ATP-Binding-Cassette Transporters in Biliary Efflux and Drug-Induced Liver Injury

JENNY M. PEDERSEN
Membrane transport proteins are known to influence the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drugs. At the onset of this thesis work, only a few structure-activity models, in general describing P-glycoprotein (Pgp/ABCB1) interactions, were developed using small datasets with little structural diversity. In this thesis, drug-transport protein interactions were explored using large, diverse datasets representing the chemical space of orally administered registered drugs. Focus was set on the ATP-binding cassette (ABC) transport proteins expressed in the canalicular membrane of human hepatocytes. The inhibition of the ABC transport proteins multidrug-resistance associated protein 2 (MRP2/ABCC2) and bile salt export pump (BSEP/ABCB11) was experimentally investigated using membrane vesicles from cells overexpressing the investigated proteins and sandwich cultured human hepatocytes (SCHH). Several previously unknown inhibitors were identified for both of the proteins and predictive in silico models were developed. Furthermore, a clear association between BSEP inhibition and clinically reported drug induced liver injuries (DILI) was identified. For the first time, an in silico model that described combined inhibition of Pgp, MRP2 and breast cancer resistance protein (BCRP/ABCG2) was developed using a large, structurally diverse dataset. Lipophilic weak bases were more often found to be general ABC inhibitors in comparison to other drugs. In early drug discovery, in silico models can be used as predictive filters in the drug candidate selection process and membrane vesicles as a first experimental screening tool to investigate protein interactions. In summary, the present work has led to an increased understanding of molecular properties important in ABC inhibition as well as the potential influence of ABC proteins in adverse drug reactions. A number of previously unknown ABC inhibitors were identified and predictive computational models were developed.

Keywords: ABC transport protein, Pgp, P-glycoprotein, ABCB1, BCRP, breast cancer resistance protein, ABCG2, MRP2, multidrug resistance-associated protein 2, ABCC2, BSEP, bile salt export pump, ABCB11, sandwich cultured human hepatocytes, SCHH, drug-induced liver injury, DILI, multivariate data analysis, OPLS

Jenny M. Pedersen, Uppsala University, Department of Pharmacy, Box 580, SE-751 23 Uppsala, Sweden.

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The beautiful thing about today is that you get the choice to make it better than yesterday.

Robin Sharma
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

3D Three dimensional
ABC ATP binding cassette
ADMET Absorption, distribution, metabolism, excretion and toxicity
ADR Adverse drug reaction
AMP Adenosine monophosphate
ANOVA Analysis of variance
AR Adverse reaction; FDA classification
ATP Adenosine triphosphate
BBB Blood brain barrier
BCRP Breast cancer resistance protein
BEI Biliary excretion index
BIC Bile intracellular correlation
Bile Acc Bile accumulation in SCHH
Bile Acc,MTA Predicted bile accumulation in SCHH
BSA Bovine serum albumin
BSEP Bile salt export pump
BW Boxed warnings; FDA classification
CL Bile Biliary clearance
CL Bile,MTA Predicted in vivo biliary clearance
CycA Cyclosporine A
CYP Cytochrome P450 enzyme
DA Discriminant analysis
DDI Drug-drug interactions
DILI Drug induced liver injuries
DMEM Dulbecco’s modified Eagle’s medium
DMSO Dimethyl sulfoxide
E17G Estradiol 17-glucuronide
E3S Estrone 3-sulfate
EDTA Ethylenediaminetetraacetic acid
EMA European Medicines Agency
FDA The Food and Drug Administration
HBSS Hank’s Balanced Salt Solution
HomProt Total protein amount in human liver homogenate
<table>
<thead>
<tr>
<th>Abbr</th>
<th>Description</th>
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<tbody>
<tr>
<td>IC&lt;sub&gt;Acc&lt;/sub&gt;</td>
<td>Intracellular accumulation in SCHH</td>
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<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Concentration corresponding to half maximal transport rate; Michaelis-Menten transport kinetics parameter</td>
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<tr>
<td>LogD&lt;sub&gt;7.4&lt;/sub&gt;</td>
<td>Octanol water partition coefficient at pH 7.4</td>
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<tr>
<td>MDR</td>
<td>Multidrug-resistance</td>
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<tr>
<td>MRP</td>
<td>Multidrug-resistance associated protein</td>
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<tr>
<td>MSD</td>
<td>Membrane spanning domain</td>
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<td>MTA</td>
<td>Maximal transport activity</td>
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<td>NBD</td>
<td>Nucleotide binding domain</td>
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<td>NMQ</td>
<td>N-methyl quinidine</td>
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<td>OPLS</td>
<td>Orthogonal partial least squares projection to latent structures</td>
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<td>PCA</td>
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<td>Pgp</td>
<td>P-glycoprotein</td>
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<td>PLS</td>
<td>Partial least squares projection to latent structures</td>
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<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
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<td>SCHH</td>
<td>Sandwich cultured human hepatocytes</td>
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<tr>
<td>SLC</td>
<td>Solute carrier transport protein</td>
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<td>TA</td>
<td>Taurocholate</td>
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<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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<tr>
<td>Total&lt;sub&gt;Acc&lt;/sub&gt;</td>
<td>Summarized intracellular- and bile-accumulation in SCHH</td>
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<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal transport rate; Michaelis-Menten kinetics parameter</td>
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<td>WP</td>
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Introduction

New drugs are continuously being developed with the intention to prevent, cure, and treat diseases. During the last century, drugs developed on the basis of important pharmacological discoveries, such as antibiotics, markedly increased human life expectancy [1]. Today, drug development is a lengthy, complex, and expensive effort. Even if exact quantifications are difficult, recent estimates (2012) state that the drug development process takes 10-15 years and costs more than one billion (~1,200,000,000) US dollars. The statistics further reveal that only two of ten marketed drugs return revenues that reach or exceed their development cost [2]. The complexity of the process is well reflected in the interdisciplinary cooperation of scientists and clinical researchers required for the successful development of a new drug. Nevertheless, the number of new drug applications approved by the American regulatory agency has increased 55% during the 1990s and the first decade of this century. Statistics from the Food and Drug Administration (FDA) show that the flow of drugs involving new molecular entities instead is decreasing [3]. The latter may indeed reflect the complexity of drug development, including the constantly increasing challenge from surpassing the efficacy and safety level of already marketed drugs.

In the 1990s, studies revealed that poor pharmacokinetic properties were the main reason for compound attrition during drug development [4, 5]. Previously, pharmacokinetics had played only a minor role in the drug development process. To ensure that substandard compounds were eliminated at an early stage from the development process, the industry began to implement rigorous strategies for detection of inadequate pharmacokinetic properties, which resulted in the development of new in silico and in vitro methods. This preventive approach has proven successful and in the year 2000 the attrition rate due to pharmacokinetic properties had dropped to less than ten percent [1]. However, new models able to predict toxicological responses are still warranted.

At the same time, studies of knock-out animal models and loss-of-function human genetic variants suggested that the bioavailability, disposition, and adverse reactions of drugs were more influenced by transport proteins than what was previously believed [6, 7]. During the last 20 years numerous studies on drug-transporter interactions have provided important information on
how transport protein interactions influence the pharmacokinetic properties of drugs [8-11]. Transport proteins are now thought to work in concert with passive permeability and other proteins, such as drug metabolizing enzymes, in the absorption and elimination of drugs [9]. As a consequence of these research efforts, several regulatory agencies including the European Medicines Agency (EMA) and the American FDA have included drug-transporter interaction studies in their industry guidelines [12, 13]. In this thesis, drug-transporter interactions were examined to better understand the influence of transport proteins on biliary excretion, drug-drug interactions (DDI), and drug-induced liver injuries (DILI). In particular, this thesis focuses on the ATP-binding-cassette (ABC) transport proteins expressed in the canalicular membrane of human hepatocytes.

Membrane Transport of Drugs in Human

Oral administration is generally the preferred administration route of drugs. In addition to low production costs, the ease of administration can improve patient compliance [14]. To achieve a systemic effect, all orally-administered drugs need to pass the intestinal epithelium to reach the circulatory system. Depending on their site of action, most drugs also have to overcome one or more additional membrane barrier, such as the plasma membranes (for intracellular effects) or the blood-brain barrier (BBB, for CNS effects).

The fundamental structure of the membrane barrier is a phospholipid bilayer with aqueous compartments on either side (Fig. 1). In cell barriers composed of adherent cells, two phospholipid bilayers and the aqueous intracellular compartment separate the two extracellular aqueous compartments on either side (Fig. 1B). In addition to the cells themselves, the integrity of a cell barrier is sustained by adherent- and tight junctions. The constituents of the phospholipid membranes and the cell junction integrity play a major role on the overall cell membrane permeability [15, 16]. Through adjusting the membrane composition, e.g., by protein expression, cholesterol load, and tight junction integrity, evolution has altered the membrane permeability to fit the purpose of a specific tissue. For instance, in the BBB that works to protect the brain from harmful compounds, the tight junction integrity is approximately 20 times higher compared to that of the jejunum [17]. The diverse membrane properties of individual tissues, e.g., transport protein expression, influence the relative importance of different transport routes, but in general, drug transport across a cell barrier depends on three distinct pathways, described below (Fig. 1).
Figure 1. The phospholipid bilayer of the cellular membrane contain hydrophilic head-groups (1) towards the outside aqueous compartments and hydrophobic carbon chains (2) in between. The cholesterol content (3) within the hydrophobic intramembranous compartment influences the membrane fluidity and passive diffusion (A). Transport proteins (B) in the cellular membranes can facilitate drug transport over cellular membranes in an ATP-dependent manner or by facilitated diffusion. Small hydrophilic compounds can pass cellular membranes through the paracellular transport route (C) between adjacent cells.

**Passive Diffusion**

For many drugs, transcellular passive diffusion (Fig. 1A) is the major transport route in absorption and tissue distribution. Passive diffusion is a chemical movement from high to low concentration driven by the gain of system entropy. The extent of passive diffusion over a cellular membrane is determined by the molecular properties of the drug and the membrane composition. Passive diffusion benefits from low molecular weight and high lipophilicity of the compound, as well as low cholesterol load of the membrane [18, 19]. Large molecules or particles can permeate a membrane barrier through transcellular transport in membrane vesicles by endocytosis. However, this transport route has little influence on transport of small molecule drugs.

