In vitro and in silico Predictions of Hepatic Transporter-Mediated Drug Clearance and Drug-Drug Interactions in vivo

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Dissertation presented at Uppsala University to be publicly examined in Sal B41, Biomedicinskt centrum, Husargatan 3, Uppsala, Friday, 27 February 2015 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English. Faculty examiner: Professor Kim Brouwer (UNC School of Pharmacy).

Abstract


The liver is the major detoxifying organ, clearing the blood from drugs and other xenobiotics. The extent of hepatic clearance (CL) determines drug exposure and hence, the efficacy and toxicity associated with exposure. Drug-drug interactions (DDIs) that alter the hepatic CL may cause more or less severe outcomes, such as adverse drug reactions. Accurate predictions of drug CL and DDI risk from *in vitro* data are therefore crucial in drug development.

Liver CL depends on several factors including the activities of transporters involved in the hepatic uptake and efflux. The work in this thesis aimed at developing new *in vitro* and *in silico* methods to predict hepatic transporter-mediated CL and DDIs *in vivo*. Particular emphasis was placed on interactions involving the hepatic uptake transporters OATP1B1, OATP1B3, and OATP2B1. These transporters regulate the plasma concentration-time profiles of many drugs including statins.

Inhibition of OATP-mediated transport by 225 structurally diverse drugs was investigated *in vitro*. Several novel inhibitors were identified. The data was used to develop *in silico* models that could predict OATP inhibitors from molecular structure. Models were developed for static and dynamic predictions of *in vivo* transporter-mediated drug CL and DDIs. These models rely on a combination of *in vitro* studies of transport function and mass spectrometry-based quantification of protein expression in the *in vitro* models and liver tissue. By providing estimations of transporter contributions to the overall hepatic uptake/efflux, the method is expected to improve predictions of transporter-mediated DDIs. Furthermore, proteins of importance for hepatic CL were quantified in liver tissue and isolated hepatocytes. The isolation of hepatocytes from liver tissue was found to be associated with oxidative stress and degradation of transporters and other proteins expressed in the plasma membrane. This has implications for the use of primary hepatocytes as an *in vitro* model of the liver. Nevertheless, by taking the altered transporter abundance into account using the method developed herein, transport function in hepatocyte experiments can be scaled to the *in vivo* situation. The concept of protein expression-dependent *in vitro*-in *vivo* extrapolations was illustrated using atorvastatin and pitavastatin as model drugs.

**Keywords:** OATP1B1, OATP1B3, OATP2B1, NTCP, drug transporters, human hepatocytes, atorvastatin, pitavastatin, proteomics, sandwich-cultured human hepatocytes, SCHH, mechanistic modeling, in vitro-in *vivo* extrapolation, transport inhibition, hepatic uptake, hepatocyte isolation, transporter contribution

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ISSN 1651-6192
urn:nbn:se:uu:diva-241376 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-241376)
“Models are not descriptions of reality; they are descriptions of our assumptions about reality.”
Jeremy Gunawardena
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, Distribution, Metabolism, and Excretion</td>
</tr>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>AUC</td>
<td>Area Under the Plasma Concentration-Time Curve</td>
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<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
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<td>CL</td>
<td>Clearance</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DDI</td>
<td>Drug–Drug Interaction</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
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<tr>
<td>IVIVE</td>
<td><em>In Vitro–In Vivo</em> Extrapolation</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten Constant</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Tandem Mass Spectrometry</td>
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<tr>
<td>MRP</td>
<td>Multidrug Resistance-Associated Protein</td>
</tr>
<tr>
<td>NTCP</td>
<td>Sodium Taurocholate Cotransporting Polypeptide</td>
</tr>
<tr>
<td>NPC</td>
<td>Non-Parenchymal Cell</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic Anion Transporting Polypeptide</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar Surface Area</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative Structure-Activity Relationship</td>
</tr>
<tr>
<td>SCHH</td>
<td>Sandwich-Cultured Human Hepatocytes</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
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<tr>
<td>TPA</td>
<td>Total Protein Approach</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5’-Diphospho-Glucuronosyltransferase</td>
</tr>
<tr>
<td>UWL</td>
<td>Unstirred Water Layer</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal Uptake Rate</td>
</tr>
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Introduction

For an orally administered drug to achieve its intended systemic effect, it must reach its therapeutic target in sufficiently high levels. Multiple biological barriers exist that limit the amount of drug that is delivered to the site of action. Examples of such barriers include the intestinal epithelium where absorption occurs and the liver where the drug is eliminated through metabolism and excretion. Consequently, not only is the activity of the drug of importance for its efficacy, but also its absorption, distribution, metabolism, and excretion (ADME) since these properties govern the amount of drug at the target site. Although this was not fully appreciated twenty to thirty years ago, characterization of the ADME properties of a new drug entity is now an integrated part of early drug development along with optimization of its potency. As a result, drug attrition rate due to poor pharmacokinetics has decreased immensely.

With an increased understanding of the ADME processes that influence the pharmacokinetics of a drug, transport proteins gained attention in the 1990s. Since then, numerous drug transporters have been identified. Research on drug transporters has improved our understanding of their tissue and subcellular localization, transport mechanisms, substrate and inhibitor specificities, genetic polymorphisms, and species differences.

Today, it is well acknowledged that transporters can have a significant role in the absorption, distribution, and excretion of drugs and their metabolites. By determining the amount of drug that enters and exits a cell, transporters also influence the rate and extent of drug metabolism. Being such important determinants of systemic and tissue exposure, transport proteins are possible targets for drug–drug interactions (DDIs) with potential changes in drug safety and/or efficacy. Hence, regulatory agencies such as the American Food and Drug Administration and the European Medicines Agency now recommend studies of transporter-mediated drug interactions in their industry guidelines. These recommendations direct drug development towards a better understanding of the determinants that account for the pharmacokinetics of new drug entities.

Transport proteins are expressed in varying abundance in all tissues in the human body including the liver. The elimination of drugs that takes place in the liver has a substantial impact on drug pharmacokinetics. Predictions of hepatic elimination in humans are therefore of crucial importance for assessing the probability of success of new drug candidates. Several different
methods to predict hepatic clearance (CL) from in vitro studies exist. However, the in vivo elimination of drugs that are dependent on transport proteins for their CL is still generally underpredicted.\textsuperscript{12, 13} Thus, new improved strategies for extrapolating in vitro data to the in vivo situation are needed and have been addressed in this thesis.

The Human Liver

The liver is the most important organ for drug elimination. With its strategic anatomical location in the human body, it connects the gastro-intestinal tract to the systemic circulation. Orally administered drugs that are absorbed in the intestine thus have to pass through the liver before they reach the systemic blood circulation. The elimination that takes place during this step is referred to as the hepatic first pass effect and may have a substantial impact on the bioavailability of the drug (i.e., the fraction of unchanged drug that reaches the systemic circulation upon oral administration).\textsuperscript{14} Once the drug has reached the systemic blood circulation, the kidneys aid the liver in continued elimination from the body. In addition to the elimination of drugs and other exogenous and endogenous compounds, the liver has several other essential functions, such as bile production and secretion, plasma protein synthesis, and glycogen storage.

Cell Composition and Organization of the Liver

Two major types of cells populate the liver, parenchymal and non-parenchymal cells (NPCs). The parenchymal cells, also known as hepatocytes, represent about 60\% of the liver cell population and 80\% of the volume of the organ.\textsuperscript{15} They carry out most of the liver functions and are responsible for the elimination of drugs. Only 6.5\% of the liver volume is formed by NPCs.\textsuperscript{15} Of these, sinusoidal endothelial cells constitute the largest fraction. The remaining NPCs of the liver include hepatic stellate cells (fat- and vitamin A-storing cells), Kupffer cells (liver macrophages), and cholangiocytes (biliary epithelial cells).\textsuperscript{15}

The human liver has a dual blood supply, where the greater part (~75\%) stems from the hepatic portal vein that carries nutrient-rich venous blood from the gastrointestinal tract and spleen. The remaining supply is from the hepatic artery, carrying fresh, oxygenated blood from the aorta. Within the liver, distributing branches of these blood vessels course in parallel and subdivide repeatedly. The terminal branches of the vessels lead to a lobule, the structural unit of the liver (Figure 1).\textsuperscript{16} The lobule has a hexagonal shape. At the midpoint of the lobule is the central vein. From here, hepatocytes radiate out in linear cords (plates of hepatocytes just one cell-layer thick) to the periphery of the lobule. Blood from the branches of the hepatic artery and por-
tal vein combines in hepatic sinusoids, located in between the hepatic cords, and exits the lobule through the central vein. Most of the hepatocytes are exposed to the sinusoidal vasculature on two sides, allowing efficient exchange between the blood and the parenchymal cells.¹⁶

Liver sinusoidal endothelial cells form the wall of the sinusoid. These cells are highly fenestrated, permitting plasma solutes to move freely into the perisinusoidal space of Disse, the space between the sinusoidal endothelial cells and the hepatocytes.¹⁷ Associated with the sinusoids are Kupffer cells and hepatic stellate cells, which reside within the space of Disse. The stellate cells are important for extracellular matrix homeostasis,¹⁸ thereby regulating the three-dimensional structure of the liver.

**Figure 1.** Schematic representation of the microarchitecture of the liver lobule. Blood from the hepatic artery and portal vein combines in the sinusoid and exits through the central vein in the center of the lobule. The hepatocytes form the cords of the lobule. Bile canaliculi are formed between adjacent hepatocytes. Through an interconnected network they transfer bile in the opposite direction of the blood, towards the periphery of the lobule. The sinusoids are lined by highly fenestrated liver sinusoidal endothelial cells (see inset). The hepatic stellate cells reside in the space of Disse between the hepatocytes and the sinusoidal endothelial cells. These cells control the three-dimensional structure of the liver by regulating the turnover of extracellular matrix. Kupffer cells, which are liver-resident macrophages, are mainly located within the sinusoids. The figure was drawn after an illustration by DH Adams and B Eksteen, Nat Rev Immunol, 2006.

At the apical membrane of hepatocytes, where adjacent hepatocytes meet, bile canaliculi are formed through invaginations of the plasma membrane.¹⁹ The apical membrane is separated from the basolateral membrane by tight junctions and desmosomes. The bile canaliculi form an interconnected network that transfers bile in the opposite direction of the sinusoidal blood, i.e., towards the periphery of the lobule. There they join to form bile ducts. The bile ducts are lined by cholangiocytes, which contribute to bile secretion by
release of water and bicarbonate. The bile ducts carry bile components to the common hepatic duct, which is connected to the gallbladder. The gallbladder, in turn, releases its content into the small intestine.

The Hepatocyte

The hepatocyte is a highly polarized cell with two distinct membranes: the basolateral membrane, facing the sinusoids and the neighboring hepatocytes of the cords (intercellular section), and the apical membrane facing the bile canaliculi. Both the basolateral membrane and the apical membrane have microvilli protruding into the space of Disse and the bile canaliculi, respectively, increasing the surface area of these membranes. Half of the surface area of a hepatocyte has been reported to be in contact with either the sinusoid (37%) or the bile canaliculi (13%). In another study, 22% and 10% of the hepatocyte surface area was found to be in contact with sinusoids and bile canaliculi, respectively.

The hepatocytes take up substances destined for the bile via the basolateral membrane and excrete them across the canalicular membrane. This vectorial transport requires a domain-specific distribution of lipid and protein components (including transport proteins) in the plasma membrane. The polarity is achieved through sorting of the proteins to the different membranes and is upheld by tight junctions.

Due to differences in local blood composition and oxygenation along the sinusoids, the hepatocytes are heterogeneous in function, resulting in the division into three zones (Figure 2). Hepatocytes close to the oxygen- and nutrient-rich periportal area are perfused first and reside in zone I. Hepatocytes in the intermediate zone II and distant zone III, close to the central vein, are perfused sequentially with blood already modified by the preceding hepatocytes. Consequently, they receive less nutrients and oxygen. Different metabolic functions are carried out in the various zones. As an example, hepatocytes in zone III have the highest expression of drug metabolizing enzymes and are the most important for biotransformation of drugs.

The hepatocytes do not only show functional heterogeneity, but also morphological. In general, the hepatocytes are cuboidal in shape with one or more cell nuclei. The fraction of polypluid hepatocytes increases from zone I to zone III. In addition, hepatocytes close to the portal vein (zone I) tend to be smaller in size than those close to the central vein (zone III).
Figure 2. Zonation of the liver. Hepatocytes are heterogeneous in appearance and function along the axis of the liver lobule. The functional zonation is a result of a gradual decrease in the concentration of oxygen and nutrients from the periportal (zone I) to the perivenous (zone III) area. The metabolism of drugs and xenobiotics is one of the processes affected by this zonation. Drug metabolism mainly occurs in the highly differentiated hepatocytes located in the perivenous area. These hepatocytes express the highest levels of drug metabolizing cytochrome P450 (CYP) enzymes.

