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**HUMAN LIVER *IN VITRO* MODELS FOR  
EVALUATION OF DRUG METABOLISM  
AND DISPOSITION**

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## ABSTRACT

The administrated dose of a drug is adjusted to give a therapeutic effect in patients without causing side-effects or toxicity. Cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT) enzymes, uptake and efflux transporters and nuclear receptors regulating these enzymes, expressed in the liver and in other tissues, are all important players in drug metabolism, disposition and elimination. Many drugs are substrates and/or inhibitors of the same enzymes and may cause drug-drug interactions (DDIs) in a patient that takes several drugs at the same time, which can result in loss of therapeutic effect, side-effects or toxicity. The detection of major metabolites, reactive metabolites, metabolizing enzymes and transporter proteins for all new drug candidates is of high importance during preclinical evaluations. Reliable *in vitro* test systems of the human liver are essential for a complete and accurate preclinical evaluation of a new drug candidate. Primary human hepatocytes lose their hepatic functions within a few hours or days when maintained in suspension or cultured in two-dimensions (2D).

In this work, important hepatic functions were investigated in the human hepatoma cell line, HepaRG, and fresh human hepatocytes in suspension and in a dynamic three-dimensional (3D) bioreactor system. Fresh human hepatocytes cultured in 3D retained P450, UGT and OATP1B1 uptake activities for at least one week. Further, all major *in vivo* metabolites of AZD6610 and diclofenac were detected in “fresh” human hepatocytes after 6 days culture in 3D. Three P450 enzymes, CYP2J2, CYP4A11 and CYP4F3B, which are normally not involved in the metabolism of drugs, were identified to take part in the hydroxylation of AZD6610. Furthermore, the UGT activity was higher and the P450 and OATP1B1 activities were lower in HepaRG cells compared to primary human hepatocytes, for the model substrates evaluated in this study. The HepaRG cells maintained P450 activities for several weeks and UGT activities for at least one week in the bioreactor culture. Moreover, effects of rifampicin and ketoconazole on P450 activities in HepaRG cells cultured in the bioreactor predicted well the effects observed *in vivo*. The primary human hepatocytes and HepaRG cells were polarized in the bioreactor and formed tissue-like structures, which resembled the human liver tissue. In addition, the detection of glucuronides in the bioreactor medium indicated an active efflux of conjugated metabolites from 7 days old primary human hepatocytes cultured in the bioreactor back to the circulating medium. Knockdown of drug transporters in Caco-2 cells using short hairpin RNA (shRNA) was shown to be a valuable tool to understand potential sites of transporter-mediated pharmacokinetic interactions and the involvement of hepatic transporters in drug disposition. This model clearly showed the involvement of P-gp but not of MRP2 in the efflux of ximelagatran, hydroxy-melagatran and melagatran. The liver bioreactor using either fresh human hepatocytes or HepaRG cells retained biotransformation and transporter capacities for at least one week. This is a compelling feature of the 3D model, which open up for long-term cultures required for detection of metabolites from slowly metabolized drugs as well as induction, DDI and toxicity investigations.



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- I. **Darnell M**, Karlsson J, Owen AD, Hidalgo JJ, Li J, Zhang E and Andersson TB. (2010) Investigation of the involvement of P-Glycoprotein and Multidrug Resistance-Associated Protein 2 in the efflux of ximelagatran and its metabolites by using short hairpin RNA knockdown in Caco-2 cells. *Drug Metab Dispos* **38**:491-497.
- II. Zeilinger K, Schreiter T, **Darnell M**, Söderdahl T, Lübberstedt M, Dillner B, Knobeloch D, Nüssler AK, Gerlach J and Andersson TB. (2011) Scaling down of a clinical three-dimensional perfusion multicompartiment hollow fiber liver bioreactor developed for extracorporeal liver support to an analytical scale device useful for hepatic pharmacological *in vitro* studies. *Tissue Eng Part C* **17**:549-556.
- III. **Darnell M**, Schreiter T, Zeilinger K, Urbaniak T, Söderdahl T, Rossberg I, Dillner B, Berg AL, Gerlach J and Andersson TB. (2011) Cytochrome P450-dependent metabolism in HepaRG cells cultured in a dynamic three-dimensional bioreactor. *Drug Metab Dispos*, **39**:1131-1138.
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- V. Ulvestad M, **Darnell M**, Molden E, Ellis E, Åsberg A and Andersson TB. Evaluation of OATP1B1 and CYP3A4 activities in primary human hepatocytes and HepaRG cells cultured in a dynamic three-dimensional bioreactor system. *Submitted*

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## ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ABC	ATP-Binding Cassette
AhR	Aryl hydrocarbon receptor
ALT	Alanine aminotransferase
ASBT	Apical sodium-dependent bile acid transporter
AST	Aspartate aminotransferase
AUC	Area under the plasma concentration versus time curve
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
Caco	Colorectal adenocarcinoma
CAR	Constitutive androstane receptor
CoA	Coenzyme A
CYP	Cytochrome P450
CK19	Cytokeratin
DDI	Drug-drug interaction
DMSO	Dimethyl sulfoxide
E17 $\beta$ G	Estradiol-17 $\beta$ -D-glucuronide
E3S	Estrone-3-sulfate
LC	Liquid chromatography
LDH	Lactate dehydrogenase
MS	Mass spectrometry
Mate	Multidrug and toxin extrusion protein
MDR	Multidrug resistance
MCT	Monocarboxylic acid transporter
MRP	Multidrug resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NTCP	Sodium/taurocholate co-transporting peptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OST	Organic solute transporter
P450	Cytochrome P450
PEPT	Peptide transporter
P-gp	P-glycoprotein
PXR	Pregnane X receptor
SiRNA	Small interfering RNA
SLC	Solute carrier
ShRNA	Short hairpin RNA
UGT	UDP-glucuronosyltransferase
URAT	Urate transporter
Q-ToF	Quadrupole Time-of-Flight



# 1 INTRODUCTION

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## 1.1 GENERAL INTRODUCTION

Drugs available on the market aim to improve health and survival of patients worldwide. However, changed efficacy, toxicity and side-effects may occur due to polymorphic enzymes or in patients using several drugs at the same time.

Most drugs are taken orally and undergo first pass metabolism in the intestine and liver before entering the systemic blood circulation. The metabolizing enzymes, uptake and efflux transporters expressed in the tissues together with the physical chemical properties of the drugs determine the pharmacokinetics of the drug including absorption, distribution, metabolism and excretion (Curatolo, 1998). Knowledge and identification of processes and certain enzymes involved in the pharmacokinetics of new drug candidates, revealed during drug development, will increase the success rate of drugs reaching the market and facilitate the design of relevant drug-drug interaction (DDI) clinical studies, that are needed to appropriately label a drug for safe and effective use (Giacomini *et al.*, 2010).

The liver is a critical organ for the bioavailability and metabolism of drugs. Freshly isolated human hepatocytes represent a good model of the liver since they are able to perform the full range of *in vivo* drug biotransformation pathways and retain many of the uptake and efflux functions of liver cells (De Bartolo *et al.*, 2006). However, the use of fresh human hepatocytes has several drawbacks such as scarce and unpredictable availability, inter-donor variability and significant variation in cell functions, especially cytochrome P450 (P450) activities (Luo *et al.*, 2002; Ohtsuki *et al.*, 2012; Rogue *et al.*, 2012; Schaefer *et al.*, 2012). The loss of liver specific functions in freshly isolated cells may partly be explained by the rupture of the three-dimensional (3D) structure of the tissue. In contrast, hepatoma cell lines can be cultured under longer periods of time and are often used for detection of acute toxicity, whereas functions important for investigation of *in vivo* relevant metabolites and chronic toxicities are absent.

Thus, preclinical drug metabolism, pharmacokinetic and safety research are lacking reliable *in vitro* tools to predict the metabolic fate, DDIs and toxicity of drugs in the liver. The limitation of the *in vitro* system described above is a major problem for the

pharmaceutical industry and can result in delayed deliveries and even withdrawal of drugs from the market.

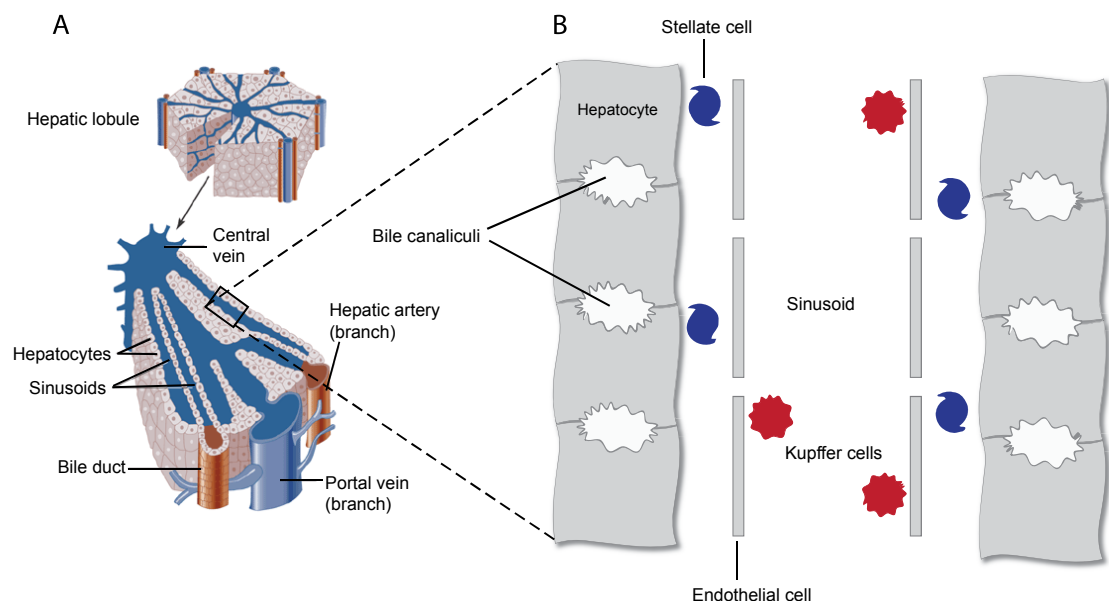
Three-dimensional cultures of human hepatocytes may help to establish an improved *in vitro* tool for drug discovery and development. It has been shown that well-perfused liver cells cultured in a 3D bioreactor retain *in vivo* like properties and form tissue like structures, enabling liver specific functionality to be extended over at least two weeks (Zeilinger *et al.*, 2004; Schmelzer *et al.*, 2009). Further, the human hepatoma cell line, HepaRG, exhibits promising features expressing important functions for drug disposition such as P450 enzymes, UDP-glucuronosyltransferase (UGT) enzymes, nuclear receptors and transporter proteins that resemble those found in primary human hepatocytes (Aninat *et al.*, 2006; Le Vee *et al.*, 2006; Kanebratt and Andersson, 2008b). Recently, HepaRG cells were evaluated as a valuable *in vitro* model for prediction of P450 induction by drugs *in vivo* in human (Kanebratt and Andersson, 2008a).

## 1.2 LIVER STRUCTURE AND FUNCTION

The liver plays a central role in several essential processes in the body, including the metabolism of cholesterol, carbohydrates, fatty acids and amino acids. The organ is also of great importance in the protection against and detoxification of endogenous and foreign substances (xenobiotics).

### 1.2.1 Cell types

The organ is composed of many different cell types, which are divided into parenchymal cells (hepatocytes) and non-parenchymal cells. 80% of the liver tissue volume consists of hepatocytes responsible for the uptake, metabolism and storage of a great variety of substances, including drugs. The non-parenchymal cells include endothelial cells, hepatic stellate cells (fat-storing cells), biliary epithelial cells and immune cells such as Kupffer cells (Figure 1) (Gumucio *et al.*, 1996).



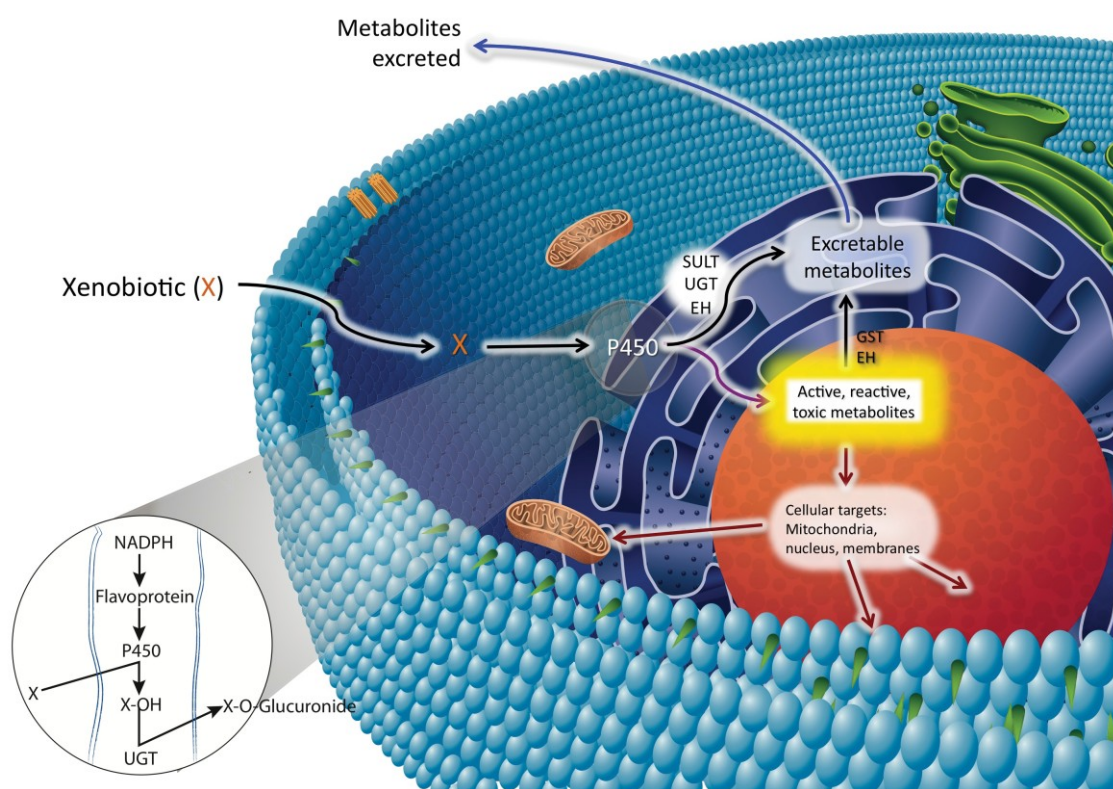
**Figure 1.** Overview of the liver structure: Hepatocytes and sinusoidal capillaries between the portal triad (bile duct, portal vein and hepatic artery) and central vein in the hepatic lobule are shown in A. Further, the bile canaliculi formed between the hepatocytes, the sinusoid between the hepatocyte cords as well as endothelial cells, stellate cells and kupffer cells are present in B.

Figure 1A is adapted from Cunningham and Van Horn (2003) with permission from the publisher.

### 1.2.2 Structure

The hepatic lobule is the structural and functional unit of the liver (Rappaport *et al.*, 1954). The hepatocytes in the lob are arranged in radial cords from the peripheral part to the centre and each hepatocyte is exposed to a mixture of venous blood (from portal

vein) and arterial blood (from hepatic artery) to provide sufficient oxygen and enabling good transport of metabolites (Figure 1). The mixed blood flow from the vein and artery to the central vein in the vascular channels, the sinusoids, formed in the space between the hepatocyte cords. The endothelial cells form the walls of the hepatic sinusoids and Kupffer cells are located in the sinusoids (Figure 1) (Rappaport et al., 1954; Angelin et al., 1988; Ishibashi et al., 2009). This structure is important in directing the excretion of the products of biotransformation out of the hepatocytes into the bile and/or the blood. The hepatocytes secrete bile into the bile canaliculi formed between the hepatocytes, which eventually ends up in bile ducts (Figure 1B). Hepatocytes facing the blood side are located at different positions between the portal vein/hepatic arteries and the central vein and are exposed to different concentrations of oxygen and nutrients, which results in different gene expressions and distinct functional capabilities (Rappaport et al., 1954; Allen et al., 2005).



**Figure 2.** Overview of the processing of xenobiotics in the hepatocyte: P450s and other hepatic enzymes such as conjugating enzymes, UGT and sulphotransferase (SULT) are important in the metabolism of xenobiotics. The endoplasmic reticulum, where the enzymes are located, is shown in dark blue. Further, the cellular membrane has many transporters, OATPs, OCTs and ABC transporters, which are important for the transmembrane flux of many xenobiotics including the products of the conjugating enzymes (Sevior *et al.*, 2012). The figure is from Sevior *et al.*, 2012, with permission from the publisher.



### 1.2.3 General functions

One of the most important functions of the liver is the transformation of carbohydrates from the diet and the storage of it as glycogen, which can be converted to glucose via glycogenolysis when needed, thus regulating the level of glucose in the blood. New glucose is also produced by converting glucose precursors such as lactate and glycerol to glucose (gluconeogenesis). The glucose homeostasis is mainly regulated by insulin and glucagon. Insulin increases the uptake of glucose to the liver and inhibits the glycogenolysis and increase the synthesis of glycogen, whereas glucagon increases the production of glucose in the liver (Angelin *et al.*, 1988). The liver also take up and metabolize amino acids, which are used for biosynthesis of protein (e.g. clotting factors, lipoprotein and albumin), with rest products such as glucose and urea. The proteins are synthesized by ribosomes in the rough endoplasmic reticulum. The rough and smooth endoplasmic reticulum constitute an extensive mesh in the cell where the latter (Figure 2), incorporates many of the biotransformation enzymes, while others are found in the cytosol (Sevior *et al.*, 2012).

