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Induction of human cytochrome P450 enzymes

Predictive *in vitro* models and
rifampicin induction *in vivo*

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The more you know, the less you understand.
from Tao Te Ching

Det är bättre att gå stapplande på den rätta vägen än att med fasta steg gå vilse.
Aurelius Augustinus (354-430)

ABSTRACT

The cytochrome P450 (P450) enzymes comprise the most important enzyme system with regard to phase I metabolism of drugs. Induction of P450s can result in decreased plasma concentrations of the drug itself or a coadminstered drug, followed by lack of effect. In the present study different *in vitro* models have been investigated for their ability to predict P450 induction in humans. It was found that human liver slices respond to prototypical inducers, although the model is not applicable to screening of large sets of compounds. High throughput screening can however be performed in a reporter gene assay. The study showed that results from a PXR reporter gene assay could be used to classify compounds as CYP3A *in vivo* inducers or non-inducers when relating *in vivo* AUC to PXR EC_{50} values. Subsequently it was shown that instead of EC_{50} values, the concentration giving a 2-fold increase of baseline levels (F_2 values) could be used for the classification of compounds. A new cell line, HepaRG cells, was also investigated for prediction of P450 induction. Results from experiments in HepaRG cells did not only classify compounds as inducers or non-inducers, but gave a strong correlation (R^2=0.863) to *in vivo* CYP3A induction, and could hence be used to quantitatively predict the extent of CYP3A induction *in vivo*.

In addition, the drug metabolising properties of the HepaRG cells were evaluated. Stable mRNA expression of drug metabolising enzymes, transporters, and liver specific factors in HepaRG cells were shown for up to six weeks in culture. Although the mRNA expression of drug metabolising P450s were lower in HepaRG cells as compared to human hepatocytes, the relative levels of the P450s were similar. The HepaRG cells could thus be used not only for induction studies but also for investigation of metabolic pattern of drugs and new chemical entities.

Furthermore, the *in vivo* induction of P450s by three different daily doses (20, 100, and 500 mg) of rifampicin was investigated. Rifampicin is perhaps the most well documented CYP3A inducer *in vivo*, and is used as a positive control in induction studies *in vitro*. Rifampicin is also an inducer of CYP1A, CYP2B6, and CYP2C enzymes. By the use of the Karolinska cocktail, the response of four P450s could be investigated at one time point in the same subject. CYP1A2 and CYP2C9 were induced after 500 mg rifampicin daily ($p<0.05$), and CYP2C19 after 100 mg rifampicin daily ($p<0.05$). A strong 4-fold induction of CYP3A4 was seen at 500 mg rifampicin daily for both quinine/3'-hydroxyquinine and 4ß-hydroxycholesterol measurements ($p<0.001$). CYP3A4 was also induced at the two lower doses of rifampicin measured by either of these two markers ($p<0.01$). A strong correlation (Spearman rank r_s=0.71; 95% C.I.=0.52-0.90; $p<0.001$; n=22) of the two CYP3A4 markers indicates that the cholesterol metabolite 4β-hydroxycholesterol could be used as an endogenous marker for CYP3A4 induction. By the use of 4β-hydroxycholesterol, CYP3A4 induction can be investigated concurrently with the pharmacokinetics of the drug candidate *in vivo*, and no separate CYP3A induction study is needed.

PUBLICATIONS

I. **Persson KP**, Ekehed S, Otter C, Lutz ESM, McPheat J, Masimirembwa CM and Andersson TB (2006) Evaluation of human liver slices and reporter gene assays as systems for predicting the cytochrome P450 induction potential of drugs *in vivo* in humans. *Pharm Res* **23:**56-69.

II. **Kanebratt KP** and Andersson TB (2008) HepaRG cells as an *in vitro* model for evaluation of cytochrome P450 induction in humans. *Drug Metab Dispos* **36:**137-145.

III. **Kanebratt KP** and Andersson TB Evaluation of HepaRG cells as an *in vitro* model for human drug metabolism studies. *Submitted to Drug Metabolism & Disposition*.

IV. **Kanebratt KP**, Diczfalusy U, Bäckström T, Sparve E, Bredberg E, Böttiger Y, Andersson TB, and Bertilsson L Cytochrome P450 induction by rifampicin in healthy subjects: determination by the Karolinska cocktail and the endogenous CYP3A4 marker 4β-hydroxycholesterol. *Submitted to Clinical Pharmacology & Therapeutics*.

CONTENTS

ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AUC	Area under the plasma concentration versus time curve
bHLH	Basic helix-loop-helix
CAR	Constitutive androstane receptor
CL_{int}	Intrinsic clearance
Ct	Threshold cycle, the cycle number where the fluorescence passes a fixed threshold
DMSO	Dimethyl sulfoxide
EC_{50}	Inducer concentration giving half the maximum effect
ELISA	Enzyme-linked immunosorbent assay
E_{max}	Maximum effect
F_2	Inducer concentration giving a 2-fold increase of baseline levels
FDA	U.S. Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
huPO	Human acidic ribosomal phosphoprotein
MR	Metabolic ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
P450	Cytochrome P450
PBREM	Phenobarbital responsive enhancer module
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PXR	Pregnane X receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
SNP	Single nucleotide polymorphism
TCDD	2,3,7,8-Tetrachloro-dibenzo-p-dioxin
UGT	UDP-glucuronosyltransferase
XREM	Xenobiotic responsive enhancer module

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

When a drug is administered to a patient, the human body recognises the drug molecule as foreign and tries to dispose the drug. This is often accomplished by biotransformation of the drug into a more hydrophilic molecule, and thereby facilitating the excretion of the drug. Biotransformation of drugs is mainly metabolic processes performed by a number of enzymes. The liver is the organ where the majority of drug metabolism takes place.

The drug metabolising enzymes are often divided into phase I and phase II enzymes, where phase I enzymes introduce functional groups to the drug molecule by reactions such as oxidation, reduction, or hydrolysis. Cytochrome P450 (P450) enzymes comprise the most important drug metabolising enzyme system. It is responsible for more than 70% of the phase I metabolism of all clinically used drugs. Phase II enzymes conjugate the drug molecule, or the product of phase I metabolism, with endogenous compounds. Examples of phase II metabolism are glucuronidation, sulfation, and acetylation.

When two or more drugs are coadministered there is a risk that they affect each other's metabolism. One drug could decrease, inhibit, or it could increase, induce, the metabolism of other drugs. These effects are commonly called drug-drug interactions and induction of human P450s is the theme of this thesis. Induction of P450s is most of the time the result of increased gene transcription, and as a consequence increased protein activity. A drug being an inducing agent often induces its own metabolism, i.e. autoinduction, but will of course result in increased metabolism of other drugs metabolised by the induced enzyme. It may also happen that the drug induce enzymes that are not important for the metabolism of the drug itself, but for other medications. In the clinical practice, enzyme induction may lead to sub-therapeutic concentrations of the drug itself or a coadministered drug, or to increased concentrations of an active or toxic metabolite.

Enzyme induction has been investigated in many *in vitro* studies, using human liver slices, human hepatocytes, and immortalised cell lines. However, few attempts have been made to use the *in vitro* data to predict the magnitude of enzyme induction *in vivo*, which probably is due to demanding techniques and the complex process by which enzyme induction occurs. The possibility to predict the magnitude of enzyme induction *in vivo* from *in vitro* studies would nevertheless be of great advantage in the drug development process.

1.2 CYTOCHROME P450 ENZYMES

The P450 superfamily consists of many related enzymes with different but overlapping substrate specificity. The P450s are heme-containing enzymes and in eukaryotic cells the P450s are membrane bound through their N-termini. P450s are generally localised on the cytosolic side of the endoplasmic reticulum, although some P450s are located on the matrix side of the mitochondrial inner membrane (Guengerich, 2005). The human xenobiotic metabolising P450s are mainly expressed in the liver and intestine, but can also be found in the kidney, skin, and lung. The P450s utilise NAPPH as a source of electrons and the co-enzyme NADPH-cytochrome P450-reductase mediates the electron transfer. Members of the P450 superfamily are also involved in important endogenous functions such as synthesis and metabolism of hormones, vitamins, bile acids, and fatty acids (Raunio et al., 1995)

In humans there are twelve P450 families and most of the drug metabolising P450s belong to the CYP1, 2, and 3 families, which are responsible for 70-80% of all phase I dependent metabolism of clinically used drugs (Bertz and Granneman, 1997, Evans and Relling, 1999). For drug metabolism CYP3A4, CYP2C9, CYP2C19, and CYP2D6 are considered to be the most relevant enzymes (Gardiner and Begg, 2006), of which CYP3A4 is responsible for the metabolism of at least 50% of drugs primarily cleared by P450s (Bertz and Granneman, 1997). Many of the enzymes involved in the metabolism of xenobiotics are characterised by broad substrate specificity. Some of the P450s are also subject to large interindividual variability in activity, which is the result of variation in both genetic and environmental factors.

1.2.1 Nomenclature

The human genome encodes 57 individual P450s (Nelson et al., 2004) and a systematic naming of the individual enzymes is required to ensure accurate communication between scientist from different laboratories and countries. The members of the cytochrome P450 superfamily are classified according to amino acid sequence homology (Nebert et al., 1987; Nelson et al., 1996) and the nomenclature system divides the superfamily into families and subfamilies. Families have more than 40% identical amino acid sequence, and are designated by the abbreviation for cytochrome P450, CYP, and an Arabic number (e.g. CYP2). Subfamilies have more than 55% identical amino acid sequence, and are designated by a letter following the Arabic number (e.g. CYP2C). The last Arabic number is defining the isoenzyme encoded by a specific gene (e.g. CYP2C19). Different alleles of the same enzyme have been discovered and these are designated by an asterisk and a number (e.g. CYP2C19*4).

1.2.2 Polymorphism

Significant genetic variation has been described for drug metabolism and several of the drug metabolising P450s, such as CYP2C19 and CYP2D6, have allelic variants and are thus polymorphic. A polymorphism is generally defined as a more than 1% frequency of an allelic variant in a population (Flockhart and Bertilsson, 2007). The majority of allelic variants are single nucleotide polymorphisms (SNPs), but also gene duplication occurs. The presence of a polymorphism can lead to reduced or enhanced enzyme activity, resulting in higher or lower plasma concentrations of the drug than expected. This explains some of the variation in drug response seen in the clinical everyday life. Individuals with decreased enzyme activity are called intermediate or poor metabolisers, and normally carry one or two defect alleles, respectively. Individuals with normal enzyme activity are called extensive metabolisers, whereas individuals with gene duplication and increased activity are called ultrarapid metabolisers.

1.2.3 Probe substrates

A probe substrate has a metabolic pathway that is catalysed by a specific enzyme, i.e. a certain metabolite is only formed by one specific enzyme. The probe substrates are therefore used to measure specific enzyme activities both *in vitro* and *in vivo*. The probe substrates used *in vivo* can be different from the probe substrates used *in vitro*, since ethical considerations to the patient/healthy volunteer have to be taken. In **Table 1** some of the most commonly used *in vitro* and *in vivo* probe substrates are listed for the most important inducible P450s.

To be able to measure more than one enzyme activity simultaneously a cocktail approach, where several probe drugs are administered concurrently, can be applied. This approach offers several advantages. *In vitro* the use of the scarce supply of human material can be optimised. *In vivo* the number of induction studies can be reduced. At the same time intraindividual variability is abolished, since environmental factors do not change during the limited time of an experiment. For a cocktail approach to be successful, it has to be established that the different probe drugs do not interact with each other, both enzymatically and analytically. *In vitro* cocktails have been developed for the assessment of as many as seven individual P450s at the same time (Dierks et al., 2001; Unger and Frank, 2004; Dixit et al., 2007). In Paper II and III a smaller cocktail with three probe substrates were applied. For cocktails in clinical studies, also any interactions on effect of the drugs have to be taken into consideration. Several cocktails has been developed also for clinical studies (Frye et al., 1997; Streetman et al., 2000; Zhu et al., 2001; Christensen et al., 2003). In Paper IV the Karolinska cocktail developed by Christensen et al. (2003) has been employed.

Table 1. Nuclear receptor controlling the expression, probe substrates and inducers for the most important inducible P450s.