Hepatic Disposition of Drugs

One of the important functions of the hepatocytes is the degradation of endogenous and exogenous compounds such as drugs. For this purpose, the liver contains a wide array of metabolizing enzymes that convert lipophilic substances into more water-soluble metabolites. The metabolites can then more easily be excreted via the bile or the kidneys. Because the metabolizing enzymes are located intracellularly, drugs first have to cross the basolateral membrane of the hepatocyte. A drug molecule can pass through the cell membrane by different modes of transport, of which the main ones for small-molecular drugs are passive transmembrane diffusion and carrier-mediated transport. Following uptake across the basolateral membrane, drugs may undergo biotransformation by phase I and phase II metabolizing enzymes. In addition, drugs and their metabolites may be excreted into the bile or transported back into the blood for renal elimination. The various processes contributing to hepatic disposition of drugs are described in more detail in the following sections.

Metabolism

The hepatocyte is well equipped for its central role in drug elimination through its high expression of drug-metabolizing enzymes. Drug metabolism can be divided into two phases. The first phase includes reactions such as reduction, oxidation, and hydrolysis, which introduce reactive or polar groups into the compound being metabolized. The majority of these reactions are catalyzed by enzymes of the cytochrome P450 (CYP) family, accounting for around 75% of total drug metabolism.27 CYP3A4, CYP2D6,
CYP2C9, and CYP2C19 are the most important for the metabolism of commercially available drugs today. Other notable CYPs in drug metabolism are CYP1A2, CYP2B6, and CYP2E1.

If the metabolites being formed during phase I metabolism are sufficiently polar, they may be directly excreted into the bile or blood. Many phase I products, however, undergo subsequent phase II metabolism, in which hydrophilic functional groups such as glutathione or glucuronic acid are conjugated to the molecules. Phase II metabolism can also occur without preceding phase I metabolism. Phase II metabolism is catalyzed by transferases including uridine 5'-diphospho-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), and sulfotransferases (SULTs). UGTs are the most important of these conjugating enzymes, accounting for approximately 10% of total drug metabolism. The main drug-metabolizing UGT enzymes are UGT2B7, UGT1A1, and UGT1A4.

Passive Transmembrane Diffusion

Drugs have to cross the cell membrane to gain access to the cell interior. The unassisted permeation of molecules through the lipid bilayer is generally considered the most important mode of transport by which a drug crosses the cell membrane. Some researchers, however, claim that active carrier-mediated uptake is the rule rather than the exception.

The process of passive transmembrane diffusion involves partitioning into/out of the cell membrane and diffusion through the cellular membrane. The fundamental principle of passive permeation is contained in Fick’s first law of diffusion: a substance diffuses from a region of high concentration to a region of low concentration at a rate proportional to the magnitude of the concentration gradient. Passive diffusion is spontaneous and requires no energy. Rather, the driving force is the increase of entropy in the system. The net movement of molecules from a region of high concentration to a region of low concentration can be explained by the random walk (Brownian motion) of individual molecules: it is more likely that any single molecule will move from an area of high concentration to an area of low concentration than in the opposite direction. Therefore, the net movement will be down the concentration gradient until equilibrium is reached.

The diffusion rate across the cell membrane is directly proportional to the permeability coefficient ($P$), the surface area of the membrane ($A$), and the concentration gradient ($dC/dx$), equation 1.

$$ v = P \times A \times \frac{dC}{dx} $$

(1)
The permeability coefficient associated with the passive diffusion of drug molecules through a cell membrane is temperature-dependent. The lower the temperature, the more rigid the cell membrane becomes and the lower the passive diffusion of molecules across it.\textsuperscript{32} The decrease in temperature also reduces the motion of the molecules themselves and thereby the permeability constant. Furthermore, membrane fluidity is influenced by the composition of the membrane, which differs for various cell types.\textsuperscript{33} Besides membrane properties, the physicochemical properties of the drug also influence the rate of passive diffusion. Significant correlations between lipophilicity and membrane permeability have been reported in several studies.\textsuperscript{34} Ionization state of the drug is another important factor, where the uncharged form of a molecule can cross the cell membrane more easily than the charged.\textsuperscript{35} Partitioning into the hydrophobic interior of the cell membrane and diffusion across it is also benefitted by a low molecular weight.\textsuperscript{36}

**Carrier-Mediated Transport**

Endogenous compounds and drugs that do not readily permeate lipid bilayers may still be able to cross the hepatic cell membrane to a considerable extent through carrier-mediated transport. This transport mechanism is assisted by transporters, which are integral transmembrane proteins that allow for selective passage of molecules. The transport proteins are categorized as either influx or efflux transporters depending on the direction in which they transport substrates across the membrane.\textsuperscript{37} Influx or uptake transporters mediate transport of molecules into the cell, whereas efflux transporters decrease intracellular exposure by transporting the compound out of the cell. Some transporters are able to mediate transport in both directions.

There are two forms of carrier-mediated transport, active transport and facilitated diffusion, of which the latter consumes no energy.\textsuperscript{37} Active transport, on the other hand, requires energy and represents a way to transport molecules against their concentration gradient. Depending on the source of energy, active transport is either categorized as primary or secondary. Primary active transport relies on the direct hydrolysis of adenosine triphosphate (ATP) for substrate translocation. In contrast, secondary active transport involves the use of another energy source than ATP such as an ion gradient generated by ATP-dependent primary transporters.

The relation between the rate of transport ($v$) and substrate concentration ($[S]$) can be described by the Michaelis-Menten equation (eq. 2), originally developed to describe enzyme kinetics.\textsuperscript{38, 39}

$$v = \frac{v_{\text{max}} \times [S]}{K_m + [S]}$$

(2)
where $v_{\text{max}}$ is the maximal transport rate and $K_m$ is the substrate concentration yielding a transport uptake rate that is half of $v_{\text{max}}$. The maximal transport rate is the product of the total amount of transport protein and the turnover number, i.e., the number of substrate molecules translocated by a transport protein per unit time when the transporter is fully saturated with substrate. $K_m$, on the other hand, is independent of transporter expression. $K_m$ is generally thought to describe the binding strength of the transporter-substrate complex, but this only holds true if the dissociation rate of the complex is much faster than the rate of substrate translocation.  

The hepatocytes express several different transport proteins that distribute drugs across the membrane. The most important ones are depicted in Figure 3.

*Figure 3.* Schematic representation of hepatocytes with selected transport proteins mediating the transmembrane flux of drugs and endogenous substances. Uptake transporters in the basolateral membrane include organic cation transporter 1 (OCT1), organic anion transporter 2 (OAT2), sodium-taurocholate cotransporting polypeptide (NTCP), and three members of the organic anion transporting polypeptide (OATP) family (OATP1B1, OATP1B3, and OATP2B1). Basolateral efflux transporters are represented by two multidrug resistance-associated proteins (MRP3 and MRP4), while the canalicular membrane contains efflux transporters such as the breast cancer resistance protein (BCRP), bile salt export pump (BSEP), P-glycoprotein (Pgp), and MRP2.
Basolateral Uptake Transporters

The basolateral membrane of hepatocytes contains a large number of influx transporters belonging to the solute carrier (SLC) family. These transporters mediate the uptake of compounds from the sinusoidal blood. The SLC family includes passive (facilitative) transporters and secondary active transporters. The most important ones for hepatic drug uptake belong to the SLC10, SLC22, and SLCO gene subfamilies.5

The sodium-taurocholate cotransporting polypeptide (NTCP, SLC10A1) is expressed exclusively in the basolateral membrane of hepatocytes. NTCP is the predominant transporter involved in the uptake of bile acids in the liver, mediating electrogenic sodium-dependent transport with a stoichiometry of two sodium ions per bile acid molecule.41, 42 Its substrate specificity is not only restricted to bile acids though. NTCP also accepts various statins (or HMG-CoA reductase inhibitors) as substrates.43-45

Statin and bile acid uptake across the hepatic sinusoidal membrane is also mediated by organic anion transporting polypeptides (OATPs). In contrast to NTCP, the OATP transporters operate in a sodium-independent manner.46 The exact transport mechanism(s) is not fully understood, but it appears to be anion exchange.47, 48 At least three different OATP transporters are expressed in the human liver: OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1).5 Both OATP1B1 and OATP1B3 are liver-specific, while OATP2B1 is expressed in other tissues as well.49 These transporters have a broad and overlapping substrate profile, including drugs such as statins, angiotensin II receptor antagonists, HIV protease inhibitors, fexofenadine, and rifampicin.50-52 OATP-mediated uptake is reported to be the rate-limiting step in the elimination of several drugs.53, 54 In addition, reduced function genetic variants of OATP1B1 and inhibition of OATP-mediated uptake have been associated with increased plasma concentrations of substrate drugs.52

The organic anion transporter 2 (OAT2, SLC22A7) is mainly expressed in the liver, in contrast to the other kidney-localized OAT transporters.55 It accepts a range of structurally diverse compounds as substrates, including allopurinol, 5-fluorouracil, and paclitaxel.56 Like the OATP transporters, OAT2 is believed to transport substrates through anion exchange.56

The uptake of organic cations in the liver is primarily mediated by the polyspecific organic cation transporter 1 (OCT1, SLC22A1). OCT1 is a facilitative transporter, preferentially expressed in the liver, which can translocate molecules in both directions across the membrane. The transport by OCT1 is sodium-independent and driven by the membrane potential.57 Preferred substrates are low molecular weight and relatively hydrophilic cations such as the anti-diabetic drug metformin and the prototypical substrates tetraethylammonium and 1-methyl-4-phenylpyridinium.5 OCT1 is also involved in
the transport of the anticancer drug oxaliplatin. Other anticancer drugs that are likely OCT1 substrates include irinotecan and paclitaxel.39

**Canalicular and Basolateral Efflux Transporters**

The canalicular membrane of hepatocytes contains several efflux transporters that actively excrete exogenous and endogenous compounds and their metabolites into the bile. These include multidrug resistance-associated protein 2 (MRP2, ABCC2), P-glycoprotein (Pgp, ABCL1), breast cancer resistance protein (BCRP, ABCG2), and the bile salt export pump (BSEP, ABCB11). A different set of efflux transporters is present in the basolateral membrane, transferring compounds into the blood, e.g., MRP3 (ABCC3) and MRP4 (ABCC4). Both the canalicular and the basolateral efflux transporters belong to the ATP-binding cassette (ABC) family of transporters that directly hydrolyze ATP to transport molecules across the membrane.5

MRP2 is a major xenobiotic efflux pump at the canalicular membrane. It recognizes a wide range of compounds including statins, HIV protease inhibitors, anticancer drugs, and conjugated phase II metabolites.60 Like the OATPs, it mainly transports organic anions. The absence of functional MRP2 protein in the liver is associated with conjugated hyperbilirubinaemia resulting from impaired excretion of conjugated bilirubin.61 Besides the hepatocytes, MRP2 is located in the apical membrane of kidney proximal tubules and intestinal epithelia, where it also clears the body from xenobiotics.5

Pgp, also known as multidrug resistance protein 1 (MDR1), is another important transporter for biliary efflux of drugs and their metabolites. It is the most studied of the drug transporters, because of its involvement in multidrug resistance of tumor cells and its widespread expression in various cell barriers of the body, including the intestine, blood-brain barrier, and kidneys. Pgp has very broad substrate specificity and shares many substrates and inhibitors with CYP3A4.62 This observation along with the intestinal colocalization has resulted in the hypothesis of a co-operative interplay between Pgp and CYP3A4 in limiting the entry of xenobiotics into the blood circulation.63 The literature is, however, not unified on how these proteins interact to attenuate the oral bioavailability. While some researchers support the hypothesis that transport and metabolism work co-operatively,64-68 others propose that Pgp-mediated efflux may in fact reduce CYP3A4 metabolism in the intestine.69, 70 Substrates of Pgp are generally bulky, hydrophobic, and cationic. Examples of drugs transported by Pgp include digoxin, loperamide, and the anticancer drug doxorubicin.5

BCRP is a half transporter that forms a functional homodimer.71 Like the other efflux transporters, it is expressed not only in the liver but also in other tissues with barrier function, such as the placenta, intestine and blood-brain barrier.72 Substrates of BCRP include statins, anticancer drugs, and sulphate conjugates.5
BSEP is not involved in drug transport to the same extent as the other canalicular efflux transporters. Nevertheless, it transports a few drugs, including pravastatin. Instead, BSEP constitutes the rate-limiting step in the secretion of bile salts across the canalicular membrane of hepatocytes. There is no compensatory mechanism for loss of BSEP, manifested by symptoms of cholestasis in individuals with inherited BSEP dysfunction. In addition, inhibition of the BSEP transporter by drugs is associated with drug-induced liver injury through intracellular accumulation of bile salts, further demonstrating its essential role in bile acid homeostasis.