The biotransformation enzymes P450s and UGTs and the membrane bound transporters such as organic anion-transporting polypeptides (OATPs) in the hepatocytes, play an important role in the first pass metabolism and bioavailability of drugs together with gut wall enzymes and bacterial enzymes (Kato, 2008; Wu *et al.*, 2011). The first pass metabolism is a protective function of the body to prevent or reduce the entry of xenobiotics. The foreign substances can be transported back into the gut or biotransformed to more polar substances and excreted by transporters into bile or urine (Figure 3). Thus, drug metabolizing enzymes and drug transporters are co-operating to reduce the bioavailability and increase excretion of administrated drugs (Benet, 2009; Wu, 2012). It is essential to understand which particular enzymes that interferes with a certain drug to avoid DDIs, side-effects as well as complications with polymorphic enzymes (Ingelman-Sundberg *et al.*, 2007; Close, 2012). The expression and function of drug transporters and metabolizing enzymes in animals used in preclinical development do not always reflect the expression and functions in human (Cao *et al.*, 2006; Katoh *et al.*, 2006; Li *et al.*, 2009). Therefore, reliable human *in vitro* models are desired for evaluation of drug metabolism and disposition *in vivo* in human.

## 1.3 DRUG METABOLISM

Drug metabolism takes place in several organs and tissues. The most important organ is the liver followed by intestine (including intestinal microflora), kidney, lungs, brain, skin, placenta, plasma and many more. Drug metabolism reactions are normally classified as phase I and phase II reactions. Phase I reaction involves functionalization and phase II reaction results in conjugation, of the drug or metabolite.

### 1.3.1 Phase I metabolism

The term functionalization implies the creation of a functional group or the modification of an existing one and it includes all important redox reactions and hydrolysis/hydrations. There are several oxidoreductases involved in xenobiotic metabolism and the P450s is the far most significant enzyme family estimated to metabolize approximately 70-90% of all drugs and drug candidates. Other important enzymes are flavin-containing monooxygenases (FMOs), known to catalyze some reactions in parallel with P450s, aldo-keto reductases, alcohol and aldehyde dehydrogenases as well as hydrolases.

#### 1.3.1.1 Cytochrome P450 (P450)

The evolution of P450 enzymes involved in drug metabolism appears to have been driven by the need for versatile enzymes capable of coping with a variety of substrates. P450 families 1, 2 and 3 mainly metabolize xenobiotics, whereas P450 families 4-51 are involved in essential physiological functions like oxidations of fatty acids, biosynthesis of biliary acid as well as biosynthesis and metabolism of cholesterol and steroid hormones (Testa and Krämer, 2008).

In humans, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Table 1) are of particular importance in the metabolism of drugs and a universal and univocal nomenclature system is used to describe the different isoforms and their evolutionary relationships. For example, the nomenclature of the CYP3A4 gene is, CYP (root), 3 (family), A (sub-family) and 4 (individual gene) (Nelson *et al.*, 2004).

Structurally, P450s are containing a rigid heme-binding core and a highly flexible distal side which is likely to be involved in substrate binding and product release, because the majority of the access/egress active site channels identified to date are located in this region. Experimental techniques and molecular dynamics simulations indicate that both CYP3A4 and its substrate binding active site exhibit a remarkably high degree of flexibility. In contrast, CYP1A2 and CYP2A6 are more rigid, while CYP2C9 and CYP2D6 exhibit intermediate flexibility. This suggests that there may be a relationship between active site flexibility and substrate promiscuity, because CYP3A4 is highly promiscuous, while CYP1A2 and CYP2A6 are more selective in binding their substrates (Otyepka *et al.*, 2012).

Further, P450s are mainly located in the endoplasmic reticulum (Figure 2) (Edwards *et al.*, 1991) together with NADPH-cytochrome P450 reductase, NADH-cytochrome b5

reductase and cytochrome b5, which are components of the electron-transfer systems (Masters and Marohnic, 2006). For many typical oxidative reactions, P450 enzymes utilize O<sub>2</sub> and two electrons supplied by NADPH to catalyze the monooxygenation of numerous exogenous and endogenous substrates (Hrycay and Bandiera, 2012).

Unexpected pharmacokinetic properties, efficacy and side-effects of drugs in patient are often related to polymorphic P450 enzymes or interactions with co-administrated drugs that are substrates, inducers or inhibitors of P450 enzyme(s) (Hisaka *et al.*, 2010). Especially elderly patients, taking several drugs at the same time, may suffer a significant harm from DDIs and thus an increased risk for hospitalization (Hines and Murphy, 2011). For example, CYP3A4, CYP2D6, CYP2B6 and CYP2C9 are often recognized as potential sites of DDIs and the CYP3A4 inhibitor ketoconazole is known to increase the area under the plasma concentration versus time curve (AUC) *in vivo* for several CYP3A4 substrates (Hisaka *et al.*, 2010). Further, the polymorphic nature of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A5 can affect the therapy outcome and polymorphic P450 enzymes are estimated to influence 20–25% of all drug therapies (Table 1) (Ingelman-Sundberg, 2004; Johansson and Ingelman-Sundberg, 2011).

**Table 1.** *In vivo* P450 probe substrate, inhibitors, nuclear receptors, inducers and functional effects of P450 polymorphisms.

P450	<i>In vivo</i> probe substrate <sup>(1)</sup>	<i>In vivo</i> inhibitor <sup>(2)</sup>	Nuclear receptor	<i>In vivo</i> inducer <sup>(3)</sup>	Functional effects <sup>(4)</sup>
CYP1A2	Caffeine, theophylline	Fluvoxamine	AhR	Tobacco smoke	Rare
CYP2B6	Bupropion, efavirenz	Clopidogrel	CAR (PXR)	Rifampicin	Reduced drug metabolism
CYP2C8	Repaglinide, paclitaxel	Gemfibrozil	PXR (CAR)	Rifampicin	Reduced drug metabolism
CYP2C9	Celecoxib, warfarin	Amidalone	PXR (CAR)	Rifampicin	Very significant
CYP2C19	Omeprazole, S-mephenytoin	Ticlopidine	PXR (CAR)	Rifampicin	Very significant
CYP2D6	Dextromethorphan, pimozide	Paroxetine		None known	Very significant
CYP3A4	Midazolam, quinidine	Ketoconazole	PXR (CAR)	Rifampicin	No or small

(1) - (3) The information is a selection from tables present in the FDA Draft Guidance for Industry - Drug Interaction Studies:

<http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm>

(4) Functional effects of polymorphic P450 enzymes. From Johansson and Ingelman-Sundberg, 2011 (Table 1).

### 1.3.1.2 Dehydrogenases/reductases

Alcohol dehydrogenases, aldehyde dehydrogenases, aldo-keto reductases, short-chain dehydrogenases/reductases and quinone reductases are all enzyme families of importance in drug metabolism (Oppermann and Maser, 2000), which are mainly found in the cytoplasm. The enzymes are expressed in several tissues including the liver, kidney and brain etc. (Penning *et al.*, 2000; Belyaeva, 2003; Nishimura and Naito, 2006; Marchitti *et al.*, 2010).

Interestingly, a recently identified enzyme in the outer mitochondrial membrane, molybdenum cofactor sulfurase C-terminal containing 2 (MOSC2), has been recognized to be directly involved in the amidoxime reductase activity. Thereby playing an important role in the activation of prodrugs containing amidoximes, such as the direct thrombin inhibitor ximelagatran (studied in Paper I) and its follow-up compound AZD0837 (Eriksson *et al.*, 2003; Deinum *et al.*, 2009), by reducing the prodrugs to the bioactive amidines (Neve *et al.*, 2012).

#### 1.3.1.3 Hydrolases

Hydrolases take part in non-redox reaction involving H<sub>2</sub>O as a reactant and are of major interest in the metabolism of drugs, prodrugs and other xenobiotics. The hydrolases with major significance in drug metabolism are carboxylesterases, cholinesterases, paraoxonase and epoxide hydrolases (Testa and Krämer, 2008). They are expressed in plasma, liver, brain, lung, small intestine etc. and located in the cytoplasm and endoplasmic reticulum (McCracker *et al.*, 1993a; McCracker *et al.*, 1993b).

### 1.3.2 Phase II metabolism

Conjugation requires a suitable functional group in the substrate, which will serve as the anchoring site for an endogenous molecule or moiety such as methyl, sulphate, glucuronic acid or glutathione. The endogenous conjugating moiety is usually carried by a cofactor and the reactions are in most cases catalysed by transferases bringing the substrate and cofactor close enough to allow the reaction to proceed. The anchoring site may already be present in the xenobiotics or created by the phase I reaction as described above. The conjugation of xenobiotics has a protective function as it often forms a less reactive product and enables excretion by increased hydrophilicity (Testa and Krämer, 2010). However, some conjugations may cause toxicity since the products are reactive or products are accumulated in the tissue as residues and reach toxic levels (McCarver and Hines, 2002). Interestingly, it is believed that a co-evolution of transferases and transporters have occurred, thus coupling the formation of polar conjugates and their active excretion by drug transporters (Jeong *et al.*, 2005).

The conjugation reactions can be divided into methylations, sulfonations, phosphorylations, glycosidations including glucuronidations, acetylations, formations of coenzyme A conjugates and glutathione conjugations. The glucuronidations and formation of glutathione conjugates are common reactions in xenobiotics metabolism (Testa and Krämer, 2010). There the glutathione and glutathione S-transferases have evolved as a major chemical protection against reactive xenobiotics and reactive

compounds produced during metabolism of endogenous and exogenous compounds and play a critical role in cellular protection against oxidative stress and radiations (Mitchell *et al.*, 1988; Okada *et al.*, 2011; Raza, 2011).

#### 1.3.2.1 Glucuronidations

UDP-glucuronosyltransferases (UGTs) are known to catalyze the highly diverse reactions of glucuronidation and facilitate the reaction by binding the substrate and cofactor uridine-5'-diphospho- $\alpha$ -D-glucuronic acid (UDPGA). The glucuronic acid is transferred from the cofactor to the substrate and attached to a nucleophile, forming O-, N-, S- or C-glucuronides (Dutton, 1980). The human UGTs are the products of four gene families, UGT1, UGT2, UGT3 and UGT8 (Mackenzie *et al.*, 2005) and are located in the membrane of the smooth endoplasmic reticulum (Figure 2) (Meech and Mackenzie, 1998). The UGTs are detected in different tissues including the liver, kidney, gastrointestinal tract, reproductive organs and the skin (Peters and Janson, 1988; Ohno and Nakaji, 2009). The isoforms found in the liver are UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B15 and UGT2B17 (Ohtsuki *et al.*, 2012; Ohno and Nakaji, 2009; Court, 2010) and some of the substrates are alcohols, phenols, carboxylic acids, amines, amides, bile acids and bilirubin (Tephly *et al.*, 1988). Similar to the CYP families, polymorphisms have been reported in the UGT 1 and 2 families (Court, 2010).

AZD6610 and diclofenac, used as model substrates in Paper IV, formed acyl glucuronides from carboxylic acids. The acyl glucuronides formed from carboxylic acids are an important class of the O-glucuronides, since these metabolites are quite reactive and may cause toxicity (Williams *et al.*, 1992). Intermolecular reactions with nucleophilic compounds include hydrolysis, transacylation with glutathione (Grillo *et al.*, 2003) and direct trans-acylation of protein (McGurk *et al.*, 1996), leading to proteins which may induce or interfere with an immune response. These reactions are in competition with intra-molecular nucleophilic rearrangements, particularly internal migration of the acyl moiety resulting in glucuronide isomers (Skordi *et al.*, 2005).

#### 1.3.2.2 CoA conjugation and $\beta$ -oxidation

Mitochondrial  $\beta$ -oxidation is primarily involved in the oxidation of fatty acids and provides energy to cellular processes. The first step in microsomal fatty acid oxidation is  $\omega$ -hydroxylation at the terminus carbon, which takes place in the endoplasmic reticulum by CYP4 enzymes and the resulting  $\omega$ -hydroxy fatty acid is then dehydrogenated to a carboxylic acid in the cytosol. Carboxylic acids are converted to carboxylic-CoAs for oxidation by the  $\beta$ -oxidation pathway and the chain is shortening by removal of two carbon units (Mortensen, 1992; Fer *et al.*, 2008). Noteworthy, the

same oxidation pathway has been recognized to be involved in the metabolism of some xenobiotics, including the PPAR  $\alpha/\gamma$  agonist, AZD6610, studied in Paper IV (Hashizume *et al.*, 2002; Kalsotra *et al.*, 2004; Kalsotra and Strobel, 2006; Jin *et al.*, 2011; Zollinger *et al.*, 2011)

## 1.4 DRUG TRANSPORT

Transporter pharmacology is a rapidly emerging field in drug discovery and development with challenges of overlapping substrate and inhibitor specificities across transporters. Although more than 400 human transporters have been identified at the molecular level, relatively few of these have, to date, been shown to be important in drug disposition (Giacomini *et al.*, 2010). Transporters are expressed in several tissues including, but not limited to, intestine, brain, liver and kidney. Some transporters are tissue specific, whereas others are detected in more than one tissue (Figure 3).

### 1.4.1 Nomenclature

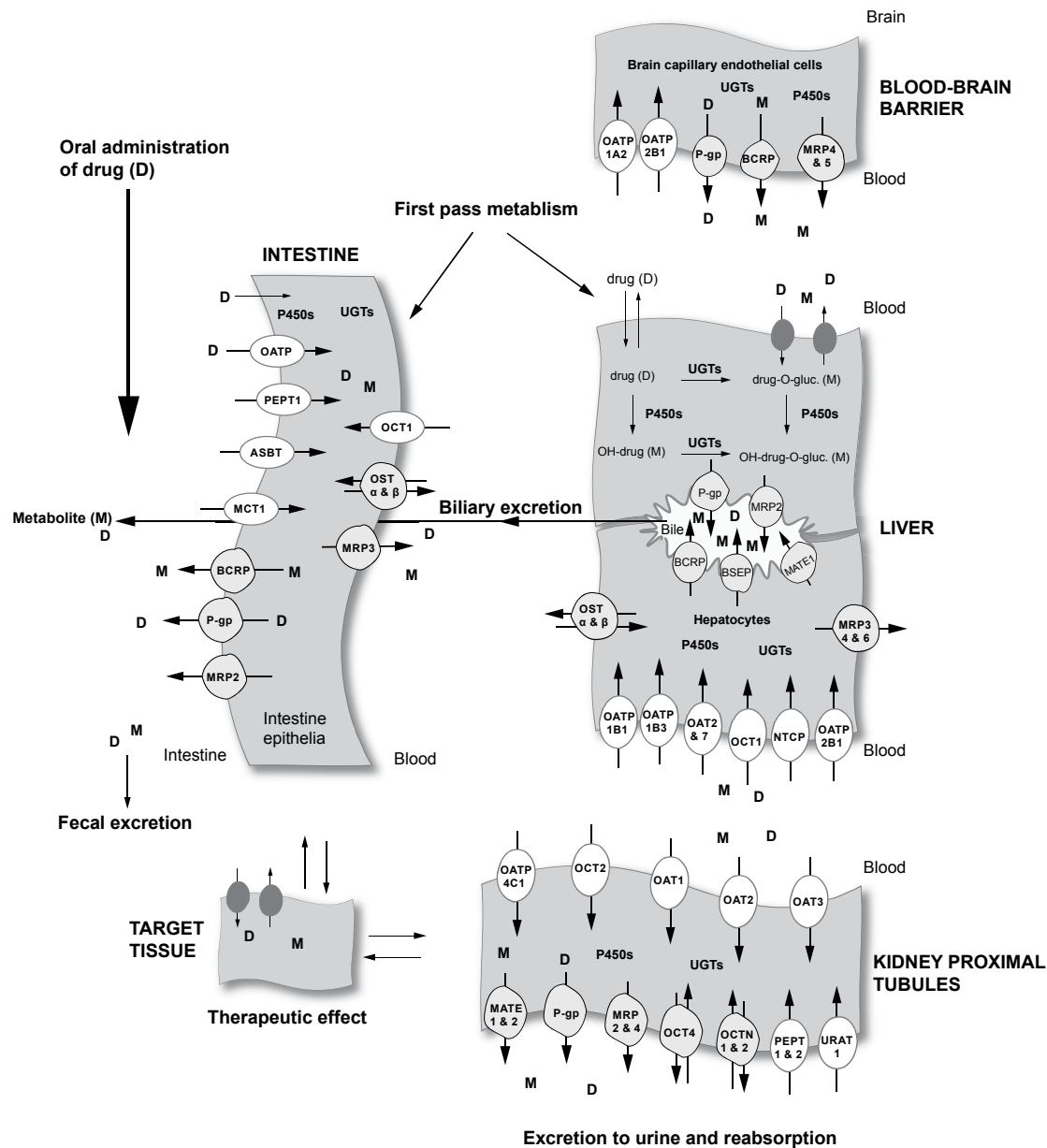
Membrane transporters are divided into two main superfamilies, ATP-Binding Cassette (ABC) (Schinkel and Jonker, 2003) and SoLute Carrier (SLC) (Hediger *et al.*, 2004) superfamily. The gene names reflect the superfamilies, e.g. ABCB11 and SLCO1B1, whereas some protein names reflect the type of substrate being transported, e.g. BSEP (Bile Salt Export Pump) and OATP1B1 (Organic Anion Transporting Polypeptide 1B1).

### 1.4.2 Function and location

Transporters consist of a number of transmembrane domains and one or more binding domain(s) which facilitate the translocations of drugs over the cell membrane. The transport is bi-directional and active, enabling transport against a concentration gradient. However, many drugs undergo both passive diffusion and active transport. The ABC transporters, also referred to as efflux transporters, are ATP-dependent and pump the drugs out from the cell. The SLC transporters may work in both direction and are driven by electrochemical gradients or gradients of counter-substrates or co-substrates (Choudhuri and Klaassen, 2006; Endres *et al.*, 2006).

Drug transporters are expressed throughout the body in all tissues and are detected both on the apical and basolateral membrane of the cells (Figure 3) (Nishimura and Naito, 2005; Endres *et al.*, 2006; Hilgendorf *et al.*, 2007; Giacomini *et al.*, 2010). The efflux transporters, located on the apical membrane have a protective function. One example is the most well known transporter, P-glycoprotein (P-gp, ABCB1/MDR1), which efflux the substrates into the gut or into bile and from brain capillary endothelial cells into peripheral blood (being a part of the blood-brain barrier), preventing the substrate to reach the blood circulation and/or sensitive organs (Figure 3) (Endres *et al.*, 2006). The SLC transporters are frequently associated with uptake of compounds from the blood into tissue or organs such as liver and kidney or involved in absorption from the

gastrointestinal tract or lung tissue into the peripheral circulation. Uptake and efflux transporters can co-operate in order to eliminate xenobiotics (Endres *et al.*, 2006).