**Paracellular Transport**

Tight junction integrity determines the size of the pores that allow paracellular transport (Fig. 1C). Mainly due to the small fraction of the total membrane area comprised of paracellular pores, this route influences the membrane permeability of small hydrophilic drugs that are less able to permeate the phospholipid bilayer [20]. It has been shown that paracellular transport mostly influences the intestinal drug permeability of poorly absorbed drugs [21].
**Active Transport**
In addition to passive diffusion, membrane transport proteins (Fig. 1B) spanning the cellular membranes are important contributors to the membrane permeability of drugs [8, 10]. The transport capacity of the active transport is dependent on protein expression levels and transport efficiency and these parameters, in combination with the extent of passive permeability, determine the influence of active transport on the membrane permeability.

**Membrane Transport Proteins**
Membrane transport proteins can be regarded as gatekeepers for all cells and organelles in the body. They control the uptake and efflux of many compounds and are crucial in maintaining membrane potential or chemical gradients essential to many cell functions. To date, more than 400 membrane transport proteins have been cloned and many of them have been localized to tissues and organs throughout the human body [22]. The primary functions for most of these transport proteins include membrane transport of endogenous substrates such as sugars, amino acids, nucleotides or vitamins, or protecting the body from dietary or environmental toxins. However, substrate specificities of the membrane transport proteins are not strictly limited to their physiological substrates, and about 20 of them are considered relevant to the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drugs [23, 24].

Membrane transport proteins are important contributors in the membrane barrier function in e.g., the intestine [25, 26]. Membrane barriers are often comprised of polarized cells with diverse protein expression in the cellular domains facing the two sides of the membrane. The expression of drug transport proteins is generally highly restricted to one side, either the basolateral or apical domain, of the polarized cells [27, 28]. Transport proteins in the two membrane domains often work collaboratively to facilitate the substrate transport over the entire cell barrier [9, 29, 30]. Transport proteins differ in their two- and three-dimensional protein structures and they transport their substrates via different transport mechanisms. On the basis of these molecular and mechanistic differences, the transport proteins are organized into protein superfamilies. The two main transport protein superfamilies involved in drug transport are the solute carriers (SLC) and ABC proteins [31, 32].

**Solute-Carrier-Transport (SLC) Proteins**
Most identified transport proteins are SLC transporters and, in total, more than 350 members of this family have been identified [31]. SLC transporters translocate their substrates across biological membranes through several
different mechanisms, including facilitated diffusion, ion coupling, and ion exchange. Depending on which driving force the SLC transport proteins use, they can be further classified into facilitated transporters and secondary active transporters. The facilitated transporters move their substrates with the concentration gradient and the transport is therefore not energy dependent. In contrast, secondary active transporters translocate their substrate using membrane gradients produced by other energy consuming transport proteins. This transport process is thus energy-dependent, even if the transport proteins themselves are unable to hydrolyze ATP to produce the energy for the transport process [33-35].

![Figure 2. Predicted membrane topology of SLC transport proteins. The pore through the membrane consists of 12 TMDs. Glycosylation of the extramembranous loops regulates protein function and cellular trafficking.](image)

Most SLC proteins are located in the plasma membrane where they translocate substrates from the extracellular to the intracellular compartment. However, some of them are expressed in other cellular membranes such as the mitochondrial membrane [36]. Generally, members of the SLC protein family have a similar protein structure that consists of 12 putative membrane-spanning domains (Fig. 2) and their molecular mass is approximately between 50 and 100 kDa [37, 38]. Several of the SLC transport proteins are ubiquitously expressed throughout the human body (e.g., SGLT/SLC5 and SNAT/SLC38) and they participate in essential transport processes including cellular uptake of glucose and amino acids [39, 40]. Other SLC proteins are specifically expressed in one or few tissues, such as the OATP1B1/SLCO1B1 that is involved in the hepatic uptake of bile acids and organic anions [41]. SLC transport proteins involved in drug transport are usually not expressed ubiquitously; rather, they are located in tissues important to the ADMET properties of drugs, such as the intestine, liver and kidney [25, 26].

**ATP-Binding-Cassette (ABC) Transport Proteins**

The ABC-transport protein family is one of the most widely expressed protein families known. In humans, 48 proteins organized into seven subfami-
lies have been identified [42]. ABC proteins in the plasma membrane translocate their substrate from the intracellular to the extracellular compartment, but some of them, e.g., the phospholipid transporter ABCB4, only facilitate transport over one of the membrane leaflets [43]. The dislocation of the substrate is energy dependent and the substrate transport often occurs against steep concentration gradients. The ABC-transport proteins produce the energy required for this transport through the binding, and subsequent hydrolysis of, ATP at their two nucleotide-binding domains (NBD). The NBDs consist of two highly conserved amino acid sequences (the Walker A and B motifs) that the ABC proteins share with other ATP binding proteins. However, the ABC proteins also consist of a third, highly-conserved amino acid sequence, located between the Walker A and B motifs. This third motif is unique to the ABC proteins, and often referred to as the ABC signature motif or the C motif [42]. High-resolution crystal structures of NDBs from the ABC proteins suggest that this motif participate in the recognition, binding, and hydrolysis of ATP [44].

Figure 3. Structure and membrane topology of ABC transport proteins. (A) The high resolution crystal structure from mouse Pgp (Protein Data Bank ID 3G5U) show the two intracellular NBD and the alphahelices constituting the TMD. The grey area shows the approximate position of the cell membrane. (B) The predicted membrane topology of ‘full transporters’ including Pgp, MRP4 and MRP5. In these proteins the entire core unit of two MSD and two NBD are encoded for in a single polypeptide. (C) In addition to the core unit MRP1-3 and MRP6 have an additional MSD containing five TMDs. (D) Half transporters such as the ABCG family, including BCRP, assemble homo- or heterodimers to form the functional core unit of the full transporters. Figure A altered from Aller et al. 2009 [45].

A high resolution crystal structure of a human ABC protein, the mitochondrial ABCB10, was recently resolved for the first time [46]. Among the determined ABC crystal structures, mouse-Pgp (Protein Data Bank ID 3G5U; Fig. 3A) has the highest sequence homology to the human multidrug resistance-associated (MDR) proteins. For instance, human-Pgp has a sequence homology of 87% with the mouse-Pgp, but only 37% sequence ho-
mology to the human ABCB10 [45, 47]. Homology models used to predict human Pgp (or other MDR) ligand interactions are therefore often based on the crystal structure of mouse-Pgp. By general consensus, the core unit in functional ABC proteins consists of two membrane-spanning domains (MSDs) and two NBDs. The MSDs contain, in total, 12 transmembrane domains (TMDs) that are believed to constitute the channel that enables the transmembrane dislocation of the substrates [32, 42]. The entire core unit can be encoded in a single polypeptide chain, as is the case of the ‘full transporters’ (e.g., Pgp and Multidrug-resistance-associated protein 5/MRP5) (Fig. 3B) [48]. Alternatively, the core unit can assemble as a homo- or heterodimer of peptides, each containing one MSD and one NBD. These transporters are often referred to as ‘half transporters’ and are exemplified by the ABCG family that includes the Breast-Cancer-Resistance Protein (BCRP; Fig. 3D) [49]. In addition to these four core functional units, MRP1-3 and MRP6 also comprise a third MSD of five transmembrane helices (Fig. 3C). The exact function of this domain has not been elucidated but it possibly aids in the membrane localization of the ABC protein [50, 51]. Despite these differences in domain organization, there is considerable overlap in the substrate specificities between several of the included transport proteins.

The ABC proteins accept a wide variety of substrates, including phospholipids, ions, peptides, steroids, polysaccharides, and bile acids [52, 53]. The physiological role of the ABC proteins becomes apparent in patients with low or no functional expression of individual ABC proteins. For instance, patients who lack MRP2 function develop chronic conjugated hyperbilirubinemia due to low hepatobiliary excretion of bilirubin [52]. Other examples include the Tangier and Stargardt diseases, where ABC malfunctions lead to disturbed cholesterol balance and macular dystrophy, respectively [54, 55]. In addition to the large number of endogenous ABC substrates, many drugs and other xenobiotics also undergo ABC transport [24]. The role that ABC proteins play in MDR was first recognized in the context of cancer therapy, but now the ABC proteins are generally believed to influence pharmacokinetic properties of drugs within most therapeutic classes [24, 56]. Three main subfamilies (ABCB, ABCC, and ABCG) are known to be involved in MDR [8]. Members of these families are distributed among all of the membrane barriers, such as the BBB, intestine and placenta. It is likely that these subfamilies, have evolved to aid in the excretion of cellular waste products and to protect the human body from xenobiotic exposure [25, 57, 58]. In this thesis, particular emphasis is placed on investigating the influence on the hepatobiliary excretion of drugs and endogenous compounds by four ABC transport proteins, the Pgp, BCRP, MRP2 and BSEP.
Drug-Drug Interactions (DDI)

To generate the intended effect, an administered drug needs to interact with its target site. This target site often consists of a protein expressed, for instance, in the plasma or nuclear membranes [59]. In addition to the target site, drugs also interact with other cellular components, including metabolic enzymes and transport proteins. These proteins are often of crucial importance to the drug bioavailability, efficacy, and safety [22, 60]. If a drug is co-administered with other drugs or some food, competitive protein interactions increase the risk of altered pharmacokinetic properties. Possibly, such interactions lead to clinically relevant DDIs where one or more of the administered drugs lose effect or generate adverse drug reactions (ADR) [60, 61].

DDI mechanisms are generally related to inhibition or stimulation of proteins involved in the drug binding to the target site or to proteins influencing the metabolism and excretion of the drug. In the latter, reversible inhibition is responsible for the majority of all DDIs with clinical relevance [61]. As mentioned previously, transport proteins and metabolic enzymes work in concert to reduce the load of drugs in the body. Examples of DDI mechanisms including these proteins are:

1) Inhibition of hepatic SLC or metabolizing enzymes that decrease drug metabolism. This may lead to increased systemic drug concentration that in turn increases the risk of ADR.

2) Inhibition of metabolizing enzymes or intestinal-, BBB-, or kidney-ABC proteins can lead to increased bioavailability and systemic drug concentrations increasing the risk of ADR.

3) Inhibition of ABC transporters in the liver and kidney may cause increased intracellular or systemic levels of possibly toxic metabolites causing cellular damage or systemic adverse reactions. This can also lead to decreased drug excretion, increased systemic drug concentration, and increased risk of ADR.

4) Inhibition of intestinal uptake transport proteins can reduce the bioavailability of an administered drug and therefore cause lack of effect.