MRP3 is a sinusoidal efflux transporter expressed by hepatocytes. It mediates the transport of drugs and their metabolites from the liver into the blood. MRP3 shares 48% amino acid similarity with canalicularly localized MRP2. Like MRP2, it is involved in the transport of glucuronide conjugates and drugs such as methotrexate. The expression of MRP3 is markedly induced during cholestasis and in individuals with dysfunctional MRP2. This indicates that it has a protective role in the setting of impaired canalicular excretion by reducing hepatocellular accumulation of anionic conjugates.

MRP4 is another sinusoidal transporter which shows increased expression levels during cholestasis. MRP4 transports a range of drugs, covering antiviral, cardiovascular, and anticancer drugs.

Co-Existence of Passive and Active Transport

Net transport across a cellular membrane is the sum of passive diffusion and active carrier-mediated transport (Figure 4). The relative contribution of each depends on the physicochemical properties and the concentration of the drug. Carrier-mediated transport is more important for drugs with low passive permeability. Hence, active transport is more readily observed for hydrophilic drugs than for lipophilic ones. Because carrier-mediated transport is saturable, its contribution to the net transport will decrease with increasing drug concentration. The saturable feature is a result of the limited number of carrier proteins in the cell membrane. Because systemic blood concentrations typically are low in comparison to the number of transport proteins, saturation of hepatic uptake transporters is not expected in vivo.
Figure 4. Drugs must cross the cell membrane to enter the cell interior. The total (net) transport across the cell membrane is the sum of passive diffusion and active transport. Passive transmembrane diffusion is non-saturable and increases in proportion to the concentration gradient across the membrane. Active transport, on the other hand, is saturable and characterized by a maximal transport rate ($v_{max}$). Saturation occurs when the number of permeant molecules exceeds the number of carrier protein binding sites available for transport.

**Drug–Drug Interactions**

Concomitant administration of drugs may cause altered exposure of them, a situation referred to as pharmacokinetic drug-drug interaction (DDI). This is a result of the interference of one of the drugs (perpetrator) with one or more of the determinants accounting for the pharmacokinetics of the other drug (victim drug). Processes that can be affected include drug absorption, distribution, metabolism, and excretion. Depending on the direction of change (inhibition or induction), drug exposure will either increase or decrease. Increased plasma concentration is associated with an increased risk of adverse events, whereas decreased drug levels may result in a loss of drug efficacy. Clinical DDIs can cause severe outcomes. For example, co-administration of cerivastatin and gemfibrozil (an inhibitor of the hepatic uptake of cerivastatin) was reported in 12 of 31 cases of fatal rhabdomyolysis in the United States before cerivastatin was withdrawn from the market.82

**In vitro Methods for Studying Hepatic Drug Transport and Transporter-Mediated Inhibition**

Several different experimental systems of varying complexity have been developed for the study of transport processes. Each has its advantages and weaknesses. The sections below presents, in order of decreasing complexity, the assay systems commonly used to assess transport function and activity. A subset of the assay systems is depicted in Figure 5.
Figure 5. Some in vitro models used to study hepatic drug transport and transporter-mediated inhibition. (A) Primary human hepatocytes cultured between two layers of extracellular matrix (a so-called sandwich configuration) form intact bile canaliculi between the cells which enables vectorial transport studies. SCHH is a complex cell system with expression of most or all transport proteins important for drug disposition in the liver. (B) Immortalized cell lines such as human embryonic kidney (HEK) 293 cells are anchorage-dependent and form monolayers when grown on a solid support. The cell lines can be transfected with one or more transporters of interest for the study of drug uptake and/or efflux. The transfected cell lines represent a simpler assay system than SCHH. (C) Inverted membrane vesicles are derived from transfected cells. They are typically used to study efflux transporters. Because of the inverted configuration, drugs can interact directly with the efflux transporter without having to cross the cell membrane. This is the simplest assay system.

Primary Human Hepatocytes

Hepatocytes isolated from human liver tissue are considered the gold standard for studies of drug metabolism and transport activity. This experimental system is one of the most physiologically relevant ones available today with the advantage of expressing the full complement of drug transporters and metabolizing enzymes present in the liver. Isolated hepatocytes are a versatile tool that can be used as both suspensions and plated monolayers. Because of the anchorage-dependence of hepatocytes, the cells only survive for a few hours in suspension. Hepatocytes plated in conventional monolayers, on the other hand, survive for about a week. The plated cells are, however, associated with a dedifferentiation over time in culture. Attempts have been made to restore or maintain hepatocyte-specific functions by optimizing the culture conditions. One such way is the use of sandwich-cultures, explained below.

Sandwich-Cultured Human Hepatocytes

The in vivo polarity of hepatocytes is lost upon isolation, leading to the inability to assess vectorial transport of compounds. The polarity can be re-established by growing the hepatocytes in a so-called sandwich configuration, i.e., between two layers of an extracellular matrix. This allows the cells to regenerate tight junctions and to form bile canaliculi-like structures between them. By modulating the tight junctions in the presence or absence of divalent cations (Ca$^{2+}$ and Mg$^{2+}$), canalicular efflux can be assessed.
Sandwich-cultured human hepatocytes (SCHH) maintain morphology and function for up to six weeks. Culture conditions may, however, alter protein expression of transporters and other liver-specific proteins. While sandwich-cultured rat hepatocytes (SCRH) show significant time-dependent down-regulation of uptake transporters, the expression and function in SCHH is better maintained. In contrast to the uptake transporters, efflux transporters are upregulated over time in SCRH. SCHH also display increased expression of certain efflux transporters with culture time, whereas others are more stable in their expression.

Human Hepatoma Cell Lines

Because of the scarcity and unpredictable availability of human liver tissue, attempts have been made to generate hepatocyte cell lines from hepatocellular carcinomas as an alternative to isolated primary human hepatocytes. The hepatocyte cell lines also benefit from a more stable phenotype than the primary hepatocytes.

One of the most widely used human hepatocyte cell lines is HepG2. Although HepG2 cells are highly differentiated and show many liver-specific functions, they express very low levels of drug-metabolizing CYP enzymes and transporters compared with the expression in primary hepatocytes and liver tissue, respectively. The low CYP expression in HepG2 cells is likely a result of altered expression of transcription factors regulating these enzymes. This might also explain the weak response to different CYP inducers. The use of HepG2 cells for drug disposition studies is therefore limited.

In contrast to the HepG2 cell line, differentiated HepaRG cells express phase I and II metabolizing enzymes at levels closer to those measured in freshly isolated human hepatocytes. Because of the expression of key nuclear receptors involved in CYP induction, HepaRG cells show similar induction patterns as primary cultured human hepatocytes. In addition, polarized expression of drug transporters has been demonstrated in the HepaRG cell line. Although HepaRG cells better reflect primary hepatocytes in their gene expression pattern than HepG2 cells, they still generally express lower levels of CYP enzymes, with the exception of CYP3A4.

Transfected Immortalized Cell Lines

Immortalized cell lines can be transfected (stably or transiently) with one or more transport proteins of interest, which enables the study of a single or a limited set of transporters at a time. This presents a more defined assay system than the more complex primary hepatocytes and hepatic cell lines. Immortalized cell lines that are commonly transfected for transport studies include human embryonic kidney (HEK) 293, Madin-Darby canine kidney
(MDCK), and Chinese hamster ovary (CHO) cells. The HEK293 cells have the advantage of being of human origin and of expressing low endogenous levels of drug transporters. Transfected cell lines are useful tools for the characterization of substrates and inhibitors of individual transporters; information that can be used to develop quantitative structure-activity relationship (QSAR) models. They can also be used to investigate transport mechanisms and differences in transport activity as a result of genetic polymorphisms.

Most transfected cell lines are grown as monolayers on a solid support. Some cell lines, such as MDCK cells, are polarized, which enables vectorial transport studies. Vectorial assay systems allow for the study of drug flux in two directions: apical-to-basolateral (A-B) and basolateral-to-apical (B-A). These polarized cells are commonly used to study efflux transporters by measuring the B-A/A-B flux ratio.

Xenopus Laevis Oocytes

*Xenopus laevis* oocytes are germ cells surgically harvested from South African clawed frogs. The *Xenopus laevis* oocyte system is a valuable expression tool for studies of membrane transporters. The cells are large in relation to other cells (diameter of 1.2 mm), which makes them easy to handle. By microinjection of mRNA, the oocytes start to synthesize proteins in high amounts. Because of the injection of each individual cell, a high proportion of cells expressing the transferred genetic material is obtained. The cells can be injected with anything from RNA material coding for a single transporter to total liver mRNA. Another great advantage with this expression system is the limited number of endogenous transporters, resulting in a very low background transport activity.

Inverted Membrane Vesicles

Inverted plasma membrane vesicles are prepared from cells or tissue expressing a transport protein of interest. The selection of cell/tissue type influences the percentage of inside-out oriented vesicles obtained from the preparation. This fraction typically varies between 30 and 40%. Inverted plasma membrane vesicles are primarily used to study efflux transporters as a complement to cell-based assay systems. The membrane vesicles allow for a direct interaction of substrate with the cytoplasmic side of the efflux transporter as a result of the inside-out vesicle orientation. This is a clear advantage over the cell-based assay system in which the substrate first has to pass the cell membrane before it can interact with the efflux transporter. The direct interaction with the transporter is particularly important for determinations of transport kinetics and transport mechanisms, which require that changes in assay conditions have a direct impact on the transport process.
instead of being restricted by the cell membrane barrier. However, transport kinetics of lipophilic drugs that easily permeate cell membranes may be difficult to determine using membrane vesicles because of a high diffusion rate out of the vesicle.\textsuperscript{115}

Predictions of Hepatic Transporter-Mediated Clearance and Drug–Drug Interactions \textit{in vivo}

Accurate predictions of hepatic drug disposition from \textit{in vitro} data are highly beneficial in drug development to identify candidates with inappropriate \textit{in vivo} pharmacokinetics at an early stage. In this context, it is not only important to estimate the rate of elimination but also to get an extensive understanding of the transporters and metabolizing enzymes involved. A characterization of major contributors to the hepatic disposition provides an early assessment of the potential for DDIs and interindividual differences in pharmacokinetics due to polymorphic genotypes. The greater the contribution of a single enzyme/transporter, the greater the potential for a clinically relevant DDI.

Drug elimination in pharmacokinetics is usually described by the term clearance (CL).\textsuperscript{116} Clearance is a measurement relating the elimination rate to drug concentration in the systemic circulation (eq. 3). It can also be interpreted as the volume of blood that is cleared of drug per unit time.

\[
\text{Elimination rate} = \text{CL} \times [S]
\]

\begin{equation}
\text{CL} = \frac{v_{\text{max}}}{K_m + [S]}
\end{equation}

\textit{In vitro–in vivo} Scaling of Hepatic Drug Transport

When studying transport processes \textit{in vitro}, a common aim is to extrapolate the data to the \textit{in vivo} situation. Because \textit{in vitro} systems may differ in transport activities compared to the tissue of interest,\textsuperscript{95} strategies for scaling the data to the \textit{in vivo} situation need to be established.

Transport data obtained from primary hepatocyte experiments are typically scaled directly to the \textit{in vivo} situation by multiplying the measured CL by the number of hepatocytes in the liver and by liver weight.\textsuperscript{54, 117, 118} This scal-
ing approach implicitly assumes that the isolated hepatocytes are identical to their *in vivo* counterpart with regard to transport function.

Transfected cell lines and membrane vesicles, on the other hand, usually differ considerably in transport activity compared to hepatocytes in the liver. Scaling data from these experimental systems therefore requires correction for this difference. This is done on an individual transporter basis. Various methods can be used to assess the difference in transport activity, including activity measurements with transporter-specific substrates and protein expression determinations. The data can then be further scaled by the number of hepatocytes in the liver and by liver weight to yield whole liver clearance.

**Separating Active and Passive Transport**

Regardless of the experimental system used, total measured flux across the membrane needs to be separated into the individual transport processes, i.e., active carrier-mediated transport and passive diffusion. Several different approaches exist.

For studies using transfected cell lines, the most common approach is to do parallel studies in mock-transfected control cells. If these cells have low endogenous expression of transporters, any uptake will be due mainly to passive diffusion. With knowledge of the passive permeability, this component can be subtracted from the total transport in the transporter-transfected cells to obtain an estimate of the active transport. The same approach can be used with membrane vesicles although an alternative method also exists. Because efflux transporters directly hydrolyze ATP for the substrate translocation, passive permeability in membrane vesicles can be determined by incubations with adenosine monophosphate (AMP) instead of ATP.

Unfortunately, for studies of drug transport in hepatocytes, there are no corresponding control cells with no or low transport activity. Traditionally, incubations at 4°C have been used to assess the passive permeability in these cells, the rationale being that active processes are inhibited at low temperature. The passive permeability is, however, also affected by temperature. The increase in membrane rigidity at low temperature leads to an underprediction of the passive diffusion. Recently, pan-transporter inhibitors have been implemented to measure the passive component of the hepatic uptake. A cocktail of inhibitors is another option. The use of inhibitors for quantifying the passive component of the uptake relies on a complete inhibition of the active transport. If this is not achieved, the passive diffusion will be either under- or overestimated depending on whether efflux or uptake transporters are still active. Mechanistic modeling approaches to simultaneously estimate active and passive processes from *in vitro* transport data have also been tried. However, the use of mathematical models is demanding in that it requires many experimental data points for parameter fitting.
Factors to Consider when Scaling Passive Permeability and Active Carrier-Mediated Transport

A number of factors affect the accuracy of in vitro–in vivo extrapolation (IVIVE) approaches. Below, some factors are discussed that may be of importance, but that are not always taken into account.