**Figure 3.** Overview of selected human transport proteins, for drugs and endogenous substances, expressed in intestine epithelia, brain capillary endothelial cells, hepatocytes and in the kidney proximal tubules (Giacomini *et al.*, 2010) and the presence of P450 and UGT enzymes in the same tissue. After oral administration of a drug, both transporters and metabolizing enzymes can take part in the first pass effect influencing the bioavailability of the drug (D). The drug (D) and/or the metabolite (M) can leave the body via excretion to urine, bile and faeces.



### 1.4.3 Clinical relevant transporters

There are a limited number of transporters that seems to have an impact on drug disposition, safety and efficacy. Researches within the academy and pharmacy industry as well as regulatory agencies such as, Food and Drug Administration (FDA), have formed the International Transporter Consortium (ITC) to draw up” to date” transporter guidelines for drug development. The ITC members emphasize the importance of dynamic guidelines, which will need to be modified regularly as the research front in this area moves forward (Giacomini *et al*, 2010). The transporters that are currently proven to be clinically important are P-gp/MDR1, BCRP, OATP1B1, OATP1B3, OCT2, OAT1, OAT3, OCT1 and BSEP (Figure 3) (ITC, European Medicines Agency (EMA) draft).

### 1.4.4 Transporter mediated drug-drug interactions

There are several clinical DDIs described in the literature that are believed to be transporter mediated. Two examples are the 890% increase of pravastatin AUC when co-administrated with cyclosporine and the 157% increase of digoxin AUC when co-administrated with dronedarone probably due to inhibition of OATPs and P-gp, respectively (Neuvonen *et al.*, 2006; US Food and Drug Administration, 2006; Kiser *et al.*, 2008).

Clinical DDI studies may not elucidate the molecular mechanism for a certain interaction but rather determines required dose adjustments. The mechanism for drug interactions are often best studied using *in vitro* systems in preclinical investigations (Giacomini *et al*, 2010).

### 1.4.5 Polymorphic transporters

Some drug transporters are polymorphic with non-synonymous mutations leading to amino acid changes/deletions. These amino acid alterations may affect membrane localization, function and capacity of the transporters (Shu *et al.*, 2003; Niemi, 2007). Transporters expressed in the liver, such as OATP1B1, OCT1 and BCRP are reported to have clinical relevant genetic polymorphisms (Pasanen *et al*, 2006; Keskitalo *et al*, 2009; Zhou *et al*, 2009)

## 1.5 METABOLISM AND TRANSPORT INTERPLAY

Both transporters and metabolizing enzymes may be involved in the elimination of the same drug, which can complicate the evaluation and understanding of drug-drug interactions studies *in vitro* and *in vivo*. A drug may be: 1) taken up into the hepatocyte by uptake transporters, 2) metabolised by phase I and/or phase II enzymes, 3) the parent drug and/or the metabolite(s) may be transported back to the blood and/or into bile (Figure 3). All these enzymes and transporters are potential sites of DDIs. There are many examples of DDIs at the level of hepatic cytochrome P450, but changes in the concentration of the drug in the cells or in the circulation can also occur by either inhibition or induction of relevant transporter proteins in the liver (Wu and Benet, 2005; Shitara *et al.*, 2003a).

### 1.5.1 Induction

Enzyme induction generally occurs at the transcriptional level and the most important nuclear receptors for regulation of drug metabolizing P450s and transporters are the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). The transcription of several metabolizing enzyme and transporter genes can be increased by the same inducer, caused by ligand binding to one or more nuclear receptor(s). The activation of AhR induce CYP1A gene expression, whereas CAR and PXR have been reported to induce the same genes by binding to DNA response elements belonging to the CYP2B6, CYP2C, CYP3A4, ABCB1 and ABCC2 genes (Lin, 2006; Xie *et al.*, 2000; Martin *et al.*, 2008; Pal *et al.*, 2011). Further, it is not unusual that the ligand binding to the transcription factors are substrates or products of the induced gene (autoinduction).

### 1.5.2 Drug-drug interactions

Rifampicin both inhibits OATP uptake transporters and induces P450 enzymes. Zheng and co-workers (2009) showed a clinical example where rifampicin interacted with the OATP/P450 substrate glyburide. The first intravenous dose of rifampicin increased the AUC of glyburide, most likely due to inhibition of OATP, whereas multiple doses of rifampicin decreased the AUC of glyburide, probably due to induction of P450 enzymes. Further, Niemi *et al.* (2003) showed that repaglinide, a substrate of both CYP3A and OATP, gave a 1.4-fold increase in AUC upon co-administration with itraconazole (CYP3A inhibitor) and 8.1-fold increase in AUC with gemfibrozil (OATP inhibitor). However, simultaneous inhibition of CYP3A and OATP resulted in a 19-fold AUC increase of repaglinide, suggesting that enzyme-transporter interplay may give rise to synergistic inhibitory effects.

## 1.6 LIVER *IN VITRO* MODELS

Both *in vitro* tools and animal experiments are used in preclinical drug development to evaluate the pharmacokinetic properties of new drug candidates. In addition, human relevant tools are needed to investigate the formation of major drug metabolites, the involvement of metabolizing enzymes and transporters in drug disposition as well as the potential site of DDIs. Since the liver is the most important organ for drug metabolism, *in vitro* models which reflect functions of the human liver are desired.

### 1.6.1 Identification of drug metabolizing enzymes

To find out if a new drug candidate or its metabolites are substrates of a certain metabolizing enzyme, human recombinant P450 and UGT enzymes expressed in e.g. *Escherichia coli* or baculovirus infected cells, can be used to investigate one enzyme at a time (Zhao *et al.*, 1996; Mano *et al.*, 2004). The fraction of the metabolic clearance via a certain enzyme is valuable information during drug development and in clinical studies to understand the basis for pharmacokinetic variability and sensitivity as a victim for drug interactions. Further, *in vitro* P450 inhibition studies are required to evaluate whether a drug candidate may act as a perpetrator and thus pose a risk to affect the kinetic profiles of co-administered drugs. The inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 by the drug candidate is routinely tested in recombinant human P450s systems (Turpeinen *et al.*, 2006).

### 1.6.2 Identification of drug metabolite profiles

The impact on efficacy and safety of drug metabolites formed and circulating *in vivo* has to be evaluated in human. Comparisons of *in vitro* metabolite profiles across species can provide an early indication if a new drug candidate has a different major metabolic pathway in human than in animals used for safety evaluations (Wang *et al.*, 2010). Therefore, radiolabeled drug candidates are incubated in suspensions of cryopreserved hepatocytes prepared from animals and humans to compare the metabolite profiles. The standard incubation time is 2 h and the metabolites are identified using liquid chromatography-high resolution mass spectrometry (LC/MS). However, longer incubation times may be applied when slowly metabolized drugs are evaluated.

### 1.6.3 Identification of drug transporter enzymes

To evaluate the contribution of a single uptake transporter in drug or metabolite disposition, human embryonic kidney 293 (HEK293) cells over expressing individual transporters may be used. The study of efflux transporters is however more difficult. First, the drug has to pass the cell membrane (excluding drugs and metabolites with poor permeability), then the cells loaded with the drug have to be washed, before the efflux can be measured. Therefore, inside-out membrane vesicles prepared from cells over expressing a specific transporter are increasingly being used to study the function of efflux transporters. In contrast to the cellular systems, the drug interacts directly with the efflux transporter in the vesicles without the need to first permeate a cell membrane. The kinetics of drug transporter interactions can thus be determined with higher accuracy in a vesicle system than in a cellular system (Karlsson *et al.*, 2010). Unfortunately, this model is not suitable for lipophilic and highly membrane-permeable drugs, which result in high passive uptake into the vesicles, masking the contribution of active transport.

### 1.6.4 Hepatic efflux

Human epithelial Caco-2 cells (colorectal adenocarcinoma cells) can be used as a model to study hepatic efflux transporter-mediated interactions. Caco-2 cells are polarized and express drug efflux transporters such as P-gp, BCRP and MRP2, which are also found in the canalicular membrane of hepatocytes. Caco-2 also express uptake transporters and to some extent metabolizing enzymes. Confluent monolayer of Caco-2 cells on filters in transwell plates enables bi-directional transport studies. Co-administration of two or more drugs acting as substrates or inhibitors of the same transporter comprises the basis for transporter-mediated interactions (Choudhuri and Klaassen, 2006). However, the inhibitors used to identify the involvement of a specific transporter of a drug compound may not be selective and the *in vitro* concentrations used in such experiments are often high. Thus, it cannot be excluded that these inhibitors block the function of several other transporter proteins expressed in Caco-2 cells and thus the information from *in vitro* experiment, investigating the involvement of specific transporters by inhibitors, may not be conclusive (Watanabe *et al.*, 2005; Wang *et al.*, 2008).

RNA silencing leading to functional inactivation of the target gene is an attractive method for down-regulation of the expression of specific genes. Short interfering RNA (siRNA) can mediate strong and specific suppression of gene expression by sequence specific cleavage of mRNA, thus blocking the translation into target protein (Watanabe *et al.*, 2005; Yue *et al.*, 2009). SiRNA is a valuable tool to investigate the contribution

of specific transporters in the transcellular transport of drug molecules and to predict potential sites of pharmacokinetic interactions (Darnell *et al.* 2010 (Paper I)).

### 1.6.5 Hepatic uptake

Although Caco-2 cells can be used as a model to study the hepatic efflux transport interactions, the evaluation of uptake activity of important hepatic drug transporter such as OATP1B1, OATP1B3 and OCT1 requires more liver like models or cell lines over expressing these enzymes (Hilgendorf *et al.*, 2007). Different hepatic *in vitro* assays have been established to evaluate the uptake kinetic of drugs and the biliary efflux as well as the loss of drugs from the incubation medium.

Primary hepatocytes express a complete set of metabolizing enzymes and transporters involved in hepatic drug clearance and are recognized to best predict relevant *in vivo* clearance parameters. However, an extensive decrease in OATP1B1/1B3 activity, already after 6 h, has been reported in plated fresh human hepatocytes (Ulvestad *et al.* 2011). Therefore, it is important to perform uptake studies within a few hours after cell isolation.

Alternatively, sandwich cultured human hepatocyte can be used if longer incubation times are required. The culture of hepatocytes in a sandwich format between collagen and matrigel allows the formation of intact canalicular networks and polarized excretory function (Bi *et al.*, 2006; Lee *et al.*, 2010). Further, the transporter protein levels are maintained for several days and both uptake and biliary efflux can be accessed through modulation of calcium ions (Hoffmaster *et al.*, 2004; Bi *et al.*, 2006; Lee *et al.*, 2010).

However, the use of fresh human hepatocytes is limited by the availability and quality. Fortunately, the activities of important hepatic drug uptake transporters OATP1B1/1B3 and to some extent also OCT1 have been reported to be present in cryopreserved human hepatocytes, which is more convenient to use than fresh human hepatocytes (Soars *et al.*, 2009; Shitara *et al.* 2003b; Umehara *et al.*, 2007).

### 1.6.6 Predictions of hepatic clearance

The pharmaceutical industry aims to develop metabolic stable drugs, which in many cases leads to a shift in drug elimination processes from metabolic, towards transporter-mediated drug excretion. Today, the clearance of up to 20% new drug candidates is under-predicted, probably due to an active uptake of drugs into the hepatocyte (Soars *et al.*, 2009). The clearance of new drug candidates is routinely assessed using

suspensions of cryopreserved human hepatocytes, by measuring the disappearance of the parent drug in a mixture of cells and medium. The transporter processes are not properly evaluated in such assays and metabolic stable drugs are predicted to have almost no clearance *in vivo*. However, the *in vivo* clearance can be high if the drug is a substrate of hepatic drug transporters, which can enable fast elimination (e.g. via excretion to bile). Soars and co-worker (2009) discuss two relatively new methods which enables the measurement of hepatic uptake. The cells are centrifuged in Eppendorf tubes or through a layer of oil and the loss of drug from the media is measured in the supernatant. In addition, the appearance of the drug in the cells can be assessed. These new *in vitro* methods, which include the hepatic drug uptake processes, have improved the *in vitro-in vivo* drug clearance correlations. In addition, the method can also be used to assess potential DDIs in the hepatic uptake by applying inhibitors to the cell incubations (Soars *et al.*, 2009).

### **1.6.7 Induction**

Metabolizing enzymes and transporters can be induced by xenobiotics and may cause loss of effect due to sub-therapeutic concentrations or unwanted side-effects due to changed concentrations of the drug or metabolite in plasma and tissue. Reporter gene assays, immortalized cell lines and cultured primary human hepatocytes have been used to evaluate the induction of P450 enzymes (e.g. CYP1A, CYP2B6, CYP2C and CYP3A4) by new drug candidates (Abadie-Viollon *et al.* 2010). Recently, the human hepatoma cell line, HepaRG, has been documented to provide reliable prediction of P450 drug induction *in vivo* in human (Kanebratt and Andersson, 2008a) and can be used as a new model to evaluate the P450 induction potential of drug candidates.

### **1.6.8 Tissue like *in vitro* model of the human liver**

As described above, primary human hepatocytes are used in most liver *in vitro* studies in drug development, since they are able to perform the full range of known *in vivo* drug biotransformation pathways and retain many of the uptake and efflux functions of liver cells (De Bartolo *et al.*, 2006). However, a high variability of P450 and transporter activities between different donors is usually observed, which can be caused by both inter-donor differences and variation in cell quality (Tostões *et al.*, 2011). The majority of liver cell culture studies have been performed using conventional two-dimensional (2D) cell culture systems, which are convenient and easy to use (Goral *et al.*, 2011). Nevertheless, both fresh and cryopreserved hepatocytes have a rapid loss of liver specific functions over time in culture, which may partly be explained by the rupture of the 3D structure of the tissue, low oxygen supply and the absent of shear stress from the blood-flow (Tilles *et al.*, 2001; Rodríguez-Antona *et al.*, 2002; Wang *et al.*, 2010; Vinci

*et al.*, 2011). Recently, unexpected plasticity of mature hepatocytes to dedifferentiate into progenitor cells, when cultured in 2D, was reported by Chen and co-workers (2012). The study revealed that hepatocytes rapidly transformed into liver progenitor cells within one week through a transient oval cell-like stage when maintained in 2D, thus explaining the loss of liver specific functions (Chen *et al.*, 2012).

Several attempts to provide physiologically relevant conditions that preserve *in vivo*-like phenotype and biological activity of hepatocytes have been published. Microfluidic platforms, co-cultures, flow based hollow fiber bioreactors and spheroids have been used to mimic the situation in the liver (De Bartolo *et al.*, 2006; Dittrich *et al.*, 2006; Khetani and Bhatia, 2008; De Bartolo *et al.*, 2009; Schmelzer *et al.*, 2009; Leite *et al.*, 2011; Prot *et al.*, 2011). Some of these new culturing approaches enable a 3D structure, cell-cell contact and also a constant medium flow and oxygen supply that all are important for the intracellular functions and the maintenance of cell polarity (Tilles *et al.*, 2001; Zeilinger *et al.*, 2004; Schmelzer *et al.*, 2009; Vinci *et al.*, 2011). It has previously been shown that fresh human hepatocytes can retain their liver specific functions such as urea and albumin synthesis, glucose metabolism and P450 activities for at least two weeks in 3D cultures (Zeilinger *et al.*, 2002; Zeilinger *et al.*, 2011 (Paper II)).

The bioreactor technology enables prolonged incubation times and may enable the prediction of clearance, metabolite profiles as well as interaction profiles of metabolites formed from slowly metabolized drugs, which are not detectable in other human *in vitro* systems. Thus, the bioreactor can be used to avoid selection of drug candidates with human unique metabolites, which are not formed in animals. Such metabolites may be formed and identified after prolonged incubation times in human *in vitro* systems before entering clinical studies.

Another important application of the bioreactor for the pharmaceutical industry is to predict human hepatic toxicity, which is not always revealed by the preclinical models used today (Leite *et al.*, 2011)





## 2 AIMS

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The overall research aim for my thesis was to evaluate the use of several *in vitro* techniques to predict drug metabolism, drug transport and drug-drug interactions *in vivo*. Special attention was directed towards long-term cultures in a dynamic three-dimensional bioreactor culture system using HepaRG cells and primary human hepatocytes.

The following studies were performed:

- Knockdown of drug efflux transporters in Caco-2 cells using short hairpin RNA to identify the involvement of efflux transporters in drug transport and to predict potential sites of transporter-mediated pharmacokinetic interactions.
- Measurement of P450 activities over time in fresh human hepatocytes cultured in a dynamic 3D bioreactor.
- Investigation of the maintenance, induction and inhibition of P450 activities in HepaRG cells cultured in a dynamic 3D bioreactor compared to *in vivo* data.
- Evaluation of the major human *in vivo* metabolic pathways of two model substrates in HepaRG cells, fresh and cryopreserved human hepatocytes using cell suspension and a dynamic 3D bioreactor system.
- Investigation of the functionality of OATP1B1 in fresh human hepatocytes and HepaRG cells using suspension and a dynamic 3D bioreactor system.



## 3 METHODOLOGICAL CONSIDERATIONS

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### 3.1 TRANSPORT STUDIES IN CACO-2 KNOCKDOWN CELLS

Usually, the detection of transporter-mediated interactions is achieved by co-administration of a compound of interest with other substrates or inhibitors that bind to and/or interact with the same transporter (Choudhuri and Klaassen, 2006). In Paper I, RNA interference was used to knockdown efflux transporters in Caco-2 cells to detect transporters responsible for the efflux of drug substances that may be involved in drug-drug interactions *in vivo*.

#### 3.1.1 siRNA knockdown

RNA interference is a natural cellular process that effects post-transcriptional gene silencing in eukaryotic cells. SiRNA molecules are the key intermediaries in this process which can inhibit or silence the expression of any given target gene by degradation of mRNA in a sequence-specific manner. SiRNA can be exogenously delivered to cells as synthetic duplexes or endogenously expressed as short hairpin RNA (shRNA), following transfection of plasmid or viral siRNA expression vector constructs. SiRNA causes only transient knockdown of target genes, whereas stable knockdown is established by using shRNA (Celius *et al.*, 2004; Yue *et al.*, 2009).

