Studies of hepatic drug transport and metabolism in different model systems

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Oslo, December 2013
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LIST OF PAPERS

I. **Ulvestad M**, Björquist P, Molden E, Asberg A, Andersson TB. OATP1B1/1B3 activity in plated primary human hepatocytes over time in culture. *Biochem Pharmacol.* 2011; 82(9): 1219-26.

II. **Ulvestad M**, Darnell M, Molden E, Ellis E, Åsberg A, Andersson TB. Evaluation of organic anion-transporting polypeptide 1B1 and CYP3A4 activities in primary human hepatocytes and HepaRG cells cultured in a dynamic three-dimensional bioreactor system. *J Pharmacol Exp Ther* 2012; 343(1): 145-56.

III. **Ulvestad M**, Nordell P, Asplund A, Rehnström M, Karlsson SJ, Holmgren G, Davidson L, Brolén G, Edsbagge J, Björquist P, Küppers-Munther B, Andersson TB. Drug metabolizing enzymes and transporter protein profiles of hepatocytes derived from human embryonic and induced pluripotent stem cells. *Biochem Pharmacol*. 2013; 86(5):691-702.

IV. **Ulvestad M**, Skottheim IB, Jakobsen GS, Bremer S, Molden E, Asberg A, Hjelmesæth J, Andersson TB, Sandbu R, Christensen H. Impact of OATP1B1, MDR1, and CYP3A4 Expression in Liver and Intestine on Interpatient Pharmacokinetic Variability of Atorvastatin in Obese Subjects. *Clin Pharmacol Ther* 2013; 93(3): 275-82.

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ABC	ATP-binding cassette
ADR	Adverse drug reaction
ASBT	Apical sodium-dependent bile acid transporter
AUC	Area under the concentration versus time curve
BCRP	Breast cancer resistance protein
BMI	Body mass index
BSEP	Bile salt export pump
CL	Clearance
CL/F	Oral clearance
$CL_{act,efflux}$	Intrinsic clearance of the transporter-mediated sinusoidal efflux process
$CL_{act,uptake}$	Intrinsic clearance of the transporter-mediated basolateral uptake process
$CL_{int,bile}$	Intrinsic clearance of the biliary efflux process
$CL_{int,m}$	Metabolic intrinsic clearance
CL_{diff}	Intrinsic clearance of the passive diffusion process
CL_{int}	Intrinsic clearance
$CL_{int,all,h}$	Overall hepatic intrinsic clearance
$CL_{int,h}$	The sum of biliary efflux intrinsic clearance and metabolic intrinsic clearance
CYP	Cytochrome P450
DDI	Drug-drug interaction
E3S	Estrone-3-sulfate
E17βG	Estradiol-17β-D-glucuronide
GST	Glutathione-S-transferase
hESC	Human embryonic stem cell
hESC-Hep	Human embryonic stem cell-derived hepatocytes
hiPSC	Human induced pluripotent stem cell
hiPSC-Hep	Human induced pluripotent stem cell-derived hepatocytes
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
K_m	Michaelis constant
k_{el}	Elimination rate
MATE	Multidrug and toxin extrusion

MCT	Monocarboxylic acid transporter
MDR	Multidrug resistance protein
MDCK	Madin-Darby canine kidney
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
NME	New molecular entity
NTCP	Sodium-taurocholate cotransporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OST	Organic solute transporter
PEPT	Peptide transporter
P-gp	P-glycoprotein
$\mathbf{PS_{inf}}$	Intrinsic clearance of the total basolateral uptake process
$\mathbf{PS_{eff}}$	Intrinsic clearance of the total sinusoidal efflux process
SCHH	Sandwich-cultured human hepatocytes
SLC	Solute carrier
SNP	Single nucleotide polymorphism
UGT	Uridine diphospho-glucuronosyltransferase
$\mathbf{U_{active}}$	Active transporter-mediated uptake
$\mathbf{U_{passive}}$	Uptake by passive diffusion
$\mathbf{U_{total}}$	Total uptake of a compound across a membrane
$\mathbf{V_0}$	Initial velocity
$\mathbf{V_{0\_active}}$	Initial velocity of the active transporter-mediated uptake
$\mathbf{V_{0\_passive}}$	Initial velocity of the passive diffusion
$\mathbf{V_{0\_total}}$	Initial velocity of the total transport including active and passive transport
$\mathbf{V_d}$	Volume of distribution
$\mathbf{V_{max}}$	Maximum velocity

ABSTRACT

Individual differences in pharmacokinetics may cause extensive variability in drug efficacy, toxicity and adverse drug reactions, and represent a major concern in drug development. The overall aim of this thesis was to evaluate the hepatic transport and metabolism in *in vitro* models used in assessments of drug pharmacokinetics and toxicity, and to investigate the contribution of membrane transporters and metabolizing enzymes to *in vivo* pharmacokinetic variability.

In plated primary human hepatocytes, uptake kinetics studies of OATP1B1/1B3-mediated transport showed an extensive and variable decrease in OATP1B1/1B3 activity and increased passive diffusion over time in two-dimensional (2D) culture. In three-dimensional (3D) bioreactor cultures of primary human hepatocytes, OATP1B1 activity was observed for at least 7 days, while CYP3A4 activity was observed at day 3 and 4 in culture. The activity data were in agreement with immunohistochemical stainings which showed OATP1B1 and CYP3A4 protein expression for at least 9 days in culture. In bioreactor cultures of differentiated HepaRG cells, the observed CYP3A4 activity was comparable to primary human hepatocytes, while OATP1B1 activity could not be detected later than day 2. In 2D cultures of hepatocytes derived from human embryonic and induced pluripotent stem cells, OATP1B1 and CYP activities were very low compared to plated cryopreserved human hepatocytes, but moderate activity of the hepatic transporters NTCP and BSEP was observed. Finally, an *in vivo* study investigating the relationship between expression of OATP1B1, MDR1 and CYP3A4 and the pharmacokinetics of atorvastatin in 21 obese patients with paired biopsies from liver and intestinal segments showed a significant positive correlation between OATP1B1 expression and oral clearance (CL/F) of atorvastatin, while no association was observed with CYP3A4 or MDR1.

In conclusion, plated primary human hepatocytes are a useful *in vitro* model for OATP1B1/1B3-mediated uptake studies, but only for a restricted period of time in culture. The preserved OATP1B1 and CYP3A4 activity in bioreactor cultures of primary human hepatocytes allows long-term *in vitro* studies of hepatic drug clearance and toxicity in this system. Differentiated HepaRG cells cultured in the same 3D system represents a useful *in vitro* tool for long-term studies of slowly metabolized drugs, but the low OATP1B1 activity is a major limitation of this model compared to human hepatocytes. Furthermore, stem-cell

derived human hepatocytes represent a potential alternative to human hepatocytes, but additional refinements of the derivation process are required in order to obtain fully functional human hepatocytes applicable in drug disposition and metabolism studies *in vitro*. Finally, the *in vivo* study shows that uptake transporters could be more important than metabolizing enzymes for the pharmacokinetic variability of certain drugs.

1 INTRODUCTION

1.1 Drug development and adverse drug reactions

In drug development, the goal is to improve health and survival of patients through development of safe and efficient drugs for treatment or prevention of disease. In addition to the positive health aspect, development of agents with limited or no susceptibility of inducing adverse drug reactions (ADRs) might have a direct impact on market penetration and risk of market withdrawal.

Interpatient variability in drug response, including drug efficacy, ADRs and toxicity, is a major concern in drug development. Approximately 90% of the new molecular entities (NMEs) that enter clinical trials fail due to inadequate safety, unfavourable pharmacokinetic profile or limited efficacy.[1] Furthermore, despite the comprehensive pre-clinical and clinical investigations of NMEs, 34 drugs were withdrawn from the market between 1990 and 2005 due to safety concerns, of which the majority was due to hepatotoxicity and pharmacokinetic drug-drug interactions (DDIs).[2] For example, troglitazone, an antidiabetic and anti-inflammatory drug, was withdrawn from the market due to hepatotoxicity.[3,4] Mibefradil, a calcium channel blocker used in the treatment of hypertension and chronic angina pectoris, showed great efficacy in monotherapy, but was withdrawn from the market due to its potent inhibition of drug-metabolizing enzymes. Combined use of mibefradil substantially increased plasma concentrations and toxicity risk of a variety of drugs such as beta blockers, digoxin, terfenadine, cyclosporine, tacrolimus and simvastatin.[5-10] Furthermore, co-administration of terfenadine, a non-sedating antihistamine, and drugs such as macrolide antibiotics and imidazole antifungals caused QT interval prolongation and subsequent cardiac arrhythmia in patients, which led to withdrawal of terfenadine.[11-14] Moreover, the lipid-lowering agent cerivastatin was withdrawn from the market due to reports of fatal rhabdomyolysis both after monotherapy and after co-administration of certain drugs, e.g. gemfibrozil.[15]

The withdrawal of these drugs around the turn of the century emphasized the importance of determining the pharmacokinetic profiles and potential toxicity of NMEs. Most importantly, this knowledge is essential to ensure patient safety and optimal drug therapy. Moreover, withdrawal of drugs from the market, as well as attrition of drugs during the clinical phase of drug development, has enormous economic implications for the pharmaceutical industry. A

single clinical trial can cost up to $100 million, and for every new drug approved, the costs for discovering, developing and launching a new drug, along with the prospective drugs that fail, range between $4 billion and $12 billion per drug.[16] Consequently, reducing late-stage drug attrition and avoiding withdrawal of drugs from the market are of great value in drug development.

To predict pharmacological complications potentially resulting in ADRs, determination of the pharmacokinetic profile and potential toxicity of new drug candidates is important at an early stage of drug development when determining whether a compound should be included in further clinical trials, and if so, which pharmacokinetic studies that should be performed. Furthermore, individualized drug treatment is a visionary goal of modern medicine, and in order to treat each patient with an optimal drug at an optimal dose, a detailed understanding of pharmacokinetic processes underlying the variability in drug response is required. Such knowledge requires robust and human relevant *in vitro* models for assessment of pharmacokinetic profiles and toxicity of new drug candidates as well as drugs on the market.

1.2 Pharmacokinetic variability

Variability in pharmacokinetics, which refers to the processes of drug absorption, distribution, metabolism and excretion, is a major cause of interpatient variability in drug response. Factors associated with pharmacokinetic variability are for example age, weight, body mass index (BMI), organ function, disease state and protein binding. Furthermore, interindividual variability in the expression or activity of membrane transporters and metabolizing enzymes may impact the pharmacokinetic profile of drugs interacting with the current protein(s). Variability in protein expression and activity could be determined by genetic factors, primarily polymorphisms in the gene encoding the protein, or by environmental factors, i.e. foods, pollutions and drugs. Co-administration of drugs or other xenobiotics affecting the same disposition pathway may impact the pharmacological profile of these substances through transport and/or enzyme inhibition or by induced expression of certain transporters and/or enzymes.