In any in vitro experimental assay, the total amount of drug added to the incubation is known. It is assumed that this concentration is the one driving both the passive and active transport. However, a compound may bind non-specifically to the incubation vessel, medium components (e.g., proteins if such are added), or the membrane of the biological system, reducing the free concentration of drug available for transport. This non-specific binding is generally higher for more lipophilic compounds.\textsuperscript{125}

Clearance by passive permeability is dependent on the membrane surface area across which transport occurs. The surface area in contact with drug solution should therefore be considered for accurate IVIVE. For example, suspended hepatocytes have a greater surface area exposed to the incubation medium than hepatocytes in intact liver tissue have to the sinusoidal blood.

Transporter-mediated CL is determined by the kinetic parameters $K_m$ and $v_{\text{max}}$. While $v_{\text{max}}$ depends on transporter expression (which usually differs between in vitro systems and hepatocytes in the liver), the Michaelis-Menten constant $K_m$ is assumed to be the same in vivo as in vitro. However, the apparent $K_m$ determination in vitro is affected by the presence of an unstirred water layer (UWL).\textsuperscript{126} The greater this UWL is, the greater the bias in the $K_m$ determination will be. Moreover, if efflux transport kinetics in a cell assay experiment is approximated on the basis of the extracellular concentration instead of the intracellular one, the apparent $K_m$ may be very different from the true $K_m$.\textsuperscript{127}

In vitro–in vivo Scaling of Transporter-Mediated Drug–Drug Interactions

In vitro–in vivo extrapolations are performed not only to predict drug CL but also to predict potential DDIs. Because the substrate specificities of transporters are broad and often overlap with each other, multiple transporters may be involved in the disposition of a drug. The relative contributions of these transporters to the total flux therefore need to be determined for accurate estimations of the change in in vivo CL upon inhibition of one or several of them.

Estimating Transporter Contributions

Several different approaches to predict transporter contributions have been developed. Many of them originated in the field of drug metabolism, but are now applied to the quantitative characterization of transporter contributions.
The relative activity factor (RAF) approach is one such method.\textsuperscript{120} It estimates the relative contributions of uptake transporters to the net active transport of a test compound by using transporter-specific substrates as reference compounds. The RAF value is determined (eq. 5) by relating the uptake clearance of the reference compound in human hepatocytes (CL\textsubscript{Hep,Reference}) to that in an experimental system (CL\textsubscript{Exp,Reference}) expressing a single transporter (e.g., an overexpressing cell line). This RAF value is a measure of the functional activity of the transporter in hepatocytes relative to that in the experimental system.

\[
\text{RAF} = \frac{\text{CL}_{\text{Hep,Reference}}}{\text{CL}_{\text{Exp,Reference}}}
\]  

(5)

The uptake clearance of the test compound in human hepatocytes (CL\textsubscript{Hep,Test}) is subsequently predicted by multiplying the uptake clearance in the experimental system (CL\textsubscript{Exp,Test}) by the RAF value (eq. 6).

\[
\text{CL}_{\text{Hep,Test}} = \text{RAF} \times \text{CL}_{\text{Exp,Test}}
\]  

(6)

For many transporters, their specific substrates are unknown, which limits the applicability of the RAF method. Instead, an analogous methodology can be used that relies on transporter expression levels as a substitute of functional activity measurements. This method, referred to as the relative expression factor (REF) approach,\textsuperscript{120} assumes a linear correlation between protein expression and transport activity. By relating the expression in human hepatocytes or liver tissue to that in the experimental system used to study transport function, \textit{in vivo} CL can be predicted.

Other tools that can be used to estimate transporter contributions include selective gene silencing or gene knock-down in hepatocytes by antisense oligonucleotides and RNA interference.\textsuperscript{113, 128} These methods reduce transporter expression by interfering with the mRNA translation. Transporter contribution can also be estimated by using selective inhibitors. For transporters dependent on specific cofactors, e.g., the sodium-dependent uptake transporter NTCP, yet another approach exists. The transporter can be studied in the absence and presence of the cofactor to determine its contribution.\textsuperscript{44}

**Predicting Change in Exposure by a Transporter-Mediated Drug–Drug Interaction**

With knowledge of the relative transporter contributions, predictions of the impact of a transporter-mediated DDI on the \textit{in vivo} transporter-mediated CL can be obtained using a static model (eq. 7).\textsuperscript{129}
where $\frac{\text{CL}_{\text{transporter}, +1}}{\text{CL}_{\text{transporter}, -1}}$ is the transporter-mediated CL in the presence and absence of inhibitor respectively, $f_{c,j}$ is the fraction of transport over a given membrane mediated by transport protein $j$, $[I]$ is the inhibitor concentration, and $K_{i,j}$ is the inhibition constant for transport protein $j$. A considerable change in CL ($> 2$-fold) is expected only if the transport protein contributes to more than 50% of the total transport across the membrane and the inhibitor concentration exceeds the $K_i$.

It is the unbound fraction of the drug which exhibits the inhibitory function; hence, the unbound concentration of inhibitor should be used in the DDI predictions. Although the inhibitor concentration at the target site changes over time in vivo, the static model maintains the maximal unbound concentration to avoid false-negative predictions. This leads to an overestimation of the DDI risk. For hepatic uptake transporters, the inhibitor concentration is described by the unbound concentration in the blood, whereas efflux transporters are exposed to the unbound concentration in hepatocytes. Given that the liver is involved in the first pass extraction of drugs, the maximal unbound concentration measured systemically (after passage through the liver) is less than that at the inlet to the liver. Practical aspects, however, limit the measurement of inhibitor concentrations in the hepatic portal vein. Instead, the maximal unbound concentration at the inlet to the liver ($I_u,in,max$) can be estimated by equation 8.

$$I_u,in,max = I_u,max \times \frac{\text{fraction unbound} \times \text{absorption rate constant} \times \text{intestine availability} \times \text{Dose}}{\text{hepatic blood flow rate}}$$

Mechanistic Pharmacokinetic Modeling

Mechanistic pharmacokinetic models are useful tools in the study of complex systems such as the liver and its role in drug disposition. These models are typically developed as a set of mathematical equations that describe the processes underlying the behavior of the system being modeled. A major challenge in mechanistic modeling is to incorporate enough detail to accu-
rately predict the output, while limiting the complexity to avoid overly long simulation times. Every model therefore relies on one or more assumptions. A substantial benefit of the mechanistic approach of modeling is that it can simulate the influence of various parameters on the final outcome.\textsuperscript{132}

Mechanistic pharmacokinetic models can be used to predict drug disposition and drug–drug interactions \textit{in vivo}.\textsuperscript{133, 134} The dynamic feature of these models allows for the assessment of DDIs on the basis of temporal changes in the free concentration of inhibitor and substrate at the site of interaction. This should provide more realistic predictions of DDI risks than those obtained using static models, provided that the dynamic model incorporates enough detail to describe the system properly. Furthermore, the dynamic models allow for simultaneous interplay of multiple processes that influence drug disposition, such as hepatic uptake and metabolic/biliary elimination.

\textbf{In silico Predictions of Transport Inhibitors}

Statistical multivariate models can identify correlations between variables in large data sets. This can be exploited to derive QSAR models that predict a given property (e.g., transport inhibition) from a set of molecular descriptors.\textsuperscript{135} The molecular descriptors describe the chemical information contained in the molecule numerically. Examples of molecular descriptors include molecular weight, number of atoms, and maximum positive charge.

The QSAR models are developed using a training set, i.e., a set of molecules for which there are experimental data on the property in question. The models are consequently only as good as the data upon which they are based. Once the model is trained, it can be used to make predictions on molecules not included in the training set. Generally, reliable predictions can only be expected for molecules that are similar to those included in the training set.\textsuperscript{136}

\textbf{Proteomics}

The extent of hepatic drug elimination is dependent on the abundance and activity of the transporters and enzymes involved in its disposition. Historically, abundance has been assessed by gene expression analysis. However, many reports have shown that protein levels correlate better with activity than mRNA levels do.\textsuperscript{137-139} This is not surprising given the many processes that influence protein abundance besides mRNA transcription. For example, translation rates vary for different mRNA transcripts and explain much of the differences in protein abundance.\textsuperscript{140} Translation is also subject to regulation by e.g., microRNAs, which can repress the translation of mRNA transcripts.\textsuperscript{141} Since proteins, rather than mRNA, are responsible for carrying out cellular functions, protein abundance measurements are considered useful in
estimating cellular activities. Traditional immunological methods are, at best, semi-quantitative. Instead, mass spectrometry has emerged as a more accurate method of quantifying protein abundance. There are two different types of quantitative mass spectrometry methods: label-based and label-free quantification. Both methods rely on the measurement of peptide levels as a surrogate for the protein. The peptides are produced by enzymatic cleavage of the proteins in the sample by different proteases. Trypsin is the most commonly used, but depending on the protein of interest another protease may produce better-suited peptides for the analysis. 

**Label-Based Quantification of Protein Abundance**

The label-based approach of protein quantification, also known as targeted proteomics, focuses on determining the concentration of a specific protein (or set of proteins) of interest. It involves the use of a labeled standard that is spiked into the sample in a known amount. Because the quantification strategy involves relative quantification against an internal standard, the accuracy is only as good as the quantification of the standard itself.

The standard can be introduced at different stages of the sample preparation, either as an isotope-labeled protein or isotope-labeled peptide. Protein standards are introduced earlier in the workflow (before protein digestion), whereas peptide standards are introduced later (during or post-digestion). The earlier the standard is introduced, the better. An early introduction can eliminate systematic errors in the sample handling, such as incomplete protein digestion. Purified transport protein standards are, however, expensive and difficult to produce. Hence, the most common approach for targeted transporter quantification is to use peptide standards. Accurate determination of protein levels with this approach requires complete protein digestion since the stoichiometric relationship between the amount of proteolytic peptide and corresponding protein is not established.

Because peptides are quantified as surrogates for protein, selection of suitable target peptides is critical for the label-based quantification. The surrogate peptide has to be unique for the target protein and have optimal properties for mass spectrometry analysis. In addition, it is important that the selected peptide does not include any known sites for post-translational modifications or single nucleotide polymorphisms.

**Label-Free Quantification of Protein Abundance**

While label-based protein quantification with spiked-in standards is restricted to a limited set of target proteins, label-free shotgun proteomics can be used for whole proteome analyses. Shotgun proteomics has historically been used for qualitative analysis of proteins (i.e., protein identification), but more recently label-free protein quantification methods have emerged. One such
example is the total protein approach (TPA), which has proven to provide accurate estimations of protein abundance.\textsuperscript{149, 150} The TPA-based quantification of proteins relies on information obtained directly from the mass spectrometer readout and does not require any standards. A prerequisite, however, is a sufficient depth of the proteomic analysis since the underlying assumption is that the detected proteins constitute the major part of the proteome by mass. The number of proteins required for an appropriate analysis depth will vary with cell/tissue type. For example, the plasma proteome is dominated by less than thirty abundantly expressed proteins,\textsuperscript{151} whereas hepatocytes express thousands of proteins at relatively high levels.\textsuperscript{152} Like other label-free protein quantification methods, the TPA approach assumes proportionality between measured peptide intensities and protein quantities. Under this assumption, the fractional value of the summed signal intensities of all peptides identifying a particular protein to the total MS-signal describes the protein abundance as a fraction of total mass.

Statins as Model Drugs

Statins are a class of widely prescribed drugs to lower cholesterol levels. They mediate their effect through inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway.\textsuperscript{153} The target is located in the liver, which is also the major eliminating organ of this drug class. Statins are generally well tolerated, but up to 10\% of persons experience myopathy.\textsuperscript{154, 155} These muscle symptoms can also, in rare cases, lead to life-threatening rhabdomyolysis.\textsuperscript{156}

In 2008, a genome-wide association study discovered that patients with reduced function genetic variants of the hepatic uptake transporter OATP1B1 are at increased risk of statin-induced myopathy.\textsuperscript{157} This association is supported by numerous clinical studies that report of increased statin exposure in individuals with reduced function genetic variants of OATP1B1.\textsuperscript{158-161} Another risk factor for statin-induced myopathy is transporter-mediated DDIs resulting in decreased hepatic uptake and, hence, increased systemic concentration of the statin.\textsuperscript{162}

Statins are administered either in the active acid form or as the more lipophilic lactone prodrug, which requires conversion to the active form via hydrolysis. These two forms co-exist \textit{in vivo} due to reversible interconversion. Although all statins are substrates of transport proteins, they differ in their interaction patterns and hence, their dependency on specific transporters for hepatic elimination. Some statins are metabolized to a large extent, rendering them vulnerable for metabolic DDIs as well. The section below summarizes the knowledge of hepatic transporters and metabolizing enzymes involved in the pharmacokinetics of the two statins used as model drugs in this thesis,
atorvastatin and pitavastatin (Figure 6). Both of these statin drugs are administered in their active acid form.