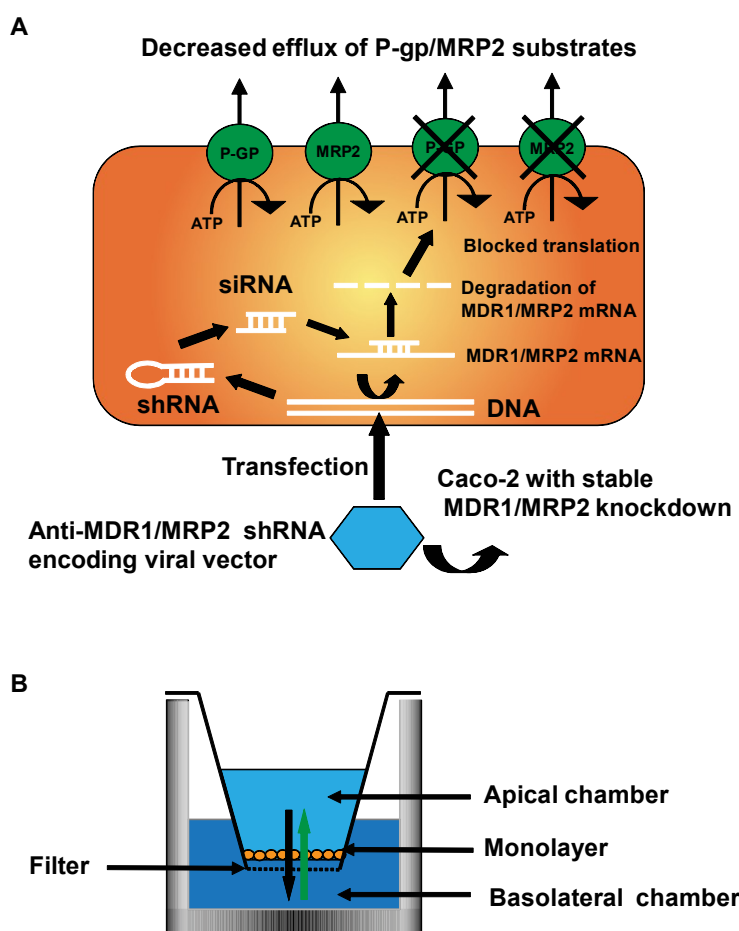
In Paper I, Caco-2 cells were transfected with Lentivirus plasmid vectors containing shRNA inserts targeting human P-gp (GenBank accession number NM\_000927) or MRP2 (GenBank accession number NM\_000392) genes (Sigma-Aldrich, St. Louis, MO) at Absorption Systems LP (Exton, Pennsylvania) to establish cell lines with stable knockdown of transporters (Figure 4A). As a transduction control, parental Caco-2 cells were also transduced with a lentivirus plasmid vector containing shRNA that does not match any known human genes (Sigma-Aldrich), and the transduction procedures were identical to those used to establish P-gp and MRP2 knockdown.

The P-gp and MRP2 mRNA expression, protein expression and transporter activity using probe substrates (Table 2) were compared in vector control cells and P-gp and MRP2 knockdown cells. In addition, the mRNA expression of several transporters,

metabolizing enzymes and transcription factors were measured in the three different cell lines to detect nonspecific effects, which can depend on both knockdown of nontarget mRNA (Jackson *et al.* 2006) or compensatory effects causing up-regulation of other genes (Chen *et al.*, 2005). The only changes observed were the 2-fold higher mRNA expression of UGT2B7 in P-gp knockdown cells and of transthyretin in MRP2 knockdown cells compared to control vector cells (Darnell et al 2010 (Paper I)). These nonspecific effects are not likely to interfere with the transport of ximelagatran and its metabolites investigated in Paper I.

### 3.1.2 Bi-directional transport studies

Caco-2 cells cultured on filters in transwell plates form a monolayer with tight junctions which separate the apical and basolateral chamber (Figure 4B). This system enables bi-directional transport studies. The knockdown of efflux transporters mimics the situation *in vivo*, when the efflux transport of the drug of interest is reduced due to co-administration of a drug, which is a substrate or inhibitor of the same transporter.



**Figure 4.** (A) Stable knockdown of P-gp and MRP2 in Caco-2 cells using shRNA. (B) Culture of Caco-2 cells in transwell plates to study the bi-directional transport of drugs.

## **3.2 PRIMARY CELLS AND CELL LINES**

### **3.2.1 Fresh human hepatocytes**

Primary human liver cells used in Paper IV and V were isolated from the liver tissue remained after partial resection by qualified medical staff following ethical and institutional guidelines at Karolinska University Hospital (Huddinge, Sweden). The isolated fresh human hepatocytes were transported from Karolinska University Hospital to AstraZeneca R&D (Mölndal, Sweden) in a cold package at the same day as tissue surgery and cell isolation.

Primary human liver cells in Paper II were isolated from donor organs excluded from transplantation due to organ injury or from liver tissue remained after partial resection at the Charité University Hospital (Berlin, Germany). Cells were isolated from whole organs or tissue pieces in accordance with European and national regulations and with the approval by the local ethics committee.

It is well known that hepatocytes suffer a rapid loss of liver specific functions after cell isolation. Therefore, the cell suspension experiments, in Paper IV and V, were performed the same day as the tissue surgery and hepatocyte isolation to evaluate the functional properties of the hepatocytes shortly after cell isolation. In addition, the hepatocytes, in Paper II, IV and V, were inoculated into the bioreactor the same day as tissue removal and cell isolation to attain as good quality of the hepatocytes as possible.

### **3.2.2 Cryopreserved human hepatocytes**

A considerable improvement of hepatocyte cryopreservation protocols has been achieved during recent years allowing storage, transport and scheduling of experiments (Li *et al.*, 2008). In Paper, IV and V, pooled cryopreserved human hepatocytes from three different batches (IRK, UMJ and PHL), each containing hepatocytes from ten donors, were used. Cryopreservation and pooling of human hepatocytes were conducted at Celsis In Vitro Technologies (Brussels, Belgium) using a controlled freezing protocol according to in-house procedures. The P450s, UGTs and drug transporters activities were well characterized and 10 different donors were selected to be included in the same batch to receive a good balance of drug metabolizing enzyme and transporter activities. Thus, avoiding the drawbacks of high inter-donor variability observed when using primary human hepatocyte from few donors.

### 3.2.3 HepaRG cells

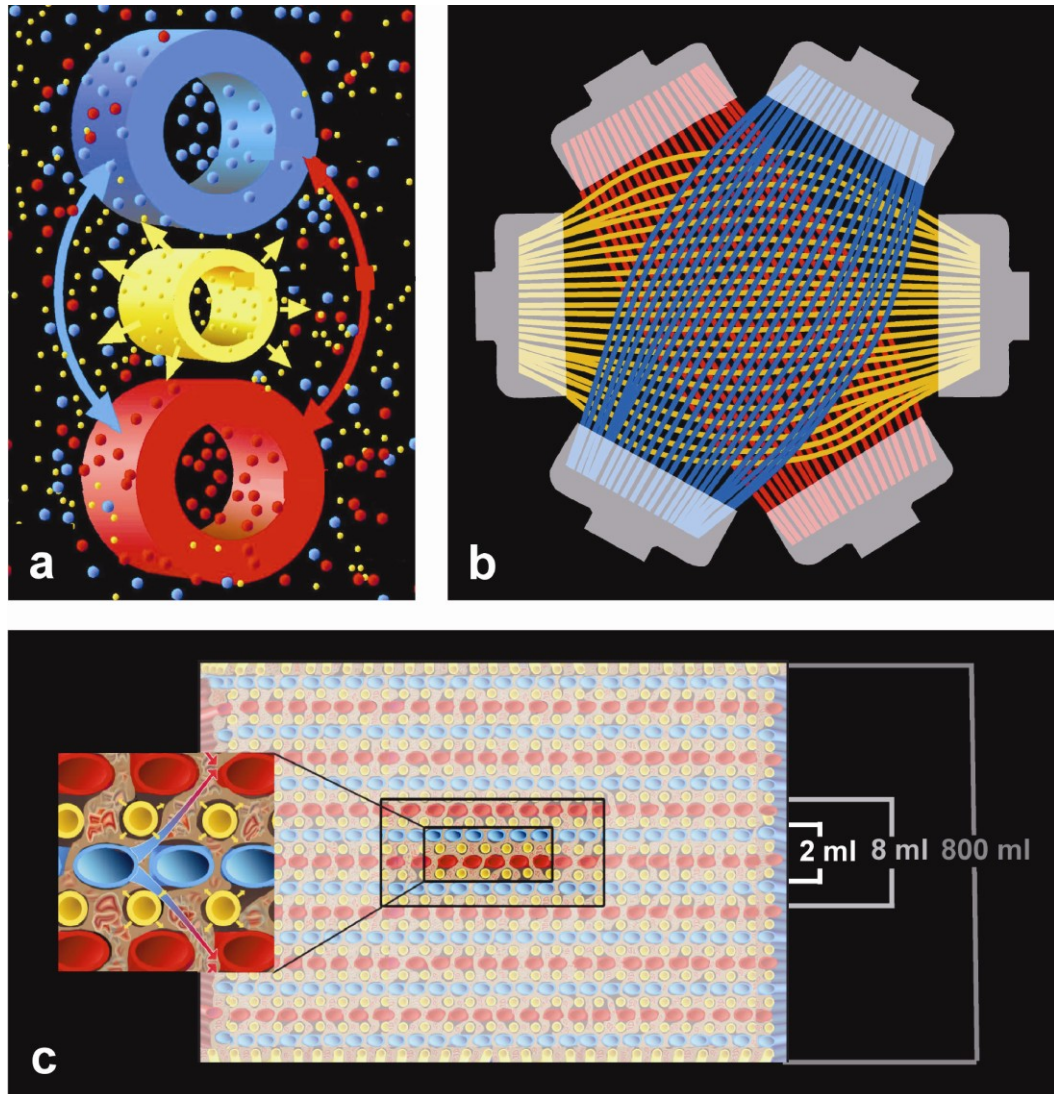
The HepaRG cells were developed from a human hepatocellular carcinoma and were purchased from Biopredic International (Rennes, France). *In vitro*, proliferating HepaRG cells differentiate toward hepatocyte-like and biliary-like cells at confluence. However, maximum cell differentiation is reached after two weeks with 2% DMSO exposure. Hepatocyte-like cells exhibit a phenotype close to that of human hepatocytes, with functional bile canaliculus-like structures as evidenced by fluorescein excretion (Cerec *et al.*, 2007). In addition, HepaRG cells exhibit important functions for drug metabolism and disposition such as P450, UGT and transporter activities (Aninat *et al.*, 2006; Le Vee *et al.*, 2006; Kanebratt and Andersson, 2008a; Hart *et al.*, 2010). If hepatocyte-like cells are selectively isolated and cultured at high cell density, they proliferate and preserve their differentiation status. However, when plated at low density, they transdifferentiate into hepatocytic and biliary lineages through a bipotent progenitor (Cerec *et al.*, 2007).

In Paper III, the cells were first proliferated in 2D flasks to gain sufficient cells ( $80 \times 10^6$  cells) for culture in a bioreactor with a cell compartment of 2 ml. Then the HepaRG cells were further proliferated in the bioreactor to reach confluence followed by two weeks differentiation with DMSO. The DMSO containing medium was washed out and the experimental phase was started nine weeks after the HepaRG cells were received from Biopredic. To shorten the experimental period, cryopreserved differentiated HepaRG cells were applied in Paper IV and V. The experiments could start already 2 days after inoculation. A two layer bioreactor with a smaller cell compartment of 0.5 mL was applied, thus reduced the number of cells needed.

### 3.3 3D CULTURE SYSTEM

#### 3.3.1 Bioreactor prototypes

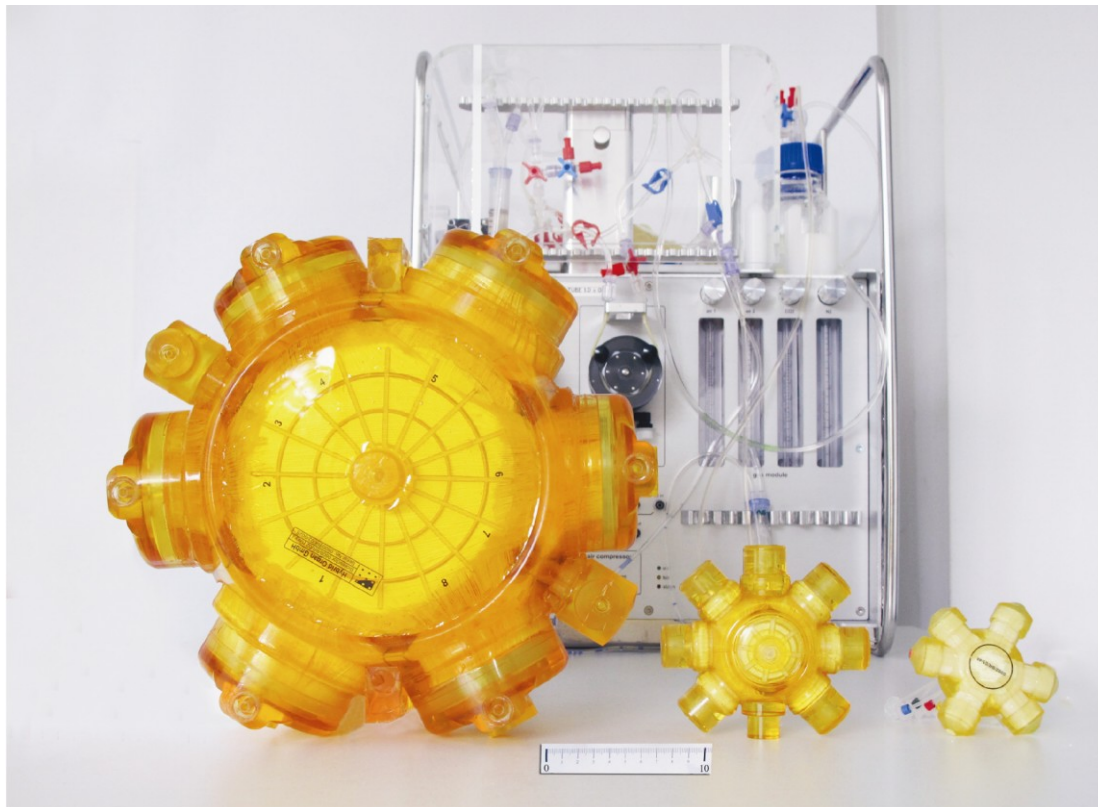
The bioreactor consists of three interwoven capillary bundles, each made of multiple hollow fiber capillaries for counter-current medium perfusion (red and blue) and gas supply (yellow), which allows decentralized nutrient and oxygen/CO<sub>2</sub> exchange with low gradients (Figure 5A, B).



**Figure 5.** (A) Smallest capillary unit with two medium capillaries that are independently perfused (red and blue) and one gas capillary (yellow); cells are cultured within the extra-capillary space (cell compartment). (B) The hollow fiber capillaries. (C) Down-scaling of the clinical-scale bioreactor prototype with a cell compartment volume of 800 mL resulting in a cell compartment volume of 8 mL and a further down-scaled model with a cell compartment volume of 2 mL. From Zeilinger *et al.*, 2011, Figure 1 (Paper II).

In Paper II, the three-dimensional multicompartiment hollow fiber bioreactor proven to function as a clinical extracorporeal liver support system was scaled down in two steps from a cell compartment of 800 mL to 8 mL and 2 mL (Figure 5C, 6). A smaller number of fresh human hepatocytes or HepaRG cells are required in bioreactors with reduced cell compartments, increasing the utility of bioreactors as a tool in preclinical studies.

In Paper III, a 2 mL bioreactor was used and in Paper IV and V, an even smaller bioreactor prototype was used with a cell compartment of 0.5 mL made of only two layers of capillaries with oxygenation and medium capillaries in the same layer. This prototype was developed to increase the throughput of experiment and enabling experiments in three parallel bioreactors in the same perfusion system.

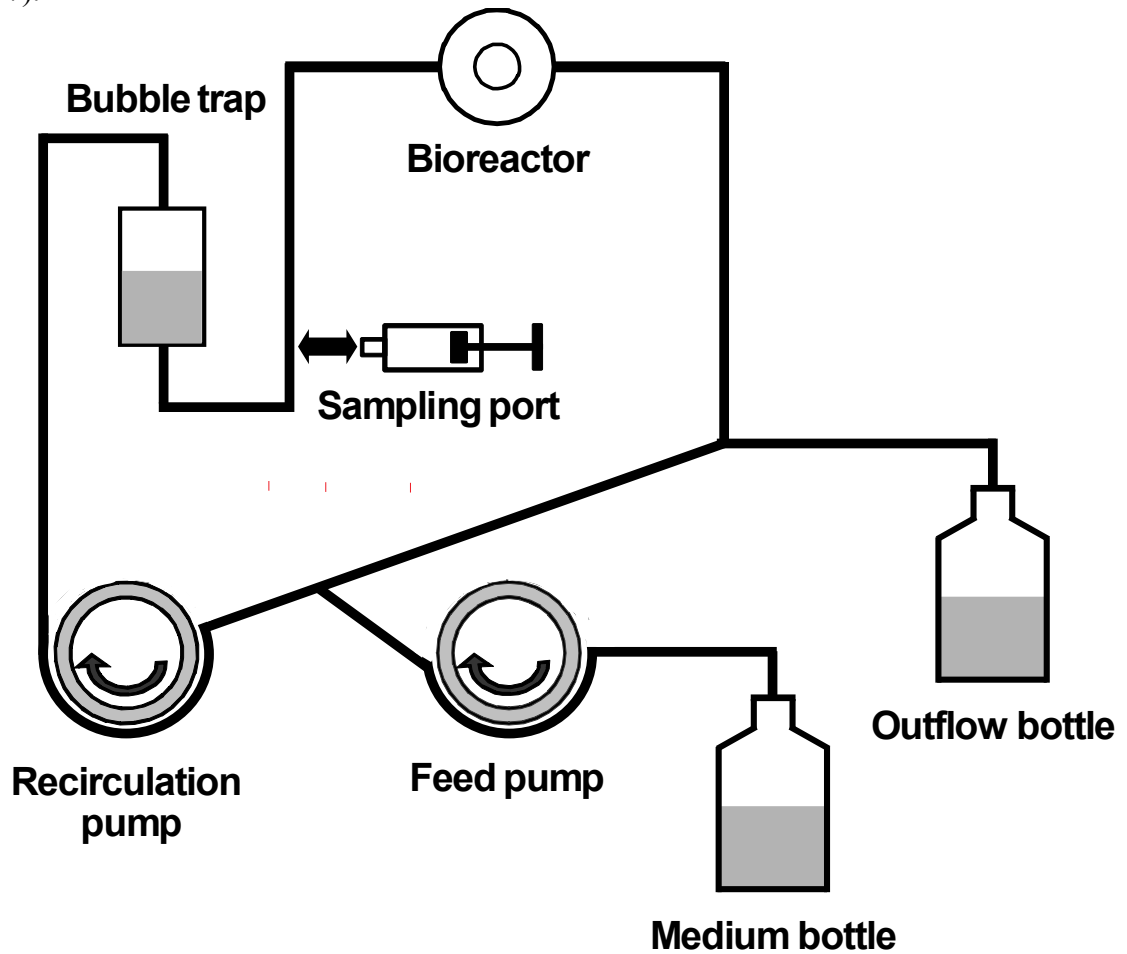


**Figure 6.** The photograph shows the clinical-scale 800 mL bioreactor (left) and two laboratory-scale variants with a cell compartment volume of 8 mL (middle) or 2 mL (right). The scale bar corresponds to 10 cm. In the background, the perfusion device for bioreactor operation can be seen. The device disposes of pump heads for medium recirculation and medium substitution with automated pressure control, an electronically regulated heating unit, rotameters for regulation of the flow rates for air, oxygen, CO<sub>2</sub>, and for the total gas mixture, and a display for digital monitoring and regulation of system parameters. From Zeilinger *et al.*, 2011, Figure 2 (Paper II).