5) Stimulation of hepatic transport proteins and metabolic enzymes can reduce systemic drug concentrations and therefore cause loss of drug efficacy.
The Human Liver

After the skin, the liver is the largest organ in the human body and comprises of up to five percent of the adult body weight. It serves as the primary site of detoxification of natural and synthetic compounds in the systemic circulation and therefore plays an important role in human physiology. In addition to its impact on systemic detoxification, the liver synthesizes and secretes important blood and bile main constituents, such as albumin, bile salts, and cholesterol. Other important functions include protein, steroid, and fat metabolism, vitamin and hormone synthesis, iron homeostasis, and sugar storage [62, 63]. The liver is a highly specialized tissue that comprises many different cell types, such as endothelial cells, macrophage Kupffer cells, and a small stem cell population called oval cells [64]. However, most of the functions attributed to the liver are performed by the parenchymal cell type, the hepatocytes. Hepatocytes compose approximately 70% of the hepatic cell population and are highly differentiated cells originating from fetal endothelial cells [63, 64]. They are highly polarized and depend on the maintenance of two distinct membrane domains to maintain normal hepatocyte functions [64, 65].

Figure 4. (A) The liver consists of 50,000 to 100,000 functional units called liver lobule [62]. The blood enters the liver at the portal triad (PT) and exits into the central vein (CV). (B) The PT comprises single branches of the portal vein (blue), hepatic artery (red) and bile duct (green). The blood from the portal vein and the hepatic artery mixes and travels through the microvasculature to a branch of the hepatic vein that continues to the CV. The bile flow is reversed and exits the liver at the PT. (C) The hepatic lobules are divided into three zones with different microenvironment and cell phenotypes. Zonal differences in hepatocyte phenotypes can be observed in e.g., expression of metabolic enzymes and transport proteins. For instance, a higher expression of the transport protein OATP1B1 can be observed in pericentral (zone III) hepatocytes, as shown in Figure 4C [66].

The liver is closely linked to the small intestine and processes the venous blood flow from the digestive tract containing all absorbed molecules such as nutrients and drugs. Most of the hepatic blood flow originates from the gastrointestinal tract and enters the liver from the portal vein. However, oxy-
genated blood also enters the liver from the hepatic artery. After passing through the sinusoidal microvasculature, the blood exits through the hepatic central vein. Histologically the liver consists of small functional units called hepatic lobules (Fig. 4A), with a centralized branch from the hepatic vein, connected to the central vein (CV), surrounded by several portal triads, each consisting of branches from the portal vein, hepatic artery, and bile ducts (Fig. 4B) [62]. Between the portal triad and the central vein, the liver is both structurally and functionally further divided into three zones with distinct differences in cell phenotype and maturation. There are also zonal variations in chemical concentrations e.g., of oxygen, nutrients, insulin, and glucagon [63]. The diverse microenvironments and cellular functions generate the zonal variations observed in many important hepatic functions. For example, hepatocytes around the central vein have a higher density of endoplasmic reticulum and express the highest levels of several metabolizing enzymes and uptake transporters involved in detoxification of e.g. drugs (Fig. 4C) [64, 66].

Drug Disposition in the Liver
Only the free fraction of a drug is available for passive or active uptake into the hepatocytes. Nonetheless, the liver has a remarkable capacity to extract protein-bound drugs from the systemic circulation. For example, atorvastatin is well absorbed in the intestine and has plasma protein binding of 98%, yet, an absolute bioavailability of 14% [67]. Until approximately 20 years ago, it was generally assumed that the hepatobiliary clearance of drugs and drug metabolites was determined by passive diffusion, governed by the physico-chemical properties of molecules. However, it is now well established that transport proteins are important contributors to the hepatic uptake and efflux over the basolateral and apical membranes of the hepatocyte [24, 31, 68]. In this way and in cooperation with the metabolic enzymes, the transport proteins significantly influence the bile and systemic blood concentration of drugs and their metabolites [30, 69, 70].

Thanks to advances in molecular biology, all the major transport systems involved in the hepatobiliary transport of drugs and endogenous compounds have been cloned and characterized during the last 20 years [71, 72]. As a result we now know the identity of and some of the preferred substrates of the transport proteins involved in the basolateral uptake, basolateral efflux and canalicular efflux of drugs in the liver [23, 24].

Basolateral Uptake
Basolateral uptake is the first step in hepatic clearance of compounds from the systemic circulation. The hepatic uptake is facilitated by SLC transport proteins abundantly expressed in the basolateral membrane of the hepato-
cytes [25]. Transport proteins with major contribution to the hepatic uptake of drugs have been identified in the SLC22A and SLCO superfamilies. They include the organic anion transporters, organic cation transporters, and the organic anion transporting polypeptides [73]. Members of these transport protein families support the uptake of a large number of substrate groups, including organic anions and cations, prostaglandins, and bile salts [74-76]. However, bile salt uptake is mainly performed by the Na\(^+\)/taurocholate transporting polypeptide in the SLC10 superfamily [53].

Hepatic uptake transporters influence the pharmacokinetic properties of drugs by increasing the amount of substrate presented to the intracellular metabolic enzymes and efflux transporters. Therefore, they act as important determinants of the metabolism and biliary excretion of drugs that are dependent on facilitated hepatic uptake. Changes in the hepatic uptake process, such as transport inhibition, alter the drug metabolism and biliary excretion pattern and potentially decrease the overall hepatic drug clearance [30, 69, 70, 77].

**Figure 5.** Transport proteins involved in hepatic drug disposition. SLC transport proteins in the basolateral membrane facilitate uptake from the blood to the intracellular compartment. Inside the cell compounds can interact with metabolic enzymes or efflux transporters. Compounds can get effluxed back into the blood or get excreted to the bile by ABC transport proteins such as Pgp and BSEP or the two SLC proteins MATE1 and OST \(\alpha/\beta\).

**Basolateral Efflux**
Basolateral efflux proteins transport endogenous and xenobiotic compounds and their respective metabolites from the intracellular to the basolateral compartment. ABC efflux transporters with basolateral membrane expression include MRP3, MRP4 and MRP6 [24]. In addition, MRP1 and 5 have been identified to have low expression levels in healthy liver [25, 78]. During e.g. cholestasis and primary biliary cirrhosis the expression levels of
MRP1, 4, and 5 increases, indicating that they may participate in the hepatic protective response mechanisms during some pathological episodes [79-83]. All the basolateral efflux transporters include anionic compounds and phase II conjugates among their substrates, thereby facilitating renal excretion [52]. Furthermore, MRP3 is co-expressed with several of the metabolizing cytochrome P450 (CYP) enzymes in the pericentral part (zone 3) of the liver lobule, further indicating that the basolateral ABCs may redirect metabolism excretion from bile to urine [84, 85]. Inhibition of the basolateral efflux transporters therefore potentially increases the risk of hepatic accumulation of metabolites.

**Canalicular efflux**

ABC transport proteins in the canalicular membrane of hepatocytes include Pgp, BCRP, MRP2 and BSEP that mediate the biliary excretion of drugs, bile salts, and metabolites against a sometimes steep concentration gradient [8, 24, 25, 42, 52]. In addition, the SLC-transporter multidrug and toxin extrusion exchanger 1 has been identified to facilitate biliary excretion of organic cations [86]. BSEP has a narrow substrate spectrum, primarily restricted to monovalent, negatively charged bile acids and is therefore not believed to influence the biliary excretion of drugs [87]. However, drug inhibition of BSEP transport may be associated with increased risk of DILI [88, 89]. In contrast, Pgp, BCRP and MRP2 have broad substrate specificities and play important roles in several protective membrane barriers [26, 90-92]. In the liver they decrease the intracellular load of many endogenous and xenobiotic compounds or their metabolites. The broad substrate specificity of these transporters increases the risk for DDI at the transporter level [93-95].

Drugs, endogenous metabolites, bile salts, and cytokines, as well as several disease states or developmental stages, affect the expression levels of these transport proteins. Disorders with decreased function or protein expression, such as progressive intrahepatic cholestasis type II or Dubin-Johnson syndrome, exemplify the importance of the canalicular-efflux transporters in human physiology [96, 97].

**Cellular Models for Hepatobiliary Transport Investigations**

Several methods have been established to elucidate drugs interactions with transport proteins in cellular barriers throughout the body, including the hepatobiliary tract [98-104]. However, each method has strengths and limitations and, to date, no individual method can adequately predict the contribution of individual hepatic transport proteins to the overall hepatic drug disposition in humans in vivo.
Membrane Vesicles

The simplest model comprises inverted membrane vesicles (Fig. 6A) produced from cells overexpressing the transport protein of interest or from the canalicular membrane domain from isolated hepatocytes [99, 105]. This model is easy to use and allows automated high-throughput screening of transport inhibitors. Inverted membrane vesicles also allow the investigated compounds to interact directly with the transport protein without the first membrane passage that is required in cellular systems. This is an advantage in studies aimed to describe compound-protein interactions at a molecular level. However, this method has limited use in screening for transport substrates among compounds of high permeability. Due to the passive diffusion out of the vesicles such compounds fail to accumulate in the vesicles despite active transport of the investigated protein.

Figure 6. Models that can be used to study transport protein interactions. (A) A schematic figure of an inverted membrane vesicle from a membrane including an ABC transport protein. Because of the inverted configuration solutes are free to interact with the transport protein directly without first diffusing into the intracellular compartment. (B) Some immortalized cell lines form high integrity cell monolayers with tight junctions restricting the paracellular transport. Such cell lines, e.g., MDCK-II and Caco-2, can be used for bidirectional transport experiments. (C) Primary hepatocytes express most or all proteins important in hepatic drug disposition. If cultured in 3D configuration with extracellular matrix they form canalicular networks simulating the biliary compartment in the in vivo liver. The arrows in figure 6C indicate three such networks.

Immmortalized Cell Lines

ABC-overexpressing cell lines are frequently used to investigate specific efflux protein interactions [106-108]. Some cell lines, such as MDCK-II, benefit from their ability to establish high-integrity cell monolayers that consequently allow experimental setups that determine the vectorial transport of the test compounds (Fig. 6B). Because of the general absence of uptake transport proteins in these models, sufficient (logP > 0.5) passive permeability is a prerequisite for ABC-protein interaction studies in these models [103].