P450	**Nuclear receptor**	***In vitro* probe substrate**	***In vitro* inducer**	***In vivo* probe substrate**	***In vivo* inducer**
CYP1A2	AhR	Phenacetin-*O*-deethylation	Omeprazole, β-naphthoflavone	Caffeine, theophylline	Tobacco smoke
CYP2B6	CAR (PXR)	Bupropion hydroxylation, efavirenz hydroxylation	Phenobarbital	Bupropion, efavirenz	Rifampicin
CYP2C8	PXR (CAR)	Taxol 6'-hydroxylation	Rifampicin	Repaglinide, rosiglitazone	Rifampicin
CYP2C9	PXR (CAR)	Diclofenac 4'-hydroxylation, S-warfarin 7'-hydroxylation	Rifampicin	Losartan, warfarin, tolbutamide	Rifampicin
CYP2C19	PXR (CAR)	S-Mephenytoin 4'-hydroxylation	Rifampicin	Omeprazole, mephenytoin, lansoprazole	Rifampicin
CYP3A4	PXR (CAR)	Midazolam 1'-hydroxylation, testosterone 6β-hydroxylation	Rifampicin	Midazolam, quinine, triazolam, simvastatin, buspirone, felodipine	Rifampicin

1.3 ENZYME INDUCTION

Repeated doses of a drug may enhance the metabolism of the drug (autoinduction). The metabolism can also be induced by another drug, given concomitantly, or an environmental constituent such as dietary ingredients or tobacco smoke. If the metabolism for a drug is induced it can lead to loss of effect due to sub-therapeutic concentrations. On the other hand, if the drug is a prodrug or has a toxic metabolite it could instead lead to unwanted side effects. During the drug development process it is essential to know as early as possible if the drug candidate is an enzyme inducer. For this purpose *in vitro* induction models have been developed. Human hepatocytes are often referred to as the "gold standard" *in vitro* model for induction studies, but also immortalised cell lines and reporter gene assays have been used. The outcome of preclinical *in vitro* studies on possible induction liability of a new drug candidate will have major impact on the design of the clinical program. Positive *in vitro* results advocate for a clinical induction investigation. Indications that drug candidates may be P450 inducers could also result in restrictions in inclusion criteria for healthy volunteers and patients in the clinical study program.

Many of the drug metabolising P450s are known to be inducible *in vitro*, e.g. CYP1A, CYP2B6, CYP2C, and CYP3A. One exception is CYP2D6, which has not been shown to be subject to induction. CYP3A4 is the most important P450 enzyme in drug

metabolism and also seems to be the enzyme with the highest potential for induction *in vivo* (Backman et al., 1996; Wang et al., 2001; Gerber et al., 2005). In contrast to CYP2C and CYP3A enzymes, clinically significant CYP1A induction has not been reported by any drugs on the market at therapeutic doses.

1.3.1 Mechanism of enzyme induction

Enzyme induction generally occurs at the transcriptional level and is then the result of ligand binding to and activation of one of the nuclear receptors controlling the P450 gene transcription. The most important nuclear receptors for drug metabolising P450s are the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR). In general, when a receptor is activated it associates with other transcription factors and the formed complex binds to the DNA and initiates the transcription. An increased activation of the receptors leads to increased enzyme activity through the cascade depicted in **Figure 1**.

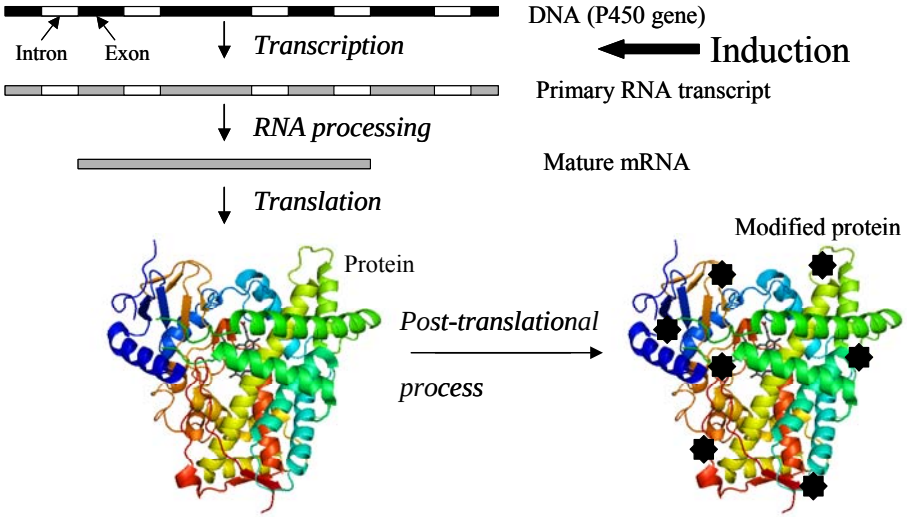

Figure 1. From gene to a functional protein. The process starts with transcription of the gene into a primary RNA, which is spliced and further processed into a mature mRNA. The mRNA is translated into the amino acid sequence comprising the protein, which then is further processed by e.g. phosphorylation into the active protein.

PXR is the major determinant of CYP3A4 gene regulation by xenobiotics (Lehmann et al., 1998; Gibson et al., 2002), and has also been established to be important for CYP2C9 induction (Chen et al., 2004). CAR has been proposed to regulate the expression of CYP2B6 (Sueyoshi et al., 1999; Wang et al., 2004), while the CYP1A enzymes are regulated by AhR (Denison and Nagy, 2003).

Although increased transcription is by far the most important mechanism behind enzyme induction, cases of non-transcriptional mechanisms have also been reported. For example, induction of CYP3A by troleandomycin in rats is a result of decreased protein degradation without increased protein synthesis (Watkins et al., 1986). Similarly, the induction of CYP2E1 following alcohol intake has been suggested to be the result of enzyme stabilisation by ligand binding to the protein (Song et al., 1987; Chien et al., 1997). The induction of CYP1A1 by primaquine in mammalian V79 cells is a result of both increased transcription and decreased protein degradation (Werlinder et al., 2001).

1.3.1.1 Aryl hydrocarbon receptor

Of the two family members of CYP1A, CYP1A2 is highly expressed in the liver, whereas CYP1A1 primarily is expressed in lung, placenta and lymphocytes. The expression of the CYP1A enzymes is regulated by AhR (Denison and Nagy, 2003; Lin, 2006). Prototypical AhR ligands are planar, hydrophobic, and halogenated hydrocarbons such as 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). AhR is a basic helix-loop-helix (bHLH) protein, where the basic region contributes to the DNA-binding, and the HLH region is responsible for protein-protein dimerisation. When AhR is not associated with a ligand it is located in the cytoplasm of the cells where it exists as a multiprotein complex. When a ligand (inducer) binds to AhR, the receptor undergoes conformational changes and translocate into the nucleus. In the nucleus, AhR dissociates from parts of the multiprotein complex and forms a heterodimer with a related nuclear protein called Arnt. The heterodimer activates the CYP1A genes through binding to one distal enhancer element and one proximal promoter element upstream of the CYP1A genes. The distal enhancer has three binding sites for the AhR/Arnt heterodimer, whereas the promoter has none. Transcription experiments suggest that there must be a mechanism for transmitting the induction signal after binding to the enhancer but the exact mechanism for this is not known (Lin, 2006).

1.3.1.2 Constitutive androstane receptor

CAR is one of the main determinants of CYP2B expression. Similarly to AhR, CAR is located in the cytoplasm when it is not activated. Contrary to AhR, translocation of CAR does not require direct ligand binding, but seems to involve a specific and sensitive dephosphorylation step (Honkakoski and Negishi, 1998). When CAR is translocated into the nucleus, it forms a heterodimer with the retinoid X receptor (RXR). The heterodimer binds to retinoic acid response elements (RAREs) and transactivates the target genes of RAREs in a constitutive manner in absence of ligands (Baes et al., 1994). Because of the constitutive transactivation, CAR has been referred

to as “constitutive androstane receptor”. The CYP2B genes are regulated by the CAR/RXR heterodimer via binding and transactivation of phenobarbital responsive enhancer modules (PBREMs) upstream of the CYP2B genes (Honkakoski et al., 1998). PBREM is a conserved arrangement, which contains two nuclear receptor binding sites (Honkakoski et al., 1998).

1.3.1.3 Pregnane X receptor

PXR is predominantly expressed in the liver and to a smaller extent in the intestine. The general belief is that PXR is located in the nucleus in its inactive form, although a recent study has questioned this dogma (Squires et al., 2004). PXR mediates the induction of CYP2C and CYP3A enzymes and is a promiscuous receptor that can be activated by several structurally diverse compounds. This is due to a highly hydrophobic and flexible ligand binding domain. When PXR is activated by ligand binding, it forms a heterodimer with RXR, similar to CAR. The PXR/RXR dimer binds to response elements upstream of the target gene. In the CYP3A genes, three binding sites have been found. Two are located in the distal enhancer region and are termed xenobiotic responsive enhancer module (XREM), and the third binding site is located in the proximal promoter. Mutation studies has indicated that all three binding sites are necessary to get full PXR activation (Sueyoshi and Negishi, 2001).

1.3.2 Cross talk

The process that an individual gene can be activated by more than one nuclear receptor is called cross talk. Although CAR and PXR have been determined to be the major regulators of CYP2B and CYP2C/3A, respectively, extensive cross talk between the nuclear receptors have been reported, indicating that CAR and PXR are affecting the regulation of all three P450 families (Lin, 2006).

The cross talk originates from a significant overlapping between the binding of CAR and PXR to DNA response elements of several genes. The CAR/RXR heterodimer is able to bind and transactivate the response element for PXR/RXR in the CYP3A genes (Xie et al., 2000b; Goodwin et al., 2002). Similarly PXR is able to transactivate CYP2B genes by binding to the response elements of the CYP2B genes (Xie et al., 2000b). PXR/CAR cross talk has also been reported for CYP2C induction (Gerbal-Chaloin et al., 2002).

1.3.3 Prototypical inducers

Due to the broad substrate acceptance of PXR, the receptor is activated by a diverse set of compounds. The most commonly used positive control in *in vitro* studies for PXR activation and CYP3A and CYP2C induction is rifampicin. Inducers recommended by the U.S. Food and Drug Administration (FDA) as positive controls in *in vitro* induction studies are listed in **Table 1**. Phenobarbital is perhaps the most potent CYP2B inducer and is commonly used as positive control for CYP2B induction. CYP2B is often induced by the same compounds as CYP3A because of PXR's and CAR's ability to activate both CYP2B and CYP3A genes. Due to the cross talk there are no selective CYP3A or CYP2B inducers, although rifampicin and phenytoin are considered to be selective activators of PXR and CAR, respectively (Moore et al., 2000; Wang et al., 2004). TCDD has historically been used as a positive control for *in vitro* studies of CYP1A induction. However, TCDD is an environmental toxin and hence clinical studies are not accessible for this compound. Omeprazole can be used as a positive control for AhR activation and CYP1A induction instead of TCDD.

1.3.4 Time dependency

Enzyme induction is a time dependent process, involving activation of transcription, biosynthesis of mRNA and eventually formation of the active protein. Both *in vitro* and *in vivo* it has been shown that the time to maximum induction varies between the different P450s. In a clinical study on the effect of carbamazepine on CYP1A2 and CYP3A4 the estimated half-lives for induction were 105 and 70 hours, respectively (Magnusson, 2007). Time dependent induction in human hepatocytes has been investigated for CYP2C8, CYP2C9, CYP2B6, and CYP3A4 mRNA. The CYP2C enzymes seemed to reach a maximum before CYP2B6 and CYP3A4 (Gerbal-Chaloin et al., 2001). The time factor is thus important to consider in the design of induction studies both *in vitro* and *in vivo*.

1.3.5 Species differences

Induction of CYP1A and CYP3A4 are subject to significant species differences. For example, omeprazole is a CYP1A inducer in humans, but has little or no effect in mice and rabbits (Diaz et al., 1990; McDonnell et al., 1992). Rifampicin is a potent inducer of CYP3A enzymes in humans and rabbits, but has little effect on CYP3A in rats (Kocarek et al., 1995). The reason for the different induction patterns of CYP3A in different species has been shown to be caused by structural differences in PXR (Jones et al., 2000; Xie et al., 2000a). Comparison of PXR from different species revealed that there is more than 95% sequence homology in the DNA binding domain, whereas the

ligand binding domain only shares 76-83% homology (Zhang et al., 1999; Wang and LeCluyse, 2003). Because of the discrepancies between animals and humans, predictions from animal models of possible induction in man are not straightforward. One of the aims of the thesis was therefore to find a human based *in vitro* model, which accurately could predict the *in vivo* induction in humans.