1.2.1 Drug transport

Passive diffusion through the membrane has been viewed as dominant in the disposition of most drugs, but it is now well recognized that carrier-mediated transport has a significant

impact on drug absorption, distribution and excretion.[17] Drug transporters are membrane proteins expressed in several tissues throughout the body including the intestine, liver, kidney and brain, where they facilitate the transport of compounds in or out of cells. Indirectly, by facilitating transport into intestine and liver cells, membrane transporters also affect drug metabolism.

Two major gene superfamilies play a prominent role in the transport of drugs across biological membranes: the solute carrier (SLC) superfamily and the ATP-binding cassette (ABC) superfamily.[17] The members of the SLC superfamily are facilitated transporters or ion-coupled secondary transporters, frequently associated with uptake of compounds from the blood into tissues or organs such as the liver and kidney, or in the absorption from the gastrointestinal tract into the circulation. The SLC superfamily include 48 subfamilies of which the most investigated are the organic anion transporting polypeptide (OATP, *SLCO*) family and the organic cation transporters (OCT) and organic anion transporters (OAT) included in the *SLC22* subfamily, as well as the more recently identified multidrug and toxin extrusion (MATE, *SLC47A*) family which is involved in drug excretion from the kidney and liver.[17]

The human OATP family are encoded by the *SLCO* genes and consists of 12 members.[18,19] The OATPs are expressed in multiple tissues including the intestine, liver, kidney and brain, where they mediate the transport of a wide variety of substrates, e.g. bile salts, hormones and steroid conjugates.[20-23] The OATP-mediated uptake is pH dependent and generally accompanied by bicarbonate efflux.[24] OATP1B1 and OATP1B3 belong to the OATP1B subfamily and are expressed predominantly in the basolateral membrane of human hepatocytes[20,21,25] where they serve as bidirectional facilitated diffusion transporters.[26] OATP1B1 and OATP1B3 have an overlapping substrate spectrum and play a key role in the hepatic uptake of many drugs, e.g. HMG-CoA reductase inhibitors (statins), angiotensin II receptor antagonists, angiotensin-converting enzyme inhibitor and anticancer agents.[23,27-31] Several OATP1B1/1B3-mediated DDIs involving the agents mentioned above have been reported,[32-36] e.g. substantially increased statin exposure and increased risk of toxicity during co-administration of the OATP1B1/1B3 inhibitor cyclosporine A.[37,38] Furthermore, several single nucleotide polymorphisms (SNPs) and haplotypes of *SLCO1B1*, the gene encoding OATP1B1, have been associated with altered transport activity of OATP1B1.[39-42] Individuals

carrying the c.521T>C allele (existing in haplotypes **5* and **15*) have shown impaired hepatic uptake and markedly increased plasma concentrations of OATP1B1 substrates,[43-49] e.g. pravastatin and rosuvastatin. Increased statin plasma concentration enhances the risk of statin-induced myopathy in patients carrying these specific alleles. Although the relatively low frequencies of haplotypes **5* and **15* (2% and 16% in Caucasians, respectively)[50], these variants may have an effect in many individuals due to the large number of patients on statin treatment.

The members of the ABC superfamily are primary active efflux transporters using energy released from ATP hydrolysis to transport substrates out of cells.[51] The ABC superfamily is divided into 7 subfamilies named ABCA to ABCG. The most important proteins involved in drug transport are multidrug resistance protein 1 (MDR1, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*), as well as members of the multidrug resistance-associated protein (MRP, *ABCC*) family.[17]

MDR1 (P-glycoprotein, P-gp) is a well-known membrane transporter expressed in several human tissues including the luminal membrane of the small intestine and blood-brain barrier, and the apical membrane of hepatocytes and kidney proximal tubule epithelia.[52,53] The tissue distribution and broad substrate specificity indicate that MDR1 plays a major role in protecting the body against xenobiotics, namely by excretion of cytotoxic agent into the gastrointestinal tract, bile and urine, as well as participating in the function of the blood-brain barrier and hence protecting the central nervous system. In the intestine, MDR1 can affect the absorption of drugs such as digoxin and paclitaxel by transporting them back into the lumen,[54,55] while in the liver, MDR1 is responsible for the biliary efflux of several drugs, e.g. statins.[56] Several MDR1-mediated DDIs have been reported, e.g. increased plasma concentrations and/or reduced clearance of digoxin during co-administration of the MDR1 inhibitors quinidine and ritonavir.[57-59] A number of different SNPs have been identified in the *ABCB1* gene, but inconsistent results have been reported with regard to their effect on MDR1 phenotype and drug pharmacokinetics of MDR1 substrates.[60] With regard to atorvastatin, minor effects have been observed in patients carrying the *ABCB1* haplotypes c.1236T-c.2677T-c.3435T and c.1236C-c.2677G-c.3535C carriers (55% greater area under the concentration versus time curve (AUC) in TTT/TTT individuals compared to CGC/CGC individuals[61]). However, Niemi (2010) states that given the high allele frequencies of the TTT

and CGC haplotypes (34% and 43% in Caucasians, respectively[61]), these haplotypes might play some role in the variability of atorvastatin pharmacokinetics at the population level.[62]

Table 1. Examples of substrates, inhibitors and inducers of membrane transporters involved in drug transport.

Transporter (*gene*)	**Substrates**	**Inhibitors**	**Inducers**
OATP1B1 (*SLCO1B1*)	Estradiol-17β-D-glucuronide*, estrone-3-sulfate*, bosentan, enalapril, methotrexate, repaglinide, statins, valsartan	Estrone-3-sulfate[a], rifampicin, cyclosporine A, ritonavir	Rifampicin
OATP1B3 (*SLCO1B3*)	Estradiol-17β-D-glucuronide*, cholecystokinin 8*, digoxin, fexofenadine, statins, valsartan	Estrone-3-sulfate[a], cyclosporine A, rifampicin, ritonavir	Chenodeoxycholic acid
MDR1, P-gp (*ABCB1*)	Digoxin*, atorvastatin, fexofenadine, indinavir, loperamide, paclitaxel, vincristine	Cyclosporine A, quinidine, rifampicin, verapamil	Rifampicin*, carbamazepine, phenytoin, ritonavir
NTCP (*SLC10A1*)	Taurocholate*, estrone-3-sulfate, micafungin, rosuvastatin	Bromosulfophthalein, cyclosporine A, furosemide, ritonavir, rifampicin, propanolol	Dexamethasone
BSEP (*ABCB11*)	Taurocholate*, micafungin, pravastatin, vinblastine	Ritonavir, cyclosporine A, rifampicin, troglitazone, bosentan, glibenclamide, vinblastine	Chenodeoxycholic acid

Adapted from[17, 67-71].

*) Often used as probe agents in phenotyping of the respective transporters

[a]) Selective OATP1B1 inhibitor, compared to OATP1B3, at 30 μM[67]

Abbreviations: BSEP, bile salt export pump; MDR, multidrug resistance protein; NTCP, sodium-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein

Vectorial transport is asymmetrical transport across a monolayer of polarized cells, and is important in the transfer of endogenous substances and xenobiotics across epithelial or endothelial barriers, e.g. in the intestinal absorption and in the hepatobiliary and urinary excretion of drugs from the blood to the lumen. The sodium taurocholate cotransporting polypeptide (NTCP, *SLC10A1*), a member of the SLC superfamily, is expressed predominantly in the basolateral membrane of human hepatocytes and is generally known as the key transporter for hepatic uptake of bile salts.[63] The ABC transporter bile salt export

pump (BSEP, *ABCB11*) is expressed in the canalicular membrane of human hepatocytes where it mediates the biliary secretion of bile salts.[64] Together, NCTP and BSEP mediate the hepatobiliary excretion of bile salts, and hence, drug inhibition of these transporters may cause cholestasis. As discussed previously, troglitazone was withdrawn from the market due to hepatotoxicity. Troglitazone sulphate has been reported to inhibit BSEP-mediated efflux of taurocholate, suggesting that troglitazone sulphate induces cholestasis by inhibition of BSEP.[65,66]

Drug transport kinetics

Drugs cross cell membranes either by passive or active transporter-mediated processes. The total transport of a compound into the cell, U_{total}, can be expressed as:

$$U_{total} = U_{active} + U_{passive} \qquad (1)$$

, where U_{active} and $U_{passive}$ represent the active transporter-mediated uptake and the passive diffusion of the compound across the membrane, respectively. Active transporter-mediated uptake is saturable, and the Michaelis-Menten model can be applied to evaluate the uptake kinetics of carrier-mediated drug transport through membranes. The initial uptake rate of the transporter-mediated uptake, V_{0_active}, at a given substrate concentration [S], is given by the following equation:

$$V_{0,active} = \frac{V_{max} \times [S]}{K_m + [S]} \qquad (2)$$

, where V_{max} is the maximum velocity of the transport process and K_m is the Michaelis constant. The uptake kinetics of an actively transported compound following the Michaelis Menten kinetics is illustrated in Figure 1.

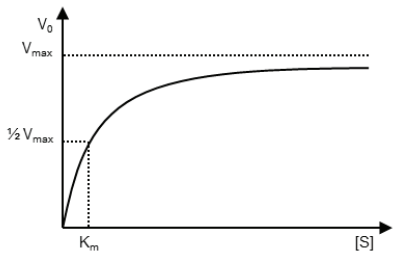

Figure 1 The uptake kinetics of an actively transported compound following the Michaelis-Menten kinetics. K_m, Michaelis constant; [S], substrate concentration; V_0, initial uptake rate; V_{max}, maximum velocity.

The passive diffusion of a compound across a membrane is a non-saturable process, and the initial velocity of the unidirectional passive diffusion, $V_{0\_passive}$, at a given substrate concentration [S], is expressed as:

$$V_{0,passive} = P \times [S] \qquad (3)$$

, where P represents a constant describing the passive diffusion. Combining equation 1, 2 and 3 gives an expression of the total initial velocity, V_{0_total}, of a transport process, including active and passive transport, at a given substrate concentration [S]:

$$V_{0,total} = \frac{V_{max} \times [S]}{K_m + [S]} + P \times [S] \qquad (4)$$

This is the extended Michaelis-Menten equation, which can be applied to evaluate uptake of compounds into whole cells or expression systems (the conventional two-step approach).[72,73] Characterization of concentration-dependent uptake is performed under initial rate conditions and in the time-linear range. Equations 2, 3 and 4 (the active, passive and total uptake, respectively) are illustrated in Figure 2 for compounds exhibiting low and high passive diffusion compared to active transporter-mediated uptake.

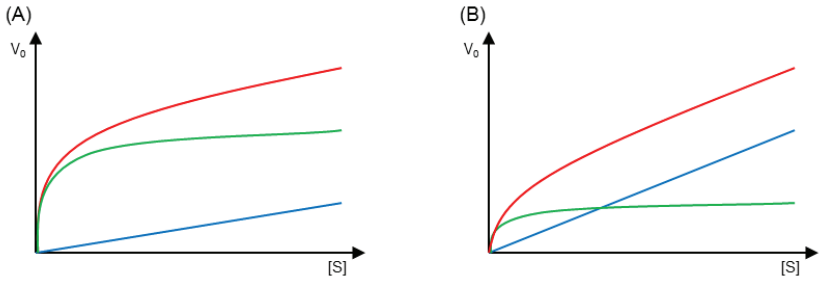

Figure 2 Total (red line), active (green line) and passive (blue line) uptake of a drug exhibiting low passive diffusion (A) and high passive diffusion (B). [S], substrate concentration; V_0, initial uptake rate.