Immortalized hepatoma cell lines provide a reliable cell source with high yield. The HepG2 cell line is the most commonly used and best character-
ized of the hepatoma cell lines [109, 110]. Compared to primary hepatocytes, HepG2 has a more stable phenotype that could benefit routine testing. In conformity with other available hepatoma cell lines, HepG2 generally expresses low levels of metabolic enzymes and transport proteins and often needs transfection or enzyme induction modifications to facilitate metabolism investigations [25, 111, 112]. The HepaRG hepatoma cell line constitutes the most promising substitute for primary hepatocytes [113]. When differentiated by treatment with dimethyl sulfoxide (DMSO), HepaRG cells express a variety of metabolic enzymes and functional receptor pathways important in drug metabolism [114-117]. In comparison to HepG2, the HepaRG cell line has a CYP expression profile and induction pattern that more resembles primary hepatocytes. However, both cell lines fail to report toxicity responses observed in primary hepatocytes [111].

Primary Hepatocytes
Primary hepatocytes can be regarded as the most relevant experimental systems for the evaluation of hepatic drug properties available today. These generally express all the transport proteins and metabolizing enzymes found in the human liver and are one of the in vitro models best mimicking in vivo liver [111, 118]. Primary hepatocytes enable investigations of most hepatic drug interactions such as transport protein interaction, phase I and II metabolism, DDI potential, and receptor response mechanisms [111, 119, 120]. They can be cultured in a sandwich configuration containing extracellular matrixes which allows a three dimensional (3D) culture with more in vivo-like cell-cell and cell-extracellular matrix interactions (Fig. 6C) [118, 121]. This configuration has been observed to maintain morphologically and functionally stable hepatocyte cultures for up to eight weeks [64]. However, not all protein expression remains stable during sandwich culture and one of the hurdles for researchers using isolated primary hepatocytes is to overcome the reduced activity of the metabolic enzymes and some basolateral uptake transporters during the first days of culture.

Stem Cell Derived Hepatic Cells
Stem cells are undifferentiated cells with the ability to develop into many different cell types [122]. They also possess unlimited replication capacity [123]. The multipotent or pluripotent properties of adult and embryonic stem cells, respectively, provide a highly interesting model system for basic research, or as a potential hepatic cell source for drug discovery or toxicological research [123, 124]. Following intricate differentiation protocols, the differentiated hepatocyte-like cells exhibit several hepatocyte specific functions, including activity of metabolic enzymes [125-127]. Nevertheless, important barriers including the often fetal phenotype of differentiated hepatocytes still remain before differentiated stem cells become a reliable substitute for primary hepatocytes [98].
Drug-induced Liver Injury (DILI)

During the last decades, DILI has been the most frequent cause of safety-related drug marketing withdrawals in the US. Hepatotoxicity occurring in the late stages of drug discovery has restricted the use, or even prevented the approval, of many drugs [128].

DILI is commonly classified into intrinsic and idiosyncratic hepatotoxicity [129-131]. Intrinsic hepatotoxicity is regarded as dose-dependent and predictable above a threshold dose, whereas idiosyncratic hepatotoxicity occurs without obvious dose-dependency and in an unpredictable fashion. Intrinsic hepatotoxic compounds are generally discovered and rejected in preclinical trials, while idiosyncratic hepatotoxicity may not be identified until post-marketing monitoring when the drug becomes available to a larger population [129]. Among drugs withdrawn from the market due to severe DILI, hepatic failure appears at frequencies less than 1 of 10 000 treated patients [128]. When compared to the few thousand individuals typically included in clinical trials for new drug applications, the risk of underestimating the occurrence of severe DILI becomes apparent.

The initial of toxicity of a drug, or often its metabolites, can be caused through several mechanisms e.g., direct cell stress, targeted mitochondrial function, or triggered specific immune reactions [130, 132, 133]. Despite the different initial mechanisms, cell stress and triggered specific immune reactions commonly lead to mitochondrial damage and altered membrane potential. This happens either through a direct pathway activated by severe cellular stress or via an indirect pathway, initiated by mild cellular stress or activated specific immune reactions [131]. Independently of the origin of the first insult, the mitochondria seem to play a critical role in the initiation of DILI. The extent of mitochondrial impairment possibly determines whether hepatocytes die by apoptosis or necrosis [134]. In most patients, this onset of hepatic injury is followed by adaptation that most often results in resolution of the injury, despite continued drug treatment. However, in some patients the stress and injury reach a critical threshold and death-signaling pathways become dominant [135]. The injury then exceeds the adaptive repair and regeneration capacity of the liver and contributes to a progressive liver injury. Therefore, DILI can be seen as an active process involving recruitment of death-signaling pathways that mediate cell death rather than a passive process due to overwhelming biochemical injury [135].
Computational Modeling of Transport Protein Interactions

Since the late 1980s computational (in silico) approaches to assess interactions between drug or drug-like molecules and transport proteins have become more widely used in early drug discovery [136]. Computational models describing protein interactions generally follow one of two approaches. **Protein based models** describe the 3D protein structure and can be used for ligand docking [137, 138]. However, the lack of high-resolution crystal structures of human transport proteins restrict these models to be based on crystal structures of ABC proteins of other species- or protein family- origin [45, 139]. **Ligand based models** describe the molecular structure of the ligand interacting with the protein. On the basis of the approach taken to describe the ligand, the ligand-based models can be further divided into subgroups including quantitative structure-activity models (QSAR) and pharmacophore models [138, 140, 141]. Pharmacophore models use the spatial location of key molecular structure elements while QSAR uses numeric values of calculated molecular descriptors to describe important molecular features of protein-interacting compounds.

Developed computational models can be used to screen large, diverse databases of molecules to identify compounds likely to be substrates or inhibitors of transport proteins. Predictions can be performed from molecular structures even before compound synthesis, and thereby aid in the selection of structures less likely to have undesirable transport-protein interactions. The dataset selection has a crucial impact on the possible fields of application of the final models. To allow for the development of predictive in silico models the dataset has to be well distributed through the chemical space that the final model aims to describe. Global models aim to describe an entire chemical space, such as that of orally administered drugs. In contrast, models aimed to describe compound series or parts of a chemical space are generally referred to as local models. In this thesis, large, diverse datasets were used to develop global models of the chemical space of orally administered drugs. The methods included in the work are described in the following sections.

**Principal Component Analysis (PCA)**

Given the large amount of variables that can be used to describe the variance within a dataset, exact knowledge of the information captured by each variable is not always available. Therefore, the correlation, or overlap in information, between the included variables is unknown. If the variable correlation is unknown or if included variables are known to correlate with each other to some extent, principal component analysis (PCA) is a good tool to describe the variance within the dataset [142].
PCA can be used to reduce the number of variables needed to describe the variance in a dataset, and to identify outliers and trends. The method combines correlated variables into fewer artificial variables, called principal components. For example chemical descriptors such as molecular weight, number of carbons, and molecular surface area, are different properties related to molecular size. These three variables could therefore be combined into one principal component describing molecular size. Each of the variables influences the principal component to different extents, depending on the way that the molecular descriptors correlate to molecular size. Chemical diversity is a good example of when PCA is a suitable tool to describe the overall diversity in a dataset using few principal components, often describing size, shape, lipophilicity or polarity.

In short, PCA is a way to extract information from many variables and combine them into in fewer principal components. PCA reduces the number of dimensions needed to describe the systematic variance and therefore can be used to display the variations in a dataset graphically.

Partial Least Squares Projection to Latent Structures (PLS)
Partial least squares projection to latent structures (PLS) is a regression extension of PCA based on the same theory that combines correlated variables into principal components. However, PLS models relate the components to a set of one or more response variables, such as experimental in vitro data. PLS models seek solutions that maximize the response variance explained by the included variables or components. Hence, these models describe the response variables using correlating, or co-varying, components [142, 143].

PLS can be performed in several different ways. If the response variable has a binary property, for instance a yes or no answer, discriminant analyses (DA) can be performed. In PLS-DA the model seeks components that distinguish one group from the other. In orthogonal PLS (OPLS), the systematic variation in the dataset is divided into two groups: the predictive and the orthogonal variables. The predictive variables co-vary with the response variable while the orthogonal ones do not. As the name implies, the orthogonal components are perpendicular to the predictive component and therefore have an average zero variance in the predictive component [142, 143].

In this thesis, molecular properties important in ABC inhibition was identified using OPLS-DA analyses of molecular descriptors and in vitro data as variables and response variables, respectively.

Ligand-Based Docking to Protein Models
Ligand-based docking requires a crystal structure or homology model of the 3D structure of the investigated protein. In 2009, the first high-resolution
crystal structure of an eukaryotic ABC protein, mouse-Pgp, was determined [45]. On the basis of this crystal structure, homology models for human ABC proteins (previously restricted to bacterial ABC crystal structures), could be developed [95] and subsequently employed in ligand-docking models [144, 145]. Protein-ligand docking is generally performed in three steps. First, the 3D protein structure is scanned for regions of tight atom packing that indicate possible ligand-binding sites (LBS). Next, energy-minimized 3D ligand configurations are determined. In the third and final step, the ligands are docked to the protein-binding site and scored according to favorable interactions with the protein-binding site (positive scores) and unfavorable protein-overlapping ones (negative scores).
Aims of This Thesis

The general objective of this thesis was to examine drug interactions with ABC transport proteins and investigate how such interactions influence the biliary excretion, DDI and DILI.

The specific aims were:

- To establish methods for the investigation of ABC-dependent transport, using membrane vesicles (Paper I-IV) and sandwich cultured human hepatocytes (SCHH) (Paper III and IV).

- To identify MRP2 and BSEP transport inhibitors within diverse datasets distributed throughout the chemical space of orally-administered, registered drugs (Papers I and IV).

- To investigate the Pgp-, MRP2-, and BCRP-inhibition in cellular and vesicular \textit{in vitro} model systems, and to identify inhibitors with specificity towards individual or multiple ABC proteins (Papers II and III).

- To develop predictive computational models describing the inhibition of Pgp (Paper II), MRP2 (Paper I and II), BCRP (Paper II) and BSEP (Paper IV).

- To predict the maximal intrinsic biliary clearance and SCHH hepatobiliary transport, using quantified protein levels and \textit{in vitro} transport kinetics (Paper III).

- To determine the role that BSEP inhibition plays in DILI, and to investigate the extent to which BSEP transport inhibition can predict such adverse events (Paper IV).
Methods

Dataset Selection

The studies in the thesis were focused on drug interactions with ABC transport proteins. Therefore, the datasets were chosen to be structurally diverse and well-distributed throughout the chemical space of orally-administered, registered drugs.