1.4 ENZYME INDUCTION *IN VITRO*

1.4.1 *In vitro* models

1.4.1.1 Human liver slices

Human liver slices comprise a model that closely resembles the human liver *in vivo*, with all cell types represented and cell-cell interactions preserved. Compared to human hepatocytes the technique to prepare the human liver slices is easier to perform and no digestive enzymes are used, which is an advantage since these enzymes can affect the viability of the cells. Apart from the preparation process, liver slices are flawed with the same drawbacks as hepatocytes, i.e. high interindividual variability and erratic supply. Some induction studies have however been performed in the liver slice model. Induction of apoprotein for the major P450s were showed by Edwards et al. (2003) and in Paper I the P450 mRNA induction was investigated in addition to enzyme activities. The enzyme activities decline significantly over time in cultured human liver slices (Paper I, Martin et al., 2003) and many times decrease below the limit of detection. Since a control activity in untreated cells is necessary to be able to determine the extent of induction the use of enzyme activity as an endpoint is limited in human liver slices.

1.4.1.2 Human hepatocytes

Primary human hepatocytes are often referred to as the "gold standard" for induction investigations *in vitro* (Li et al., 1997; LeCluyse, 2001; Madan et al., 2003). However, human hepatocytes are flawed with some serious drawbacks. In general there is a time-dependent decrease in the expression of mRNA for all major P450s, which results in deterioration of P450 activities (LeCluyse, 2001). Advanced culture conditions such as Matrigel sandwich cultures have been used to counteract these declines. Although CYP3A4 and CYP2D6 mRNAs return to levels nearly equal to or greater than those at time of isolation, other enzymes like CYP1A2 and CYP2E1 do not return to original values (LeCluyse, 2001). Moreover, the use of complex culture conditions do not affect the induction response (LeCluyse, 2001).

Human hepatocytes are also subject to high interindividual variability both in basal P450 levels and in the extent of induction. The high variability in induction response seems to be correlated to the basal P450 activities of the hepatocytes, inasmuch low basal levels result in high induction response and *vice versa* (Kostrubsky et al., 1999). The consequence of the high variability between batches is that multiple individuals need to be investigated in order to get an adequate average result. The time line for repeated experiments can however be unpredictable, since supply of fresh human hepatocytes is erratic.

1.4.1.3 Cell lines

Over the years, considerable efforts have been made to develop cell lines, both immortalised hepatocytes and human hepatoma cell lines, to supersede human hepatocytes in induction studies. Unfortunately the cell lines many times degenerate to immature cells that do not express P450s and required transcription factors. The perhaps most well known human hepatoma cell line is the HepG2 cells. The HepG2 cells express CYP1A enzymes and have therefore been used to investigate the mechanism for CYP1A induction (Fontaine et al., 1999; Backlund and Ingelman-Sundberg, 2004).

A newly developed cell line is the Fa2N-4 cells, which is immortalised human hepatocytes. CYP1A2, CYP2C9, and CYP3A4 have been shown to be induced by prototypical inducers in the Fa2N-4 cells, although the utility for induction of CYP2B6 has yet not been shown for this cell line (Mills et al., 2004; Ripp et al., 2006).

A cell line with promising qualities is the human hepatoma HepaRG cell line, which is investigated in Paper II and III. HepaRG cells can differentiate into a hepatocyte-like morphology and display several hepatocyte-like functions (Aninat et al., 2006; Le Vee et al., 2006). The HepaRG cells express drug metabolising enzymes, nuclear receptors and hepatic drug transporters and have been shown to respond to P450 inducers such as 3-methylcholantrene, rifampicin, and isoniazid (Aninat et al., 2006).

A cell line expressing the necessary transcription factors, drug metabolising enzymes and transporter proteins could be a valuable substitute for human hepatocytes in induction studies. Such a cell line would also have the potential to be used for investigations of drug metabolism. Since the HepaRG cells showed very promising metabolic properties, this was investigated in Paper III.

1.4.1.4 Reporter gene assays

Reporter gene assays are cell-based models where the activation of nuclear receptors regulating the P450 expression can be measured. Reporter gene assays have been developed for both PXR and AhR and were evaluated as induction models in Paper I. In the PXR assay the DNA sequence coding for PXR is transiently transfected into HepG2 cells together with a DNA construct including the distal enhancer and proximal promoter region for CYP3A4, followed by the luciferase gene (Goodwin et al., 1999). After transfection the cells are treated with the potential PXR activator. Receptor activation can be detected as increased luminescence from increased transcription of the luciferase gene, as schematically described in **Figure 2**.

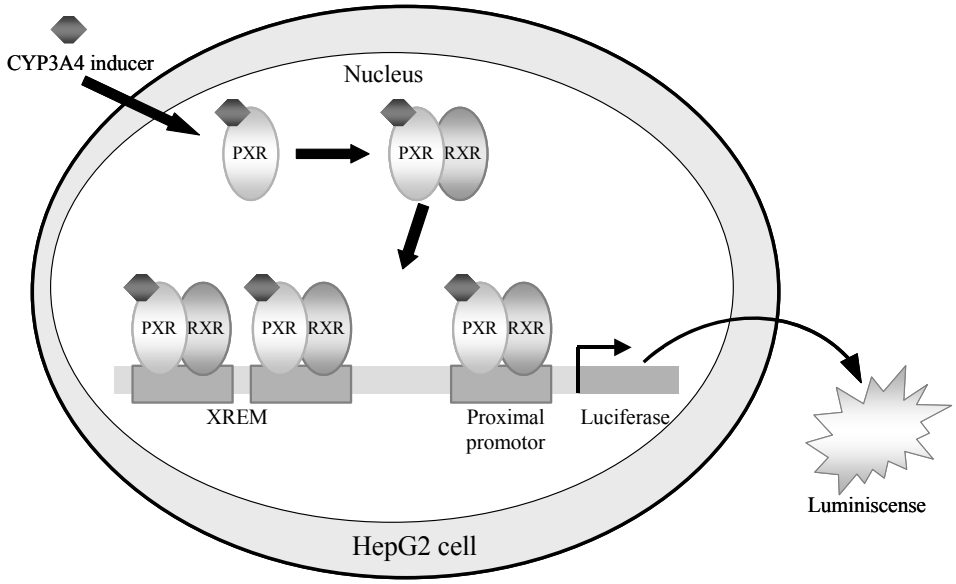

Figure 2. Schematic description of PXR reporter gene assay in HepG2 cells. The inducer enters the cell and the nucleus and binds to PXR, which upon activation forms a heterodimer with RXR. The PXR/RXR complex binds to the DNA binding sites, which earlier has been transfected into the cell. The binding of PXR/RXR to the DNA initiates the transcription of luciferase, which can be can be detected as increased luminescence in cells where PXR has been activated.

PXR is known to be the major transcription factor determining the induction of CYP3A4. A good correlation between activation of PXR in a reporter gene assay and the induction of CYP3A4 mRNA and activity in primary human hepatocytes was reported by (Luo et al., 2002). Thus there is a mechanistic basis for investigating whether PXR activation could predict CYP3A induction *in vivo*.

An equivalent model has also been developed for AhR, where the CYP1A distal enhancer and the luciferase gene is stably transfected into HepG2 cells (Gradin et al., 1993).

Creating a similar model for CAR has been a much more intriguing task. CAR spontaneously translocate into the nucleus after being transfected into immortalised cell lines, resulting in constitutive translocation of CAR (Kawamoto et al., 1999; Wang et al., 2004). However, recently a model was developed with an alternative splicing variant for human CAR, which exhibited significantly lower activity in immortalised cells than wild type CAR. This model could potentially be used for evaluation of CAR activation (Auerbach et al., 2005; Faucette et al., 2007).

The reporter gene assays have some advantages over primary human hepatocytes. For example, they are based on inexpensive immortalised cell lines such as the human hepatoma HepG2 cells, which lead to lower cost. Furthermore, planning of experiments is straightforward since a reporter gene assay is ready to use and the reporter gene assays can be developed for screening of larger sets of compounds.

1.4.2 Assessment of induction potential *in vitro*

The endpoint in a reporter gene assays is often increased luminescence in cells where the nuclear receptor has been activated. When employing cell lines or human hepatocytes for induction studies, the results can be detected at different points between receptor activation and increased enzyme activity. Since variation between experiments always can be expected, inclusion of appropriate controls is essential. Examples of inducers recommended as positive controls are listed in **Table 1**.

1.4.2.1 mRNA

As discussed above, induction is in general caused by increased transcriptional activation, which leads to increased levels of mRNA in the cell. Today rapid and sensitive techniques for measurement of mRNA are available [e.g. real-time polymerase chain reaction (PCR)]. Measurement of mRNA can therefore be readily used to assess the induction potential of new drug candidates. A great advantage with this method is that induction of several genes can be analysed from a relatively small amount of cells.

1.4.2.2 Apoprotein

The determination of apoprotein levels is often performed with Western blot analysis, which is at best semi-quantitative and only gives rough estimates of the induction. Protein levels could also be detected with ELISA (enzyme-linked immunosorbent assay), which gives better quantitative data than Western blot. A new technique, where

immunocytochemical staining with specific P450 antibodies are used, is under development and the method could potentially give a quantitative measure of protein levels, even in individual cells. However, a thorough evaluation of the method is needed before routine use. Determination of protein levels is therefore not the first choice of endpoint for the time being.

1.4.2.3 Enzyme activity

Increased enzyme activity is the final outcome of any induction and therefore the most pertinent endpoint for an induction assay. However, if a relationship between increased enzyme activity and increased mRNA levels has been established for a potential inducer, measurement of enzyme activities could be excluded in favour of the more convenient mRNA determinations. Enzyme activity could be measured directly in cell monolayers or in microsomes isolated from induced cells. Although the former technique requires less cells and is amenable to high-throughput screening, the distribution of probe substrates and formed metabolites has to be taken into consideration when using cell monolayers. In some cases it has been seen that a compound is both an inducer and an inhibitor of the same P450. In these cases it is advisable to measure both mRNA levels and enzyme activity. If this is the case, microsomes or cells might not show any change in enzyme activity, yet an increase in mRNA levels would be detected.

1.5 ENZYME INDUCTION *IN VIVO*

In vivo induction is measured by administration of probe substrates (**Table 1**). The probe is administered before and after repeated administration of the potential inducer, and the induction is in general measured as the decrease in the area under the plasma concentration versus time curve (AUC) of the probe drug. The activity of different P450s *in vivo* is highly variable. In a study of liver microsomes from 30 Japanese and 30 Caucasian individuals it was shown that all the major drug metabolising P450s varies considerably (Shimada et al., 1994). The interindividual variability depends on genetic factors as discussed above, and on environmental factors such as diet and pollutants. As a consequence, the extent of enzyme induction *in vivo* in humans, similar to human hepatocytes, is highly variable (Lin and Lu, 2001).

The bioavailability, and hence the AUC, will be affected not only by induction of hepatic P450s but also by induction of P450s in the intestine. Some reports have even suggested that the intestinal first-pass metabolism is quantitatively more important than the hepatic first-pass metabolism (Holtbecker et al., 1996; Gorski et al., 2003), although this has been critically questioned (Lin et al., 1999). In addition to induction of P450s,

rifampicin has also been shown to induce the ATP-binding cassette transporter P-glycoprotein (P-gp) (Greiner et al., 1999), which also may lead to decreased bioavailability.

1.5.1 Clinical implications

The rationale to study enzyme induction is that it can affect the clinical outcome of drug treatment and in some cases changes in drug dosage is required to maintain efficacy during coadministration with a potent P450 inducer. In theory, P450 induction is expected to give a significant decrease in AUC of a drug with high P450 dependent hepatic clearance after an oral dose, whereas little effect on AUC is expected for such a compound after intravenous administration. This is because AUC_{iv} is dependent on systemic clearance, which in the case of a high clearance drug is limited by hepatic blood flow and therefore not sensitive to changes in P450 activity. On the other hand, the AUC_{po} is highly dependent on the bioavailability, which is determined by the first-pass metabolism and therefore influenced by P450 induction. For a drug with low hepatic clearance, significant effects are expected independent of administration route. The first-pass metabolism is low and AUC_{iv}, which is dependent on systemic clearance, is in this case limited by enzyme activity and thus sensitive to changes in P450 activity. However, the magnitude of decrease in AUC seems to be higher for moderate and high clearance drugs as compared to low clearance drugs (Lin, 2006).