The intrinsic clearance of the transporter-mediated uptake process, $CL_{act,uptake}$, is defined as the capacity of a membrane transporter to mediate the active transport of a compound across the membrane, and is calculated by the following equation:

$$CL_{act,uptake} = \frac{V_{max}}{K_m}$$

, where V_{max} is the maximum velocity of the transport process and K_m is the Michaelis constant, assuming first order kinetics.

1.2.2 Drug metabolism

In drug metabolism, drugs are chemically altered to more hydrophilic chemicals to facilitate their elimination from the body. Most drugs are hydrophobic compounds that, in the absence of metabolism, would not be efficiently eliminated, and thus would accumulate in the body, potentially causing toxicity. Drug-metabolizing reactions are classified into phase I and phase II. Phase I reactions involves oxidation, reduction or hydrolysis of the drug, and are primarily mediated by the cytochrome P450 (CYP) family of enzymes. Phase II reactions involve covalently binding of an endogenous compound, most often glucuronide acid, glutathione or sulphate, to the phase I metabolite. Examples of phase II enzymes are uridine diphospho-glucuronosyltransferases (UGTs) and glutathione-S-transferases (GSTs). The phase II conjugation produces a more polar metabolite and promotes excretion of the drug from the tissue, normally via efflux transporters.

The CYP enzymes responsible for metabolism of xenobiotics are expressed primarily in the liver and intestines, and to less extend in the lung, kidney and central nervous system. CYP enzymes are located in the endoplasmic reticulum of cells where they carry out the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent oxidation of a diversity of substrates. The superfamily of CYP enzymes comprises 57 genes which have been organized into families (denoted by the first identification number, e.g. CYP3) and subfamilies (denoted by letters, e.g. CYP3A).[74] The individual isoenzymes within each subfamily are further denoted by numbers, e.g. CYP3A4. CYP enzymes are in general promiscuous in their capacity to bind and metabolite substrates, and thus, there is significant overlapping substrate specificity among CYP enzymes. The human isoenzymes CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 are considered of particular importance in drug metabolism.[75]

Estimates suggest that the metabolism of approximately 40-50% of all drugs on the market involves CYP3A-mediated oxidation.[76] For the majority of individuals, the human CYP3A isoform CYP3A4 are the most abundant CYP enzyme expressed in the liver and intestine,[77-80] and the main drug-metabolizing enzyme in human. Consequently, a large number of clinical DDIs involving CYP3A4 agents have been reported,[81] e.g. significantly increased plasma concentration of cyclosporine A in the presence of the CYP3A4 inhibitor ketoconazole,[82,83] or significantly reduced plasma concentrations of midazolam in the presence of the potent

CYP3A4 inducer rifampicin.[84,85] Although CYP3A4 expression and activity is characterized by large interindividual variability, the contribution of genetic factors has remained unclear.[79, 86-88] Several SNPs in the gene encoding CYP3A4 have been reported, but no clear association between SNPs and altered phenotype has been shown. However, recently, an SNP (rs35599367, C>T) with corresponding allele name *CYP3A4*22* (frequency of 5-7% in Caucasian) was discovered.[89] Carriers of the *CYP3A4*22* variant allele are associated with reduced hepatic CYP3A4 expression and activity[89-91] and lower dose requirements of CYP3A4 substrates such as certain statins and calcineurin inhibitors compared to *CYP3A4*1/*1* carriers.[91,91-94] The CYP3A isoform CYP3A5 is closely related to CYP3A4 and show significant overlap in substrate specificity, although the substrate affinity may differ.[86] CYP3A5 is a polymorphic protein expressed at significant levels in 10-40% of Caucasians, and may represent as much as 50% of the total CYP3A content in these individuals.[86,95]

Table 2. Examples of substrates, inhibitors and inducers of CYP enzymes important for drug metabolism.

Metabolizing enzyme	Substrates	Inhibitors	Inducers
CYP1A2	Phenacetin*, caffeine, clozapine, naproxen, propranolol, olanzapine, theophylline	Furafylline*, cimetidine, ciprofloxacin, fluvoxamine	Omeprazole*, lanzoprazole*, coffee, phenobarbital, phenytoin, rifampicin, ritonavir,
CYP2B6	Bupropion*, efavirenz, ketamine, sertraline, tramadol	Clopidogrel*, sertraline	Phenobarbital*, phenytoin, rifampicin, ritonavir, statins
CYP2C9	Diclofenac*, tolbutamide*, fluoxetine, ibuprofen, rosuvastatin, valsartan, warfarin	Sulphenazole*, amidorone, fluconazole	Rifampicin*, barbiturates, bosentan, carbamazepine, ritonavir, statins
CYP2C19	Mephenytoin*, fluoxetine*, omeprazole*, amitriptyline, clopidogrel, ranitidine	Ticlopidine*, clopidogrel, fluoxetine, omeprazole	Rifampicin*, artemisinine, barbiturates, carbamazepine, ritonavir
CYP2D6	Bufuralol*, dextromethorphan*, amphetamine, carvediol, codeine, metoprolol, paroxetine	Quinidine*, bupropion, fluoxetine, haloperidol	None identified
CYP3A4/3A5	Midazolam*, testosterone*, atorvastatin, carbamazepine, claritromycin, cyclosporine A, erythromycin, felodipine, tacrolimus, verapamil	Ketoconazole*, itraconazole*, clarithromycin, ritonavir, saquinavir, verapamil,	Rifampicin*, barbiturates, bosentan, carbamazepine, dexamethasone, phenytoin, ritonavir, statins, St. John's wort

Adapted from[70,96].

*) Often used as probe agents in phenotyping of the respective enzymes

Abbreviations: CYP; cytochrome P450

1.3 Transporter and enzyme contribution to pharmacokinetic variability *in vivo*

For the majority of drugs, systemic exposure is determined by an interplay between presystemic transporters and enzymes expressed in the intestine and liver (Figure 3).[97] Furthermore, membrane transporters contribute to the tissue-selective distribution of many drugs, and indirectly affect drug metabolism by controlling the access to metabolizing enzymes in certain organs. The contribution of specific transporters and/or enzymes to drug disposition and clearance is difficult to estimate due to the significant interplay between these proteins. Such estimates are necessary for the prediction of the extent to which variability in activity of certain transporters and/or enzymes will affect drug concentrations in plasma and tissues.

1.3.1 Transporter and enzyme interplay

There is considerable overlap in substrate specificity and tissue distribution among membrane transporters and metabolizing enzymes throughout the body.[97] Recently, a significant substrate overlap between hepatic uptake transporters and enzymes has been recognized,[97] e.g. between CYP3A4 and OATPs.[98,99] However, the interactive nature of CYP3A and MDR is the most extensively studied interplay between enzymes and transporters.[97,100-102] CYP3A and MDR1 act as a coordinated barrier for xenobiotics.[102-104] However, clinical studies have demonstrated that the role of intestinal MDR1 extends beyond simply limiting absorption of the parent drug.[100-102] In the intestine, where the drug enters the enterocytes from the luminal side, MDR1 is controlling the access of the drug to the enzyme through repeated cycles of absorption and efflux, giving CYP3A multiple opportunities to prevent the intact xenobiotic from entering the bloodstream. That is, after penetration into enterocytes, molecules that escape metabolism may be transported back into the lumen via MDR1 or other apical efflux transporters thereby allowing re-entry into enterocytes and increased chance of metabolic conversion by CYP3A.[82,105] In contrast, in the liver, where the drug enters the hepatocytes from the basolateral side and encounters CYP3A prior to MDR1-mediated efflux into the biliary canaliculi, drugs will not re-enter the cells because it would be against a concentration gradient, thus less metabolites are formed and more parent traverses the membrane.

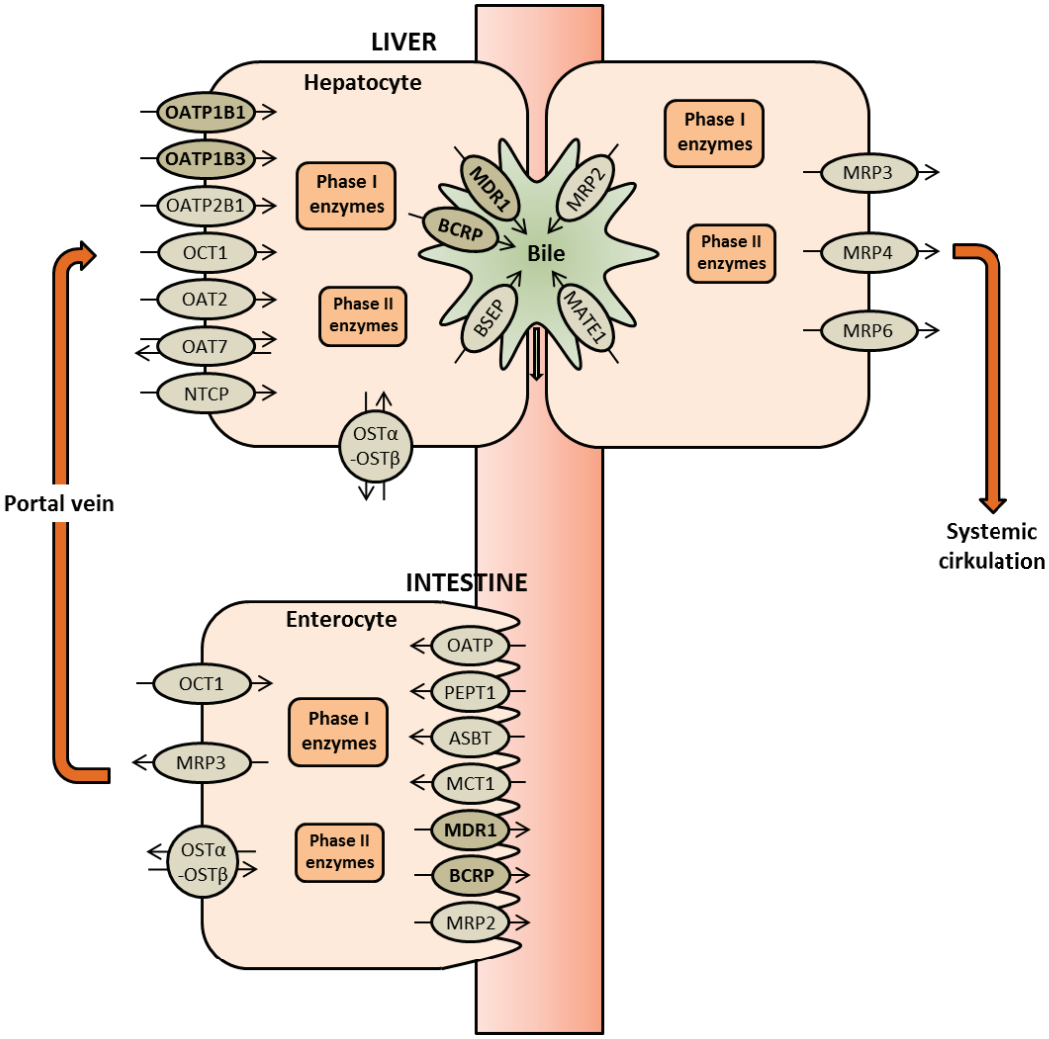

Figure 3. Selected membrane transporters involved in first pass transport of drugs and xenobiotics. The systemic drug exposure is determined by an interplay between these transporters and phase I and II enzymes expressed in the intestine and liver. The membrane transporters outlined in bold text are considered especially important for drug transport.[17] Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; MATE, multidrug and toxin extrusion; MCT, monocarboxylic acid transporter; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NTCP, sodium-taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; PEPT, peptide transporter.