Figure 6. Molecular structures of atorvastatin and pitavastatin acid.

Hepatic Disposition of Atorvastatin

Hepatic clearance of atorvastatin occurs through transporter-mediated uptake followed by metabolism. The metabolites are mainly eliminated by excretion into bile, while renal secretion represents a minor elimination pathway (< 2%).

At least four transporters on the basolateral membrane of human hepatocytes recognize atorvastatin as a substrate. These are OATP1B1, OATP1B3, OATP2B1, and NTCP. At the onset of this work, OATP1B1 was known to play an important role in the hepatic uptake as demonstrated by altered pharmacokinetics of atorvastatin in individuals with SLCO1B1 polymorphism. Shitara and colleagues estimated a 47% contribution of OATP1B1 to the total hepatic uptake from the observed change in the area under concentration-time curve (AUC). A similar contribution of OATP1B1 (50%) was reported in a recently published study as predicted from protein abundance and activity data. The same study predicted OATP1B3-mediated uptake to represent around 13% of the total active uptake of atorvastatin.

Following hepatic uptake, atorvastatin is almost completely metabolized by CYP3A4. Because the lactone form of atorvastatin has a much higher affinity for CYP3A4 than the acid form, metabolism of the lactone form is expected to be the relevant metabolic clearance pathway. The lactone metabolites can be interconverted to the active open acid metabolites ortho- and parahydroxylated atorvastatin. Both the parent compound and its two active metabolites can also undergo glucuronidation by UGT1A1 and UGT1A3 followed by subsequent lactonisation. In addition, the glucuronidated metabolites may undergo deconjugation back to the active metabolites.

Atorvastatin and its metabolites are primarily eliminated into the bile. Transporter knock-down studies in Caco-2 cells indicate that atorvastatin is transported by Pgp, BCRP, and MRP2. ATPase activity in membranes expressing MRP2 also suggests MRP2-mediated transport. Experiments in a BCRP-expressing cell line confirmed atorvastatin to be a BCRP
substrate. In addition, clinical studies show that \textit{ABCB1} and \textit{ABCG2} polymorphisms are associated with increased AUC of atorvastatin, indicating that both Pgp and BCRP are important for the \textit{in vivo} disposition.

**Hepatic Disposition of Pitavastatin**

In contrast to atorvastatin, which is heavily metabolized, pitavastatin is mainly eliminated unchanged into the bile. Renal excretion is involved in the elimination to a lesser extent. Because of its efficient biliary excretion and high bioavailability compared to most other statins, pitavastatin has been suggested to undergo enterohepatic recirculation. 

Pitavastatin is an \textit{in vitro} substrate of OATP1B1, OATP1B3, OATP2B1, and NTCP. Predictions of the OATP1B1-mediated contribution to the total active uptake \textit{in vivo} range from 32\% to 98\%. The large (162–191\%) increase in pitavastatin AUC in individuals with OATP1B1 polymorphisms compared to those expressing the wildtype variant suggests that OATP1B1 is critical to the \textit{in vivo} disposition. In line with this, the \textit{in vivo} data indicate that 70\% of pitavastatin is taken up by OATP1B1. OATP1B3 is predicted to play a less important role in the uptake with an estimated contribution of 1.8-25\%. Early studies of the hepatic uptake of pitavastatin in the presence and absence of sodium suggested that pitavastatin is mainly taken up in a sodium-independent manner. This indicated that NTCP was not important for the uptake. More recent data, however, assign 29\% of the total active uptake across the basolateral membrane of hepatocytes to NTCP. The role of OATP2B1 in pitavastatin disposition has not been investigated.

Pitavastatin is metabolized to a very limited extent. The major metabolite formed is pitavastatin lactone, which can be reversibly converted to the parent drug. The lactone metabolite is formed by UGT-mediated glucuronidation, followed by non-enzymatic conversion of the glucuronidated metabolite to the lactone form. Pitavastatin is also subjected to oxidative metabolism by CYP2C9, producing the minor metabolite 8-hydroxypitavastatin.

Transporter-mediated biliary excretion is the major clearance route for pitavastatin. While Pgp did not interact with the acid form of pitavastatin in one \textit{in vitro} study, other research groups have observed Pgp-mediated transport of pitavastatin \textit{in vitro}. A recent clinical study found an association between a single nucleotide polymorphism in \textit{ABCB1} and pitavastatin AUC, validating the role of Pgp in pitavastatin disposition. Pitavastatin is also a substrate of BCRP \textit{in vitro}, but its pharmacokinetics is not influenced by genetic variants of BCRP \textit{in vivo}. In addition, MRP2 has been shown to transport pitavastatin \textit{in vitro}. A genetic variant of MRP2 is associated with altered pitavastatin exposure.
Aims of the Thesis

The general objective of this thesis was to provide new predictive *in silico* and *in vitro* models of *in vivo* hepatic transporter-mediated drug clearance and drug–drug interactions.

The specific aims were:

- To investigate the inhibition overlap of OATP1B1-, OATP1B3-, and OATP2B1-interacting drugs
- To develop *in silico* models for the prediction of OATP inhibitors
- To develop static models for the prediction of *in vivo* transporter-mediated clearance and drug–drug interactions from *in vitro* experiments and protein abundance data
- To map and compare the abundance of transporters and other proteins of importance for drug disposition in human liver tissue and isolated human hepatocytes
- To develop a dynamic mechanistic pharmacokinetic model for the prediction of hepatic drug disposition from *in vitro* experiments and protein abundance data
Methods

Compound Selection
In paper I, the aim was to develop in silico models that could predict OATP inhibitors on the basis of their physicochemical properties. For the model to be applicable to a wide range of drugs, compounds were chosen to represent the chemical space of orally administered drugs. In addition to the drug-likeness and molecular diversity, compounds known to interact with the OATP transporters were included as positive controls and to increase the number of hits for computational modeling. To enable high-throughput screening of OATP inhibition, radioactively labeled compounds (estradiol-17β-glucuronide and estrone-3-sulphate) were used as probe substrates.

Papers I-II investigated the hepatic uptake clearance of atorvastatin in absence and presence of inhibitors. Atorvastatin was chosen as a model drug due to its dependence on multiple transporters (OATP1B1, OATP1B3, OATP2B1, and NTCP) for its hepatic uptake. Although atorvastatin was known to be a substrate for these transporters in vitro, their relative contribution to the uptake in vivo was at the time unknown.

In papers III-IV, pitavastatin was used as a model drug. Unlike atorvastatin, which is heavily metabolized in vivo, pitavastatin is mainly excreted unchanged in the bile. Consequently, its hepatic disposition is almost completely dependent on active transport processes, which was the reason for its selection. Paper III characterized the uptake of pitavastatin in human liver and hepatocytes. For a further understanding of hepatic disposition of pitavastatin, the interplay between uptake and efflux in sandwich-cultured human hepatocytes was investigated in paper IV using a mechanistic pharmacokinetic model.

Kinetic Characterization of Transporter-Mediated Uptake and Efflux
Papers I-IV used HEK293 cell lines stably transfected with a single transporter (OATP1B1, OATP1B3, OATP2B1, or NTCP) to study transporter-mediated uptake of drug compounds and probe substrates. Active transport via the efflux transporters BCRP, Pgp/MDR1, MRP2, MRP3, and MRP4
was studied using inverted membrane vesicles derived from HEK293 and Sf9 cells (paper IV).

The kinetic parameters describing transporter-mediated uptake/efflux were determined by measuring initial transport rate at varying substrate concentrations. In brief, cells or inverted membrane vesicles were incubated with substrate for a short interval. All experiments were performed in the initial linear range of the time-dependent uptake. Transport was stopped by the addition of cold buffer and the cells/membrane vesicles were washed before analysis of accumulated substrate by either scintillation counting (probe substrates) or liquid chromatography-tandem mass spectrometry (LC-MS/MS, drug compounds).

The relation between measured transport rate \( (v) \) and substrate concentration \( ([S]) \) was described by the Michaelis-Menten equation with the addition of a non-saturable passive diffusion \( (P_{\text{dif}}) \) rate component (eq. 9). Using non-linear regression, the maximal transport rate \( (v_{\text{max}}) \) and the substrate concentration at which the uptake rate is half of \( v_{\text{max}} \) \( (K_m) \) were determined and used to describe transport kinetics.

\[
v = \frac{v_{\text{max}} \times [S]}{K_m + [S]} + P_{\text{dif}} \times [S]
\]

\( (9) \)

**In vitro Screening of Transport Inhibition**

In papers I and II, transfected HEK293 cells and isolated human hepatocytes were used to study inhibition of transporter-mediated uptake, respectively. Cells were incubated with a substrate concentration at or below \( K_m \) in the presence or absence of a potential inhibitor, allowing for the calculation of percent inhibition. In paper I, inhibition was screened at a single concentration of 20 µM. Compounds that inhibited the uptake by at least 50% were classified as inhibitors. In paper II, inhibition was screened at in vivo-relevant concentrations. In addition, concentration-dependent inhibition in HEK293 cells was studied in both papers I and II. The half maximal inhibitory concentration \( (IC_{50}) \) was determined by fitting the data to equation 10.

\[
\text{Transport (% of control)} = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{[log[I] - log IC_{50}] \times \text{Hill slope}}}
\]

\( (10) \)

where \( [I] \) is the inhibitor concentration and the Hill slope describes the steepness of the curve. A top plateau value of 100% and a bottom plateau value of 0% were used as constraints when fitting the data. This was to allow for estimations of IC_{50}-values despite occasionally incomplete inhibition curves due to solubility limitations of the perpetrator drug. The IC_{50}-values
were used to calculate corresponding inhibition constants, $K_i$, with the relationship described by equation 11 (assuming competitive inhibition).

\[
K_i = \frac{\text{IC}_{50}}{1 + \frac{S}{K_m}}
\]  

(11)

Quantifying Isoform Specificity of Inhibitors

The promiscuity (or inversely, the specificity) of the inhibitors in paper II was calculated using the index of inhibition promiscuity introduced by Nath et al.\cite{180} This entropy-based metric of drug promiscuity requires the definition of inhibitor potencies ($x_i$) such that $x_i$ is higher for more potent inhibitors and always greater than zero. Inhibitor potency was defined by equation 12.

\[
x_i = -\log \frac{\text{IC}_{50}}{1000}
\]  

(12)

For pairs of transporters and perpetrator drugs that did not display sufficient inhibition for accurate IC$_{50}$ determinations, the IC$_{50}$-value was set to 999 µM. The inhibition promiscuity ($I_{inh}$) for each perpetrator drug was subsequently calculated using equation 13.

\[
I_{inh} = -\frac{1}{\log N} \sum_{i=1}^{N} \frac{x_i}{\sum_{j=1}^{N} x_j} \log \frac{x_i}{\sum_{j=1}^{N} x_j}
\]  

(13)

where $N$ is the number of transporters in the panel, $x_i$ is the inhibitory potency toward transporter $i$, and $x_j$ is the inhibitory potency toward transporter $j$. The index of inhibition promiscuity can take on any value between 0 (indicating a completely specific inhibitor) and 1 (indicating a completely promiscuous inhibitor with equal potency against all transporters). The specificity was calculated as $1 - I_{inh}$.

Isolation and Culture of Primary Human Hepatocytes

Non-tumorous excess human liver tissue was obtained from liver resections under ethical approval (ethical approval no. 2009/028 and 2011/037). All donors gave their informed consent. Human hepatocytes were isolated from
the liver tissue according to the two-step collagenase perfusion method. In brief, the tissue was first perfused with a calcium-free buffer containing the chelating agent EGTA (ethylene glycol tetraacetic acid). Calcium was removed to rapidly disrupt intercellular junctions, resulting in the loss of cell-cell contacts. In the second step, the tissue was perfused with collagenase to digest the connective tissue of the liver, resulting in the release of dispersed hepatocytes after completed digestion. The crude hepatocyte preparation was subsequently filtered to remove undigested material and washed three times by centrifugation to remove dead and damaged cells. The second wash consisted of a Percoll density centrifugation to remove NPCs and enrich for viable hepatocytes.

Isolated hepatocytes were suspended in Dulbecco’s Modified Eagle’s Medium supplemented with fetal bovine serum, penicillin-streptomycin (PEST), insulin, and dexamethasone. Cells were seeded in 24-well collagen-coated plates at a density of 375,000 viable cells per well and were allowed to attach for 2-3 h at 37°C and 5% CO₂ atmosphere. After attachment, medium was replaced with Hepatocyte Maintenance Medium supplemented with PEST, insulin-transferrin-selenium, and dexamethasone. For cultures kept longer than 24 h, hepatocytes were overlaid with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Sandwich-cultured hepatocytes were maintained in culture for an additional 5 days post-overlay to allow for bile canaliculari formation. Culture medium was replaced daily.