The perhaps most potent and well studied inducer *in vivo* is rifampicin. Rifampicin is an antibiotic agent used for treatment of tuberculosis and it is a potent CYP3A, CYP2B, and CYP2C inducer (Venkatesan, 1992; Grange et al., 1994; Lopez-Cortes et al., 2002; Niemi et al., 2003; Loboz et al., 2006). Because many drugs are metabolised by these enzymes, the list of potential interactions is very long (Niemi et al., 2003). Rifampicin significantly reduces the plasma concentrations of e.g. methadone, midazolam, phenytoin, verapamil, warfarin, and efavirenz (Lopez-Cortes et al., 2002; Niemi et al., 2003). Due to the wide variety of drugs that can be affected, the therapeutic outcome of induction interactions also differs. One of the more serious interactions is between rifampicin and cyclosporin. In a clinical study using 600 mg rifampicin once daily for 11 days, a more than 3-fold decrease in oral AUC of concomitantly administered cyclosporin was observed (Hebert et al., 1992). This reduction in AUC for cyclosporin could have serious consequences for transplant patients infected with tuberculosis. The rifampicin-warfarin interaction could also have serious implications on anticoagulant therapy in many patients (Cropp and Bussey, 1997). The antiepileptic drugs carbamazepine and phenytoin are two other drugs well known to reduce plasma concentrations of drugs metabolised by CYP3A (Kut, 1995; Levy and Wurden, 1995; Backman et al., 1996; Spina et al., 1996). Induction of CYP3A by rifampicin, carbamazepine, and phenytoin has been reported to increase the metabolism of oral

contraceptives. The interaction may lead to breakthrough bleedings and failure of contraception and thus non-hormonal contraception should be used during treatment with these drugs.

In recent years awareness has been drawn to the effects of herbal remedies on P450 activities. Many patients consider herbal remedies as natural and harmless and omit to inform their physician that they consume such preparations. Hyperforin, a constituent of St John's wort, has been shown to be a potent CYP3A inducer and interactions with drugs metabolised by CYP3A could therefore be anticipated. In cases where St John's wort has been coadminstered with oral contraceptives, this has lead to breakthrough bleedings and in some cases unwanted pregnancies (Henderson et al., 2002). Reason for awareness of enzyme induction is therefore called for also for herbal remedies.

1.6 *IN VITRO-IN VIVO* CORRELATIONS

The ultimate goal, but a very complex task, of *in vitro* induction studies is to quantitatively predict *in vivo* induction. As discussed above, P450 induction is not a direct effect on the enzyme, but rather a receptor-mediated effect, and hence both time and concentration dependent. The duration of treatment with the inducer *in vivo* should preferably be sufficient to reach a steady state in the induction response. If possible it is desirable to study the induction at more than one dose, covering the clinically most commonly used doses. Furthermore, *in vivo* data included in an *in vitro-in vivo* correlation should be from studies utilising probe substrates for which similar effects of induction could be assumed (see section 1.5.1)

Classifications of compounds as inducer or non-inducer *in vivo* have been made from reporter gene assays (Paper I, Sinz et al., 2006). The reporter gene assays only detect the activation of one receptor and the interplay of different transcription factors is not complete in the assay. Quantitative predictions probably require a more advanced *in vitro* model where all transcription factors are functional. It could also be of importance that the whole cascade from transcription to active protein is represented because of possible regulating feedback mechanism. Quantitative predictions have been performed by using E_{max} and EC_{50} results from human hepatocytes and Fa2N-4 cells, together with the unbound plasma concentration of the inducer *in vivo* (Kato et al., 2005; Ripp et al., 2006). In our reporter gene assay and HepaRG cell studies (Paper I and II) predictions were made using EC_{50} or F_2 values and the *in vivo* AUC of the inducer. Good correlations were achieved in all four studies although different approaches were applied. In the study with Fa2N-4 cells eight compounds were used for the *in vitro-in vivo* correlation, whereas in the HepaRG cell study (Paper II) ten compounds were used. It would of course be preferable to include more compounds in such a correlation, although this is hampered by the availability of *in vivo* data.

2 AIMS

The present studies were undertaken to gain further insight into the relationship between *in vitro* and *in vivo* P450 induction and to find a method for quantitative predictions of the magnitude of *in vivo* induction from *in vitro* studies. The specific aims were:

- To evaluate PXR and AhR reporter gene assay as models for prediction of P450 induction properties *in vivo*.

- To evaluate human liver slices as a model for prediction of P450 induction properties *in vivo*.

- To characterise the induction properties of the HepaRG cell line and to investigate if the HepaRG cells could be used for predictions of P450 induction *in vivo*.

- To characterise the drug metabolising properties of the HepaRG cells and the stability of the cell line over time in culture.

- To investigate the dose-dependent induction by rifampicin *in vivo* by the use of the Karolinska cocktail and to evaluate 4β-hydroxycholesterol as an endogenous marker for CYP3A4 induction *in vivo*.

3 METHODOLOGICAL CONSIDERATIONS

3.1 *IN VITRO* METHODS

3.1.1 Human liver tissue and human hepatocytes

Human liver tissue used for preparation of human liver slices was obtained as surgical waste from Sahlgrenska University Hospital (Göteborg, Sweden). All tissues were obtained through qualified medical staff, with donor consent and with the approval of the local ethics committee. Cryopreserved primary human hepatocytes were purchased from In Vitro Technologies (Baltimore, Md, USA). The demographic data for human liver tissue and human hepatocytes are presented in Paper I and III, respectively. As discussed previously, both drugs, genetics and environmental factors can affect the activity of P450s in human tissue samples. For batches of cryopreserved human hepatocytes both demographic data and phenotypic characterisation were available at purchase. Batches with known phenotypic deviations such as significantly low activity of polymorphic enzymes were excluded. Since the human liver slices were used fresh, only demographic data were available and selection based on phenotypic characterisation could not be performed.

3.1.2 Reporter gene assays

The reporter gene assays are described in detail in section 1.4.1.4. The reporter gene assays evaluated in Paper I used HepG2 cells stably transfected with the CYP1A distal enhancer reporter construct to study AhR activation, whereas the CYP3A4 distal enhancer and proximal promoter reporter construct was transiently transfected into HepG2 cells to study PXR activation. Transient transfection could result in variation in transfection efficiency between different experiments in the PXR assay. However, results were always normalised to rifampicin results, which was included as a positive control in all experiments. The normalisation against rifampicin should adjust for any variation in transfection efficiency.

3.1.3 HepaRG cells

The HepaRG cells were developed from a human hepatocellular carcinoma and are purchased from Biopredic International (Rennes, France) as differentiated cells plated in 96- or 24-well plates. After arrival the cells were always allowed to recover for 24 hours before initiation of experiments. The HepaRG cells are differentiated into a hepatocyte-like morphology by addition of 2% dimethyl sulfoxide (DMSO) to the culture medium when the cells reach confluence (Cerec et al., 2007). DMSO has been used as a differentiation-inducing agent for many tumour cell lines (Yu and Quinn, 1994), although the mechanism by which DMSO acts is poorly understood. It is known that DMSO increases the expression of CYP3A4 (Nishimura et al., 2003), and a previous study has shown that rifampicin do not increase CYP3A4 activity in HepaRG cells cultured in 2% DMSO (Aninat et al., 2006). This indicates that CYP3A4 is already induced in these cells. Hence, HepaRG cells used for induction studies in Paper II were cultured in DMSO free medium for 5 days in order to avoid any possible P450 induction by DMSO, which otherwise would overthrow the induction by other inducers. Although DMSO was used to solve the inducers employed in Paper II, the final DMSO concentration, 0.1%, should be too low to result in any induction (Nishimura et al., 2003).

3.1.4 Human liver slices

Although the preparation of human liver slices is straightforward, there are some concerns about the method. The preparation of the slices is rather tissue demanding. A cylinder is drilled from the tissue, which is cut into thin slices. This means that a lot of tissue is left over and has to be discarded. When preparing hepatocytes the whole piece of tissue can be used. Another concern is the thickness of the liver slice. This should be thick enough to hold the tissue together but as thin as possible for medium, oxygen, and substance to diffuse into the core of the slice. After the preparation the slices are incubated for up to three days and as a consequence the incubation conditions are important. The slices should be incubated at 37 °C in a humidified atmosphere with 5 % CO_2. In addition, the liver slice has to be in motion. If the slice is let to lie on the bottom of the culture dish, there will be no access of medium and oxygen to the cells on the bottom side, which then will die. In Paper I this was solved by an orbital shaker, which allowed the slices to move slowly around in the medium without touching the sides of the culture dish. In the experiments with human liver slices the inducers was dissolved in DMSO, final concentration 1%. This DMSO concentration could induce CYP3A4 (Nishimura et al., 2003). However, induced samples were compared to control samples, treated with 1% DMSO and hence false positive induction should be avoided.

3.1.5 Measurement of mRNA

In Paper I-III the mRNA levels were measured by real-time PCR. The technique uses a fluorogenic probe with a reporter dye and a quencher dye, which suppresses the reporter dye. The probe is designed to bind to the target gene sequence, preferably spanning over an exon-exon boundary. During elongation of the PCR primers, the probe is cleaved, the reporter and quencher are separated and the fluorescence increased. The threshold cycle (Ct) is reported as the cycle number where the fluorescence passes a fixed threshold, set in the exponential phase of the amplification curve. The amount of target normalised to an endogenous control and relative to a calibrator is then calculated as $2^{-\Delta\Delta Ct}$ (Applied Biosystems, 1997).

In Paper I-III normalisation to endogenous control was performed to adjust for sample DNA added to the reaction. huPO (human acidic ribosomal phosphoprotein) (Paper I and II) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Paper II and III) were used as endogenous controls. In Paper I equal amplification efficiency in the product formation of CYP1A1, CYP1A2, CYP2C9 and CYP3A4 by in-house designed primers and probes were achieved. For the use of Assay on Demand in Paper II and III it was assumed that the reverse transcription efficiency of the target gene and the endogenous control was approximately equal.

3.1.6 Enzyme activity

3.1.6.1 Substrate depletion

The substrate depletion method is commonly used in drug discovery to screen the metabolic stability of drug candidates. Human liver microsomes have historically been the most common source of enzymes in this assay. Because of the increasing knowledge of the importance of drug transporters and other enzyme systems than P450s for drug clearance, the drug industry is currently turning to the use of primary human hepatocytes instead of human liver microsomes. From the depletion curve the intrinsic clearance (CL_{int}) is calculated as:

$$CL_{\text{int}}\ (\mu l/\min/mg) = \frac{k\ (\min^{-1})}{protein\ concentration\ (mg/\mu l)}$$

where k is the slope of the depletion curve. In Paper III the CL_{int} for six compounds in HepaRG cells at different culture conditions were compared with CL_{int} for the same compounds in cryopreserved human hepatocytes. The compounds were chosen to cover metabolism by a broad range of enzymes, both P450s and phase II enzymes. The assay in HepaRG cells was performed as identical to the human hepatocyte assay as possible

with the exception that human hepatocytes are in suspension and HepaRG cells are attached to the culture plate. This could possibly affect the distribution of the compounds to the cells.

3.1.6.2 Metabolite formation

The activity of a specific enzyme is determined by formation of a metabolite that is formed only by the enzyme of interest (see section 1.2.3). No interactions of the three compounds incubated as a cocktail, phenacetin, diclofenac, and midazolam, were seen in preliminary studies (unpublished data). The bupropion hydroxylase activity was not investigated in Paper I, but was measured in separate incubations in Paper II and III.
It is known that commonly used organic solvents can inhibit P450 activities (Busby et al., 1999). In Paper I the probe substrates were solved in methanol, final concentration 2.5%, which resulted in less than 20% inhibition of any tested activity. For activity measurements in Paper II and III the methanol was evaporated and compounds were thus directly solved in medium.

3.1.7 Derivation of EC_{50} and F_2 values

P450 induction experiments *in vitro* should be run over a concentration interval to establish maximum response. However, many times it can be difficult reach a maximum response due to low solubility or cell toxicity of the inducer, resulting in curves exemplified in **Figure 3B**.

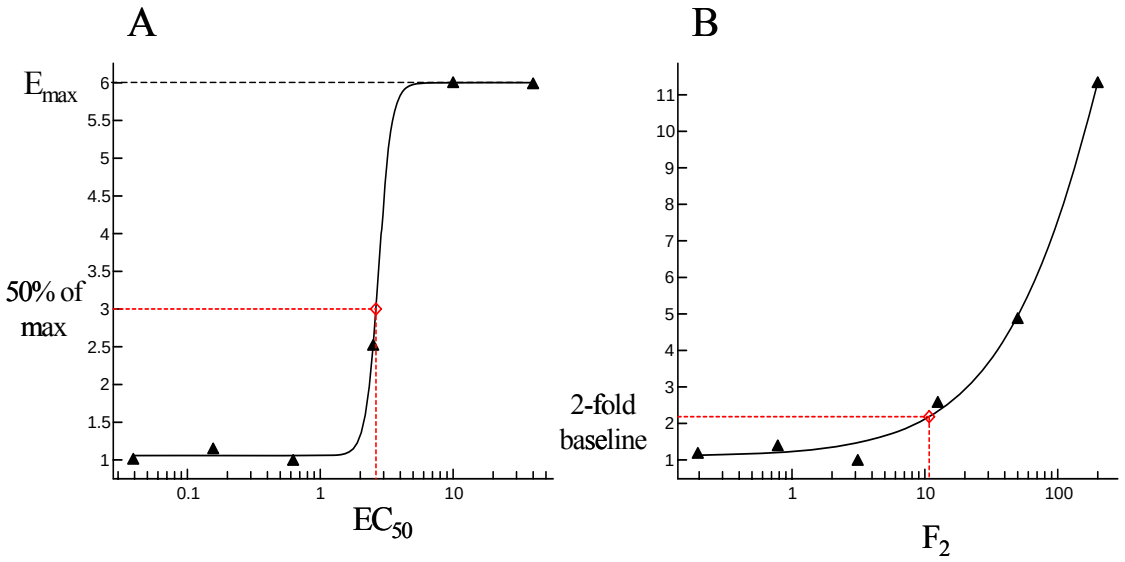

Figure 3. Examples of a full dose-response curve (A) and dose-response curve where maximum response is not achieved (B).