In papers I and IV, the aim was to describe the affinity pattern for MRP2 and BSEP inhibition, respectively. In addition to molecular diversity and drug-likeness, compounds in these studies were selected on the basis of previous knowledge of their affinities for ABC proteins. The aim of paper II was to compare the inhibition affinity pattern for Pgp, BCRP, and MR2 in a diverse dataset. Compounds were included on the basis of high-quality literature data on Pgp inhibition in combination with in-house BCRP and MR2 inhibition data. In paper III, the dataset was selected on the basis of the previous results with the aim to further explore the general and more specific affinity patterns of Pgp, BCRP, and MR2 inhibitors.

Experimental Methods

ABC Transport in Membrane Vesicles

Inverted membrane vesicles from Sf9 cells overexpressing human MRP2 or BSEP were used in papers I, II and IV. Membrane vesicles prepared from HEK293 cells transiently transfected to overexpress Pgp, BCRP or MR2 were used in paper III. Incubation times and substrate concentrations were selected to be within linear regions of transport kinetics. MR2 transport was studied using estradiol 17-β-glucuronide (E17G) at 50 µM or 10 µM (Paper III). Both concentrations were in the linear transport range and. The higher concentration was used to minimize the occurrence of transport stimulation effects observed for E17G [146, 147]. BCRP transport in vesicles was investigated using 1 µM estrone 3-sulfate (E3S) or 10 µM E17G, both used within linear transport kinetics. For the same reason Pgp transport was investigated using 0.04 µM N-methyl quinidine (NMQ) and 10 µM E17G. In general, substrate concentrations three to ten times below $K_m$ were used.
The transport experiments were conducted using the rapid filtration technique previously described [148]. In brief, membrane vesicles were quickly thawed from -85°C to 37°C and a volume corresponding to 10 – 20 µg total protein per well was pre-incubated with the substrate. Transport was initiated by the addition of ATP and creatine kinase or exchanged to AMP in control wells for the determination of passive permeability. Transport was stopped by the addition of ice-cold stop solution and after filtration and filter wash steps the vesicles were analyzed for substrate content through scintillation counting for radiolabeled substrate or HPLC-MS/MS for unlabeled substrates.

For scintillation analysis, the filters were completely dried before scintillation cocktail was added after which the filters were analyzed for radioactivity, using a TopCount NXT scintillation counter (PerkinElmer, Waltham, MA). LC-MS/MS quantifications were performed with Waters Xevo TQ MS with electrospray ionization coupled to Acquity HPLC or UPLC systems (Waters, Milford, MA). For HPLC-MS/MS analyses, the test compounds were extracted from the washed filter plates with acetonitrile and water (20:80) containing an internal standard. After centrifugation, the filtrates were injected and intravesicular compound levels were quantified. ATP-dependent transport was calculated by subtraction of passive permeability determined from AMP incubations and ATP transport kinetics was determined using non-linear regressions (Prism, GraphPad, San Diego, CA).

Inhibition of ABC Transport in Membrane Vesicles

The inhibition experiments were performed as normal transport assays (described above) but co-incubated with inhibitors at concentrations suitable for the purpose of the study. MRP2 (paper I and II) and BSEP (paper IV) inhibition were screened at test compound concentrations of 80 µM and 50 µM, respectively. In IC_{50} determinations, an inhibitor concentration interval from 1 to 500 µM was generally used. However, due to the inhibition potency of ko143, a lower concentration interval (0.001 to 10 µM) was used for this compound. IC_{50} values were calculated from nonlinear regressions of the dose-response curves using Prism version 5 (GraphPad, San Diego, CA). Subsequent K_i values were calculated according to equation 1.

\[ K_i = \frac{IC_{50}}{\left(\frac{[S]}{K_m} + 1\right)} \]

where the IC_{50} denotes the inhibitor concentration that inhibits 50% of the substrate transport, [S] is the substrate concentration in the medium, and K_m represents the concentration that corresponds to half the maximal substrate transport rate. In papers II and III, an inhibitor was regarded as specific for
one of the transport proteins if the IC\textsubscript{50} or K\textsubscript{i} was at least 10-fold lower compared to the other two transporters.

Inhibition of Efflux in BCRP-Overexpressing Cells

In paper II, inhibition of BCRP-mediated efflux was investigated in Saos-2 cells, stably transfected to express human BCRP. To determine compounds inhibitory effects on BCRP transport, the cells were incubated with 1 µM mitoxantrone for 60 min, with or without the addition of 50 µM of the test compounds. Transport was terminated by the addition of ice-cold buffer, the cells were washed, trypsinated, and then analyzed by flow cytometry (Beckman Coulter FC500, CA) to determine intracellular mitoxantrone concentrations. The BCRP inhibition obtained by 0.5 µM of the potent BCRP inhibitor ko143 was set to 100% and used to normalize the results of the test compounds.

Isolation of Primary Human Hepatocytes

Liver tissues were obtained from human donors undergoing partial liver resections at the Department of Surgery, Uppsala University Hospital, Sweden. Resected tissues from patients not suffering from primary hepatic cancers or hepatitis infections were included. All donors gave informed consent, in accordance with the ethical approval from the Uppsala Regional Ethical Review Board (Ethical Approval no. 2009/028). Hepatocytes were isolated using the two-step liver digestion technique of LeCluyse and Alexandre (2010) [149]. Briefly, tissue perfusion and cell isolations were initiated within two hours after tissue resection. During this time the tissues were kept in ice-cold Dulbecco’s modified Eagle’s medium (DMEM). The tissues were flushed free from blood with ice-cold DMEM and cannulated to support two perfusion lines. The first perfusate, containing ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), and ascorbic acid, was maintained at 37°C and circulated for approximately 10 min. After terminating the first perfusion, the second perfusate including collagenase, protease, BSA, and ascorbic acid, was immediately started. When clear signs of tissue digestion were visible, the perfusion was stopped and the cells harvested. Three centrifugation steps, with the second one including 27% Percoll (GE-Healthcare, Sweden), were performed to enrich viable hepatocytes suitable for culture.

Culture of Human Hepatocytes

Isolated primary hepatocytes with a viability > 85% were seeded in collagen-1 coated 24-well plates (BD Biosciences, Bedford, MA) at a density of 3.75 x 10\textsuperscript{5} cells per well and maintained at 37°C and 5% CO\textsubscript{2}. To allow the
cells to attach to the plate, 500 µl DMEM supplemented with 5% (v/v) fetal bovine serum, 4 µg/ml insulin, 1 µM dexamethasone, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin was added to each well. After two to three hours this medium was replaced with 500 µl culture medium (HMM (Lonza, Basel, Switzerland) supplemented with insulin 10 µg/ml, transferrin 5.5 µg/ml and selenium 5 ng/ml, 0.1 µM dexamethasone, 100 U/ml penicillin and 100 µg/ml streptomycin). After overnight incubation, the cells were overlaid with 500 µl ice-cold 0.25 mg/ml Matrigel (BD Biosciences, Bedford, MA) in culture medium. To allow the formation of bile canaliculi, the cells were cultured for an additional four to six days during which time, the culture medium was exchanged every 24 hours.

Biliary Excretion in SCHH

Biliary excretion in SCHH was determined using a method modified from that described by Liu et al 1999 [150]. In short, SCHH were rinsed twice with either standard Hank's Balanced Salt Solution (HBSS), to maintain the integrity of tight junctions and bile canaliculi, or with Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS, to disrupt the tight junctions. Cultures were pre-incubated with standard or Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS, including the investigated inhibitor (10 or 50 µM) in inhibition experiments. Substrate (1 µM E17G and TA in Paper III and IV, respectively) was added and the uptake and excretion into the bile canaliculi was studied. Transport was terminated after 10 min by removing the incubation medium and rinsing the cells with ice-cold standard or Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS. Cells were lysed with 1 M NaOH and kept at 4°C overnight before LC-MS/MS analysis. Total protein content was determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL), according to manufacturer’s instructions. Substrate uptake was converted to molar amounts by normalizing to measurements in substrate solutions of known concentrations and normalized to total protein content.

Total accumulation (TOTAL\(_{Acc}\); intracellular + bile from HBSS incubations) and intracellular accumulation (IC\(_{Acc}\); from Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS incubations) were used to calculate biliary accumulation (BILE\(_{Acc}\)) according to equation 2.

\[
BILE_{Acc} = TOTAL_{Acc} - IC_{Acc}
\]  

(2)

Biliary excretion index (BEI; Eq.3) is a ratio describing the biliary accumulation of a compound in relation to its total accumulation [150]. BEI decreases if a test compound inhibits the canalicular efflux. The BEI parameter includes the bile accumulation in both the numerator and denominator. Thus
a decrease in BEI need not be directly proportional to a decrease in canalicular efflux caused by an added transporter inhibitor. BEI was calculated according to equation 3.

\[
BEI = \frac{BILE_{Acc}}{TOTAL_{Acc}} \times 100
\]  

Bile intracellular correlation (BIC) describes the biliary accumulation in relation to the intracellular accumulation. Thus, BIC compensates for variability in intracellular substrate accumulation, which is of interest since it governs efflux kinetics. Because BIC is calculated using the accumulation in the adjacent intracellular and bile compartments, any decreases in BIC is directly proportional to decreases in the canalicular efflux. BIC was calculated according to equation 4.

\[
BIC = \frac{BILE_{Acc}}{IC_{Acc}}
\]  

The in vitro biliary clearance (CL\textsubscript{Bile}) was calculated according to equation 5. CL\textsubscript{Bile} is dependent on the net substrate flux across the cell, i.e., on the net basolateral uptake, including basolateral efflux, and canalicular efflux. Therefore, CL\textsubscript{Bile} decreases if a test compound inhibits the basolateral net uptake or the canalicular efflux. CL\textsubscript{Bile} was calculated according to equation 5.

\[
CL_{Bile} = \frac{BILE_{Acc}}{AUC}
\]

where AUC represents the dose calculated from substrate concentration in the medium and incubation time.

Identification of Compounds Causing DILI

In paper IV, the influence of BSEP inhibition on DILI was investigated. The ADR classification system implemented in drug labels issued by the FDA was considered to be a suitable data source for indication of the drug’s potential to inflict DILI. The FDA drug labels represent a consensus of regulatory and industry experts and they aim to include all known adverse effects of a drug, from in vitro results to post-marketing monitoring. The three ADR sections within the drug labels categorize ADRs by increasing severity, ranging from the least severe ‘Adverse Reactions’ (AR), through the intermediate ‘Warnings and Precautions’ (WP), to the most severe ‘Boxed Warnings’ (BW). Category inclusion criteria are regulated by US federal regulation 21CFR201.57 [151]. The AR section describes the overall adverse reaction
profile of a drug and includes those adverse events believed to have a causal relationship with the drug. As soon as reasonable evidence of a causal association between an ADR and a drug is established, it should be included in the WP or BW section, depending on the severity of the ADR.