1.3.2 Contribution of transporters and enzymes in hepatic drug clearance

As discussed above, drugs may be substrates of several transporters and/or enzymes which work in concert to eliminate the drugs from the body. Thus, it is of importance to determine which processes that are important for *in vivo* systemic exposure and tissue distribution, and to assess the rate-limiting step(s) in drug elimination. Sugiyama and co-workers have characterized the interplay of enzymes and transporters to understand the importance of parameters that determine the intrinsic drug clearance in the intestine, liver and kidney.[106] The hepatic elimination of drugs is mediated mainly by four intrinsic processes; basolateral uptake into the hepatocytes, sinusoidal efflux from the hepatocytes into the blood, biliary secretion and hepatic metabolism. Thus, the overall hepatic intrinsic clearance, $CL_{int,all,h}$, can be described by the following equation:[73,106-108]

$$CL_{int,all,h} = PS_{inf} \times \frac{CL_{int,h}}{CL_{int,h} + PS_{eff}}$$

,where PS_{inf}, PS_{eff} and $CL_{int,h}$ represent the basolateral uptake intrinsic clearance ($CL_{act,uptake}$ + CL_{diff}), the sinusoidal efflux intrinsic clearance ($CL_{act,efflux}$ + CL_{diff}), and the sum of biliary efflux clearance and metabolic intrinsic clearance ($CL_{int,bile}$ + $CL_{int,m}$), respectively. Depending on the drug, each of these processes can be rate-limiting for the overall hepatic clearance. Theoretically, for highly lipophilic compounds which mainly cross the membrane by passive diffusion, both PS_{inf} and PS_{eff} reflect passive diffusion (CL_{diff}). In this case, PS_{inf} is assumed to be equal to PS_{eff}, and $CL_{int,all,h}$ approximates $CL_{int,h}$. For these compounds, total hepatic clearance can be described by the traditional organ clearance model incorporating blood flow, extend of protein binding and $CL_{int,h}$.[109] However, most anionic drugs, and some hydrophobic organic cations, exhibit poor membrane permeability and require active transport across the cell membrane, both in and out of hepatocytes. For these compounds, $CL_{int,all,h}$ is directly affected by the transporter activity of the uptake transporters (PS_{inf}). Furthermore, when PS_{eff} is negligibly compared to $CL_{int,h}$ ($PS_{eff} << CL_{int,h}$), $CL_{int,all,h}$ approximates PS_{inf}, and a change in $CL_{int,bile}$ or $CL_{int,m}$ does not directly affect the overall hepatic intrinsic clearance. On the other hand, when PS_{eff} is considerably higher than $CL_{int,h}$ ($PS_{eff} >> CL_{int,h}$), all intrinsic processes (PS_{inf}, PS_{eff}, $CL_{int,bile}$ and $CL_{int,m}$) affect the total hepatic intrinsic clearance. For such drugs, variability in both transporter and enzyme activities due to polymorphisms or interactions with co-administrated drugs could cause interindividual variability in drug pharmacokinetics.

Atorvastatin, one of the most prescribed lipid-lowering agents, shows low oral bioavailability and is a substrate for OATP1B1, MDR1 and CYP3A4.[39,110-113] Atorvastatin is administered in the pharmacodynamic active acid form, but is partly interconverted *in vivo* to an inactive lactone metabolite.[111] Although the lactone form is inactive towards HMG-CoA reductase, its systemic levels have been linked as a marker of myotoxicity of atorvastatin treatment.[114-116] Both atorvastatin acid and lactone exhibit highly variable pharmacokinetics.[110,114,117] The acid form is the primary substrate for OATP1B1,[37,56] while the lactone form exhibits higher affinity for CYP3A4.[118] A recent *in vitro* study by Neve *et al.* (2013) showed that CYP3A4-mediated metabolism of atorvastatin acid was dependent on OATP1B1 uptake and influenced by MDR1 efflux, while the metabolism of atorvastatin lactone was not affected by OATP1B1 or MDR1-mediated transport.[119] Given its extensive CYP3A4 metabolism, as well as OATP1B1 and MDR1-mediated transport, atorvastatin is a suitable model drug for investigating the contribution of hepatic and intestinal expression of these proteins to the interindividual variation in drug pharmacokinetics *in vivo*.

1.3.3 Transporter and enzyme interplay in DDIs

Due to the considerable overlap in substrate specificity and tissue distribution, DDIs may involve inhibition and/or induction of several transporters and/or enzymes in multiple tissues at the same time (Figure 3), and the consequences of co-administration of drugs in terms of changes in plasma- and tissue concentrations could be difficult to interpret. Co-administration of rifampicin, which has been reported to induce the expression of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5, MDR1, MRP2, MRP3, MRP4 and OATP1B1 through activation of the nuclear receptor PXR,[68,120] can affect the pharmacokinetic profile of drug substrates of these transporters and enzymes in multiple ways. With regard to inhibition, Niemi *et al*. (2003) showed that AUC of repaglinide, a substrate of both OATP and CYP3A, increased 8.1-fold upon co-administration of the OATP inhibitor gemfibrozil, while a 1.4-fold increase in AUC was observed in the presence of the CYP3A inhibitor itraconazole. However, AUC of repaglinide increased almost 20-fold upon concomitant administration of both gemfibrozil and itraconazole, suggesting that transporter and enzyme interplay may give rise to synergistic inhibitory effects.[121]

1.4 Human *in vitro* liver models for evaluation of drug transport and metabolism

The liver is the most important organ in drug metabolism and excretion, and hepatic CYP enzymes and membrane transport proteins are recognized as major determinants of pharmacokinetic variability of many drugs.[17,75,97] Thus, detailed characterization of the isolated hepatic transport and metabolism processes of NMEs are needed at an early state of drug development, as well as knowledge of the total hepatic drug clearance which reflects the interplay between transporters and metabolizing enzymes. In animals, the expression and function of drug transporters and metabolizing enzymes do not always reflect the situation in human.[122-124] Thus, human liver *in vitro* models expressing functional transporters and enzymes reflecting the hepatic *in vivo* situation are needed for reliable predictions of *in vivo* drug metabolism, disposition and clearance. Furthermore, *in vitro* models with maintained hepatic functions over an extended period of time are desirable to enable studies of potential long-term toxicity. To determine the contribution of single transporters and enzymes to total hepatic clearance and to characterize the mechanism of transporter- or enzyme-mediated DDIs, specific inhibitors are required. Although much effort has been made to identify specific inhibitors for important membrane drug transporters,[98,99,125-128] selective inhibitors have not been identified for most transporters. In these cases, transfected *in vitro* systems over-expressing certain transporters and/or enzymes are probably the best alternative.

1.4.1 Transfected *in vitro* systems

Recombinant transporters that are stably or transiently expressed in cell lines, e.g. HEK293 cells,[129] can be used to determine whether a drug is substrate or inhibitor of a certain transporter. A drug substrate is sensitive (victim) to DDIs, while a drug inhibitor (perpetrator) may affect the kinetic profile of co-administered drugs. Single-transfected cell lines, or *Xenopus leavis* oocytes injected with cDNA encoding an uptake transporter of interest,[23] are mainly used to determine drug interaction with uptake transporters. For the assessment of drug efflux, membrane vesicles prepared from transfected cells are commonly used.[130] Due to the inverted configuration, influx rather than efflux is determined, which enables assessment of substrate or inhibitor interaction with the target efflux transporter. However, hydrophobic compounds exhibit high degree of binding to cell membranes and highly membrane-permeable drugs undergo extensive passive uptake into vesicles masking the active transport, limiting the use of membrane vesicles.

With regard to metabolizing enzymes, human recombinant CYP enzymes expressed in e.g. Escherichia coli or baculovirus infected cells can be used to identify compounds that are substrates or inhibitors of specific CYP enzymes and thus may act as victim or perpetrator drugs in DDIs.[131,132]

Apart from recombinant systems expressing a single transporter or enzyme, double or multiple-transfected cell lines can be used to investigate the interplay between certain transporters and/or enzymes. Double-transfected, polarized cell lines that stably express canalicular and biliary transporters can be used for the assessment of hepatic vectorial transport from the blood to the bile.[122,133,134] Furthermore, Neve *et al*. (2013) recently developed a transfected HEK293 cell line expressing both an uptake transporter (OATP1B1), a metabolizing enzyme (CYP3A4) and an efflux transporter (MDR1), allowing studies of the interplay between these three proteins.[119] Similarly, a stable MDCK cell model has been developed expressing OATP1B1, CYP3A4 and MRP2, as well as the phase II enzyme UGT1A1.[135] Transfected systems may not reflect the relative amount of transporters and enzymes expressed *in vivo*, but combined with quantitative protein analysis of the relevant transporters and enzymes for accurate scaling of the *in vivo* situation,[99] these multiple-transfected models represent new valuable screening tools in drug discovery and development. However, compounds may be substrates of additional transporters or enzymes not expressed in these models, and, in the case of DDIs, undergo disposition or clearance by compensatory pathways if inhibition of the "regular" pathway is present. Thus, multiple-transfected cell lines may not predict the true *in vivo* situation.

1.4.2 Human hepatocytes

Freshly isolated primary human hepatocytes, plated or in suspension, represent the current standard *in vitro* model for evaluation of hepatic drug disposition, metabolism, clearance and toxicity in the pharmaceutical industry.[136] Primary human hepatocytes are derived from intact liver tissue, and, at the time of isolation, these cells express a complete set of enzymes and transporters involved in hepatic drug clearance.[137-141] However, their spontaneous dedifferentiation and loss of enzyme and transporter expression in 2D culture is a major concern and limits its application.[141,142]

Restricted tissue availability and inter-donor variability further limits the utility of fresh primary human hepatocytes. A considerable improvement of hepatocyte cryopreservation protocols, allowing storage, transport, scheduling of experiments and repeated experiments using hepatocytes isolated from the same donors, has been achieved during recent years.[143] Cryopreserved human hepatocytes have been reported to exhibit both CYP, UGT and transporter activities,[137,144-147] and is now a routinely used model in studies of hepatic drug disposition, metabolism, clearance and toxicity.[136] However, as in non-frozen primary human hepatocytes, a rapid loss of enzyme and transporter expression is observed when these cells are cultured in 2D models.[141]

Unfortunately, the polarity of hepatocytes *in vivo* is rapidly lost upon isolation, leading to the inability to assess a potential canalicular efflux. However, the polarity can be regenerated when hepatocytes are cultured in a sandwich configuration between two layers of gelled collagen.[148,149] Hoffmaster *et al.* (2004) reported that the expression and function of several canalicular and biliary transporters are retained in sandwich-cultured human hepatocytes (SCHH),[150] and for some compounds, good correlations have been demonstrated between *in vitro* and *in vivo* biliary clearance.[151] However, the metabolic capacity of this model has been questioned.