From full dose-response curves the maximum effect, E_{max}, and the concentration giving half the maximum effect, EC_{50}, are calculated (**Figure 3A**). Curve-fitting in Paper I and II were performed with XLfit v2.0.9 and v4.1.1. When deriving EC_{50} values with this program the curve should be carefully examined since the program calculates an EC_{50} even if a true maximum is not reached. Instead of EC_{50} values the F_2 values could be used (**Figure 3B)**. This is the concentration giving a 2-fold increase over the baseline, which means that maximum response is not needed for the calculation.

3.2 INDUCTION *IN VIVO*

In the clinical study (Paper IV), the difference in dose-dependent induction by rifampicin of CYP3A4 and CYP2C9 and the possible induction of CYP1A2 and CYP2C19 was investigated. The induction was measured by the use of the Karolinska cocktail and the endogenous CYP3A4 marker 4β-hydroxycholesterol. In order not to study a mixture of CYP3A4 and CYP3A5 activities, individuals expressing CYP3A5 were excluded from this investigation. The decision was based on the fact that CYP3A5 is a polymorphic enzyme and only expressed in ~10% of Caucasian Swedes (Mirghani et al., 2006). Furthermore, subjects were only included if they had at least one CYP2C9*1 and one CYP2C19*1 allele, to assure the possibility of induction of these polymorphic enzymes by rifampicin.

3.2.1 Rifampicin

Rifampicin is an antibiotic used for treating e.g. tuberculosis, and is perhaps the most well documented P450 inducer *in vivo*. Rifampicin is known to induce CYP3A4 and CYP2C9 (Heimark et al., 1987; Backman et al., 1998), with clinically significant interactions as a result (see section 1.5.1). It has also been suggested that rifampicin is an inducer of CYP1A2, CYP2C8, and CYP2C19 (Zhou et al., 1990; Niemi et al., 2004; Park et al., 2004; Backman et al., 2006). It would therefore be very interesting to study the possible dose-dependent induction of all the aforementioned P450s simultaneously. To our knowledge this has not been performed previously, probably due to the risk of interactions of the different probe drugs. The development of the Karolinska cocktail (see section 3.2.2) has made such an investigation possible.

Several aspects need to be considered in the design of a study of dose-dependent induction. The Karolinska cocktail overcomes interactions between the probe substrates for the different P450s but also any possible effect of the inducer, rifampicin, needs to be considered. In a Danish study on the effect of rifampicin on repaglinide metabolism (Bidstrup et al., 2004) it was shown that the reduction of repaglinide AUC was greater when this drug was administered the day after compared to given together with the last

rifampicin dose. This was suggested to be caused by rifampicin acting as both an inducer and an inhibitor. In the present study the cocktail drugs were therefore administered one day after the last rifampicin dose, when rifampicin is eliminated and the inhibition has decreased, but the induction is still present.

Since we wanted to study a dose-response effect it was decided to give three different doses of rifampicin. When surveying the literature, 600 mg rifampicin daily is the most frequently used dose in clinical interaction studies, although CYP3A4 seems to be strongly induced already at 450 mg daily (Niemi et al., 2003). The three doses of 20, 100, and 500 mg rifampicin daily were therefore chosen to cover a pronounced span of induction. The duration of the rifampicin treatment is of great importance. From studies on the effect of rifampicin on pharmacokinetics of verapamil, which is metabolised by CYP3A4 and CYP2C9, it was estimated that full induction of these enzymes was reached after one week of treatment with rifampicin (Niemi et al., 2003). In a study by Magnusson et al., the half-life of induction of CYP3A4 was found to be 70 hours (Magnusson, 2007). If approximating four half-lives to steady state, this would be established after 12 days. To make sure that a pronounced induction was reached, the duration of rifampicin treatment was 14 days in Paper IV.

3.2.2 The Karolinska cocktail

The Karolinska cocktail has previously been developed as a tool for phenotyping five of the most important human drug metabolising P450s (Christensen et al., 2003). Paraxanthine/caffeine in a 4 hour plasma sample, and metabolic ratios (MRs) for losartan/E-3174 (metabolite of losartan) in 0 to 8 hours urine, omeprazole/5'-hydroxyomeprazole in a 3 hour plasma sample, debrisoquine/4'-hydroxydebrisoquine in 0 to 8 hours urine, and quinine/3'-hydroxyquinine in a 16 hour plasma sample were used as phenotypic indices of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 activity, respectively. In the study by Christensen et al., losartan, debrisoquine, caffeine, and omeprazole were given simultaneously, whereas quinine was given after the samplings of the other drugs were finished. Quinine inhibits CYP2D6 and both quinine and omeprazole are P-gp substrates and therefore the administration of quinine was separated from the other drugs.

No analytical, kinetic or dynamic interactions between the drugs were seen except for debrisoquine (Christensen et al., 2003). When debrisoquine was administered in a cocktail a statistically significant increase of debrisoquine MR was seen, suggested to be caused by inhibition of debrisoquine metabolism by any of the three coadminstered drugs. Subsequently debrisoquine has been administered the evening before giving the other three drugs the next morning. Since CYP2D6 seems not to be inducible, debrisoquine was excluded from the cocktail in the study in Paper IV. To minimise the

sampling occasions the recommendations of Christensen et al. was followed, and caffeine was administered one hour before losartan and omeprazole. The time period for caffeine restriction was also extended to 36 hours before and 4 hours after caffeine intake. In addition to the original measurements in the Karolinska cocktail, the omeprazole/omeprazole sulfone ratio was analysed. This ratio has been shown to represent the CYP3A4 activity when omeprazole was administered without the other cocktail drugs (Böttiger, 2006). We therefore evaluated if the omeprazole/omeprazole sulfone ratio could be used as a marker for CYP3A4 induction within the setting of the Karolinska cocktail.

3.2.3 4β-hydroxycholesterol

4β-hydroxycholesterol is a metabolite of cholesterol formed by CYP3A4 catalysed metabolism (Bodin et al., 2001) for which plasma concentrations show no diurnal variation and have low intraindividual variability (unpublished data). Previous studies have shown that plasma concentrations of 4β-hydroxycholesterol are increased in patients treated with rifampicin (Niemi et al., 2006). Carbamazepine and phenytoin, which are antiepileptic drugs known to induce CYP3A, also increased plasma concentrations of 4β-hydroxycholesterol (Bodin et al., 2001). Recently it was shown that 4β-hydroxycholesterol could be used as a marker for CYP3A in Swedes, Tanzanians, and Koreans (Diczfalusy et al., 2008) and therefore also should be useful as a marker for CYP3A induction.

4 RESULTS AND DISCUSSION

4.1 P450 INDUCTION IN HUMAN LIVER SLICES (PAPER I)

The P450 activities and mRNA levels were investigated in human liver slices over the incubation period of three days. A substantial decrease was seen for the P450 dependent phenacetin *O*-dealkylase, diclofenac 4'-hydroxylase, and midazolam 1'-hydroxylase activities (**Figure 4A**) and CYP1A1, CYP1A2, CYP2C9, and CYP3A4 mRNA (**Figure 4B**). Previous studies have reported a decrease of P450 apoprotein levels in human liver slices (Martin et al., 2003) as well as decrease of P450 activities in human hepatocytes over a period of time in culture (Meunier et al., 2000). After the initial experiments it was decided to use mRNA levels as endpoint since mRNA results were more consistent and, contrary to P450 activities, detectable in all samples.

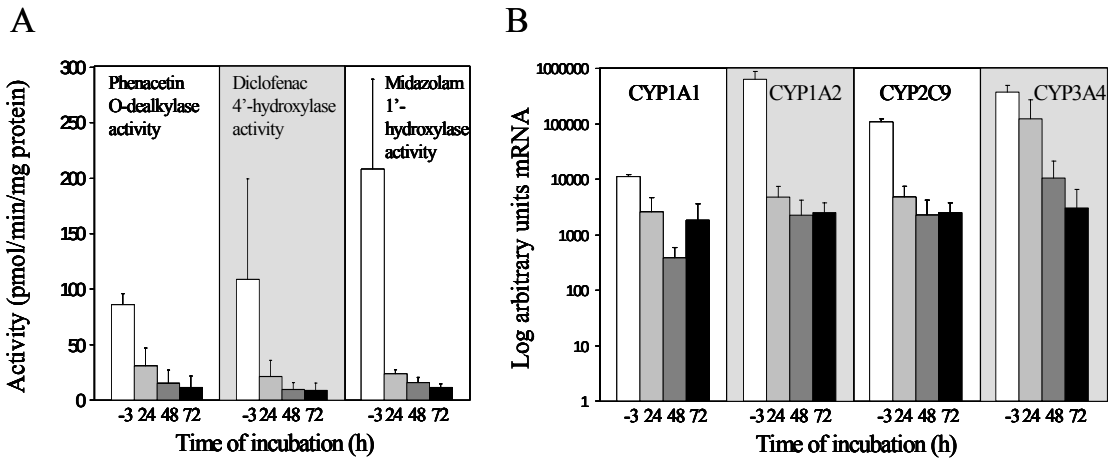

Figure 4. Specific enzyme activities (A) and normalised amount (arbitrary units) of mRNA levels (B) in human liver directly after resection (time = -3 hours) and in human liver slices after incubation with control media for 24, 48 and 72 hours. All values are mean +SD from 3 individual livers. From Paper I.

The time point for maximum fold induction of CYP1A1 and CYP1A2 mRNA in human liver slices was drug dependent, although for CYP3A4 the maximum induction was reached after 72 hours exposure independent of compound. These results are in accordance with time profiles in human hepatocytes (Meunier et al., 2000). The different peak times could be due to different induction half-lives for the different enzymes. These results emphasise the need to follow the induction over a period of time when investigating P450 induction of new drug candidates in human liver slices and hepatocytes.

TCDD was used as a positive control for CYP1A induction in human liver slices. Besides TCDD, omeprazole and primaquine exhibited a significant induction of CYP1A1 and CYP1A2 mRNA. Clotrimazole, phenytoin, rifampicin, and troglitazone gave a less than 3-fold induction of CYP1A mRNAs in human liver slices, which is very small as compared with the 137- and 86-fold induction of CYP1A1 and CYP1A2 mRNA levels by TCDD. Furthermore, clotrimazole, phenytoin, rifampicin, and troglitazone did not activate AhR (see section 4.2.1), which indicates that the increase in mRNA may be via other mechanisms than receptor activation.

In the human liver slices the rifampicin (used as a positive control) induction of CYP2C9 mRNA was lower than for CYP3A4 mRNA. The results are in accordance with the *in vivo* response and results for induction of mRNA in human hepatocytes and apoprotein in human liver slices (Rae et al., 2001; Edwards et al., 2003). Known CYP3A4 *in vivo* inducers such as carbamazepine, St. John's wort, phenobarbital, phenytoin, and troglitazone did not significantly induce CYP3A4 mRNA in the human liver slices. The reason for this is most likely the high interindividual variability in the liver slice experiments. Omeprazole is not reported to be an *in vivo* inducer of CYP3A4 (Ching et al., 1991) but significantly induced CYP3A4 mRNA in human liver slices. However, this can be explained by the supra-therapeutic concentrations of omeprazole used in the study.

The experimental setting of human liver slices is not applicable to high throughput screening, which thus limits the use of liver slices in early drug discovery. Another concern is the interindividual variability in response of P450s to potential inducers in human liver slices reported in Paper I and elsewhere. As a consequence, experiment needs to be performed in several individuals in order to get a proper average. Human liver slices could still be used as a model to study the regulation of a larger number of enzymes by a single compound identified as a possible P450 inducer.