Characterization of NTCP Contribution to Hepatic Transporter-Mediated Uptake

The contribution of the sodium-dependent transporter NTCP to the net uptake of atorvastatin and pitavastatin in plated hepatocytes was assessed by comparing uptake in the presence and absence of sodium. The hepatocytes were incubated with 1 µM substrate in sodium-containing or sodium-free (NaCl and NaCHO₃ replaced by choline chloride and KHCO₃) Krebs-Henseleit bicarbonate buffer. After 2 min incubation, uptake was stopped by addition of cold buffer and the cells were washed. Intracellular accumulation of drug was quantified by LC-MS/MS.

Characterization of Drug Disposition in Sandwich-Cultured Human Hepatocytes

Paper IV investigated time-dependent uptake and efflux of pitavastatin in SCHH. In brief, SCHH cells were pre-incubated in the presence of divalent cations, to maintain the integrity of tight junctions, or in their absence, to
disrupt the tight junctions and open up the bile canaliculi. Substrate was added and the cells were incubated for a specified time. The incubation medium was removed and the cells were washed in cold buffer before analysis of accumulated substrate by LC-MS/MS. The amount of drug accumulated intracellularly was calculated from the incubations in the absence of Ca$^{2+}$ and Mg$^{2+}$, whereas net accumulation in cells and bile canaliculi was determined from the incubations in the presence of divalent cations. Substrate depletion from the medium was also measured by taking samples from the medium at representative time points.

Quantitative Analysis of Protein Abundance by Liquid Chromatography-Tandem Mass Spectrometry

Transporter abundances were determined by LC-MS/MS. The two different methods are described in more detail below. Both methods rely on the measurement of peptide levels as a surrogate for protein abundance. The general workflow applied to the samples consisted of two steps. First, samples were fractionated to enrich the membrane proteins. Second, the proteins were enzymatically cleaved into peptides by trypsin for LC-MS/MS analysis.

Targeted Proteomics

The abundance of selected transport proteins in human liver tissue, isolated human hepatocytes, SCHH, HEK293 cell lines, and inverted membrane vesicles were determined by targeted proteomics. Unique peptides representing each transporter were selected for their favorable LC-MS/MS properties. Stable isotope-labeled peptides with sequences identical to the selected target peptides were synthesized. The tissue and cell samples were lysed and membrane fractions were extracted using the ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem, San Diego, CA, USA). The membrane vesicles, which are already an enriched membrane fraction, were used as they were. The membrane proteins were trypsin-digested into peptides, and isotope-labeled peptides were added as internal standards. The peptide samples were analyzed by LC-MS/MS with selective monitoring of the transitions corresponding to the target peptides. The abundance of each transport protein was determined by comparing the peak area of the unlabelled target peptide with that of the heavy isotope-labeled internal standard.

Global Proteomics

The abundance of transporters and other membrane proteins in liver tissue and isolated hepatocytes was also determined by untargeted, label- and
standard-free LC-MS/MS. Here, ultracentrifugation was used to isolate crude membrane fractions. The membrane proteins were solubilized with a high concentration of SDS followed by depletion of the detergent using the filter-aided sample preparation (FASP) method.\textsuperscript{181} In brief, SDS was dissociated from proteins by addition of urea and subsequently removed by centrifugation within a filtration device. Proteins on the filter were digested with trypsin and the resulting peptides were analyzed by LC-MS/MS. Protein abundance levels were calculated by the total protein approach (TPA), a method developed for protein quantification from in-depth shotgun proteomic data. Under the condition that peptide intensity is proportional to protein quantity, the fraction of the MS-signals of all peptides identifying a particular protein to the total MS-signal describes its abundance as a fraction of total mass.

**In vitro–in vivo Scaling of Hepatic Transport and Transporter-Mediated Drug–Drug Interactions**

For scaling \textit{in vitro} transport capacity (\(v_{\text{max,in vitro}}\)) to the \textit{in vivo} situation, transporter abundance in the \textit{in vitro} system was compared to that in human liver tissue using targeted proteomics. By assuming that the maximal transport rate is directly related to protein abundance, we used equation 14 to predict the maximal transport rate \textit{in vivo}. Transporter-mediated drug clearance (\(\text{CL}_{\text{transporter}}\)) was then predicted using equation 15. Scaling factors of 88 mg protein/g liver\textsuperscript{182} and a liver weight of 1800 g\textsuperscript{183} were used to extrapolate to whole liver.

\[
\text{MTA} = \frac{\text{Transporter abundance}_{\text{in vitro}}}{\text{Transporter abundance}_{\text{liver}}} \times v_{\text{max,in vitro}}
\]  

(14)

\[
\text{CL}_{\text{transporter}} = \frac{\text{MTA}}{K_m + [S]}
\]  

(15)

where MTA is the maximal transport activity or \(v_{\text{max, in vivo}}\).

With knowledge of the transporter-mediated CL, predictions of the extent of drug–drug interactions were made using equation 16. The predictions were based on the underlying assumption of competitive inhibition.

\[
\text{CL}_{\text{transporter,+I}} = \frac{\text{MTA}}{K_m \times \left( \frac{[I]}{K_i} + 1 \right) + [S]}
\]  

(16)
Mechanistic Pharmacokinetic Modeling of Hepatic Drug Disposition

Hepatic drug clearance in vivo is a dynamic parameter that changes over time as a result of alterations in the blood and tissue concentrations of the drug. To capture the dynamic feature of drug distribution, a mechanistic pharmacokinetic model was built in R (www.r-project.org) describing the relation between drug uptake, binding, and efflux in SCHH experiments (Figure 7).

![Diagram of pitavastatin disposition model](image)

\[
\frac{dS_{medium}}{dt} = (CL_{passive} + CL_{NTCP} + CL_{OATP1B1} + CL_{OATP1B3} + CL_{OATP2B1}) \cdot S_{medium} + (CL_{passive} + CL_{MRP3}) \cdot S_{cell} + K_{flux} \cdot S_{bile}
\]

\[
\frac{dS_{cell}}{dt} = (CL_{passive} + CL_{NTCP} + CL_{OATP1B1} + CL_{OATP1B3} + CL_{OATP2B1}) \cdot S_{medium} - (CL_{passive} + CL_{MRP3} + CL_{BCRP} + CL_{Pgp}) \cdot S_{cell}
\]

\[
\frac{dS_{bile}}{dt} = (CL_{BCRP} + CL_{Pgp}) \cdot S_{cell} - K_{flux} \cdot S_{bile}
\]

**Figure 7.** Model used to simulate pitavastatin disposition in sandwich-cultured human hepatocytes (SCHH). The model included three different compartments describing the amount of drug in the medium, cell and bile. Drug distribution was described by non-saturable passive diffusion clearance ($CL_{passive}$) and saturable transporter-mediated clearance ($CL_{NTCP}$, $CL_{OATP1B1}$, $CL_{OATP1B3}$, $CL_{OATP2B1}$, $CL_{MRP3}$, $CL_{BCRP}$, $CL_{Pgp}$). Dynamic pulsing of the bile canaliculi, causing release of accumulated drug in the bile compartment to the medium, was described by the $K_{flux}$ parameter. The change of drug amount in the various compartments over time was described by the differential equations shown in the figure.

Kinetic parameters describing the transporter-mediated uptake and efflux of pitavastatin were derived by in vitro experiments in cell lines and membrane vesicles overexpressing a single transporter. The maximal transport velocity was scaled to SCHH by the difference in transporter expression using the MTA-based approach described above. Transporter abundance levels in SCHH cultures at day 5 post-overlay, transfected HEK293 cells, and membrane vesicles were quantified by targeted LC-MS/MS. Intracellular drug
binding was described by the fraction unbound value reported by Menochet and colleagues.\textsuperscript{184}

The impact of changes in transporter activities on simulated time profiles was investigated by varying the maximal transport rate of each transporter 10 fold above and below its experimental value while keeping the other parameters fixed.

**Statistical Analysis**

All experiments were performed in duplicate or triplicate and on at least two separate occasions. Statistically significant differences were assessed by unpaired t-test, one-way or two-way ANOVA with post-hoc test as appropriate. Corrections were made for multiple comparisons.

**Correlations in Protein Expression**

Spearman rank correlation tests were applied in R with the additional package Hmisc to investigate correlations in protein expression levels among drug metabolizing CYP and UGT enzymes. Only those enzymes with quantified abundance levels in at least half of the liver and hepatocyte samples were included. The correlation coefficients were adjusted for multiple testing using the Benjamini-Hochberg correction.

**Pathway Analysis**

Proteins with a significant change in abundance for liver tissue and isolated hepatocytes were identified by a two-sample t-test as implemented in Perseus. A permutation-based false discovery ratio of 0.05 (1000 permutations) was used to define statistical significance. Bioinformatic analysis of the differently expressed proteins was performed with the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com). The analysis was restricted to human experimental observations for liver tissue or hepatocytes. Canonical pathways, from the IPA library of canonical pathways, most significantly associated with the significantly altered proteins were identified.
Identification and Prediction of Inhibitors of the Hepatic Organic Anion Transporting Polypeptide (OATP) Transporters

In paper I, 225 compounds were screened for inhibition of OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake of probe substrates with the aim of developing predictive *in silico* models. In total, 91 compounds were classified as inhibitors, characterized by $\geq 50\%$ reduction of the transporter-mediated uptake at a concentration of 20 µM (Figure 8). Over half of these compounds were inhibitors of more than one OATP isoform, revealing a large overlap for these important uptake transporters (Figure 9). Screening identified 4 novel OATP1B1 inhibitors, 16 previously unpublished inhibitors of OATP1B3, and as many as 29 OATP2B1 inhibitors unreported elsewhere. At a concentration of 20 µM, 27, 3, and 9 compounds were identified as selective inhibitors of OATP1B1, OATP1B3, and OATP2B1, respectively. Two selective compounds, pravastatin (OATP1B1-selective) and erlotinib (OATP2B1-selective), were confirmed by assessing their concentration-dependent inhibition of the three OATP transporters. Interestingly, the concentration-dependent inhibition screen showed that both cyclosporine and rifampicin interacted only weakly with OATP2B1, yet the International Transporter Consortium recommends them as general OATP inhibitors. Cyclosporin and rifampicin should therefore be considered OATP1B inhibitors rather than general OATP inhibitors. Moreover, three compounds commonly used as efflux transporter inhibitors (Hoechst33342, KO143, and MK571) inhibited OATP-dependent uptake as well. MK571 inhibition of the OATP-mediated uptake was even more potent than that reported for the efflux transporters. This emphasizes the difficulty of interpreting transporter interactions in complex systems such as hepatocytes, due to the large overlap of interacting drugs. With this in mind, cell lines overexpressing a single transporter are useful complements to the more complex cell models by pinpointing the transporter(s) of importance.
Figure 8. Interaction of 225 drugs and drug-like compounds with the OATP1B1-, OATP1B3-, and OATP2B1-mediated transport of probe substrate. Compounds were classified as inhibitors if they reduced the transporter-mediated uptake by at least 50% at a concentration of 20 µM. Almost twice as many OATP1B1 inhibitors as OATP1B3 or OATP2B1 inhibitors were identified. Modified from Karlgren et al., J Med Chem, 2012, 55(10):4740-63.

Figure 9. Overlapping inhibitor specificities of the hepatic uptake transporters OATP1B1, OATP1B3, and OATP2B1. There was a large overlap in drug interactions, in particular for OATP1B1 and OATP1B3. The majority of OATP1B3 inhibitors inhibited OATP1B1 as well, making it difficult to identify OATP1B3-specific inhibitors. Modified from Karlgren et al., J Med Chem, 2012, 55(10):4740-63.

Discriminative multivariate models developed from the inhibition data predicted inhibitors of each OATP with high sensitivity (>75%) and high specificity (>70%), Table 1. High lipophilicity and large polar surface area (PSA) were identified as distinguishing molecular features of OATP inhibitors. On the basis of these two properties, an easily interpreted predictive
model was developed that could predict inhibitors of any OATP transporter with a sensitivity of 85% for the test set. The finding that OATP inhibition was favored by both high lipophilicity and large PSA, which are negatively correlated molecular features, may be explained by the presence of multiple binding sites on the transport proteins.\textsuperscript{185, 186}

Table 1. Performance of the developed binary classification models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Type of data set</th>
<th>Inhibitors (%)</th>
<th>Non-inhibitors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>Training</td>
<td>73</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>79</td>
<td>73</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Training</td>
<td>81</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>92</td>
<td>72</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Training</td>
<td>77</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>75</td>
<td>77</td>
</tr>
</tbody>
</table>

Our study was the first to comprehensively analyze inhibition of each hepatic OATP isoform from a physicochemical molecular perspective. Since then other studies have been published on the same topic, all of them confirming the importance of lipophilicity and PSA for OATP inhibition.\textsuperscript{187, 188} The study by De Bruyn and colleagues\textsuperscript{188} included 151 compounds that were also part of our inhibition screen. When comparing the identified OATP1B1 inhibitors and non-inhibitors among the overlapping compounds, the classification agreed for 79% of them. For OATP1B3, as many as 87% of the overlapping compounds were given the same classification. Discrepancies in the classification may be explained by differences such as the probe substrates used or concentration of perpetrator drug in the screens.

