoin	1500	-	•••	•	•• / -	••• / ••••	• / •••	*Sueyoshi 1999, Moore et al. 2000,* ***III***
Phenobarbital								

The results are from studies I, III, IV or unpublished observations (*). [a]M2H assay, [b]20µM [c] The inverse agonists were able to repress the hCAR activity in the following order: EE2>S07662> PK11195>Androstenol, N.D. = not done, • = 1-2.5 -fold, •• = 2.5-5 -fold, ••• = 5-10 -fold, •••• = 10-50-fold, ••••• = 50-100 fold, + = over 100-fold, - = less than 1-fold

number of chemicals or chemical groups and on the "rigid" docking procedure, the predictions may differ from those obtained with flexible docking. Based on the results from MD simulations (**III, IV**), both the C-terminal end of hCAR (harboring HX and H12) and the H2/H3 loop were stabilized and destabilized in different ways by different ligands. As described in ***Section 2.2.1,*** both H3 and HX may be involved in retaining H12 in the active position and contribute to the high constitutive activity. The H2/H3 loop is thought to be one of the ligand entry sites (Martinez et al. 2005).

Strong agonists CITCO and FL81, and to some extent also permethrin, stabilized HX and H12 in the active position. Permethrin appeared to stabilize the H2/H3 loop better than the other ligands. The poorest stabilization of the loop was observed with FL81 and this might explain the lower SRC1 recruitment. However, the recruitment of other coactivators by FL81 (or any of the other ligands) was not studied in this work, and thus this result may not be conclusive. Nonetheless, these contrasting results show that different strong agonists can exhibit very different stabilizing effects on the LBP, all of which can lead to the activation of the receptor. Weaker agonists, such as clotrimazole and PHN, seem to push H12 towards H10 unfavourably for coactivator binding and only stabilize the C-terminal part of the H2/H3 loop.

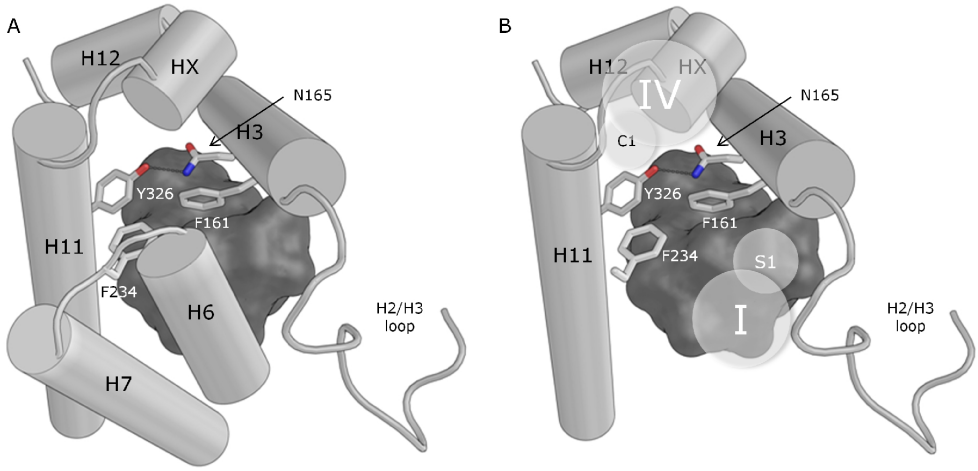

Figure 10. Schematic presentation of features of the hCAR LBP important for ligand binding and hCAR activity (adapted from Xu et al. 2004). **A**) Barrier residues, important helices and loops. The LBP is presented in dark grey. **B**) The approximate locations of positive coefficient region I, subsite S1 (framed by H2', H6 and β3 strand), the negative coefficient region IV and channel C1, connecting the LBP and H12, are presented as light gray circles (Jyrkkärinne et al. 2008). Positive coefficient regions II and III, as well as helices 6 and 7 (A) have been omitted for clarity.

Reporter assays with selected alanine mutants revealed that the mutation of Y326, which plays a central role in stabilizing H12 and also in the constitutive activity of hCAR, abolished or reduced (50% or more) hCAR activation by the agonists tested. In contrast, mutation of N165, which forms a hydrogen bond with Y326, resulted in the enhancement (*e.g.* CITCO, FL81, TPP, clotrimazole) or reduction (*o,p'*-DDT) of hCAR activity, while permethrin caused only negligible effects. F161 and F234 are central amino acids in the ligand-dependent activation of hCAR (Jyrkkärinne et al. 2008). Clotrimazole was the only compound able to activate the F161A, possibly due to its rigid structure and its central position in the pocket. In contrast, all agonists were able to activate the F234A mutant and the activity was enhanced in comparison to the activation of the wild type receptor obtained by treatment with CITCO, *o,p'*-DTT, clotrimazole and permethrin.