### 3.3.2 Perfusion system

The bioreactors are operated by means of an electronically controlled perfusion device allowing two to three bioreactors to be run in parallel. Temperature, medium feed and medium recirculation rates are monitored and regulated via a connected computer. A valve-controlled gas mix unit is used for air/oxygen/CO<sub>2</sub> supply. Bioreactors and tubing are sterilized before use and rinsed with phosphate buffered saline and medium before cell inoculation. The sampling port enables substance injection and sampling (Figure 7).



**Figure 7.** Bioreactor perfusion system with sampling port and tubing for medium recirculation, feeding and outflow. From Darnell *et al.*, Figure 1, (Paper IV).

### 3.3.3 Bioreactor culture

In Paper II, IV and V, the medium used for the long term culture of fresh human hepatocyte was enrichment with amino acids, free fatty acids and trace elements. Medium containing 5-10 times higher amino acid concentrations compared to most standard media are superior for the maintenance of cell survival, preserving cellular protein levels and liver-specific functions (Jauregui *et al.*, 1986; Sawada *et al.*, 1987). Higher levels of amino acids have been suggested to aid in the recovery of hepatocytes following collagenase digestion, arrest lysosomal protein degradation (Jauregui *et al.*, 1988), and inhibit RNA degradation while stabilizing the activity of some liver-specific enzymes (Balavoine *et al.*, 1992; Lee *et al.*, 1992).

In Paper II, the hepatic functionality and cell quality were assessed during the culture period by measuring the daily production of glucose, urea and albumin and the release of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the recirculating and outflow medium. The production of urea, glucose and albumin are important functions of the liver *in vivo* and the detection of these products in the bioreactor medium indicate maintenance of hepatocyte functions. Further, the leakage of AST and LDH into the culture medium indicates hepatocellular toxicity or tissue break down. AST and LDH are normally high in the beginning of the culture due to stress after cell isolation, but a low level is usually obtained within a few days.

## 3.4 DRUG-DRUG INTERACTIONS IN HEPARG CELLS CULTURED IN BIOREACTOR

In Paper III, the P450 activity over time was accessed in HepaRG cells cultured in bioreactors by measuring metabolites formed from a cocktail of P450 probe substrates including phenacetin, midazolam, bupropion and diclofenac (Table 2). Further, to evaluate the prediction of DDIs, the effect of the model P450 inducer rifampicin and inhibitor ketoconazole were sequentially investigated in the same bioreactor culture (Table 2). The change in metabolite formation rates was calculated to enable the comparison with *in vivo* data from the literature.

## 3.5 METABOLITE PROFILING

The metabolite profiles of two model substrates were evaluated in Paper IV in cryopreserved human hepatocyte, fresh human hepatocyte and in HepaRG cell suspensions on day 0 and in bioreactors on culture day 5 and 6. The same procedure as used within the pharmaceutical industry was conducted for metabolite profiling in suspension. The suspension experiment was performed in 96-well plates and

metabolites were analyzed from both the medium and lysed cells. However, only the medium was analysed in the bioreactor experiments. The levels of metabolites retained in the cells cultured in the bioreactor were not analyzed and the metabolite profile may be different from that detected in the medium. In future studies, a centrifugation step could be included in the suspension experiments to facilitate the measurement of metabolites in the medium, which would be more comparable to the bioreactor experiments.

### 3.6 TRANSPORTER UPTAKE ACTIVITY

In Paper V, the OATP1B1 activity was evaluated in cell suspension and in bioreactor using the same cells as in Paper IV. To study a transporter-mediated drug uptake in cell suspension, either the loss of drug from the medium or the accumulation of drug in the cells can be measured. In this study, both the intracellular accumulation and the concentration in the medium were assessed in the same experiment at different time points. Several washing and centrifugation steps were performed to enable the separation of the cells from the medium. In the bioreactor, the cells and the medium are already separated and medium samples were easily removed via the sampling port to measure the loss from medium (Figure 7). To assess the OATP1B1-mediated uptake of estradiol-17 $\beta$ -D-glucuronide (E17 $\beta$ G) and atorvastatin (Table 2), the experiments were performed with and without 30  $\mu$ M estrone-3-sulfate (E3S), which selectively inhibit OATP1B1-mediated uptake with little effect on OATP1B3 (Ishiguro *et al.*, 2006).

**Table 2.** *In vitro* probe substrates, inhibitors and inducers of metabolizing enzymes and transporters.

Compound	Function	Enzyme	Metabolite	Conc. [ $\mu$ M]	Paper
Midazolam	Substrate	CYP3A	1'-hydroxymidazolam	3	II, III
Bupropion	Substrate	CYP2B6	Hydroxybupropion	100	II, III
Phenacetin	Substrate	CYP1A1/2	Paracetamol	26	II, III
Diclofenac	Substrate	CYP2C9	4'-hydroxydiclofenac	9	II, III
Diclofenac	Substrate	UGT2B7/1A3/1A9	Diclofenac acyl glucuronide	10	IV
Digoxin	Substrate	P-gp		10	I
Bromosulphophthalein	Substrate	MRP2		0.027	I
Atorvastatin	Substrate	OATP1B1/CYP3A4	Hydroxyatorvastatin	5	V
E17 $\beta$ G	Substrate	OATP1B1/1B3		1	V
Ketoconazole	Inhibitor	CYP3A4		3	III
Estrone-3-sulfate	Inhibitor	OATP1B1		30	V
Rifampicin	Inducer	CYP3A4/2B6/2C		20	III

### 3.7 LC/MS/MS ANALYSIS

In Paper I, II, III and V LC/MS/MS analysis was conducted to measure the concentration of substrates of transporters and metabolizing enzymes as well as the formed metabolites in suspensions, 2D and 3D cultures. Standard curves with known concentration of the substrates and metabolites were included in each run and used for

quantification. Ximelagatran, hydroxyl-melagatran, ethyl-melagatran and melagatran and the co-eluted respective isotope-labeled internal standards were measured in the same injection. Moreover, the P450 cocktail metabolites, 1'-hydroxymidazolam, hydroxybupropion, paracetamol and 4'-hydroxydiclofenac, were analyzed at the same time in one injection and the same procedure was used for atorvastatin and its metabolites. However, different sample dilutions were required to measure both the substrate and the metabolites in the linear range of the standard curve.

### **3.8 Q-TOF LC/MS ANALYSIS**

In Paper IV, the metabolite profiles of two model substrates were assessed using Q-ToF LC/MS analysis. The instrument enables the detection of several protonated molecules in the same injection, with a mass accuracy up to four decimals. Radiolabeled substrates were used to facilitate the quantification of the formed metabolites. Detection of metabolites was based on the full-scan MS acquisition, corresponding retention times for the MS peak and a peak in the radiochromatogram and the absence of the potential metabolite peak in the MS chromatogram of the 0 min sample. To facilitate identification of metabolite M6 of AZD6610, MS and retention times were compared to the authentic standard.

Some metabolites co-eluted with other metabolites and could not be separated in the radiochromatogram. To estimate the contribution of each metabolite in %, the MS peak area in the extracted ion chromatogram for the respective protonated molecule was used. The calculation of metabolite proportions in the radiochromatographic peak is based on the assumption that the MS responses of all metabolites are equal. While the MS response is in fact unknown for all metabolites, the proportions calculated should be regarded as estimates, but nonetheless useful in the comparison of metabolite profiles between the different cell systems investigated.

## 4 RESULTS AND DISCUSSION

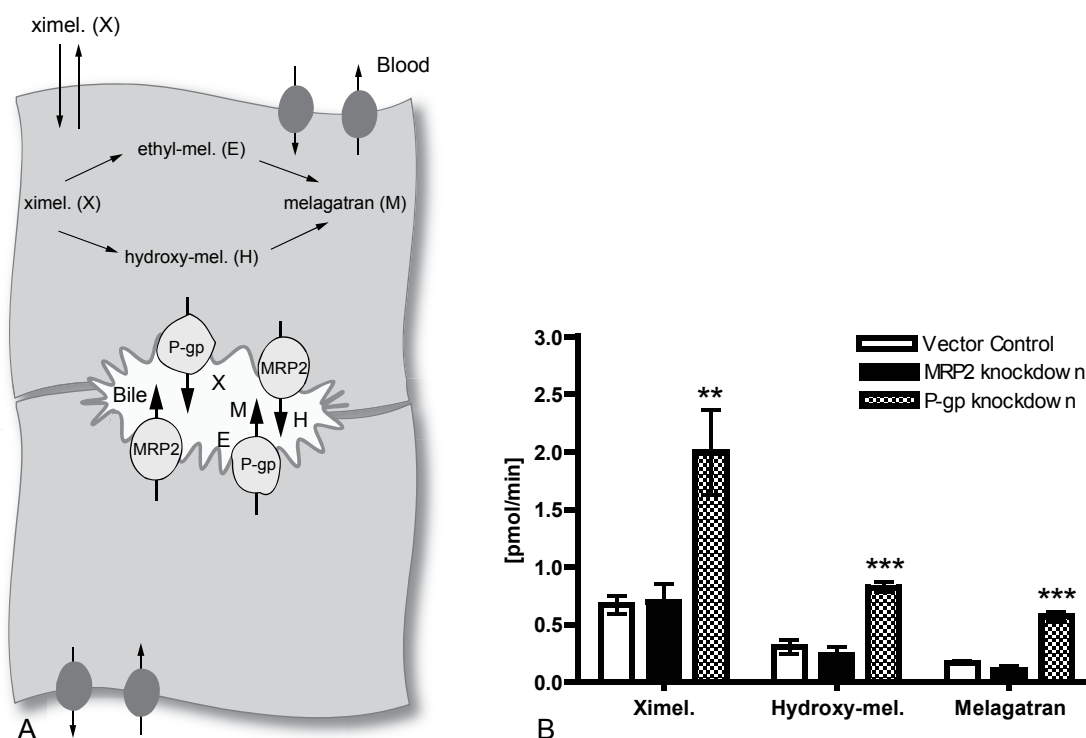
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### 4.1 PAPER I - PREDICTION OF HEPATIC TRANSPORTER INTERACTION *IN VIVO*

The human *in vivo* interaction between ximelagatran and erythromycin was suggested to be mediated by inhibition of hepatic efflux transporters (Figure 8A) (Eriksson *et al.*, 2006). In Paper I, Caco-2 cells were used as a model to study drug efflux transporters expressed both in the intestine and in the liver. Stable knockdown of P-gp or MRP2 in Caco-2 cells were used to evaluate the involvement of these drug efflux transporters in the disposition of ximelagatran and its metabolites.

In the Caco-2 cell monolayers, ximelagatran was metabolized to its intermediate metabolites, hydroxy-melagatran and ethyl-melagatran, and its active end product melagatran. The appearance rates of ximelagatran, hydroxyl-melagatran and melagatran on the basolateral side (Apical-to-Basolateral direction) were significant higher ( $p < 0.01$ ) in P-gp knockdown cells compared to control vector cells and MRP2 knockdown cells (Figure 8B). In addition, the accumulated amount of the metabolites was highest in the P-gp knockdown cells. The *in vitro* results mimic the effects observed *in vivo*, where the plasma concentrations (“basolateral/blood side”) of melagatran, hydroxy-melagatran and ximelagatran were higher after ximelagatran was co-administrated with the P-gp inhibitor erythromycin compared to administration of ximelagatran alone (Eriksson *et al.*, 2006).

Thus, the results indicated a clear involvement of P-gp but not of MRP2 in the efflux of ximelagatran, hydroxy-melagatran and melagatran. P-gp-mediated efflux can therefore be concluded to be important for the biliary secretion of ximelagatran and its metabolites. However, this study does not rule out the possibility that other hepatic transporters, e.g., breast cancer resistance protein, bile salt export pump, and multidrug and toxic extrusion 1 may be of importance in the disposition of ximelagatran and its metabolites.



**Figure 8.** (A) The figure shows the hepatic clearance of ximelagatran (ximel.) and its metabolites *in vivo* via metabolism and secretion into bile and (B) a higher appearance rate of ximelagatran and the metabolites on the basolateral (blood) side due to less efflux on the apical (bile) side in P-gp knockdown Caco-2 cells. Each bar represents mean  $\pm$  S.D.;  $n=3$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . From Darnell *et al.*, 2010, Figure 2 and 7B (Paper I).

Results from *in vitro* and *in vivo* studies may not easily identify which enzymes or transporters that cause a specific interaction by another drug due to the plethora of possible interactions (Choudhuri and Klaassen, 2006). Thus, a more specific strategy, where the target enzyme or transporter have been specifically knocked down or knocked out *in vitro* or in animals may be applied or, where possible, studies in healthy volunteers with changed activity due to polymorphism of a specific enzyme or transporter can be performed (Schinkel *et al.*, 1995; Pasanen *et al.*, 2006; Yue *et al.*, 2009).

The siRNA gene silencing technique, used in this study, has a great potential in transport-mediated DDIs studies for elucidating the function of specific transporters in drug disposition.



## 4.2 PAPER II - MAINTENANCE OF HEPATIC FUNCTIONS IN BIOREACTOR CULTURES

In Paper II, a three-dimensional multicompartiment hollow fiber bioreactor proven to function as a clinical extracorporeal liver support system was scaled down in two steps from 800 mL to 8 mL and 2 mL bioreactors. The fresh human hepatocytes showed maintained and comparable liver specific functions, such as glucose and urea production, over 2 weeks, when cultured in the three different bioreactor prototypes (Figure 9A).

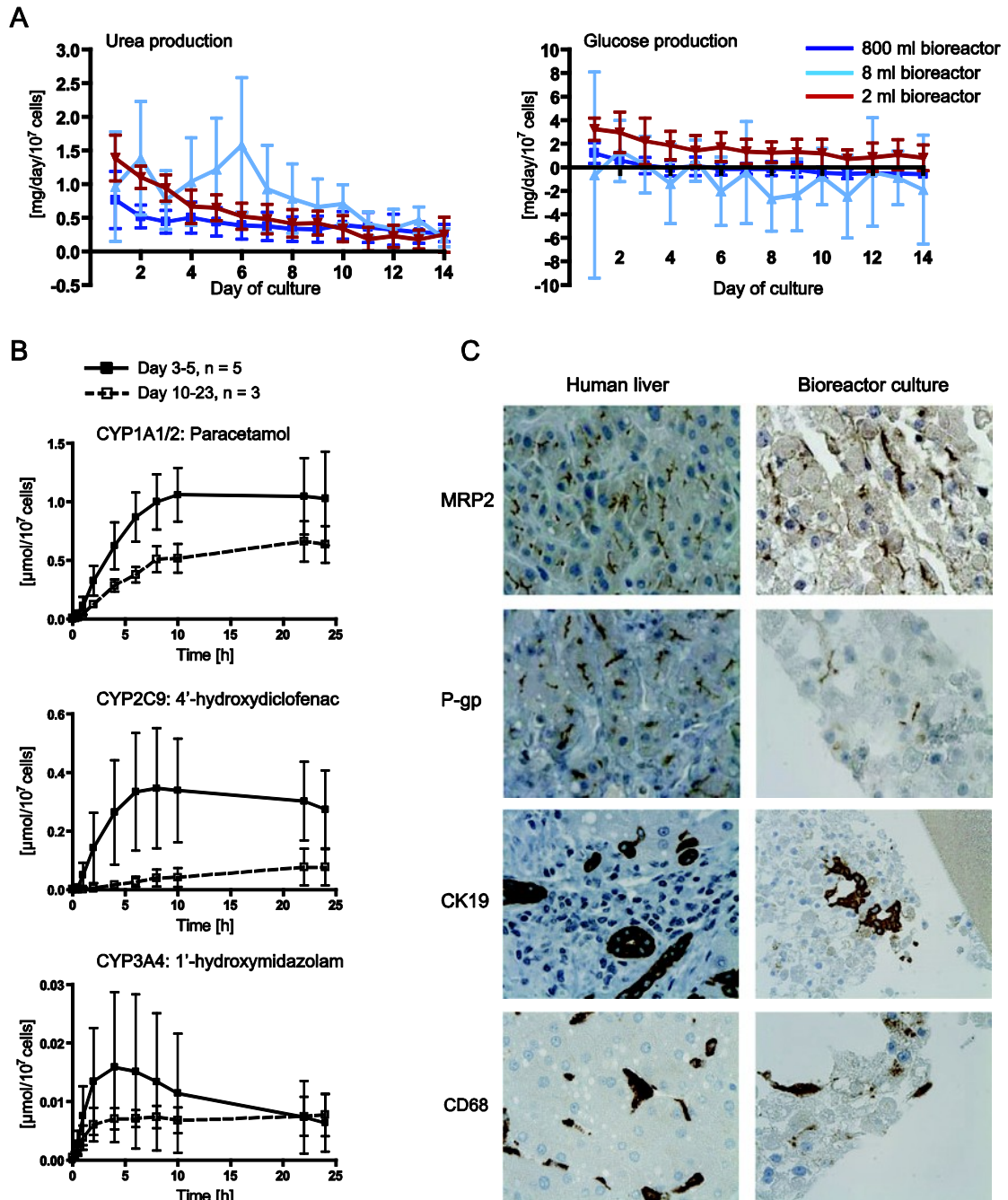
In addition, the 2 ml bioreactor preserved the activity of major drug metabolizing P450 enzymes up to 23 days (Figure 9B). A P450 substrate cocktail was injected to the bioreactor medium and the initial formation rates of paracetamol (CYP1A1/2), 4-hydroxydiclofenac (CYP2C9), and 1'-hydroxymidazolam (CYP3A4) were maintained at 43%, 8%, and 35%, respectively, in the late culture phase (day 10-23) as compared to values from the early culture time (day 3-5). Metabolism rates varied between cell preparations from different donors, which probably reflect human variation in drug metabolism due to human phenotypes and/or genotypes as well as differences in the quality of liver tissue and cells after isolation (Goyak *et al.*, 2008; LeCluyse, 2001).