Prediction of Interindividual Uptake of Atorvastatin and Pitavastatin in Human Liver

Papers I and II identified atorvastatin as an \textit{in vitro} substrate of OATP1B1, OATP1B3, OATP2B1, and NTCP. Pitavastatin was also found to be transported by all four hepatic transporters (papers III and IV). The kinetics of the transporter-mediated uptake of the two statin drugs was determined in HEK293 cell lines overexpressing each of the transporters. The uptake CL in the liver was predicted by relating the transporter expression in human liver tissue to that in the transfected HEK293 cells. This approach allowed for estimations of the relative contributions of the four transporters to the total active uptake.
From measured transporter expression levels in twelve liver-tissue samples, we predicted a two-fold interindividual variability in transporter-mediated uptake of atorvastatin. Despite the low interindividual variability in overall uptake CL, there were large differences in the contribution of the four uptake transporters (Figure 10). Nevertheless, OATP1B1 and OATP1B3 were identified as the major transporters responsible for the uptake of atorvastatin in the liver. The OATP1B1-mediated contribution was estimated to be 53% on average, which is very close to the reported value of 47%, calculated from in vivo AUC changes in individuals with reduced function genetic variants of OATP1B1.\textsuperscript{165} The good agreement with in vivo data supports our in vitro to in vivo scaling approach. OATP1B3 contribution was predicted to be 35% on average. OATP2B1 and NTCP were found to be less important for the hepatic uptake of atorvastatin than OATP1B1 and OATP1B3. The minor contribution of the sodium-dependent transporter NTCP was confirmed by human hepatocyte experiments in the presence and absence of sodium (Figure 11).

\textbf{Figure 10.} Prediction of interindividual uptake clearance (CL\textsubscript{uptake}) of atorvastatin by four transporters in the liver. Hepatic uptake CL was predicted from in vitro transport kinetics by correcting for the difference in membrane transport protein expression between in vitro cell models and human liver tissue. Modified from Vildhede et al., DMD, 2014.
NTCP-mediated uptake of atorvastatin and pitavastatin in human hepatocytes. The sodium-dependent, NTCP-mediated contribution to the overall uptake was determined by comparing the uptake in the presence (+Na+) with that in the absence (-Na+) of sodium. NTCP did not contribute significantly to the transport of atorvastatin or pitavastatin. Uptake of the control substrate taurocholate, on the other hand, was significantly less (p < 0.001) in the absence of sodium.

Pitavastatin uptake in human liver was predicted to be very similar for the twelve individuals (Figure 12). Transporter contributions, however, varied to a larger extent. OATP1B1 was identified as the major transporter with 74% contribution on average (range of 49-94%). This estimation agrees with the 70% contribution calculated from clinical data.\textsuperscript{164} Of the three remaining uptake transporters, OATP1B3 was the second most important with an average contribution of 16%. As for atorvastatin, OATP2B1 and NTCP were predicted to play minor roles in the hepatic uptake of pitavastatin. Uptake studies in the presence and absence of sodium in human hepatocytes confirmed the negligible contribution of NTCP (Figure 11).

Pitavastatin was more dependent on OATP1B1 for its hepatic uptake than atorvastatin, a finding also supported by clinical data. Individuals with reduced function genetic variants of OATP1B1 display a greater increase in exposure to pitavastatin than atorvastatin compared to individuals with wild-type OATP1B1.\textsuperscript{164} Because of its greater dependence on a single transporter, pitavastatin is expected to be more sensitive to transporter-mediated drug–drug interactions than atorvastatin.
Figure 12. Prediction of interindividual uptake clearance (CL\textsubscript{uptake}) of pitavastatin by four transporters in the liver. Hepatic uptake CL was predicted from \textit{in vitro} transport kinetics by correcting for the difference in membrane transport protein expression between the \textit{in vitro} cell models and human liver tissue.

Prediction of Interindividual Differences in Transporter-Mediated Drug–Drug Interactions Involving Atorvastatin

Paper II studied concentration-dependent inhibition of atorvastatin uptake via OATP1B1, OATP1B3, OATP2B1, and NTCP in transporter-transfected HEK293 cells. The \textit{in vitro} inhibitory potencies of atazanavir, cyclosporine, gemfibrozil, lopinavir, and rifampicin are summarized in Table 2. All five drugs were found to inhibit OATP1B1-mediated transport with lopinavir demonstrating the highest potency and gemfibrozil the lowest. OATP1B3, OATP2B1, and NTCP were only inhibited by some of the perpetrator drugs (see Table 2).
Table 2. Inhibition constants ($K_i$) of five compounds in interaction with OATP1B1-, OATP1B3-, OATP2B1-, and NTCP-mediated transport of atorvastatin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>OATP1B1 ($K_i$ (µM))</th>
<th>OATP1B3 ($K_i$ (µM))</th>
<th>OATP2B1 ($K_i$ (µM))</th>
<th>NTCP ($K_i$ (µM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>0.42</td>
<td>7.3</td>
<td>&gt;30</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>0.66</td>
<td>1.3</td>
<td>&gt;13</td>
<td>4.8</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>58</td>
<td>&gt;420</td>
<td>570</td>
<td>89</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>0.34</td>
<td>&gt;4.2</td>
<td>&gt;7.4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2.2</td>
<td>82</td>
<td>&gt;470</td>
<td>&gt;630</td>
</tr>
</tbody>
</table>

The $K_i$ values were used to predict the extent of transporter-mediated drug–drug interactions in vivo. Because of the large contribution of OATP1B1 and OATP1B3 to the uptake of atorvastatin, inhibitors of these two transporters were predicted to have a larger effect on in vivo hepatic disposition, and hence drug exposure, than equally potent OATP2B1 and NTCP inhibitors. In addition, the large interindividual variability in transporter contribution also affected the extent of drug–drug interactions. Drugs with high OATP1B1 isoform specificity in their inhibition pattern were predicted to cause larger interindividual variability in the remaining atorvastatin uptake than the more general inhibitors (Figure 13).

![Figure 13](image-url)  
*Figure 13.* Interindividual differences in the extent of drug–drug interaction (max/min inhibition) as a function of the isoform specificity of the perpetrator drug. OATP1B1- and OATP1B3-mediated uptake of atorvastatin was predicted to vary considerably between individuals despite a similar summed transporter-mediated uptake. Consequently, the extent of inhibition by the OATP1B1-specific inhibitor lopinavir was associated with a large interindividual variability. Cyclosporine, on the other hand, showed comparable in vitro inhibition potency for OATP1B1 and OATP1B3; it was predicted to inhibit the atorvastatin uptake to a similar degree in all individuals.
Comparison of ADME Protein Abundance in Isolated Human Hepatocytes and Human Liver Tissue

Isolated human hepatocytes are common tools for the study of hepatic drug disposition, including transport and metabolism. In paper III, the global membrane proteome of isolated human hepatocytes was compared with that of liver tissue. The analysis revealed that drug-metabolizing CYP and UGT enzymes in the intracellular membrane of the endoplasmic reticulum had comparable abundance levels in isolated hepatocytes and liver (Figure 14A-B).

**Figure 14.** Interindividual protein expression of drug-metabolizing cytochrome P450 (CYP) (A) and UDP-glucuronosyltransferase (UGT) enzymes (B) in human liver (black) and isolated hepatocytes (grey).
This allowed for analysis of correlations in protein expression levels between the enzymes (Figure 15). Most UGT enzymes were found to show positive correlations with one another and with CYP2C8 and CYP2C9. This may indicate common regulation factors. Several of the correlations among the UGT enzymes are supported by a meta-analysis on UGT protein abundance in the liver,\textsuperscript{189} while some were only detected in this study. Few CYP enzymes were found to show correlations in abundance. Nevertheless, the significant correlations in our material between CYP2C8 and CYP2C9 ($r_s = 0.68$), between CYP1A2 and CYP2J2 ($r_s = 0.69$), and between CYP1A2 and CYP3A4 ($r_s = 0.67$) are in agreement with the results from a recent meta-analysis.\textsuperscript{190} The only negative correlation identified was between CYP2C9 and CYP3A7 ($r_s = -0.61$). CYP3A7 is mainly expressed in fetal liver,\textsuperscript{191} while CYP2C9 is expressed at much higher levels in adult than fetal liver.\textsuperscript{192} The negative correlation may thus reflect a change in CYP expression with liver maturation.

\textbf{Figure 15.} Correlations in protein abundance of 24 drug-metabolizing enzymes. Only statistically significant correlations ($p < 0.05$) are shown. The blue and red colors indicate a positive or a negative correlation in expression, respectively.

In contrast to the metabolizing enzymes, many drug transporters and other integral plasma membrane proteins were significantly less abundant in the isolated hepatocytes than liver tissue (Figure 16). The reduced transporter abundance may explain frequently reported underpredictions of \textit{in vivo} uptake clearance from hepatocyte experiments. Indeed, we predicted a three-fold lower uptake clearance of both atorvastatin and pitavastatin in freshly isolated hepatocytes than in liver.
The transport proteins were not equally affected by the isolation process. OATP1B1, OATP1B3, and NTCP belonged to the drug transporters that were most affected with 3- to 5-fold lower abundance in isolated hepatocytes than liver tissue, whereas OATP2B1 was not affected at all. A possible explanation to this is that the fraction of transporter remaining in the membrane after isolation varies from protein to protein depending on the degree of internalization and degradation. This imbalance in functional protein, compared to *in vivo*, would result in over- or underestimations of the roles of specific transport proteins in drug disposition, with direct implications on DDI predictions. Differences in transport protein loss between batches may (at least in part) explain the highly variable results that have been published on transporter contributions to the uptake of various compounds. For example, a study on NTCP and the uptake of taurocholate reported an almost 7-fold difference for five different batches of cryopreserved hepatocytes.193

**Figure 16.** Interindividual protein abundance of 16 drug transporters in human liver and isolated hepatocytes. MATE1, MRP1, and MRP4 were only detected in the liver samples.

Analysis of pathways associated with proteins whose abundance was significantly altered in hepatocytes as compared to liver tissue indicated that the hepatocytes were exposed to oxidative stress during the isolation. The hepatic exposure to oxidative stress is common to the isolation process as a result of cellular hypoxia-reoxygenation.194 Interestingly, the isolation process was also associated with ubiquitination-dependent proteolysis, providing a possible explanation to the loss of transporters and other plasma membrane-associated proteins.
Prediction of Time-Dependent Pitavastatin Disposition in Sandwich-Cultured Human Hepatocytes

The methodology developed in paper I, for predicting transporter-mediated drug clearance from kinetics and relative protein abundance, was integrated into a dynamic mechanistic model in paper IV. The model was successfully used to simulate time-dependent uptake and efflux of pitavastatin in three different batches of SCHH (Figure 17). The results showed that functional studies in simple in vitro systems, allowing the study of a single transporter, could be used to simulate drug disposition in more complex systems such as SCHH.

In agreement with the liver predictions, OATP1B1 was identified as the major uptake transporter of pitavastatin with a contribution of 57-87% in the sandwich-cultured hepatocytes. However, in contrast to the liver predictions, NTCP was the second most important transporter instead of OATP1B3. Of the canalicular transporters, BCRP was the major contributor to the biliary efflux with more than 75% contribution across the three SCHH batches.

By altering the transporter activities in the simulations, OATP1B1 was shown to have the largest impact on medium and intracellular drug accumulation. Transport activities of the canalicular efflux transporters BCRP and Pgp influenced the amount of drug in bile, but not intracellularly or in the medium.

Figure 17. Comparisons of simulations (lines) and experimental observations (circles) of pitavastatin disposition in three different batches of sandwich-cultured human hepatocytes (SCHH). SCHH (with or without disrupted bile canaliculi) were incubated with pitavastatin at a concentration of 0.1 µM (light grey), 0.6 µM (medium grey), or 1 µM (dark grey) for 1-45 min. Note that different scales are used on the y-axis.
Conclusions

This thesis developed \textit{in vitro} and \textit{in silico} methods for improved predictions of hepatic transporter-mediated drug clearance and drug–drug interactions \textit{in vivo}. Particular emphasis was placed on interactions involving the hepatic OATP transporters that influence plasma concentration-time profiles of substrates such as the commonly administered statins. Several novel inhibitors of the OATP transporters were identified. Discriminative \textit{in silico} models were developed that could predict OATP inhibitors with high specificity and high sensitivity. These models can be used in early drug discovery to guide compound selection away from unwanted OATP interactions. In addition, a protein abundance-dependent method for prediction of \textit{in vivo} transporter-mediated clearance and drug–drug interactions was developed. This method can contribute to improved predictions of drug disposition in the human liver and estimations of transporter contributions. Because transporters are promiscuous with overlapping substrate profiles, estimations of their contribution to the overall uptake and efflux of new drug entities is important for accurate predictions of DDI risk. In summary, the tools here can contribute to the development of safe and effective drugs as well as to appropriate labeling of drugs for their safe and effective use.