1.4.3 HepaRG cells

HepaRG is a human hepatoma cell line derived from a human hepatocellular carcinoma.[152] When seeded at low density, HepaRG cells acquire an elongated undifferentiated morphology, actively proliferating until they reach confluency after approximately ten days.[152,153] HepaRG cells exhibit an epithelial phenotype at an early stage in culture, but after reaching confluency, the bi-potent property allows them to undergo two distinct differentiation programs leading to the formation of typical hepatocyte-like colonies surrounded by epithelial cells, including bile-canalicular structures.[152-154] Maximum cell differentiation is reached after two weeks exposure to dimethyl sulfoxide. The differentiated hepatocyte-like HepaRG colonies exhibit both morphological and functional characteristics of mature hepatocytes, including important functions for drug metabolism and disposition, e.g. activity of CYP and UGT enzymes and polarized expression of certain canalicular and biliary transporters.[155-158] Furthermore, differentiated HepaRG cells have been reported to maintain expression and activity of these enzymes and transporters over several weeks in 2D

culture.[156,159,160] However, the expression levels of hepatic uptake transporters have been reported to be considerably lower in HepaRG cells than in primary human hepatocytes, while the observed expression of efflux transporters are comparable to or higher than in human hepatocytes, with a few exceptions.[156-158,160,161] The lack of donor variability, no limitations by donor tissue availability and less variation in functional activities, especially in CYP activities, are advantages of HepaRG cells compared to primary human hepatocytes, and 2D cultured HepaRG cells have been evaluated as a valuable *in vitro* tool for prediction of CYP induction and CYP inhibition *in vivo*.[162,163] However, the lack of genetic variability is a limitation of this *in vitro* model.

1.4.4 Human pluripotent stem cell-derived hepatocytes

Human embryonic stem cell-derived hepatocytes (hESC-Hep) and human induced pluripotent stem cell-derived hepatocytes (hiPSC-Hep) represent potential alternatives to human primary hepatocytes as *in vitro* liver models. Human embryonic stem cells (hESCs) are cells derived from the inner cell mass of a blastocyst, an early-stage embryo.[164] hESCs are characterized by two distinct properties; their pluripotency and their ability to replicate indefinitely. Pluripotency refers to the ability to differentiate into all three embryonic germ layers, endoderm, ectoderm and mesoderm, and further on, to all somatic germ cells. Research on hESCs is controversial because derivation of hESCs involves destruction of embryos, and is prohibited or restricted in some countries.[165] In 2006, Yamanaka *et al*. (2006) demonstrated that it is possible to reprogram a mature adult cell to the state of an embryonic stem cell by transfection of certain stem cell-associated genes into non-pluripotent cells.[166] These cells are termed induced pluripotent stem cells (iPSCs). In 2007, two independent research teams reported a successful derivation of iPSCs from human adult cells.[167,168] Human iPSCs (hiPSCs) are an important advance in stem cell research as pluripotent stem cells now can be derived from any individual and without the ethically controversial use of embryos.

Because of their plasticity and potentially unlimited capacity for self-renewal, hESCs and hiPSCs present useful tools in both research and regenerative medicine. As mentioned above, both hESCs and hiPSCs have the potential to differentiate into various cell types, and several publications have described the differentiation of hESCs and hiPSCs into hepatocyte-like cells.[169-182] These hepatocyte-like cells display characteristic hepatic morphology and express liver markers such as albumin, hepatic nuclear factor 3β, α_1-antitrypsin, liver fatty acid

binding protein and cytokeratin 18.[169,175,183] Furthermore, the cells accumulate glycogen and secrete albumin and urea, typical features for hepatocytes,[170,171,174,175] and exhibit GST expression and activity that closely resemble that of human hepatocytes.[180] Moreover, expression and activity of certain CYP enzymes have been reported in both hESC-Hep and hiPSC-Hep.[169-172,174-177,179] However, a detailed evaluation of both CYP and transporter activities in these cells is warranted.

The field of human pluripotent stem cell-derived hepatocytes is rapidly taking significant steps forward, and new and more efficient differentiation protocols are constantly being developed. Thus, hopefully, both hESC-Hep and hiPSC-Hep will play important roles in studies of drug metabolism and disposition *in vitro* in the future.

1.4.5 Cell culturing systems

Up until recently, human hepatocytes, as well as HepaRG cells and stem cell-derived hepatocytes, have primarily been cultured in 2D monolayers. However, as mentioned above, a major disadvantage of human hepatocytes in 2D culture is their spontaneous dedifferentiation and rapid loss of enzyme and transporter expression.[141,142] This prevents the possibility of long-term studies which is of particular importance in toxicity testing and in investigations of slowly metabolized drugs. Thus, novel cell culture systems with improved conservation of hepatocyte functions allowing predictive long-term *in vitro* pharmacokinetic or toxicological studies are warranted.

In 1994, Gerlach *et al*. introduced the multicompartment bioreactor technology for dynamic 3D perfusion culture of human liver cells.[184] This technique uses interwoven hollow-fiber capillary membranes that provide independent, decentralized medium and gas exchange to the cells located between the capillaries (Figure 4). When cultured in a perfused 3D bioreactor, human liver cells retain *in vivo*-like properties and are arranged in tissue-like structures.[185-187] Zeilinger *et al*. (2002) showed that liver-specific functions such as urea and albumin synthesis, glucose metabolism and CYP activities were all maintained for at least 14 days in bioreactor culture.[187] Recently, Zeilinger *et al*. (2011) reported preserved activity of CYP enzymes important for drug metabolism for up to 23 days in a miniaturized bioreactor.[188] However, the maintenance of transporter activities in human hepatocytes cultured in this system has not been investigated.

Differentiated HepaRG cells have been reported to exhibit CYP and UGT activities over several weeks in the perfused 3D bioreactor.[162,190] Furthermore, HepaRG cells predicted well the CYP inhibition and induction observed *in vivo*.[162] Although polarity of transporter expression and formation of tissue-like structures including bile canaliculi have been demonstrated using immunocytochemistry,[162] an evaluation of the transporter activities in HepaRG cells cultured in a bioreactor is still warranted.

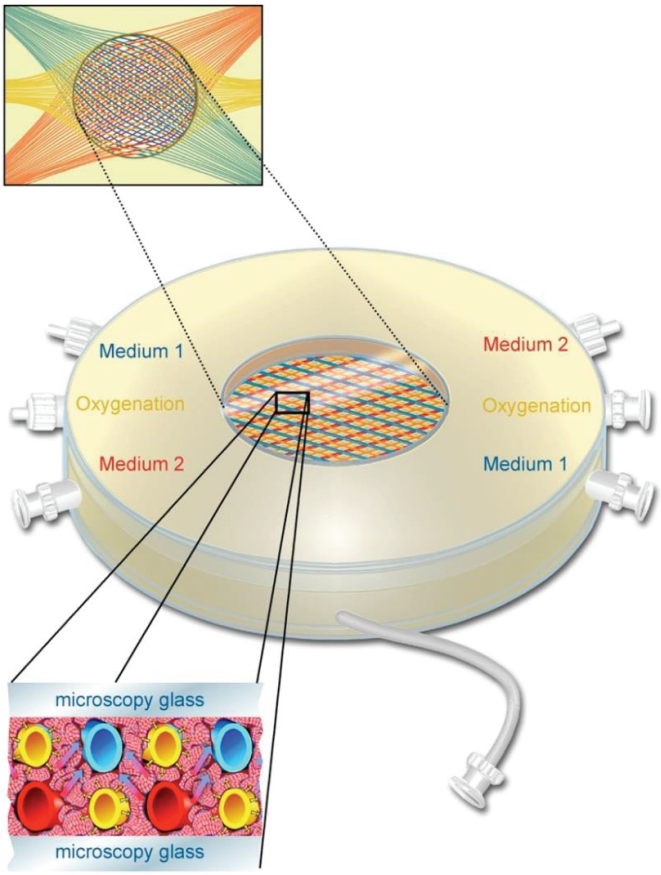

Figure 4. Schematic view of the miniaturized bioreactor. Three independent bundles of hollow fiber membranes are interwoven in two layers, serving for perfusion of the cells cultured in the space between the fibers. The upper panel shows the spatial arrangement of the three independent capillary bundles seen from above. The lower panel depicts a cross-section with cellular clusters among fibers and mass exchange between medium capillaries of different layers.[189] This figure is reproduced from Hoffman *et al*. (2012),[189] with permission from the publisher (John Wiley and Sons).

As mentioned above, hESCs and hiPSCs have the potential to provide a stable and unlimited supply of human hepatocytes, and much effort has been made to differentiate hESCs and hiPSCs toward the hepatic lineage, primarily using 2D cultures systems. Although the results are encouraging, several factors still limit the general use of stem cell-derived hepatocytes in drug discovery, including satisfactory levels of drug metabolizing enzymes.[191] Sivertsson *et al.* (2012) have reported improved hepatic differentiation of hESCs, evaluated by a global transcriptional analysis, in the perfused 3D bioreactor as compared to in 2D culture systems.[192]

2 AIM OF THE THESIS

The overall aim of this thesis was to evaluate the hepatic transport and metabolism in *in vitro* models used in assessments of drug pharmacokinetics and toxicity, and to investigate the contribution of membrane transporters and metabolizing enzymes to *in vivo* pharmacokinetic variability.

Specific aims were as follows:

- evaluate the activity of OATP1B1/1B3 in plated primary human hepatocytes over time in culture (**paper I**).
- evaluate the activity of OATP1B1 and CYP3A4 in fresh primary human hepatocytes and differentiated cryopreserved HepaRG cells cultured in a 3D bioreactor system (**paper II**).
- determine the expression and function of important membrane transporters and CYP enzymes in hESC-Hep and hiPSC-Hep compared to cryopreserved human primary hepatocytes (**paper III**).
- investigate the impact of OATP1B1, MDR1, and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects (**paper IV**).

3 SUMMARY OF PAPERS

Paper I

OATP1B1/1B3 activity in plated primary human hepatocytes over time in culture.