To assess the DILI potential of registered drugs, information on hepatic ADRs in FDA-approved drug labels was obtained from DailyMed (http://dailymed.nlm.nih.gov/) [152]. Briefly, FDA drug labels were reviewed for hepatic adverse reactions by searching for keywords related to liver injury (see supporting information, Paper IV). A compound was regarded to have the potential to inflict DILI if the keywords were identified in the BW, WP or AR sections within a drug label. If a keyword was mentioned in several sections, the compound was classified in the most severe category (BW>WP>AR). Drugs where no hepatic ADRs could be identified were categorized as ‘Not mentioned’ (NM). The ADR sections in FDA drug labels describe ADRs in a hierarchical way on the basis of their severity. To assure that the ADR sections described DILI severity in the same hierarchical way, without influence from other ADRs reported for the same drug, the severity of DILI reported in the different sections was evaluated using the method described by Chen et al. 2011 [153].

**Pgp, BCRP and MRP2 Protein Expression**

Immunohistochemical data were determined as a part of the Human Protein Atlas project [66]. In brief, protein epitope signature tags (PrEST) were identified and the corresponding recombinant protein was expressed to produce antibodies in New Zealand rabbits. Monospecific antibodies were purified through depletion of tag-specific antibodies after which affinity columns were loaded with the protein-specific PrEST. Quality assurance was confirmed by sequence verification of the PrEST clone, size determination of the recombinant protein, and cross-reactivity with other identified PrESTs. Validations of the antibodies were investigated by matching the western blot staining pattern to those from reference antibodies [154, 155].

The protein expressions of Pgp, BCRP and MRP2 in liver tissues and the overexpressing membrane vesicles were quantified using targeted proteomics. Membrane fractions from four snap-frozen human liver tissue samples were retrieved using the ProteoExtract Native Membrane Protein Extraction Ki (Calbiochem, Temecula, CA) according to the manufacturer’s instructions. Peptides were generated through protein extraction and tryptic digestion of the membrane fractions and the membrane vesicles preparations expressing Pgp, BCRP and MRP2. The peptides, and subsequent proteins, were quantified by LC-MS/MS with the addition of stable isotope labeled internal standard peptides, as previously described [156-158].
Canalicular Efflux Predicted by the Maximal Transport Activity

The maximal transport activity (MTA) is the estimated maximal in vivo transport rate predicted from the in vitro maximal transport rate ($V_{\text{max}}$) and the in vivo and in vitro quantified protein levels [140]. MTA is directly proportional to the $V_{\text{max}}$, in vivo assuming that all quantified protein contributes to the active substrate transport and that the compared cell types contain similar amounts of total membrane proteins. The maximal Pgp, BCRP and MRP2 dependent canalicular efflux of E17G was calculated according to equation 6.

$$MTA = \frac{\text{Prot.Exp.}_{\text{in vivo}}}{\text{Prot.Exp.}_{\text{in vitro}}} * V_{\text{max, in vitro}} \quad (6)$$

where the Prot.Exp. in vivo and Prot.Exp. in vitro denotes the quantified protein levels of the transport proteins in human liver tissues (n = 4) and inverted membrane vesicles, respectively. $V_{\text{max, in vitro}}$ is the maximal substrate transport rate determined in the membrane vesicles.

The MTA was then used to predict the intrinsic in vivo biliary clearance (CL$_{\text{Bile, MTA}}$), according to equation 7.

$$\text{CL}_{\text{Bile, MTA}} = \frac{\text{MTA}}{[S]+K_m} * \text{HomProt} \quad (7)$$

where [S] represents the in vivo hepatocellular substrate concentration, that was estimated to be << than Pgp, BCRP and MRP2 $K_m$ and was therefore disregarded. $K_m$ denotes the Michaelis-Menten constant determined in the membrane vesicles and HomProt the total protein amount in human liver homogenate, 88 ± 14 mg/g tissue.

Finally, the in vivo biliary clearance (CL$_{\text{Bile, MTA}}$) was scaled to SCHH biliary clearance (CL$_{\text{Bile, SCHH}}$) with the following assumptions: 133 x 10$^6$ hepatocytes/g human liver tissue and 375 000 hepatocytes per well. The obtained values were subsequently used to predict the biliary accumulation (Bile$_{\text{Acc, MTA}}$) of E17G in SCHH, according to equation 8.

$$\text{Bile}_{\text{Acc, MTA}} = \text{CL}_{\text{Bile, SCHH}} * \text{Inc.Time} * \text{IC}_{\text{Acc}} \quad (8)$$

where Inc.Time and IC$_{\text{Acc}}$ denotes the SCHH incubation time (10 min) and the experimentally determined intracellular accumulation (12 µM), respectively.
Computational Methods

Generation of Molecular Descriptors
The two-dimensional (2D) molecular structures of the compounds in the datasets were obtained using Sci Finder Scholar (CAS, OH) (Papers I and II) or PubChem [159] (Paper IV), and converted to 3D structures using Corina (Molecular Networks, Germany). The molecular descriptors used to develop the computational models were calculated from the 3D structures using Dragon X (Talete, Italy), ADMET Predictor (SimulationsPlus, CA), and MAREA [160].

Principal Component Analysis - PCA
To evaluate and visualize the dataset selection, a PCA model describing the chemical space of orally-administered drugs was developed, using Simca-P (Umetrics, Sweden). Molecular descriptors calculated for 652 orally-administered drugs were used in the model development. Variables with subzero prediction values during the cross-validation were excluded. The resulting PCA model was then used as a background template on which the datasets were projected to evaluate structure diversity and drug-likeness.

Partial Least Squares Projection to Latent Structures (PLS)
Throughout the computational studies, discriminant analysis using PLS regressions (PLS-DA) or extensions thereof (OPLS-DA) were used to describe and predict the inhibition of ABC transport proteins. All models were developed using Simca-P (Umetrics, Sweden).

Dataset Preparation
After generating the molecular descriptors and obtaining the experimental protein interaction results, the datasets were prepared for the model development in two steps. First, the datasets were randomly divided into a training set (for model development), and a test set (for the model validation). Then the differently sized groups of inhibitors and non-inhibitors were balanced to produce equally sized groups in the data set and hence, reduce the possibility of one of the groups weighting the model to be developed.

Model Development and Validation
As a first step in model development, molecular descriptors were mean centered and scaled to unity-of-variance. Skew descriptors and those with zero variance were excluded. The models were optimized by a variable selection procedure in which groups of molecular descriptors that did not contain in-
formation relevant to the response (i.e., noise) were removed in a stepwise manner. Descriptors were not included in the model if removing them resulted in a statistically improved model, based on the classification accuracy for the training set. The statistical validity of the models was tested using a random permutation test (n = 100). All presented models collapsed to subzero cross-validated coefficients of determination (Q^2) when the response variables were permuted, demonstrating that they were indeed describing the response variables. More importantly, all models were challenged with test sets composed of one-third of the experimentally investigated compounds.

Virtual Docking

Virtual docking of general ABC inhibitors to a homology model of the nucleotide-binding domain 1 (NBD1) was performed in Maestro version 8.5 (Schrödinger, CA). The human MRP1 (ABCC1) NBD1 (Protein Data Bank ID 2CBZ) was used as a docking template for the 3D molecular structures of the test compounds. The protein and ligands were prepared using the default settings of the Maestro protein and ligand preparation procedures, respectively. The ligands were docked using default docking parameters in Glide XP version 5.027 (Schrödinger, CA) and the alignments of ATP and docked ligand were visualized using Accelrys DS Visualizer version 2.0.1 (Accelrys, CA), and QuoteMol version 0.4.1 [161].

Statistical Analysis

Data are expressed as means with standard deviations or standard error of the mean, as indicated in the text. In the MRP2 and BSEP inhibition screens, compounds that significantly decreased substrate transport were identified by one-way ANOVA with Bonferroni’s post hoc test, as implemented in Prism 5 (GraphPad Software, Inc., La Jolla, CA). Differences in the proportions of DILI-inducing agents in each ADR class were determined using normality tests. The Marascuillo procedure was used to determine statistical differences of mild and severe DILI distribution in the different ADR sections. In paper IV, analysis of SCHH accumulation data was conducted using two-way ANOVA with Bonferroni’s post hoc test.
Results and Discussion

Dataset Diversity
The aim of this thesis was to describe the inhibition of ABC transport proteins involved in the biliary excretion by using large, diverse datasets evenly distributed through the chemical space of orally-administered drugs. The dataset selection process plays a crucial role for the predictivity of the final model and particular care must therefore be taken to assure good coverage of the chemical space that is to be modeled.

The final dataset used to investigate MRP2 inhibition (Paper I) included 191 compounds. The overlapping Pgp, BCRP and MRP2 inhibition (Paper II) was studied among 122 compounds, and 250 compounds were investigated for BSEP inhibition. The dataset used in Paper II (n = 122) was included in the MRP2 and BSEP datasets. The chemical diversity of the datasets was evaluated by superimposing them on the chemical space of orally administered, registered drugs. The datasets were proven suitable for the intended investigations as they covered the relevant chemical space.

Identification of MRP2 and BSEP Inhibitors
In Papers I and IV, inverted membrane vesicles from Sf9 cells overexpressing human MRP2 and BSEP, respectively, were used to study the inhibition of the active transport of estradiol 17β-glucuronide (E17G) and taurocholate (TA), respectively.

Before investigating the transport inhibition, substrate transport in the vesicles was characterized with regard to affinity, time dependence and sensitivity for potent MRP2 and BSEP inhibitors. For MRP2, ATP-dependent transport was linear for up to 30 min. The transport kinetics from non-linear regression showed an apparent $K_m$ of $94 \pm 7 \mu M$ and a maximal transport rate of $680 \pm 30 \text{ pmol/mg/min}$. The kinetics revealed an initial lower slope for the E17G transport rate, indicating that the substrate stimulates its own transport. The MRP2 dependent transport was inhibited by the established MRP inhibitor MK571, with an IC$_{50}$ of $10 \pm 1.5 \mu M$ (Fig. 7A-C). For BSEP, the results indicated linear transport for up to 15 min with $K_m$ and $V_{max}$ of
17.8 ± 5 µM and 286.2 ± 28.2 pmol/mg/min, respectively. The established BSEP inhibitor Cyclosporine A (CycA) had an IC₅₀ of 4.6 ± 1.2 µM in this system (Fig. 7 D-F).