From the work presented herein, it can be concluded that:

- OATP1B1, OATP1B3, and OATP2B1 overlap considerably in their drug interactions as illustrated by the large number of inhibitors in common

- OATP1B1 appears more sensitive to drug–drug interactions than OATP1B3 and OATP2B1

- Inhibition of the hepatic OATP transporters is governed by and can be predicted from the molecular descriptors lipophilicity and polar surface area

- Quantification of differences in protein expression for \textit{in vitro} assay systems and human liver tissue can be used as scaling factors in static and dynamic models to improve predictions of transporter-mediated clearance
• Estimations of transporter contributions to the net transport *in vivo* are essential for quantitative predictions of drug–drug interactions.

• The isolation of hepatocytes from liver tissue is associated with mitochondrial oxidative stress and degradation of transporters and other proteins expressed in the plasma membrane. This has implications for the use of primary hepatocytes as an *in vitro* model of the liver.
Future Perspectives

This thesis describes new tools for the prediction of hepatic transporter-mediated clearance and drug–drug interactions. These tools rely on targeted quantifications of transport proteins. Mass spectrometry-based quantification of proteins is an evolving technique anticipated to advance our understanding of transporters as determinants of drug pharmacokinetics and possibly pharmacodynamics. Because it is a relatively new technique, methodological improvements can be expected. Such improvements include the use of purified protein standards at known concentrations to address the knowledge gap of protein-peptide stoichiometry. With incomplete protein digestion, surrogate peptide levels do not reflect the true protein levels. More importantly, digestion efficiency may vary from peptide to peptide and protein to protein. The current lack of protein standards therefore warrants caution when comparing protein levels determined on the basis of surrogate peptide levels for different studies and different transporters. However, relative expression levels of individual transporters in various samples (as used in this thesis) are reliable as long as sample preparation and digestion conditions are similar across the samples. To improve the accuracy of label-based transporter quantifications, future efforts should focus on the production of transport protein standards of high quality or, alternatively, on optimizing protein digestion conditions to maximize the conversion to peptides. An analysis of the current methods could help identify the ones with the most optimal digestion conditions for standardization of sample preparation.

An additional research area that needs more focus is the analysis of splice variants and post-translational modifications (PTMs) of proteins. These phenomena of protein biosynthesis increase the complexity of the protein space, but have so far received little attention.

The growing body of transporter abundance data will aid in creating virtual populations for pharmacologically-based pharmacokinetic modeling purposes. To further improve predictions of liver clearance, models replicating the zonal diversity in the liver are required. It is well-known that the hepatocytes in different zones of the liver express metabolizing enzymes and transporters at varying levels. A thorough characterization of zonal differences in expression levels of proteins determining hepatic clearance would thus be a useful complement to the existing studies on whole liver abundance.
In the human liver, the transport proteins act in concert with metabolizing enzymes. This highlights the need for a more holistic approach for predicting overall liver clearance and DDI potential. A major challenge for future investigations is to develop mechanistic models capable of describing the transporter-enzyme interplay well enough to allow accurate predictions of drug clearance and DDIs solely from \textit{in vitro} data. Recently published methods to determine unbound tissue concentrations will be useful in this regard since this is the concentration of drug to which the metabolizing enzymes and efflux transporters are exposed.

Although transport inhibitors can be recognized as such reasonably well from molecular structures, predictions of ligand-protein interactions would be further enhanced by integrating knowledge of the three-dimensional structures of the transport proteins into the modeling. Such structure-based modeling would not only offer information about the potential for inhibition but also the type (competitive or noncompetitive). The latter feature is advantageous given the substrate-dependent inhibition that several hepatic drug transporters display. Structures of human drug transporters are, however, lacking. Hence, efforts to obtain the three-dimensional structures of these proteins by X-ray crystallography or nuclear magnetic resonance spectroscopy are warranted.


Genom sin roll i nedbrytningen av läkemedel styr levern hur hög nivån av läkemedel i blodet blir. När ett läkemedel ges tillsammans med ett eller flera andra läkemedel kan dess nedbrytning hämmas genom att interaktionen med transportproteiner eller de metaboliserande enzymen störs. Detta kallas för en läkemedelsinteraktion och får till följd att läkemedlets koncentration i blodet ökar, vilket i sin tur kan leda till biverkningar av mer eller mindre allvarlig grad. Läkemedelsinteraktioner med metaboliserande enzym är ett välstudierat område. På bipacksedeln i läkemedelsförpackningar finns varningar för vilka läkemedel som inte bör tas samtidigt på grund av risken för
hämmad metabolism. Läkemedelsinteraktioner som involverar transportörer är däremot ett mindre studerat område och har därför undersöks i denna avhandling.

Arbetet i denna avhandling har fokuserat på fyra transportproteiner i lever som är involverade i upptaget av läkemedelsgruppen statiner. Statiner används för att minska mängden kolesterol i blodet och därmed risken för hjärt-och läsjsukdomar. Det är en vanligt förekommande grupp av läkemedel, framför allt hos äldre. Muskelsvår är en av de vanligare biverkningarna vid statinanvändning. Denna muskelpåverkan kan i värsta fall utvecklas till ett livshotande tillstånd även om detta är mycket ovanligt. Risken för biverkningar ökar om statinbehandling kombineras med andra läkemedel som ökar nivån av statiner i blodet, t.ex. genom hämning av upptaget till levern.

I första delen av denna avhandling undersöktes förmågan hos 225 läkemedel och liknande ämnen att hämma tre av de transportproteiner i lever som tar upp statiner från blodet. En stor andel av de undersökta ämnena (40%) minskade upptaget via åtminstone ett av de tre transportproteiner. Med hjälp av dessa experimentella data utvecklades datormodeller som kan användas för att förutsäga om ett nyframtaget läkemedel kommer att tävla med statiner om att tas upp via dessa transportproteiner. Datormodellerna är användbara i ett tidig skede av läkemedelsutvecklingen för att på ett billigt och enkelt sätt styra utvecklingen bort från oönskade läkemedelsinteraktioner.

I den andra delen av denna avhandling utvecklades en metod som kan användas för att kartlägga hur varje transportprotein bidrar till det sammanlagda upptaget av statiner (eller andra läkemedel) i levern. Metoden bygger på studier av transportfunktionen i speciella cellmodeller där varje protein kan studeras enskilt samt på bestämning av uttrycket av transportproteiner i dessa cellmodeller och i människans lever. Denna metod applicerades på statinerna atorvastatin och pitavastatin, vilka visade sig vara beroende av främst två transportproteiner för upptaget i levern. Information av denna typ är användbar för att förutsäga hur stor betydelse hämning av ett specifikt transportprotein får på det sammanlagda upptaget i levern. Ju högre bidraget av transportproteinet är till det totala upptaget, desto större påverkan på läkemedelskoncentrationen i blodet om detta transportprotein hämmas. Genom att undersöka hur mängden av transportproteinerå varierar mellan olika individer kunde även skillnader mellan olika individer förutsägas.

I den tredje delen av denna avhandling jämfördes uttrycket av tusentals proteiner (inklusive transportproteiner) i levervävnad och i hepatocyter som har isolerats från levervävnad. De isolerade hepatocyterna anses vara den bästa cellmodellen för studier av läkemedelsnadbrytning. Trots detta visade det sig att mängden transportproteiner generellt var lägre i de isolerade hepatocyterna än i levervävnad. De läkemedelsmetaboliserande enzymerna som är lokaliserade inne i hepatocyterna uppvisade däremot likvärdiga mängder i hepatocyter och vävnad. Analys av alla de proteiner som mängdmässigt
skiljde sig åt mellan de isolerade hepatocyterna och levervävnad pekade på att förlusten av transportproteiner är ett resultat av oxidativ stress som uppkommer under isoleringen och som leder till nedbrytning av de proteiner som finns i cellmembranet. Resultaten förklarar vanligt förekommande svårigheter i att förutsäga transportörerörende nedbrytning i levern från försök i hepatocyter. De visar också på fördelen med att använda metoder som tar hänsyn till skillnader i proteinmängd då cellmodeller används för att förutsäga hur mycket nedbrytning som sker i vår lever.

Sammanfattningsvis har arbetet i denna avhandling resulterat i nya dator- och cellbaserade metoder som kan användas till att förutsäga läkemedelstransport i levern samt utvärdera risken för läkemedelsinteraktioner. De framtagna metoderna kan användas för att främja utvecklingen av nya, säkra läkemedel.
Acknowledgements

The work presented in this thesis was carried out at the Department of Pharmacy, Faculty of Pharmacy, Uppsala University, Sweden.

The studies were financially supported by the Swedish Research Council, the Swedish Fund for Research without Animal Experiments, the Swedish Agency for Innovation Systems, the Lars Hierta Memorial Foundation, and O.E. and Edla Johansson’s Scientific Foundation.

IF:s stiftelse, Apotekare C D Carlssons stiftelse, Anna Maria Lundins stipendiefond och Leffmans resestipendium tackas för de generösa bidrag som gjort det möjligt för mig att delta i kurser samt åka på internationella konferenser.

There are several people who deserve my sincere appreciation and gratitude for their contribution to this thesis. I would like to thank all of you who made this thesis possible in one way or another.

My supervisors:

Per Artursson för att du antog mig som doktorand i din forskargrupp och för att du i och med detta gav mig en unik möjlighet att lära mig en massa om både mig själv och om den intressanta forskning som innefattar läkemedelstransportörer. Även om vi inte alltid förstår varandra fullt ut så har jag lärt mig oerhört mycket av min tid i din grupp och under din handledning. Jag skulle aldrig vara där jag är idag om det inte var för din strävan efter att bedriva förstklassig forskning. Jag är väldigt tacksam för att du har tagit dig tid att guida mig på vägen till att bli en självständig forskare.

Maria Karlgren för all din hjälp i labbet och för att du introducerade mig för den magiska cellvärlden. Tack för bra samarbete i våra gemensamma projekt och för all stöttning och uppmuntran längs vägen. Ditt systematiska och effektiva arbete är beundransvärt. Vårt gemensamma matintresse har även det varit ett positivt inslag med trevliga middagar på konferensresor och efter accepterat manus. 
Maria Kjellsson för din vägledning under modellerandet. Tack vare dig gick det minst 10 ggr snabbare att få klart modellen än vad det hade gjort om jag hade försökt helt på egen hand. Våra möten var alltid väldigt trevliga och produktiva; även de som inte var planerade, t.ex. när vi stötte på varandra i korridoren på BMC eller på ett träningsspass.

Ulf Norinder för din insats med den multivariata modelleringen i första manusriptet. Dina typiskt göteborgshumoristiska skämt var uppskattade även om jag inte alltid var så snabb på att förstå dem.

All my other co-authors:
Jacek Wisniewski, many thanks for giving us the opportunity to collaborate with you and for the sophisticated method developments over the years of our collaboration. I have also highly appreciated your hospitality during my visits to the proteomics lab at the MaxPlanck Institute.

Emi Kimoto and Yurong Lai for your valuable quantitative analysis of transport proteins and helpful comments on the manuscripts that you have co-authored.

Ulf Haglund och Agneta Norén för er kirurgiska skicklighet och er positiva inställning till det vetenskapliga arbetet. Utan er insats hade mina studier inte haft samma betydelse.


All the former and present members of the drug delivery group for interesting research discussions, useful feedback on my work, and for all the fun in the lab as well as outside of the lab. Work would not have been nowhere near as fun without you. I will truly miss you all.

All the former and present PhD students at the Department for all the fun at conferences, after works, dissertation parties, coffee breaks, and events outside of work. I have particularly enjoyed all the competitive elements of our company, including games, curling, music quizzes and Knally 😊.

My office mates Jonas och André. Jonas, tack för att du orkade lyssna på mitt konstanta prat under de år vi delat kontor ihop och för att du alltid har tid för uppmuntrande ord när det är lite tufft och jobbigt. Thank you André for being an excellent research buddy and for taking the time to help me out when needed. I have really enjoyed our research discussions as well as our discussions on non-research related topics.
My greatest appreciation goes to Terese Bergfors for your invaluable proof-reading and language revision of my thesis and papers II-III. Special thanks also go to Andrea, André, Christine, and Magnus for your thorough proof-reading of this thesis and of paper IV.

Stort tack till min familj och mina vänner för att ni alltid finns där. Framför allt vill jag tacka mina föräldrar för att ni alltid har hjälpt till och stöttat mig i allt som jag har gjort.

Sist, men absolut inte minst, vill jag tacka min älskade Björn för allt stöd under denna tid. Tack för att du finns där när det är jobbigt och jag behöver få prata av mig, höra några uppmuntrande ord eller få en pussbild skickad till mig.
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