The aim of the present study was to evaluate the activity of OATP1B1 and OATP1B3 in plated primary human hepatocytes over time in culture. The uptake kinetics of the OATP1B1/1B3 substrate [^{3}H]-estradiol-17β-D-glucuronide ([^{3}H]-E17βG) was determined in cells from five donors. An extensive and variable decrease in OATP1B1/1B3 activity and/or increase in passive diffusion were observed over time. Already after 6 hours in culture, the OATP1B1/1B3 activity was not possible to determine in liver cells from one donor, while after 24 hours, the uptake activity was not measureable in one additional donor. In the other three donors, the decrease in $CL_{act,uptake}$ (V_{max}/K_m) values ranged from 15% to 86% after 24 hours in culture compared to the values measured at 2 hours. Visual examination of OATP1B1 protein expression by confocal microscopy showed localization to the plasma membrane as expected, and an extensive decrease in OATP1B1 expression over time in culture supported the decline in activity. The significant reduction in *SLCO1B1* and *SLCO1B3* gene expression over time also supported the loss of OATP1B1/1B3 activity. In conclusion, plated primary human hepatocytes are useful as an *in vitro* model for OATP1B1/1B3-mediated uptake studies, but the culture time may substantially change the uptake kinetics.

Paper II

Evaluation of OATP1B1 and CYP3A4 activities in primary human hepatocytes and HepaRG cells cultured in a dynamic three-dimensional bioreactor system.

The aim of the present study was to investigate the OATP1B1 and CYP3A4 activities in fresh primary human hepatocytes and differentiated cryopreserved HepaRG cells when cultured in a 3D bioreactor system. The OATP1B1 activity was determined by loss from media experiments of [^{3}H]-E17βG and atorvastatin for up to 7 days in culture. Atorvastatin metabolite formation was determined at day 3 to 4 to evaluate the CYP3A4 activity. Overall, the results showed that freshly isolated human hepatocytes inoculated in the bioreactor retained OATP1B1 activity for at least 7 days, while in HepaRG cells, no OATP1B1 activity were observed beyond day 2. The activity data were in agreement with immunohistochemical stainings, which showed that OATP1B1 protein expression was preserved for at least 9 days in fresh human hepatocytes, while OATP1B1 was almost absent in HepaRG cells after 9 days in culture. Fresh human hepatocytes and HepaRG cells exhibited similar CYP3A4 activity in

bioreactor culture, and immunohistochemical stainings supported these findings. Comparisons of activity and gene expression of OATP1B1 and CYP3A4 in fresh suspensions of primary human hepatocytes and HepaRG cells were in agreement with data obtained in bioreactor culture. In conclusion, freshly isolated human hepatocytes cultured in a 3D bioreactor system, preserves both OATP1B1 and CYP3A4 activities, allowing long-term *in vitro* studies on drug disposition and toxicity.

Paper III

Drug metabolizing enzymes and transporter protein profiles of hepatocytes derived from human embryonic and induced pluripotent stem cells.

In this study, the expression and function of important CYP enzymes and membrane transporters in hESC-Hep and hiPSC-Hep were compared to cryopreserved human primary hepatocytes (hphep) and HepG2 cells. Overall, CYP activities in hESC-Hep and hiPSC-Hep were much lower than in hphep cultured for 4 h, but CYP1A and 3A activities were comparable to levels in hphep cultured for 48 h (CYP1A: 35% and 26% of 48 h hphep, respectively; CYP3A: 80% and 440% of 48 h hphep, respectively). Importantly, in hESC-Hep and hiPSC-Hep, CYP activities were stable or increasing for at least one week in culture which was in contrast to the rapid loss of CYP activities in cultured hphep between 4 and 48 h after plating. With regard to transporters, in hESC-Hep and hiPSC-Hep, pronounced NTCP activity (17% and 29% of 4 h hphep, respectively) and moderate BSEP activity (6% and 8% of 4 h hphep, respectively) was observed, but only low OATP1B1 activity (both 2% of 4 h hphep). Analyses of gene expression and immunocytochemistry supported the observed CYP and transporter activities and showed expression of additional CYP enzymes and transporters. In conclusion, the stable expression and function of CYP enzymes and transporters in hESC-Hep and hiPSC-Hep for at least one week opens up the possibility to reproducibly perform extensive, long-term studies, e.g. chronic toxicity testing, in a stem cell-derived hepatic system.

Paper IV

Impact of OATP1B1, MDR1 and CYP3A4 expression in liver and intestine on interpatient pharmacokinetic variability of atorvastatin in obese subjects.

In this study, we investigated the relationship between expression of OATP1B1, MDR1 and CYP3A4 and the pharmacokinetics of atorvastatin in 21 obese patients with paired biopsies

from liver and intestinal segments. The patients were also screened for the *SLCO1B1* c.521T>C variant alleles. The results showed a significant positive correlation between OATP1B1 protein expression in the liver and CL/F of atorvastatin (r = 0.53, $p = 0.041$). The estimated correlation indicated that approximately 30% ($r^2 = 0.28$) of the variation in CL/F of atorvastatin was explained by hepatic OATP1B1 protein expression. Patients carrying the *SLCO1B1* c.521C variant allele (homozygous, n = 4; heterozygous, n = 2) exhibited 45% lower CL/F of atorvastatin than the c.521TT carriers ($p = 0.067$). No association between hepatic and intestinal expression of MDR1 or CYP3A4 and atorvastatin pharmacokinetics was found (p > 0.149). Of note, a significant negative correlation between BMI and CYP3A4 expression in both the liver (r = -0.77, $p < 0.001$) and small intestine (r = -0.56, $p = 0.011$) was observed, which was in line with a significant negative correlation between BMI and CL/F of atorvastatin lactone (r = -0.59, $p = 0.004$). In conclusion, this study suggests that OATP1B1 phenotype is more important than CYP3A4 and MDR1 phenotypes for the individual pharmacokinetic variability of atorvastatin. Furthermore, high BMI is associated with low CYP3A4 expression in both the liver and intestine, which decreases CL/F of atorvastatin lactone, a metabolite of potential importance for the risk of myotoxicity.

4 DISCUSSION

4.1 Hepatic transport and metabolism in human hepatocytes

4.1.1 OATP1B1 transport

In **paper I**, we investigated the OATP1B1/1B3-mediated uptake kinetics in plated fresh primary human hepatocytes over time in culture. Overall, the results showed that plated primary human hepatocytes cultured for a restricted period of time is a useful model for *in vitro* studies of OATP1B1/1B3-mediated uptake. However, the time in culture substantially changes the uptake kinetics of OATP1B1/1B3 substrates, and profoundly limits the time possible to study OATP1B1/1B3-mediated uptake in this system. After 2 hours in culture, an active OATP1B1/1B3-mediated uptake was observed in all donors, but an extensive decrease in OATP1B1/1B3 activity, along with an increased variability between donors, was observed during longer culturing times. The extensive increase and variability in the passive uptake of E17βG into plated human hepatocytes contributed to the large variability in OATP1B1/1B3 activity observed after longer culturing time. Additionally, the change in membrane integrity over time led to increased uncertainty in the K_m and V_{max} estimations, which affects the reliability of 2D cultured human hepatocytes as an *in vitro* model for estimations of hepatic uptake clearance *in vivo*.

In the human liver, OATP1B1 and OATP1B3 are localized to the basolateral membrane of hepatocytes.[20-22,25] However, during isolation, the polarity of the hepatocytes is rapidly lost.[193] In **paper I,** the protein expression of OATP1B1 studied by confocal microscopy showed that OATP1B1 was evenly distributed over the whole cell surface at 2 hours. The decline in protein expression of OATP1B1 on the cell surface over time in culture supported the present decrease in activity. In addition, the significant reduction in gene expression of both *SLCO1B1* and *SLCO1B3* in plated human hepatocytes also supported the loss of OATP1B1/1B3 activity over time in culture. These results are consistent with previous results by Richert *et al*. (2006), who reported a decrease in *SLCO1B1* and *SLCO1B3* expression in plated human hepatocytes after 24 hours in culture.[141] No functional data was presented in the study by Richert *et al*.

In **paper I**, the functional data after 2 hours in culture did not reveal any obvious effects of the *SLCO1B1* c.521T>C genotype on the V_{max} or K_m values. The number of individuals in this study is of course too low to provide any conclusive evidence in this matter. However, a possible explanation for the apparent lack of impact of the c.521T>C polymorphism on the uptake activity of E17βG might be that factors associated to cell isolation and plating provide greater variability *per se* than the genotype, e.g. collagenase treatment, differences in attachment status[194] and/or the previously discussed inter-lot differences in passive diffusion of E17βG.

As indicated above, the use of plated primary human hepatocytes as an *in vitro* tool for predictions of hepatic drug clearance is impaired by the rapid decrease in OATP1B1 activity in culture (**paper I**). For drugs being OATP1B1 substrates, uptake transport activity may have important implications on hepatic clearance, e.g. OATP1B1 phenotype is a major determinant of the individual variability in systemic exposure of atorvastatin after oral administration (**paper IV**). Although primary human hepatocytes cultured for longer periods still could be used to evaluate hepatic clearance, experiments in suspensions of freshly isolated hepatocytes or newly thawed cryopreserved human hepatocytes, which possess both metabolism and transport activities (**paper II and III**),[137-139] will probably give more reliable predictions.

In **paper I**, we did not explore the mechanisms behind the decline in OATP1B1 activity with time. A current hypothesis is however that the OATP1B1 activity will change when cells lose their natural cell contacts and are not organized in a 3D structure. Other culturing conditions, e.g. sandwich-cultures or 3D culture systems such as a bioreactor, may better retain and stabilize the transporter activity. This was supported by findings in **paper II** where freshly isolated human hepatocytes inoculated in the perfused 3D bioreactor system exhibited preserved OATP1B1 activity for at least 7 days in culture. The activity data were in agreement with immunohistochemical stainings which showed that OATP1B1 protein expression was preserved for at least 9 days in bioreactor culture. As discussed previously, for OATP1B1 substrates, uptake transport activity may have important implications for cellular drug concentrations, which in turn may affect drug metabolism and hepatotoxicity. **Paper II** showed that freshly isolated human hepatocytes cultured in a bioreactor system preserved functionality of OATP1B1, which, together with the sustained activity of metabolizing enzymes (discussed below), allows long-term preclinical investigations on hepatic drug

clearance and toxicity. Human hepatocytes cultured in a sandwich configuration represent a potential alternative to bioreactor cultures of human hepatocytes. Hoffmaster *et al*. (2004) have previously reported that the OATP1B1 protein expression is retained in SCHH for up to six days,[150] and recently, Kimoto *et al*. (2013) reported similar OATP1B1 protein expression levels in cryopreserved human hepatocyte suspensions and SCHH at day five in culture,[195] suggesting that SCHH is another useful *in vitro* model for characterization of hepatic disposition. However, another recent study by Kimoto *et al*. (2012) showed that culturing of human hepatocytes in a sandwich configuration had a significant impact on the functional expression of CYP enzymes, with differential effects of specific CYP isoforms, suggesting that SCHH may not be considered a suitable tool for assessments of hepatic clearance.[196]

Paper II was the first to show preserved protein expression and activity of a basolateral transporter, OATP1B1, in primary human hepatocytes cultured in the bioreactor system. In previous studies, immunohistochemical stainings have shown expression of the apical efflux transporters MRP2 and MDR1 in bioreactor cultures of fresh human hepatocytes for at least two weeks.[188] In the liver, hepatic uptake of E17βG and atorvastatin is mediated by OATP1B1 and OATP1B3.[23,25,133,197] MRP2, and to less extent MDR1, are responsible for the biliary efflux of E17βG, while atorvastatin is excreted via MDR1, MRP2 and BCRP.[56,113,198,199] Formation of bile structures in hepatocytes cultured in a 3D bioreactor has been reported,[185,186] but the organization of these bile structures is not known and the bioreactor technique does not allow collection of bile at this point. In the bioreactor experiments performed in **paper II**, intracellular E17βG is probably partly effluxed back into the medium via MRP2 (and MDR1), while atorvastatin is effluxed by MDR1, MRP2 and BCRP. The curve plateau that is observed in the loss from media experiments over time could possibly be explained by equilibrium between uptake and efflux of E17βG and atorvastatin.