Figure 7. Kinetics determined for the MRP2 (Paper I) and BSEP (Paper IV) assays. Time dependent transport (A, D), transport kinetics (B, E) and concentration dependence of model inhibitors (C, MK571 for MRP2 and D, Cyc A for BSEP) was determined for MRP2 and BSEP model substrates E17G and TA, respectively.

Of the 191 compounds screened for inhibition of MRP2-mediated E17G transport, 42 reduced E17G transport by more than 50% compared to the controls (Fig. 8 A). A similar hit rate (21%) was observed for BSEP, where 53 compounds inhibited the BSEP-mediated TA transport by more than 50% (Fig. 8 B).

BSEP substrates are typically monovalent, negatively-charged bile acids. Of the few non-bile acid substrates identified to date, all carry a negative net charge at physiological pH [162, 163]. This is in agreement with the molecular features of importance for BSEP inhibition as well, where positive charge is strongly associated with a lack of inhibition and only 6% of the inhibitors are positively charged (Fig. 9A). However, unlike substrate interactions with BSEP, which are clearly disfavored by the lack of a negative charge [87], the majority of the BSEP inhibitors (58%) were un-ionized. MRP2 also has a clear preference for negatively charged substrates and approximately 80% are anionic compounds. However, the inhibitor charge pattern differed significantly from that of BSEP, and almost one-third of the MRP2 inhibitors carried a positive net charge at physiological pH (Fig. 9B). This highlights an important difference between MRP2 and BSEP inhibition affinity and may support the theory of an additional inhibitory binding site with a separate mechanism in MRP2.
Figure 8. Inhibition of the ATP-dependent transport of MRP2 and BSEP. Inhibition of MRP2 mediated E17G transport was investigated for 191 compounds and 42 inhibitors were identified. In addition, 13 compounds were found to stimulate MRP2 transport. (B) BSEP inhibition was investigated for 250 compounds using TA as a model substrate. Fifty-three compounds was found to inhibit more than 50% of the transport.

Figure 9. The charge distribution among compounds observed to inhibit more than 50% of the MRP2 and BSEP mediated E17G and TA transport, respectively. In contrast to the shared preference for negatively charged substrates, BSEP (A) and MRP2 (B) show different inhibitor charge patterns.
Overlapping Inhibition of the Canalicular ABC Proteins

Pgp, BCRP, and MRP2 are co-localized in several tissues or barriers important in the protection of the body from foreign or toxic compounds. Overlapping inhibition of the ABC transport proteins can therefore lead to serious adverse effects due to accumulation of toxic compounds or the introduction of compounds into tissues normally protected e.g., the brain. In Paper II, the inhibition overlap between Pgp, BCRP, and MRP2 was shown for the first time using a large diverse dataset (n = 122). In total, 66 compounds inhibited at least one of the transport proteins, and as many as 19 (29%) of these inhibitors significantly influenced the substrate transport of all three proteins. Interestingly, among these overlapping ABC inhibitors, 73% of all MRP2 inhibitors identified in the dataset were found. The dataset explored in Paper II was expanded with BSEP inhibition data (Paper IV), for all but one compound (gefitinib). In the new dataset (n = 121), 45 of the 68 identified inhibitors influenced the transport of at least two of the ABC proteins (Fig. 10). It could be hypothesized that the totally overlapping ABC inhibitors (n = 11) interact with the proteins at, for instance, the ATP-binding sites. Furthermore, the physicochemical properties of the general inhibitors show that they, in general, are more lipophilic and more often positively charged at physiological pH. These properties make them more likely to partition into the membrane and may therefore reflect other protein interaction mechanisms at the TMD.

Figure 10. Overlapping inhibition of MRP2, BCRP, Pgp and BSEP. A majority (n = 68) of the 121 compounds investigated for ABC transport inhibition was found to inhibit at least one of the four canalicular transport proteins. BCRP and Pgp inhibition was shared with MRP2 or BSEP for eight and nine compounds, respectively, but only one inhibitor was shared by only MRP2 and BSEP.
With the exception of the totally overlapping inhibitors, few compounds shared BSEP and MRP2 inhibition (n = 3). At the same time both proteins showed a significant inhibitor overlap with Pgp or BCRP or both, and eleven and nine BSEP and MRP2 inhibitors, respectively, were shared with the other two ABC proteins only. This further supports the theory that BSEP and MRP2 inhibitor interaction clearly differ despite their mutual preference for negatively charged substrates.

Molecular Properties Important in ABC Inhibition

One of the aims of the thesis work was to define molecular properties important for the inhibition of ABC transport proteins. For this purpose, computational models that discriminated between inhibitors and non-inhibitors were developed. One of the benefits of the PLS/OPLS-DA models compared to other computational models, such as neural networks, is the transparency of the developed models. Such models include relatively few molecular descriptors and allow for the interpretation of how individual molecular descriptors influence the ABC inhibition. Despite the simplicity of the final models, they correctly classified the absolute majority of the inhibitors or non-inhibitors in the models describing MRP2-, general ABC-, and BSEP inhibition (Table 1).

Table 1. Predictions of MRP2-, general ABC-, and BSEP inhibition by the developed computational models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Dataset</th>
<th>Correctly Classified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibitors</td>
<td>Non-inhibitors</td>
</tr>
<tr>
<td>MRP2</td>
<td>Training set</td>
<td>86 %</td>
</tr>
<tr>
<td>(Paper I)</td>
<td>Test set</td>
<td>72 %</td>
</tr>
<tr>
<td>General ABC</td>
<td>Training set</td>
<td>79 %</td>
</tr>
<tr>
<td>(Paper II)</td>
<td>Test set</td>
<td>79 %</td>
</tr>
<tr>
<td>BSEP</td>
<td>Training set</td>
<td>84 %</td>
</tr>
<tr>
<td>(Paper IV)</td>
<td>Test set</td>
<td>82 %</td>
</tr>
</tbody>
</table>

Molecular properties predictive for general ABC inhibition, describing Pgp, BCRP, and MRP2 inhibition, were lipophilicity, aromaticity, and molecular size. These properties also dominate the model developed for MRP2 inhibition. For BSEP, the single most important molecular property was lipophilicity, but other descriptors related to charge and hydrophobicity were also important. This indicates that even though the ABC transport proteins share many of the ligand interactions properties with each other, individual aspects must still be considered. Out of the four ABC transport proteins investigated herein, BSEP seems to have the most diverse inhibitor interactions. This is a
logical result given that the bile-acid transport protein BSEP lacks the wide substrate interaction profiles of the other three ABC proteins.

ABC Inhibition in Cellular and Vesicular Models

Previous studies have indicated that the concentration range where various inhibitors display selectivity towards an ABC transport protein varies between different model systems. In Paper III we investigated Pgp, BCRP, and MRP2 inhibition in HEK293 membrane vesicles and compared the results to those from investigations using other cellular or vesicular systems.

The results indicated that the inhibitor rank order was the same in cellular and vesicular systems (Fig. 11A-C). However, the cellular systems showed a general trend of higher IC<sub>50</sub>-values compared to those observed in the vesicular systems (Fig. 11D-F). This general decrease in inhibition potency was not observed between different vesicular systems or between different substrates in the HEK293 vesicles, where the IC<sub>50</sub> instead varied individually for different inhibitors. These results indicate that the general increase in IC<sub>50</sub>-values for the cellular systems is dependent upon the system (cell versus vesicle) and not the substrate. A possible explanation for this system dependency is the initial membrane passage required before ABC protein interaction in cell based models decreases the ABC-interacting inhibitor concentration and therefore the inhibition.

Figure 11. Inhibition rank order in (A) MDCKII-Pgp and HEK293-Pgp vesicles; (B) Saos2-BCRP and HEK293-BCRP vesicles; and (C) between Sf9 and HEK293 MRP2 vesicles. Inhibition potencies observed between the same model systems for Pgp (D), BCRP (E) and MRP2 (F).
Interestingly, several of the investigated compounds showed IC₅₀-values below 10 µM in the HEK-vesicles. This is a concentration that may be well within the range of in vivo intracellular concentration, e.g., for compounds with SLC facilitated uptake. For such compounds, the risk of in vivo DDI and DILI is considerable.

Prediction of Hepatobiliary Transport of E17G

In Paper III, a method [164] suggested for the prediction of individual transport protein contribution to the in vivo or in vitro membrane transport was evaluated experimentally for the first time. In this method maximal transport activity (MTA; Eq. 6) is used to predict the activity of individual transport proteins in target organs or in vitro model systems including all the investigated transport proteins, such as SCHH.

![Protein expression and predicted MTA for MRP2, Pgp and BCRP.](image)

**Figure 12.** Protein expression and predicted MTA for MRP2, Pgp and BCRP. Immunohistochemical staining of MRP2 (A), Pgp (B) and BCRP (C) showed clear canalicular staining of MRP2 and Pgp, but only weak staining for BCRP was observed. (D) BCRP, Pgp and MRP2 protein expression in human liver tissue (n = 4) quantified by targeted proteomics. (E) Biliary excretion of E17G in SCHH and predicted by MTA on the basis of protein expression and in vitro transport kinetics.

The MTA predictions are based on the levels of protein expression (Fig. 12A-D) and the in vitro transport kinetics determined in transporter overexpressing models, such as the membrane vesicles. The results presented in Paper III show, in line with literature data, that the biliary excretion of E17G was mainly dependent on MRP2 transport, and the MRP2 dependent biliary
excretion of E17G was predicted to be 49 and 182 times higher than that mediated by Pgp and BCRP, respectively. More importantly, using this method we were able to predict the biliary excretion of E17G in SCHH. The E17G biliary excretion was predicted to 90 pmol/mg, in good agreement with the experimental results of 71.5 pmol/mg (Fig. 12E).

**BSEP Inhibition and DILI**

Several drugs reported to cause DILI have been identified as BSEP inhibitors [89, 165, 166]. In Paper IV, the association of BSEP inhibition and DILI was investigated for the first time in a dataset with a DILI incidence comparable to that found among registered drugs.