Collectively, the hCAR agonists occupy distinct but partly overlapping regions of the LBP and are able to increase its volume. Based on the accumulation of structurally variable hCAR ligands (*e.g.* ***Figure 9.***), the LBP is most likely rather flexible and interactions with certain residues are able to reshape the binding cavity.

The inverse agonists also seem to stabilize the LBP, especially around the H2/H3 loop, but in comparison to the agonists, the LBP is opened more in the direction of H12. The two strong inverse agonists, S07662 and PK11195, both affect the position of H12: While the effect of S07662 is more direct, PK11195 seems to influence the conformation HX by affecting the H6/H7 loop. In contrast to CITCO, neither of these inverse agonists increased the helical content of HX. Due to its larger size and Y-shaped structure, PK1195 may occupy and interact with the LBP more effectively than for example the smaller and L-shaped S07662, and this might be connected to its possible partial agonism (see above, ***Figure 9.***). Although the present data and the results from further studies (see below, Jyrkkärinne et al. 2012) suggest some features and mechanisms connected with inverse agonism, in part due to the fact that no hCAR crystal structure with bound inverse agonist and corepressor is available, the exact mechanism of inverse agonism for CAR remains unclear.

Comparison and limitations of different assays. The M2H assay is more sensitive in detecting weak hCAR agonists than the M1H assay (approx. 11-fold difference), since most of the compounds showed increased reporter activity at low (< 1μM) concentrations. In general, the results from M1H and M2H correlate fairly well. The drawback of the M2H assay, and also one possible explanation for the discrepancies between the M1H and M2H results, is that only one coactivator is included in the assay, whereas several cellular coactivators are present in the M1H assay. Thus, the choice of cell line for M1H assays is of great importance since the activation of NRs or AhR depend on the cofactor content of the cells being used. As mentioned in ***Section 5.2.1***, the cofactor content may play a role in the low basal activity of hCAR encountered in the C3A-based M1H assay. Similarly, competition for the recruitment of NCoR could be one of the explanations for the low corepressor recruitment by EE2 and androstenol in M2H, whereas in Y2H, both of these ligands induce a strong hCAR/NCoR interaction. There may be other explanations, such as differential transport or metabolism of these ligands or differences in the post-translational modification of the coregulator peptides. Furthermore, since both EE2 and androstenol decrease the activity of the endogenous AhR, these compounds may act through some unknown pathways or processes in C3A cells, resulting in the low hCAR/NCoR interaction.

In general, even though good correlation between reporter assays and CYP expression for hPXR and hAhR has been shown (Luo et al. 2002, Sugihara et al. 2008) direct comparisons between these two methods are problematic. The poor correlation, seen for hCAR and CYP2B6/3A4, may result from several factors: 1) The differential DT expression profiles of hepatoma cells and primary hepatocytes could lead to differences in the intracellular levels of the compounds (Teng et al. 2003), 2) the coregulator specificity of the receptor may depend on which ligand is bound as well as the coregulator profile of the cells, 3) the test compounds may be differentially metabolized in hepatoma cells and primary hepatocytes and 4) the test compounds may inhibit the measured CYP enzymes in primary hepatocytes. Compounds 3 and 6 contain ester groups, which can be easily hydrolyzed in hepatocytes, in contrast to the non-ester compounds 9 and 14 (**I**). FL81 and permethrin are also metabolized in HLMs but the specific metabolizing enzymes or metabolites have not been identified (unpublished results). Most of the compounds tested in study **I** contain chiral centers and since racemic mixtures were used in the assays, neither the isoform specificities in hCAR activation nor the possible metabolism in hepatocytes are known. Similarly, *trans*-permethrin has previously been shown to be metabolized by esterases both *in vivo* and *in vitro* (Takaku et al. 2011). Preliminary inhibition studies revealed that several of the hCAR activators were inhibitors of CYP2B6 and some CYP3A4 inhibition was also observed, with

permethrin and FL81 showing especially strong concentration-dependent CYP3A4 inhibition and a moderate inhibition of CYP2B6 with the recombinant enzymes.