Lau *et al*. (2007) reported an acute 2.7-fold increase in elimination half-life of atorvastatin *in vivo* after oral single dose co-administration of rifampicin, a potent OATP1B1 inhibitor.[42] This was in line with our observations in the bioreactor experiments with fresh human hepatocytes (**paper II**), where AUC of atorvastatin increased 1.2-fold in the medium during co-administration of estrone-3-sulfate (E3S), another OATP1B1 inhibitor. Possible explanations for the lower interaction effect *in vitro* include efflux of atorvastatin back into the media, instead of out into the bile, and less potent OATP1B1 inhibition of E3S compared

to rifampicin. Nevertheless, the *in vitro* findings demonstrate the applicability of the bioreactor as a model for preclinical interaction studies. However, *in vitro-in vivo* extrapolations should be performed to determine whether the bioreactor cultures are preferable to suspensions of human hepatocytes, which is a more cost efficient model, for this purpose.

In **paper II**, comparable OATP1B1 activities were observed in suspensions of freshly isolated and cryopreserved human hepatocytes, which support previous findings.[137] Furthermore, in **paper III**, we determined the OATP1B1 activity in cryopreserved hepatocytes 4 hours after plating, and considerable OATP1B1 activity was observed in all three batches. Hence, cryopreserved human hepatocytes, both in suspension and platable, represent good alternatives to primary human hepatocytes for evaluation of OATP1B1-mediated uptake. However, as in non-frozen primary human hepatocytes, a rapid loss of transporter expression is observed when these cells are cultured in 2D models.[141] In **paper II**, cryopreserved human hepatocytes were, however, not applied in the bioreactor system based on prior experience showing that non-platable cryopreserved human hepatocytes are not applicable in the bioreactor (data not shown). The bioreactor technique requires cell attachment to the hollow-fiber capillary membranes, and the incapability of non-platable cryopreserved hepatocyte to attach to surfaces probably explains the negative findings. In future experiments, platable cryopreserved human hepatocytes should be applied in the bioreactor to test this hypothesis.

4.1.2 CYP3A4 metabolism

It is well known that isolated primary human hepatocytes exhibit significant CYP3A4 activity.[139,147,200] In **paper II**, suspensions of freshly isolated and cryopreserved human hepatocytes exhibited comparable CYP3A4 activities. In line with previous reports,[141,142] the expression and activity of CYP3A4 are however rapidly decreasing when hepatocytes are cultured in 2D (**paper III**).

In **paper II**, we also investigated the CYP3A4 activity (atorvastatin hydroxylation) in fresh primary human hepatocytes cultured in a bioreactor system. Atorvastatin was metabolized to both 2-OH-atorvastatin and 4-OH-atorvastatin, demonstrating an active CYP3A4 metabolism at day 3 to 4.[111,201] Furthermore, immunocytochemical stainings showed CYP3A4 protein expression in primary human hepatocytes at day 9 in bioreactor culture. These findings are in

agreement with previous studies on fresh human hepatocytes in bioreactor culture where CYP3A4 activity was observed for at least two weeks.[187,188]

In **paper II**, an apparent induction of CYP3A4 in bioreactor cultures of primary human hepatocytes was observed, which is in agreement with the previously reported CYP3A4 induction by atorvastatin *in vitro*.[202-204] This suggests that primary human hepatocytes cultured in the bioreactor system might serve as an *in vitro* model for evaluation of both enzyme inhibition and induction, as well as identification of slowly metabolized drugs.

In **paper IV**, we found large interpatient variability in both mRNA and protein expression of hepatic CYP3A4 and OATP1B1, which is consistent with previous studies.[44,205-207] In agreement with this, large interindividual variability in CYP3A4 and OATP1B1 activities was observed in primary human hepatocytes in suspension, 2D and 3D cultures (**paper I and II**), which probably could be explained by interdonor variability, but maybe also by the hepatocyte isolation procedure. Furthermore, substantial interindividual variability in CYP3A4 and OATP1B1 activities was observed in platable cryopreserved human hepatocytes investigated in **paper III**, where each batch contained cells from a single donor. Although pooled batches of cryopreserved human hepatocytes from several donors exhibit less inter-batch differences (**paper II**), the restricted liver tissue availability along with the variability in quality and hepatic functions limits the utility of human hepatocytes as an *in vitro* model in drug development, and alternative hepatic *in vitro* models are warranted.

4.2 Hepatic transport and metabolism in HepaRG cells

4.2.1 OATP1B1 transport

In **paper II**, OATP1B1-mediated transport of E17βG was observed in suspensions of differentiated cryopreserved HepaRG cells, which is supported by later reports on uptake of E17βG and E3S in 2D cultured HepaRG cells.[158,161] However, **paper II** clearly showed that the OATP1B1 activity is lower in HepaRG cells than in suspensions of both fresh and cryopreserved human hepatocytes, which is in line with previous reports on *SLCO1B1* expression in 2D cultures of HepaRG cells.[156,157] These findings suggest that human hepatocytes are superior to HepaRG cells as a model for *in vitro* studies of OATP1B1-mediated transport.

In **paper II**, we also investigated the OATP1B1 activity in HepaRG cells cultured in the 3D bioreactor system for 9 days. OATP1B1-mediated transport was observed at day 2, but no OATP1B1 activity was detected beyond day 2. The activity data were in agreement with immunohistochemical stainings which showed that the OATP1B1 protein expression was almost absent in HepaRG cells at day 9 in bioreactor culture. These findings suggest that culturing of HepaRG cells in a 3D system, in contrast to human hepatocytes, does not preserve OATP1B1 activity over time. Notably, in **paper II**, differentiated cryopreserved HepaRG cells, which were recently available from the purchaser, were inoculation directly into the bioreactor in order to shorten the experimental period. Inoculation of undifferentiated HepaRG cells followed by two weeks differentiation within the bioreactor prior to experiments may improve hepatic functions such as OATP1B1 activity, and should be further investigated. In a previous study by Darnell *et al*. (2011) where HepaRG cells were differentiated in the bioreactor as described above, immunohistochemical stainings of the apical efflux transporters MRP2 and MDR1 showed expression of both transporters for at least two weeks in culture.[162] However, these efflux transporters are also highly expressed in 2D cultured HepaRG cells, in opposite to uptake transporters.[156-158,160,161] Therefore, the conclusion that differentiation of HepaRG cells in the bioreactor system will improve functional expression of uptake transporters cannot be drawn from these data.

In the bioreactor experiments in **paper II**, the OATP1B1 activity was determined by assessment of loss from media of the probe substrate. This approach is less sensitive compared to assessments of intracellular concentration due to the relatively high start concentration of substrate in the media. Thus, the loss from media approach may not detect low levels of functional transporter expression, which may explain the negative results with regard to OATP1B1 activity in bioreactor cultures of HepaRG cells beyond day 2. Hopefully, future generations of the bioreactor system will allow cell removal from the bioreactor culture for determination of intracellular concentrations of xenobiotics or endogenous compounds, as well as mRNA and protein expression levels, during the experiment run.

4.2.2 CYP3A4 metabolism

In **paper II**, suspensions of differentiated cryopreserved HepaRG cells exhibited substantial CYP3A4 activity. The observed CYP3A4 activity level in HepaRG cells was comparable to suspensions of freshly isolated human hepatocytes and cryopreserved human hepatocytes.

The suitability of suspended HepaRG cells as an *in vitro* model for studies of CYP3A4-mediated drug metabolism was supported by the observed *CYP3A4* gene expression data in **paper II** showing no significant difference between suspensions of HepaRG cells, fresh and cryopreserved human hepatocytes.

When cultured in the bioreactor, HepaRG cells were shown to retain their CYP3A4 activity in terms of hydroxylated metabolites following incubations with atorvastatin at day 3 and 4 (**paper II**), which is consistent with studies on HepaRG cells in bioreactor culture where CYP3A4 activity was observed for at least two weeks.[162] Similar levels of CYP3A4 activity were observed in HepaRG cells and human hepatocytes when cultured in the bioreactor, which were consistent with the comparable CYP3A4 protein expression levels observed at day 9 in culture. Furthermore, a CYP3A4 induction response by atorvastatin was observed in HepaRG cells cultured in the bioreactor, as previously described for bioreactor cultures of human hepatocytes. Overall, these findings support that HepaRG cells cultured in the bioreactor may serve as a valuable model for studies of CYP3A4-mediated drug clearance, particularly of slowly metabolized drugs.

As HepaRG cells cultured in 2D or 3D systems have been shown to express similar CYP activities,[155,156,159,160,162] 2D cultures may be a more convenient and less expensive *in vitro* model for long-term studies of hepatic CYP induction and metabolism. It should be noted though, that the limited OATP1B1 activity is a potential drawback of HepaRG cells in metabolism studies in cases where drugs are combined CYP/OATP1B1 substrates. The low functional expression of OATP1B1 and other uptake transporters in HepaRG cells[156-158, 160,161] may confound the *in vitro-in vivo* extrapolations. Furthermore, in an additional paper, we reported that the proportions of relevant hydroxylation and glucuronidation biotransformation pathways of two model substrates metabolized by both CYP and UGT enzymes were different in bioreactor culture of HepaRG cells compared to human hepatocytes.[190] These findings reflect the differential expression levels of CYP and UGT enzymes in HepaRG cells and human hepatocytes, and should be considered when performing *in vitro-in vivo* extrapolations from HepaRG data.

4.3 Hepatic transport and metabolism in human pluripotent stem cell-derived hepatocytes

4.3.1 OATP1B1 transport

In **paper III**, the activity and expression of several membrane transporters important for drug disposition and toxicity in human pluripotent stem-cell derived hepatocytes were evaluated. The OATP1B1 activity in hESC-Hep and hiPSC-Hep was only about 2% of the activity in plated (4 h) cryopreserved human hepatocytes. These findings were consistent with low *SLCO1B1* gene expression in hESC-Hep and hiPSC-Hep. Although a moderate OATP1B1 staining in hiPSC-Hep was shown, there is no doubt that the currently available human pluripotent stem cell-derived hepatocytes are not yet an appropriate model for studies of OATP1B1-mediated transport.