Moreover, hepatocytes and nonparenchymal cells formed tissue-like structures between the capillary fibers in the small-scale bioreactor. Biliary cells characterized by cytokeratin (CK) 19 immunoreactivity formed channel-like structures that were similar in size to those found in intact liver tissue (Figure 9C). Further, the localization and distribution of hepatic efflux transporter proteins, such as MRP2 and P-gp/MDR1, to one side of the plasma membrane, revealed that the hepatocytes were polarized in the bioreactor (Figure 9C). Furthermore, the P-gp/MDR1 and MRP2-positive sides of the hepatocytes were facing each other, thus forming bile canaliculi-like structures similar to those found in the human liver tissue used as a control (Figure 9C). The finding of hepatocytes after two weeks culture in 3D (Paper II), is in sharp contrast to the total lack of hepatocytes, due to dedifferentiation into liver-derived progenitor cells, after two weeks culture of primary rat hepatocytes in 2D (Chen *et al.*, 2012).

Many new drug candidates have been selected on the basis to be slowly metabolized by the liver for a suitable pharmacokinetic profile. However, the experimental models available today do not function over a period sufficient to be able to properly investigate critical metabolic parameters and toxicity and do not provide the complexity of the liver tissue. The preserved liver specific functions, the liver-like tissue and the reduced number of cells required for the small scale 2 ml bioreactor, evaluated in this study, open up for new and useful application within pharmaceutical research. The



results suggest that the system could be used for studies requiring long-term performance of cultures, for example, to study kinetic profiles, slowly metabolized drugs, DDIs, induction of drug metabolism, and toxicity.

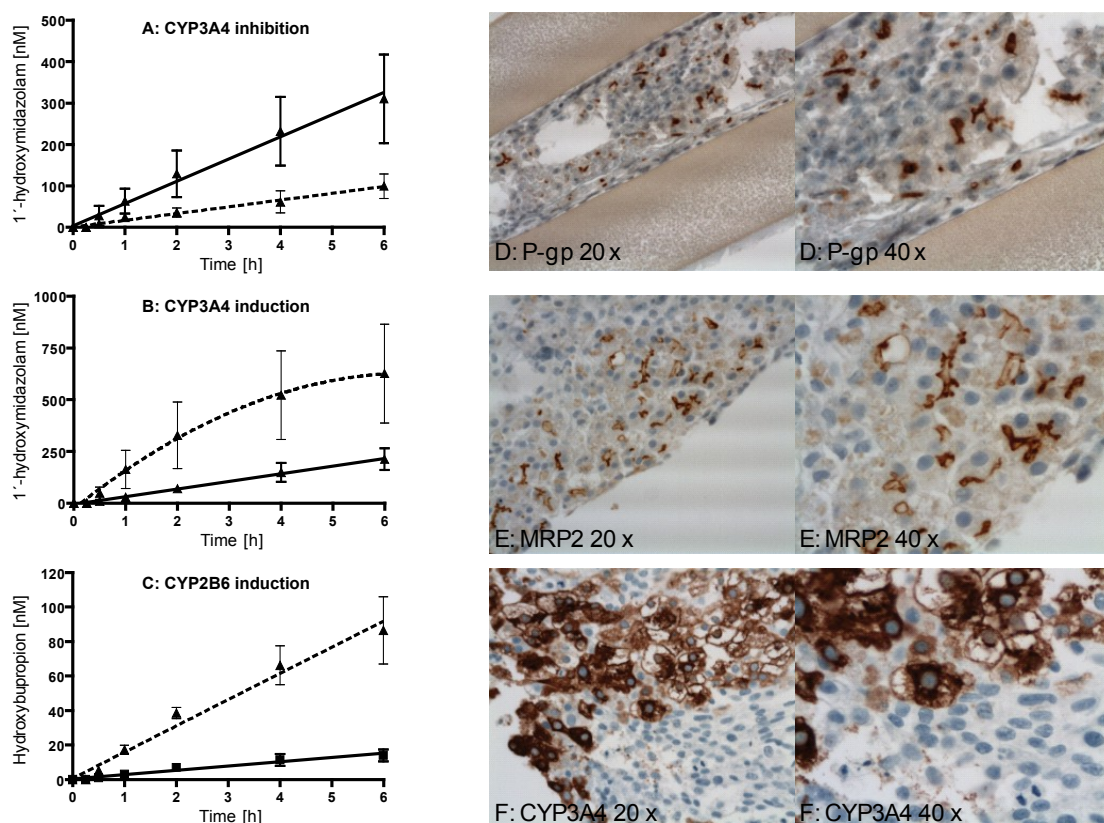


**Figure 9.** The figure shows (A) the retained urea production and glucose production/uptake in the 2 mL, 8 mL and 800 mL bioreactors and (B) the maintained CYP1A1/2, CYP2C9 and CYP3A4 activities in the 2 mL bioreactor with fresh human liver cells. In addition, (C) the immunohistochemical characterization of fresh human liver cells cultivated in the 2 mL bioreactor over 2 weeks (C, right lane) showed polarized cells with hepatic efflux transporters MRP2 and P-gp in the cell membrane as well as biliary cells (CK19 positive) and Kupffer cells (CD68 positive). Staining of intact human liver is shown for control (C, left lane). Magnification: 200-fold (bioreactor culture, CK19) or 400-fold. From Zeilinger *et al.*, 2011, Figure 3, 4 and 5 (Paper II).

### 4.3 PAPER III - PREDICTION OF P450 INDUCTION AND INHIBITION USING BIOREACTOR CULTURED HEPARG CELLS

To evaluate an alternative cell source to fresh human hepatocytes for bioreactor culture, the stability of P450 activity and prediction of *in vivo* relevant DDIs were investigated in HepaRG cells cultured in the 2 ml bioreactor. We showed that the CYP1A1/2, CYP2C9, CYP2B6, and CYP3A4 activities were maintained over several weeks by measuring the formation of paracetamol, 4'-hydroxydiclofenac, hydroxybupropion and 1'-hydroxymidazolam from a cocktail of P450 probe substrate (Table 2).

Further, the co-administration of ketoconazole with midazolam reduced the CYP3A4 activity by 69% in HepaRG cells cultured in the bioreactor (Figure 10A), which reflected well the 85% reduction of midazolam clearance in human when



**Figure 10.** The inhibition of CYP3A4 activity (A) in bioreactor cultured HepaRG cells, before (solid line) and during (dotted line) ketoconazole treatment and the induction of CYP3A4 (B) and CYP2B6 (C) activities in bioreactor cultured HepaRG cells before (solid line) and directly after (dotted line) rifampicin treatment. Results are given as means  $\pm$  S.D,  $n = 3$  bioreactors. Immunohistochemical staining (brown) in HepaRG bioreactor tissue showed bile canaliculi-like structures, where both P-gp (D) and MRP2 (E) were located to one side of the hepatocyte-like cells. The metabolic enzyme CYP3A4 was also present (F). The distribution of the HepaRG cells between two capillaries is shown in D. From Darnell *et al.*, 2011, Figures 3, 7 and 8 (Paper III).

co-administrated with ketoconazole (Tsunoda *et al.*, 1999; Tham *et al.*, 2006; Yong *et al.*, 2008; Krishna *et al.*, 2009).

Furthermore, the 6-fold increases of CYP3A4 and CYP2B6 activities (Figure 10B, C; Paper III) after 60 h treatment with the P450 model inducer rifampicin were within the induction range observed *in vivo* in human, where rifampicin increased midazolam clearance (CYP3A) by 1.4 to 7.4-fold (Gorski *et al.*, 2004) and the hydroxybupropion formation rates (CYP2B6) by 3 to 5-fold (Kharasch *et al.*, 2008). A larger variation in the induction range (0 to 21-fold) of CYP3A and CYP2B6 was reported in 2D cultured primary human hepatocytes (Abadie-Viollon *et al.* 2010) compared to *in vivo* and HepaRG cells cultured in 3D (Paper III). Thus, the range of CYP3A and CYP2B6 induction *in vivo* in human was better represented by HepaRG cells in bioreactor culture than by human hepatocytes in 2D cultures. The larger variation in the induction response observed in 2D cultured hepatocytes may be caused by variation in cell quality and the erratic loss of liver-specific functions resulting in low basal levels of P450 enzymes before exposing the cells to potential inducers (Luo *et al.*, 2002).

Confluent HepaRG cells are known to differentiate into both hepatocyte-like and biliary-like cells (Aninat *et al.*, 2006; Guillouzo *et al.*, 2007), which was clearly shown also in the bioreactor where tissue-like cell aggregations between the capillaries in the bioreactor contained both cell types (Paper III). The hepatocyte-like cells were polarized as revealed by the location of the transporter proteins P-gp and MRP2 to one side of the membrane. The P-gp and MRP2-positive sides of the cells were also facing each other (Figure 10D, E), which resembles the histology of the primary human hepatocyte cultured in the same bioreactor prototype described in Paper II as well as the situation *in vivo*. In addition, CYP3A4 was found to be expressed in the hepatocyte-like cells (Figure 10F) and strand-like formations of CK19-positive cells indicated the formation of biliary structures (Darnel *et al.*, 2011 (Paper III)).

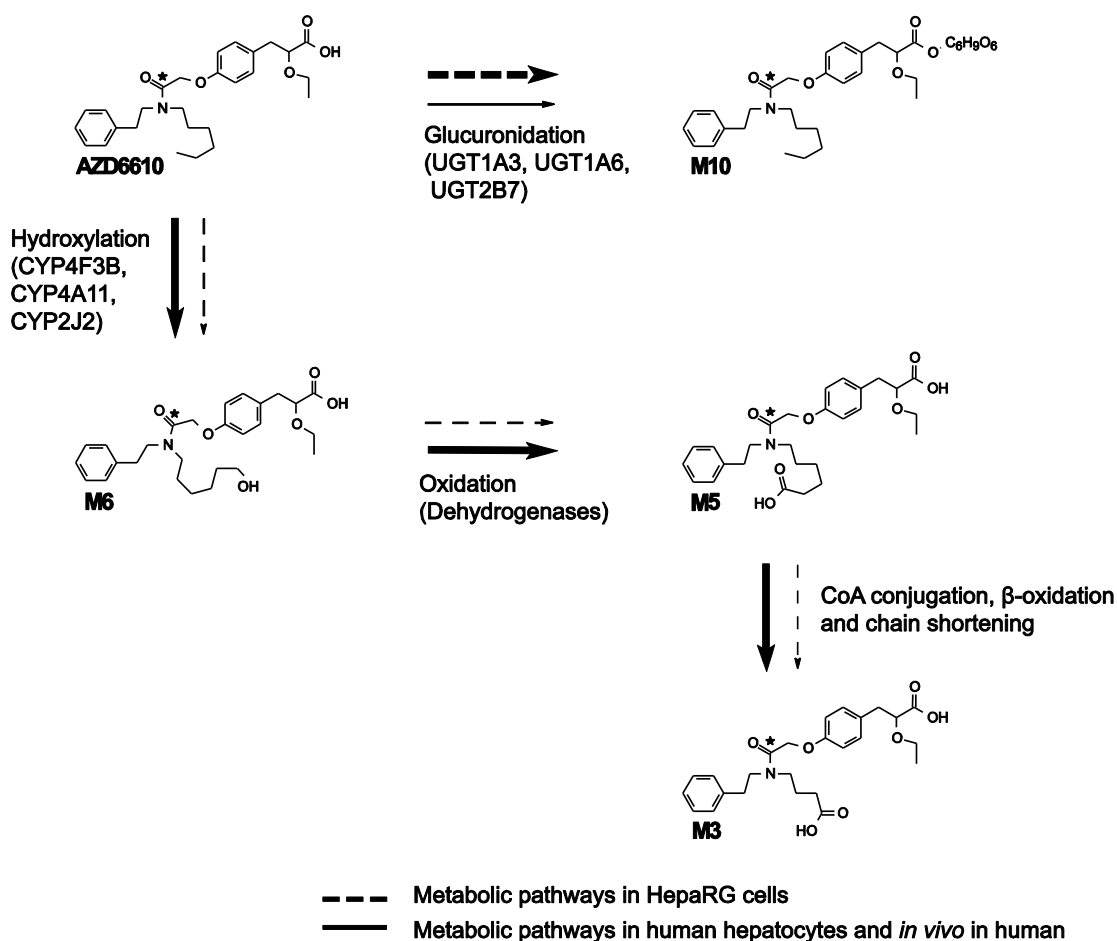
The HepaRG cells have previously been shown to maintain P450 activities in 2D cultures for several weeks (Jossé *et al.*, 2008; Antherieu *et al.*, 2010). In addition, the HepaRG cells have been documented to provide reliable prediction of P450 drug induction and drug clearance *in vivo* in human (Kanebratt and Andersson, 2008a; Zanelli *et al.*, 2012). In this study, we showed that HepaRG cells cultured in 3D also have a stable P450 activity over several weeks and that the induction and inhibition of P450 activities predicted well the magnitude of changes observed *in vivo* (Gorski *et al.*, 2004, Kharasch *et al.*, 2008). In addition, polarity of transporter expression and formation of tissue-like structures including bile canaliculi were demonstrated by immunohistochemistry.

The long-lasting bioreactor system using HepaRG cells thus provides a promising and stable liver-like *in vitro* model for continuous investigations of the hepatic kinetics of drugs and DDIs, which well predict the situation *in vivo* in human.

#### **4.4 PAPER IV - *IN VIVO* DRUG METABOLIC PATHWAY IN HEPATIC *IN VITRO* SYSTEMS**

To investigate the metabolite profile in HepaRG cells compared to primary human hepatocytes and human *in vivo* data, AZD6610 and diclofenac, showing both phase I and phase II metabolism, were used as model substrates. The metabolite profiles were evaluated in suspension, on day 0, and in bioreactor culture 6-7 days after cell isolation/thawing (Paper IV).

One of the model substances, AZD6610, is mainly metabolized via hydroxylation to M6, followed by further oxidation to M5 and M3, while low levels of the glucuronide M10 are detected in human *in vivo* (unpublished results; Figure 11). In Paper IV, all major human *in vivo* relevant AZD6610 metabolites were detected in fresh and cryopreserved human hepatocyte suspensions and the hydroxylation pathway was found to be the major route of metabolism. In HepaRG cells, a high level of the glucuronide M10 was detected, whereas the level of metabolites in the hydroxylation pathway was low or absent. Thus, the main difference between primary human hepatocytes and HepaRG cells was the balance between the hydroxylation route, which was favoured in human hepatocytes, and the glucuronidation route, which was favoured in HepaRG cells (Figure 11). Three P450 enzymes, which are normally not involved in the metabolism of drugs, were identified to take part in the hydroxylation of AZD6610 to M6. The lower expression of two of these enzymes, CYP2J2 and CYP4A11, in the HepaRG cells compared to primary human hepatocytes, may be the reason for the difference in the capacity to produce the major *in vivo* metabolites in the hydroxylation pathway in the two cell systems. Further, the much higher expression of UGT1A6 in HepaRG cells as compared to primary human hepatocyte may explain the efficient glucuronidation pathway in the HepaRG cells. Moreover, the metabolite profiles of AZD6610 in fresh human hepatocytes and in HepaRG cells cultured in the bioreactor for 6 days were similar compared to the metabolite profile in suspension on day 0, although the relative levels of the metabolites in the bioreactor were lower.



**Figure 11.** Overview of tentative structures and suggested major metabolic pathways of AZD6610. The hydroxylation pathway was major *in vivo* in human and in primary human hepatocytes, whereas the glucuronidation pathway was major in HepaRG cells. Enzymes involved in the hydroxylation and glucuronidation of AZD6610 are shown in the figure. The asterisk denotes the position of the  $^{14}\text{C}$  label. From Darnell *et al.*, Figure 4 (Paper IV).

Diclofenac is a carboxylic acid metabolized by both P450s and UGTs. The main human *in vivo* metabolites reported are 4-hydroxydiclofenac, diclofenac acyl glucuronide and 4-hydroxydiclofenac acyl glucuronide (Riess *et al.*, 1978; Stierlin *et al.*, 1979; Stierlin and Faigle, 1979). The initial hypothesis was that the clearance of diclofenac *in vivo* in human was dominated by the formation of 4-hydroxydiclofenac catalyzed by hepatic CYP2C9 (Stierlin and Faigle, 1979; Transon *et al.*, 1995; Kumar *et al.*, 2006b; Kumar *et al.*, 2006a). However, more recent studies revealed that the diclofenac acyl glucuronide was hydroxylated *via* CYP2C8 *in vitro* (Kumar *et al.*, 2002). Thus, the excreted 4-hydroxydiclofenac acyl glucuronide may be formed *via* two separate pathways *in vivo* and the glucuronidation pathway might play an important role in the clearance of diclofenac (Figure 12B).