4.2 ACTIVATION OF AHR AND PXR IN REPORTER GENE ASSAYS (PAPER I)

In contrast to human liver slices, reporter gene assays are amenable to high throughput screening, and can be used to investigate a large number of substances over a concentration range at controlled conditions. In the present study the maximum concentrations used in the dose-response curves were limited by solubility or cell toxicity of the compounds.

4.2.1 AhR activation

Twenty different compounds were tested for their ability to activate AhR in the reporter gene assay. Of these only three, lansoprazole, omeprazole, and indole-3-carbinol, displayed a positive response. However, all three displayed a higher EC_{50} than the positive control, TCDD, and none of them reached more than 50% of TCDD E_{max}. Indole-3-carbinol, a constituent of cruciferous vegetables, has previously been shown to induce CYP1A *in vitro* and in animals (Katchamart and Williams, 2001; Ociepa-Zawal et al., 2007). Nevertheless, few clinical studies have been performed with indole-3-carbinol and no reports on induction *in vivo* in humans have been found. Also omeprazole and lansoprazole have previously been shown to induce CYP1A enzymes in human *in vitro* systems (Curi-Pedrosa et al., 1994; Bowen et al., 2000) although *in vivo* studies of omeprazole and lansoprazole, given at therapeutic doses, reports no induction of CYP1A (Andersson et al., 1998; Dilger et al., 1999). The lack of *in vivo* induction by omeprazole is most likely because the EC_{50} in the AhR reporter gene assay was 18.1 μM, which is well above the therapeutic plasma concentrations for omeprazole (1.1-3.2 μM) (Li et al., 2004). However, the EC_{50} for lansoprazole was 5.9 μM, which is close to plasma C_{max} levels (2.0-4.8 μM) (Li et al., 2004) especially in poor metabolisers.

Primaquine did not activate AhR in the reporter gene assay although it induced CYP1A mRNAs in the human liver slices. Previous studies report conflicting results on the mechanism of CYP1A induction by primaquine. Fontaine et al. showed that primaquine is not a ligand for human AhR (Fontaine et al., 1999), whereas Backlund and Ingelman-Sundberg report primaquine as a low-affinity ligand for this receptor (Backlund and Ingelman-Sundberg, 2004). Furthermore, Werlinder et al. (2001) showed that primaquine regulate CYP1A1 both on the transcriptional as well as on the post-transcriptional level and actually inhibits CYP1A1 dependent ethoxyresorufin *O*-deethylase activity. The multiple effects of primaquine on CYP1A1 regulation contribute to difficulties in interpretation of results for this compound.

4.2.2 PXR activation

Of 26 compounds tested in the PXR reporter gene assay 18 activated the receptor. The most potent compound was hyperforin, a constituent of St John's wort, with an EC_{50} of 0.003 μM, which is considerably lower as compared to the positive control (rifampicin, EC_{50} = 0.20 μM). Pantoprazole, lansoprazole, omeprazole, and hyperforin all exhibited higher E_{max} values than rifampicin.

Several approaches based on results from the PXR reporter gene assay were used in attempt to classify the compounds as inducers or non-inducers. The simplest, EC_{50} from the PXR reporter gene assay, did not rank the test compounds according to known *in vivo* induction properties (**Figure 5**).

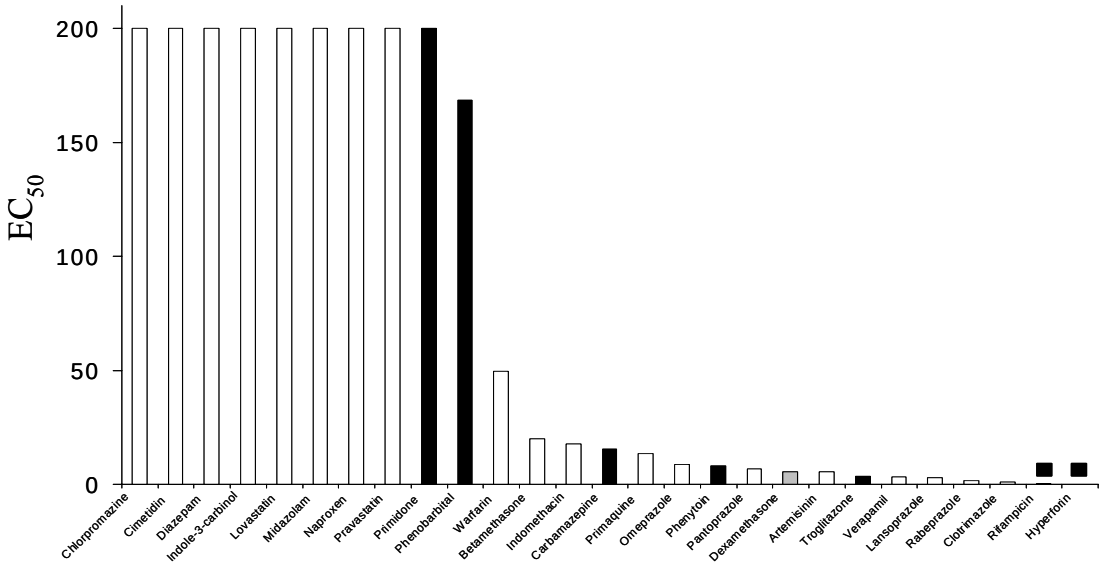

Figure 5. Ranking of EC_{50} from PXR reporter gene assay. Closed bars, *in vivo* inducers; shaded bars, weak *in vivo* inducers; open bars, *in vivo* induction of CYP3A not found in the literature.

The compounds were also ranked according to E_{max}/EC_{50} values from the PXR reporter gene assay, which reflects the overall ability of the compound to induce the enzyme and incorporates both potency and extent of induction (LeCluyse, 2001; Gibson et al., 2002). Nonetheless, this did not improve the ranking. Instead of E_{max}, *in vivo* concentrations were related to PXR EC_{50}, but this did neither rank the compounds properly. Since induction is a slow process, induction could be connected to *in vivo* exposure rather than maximum concentration *in vivo*. Therefore, the AUC was related to PXR EC_{50}. When the compounds were ranked according to AUC_u/EC_{50}, the CYP3A *in vivo* inducers were grouped together with the exceptions of primidone and

troglitazone. Ranking the compounds by AUC_{tot}/EC_{50} clustered all *in vivo* inducers except primidone (**Figure 6**).

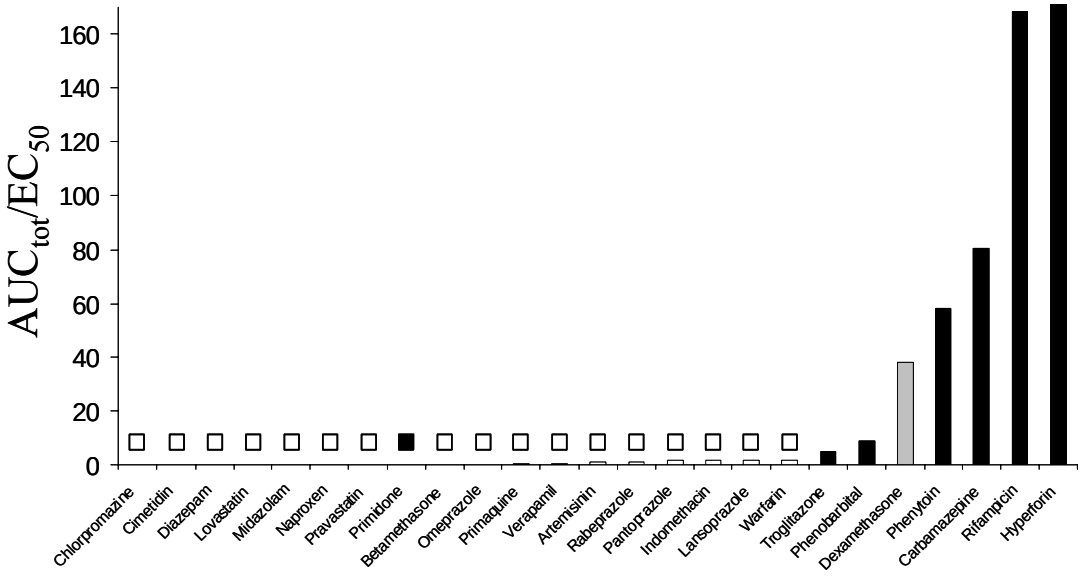

Figure 6. Ranking of AUC_{tot}/EC_{50} from PXR reporter gene assay. Closed bars, *in vivo* inducers; shaded bars, weak *in vivo* inducers; open bars, *in vivo* induction of CYP3A not found in the literature.

Primidone is metabolised to phenobarbital *in vivo* (Pisani et al., 1984), which most likely is the principal inducer. The metabolism of primidone probably does not occur in the PXR assay and thus explains the lack of effect by primidone. We also calculated the induction factor, I, based on an equation for dose-response since this takes both potency and extent of induction in the PXR reporter gene assay into consideration and also relates the *in vitro* results to the *in vivo* kinetics (see Paper I). Although the induction factor was calculated using both total and unbound C_{max} and AUC, neither of these improved the classification of the compounds. Based on the EC_{50} results from the PXR reporter gene assay and reported *in vivo* exposure an AUC_{tot}/EC_{50} value above 3 would indicate that the substance is a potential CYP3A inducer *in vivo*.

From the results in Paper I it was observed that full dose-response curves were not always achieved due to low solubility or cell toxicity of the test compound. Interest was therefore brought to a method where full dose-response curves not are compulsory. Weiss et al. reported a method where F_2 values were used instead of EC_{50} values (Weiss and Haefeli, 2006). The method was originally developed for inhibition of P-gp, but should be applicable also in induction studies. The EC_{50} and F_2 values are described in section 3.1.8. To evaluate the hypothesis that F_2 values could be used instead of EC_{50} values the results from the PXR reporter gene assay were recalculated to F_2 values

(Paper II). Similar ranking of the compounds were achieved when ranking according to AUC_{tot}/F_2 as compared to AUC_{tot}/EC_{50}. The results suggest that instead of EC_{50} values from full dose-response curves, F_2 values could be used to evaluate the induction response.

4.3 P450 INDUCTION IN HEPA RG CELLS (PAPER II)

Based on initial experiments with prototypical inducers, HepaRG cells were treated for 24 hours for mRNA and 48 hours for activity analysis. Contrary to human liver slices no differences in peak time were seen for different compounds or enzymes. As expected no induction was seen for CYP2D6 mRNA, and the mRNA for CYP2D6 was not analysed in subsequent experiments. F_2 values, fold induction at highest test concentration, and, when available, EC_{50} values are shown in Table 1-5 in Paper II.

Overall, induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 showed expected results. CYP1A1 and CYP1A2 mRNA were induced by the known *in vitro* inducers omeprazole and primaquine. CYP2B6 and CYP3A4 mRNA and corresponding activities were induced by rifampicin and phenobarbital. The CYP2C mRNAs were also induced by rifampicin and phenobarbital although to a lower degree as compared to CYP3A4, which corresponds to earlier results from Paper I and from human hepatocytes (Gerbal-Chaloin et al., 2001). However, even though primaquine induced CYP1A2 mRNA no effect was seen on CYP1A2 dependent phenacetin *O*-dealkylase activity. Primaquine has been shown to inhibit CYP1A1 dependent ethoxyresorufin *O*-deethylase activity with a K_i of 1.3 μM by binding to the active site (Werlinder et al., 2001). If a similar inhibition of CYP1A2 activity by primaquine remains after washing the cells, this could explain that no increase of CYP1A2 activity were seen by primaquine treatment in HepaRG cells. Similar to primaquine, phenytoin induced CYP3A4 mRNA but did not affect CYP3A4 dependent midazolam 1'-hydroxylase activity. Phenytoin is considered as a selective CAR activator and induced CYP2B6 mRNA and also CYP2B6 catalysed bupropion hydroxylase activity. The reasons for phenytoin discrepancies are thus unknown.

The results for CYP1A and CYP3A4 induction were comparable to results from AhR and PXR reporter gene assay in Paper I and it has earlier been reported that HepaRG cells express mRNA for AhR, CAR, and PXR (Aninat et al., 2006). Together with studies showing that rifampicin is a selective PXR activator (Moore et al., 2000) while phenytoin is a selective CAR activator (Wang et al., 2004), the results support the hypothesis that functional AhR, CAR, and PXR are present in the HepaRG cells.