Despite almost absent OATP1B1 activity, the pronounced activity of the liver-specific uptake transporter NTCP in hESC-Hep and hiPSC-Hep (~15-30% of 4 h plated human hepatocytes, **paper III**) brings hope that stem cell-derived hepatocytes can serve as a model for studies on typical drug transporters in the future. In **paper III,** the positive findings of NTCP uptake in hESC-Hep and hiPSC-Hep were accompanied by moderate activity of the hepatic efflux transporter BSEP (~7% of 4 h plated human hepatocytes). The gene expression of *SLC10A1* and *ABCB11* were consistent with the NTCP and BSEP activity data, respectively, and immunohistochemical stainings indicated membrane localization of these transporters.

In the liver, NCTP and BSEP mediates the vectorial transport of bile acids across hepatocytes from the blood to the bile,[63,64] and thus, functional NTCP and BSEP represent important liver functions present in both hESC-Hep and hiPSC-Hep. As described above, inhibition of NTCP and BSEP has been reported to mediate drug-induced cholestasis and hepatotoxicity.[65,66] Hence, hESC-Hep and hiPSC-Hep could represent a useful *in vitro* tool for predictions of *in vivo* hepatotoxicity caused by inhibition of bile acid transport. Importantly, the expression levels of NTCP and BSEP was stable or slightly increasing for at least one week in culture (**Paper III**), which enables long-term studies.

Overall, human pluripotent stem cell-derived hepatocytes exhibit a substantially low transporter expression profile compared to human hepatocytes, although improved expression

and activity of several drug transporters have been observed since comparable studies were conducted in 2009 (data not shown). Hence, additional refinements of the derivation protocol are required before hESC-Hep and hiPSC-Hep are applicable in studies of hepatic drug disposition.

4.3.2 CYP3A4 metabolism

In **paper III**, we evaluated the activity and expression of several CYP enzymes important for drug metabolism in hESC-Hep and hiPSC-Hep. Overall, the CYP activities in hESC-Hep and hiPSC-Hep were low compared to cryopreserved human hepatocytes 4 h after plating. However, the new generations of hESC-Hep and hiPSC-Hep presented in **paper III** had considerably higher CYP activity levels compared to earlier reports on stem cell-derived hepatocytes.[170,179] Compared to 48 h hepatocyte cultures, the CYP3A activity in hESC-Hep and hiPSC-Hep investigated in **paper III** was at a similar level or even higher, respectively. Investigating the gene expression of the CYP3A enzyme family revealed that hESC-Hep and hiPSC-Hep expressed only low levels of *CYP3A4* which is the major isoenzyme found in adult hepatocytes.[79] However, considerable mRNA levels of the CYP3A isoenzyme *CYP3A5* were observed in both hESC-Hep and hiPSC-Hep which indicate that the observed midazolam metabolism was mainly due to CYP3A5 activity. Moreover, compared to previous reports,[170,179] the *CYP1A* levels are also approaching levels found in plated human hepatocytes after 48 h in culture. However, hESC-Hep and hiPSC-Hep expressed higher *CYP1A1* than *CYP1A2* levels, which is opposite to in adult livers.[208] Furthermore, the activity of CYP2B6 and CYP2C9 was considerably lower than in hepatocyte cultures, which emphasize that improved differentiation protocols are needed to obtain fully functional hepatocytes.

In **paper III**, the CYP activity levels in hESC-Hep and hiPSC-Hep were in general stable or slightly increasing for at least one week in culture, in contrast to the extensive decrease in CYP activities observed in cultures of plated cryopreserved human hepatocytes. This is a promising feature with regard to long-term *in vitro* studies of e.g. chronic toxicity. Furthermore, the high robustness of the differentiation protocol and subsequently low inter-batch variability of hESC-Hep and hiPSC-Hep provides a continuous, reliable supply of hESC-Hep and hiPSC-Hep from defined genetic backgrounds.

Although the new generations of hESC-Hep and hiPSC-Hep presented in **paper III** exhibit improved hepatic features compared to earlier generations of stem cell-derived hepatocytes,[170,179] hESC-Hep and hiPSC-Hep differentiated in 2D systems seem to adapt a "plated" phenotype different from human hepatocytes with regard to expression of CYP enzymes and transporters. Thus, further improvement is required before hESC-Hep and hiPSC-Hep can be used in e.g. drug metabolism studies. A more hepatocyte-like CYP and transporter profile may be obtained through hepatic differentiation of hESC and hiPSC in a more tissue like environment, e.g. in the perfused 3D bioreactor system.[192]

4.4 Transporters and enzymes as pharmacokinetic determinants *in vivo*

4.4.1 OATP1B1 and atorvastatin pharmacokinetics

In **paper IV**, we investigated the relationship between individual expression of OATP1B1, MDR1 and CYP3A4 in paired liver and small intestinal biopsies and the pharmacokinetics of atorvastatin in morbidly obese patients. The main finding was a significant correlation between OATP1B1 protein expression in the liver and CL/F of atorvastatin in obese patients, indicating that OATP1B1 phenotype determines about 30% of the individual variability in pharmacokinetics of atorvastatin. The importance of OATP1B1 in this matter was further substantiated by the almost 50% lower CL/F of atorvastatin observed in carriers of the *SLCO1B1* c.521C variant allele, which is in line with a previous study investigating the impact of *SLCO1B1* genetics on systemic exposure of atorvastatin.[209] Our *in vivo* data clearly indicate that this mutation affects activity rather than expression of OATP1B1, which later has been supported by Nies *et al.* (2013).[210] Further, when examining the data in more detail, no association was observed between hepatic OATP1B1 expression and k_{el} of atorvastatin. The most plausible explanation would be a corresponding impact of hepatic OATP1B1-mediated uptake on both clearance and apparent volume of distribution (V_d) of atorvastatin.

No significant correlations were observed between atorvastatin pharmacokinetics and any of the other tested variables, e.g. CYP3A4 or MDR1 expression in liver and intestine. Thus, it seems evident that the hepatic expression of OATP1B1 is more important than CYP3A4 and MDR1 expression in small intestine and liver for the individual variability in pharmacokinetics of atorvastatin. These findings indicate that uptake transporters may be more important than metabolizing enzymes for interindividual variability in pharmacokinetics

and response of certain drugs. Moreover, **paper IV** supports that genotyping of *SLCO1B1* may be relevant to identify patients at risk of ADRs or impaired efficacy during treatment with certain drugs, e.g. statins.

4.4.2 CYP3A4 and atorvastatin lactone pharmacokinetics

The lactone metabolite of atorvastatin is pharmacologically inactive, but the level of this metabolite has been linked to the risk of muscular side effects.[115,116,211] Due to its high lipophilicity, the lactone form of atorvastatin penetrates passively across cell membranes into peripheral tissues, where it can be converted to active atorvastatin acid by hydrolysis.[111] **In paper IV**, we found no association between the OATP1B1 expression level and CL/F of atorvastatin lactone in the present study, which is in line with the low affinity to OATP1B1 for the lactone form.[37,56] However, regarding affinity to CYP3A4, the situation is opposite, i.e. atorvastatin lactone displays a much higher affinity to CYP3A4 than atorvastatin acid.[118] In accordance with this, a significant association was observed between CYP3A4 expression in the small intestine and the pharmacokinetics of atorvastatin lactone, but not for atorvastatin acid.

Obesity is a rapidly growing health care problem in modern societies, and it is often associated with various co-morbidities and need of drug therapy (e.g. cardiovascular, antidiabetic and antidepressant agents).[212-214] However, little is known about the effect of obesity on drug metabolism and transporter. Interestingly, in **paper IV**, we observed that the CYP3A4 expression in both liver and small intestine was strongly associated with BMI in the present population, i.e. the higher BMI, the lower CYP3A4 expression. This observation was in line with a significant negative correlation between BMI and CL/F of atorvastatin lactone. Obesity has previously been reported to be associated with reduced clearance of CYP3A4 substrates,[215] e.g. midazolam.[216] CYP3A4 is quantitatively the most important enzyme in drug metabolism, and the low expression of this enzyme in obese patients likely implies higher bioavailability and lower clearance of many drugs. Thus, obese patients might be at risk of being over-exposed to several drugs, especially when dosed according to body weight. However, additional studies are required to investigate pharmacokinetic alterations and dose requirements in obese patients in more detail.

In **paper IV**, the significant correlation observed between CYP3A4 in the liver and intestine indicates that CYP3A4 expression is coordinately regulated in the liver and intestine. These results are inconsistent with previous studies in non-obese patients.[105,205-207] However, the parallel decrease in CYP3A4 expression in paired biopsies from the liver and intestine with increasing BMI strengthens our findings that CYP3A4 are coordinately regulated in these tissues. The mechanisms behind this inverse relationship have not been investigated in this thesis, but may at least partly be due to the general inflammatory state of obese patients.[217-219] Inflammatory response involves release of cytokines which have been associated with suppression of several CYP enzymes including CYP3A4.[220]

5 CONCLUSION

In conclusion, plated primary human hepatocytes were found to be a useful *in vitro* model for OATP1B1/1B3-mediated uptake studies, but only for a restricted period of time in culture. Longer culturing times led to an extensive and variable decrease in OATP1B1/1B3 activity and/or increase in passive diffusion, which limits the time possible to study hepatic uptake and clearance in this system. Culturing of primary human hepatocytes in a dynamic 3D bioreactor system resulted in preserved OATP1B1 and CYP3A4 activities for at least one week in culture, which allows studies of chronic hepatotoxicity and slowly metabolized drugs. Furthermore, differentiated HepaRG cells exhibited maintained CYP3A4 activity in bioreactor culture similar to primary human hepatocytes. However, OATP1B1 activity could not be detected later than day 2 in bioreactor culture which is a major limitation of this *in vitro* model compared to human hepatocytes, especially for *in vitro-in vivo* extrapolations. Moreover, the hESC-Hep and hiPSC-Hep presented in this thesis exhibited improved transporter and CYP activity levels compared to previous studies. However, the overall transporter and CYP activities were considerably lower than in primary human hepatocytes. Additional refinements of the derivation process in combination with a more physiological culturing environment will hopefully, in the future, generate stem cell-derived hepatocytes with adequate levels of hepatic functions that could be applicable in *in vitro* studies of drug metabolism and disposition.

Finally, this thesis indicates that OATP1B1 phenotype is the major determinant of individual variability in atorvastatin pharmacokinetics, suggesting that hepatic uptake transporters are more important than metabolizing enzymes in the small intestine and liver for the individual variability in pharmacokinetics of certain drugs. Moreover, high BMI was associated with low CYP3A4 expression which implies higher bioavailability and lower clearance of CYP3A4 substrates. Hence, obese patients might be at risk of overdosing of such drugs. These findings may be of clinical importance for drug treatment in obese patients.

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