In Paper IV, a clear difference was observed when comparing the metabolic pathways of diclofenac in HepaRG cells and primary human hepatocytes. The diclofenac acyl glucuronide level was high in HepaRG cell suspension but absent in human hepatocyte

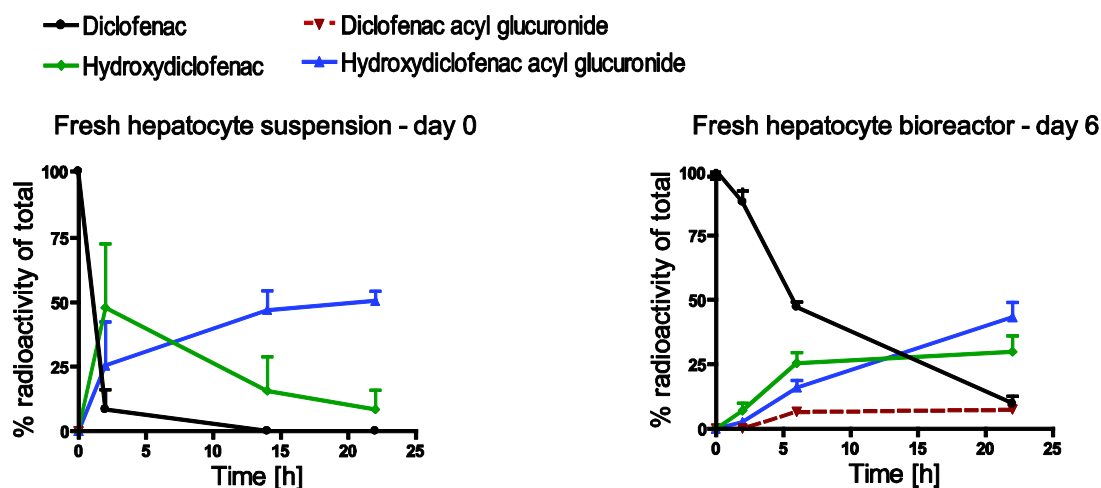
suspension. Previous studies have shown that CYP2C8 mRNA expression correlated well with CYP2C8 activity (Ohtsuki *et al.*, 2012). Therefore, the low mRNA expression of CYP2C8 in differentiated HepaRG cells (Kanebratt and Andersson, 2008b; Antherieu *et al.*, 2010) may result in a low capacity to hydroxylate the diclofenac acyl glucuronide. In contrast, the higher expression of CYP2C8 in human hepatocytes may result in depletion of diclofenac acyl glucuronide, the intermediate metabolite, in human hepatocytes. Although notable higher level of hydroxydiclofenac was observed in human hepatocytes compared to HepaRG cells in Paper IV, the hydroxydiclofenac acyl glucuronide was detected at similar levels in HepaRG cells and human hepatocytes, which may be explained by the two different pathways, which both end up in hydroxydiclofenac acyl glucuronide.

In the bioreactor experiments, 6 days after cell inoculation, hydroxylated and glucuronidated metabolites were detected in both fresh human hepatocyte and HepaRG bioreactors but the clearance of diclofenac and formation rates of metabolites were lower compared to suspension experiments (Figure 12A). In addition, diclofenac acyl glucuronide, which was not detected in human hepatocyte suspension, was detected in human hepatocyte bioreactor. The high level of diclofenac metabolites detected in 7 days old “fresh” human hepatocytes cultured in bioreactors in Paper IV is in sharp contrast to the decrease of hydroxydiclofenac in human hepatocytes, by approximately 90%, after 3 days culture in 2D, when compared to fresh hepatocytes (Rodríguez-Antona *et al.*, 2002). Furthermore, glucuronides are generally too polar to pass membranes via passive diffusion (Zamek-Gliszczynski *et al.*, 2006; Lagas *et al.*, 2010), thus, the detection of glucuronides in the bioreactor medium indicated an active efflux of conjugated metabolites from 7 days old human hepatocytes cultured in the bioreactor back to the circulating medium.

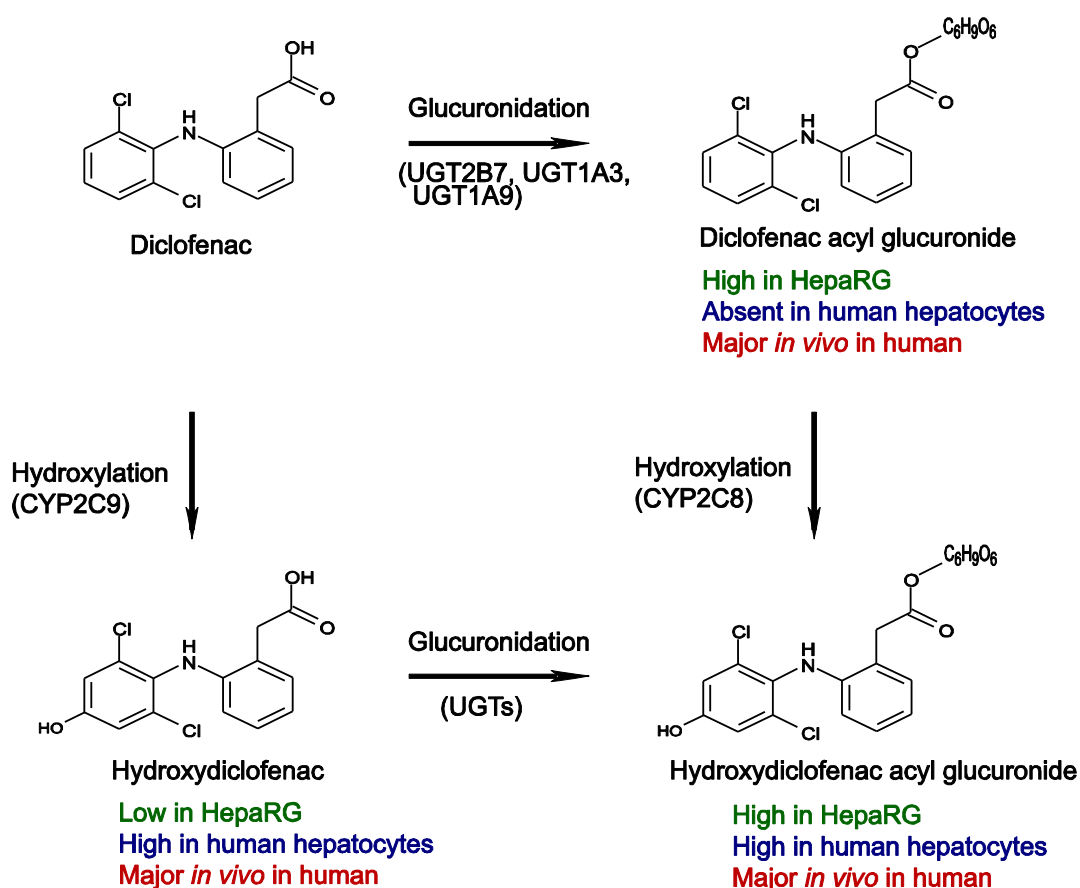
Metabolism in the liver is one of the important determinants of the overall disposition of drugs and metabolites formed can have an impact on efficacy and safety in humans. Comparisons of *in vitro* metabolite profiles across species can provide an early signal if new drug candidates could have different major metabolic pathways in human than in the animal species used for safety evaluations. Thus, liver *in vitro* systems that reliably can predict human *in vivo* metabolic pathways are highly desired.

Freshly isolated human hepatocytes are known to rapidly lose liver phenotypic functions including drug metabolism capacity *in vitro* (Rodríguez-Antona *et al.*, 2002). The liver bioreactor using both fresh human hepatocytes and HepaRG cells retained biotransformation capacity for at least one week, which is a compelling feature of the 3D model enabling detection of metabolites from slowly metabolized drugs.

A



B

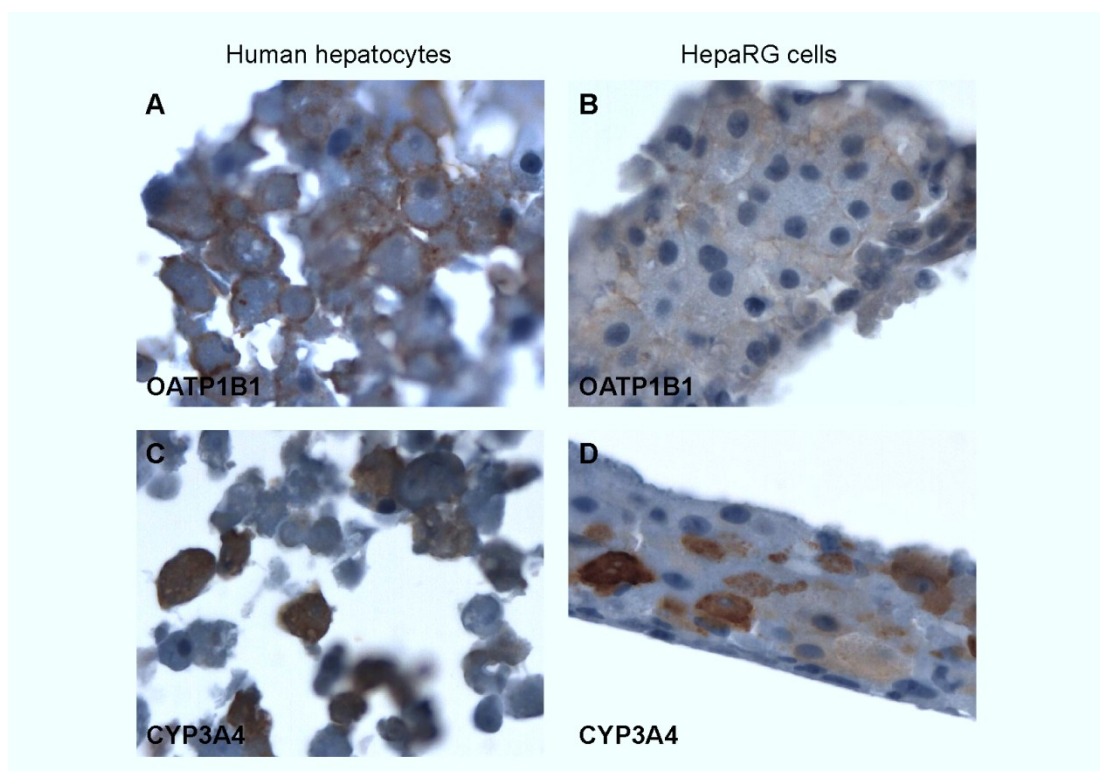


**Figure 12.** (A) The retained P450 and UGT activities in “fresh” human hepatocytes on culture day 6 in bioreactors compared to fresh human hepatocytes in suspensions on day 0. The results are given as percent of total detected radioactivity and the data points are mean values  $\pm$  SD,  $n=3$ . (B) Major metabolic pathways of diclofenac in HepaRG cell and human hepatocyte suspensions and *in vivo* in human. The main P450 and UGT enzymes (Kumar *et al.*, 2002; Sakaguchi *et al.* 2004; Kuehl *et al.*, 2005; Kumar *et al.* 2006a) involved in the biotransformation and the relative level of metabolites detected in each system are presented in B. From Darnell *et al.*, Figure 6 and 7 (Paper IV).



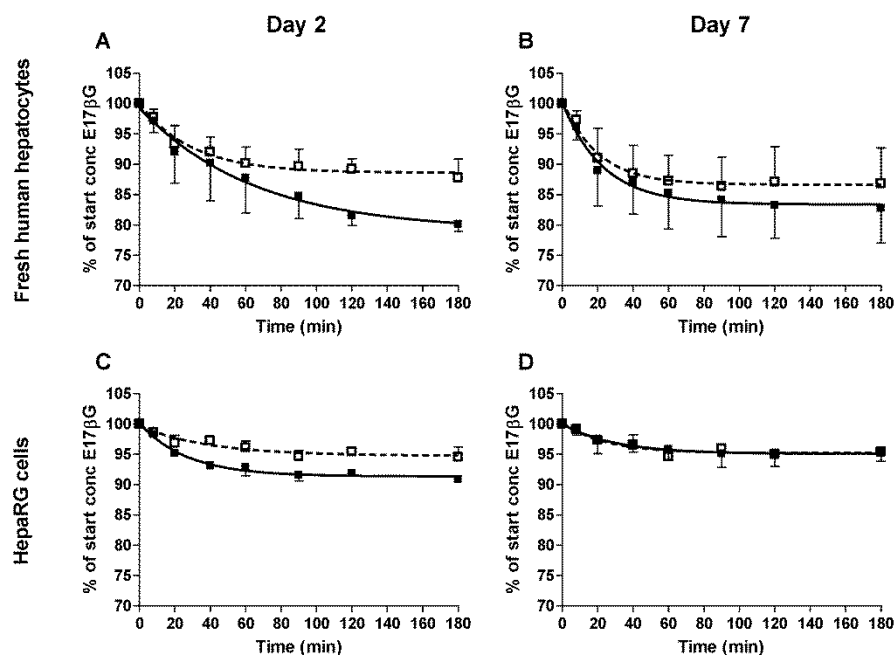
#### 4.5 PAPER V - OATP1B1 AND CYP3A4 ACTIVITIES IN 3D HEPATOCYTE BIOREACTORS

In Paper V, the OATP1B1 and CYP3A4 protein expression and activities were evaluated in up to 9 days old “fresh” human hepatocytes and cryopreserved differentiated HepaRG cells cultured in a bioreactor. The OATP1B1-mediated transport was assessed by measuring the time-dependent loss from media of E17 $\beta$ G, an OATP1B1 substrate, with and without E3S, which is an OATP1B1 inhibitor. A significant loss of E17 $\beta$ G from the bioreactor media was observed in fresh human hepatocytes on culture day 2 ( $p < 0.05$ ) (Figure 14). On day 7, two out of three donors showed an OATP1B1-mediated loss from media of E17 $\beta$ G ( $p > 0.05$ ). In HepaRG cells, a significant OATP1B1-mediated loss from media was observed on culture day 2 ( $p < 0.05$ ), but the results showed no OATP1B1 activity on day 7 ( $p > 0.05$ ) (Figure 14).



**Figure 13.** Immunohistochemical staining (brown) of the basolateral uptake transporter OATP1B1 and the metabolic enzyme CYP3A4 in bioreactor tissue of fresh human hepatocytes from donor 3 (A and C) and HepaRG cells (B and D) cultured for 9 days. OATP1B1 is evenly distributed throughout the whole cell membrane in the bioreactor tissue. Magnification: 40-fold. From Ulvestad *et al.*, Figure 8 (Paper V).

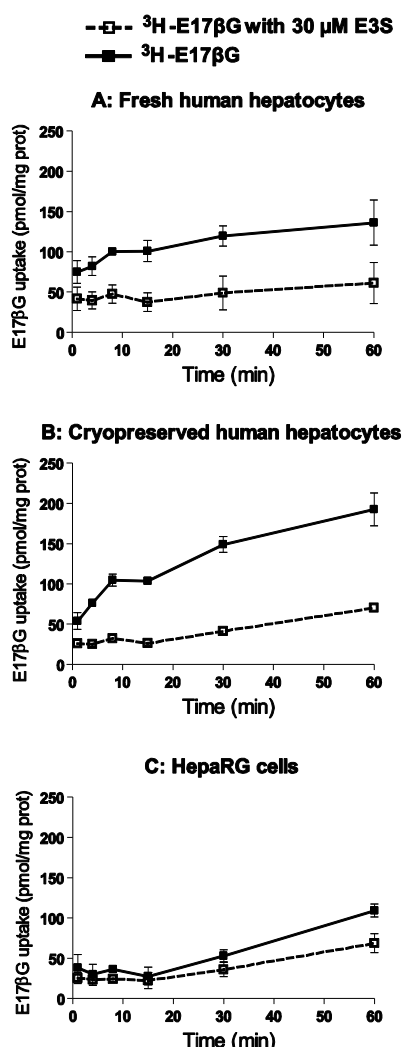




**Figure 14.** Time-dependent loss from media of  $^3\text{H}$ -E17 $\beta$ G in fresh human hepatocytes (A and B) and HepaRG cells (C and D) without (■; solid lines) and with (□; dotted lines) co-incubation of E3S at day 2 and day 7 in bioreactor culture. The medium concentration is expressed as % of start concentration E17 $\beta$ G. Each point represents the mean  $\pm$  S.D.,  $n = 2-3$ . Where the vertical error bars are not shown, the S.D. values are within the limits of the symbols. From Ulvestad *et al.*, Figure 4 (Paper V).

The activity data were in agreement with immunohistochemical staining, which showed that OATP1B1 protein expression was preserved for at least 9 days in fresh human hepatocytes cultured in 3D (Figure 13A), while OATP1B1 protein expression was almost absent in HepaRG cells on culture day 9 (Figure 13B). The maintained OATP1B1 activity and protein expression in fresh human hepatocytes is in sharp contrast to the extensive decrease in OATP1B1/1B3 activity and protein expression in plated (2D) fresh human hepatocytes, when cultured for more than 2 hours (Ulvestad *et al.*, 2011).

Further, a significant OATP1B1-mediated uptake of E17 $\beta$ G was observed in HepaRG cells, fresh and cryopreserved human hepatocytes in suspension on day 0 (Figure 15A-C). However, the OATP1B1-mediated transport was significantly higher in fresh and cryopreserved human hepatocytes than in HepaRG cells. The OATP1B1 activity data are consistent with the mRNA expression of OATP1B1 in cell suspensions showing a significantly lower gene expression in HepaRG cells than in both fresh and cryopreserved human hepatocytes. These data are in agreement with studies comparing transporter activity in fresh and cryopreserved human hepatocytes (Badolo *et al.*, 2011), and shows the improved functions and properties of cryopreserved cells (Li, 2008).



**Figure 15.** The graphs show a much higher OATP1B1-mediated uptake in suspension of fresh human hepatocytes (A), and cryopreserved human hepatocytes (B) compared to HepaRG cells (C). Each point represents the mean  $\pm$  S.D.,  $n = 3$ . From Ulvestad *et al.*, Figure 3, (Paper V).