The LPD assay, measuring increased protection of the NR from degradation due to ligand binding *in vitro*, can be used to study the association of ligands with the hCAR LBD. Even though no specific protected fragment can be associated with agonists or inverse agonists, the presence of different fragments with different ligands suggests that the ligands have different binding orientations within the LBP.

The initial pharmacophore search in study **I** limited the structural variability of the resulting database subset, which is evident by all of the identified compounds belonging to two distinct chemical classes. Furthermore, the lack of flexibility in the first docking approaches (*e.g.* Windshügel et al. 2007, **I**), the lack of coregulator peptide (**I, III, IV**) and the short length of the MD simulations (**III, IV**) at least partly made it difficult to identify the distinguishing features of agonists and inverse agonists *in silico* (Windshügel & Poso 2011, Jyrkkärinne et al. 2012). In later simulations, which contained also the SMRT peptide, it was shown that the effects of agonists on the H2-H3 loop were very modest, whereas the inverse agonists tended to destabilize the loop (Jyrkkärinne et al. 2012). In addition, the stabilization and orientation of H12 (moves towards H10 with inverse agonists) and HX (less stable with inverse agonists) differed between agonists and inverse agonists. In addition, the changes elicited by the inverse agonists were subtle perhaps due to the lack of the corepressor peptide in the simulation. The subsequent study showed that the inverse agonists caused a clearer shift of H12 when the SMRT corepressor peptide was included in the MD simulations (Jyrkkärinne et al. 2012). The ligand binding as such also induced large movements in the protein, not only reshaping and increasing the volume of the LBP but also evoking allosteric effects and thus, at least docking studies alone are not sufficient to define the activation or inactivation of the receptor seen after incubation with specific compounds.

6 Conclusions and future prospects

The following conclusions can be drawn from this study:

1. The results from study **II** demonstrate the proof of principle that the expression of multiple CYPs can be simultaneously upregulated by the constitutively active chimeric CAR and PXR, in hepatoma cells. A similar approach could be used for other cell lines lacking expression or function of the genes involved in xenobiotic metabolism. One of the established cell lines, hCAR28, has also been shown to exhibit higher CYP3A4 activity than the parent cell line (Ma et al. 2012). The obtained cell lines, especially the subline hCAR28, could be used in studies modeling the interindividual differences in CYP activities. However, further studies will be needed to determine the impact of culture conditions on DME expression in these cells.

2. The optimized and validated xenosensor assays provide a robust and reproducible method for screening potential xenosensor ligands and, together with other methods, predict the possible CYP induction resulting from the activation of the xenosensors. The hCAR assay is the first protocol which does not require the addition of supplemental chemicals or mutations to decrease the high basal activity of CAR and thus, can provide reliable information on the hCAR ligand binding specificity and affinity. The assays enable the use of different receptors to study the ligand specificities between xenosensors and other NRs or to compare the responses to ligands between different species. The sensitive M2H assay can be used to identify and to study the properties of weak hCAR ligands. In this study, only two cofactors (SRC1 and NCoR) and one NR (hCAR) were used but different NRs and cofactors could be cloned into respective vectors to make the assay more versatile and to study the cofactor specificity of different receptors.

3. The established novel hCAR ligands, FL81 and S07662, are suitable and stable reference compounds for different reporter assays, although they do not perform equally well in CYP mRNA expression studies. Together with other established hCAR ligands from novel chemical classes, they can also serve as lead compounds for the generation of new ligands for research and for the development of new selective compounds targeting hCAR. In general, this study highlights the problems encountered in evaluating specific xenosensor, especially hCAR, ligands. Thorough analyses are required before any novel chemical entity can be used as a reference or a lead compound.

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Use of Xenosensors for Drug Metabolism Studies

Focus on Constitutive Androstane Receptor

Three xenosensors, constitutive androstane receptor (CAR), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR), are main regulators of enzymes and transporters important for xenobiotic metabolism and disposition. In this thesis, modified xenosensors were used to increase the cytochrome P450 (CYP) expression in hepatoma cells. Xenosensor reporter assays were validated and used together with other biological assays and molecular modeling techniques to find ligands and to study the function of human CAR. Novel hCAR ligands are presented and their properties are compared to previously described compounds.

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