The results from induction of CYP3A4 were processed in a similar way as the results from the PXR reporter gene assay in Paper I. The ranking of compounds by AUC_{tot}/F_2 from CYP3A4 mRNA induction were the same as the ranking from the PXR reporter gene assay. However, this is only a classification of compounds as inducer or non-inducer and a quantitative correlation of *in vitro* and *in vivo* data was desirable. To be able to make a correlation of *in vitro* and *in vivo* induction, the set of compounds for investigation of CYP3A4 mRNA induction was extended. The choice of compounds was based on availability of *in vivo* data. As discussed in section 1.5.1 comparable

effect of enzyme induction is expected for medium and high clearance drugs, hence only studies with such drugs were used for the correlation. The correlation of AUC/F_2 values from CYP3A4 mRNA induction in HepaRG cells and *in vivo* induction described by % decrease in AUC of CYP3A probe drugs (**Figure 7**) shows an excellent correlation ($R^2 = 0.863$).

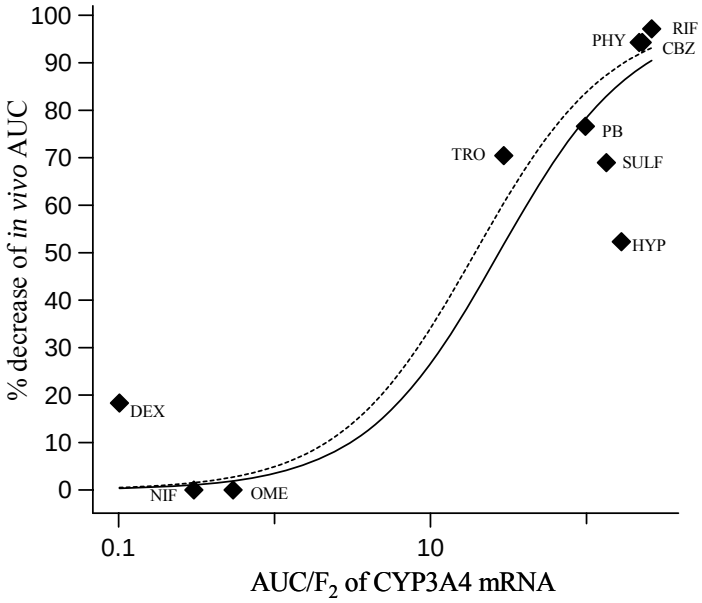

Figure 7. Correlation of AUC/F_2 of CYP3A4 mRNA in HepaRG cells with % decrease of *in vivo* AUC for CYP3A probe drugs (whole line), $R^2 = 0.863$. Correlation when hyperforin is excluded (dashed line), $R^2 = 0.943$. Compound abbreviations: CBZ, carbamazepine; DEX, dexamethasone; HYP, hyperforin; NIF, nifedipine; OME, omeprazole; PB, phenobarbital; PHY, phenytoin; RIF, rifampicin; SULF, sulfinpyrazone; TRO, troglitazone. From Paper II.

Hyperforin and dexamethasone are the two compounds deviating the most from the correlation. Dexamethasone is a more potent inducer *in vivo* than what was predicted from AUC/F_2 values. The reason for this is obscure and proposes further investigations of this compound. Regarding hyperforin the *in vivo* data for this compound could be confounded by varying concentrations of hyperforin in different extracts used in different studies. The present method correctly classified the strong *in vitro* inducers omeprazole and nifedipine as non-inducers *in vivo*. Furthermore, the degree of *in vivo* induction for the seemingly weak *in vitro* inducer phenytoin (less than 40% of rifampicin induction) was correctly predicted. These results emphasise the importance to relate the *in vitro* results to *in vivo* exposure for both seemingly "strong" and "weak" inducers in the *in vitro* system.

4.4 METABOLIC PROPERTIES OF HEPA RG CELLS (PAPER III)

In the development of new drug candidates investigations such as metabolic stability, metabolite identification, and identification of metabolising enzymes are required (Pelkonen et al., 2005). Encouraged by the very good results on P450 induction in HepaRG cells we also wanted to investigate whether the HepaRG cells could be used for such metabolism studies. For newly developed cell lines it is important to establish that the cells are stable over time in culture. The mRNA expression of drug metabolising enzymes, transporters, and liver specific factors were therefore measured up to eight weeks after seeding the HepaRG cells. When the cells reached confluence and DMSO was added to the culture medium, the expression of several P450s was increased. After differentiation of the cells only minor changes in mRNA expression for the investigated genes were seen over a period of 6 weeks in culture. The mRNA expression was compared to the expression in human hepatocytes. The most prominent difference between the two cell systems was the high expression of the two efflux transporters MDR1 and MRP1, and glucose 6-phosphatase in HepaRG cells at all culture conditions. In addition, the HepaRG cells express very low levels of CYP2D6 mRNA, less than 0.1-fold of expression in primary human hepatocytes. The CYP2D6 results are consistent with the suggestion that the cell line is derived from a patient that is a poor metaboliser of CYP2D6 (Guillouzo et al., 2007).

Because of the known inductive effect of DMSO on CYP3A4 and CYP2E1 (Nishimura et al., 2003) we wanted to investigate any possible effect on mRNA expression of P450s, transporters, and liver specific factors in differentiated HepaRG cells when DMSO was withdrawn from the culture medium. This was performed after 1, 5, and 14 days without DMSO. At the same time points the metabolic properties were investigated and compared to cryopreserved human hepatocytes. The expression of several P450s decreased considerably already after 1 day without DMSO in the medium, the most distinct being CYP1A1 and CYP3A4. Between 1 and 14 days without DMSO in the medium the expression of most of the P450s was relatively stable. The expression of transporters and liver specific factors in differentiated HepaRG cells were in general not affected when DMSO was removed from the medium. Notable exceptions were the plasma proteins albumin, which increased, and transthyretin, which decreased, when DMSO was removed from the culture medium.

For metabolism studies the relative content of the individual P450 is as important as the absolute levels of the enzymes. The percentage of individual P450s of total expression of the drug metabolising P450s were therefore calculated in human hepatocytes and HepaRG cells (**Figure 8**). The relative content in HepaRG cells cultured with 2% DMSO is dominated by CYP3A4. The HepaRG cells cultured without DMSO for 1 day better reflects the relative content of drug metabolising P450s in human

hepatocytes, except for a relatively high CYP2C19 content and a relatively low CYP1A2 content.

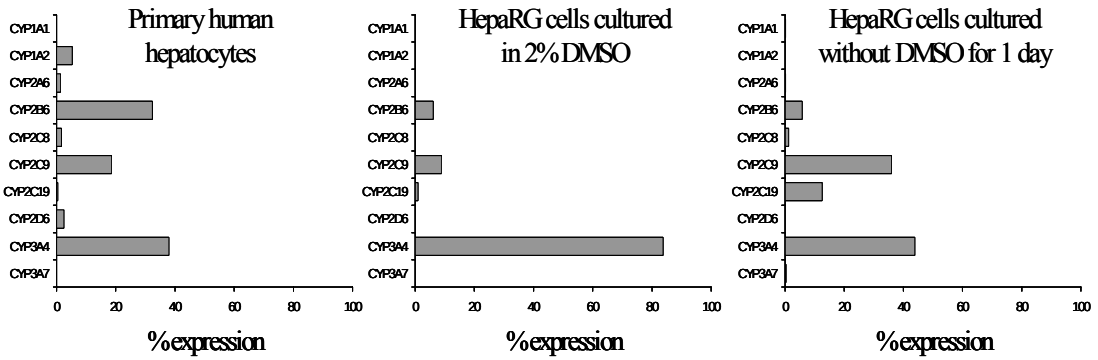

Figure 8. Relative mRNA expression denoted as % of total expression of drug metabolising P450s in primary human hepatocytes, differentiated HepaRG cells cultured with 2% DMSO, and differentiated HepaRG cells cultured without DMSO for 1 day. From Paper III.

The metabolic properties of the HepaRG cells were investigated by measuring the CL_{int} by substrate depletion of six well-known compounds, as well as the metabolite formation by specific P450s. Measurement of the metabolism of the model substrates phenacetin, bupropion, diclofenac and midazolam all indicated a rapid decrease in CYP1A2, CYP2B6, CYP2C9 and CYP3A4 enzyme activities in the HepaRG cells when DMSO was removed from the differentiated cells. The CYP1A2 and CYP2B6 activities were low in HepaRG cells at all culture conditions as compared with human hepatocytes. The metabolism in the three investigated batches of human hepatocytes varied considerably for CYP2B6 and CYP3A4. Compared with primary human hepatocytes the CYP2C9 and CYP3A4 enzyme activities were 30 and 70%, respectively, in HepaRG cells cultured with DMSO.

The compounds used in the substrate depletion assay were chosen to cover a broad range of drug metabolising enzymes, such as CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP3A, and several UDP-glucuronosyltransferases (UGTs). Propranolol, 7-ethoxycoumarin, clozapine, and dextromethorphan all have P450 dependent metabolism. For these compounds the CL_{int} were lower in HepaRG cells as compared to human hepatocytes, which is in accordance with the corresponding lower mRNA expression for the responsible P450s. The exception is clozapine, which displays similar CL_{int} in HepaRG cells as in human hepatocytes. Naloxone is mainly metabolised by UGTs (Di Marco et al., 2005) and the metabolism of naloxone was comparable in human hepatocytes and in HepaRG cells, indicating that the responsible UGTs have similar activities in the two *in vitro* models. UGT dependent metabolism could also contribute to the CL_{int} for propranolol in HepaRG cells.

Midazolam is mainly metabolised by CYP3A (Gorski et al., 1994) and was used in both substrate depletion and metabolite formation assays. In the substrate depletion assay a considerably higher midazolam CL_{int} was found in HepaRG cells cultured in 2% DMSO as compared to human hepatocytes, whereas the metabolite formation was in the same range in the two *in vitro* systems. However, midazolam CL_{int} comprise 4'-hydroxylation in addition to the measured 1'-hydroxylation, and this is probably why the midazolam results differ between the two assays. An additional explanation could be the interindividual variability in the human population, since different batches of human hepatocytes were used in the two assays (Table 1, Paper III).

4.5 RIFAMPICIN INDUCTION *IN VIVO* (PAPER IV)

The CYP1A2, CYP2C9, CYP2C19, and CYP3A4 activities were measured by use of the Karolinska cocktail as the paraxanthine/caffeine ratio, losartan/E-3174 MR, omeprazole/5'-hydroxyomeprazole MR, and quinine/3'-hydroxyquinine MR. Furthermore, omeprazole/omeprazole sulfone MR and plasma concentrations of 4β-hydroxycholesterol were measured as additional markers for CYP3A4 activity. In **Figure 9** the individual values for the three CYP3A4 markers are displayed before and after rifampicin treatment. The paraxanthine/caffeine ratio and the plasma concentrations of 4β-hydroxycholesterol are expected to increase in case of induction, whereas the MRs are expected to decrease.

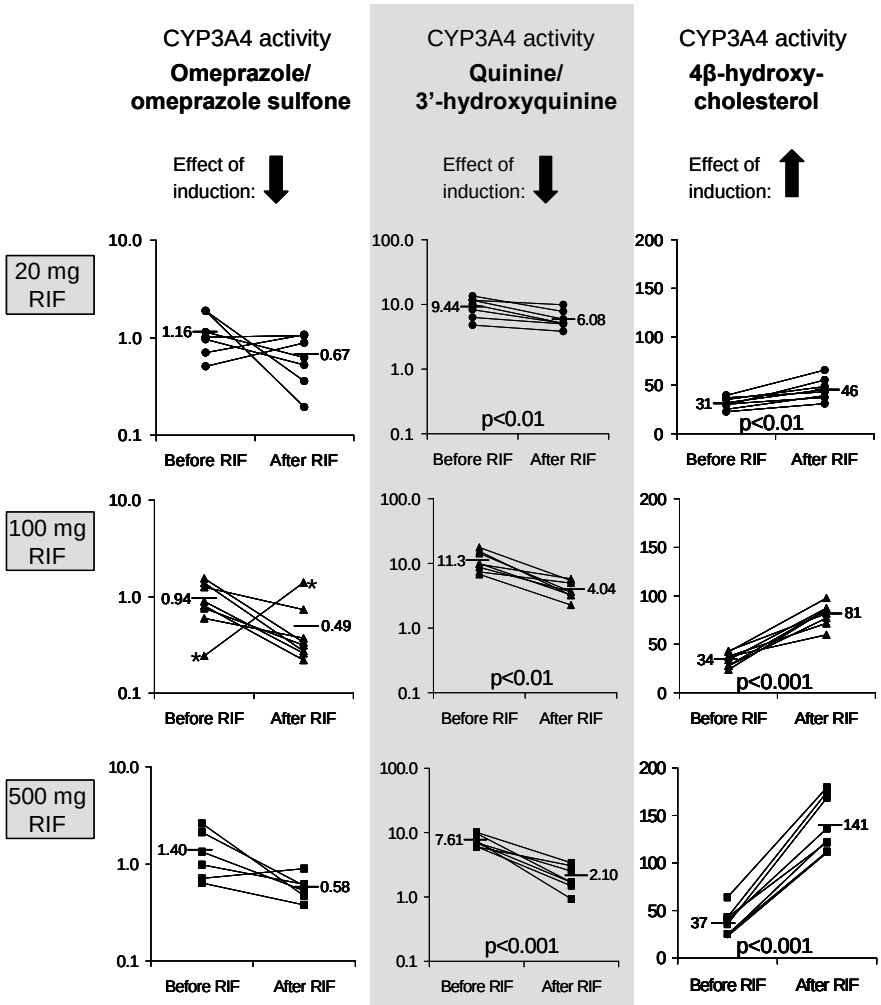

Figure 9. Omeprazole/omeprazole sulfone MR, quinine/3'-hydroxyquinine MR, and plasma concentrations of 4β-hydroxycholesterol before and after 14 days treatment with daily doses of 20, 100, or 500 mg rifampicin. Mean values are displayed and marked with a line. Statistically significant changes are shown. An outlier with very low omeprazole concentrations before taking 100 mg rifampicin daily is indicated with an asterisk.