Atorvastatin is a substrate of OATP1B1 and CYP3A4 and has previously been shown to induce CYP3A4 expression (Kocarek *et al.*, 2002; Monostory *et al.*, 2009). In Paper V, inhibition of atorvastatin uptake by E3S in both fresh and cryopreserved human hepatocyte suspension resulted in a significant decreased loss of atorvastatin and decreased formation of atorvastatin metabolites.

In the bioreactor, atorvastatin actually increased CYP3A4 expression over time, which resulted in an autoinduction in metabolism and increased metabolite formation in the consecutive atorvastatin plus E3S experiment (Paper V). Induction of CYP3A4 in HepaRG cells in bioreactor culture have previously been demonstrated as discussed in Paper III. In Paper V, an induction response by atorvastatin was observed in both primary human hepatocytes and HepaRG cells cultured in the bioreactor for 4 days after hepatocyte isolation or HepaRG thawing. Further, the CYP3A4 mRNA expression and activity in suspension on day 0, the CYP3A4 activity on culture day 3 and the

CYP3A4 protein expression (Figure 13C, D) on culture day 9 in the bioreactor were at similar levels in fresh human hepatocytes and HepaRG cells (Paper V).

The study in Paper V indicates that fresh human hepatocytes cultured in a 3D bioreactor system retain both OATP1B1 transporter and CYP3A4 metabolizing activities and protein expression longer than in currently available *in vitro* models. The rapid loss of transporter and enzyme activities in 2D cultures of human hepatocytes are a major concern when studying drug uptake, metabolism and extrusion from cells *in vitro*, and prevents reliable long-term studies to be performed (Richert *et al.*, 2006; Rodriguez-Antona *et al.*, 2002; Ulvestad *et al.*, 2011). For OATP1B1 substrates, uptake transport activities may have important implications for cellular drug concentrations, which also effect drug metabolism and possible toxic effects. The 3D bioreactor system allows long-term preclinical studies on drug uptake and metabolism, which are especially important for slowly metabolized drugs.



## 5 GENERAL DISCUSSION - FUTURE PERSPECTIVES

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Scientists within the field of pharmaceutical research are continuously exploring and validating new *in vitro* methods to establish reliable assays, which predict the fate of the drug in the human body. High throughput methods are required at an early stage, whereas the reduced numbers of drug candidates, in the later drug development phase, allow the use of more complex models. The main aim of this thesis was to characterize the following *in vitro* models of the human liver:

- Caco-2 cells
- HepaRG cells
- 3D culture of fresh human hepatocytes and HepaRG cells

### 5.1 CACO-2 CELLS

Caco-2 cells are polarized cells expressing drug efflux transporters such as P-gp, BCRP and MRP2, which are also found in the canalicular membrane of hepatocytes. The Caco-2 cells can be used as a model to study hepatic transporter-mediated DDIs of new drug candidates. The inhibitor used to block a certain efflux transporter may inhibit several other transporters expressed in the Caco-2 cells (Watanabe *et al.*, 2005; Wang *et al.*, 2008). The siRNA gene silencing technique, used to knockdown P-gp and MRP2 in Caco-2 cells in Paper I, was shown to have a great potential in transport-mediated DDIs studies to elucidate the function of specific transporters in drug disposition.

### 5.2 HEPARG CELLS

Primary human hepatocytes are the gold standard for hepatic drug metabolism and disposition investigations. Due to the restrictions and difficulties involved in the use of fresh human hepatocytes, attempts to replace these cells are continuously ongoing. Pooled cryopreserved primary human hepatocytes are of good quality, convenient to use and provide an average activity of important hepatic enzymes from several donors in the same experiment. Anyhow, hepatic immortalized cell lines, human embryonic and induced pluripotent stem cell derived hepatocytes would be the ultimate cell system

for pharmaceutical research, provided that these cells exhibit all important functions found in fresh human hepatocytes. A highly reproducible platform and continuous supply of material with maintained hepatic functions over long period of time are highly needed. Unfortunately, hepatoma cell lines such as HepG2, C3A and Huh7 cells and stem cell derived hepatocytes do not yet fully attain important hepatic functions and their utilities and applications within drug development research are limited. However, the highly differentiated human hepatoma cell line, HepaRG, has attracted great attention after its introduction 2002 (Gripon *et al.*, 2002). The HepaRG cells have been extensively evaluated the last decade and the cells exhibit several important hepatic functions. The HepaRG cells provide reliable prediction of P450 drug induction and drug clearance *in vivo* in human (Kanebratt and Andersson, 2008a; Zanelli *et al.*, 2012) and maintain P450 activities in both 2D and 3D culture for several weeks (Jossé *et al.*, 2008; Antherieu *et al.*, 2010; Darnell *et al.*, 2011 (Paper III)).

However, the UGT phase II enzyme activity and detailed studies of metabolite profiles of drugs in HepaRG cells needed further investigations (Aninat *et al.*, 2006; Jossé *et al.*, 2008; Antherieu *et al.*, 2010). In Paper IV, we showed that cryopreserved differentiated HepaRG cells exhibit UGT activity both in suspension on day 0 and in 3D culture for at least one week. The proportion between relevant hydroxylation and glucuronidation biotransformation pathways of the two model substrates in Paper IV was clearly different in HepaRG cells compared to hepatocytes. The glucuronidation pathways were favoured in HepaRG cells, whereas the hydroxylation pathways were favoured in primary human hepatocytes. These findings reflects the different mRNA expression levels, in HepaRG cells and primary human hepatocytes, of the P450 and UGT enzymes involved in the metabolism of the model substrates used in Paper IV.

Although the P-gp and MRP2 efflux activities are high in HepaRG cells, one of the most important hepatic uptake function of drugs, the OATP-mediated uptake, was low in 2D cultured HepaRG cells (Le Vee *et al.*, 2006). In Paper V, we showed that the OATP1B1 activity in cryopreserved differentiated HepaRG cells in suspension on day 0 and in 3D culture on day 2 was present, but low compared to primary human hepatocytes. The low OATP-mediated uptake in HepaRG cells may affect drug clearance, induction, metabolism and toxicity predictions, if the drug is dependent or partly dependent of active uptake to enter the cell. Thus, an improved OATP-mediated uptake in HepaRG cells would probably increase the applications and improve the *in vivo* predictions in preclinical studies.

### 5.3 3D CULTURE OF FRESH HUMAN HEPATOCYTES AND HEPARG CELLS

The high throughput capacity of the conventional 2D cell culture systems and suspension experiments is of high importance early in the preclinical phase of drug development, when selecting drugs with the right properties for further evaluations. However, the selection of metabolic stable drugs requires *in vitro* systems with preserved uptake processes and increased incubation times, which facilitate the prediction of clearance and metabolite profiles of slowly metabolized drugs. In addition, maintained hepatic functions and co-culture with all relevant cells in the liver tissue open up for long term toxicological investigations.

The multicompartiment bioreactor evaluated in Paper II, III, IV and V retained hepatic functions important for drug metabolism and disposition in the human liver. The P450 and UGT activities were preserved in fresh human hepatocytes and HepaRG bioreactors for at least one week. In addition, the OATP1B1 protein expression and uptake activity were detected in fresh human hepatocytes after one week culture in the bioreactor. This is to be compared with the rapid loss of P450 and OATP1B1/1B3 activities reported previously in 2D cultured hepatocytes (Rodríguez-Antona *et al.*, 2002; Ulvestad *et al.*, 2011). However, although metabolism and transporter functions were maintained, the activities decreased with time. Thus, further development and improvement of the bioreactor culture may result in even higher enzyme activity and prolonged incubation times. The development may include further medium optimization, coating of capillaries and co-culture with other cell types. Furthermore, the quality of the fresh human hepatocyte preparations is important for the preservation of hepatic functions in the bioreactor. In Paper VI and V, the initial release of AST and ALT was much higher in donor 2 compared to donor 1 and 3, which resulted in lower metabolism and uptake activity in the bioreactor culture. Immortalized cell lines, such as HepaRG cells, provide a more reliable and stable quality, but do not exhibit as high P450 and OATP1B1 activities in the bioreactor as the human hepatocytes.

Moreover, in Paper III a different protocol for the culture and differentiation of HepaRG cells in the bioreactor was applied as compared to Paper IV and V. In Paper III, the cells were proliferated and differentiated in the bioreactor, whereas cryopreserved differentiated HepaRG cells were directly inoculated at high density in the bioreactors used in Paper IV and V, to shorten the experimental period. Only hepatocyte-like cells seems to be selected in the cryopreservation of HepaRG cells and previous studies have revealed that no or few biliary-like cells are detected when hepatocyte-like cells are seeded at high density (Aninat *et al.*, 2006; Cerec *et al.*, 2007). Thus, the co-culture of hepatocyte-like and biliary-like cells seen in the bioreactor in Paper III, when undifferentiated cells were proliferated and differentiated in the bioreactor, may not have been present in Paper IV and V, where cryopreserved

HepaRG cells were inoculated at high density. The co-culture of the two cell types in 3D may be important for the functionality of the HepaRG cells, which should be further investigated.

In addition to the evaluation of the retained hepatic function in the bioreactors, the prediction of DDIs was investigated. 3D cultured HepaRG cells predicted well the P450 inhibition and induction observed *in vivo*. Further, OATP1B1 inhibition and CYP3A4 autoinduction were observed in both fresh human hepatocytes and in HepaRG cells cultured in the bioreactor in Paper V.

However, there are other promising 3D culture systems, besides the hollow fiber bioreactor investigated in this thesis, which retain important hepatic functions for drug metabolism and toxicity investigations (Domansky *et al.*, 2010; Wang *et al.*, 2010; Leite *et al.*, 2011; Tostões *et al.*, 2011).

Tostões *et al.* (2011) showed that alginate encapsulated primary rat hepatocyte spheroids cultured in controlled stirred bioreactors with perfusion and constant pH, temperature, and oxygen levels maintained urea, albumin synthesis and P450 activity for 3 weeks. The same culture system was used to culture primary human hepatocytes, which maintained gene expression of phase I and phase II drug metabolizing enzymes as well as albumin and urea synthesis for two weeks, although the urea synthesis was decreased with time (Tostões *et al.*, 2012). Further, immunostaining showed that CYP3A enzymes and bile canaliculi function were retained in human hepatocyte spheroids after 2 weeks culture (Tostões *et al.*, 2012). Leite *et al.* (2011) showed that co-cultures of freshly isolated rat hepatocytes with mouse embryonic fibroblast in spheroids in bioreactors improved the albumin secretion rate and the phase I and phase II enzymatic activities compared to monocultures of hepatocytes in an identical bioreactor.

Furthermore, Domansky and co-workers (2010) described a perfused liver cell culture in a multiwell plate format suitable for higher throughput applications. Immunostaining of the formed 3D microtissue units showed albumin in rat hepatocytes on day 7 and the presence of Kupffer cells and stellate cells on day 13. Further, the retention of the liver sinusoidal endothelial cell phenotype up to day 13 was dependent on the flow rate and the oxygen concentration in the perfused multiwell system.



In addition to the 3D systems reported in the literature, there are a number of companies that have developed liver 3D systems, which are commercially available. Cellasic<sup>(1)</sup>, a company in California, present a high throughput microfluidic perfusion array capable of maintaining liver-specific activity in cultured primary hepatocytes for over 12 days after plating. Microfabricated “endothelial-like” barriers are separating the cords of cultured hepatocyte from the continuous medium flow in micro-capillary channels and the P450 activity and inducibility were maintained over time. (Lee *et al.*, 2010, Poster<sup>(2)</sup>).

Moreover, InSphero<sup>(3)</sup>, a company in Switzerland, offer a static 3D rat liver microtissue (rLiMT) system with hanging drop formations, which retains albumin secretion and CYP3A4 induction up to three weeks. Moreover, immunofluorescence imaging of the hanging drop showed bile canalicular networks. In addition, co-culture of primary rat hepatocytes with Kupffer cell enabled the detection of a toxicological effect in lipopolysaccharides (LPS)-treated cultures. Another static 3D system, that offers functional human liver tissue over a long period of time, is provided by Regenemed<sup>(4)</sup>. Co-cultures of liver cells are grown in a transwell system and long term toxicity can be assessed.

Some of the 3D *in vitro* liver systems available today for culture of primary hepatocyte provide a controlled environment, oxygen supply, a perfused 3D culture, cell-cell contact and co-culture with other cell types. Parameters that seem to be important for the retention of urea and albumin synthesis, phase I and phase II activities, *in vivo*-like tissue formation and detection of drug toxicity. However, the localization and function of important drug transporters need further investigations and validation of the 3D systems to predict DDIs and toxicity is still required.

The 3D bioreactor used in this thesis, together with other available 3D culture systems, open up for long-term cultures required for detection of metabolites from slowly metabolized drugs as well as induction, DDI and toxicity investigations. Hopefully, the use of liver 3D culture system within the pharmaceutical research will contribute to a more effective drug development program, which results in safer drugs for the patients.

(1) [www.cellasic.com](http://www.cellasic.com)

(2) Lee P, Allen M, and Hung P. (2010) Microfluidic Hepatocyte Array for Long Term Drug Exposure Screening.

(3) [www.insphero.com](http://www.insphero.com)

(4) [www.regenemed.com](http://www.regenemed.com)



## 6 CONCLUSIONS

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In this thesis, the presence and stability of important hepatic functions of the human liver were evaluated in HepaRG cells and in primary human hepatocytes using both suspension and a dynamic three-dimensional bioreactor system. In addition, *in vivo* relevant drug-drug interaction predictions in the bioreactor and in P-gp and MRP2 Caco-2 knockdown cells were investigated. The following conclusions can be drawn from the results:

- P450, UGT and OATP1B1 activities were maintained for at least one week in fresh human hepatocyte cultured in bioreactors.
- CYP1A1/2, CYP2B6, CYP2C9 and CYP3A4 activities were retained in HepaRG bioreactors over several weeks allowing the performance of long term, sequential studies using the same system.
- Effects of rifampicin and ketoconazole on P450 activities in HepaRG bioreactor predicted well the effects observed *in vivo*.
- UGT activity was present in cryopreserved differentiated HepaRG cell suspension and retained for at least one week in HepaRG bioreactor.
- The proportion between relevant hydroxylation and glucuronidation biotransformation pathways of the two model substrates in Paper IV was clearly different in HepaRG cells compared to hepatocytes. The glucuronidation pathways were favoured in HepaRG cells, whereas the hydroxylation pathways were favoured in primary human hepatocytes.
- OATP1B1 protein expression and activity were lower in HepaRG cells compared to primary human hepatocytes.
- Immunohistochemical characterization of fresh human liver cells and HepaRG cells cultivated in bioreactors over two weeks showed formation of tissue-like structures and a co-culture of hepatocytes and biliary cells. The hepatocytes were polarized and resembled the histology of human liver tissue.

- Knockdown of drug transporters using shRNA is a valuable tool to predict potential sites of transporter-mediated pharmacokinetic interactions and the involvement of hepatic transporters in drug disposition.
- The 3D model is a compelling feature, which open up for long-term cultures required for detection of metabolites from slowly metabolized drugs as well as induction, DDI and toxicity investigations.

## 7 POPULÄRVETENSKAPLIG SAMMANFATTNING

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Syftet med läkemedel är att förbättra hälsa och öka överlevnad hos patienter världen över. Läkemedel kan dock ge biverkningar, toxiska effekter samt för låg terapeutisk effekt, särskilt när patienter tar många läkemedel samtidigt. Kroppen har en skyddsmekanism för att avlägsna främmande och potentiellt giftiga ämnen, där levern, kroppens reningsverk, utgör en viktig roll. Det är samspelet mellan läkemedelstransportörer och metaboliserande enzymer i levern, tarmen, njurarna och i andra vävnader som möjliggör att läkemedel tas upp, omvandlas eller bryts ner (metaboliseras) till mer lösliga produkter som lättare utsöndras i galla och urin. Många läkemedel transporteras av samma transportörer och metaboliseras av samma enzymer, vilket kan orsaka läkemedelsinteraktioner när en patient får två eller fler läkemedel samtidigt. Interaktioner inträffar exempelvis när kroppen bryter ned två läkemedel på samma sätt med hjälp av samma leverenzym eller när flera läkemedel utsöndras via samma transportör, vilket kan medföra att koncentrationer av läkemedlet blir för höga i blod och vävnader. För höga läkemedelskoncentrationer kan leda till biverkningar och toxicitet. För att förutsäga och studera samspelet mellan dessa komplicerade processer behövs ett testsystem som bevarar leverns funktioner. Eftersom läkemedelsmetabolism skiljer sig åt mellan djur och människor är det viktigt att använda humana celler. Problemet är att leverceller förlorar sin förmåga att metabolisera och transportera läkemedel inom några få timmar eller få dagar efter det att levercellerna har överförts från sin naturliga miljö i levern till en odlingsplatta. Läkemedelstester i leverceller som har minskad eller förlorad funktion kan leda till felaktiga slutsatser som kan medföra risker för patienter som tar läkemedlet samt orsaka att läkemedelsprojekt stoppas sent i utvecklingen eller dras tillbaka från marknaden.

En idé för att bevara levercellernas funktioner är att odla cellerna i en miljö som efterliknar levern så mycket som möjligt. I mitt projekt har vi odlat humana primära leverceller och en levercellinje (HepaRG) i en bioreaktor som möjliggör en tredimensionell odling. Bioreaktorn är uppbyggd av kapillärer där medium och luft cirkulerar för att förse cellerna, som är placerade mellan kapillärerna, med energi och syre, samt transportera bort slaggprodukter. Mellan kapillärerna bygger cellerna upp en vävnadslig struktur. Cellerna får då den viktiga cell-cell kontakten som behövs för att bevara viktiga funktioner och uttrycker läkemedelstransportörer i cellmembranen. Vi har sett att levercellerna i bioreaktorn transporterar och metaboliserar läkemedel i minst en vecka till skillnad från några timmar till några dagar i odlingsplattor. Det var också möjligt att förutse läkemedelsinteraktioner som tidigare upptäckts när patienter tagit flera läkemedel samtidigt. Bioreaktorn öppnar upp för långtidsstudier av långsamt metaboliserande läkemedel som kräver långa inkubationstider samt studier av induktion, läkemedelsinteraktioner och kronisk toxicitet, vilket kan bidra med viktig information för att kunna utveckla ett säkert läkemedel som används på rätt sätt.



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