A dose-dependent induction was seen for CYP3A4 measured by quinine/3'-hydroxyquinine MR and plasma concentrations of 4β-hydroxycholesterol, and the two indicators displayed a strong correlation (Spearman rank r_s=0.71; 95% C.I.=0.52-0.90; p<0.001; n=22) (**Figure 10**).

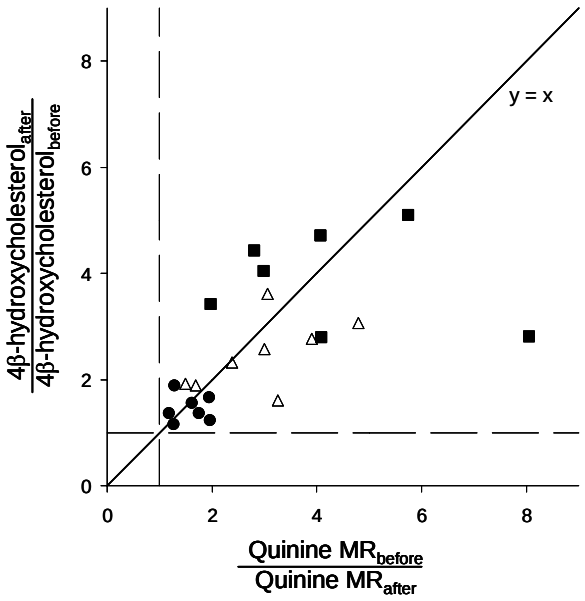

Figure 10. Correlation of induction ratios for plasma concentrations of 4β-hydroxycholesterol against quinine/3'-hydroxyquinine MR after three different daily doses of rifampicin, ● 20 mg (7 individuals), Δ 100 mg (8 individuals), ■ 500 mg (7 individuals). Spearman rank r_s=0.71; 95% C.I.=0.52-0.90; p<0.001; n=22. From Paper IV.

The results indicate that the plasma concentration of the endogenous 4β-hydroxycholesterol is a useful marker for CYP3A4 induction. Clinical studies investigating CYP3A induction could thus be considerably simplified since 4β-hydroxycholesterol could be measured in the same plasma sample as the drug candidate and thus induction could be investigated simultaneously with pharmacokinetics of the drug candidate. The only extra sample needed should be taken before start of the study to determine uninduced plasma concentrations of 4β-hydroxycholesterol. The use of an endogenous biomarker would also reduce the risk for interactions between the drug candidate and probe substrates and possible risk for the study subjects that can arise from administration of a probe drug.

The degree of induction of CYP1A2 and CYP2C9 was lower than for CYP3A4 and only reached statistical significance after treatment with 500 mg rifampicin daily. This is in accordance with earlier *in vitro* and *in vivo* findings (Williamson et al., 1998; Madan et al., 2003; Backman et al., 2006).

Omeprazole/omeprazole sulfone MR and omeprazole/5'-hydroxyomeprazole MR were used in an attempt to assess induction of CYP3A4 and CYP2C19. Based on previous studies a lower degree of induction for CYP2C19 than for CYP3A4 was expected (Zhou et al., 1990; Madan et al., 2003). However, in the present study the induction of CYP2C19 was in the same order as the induction of CYP3A4 (Paper IV). Furthermore, the induction of CYP3A4 as measured by omeprazole/omeprazole sulfone MR displayed similar changes at all three rifampicin concentrations, suggesting no dose-dependent induction as indicated by quinine/3'-hydroxyquinine MR and plasma concentrations of 4β-hydroxycholesterol. The unexpected results for omeprazole could be due to the metabolic pattern of omeprazole, which is in part displayed in **Figure 11**.

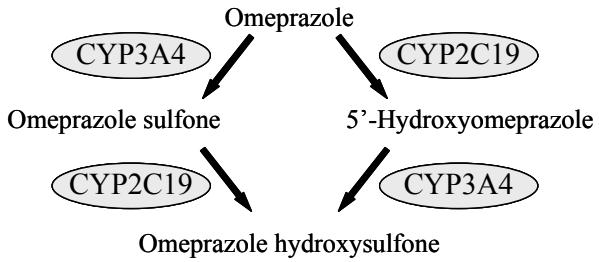

Figure 11. Metabolism of omeprazole to omeprazole sulfone and 5'-hydroxyomeprazole by CYP3A4 and CYP2C19 and further metabolism to omeprazole hydroxysulfone. Adapted from (Andersson et al., 1994).

After the first metabolic step, omeprazole sulfone and 5'-hydroxyomeprazole are further metabolised by CYP2C19 and CYP3A4, respectively (Andersson et al., 1993; Andersson et al., 1994). This means that the omeprazole/omeprazole sulfone MR and omeprazole/5'-hydroxyomeprazole MR is affected by changes of two enzyme activities. Hence the interpretation of the results for omeprazole is complex and omeprazole is thus not an optimal indicator for CYP3A4 and CYP2C19 induction.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The current study has evaluated *in vitro* models for prediction of P450 induction in humans. One of the *in vitro* models, the HepaRG cells, was also evaluated for drug metabolising properties. Further, the dose-dependent induction by rifampicin *in vivo* for four different P450s was investigated together with an endogenous CYP3A4 marker.

The most undemanding of the investigated *in vitro* induction models is the reporter gene assay, which could be used as a high throughput model. It was shown that the PXR reporter gene assay could be used to distinguish between *in vivo* CYP3A inducers and non-inducers and thus could be used as a screening tool in early drug discovery. Since many P450s are regulated by both PXR and CAR, unforeseen results may be found in later development if only screening for PXR activation. It would therefore in the future be interesting to study drugs in a CAR reporter gene assay to evaluate whether specific CAR dependent inducers will be found or if a combination of CAR and PXR results would increase the predictive value of the reporter gene assays.

Human liver slices gave expected response after treatment with prototypical inducers and could be used as a model to study induction of several P450s of a compound indicated as an inducer. However, liver slices are flawed with the same drawbacks as primary human hepatocytes. When choosing between these two models for induction investigations, the human hepatocytes are probably the model of choice, since the use of human hepatocytes are more widespread and the literature on the topic is vast.

The HepaRG cells were shown to respond to PXR, CAR, and AhR activators and *in vivo* CYP3A induction was successfully correlated to HepaRG results. The HepaRG cells are thus a valuable *in vitro* model as alternative to human hepatocytes in later stages of drug discovery when a more complex *in vitro* system is required. The drawback of the present *in vitro-in vivo* correlation, and others reported in the literature, is that *in vivo* AUC or plasma concentrations are used in the calculations. These values then need to be known or predicted. Today very few compounds with inducing properties are developed all the way through the clinical phases, and the predictive value of any model is thus difficult to evaluate.

It was also shown that *in vivo* induction could be predicted by using F_2 values instead of EC_{50} values from the CYP3A4 mRNA dose-response results in HepaRG cells.

Due to lack of *in vivo* data, *in vitro-in vivo* correlation was only performed for CYP3A4. However, induction of CYP1A2, CYP2B6, and CYP2C9 should also be measured during drug discovery. Continued monitoring of *in vitro* and *in vivo* induction of these enzymes could in the future contribute to a better understanding of when a significant *in vivo* induction will occur. To exclude non-transcriptional induction both mRNA and enzyme activities should be measured until a relationship between the two endpoints has been established. This approach would also unmask compounds acting as both inducers and inhibitors.

Further, it was shown that the HepaRG cells also could be used for metabolism studies. Although the enzyme activities in HepaRG cells are lower than in human hepatocytes, the relative contributions of several of the different P450s are comparable. The HepaRG cells could thus be used to determine which metabolites that could be formed in humans, and the relative formation of each metabolite. Increasing knowledge of the transporters indicate that the interplay between drug transport and metabolism is of importance for clearance of some compounds. It would therefore be of interest to further investigate the drug transporter activities in the HepaRG cells to determine if the cell line could be used also for transporter studies.

It was shown that the Karolinska cocktail could be used as a tool to determine CYP1A2, CYP2C9, and CYP3A4 induction. However, to achieve a reliable measure of CYP2C19 induction it is advisable to use a probe substrate other than omeprazole, since the metabolism of omeprazole is affected by changes of both CYP3A4 and CYP2C19 activities. A further development of the cocktail for use in induction studies would be the addition of a probe substrate for CYP2B6, e.g. bupropion.

Furthermore it was established that plasma concentrations of 4β-hydroxycholesterol could be used as marker for CYP3A4 induction. This would reduce the number of clinical studies since 4β-hydroxycholesterol could be measured simultaneously with pharmacokinetic investigations of the drug candidate. For this purpose it would be interesting to follow the time course of 4β-hydroxycholesterol changes and determine the treatment duration that is needed to detect CYP3A4 induction, and also the time to reach basal 4β-hydroxycholesterol levels after dosing is finished.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Läkemedel är för den mänskliga kroppen främmande ämnen som den behöver eliminera. Detta sker oftast genom utsöndring i urin och galla. För att underlätta utsöndringen omvandlar kroppen läkemedlet först till en mer vattenlöslig molekyl. Denna omvandling, kallad metabolism, sker med hjälp av enzymer, vanligen i levern. Det viktigaste enzymsystemet för nedbrytning av läkemedel är cytochrom P450 (P450) enzymerna, som bryter ner mer än 70% av de läkemedel som används idag. Vissa läkemedel inducerar, dvs. ökar aktiviteten av, P450 enzymerna. Induktion av P450 enzymer kan leda till att läkemedel bryts ner och försvinner ur kroppen fortare än vad som var tänkt. Läkemedlet hinner då inte ha den effekt som var avsedd. Om ett läkemedel inducerar P450 enzymerna kan det påverka både den egna eliminationen men även elimination av andra läkemedel som tas samtidigt.

När man utvecklar nya läkemedel är det viktigt att kunna studera induktion av P450 enzymer innan man ger det nya läkemedlet till människa. Detta gör man i *in vitro* studier (försök i provrör). Resultaten från dessa *in vitro* studier används för att förutsäga om P450 enzymerna kommer att induceras i människa eller inte. I denna avhandling har flera olika *in vitro* modeller studerats för att förbättra de förutsägningar som görs. En cellmodell där man kan mäta induktion av ett stort antal substanser visades kunna klassificera substanserna som inducerare eller icke inducerare *in vivo* i människa. Resultaten från en ny cell linje, HepaRG celler, visade dessutom kvantitativt mycket god korrelation med den induktion man har sett *in vivo* i människa. HepaRG cellerna visade sig även kunna användas för att utreda vilka P450 enzymer som bryter ner nya läkemedels substanser.

I kliniska studier av rifampicin, ett läkemedel mot tuberkulos, har man tidigare enbart studerat induktion av ett P450 enzym åt gången. I den studie som gjordes här påvisades induktion av fyra P450 enzymer samtidigt. Möjligheten att mäta induktion av flera P450 enzymer samtidigt *in vivo* är en stor fördel då det förenklar de kliniska studierna. Vidare visades att koncentrationerna av det kroppsegna ämnet 4β-hydroxykolesterol kan användas som markör för det viktigaste P450 enzymet, CYP3A4. Detta betyder att man kan mäta CYP3A4 induktion utan att ge något markörläkemedel vilket reducerar antalet kliniska studier och ökar säkerheten för deltagande individer.

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And when turning from the scientific world to the private, så byter jag språk från det vetenskapliga engelska till det personliga svenska…

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