*Figure 13. DILI potential of drugs associated with weak or strong BSEP inhibition. The FDA drug-label sections, ‘boxed warnings’ (BW), ‘warning and precautions’ (WP), and ‘adverse reactions’ (AR), were used to classify DILI potential of the drugs (n = 182) in the dataset also screened for BSEP inhibition in membrane vesicles. Bars show the fraction of all compounds in the same BSEP inhibition class (strong/weak/non-inhibitor) that are associated with DILI of a certain severity. BSEP inhibition was shown to have a significant influence on the DILI potential. Normality tests, using BSEP non-inhibitors as control, was used to test the significance of the frequency differences; illustrated by * if p < 0.05 and ** if p < 0.01."

The results gave further support to the hypothesis that BSEP inhibition is linked to DILI and a majority of the BSEP inhibitors increased the risk of more severe (BW and WP) DILI (Fig. 13). A significant proportion (18%) of the strong inhibitors were associated with potentially life-threatening, BW classified DILI, in comparison to 5% of BSEP non-inhibitors or weak inhibitors (Fig. 13). Interestingly, only the strong inhibitors increased the risk of BW-classified DILI, while the weaker inhibitors only increased the risk of
intermediate severity (WP) DILI (Fig. 13). Notably, nine compounds associated with severe DILI had not previously been identified as BSEP inhibitors (amiodarone, atazanavir, celecoxib, clarithromycin, dipyridamole, erythromycin, ezetimibe, lovastatin, tipranavir), suggesting that this mechanism may contribute to their observed clinical toxicity.

In total, 38 out of the 61 drugs (62%) identified as inhibitors of BSEP-mediated transport increased the risk of the more severe BW- or WP-class DILI, while only 37 out of the 121 (31%) BSEP non-inhibitors had BW or WP-class DILI (Fig. 14). The analysis further showed that of all compounds with reasonable evidence of a causal relationship to DILI, 51% were associated with BSEP inhibition.

Many cellular factors unassessed in BSEP-expressing membrane vesicles influence compounds ability to cause DILI. Therefore SCHH, a physiologically more relevant model, was used to assess the correlation of BSEP inhibition with DILI. A subset of twelve model compounds, with different BSEP effects in the vesicle assay (inhibitors or non-inhibitors) and DILI potential (BW/WP or AR/NM), was selected for the SCHH studies. Three compounds were selected to represent each group.

Our results on TA transport and accumulation in SCHH supported and extended the observations obtained in the membrane vesicles. As expected from the vesicle results, the three BSEP inhibitors associated with severe
DILI (BW or WP) caused a major reduction in the canalicular efflux of TA in SCHH (Fig. 15A). Notably, this group of compounds had a significantly greater impact on the biliary TA excretion than any of the other groups investigated. In contrast, the BSEP inhibitors with less severe or no reported DILI (AR or NM) only marginally affected the canalicular TA efflux in SCHH, despite showing similar degrees of inhibition in the vesicle assay (Fig. 15B). These results support the utility of SCHH in distinguishing BSEP inhibitors associated with severe DILI from those with no or mild DILI. Importantly, compounds inhibiting BSEP but which have not been reported to cause severe DILI (Fig. 15B) still resulted in modest alterations of the canalicular efflux. Such compounds may contribute to an increased ADR incidence if co-administered with other drugs also interacting with transporters involved in canalicular efflux.

Figure 15. The impact on TA transport in SCHH of 12 drugs with different BSEP inhibition and DILI potential. (A-D) Drug impact on TA disposition is shown for each of the four combinations of BSEP inhibition (yes or no) in inverted membrane vesicles and DILI classifications obtained from the FDA drug labels (severe or mild/no), as described in Figure 6D: (A) BSEP inhibitors associated with severe (BW/WP) DILI, (B) BSEP inhibitors associated with mild or no DILI (AR/NM), (C) BSEP non-inhibitors associated with BW/WP DILI, and (D) BSEP non-inhibitors associated with AR/NM DILI. Each panel shows, from left to right, the effect of the compounds on BSEP transport in inverted membrane vesicles (dark blue for inhibitors and light blue for non-inhibitors), and on the canalicular efflux of TA transport in SCHH using three complementary parameters: BEI (white), BIC (gray), and CLbile (black), the latter of which describes the effect of the basolateral net TA uptake in addition to the canalicular efflux. Data are presented as the mean ± standard error.
Analyzing Bile Excretion in SCHH

Different parameters for the analysis of transport in SCHH describe different aspects of the cellular flux in this model system; for instance, the total net transport from the medium to the canalicular compartment is described by $\text{CL}_{\text{Bile}}$. In this thesis, BIC (Eq. 4) is proposed as a parameter that facilitates analyses of the canalicular transport, or alterations thereof, in SCHH. BIC relates the biliary efflux process to the intracellular amount of substrate. Therefore, it will describe the canalicular efflux process both in stable and altered intracellular amounts of substrate. This is exemplified in Figure 16 A and B, where the intracellular substrate amounts are stable and increased, respectively. Furthermore, a parameter directly proportional to BIC, $\text{CL}_{\text{Bile, int}}$, was recently shown to significantly improve predictions of human in vivo biliary clearance [167].

Figure 16. BEI, BIC, $\text{CL}_{\text{bile}}$ and $\text{CL}_{\text{bile, int}}$ correlation with TA accumulations in SCHH in the presence (A) and absence (B) of basolateral efflux. If the intracellular concentration remains unchanged (A) despite canalicular efflux BEI, $\text{CL}_{\text{bile}}$ and $\text{CL}_{\text{bile, int}}$ correlates with the canalicular efflux inhibition. If the canalicular efflux inhibition result in increased intracellular substrate concentration BEI and $\text{CL}_{\text{bile}}$ correlates with the canalicular efflux inhibition. However, because of the increased intracellular concentration it could be argued that the canalicular efflux inhibition exceeds the decrease observed in the absolute amount in the bile, if the transport follows linear kinetics. Furthermore, BIC coincide with $\text{CL}_{\text{Bile, int}}$, recently shown to result in more accurate predictions of in vivo biliary clearance, indicating that BIC is a valid parameter to use in the interpretations of SCHH data [167].
Conclusions

This thesis investigated the inhibition of the ABC transport proteins important in the biliary excretion of drugs. Particular emphasis was placed on establishing stable in vitro techniques for investigations of drug-ABC protein interactions, and defining the molecular properties important to these interactions. Previously unknown inhibitors of MRP2 and BSEP were identified and the inhibition overlap between Pgp, BCRP and MRP2 was described for the first time using a large, diverse dataset. A majority of the inhibitors were found to affect more than one of the ABC proteins. Finally, BSEP inhibition was shown to have a pronounced influence on the occurrence of DILI, highlighting the importance of BSEP inhibition determination in early drug discovery. The work in this thesis demonstrates the usefulness of simple in vitro and in silico methods to provide the basis for understanding the cooperative mechanisms influencing cellular drug disposition. The specific conclusions were:

- **In vitro** models for studies of ABC dependent transport was established. Membrane vesicle models can be used to examine individual drug-protein interactions while SCHH allow investigation of the hepatic drug disposition.

- Approximately every fifth drug in datasets representative of the chemical space of orally-administered drugs was found to inhibit MRP2 and BSEP transport, respectively.

- Compounds with a general inhibition of Pgp, BCRP, and MRP2 transport were, in general, highly lipophilic weak bases.

- ABC inhibition could be modeled with easily interpretable molecular descriptors. These models have the potential to work as predictive filters in early drug discovery to guide compound selection away from unwanted ABC interactions.

- The individual ABC protein contribution to the biliary excretion of E17G in SCHH was well predicted from transport kinetics determined in membrane vesicles and quantified protein expression levels.

- BSEP inhibition doubles the risk of severe DILI, and half of the drugs reported to inflict severe DILI were identified as BSEP inhibitors.
Future Perspectives

The research described in this thesis has expanded the knowledge of the molecular characteristics important in the inhibition of the major hepatobiliary ABC efflux transporters BSEP, MRP2, Pgp, and BCRP.

In the human liver, the transport mechanism studied here acts in concert with SLC and other ABC transport proteins as well as intracellular metabolic phase I and II enzymes. A drug can be a substrate or inhibitor of several of these proteins and can thereby influence the protein interactions with other co-administered drugs or endogenous compounds, e.g., bile acids. The resulting change in hepatic clearance or metabolic profile may cause clinically relevant DDIs or altered disposition of endogenous compounds, potentially leading to ADRs. A major challenge for future investigations is therefore to clarify the interplay between the different transport and metabolic proteins, and to develop in vitro or in silico models capable of predicting this interplay.

Furthermore, hepatotoxicity discovered in the late stages of drug development or even after marketing approval has restricted the use of many drugs during the last two decades. The large inter-individual differences in susceptibility to DILI and the low incidence of severe DILI make these events hard to predict. An expanded knowledge of how cellular mechanisms or intercellular events contribute to DILI could improve DILI predictions. To date, it has not yet been clarified how hepatic NPCs, such as the macrophage Kupffer cells or the endothelial cells, influence hepatotoxic events. Evidence suggests that these cells are key contributors to the initiation of hepatotoxicity. Compared to what is observed today in hepatocyte monocultures, hepatocyte-NPC cocultures could have the potential to provide a more sensitive DILI model that may report a wider range of hepatic insults at clinically relevant drug concentrations. Therefore, to establish such cocultures is an important future challenge in hepatotoxicity research.
Svensk populärvetenskaplig sammanfattning


I den här avhandlingen har arbetet fokuserat på hur läkemedel och andra kemiska ämnen påverkar fyra transportproteiners förmåga att utsöndra ämnen till gallan. Tre av transportproteinerna (Pgp, BCRP och MRP2) transporterar väldigt många olika ämnen och läkemedel och är bl.a. inblandade i läkemedelsresistens vid cancerbehandlingar. Det fjärde proteinet (BSEP) finns bara i levern och är den viktigaste orsaken till att gallsalter utsöndras till gallan.

De experimentella metoderna som använts under avhandlingsarbetet inkluderar försök i cellmembran som innehåller ett av de fyra transportproteinerna och i isolerade leverceller som innehåller de flesta eller alla proteiner som uttrycks i levern i kroppen. Levercellerna har odlats på ett sätt som gör att de bildar tredimensionella strukturer som liknar de som finns i levern, med en gallsida och en blodsida.

Resultaten visar att drygt vart femte läkemedel av de över 200 substanser som undersöktes för MRP2 och BSEP interaktion, minskar eller blockerar dessa transportproteiners utsöndring till gallan. Inhibition av dessa proteiner kan leda till ibland allvarliga biverkningar och leverskador. Detta är idag en av de vanligaste orsakerna till att läkemedel dras tillbaka från marknaden eller får inskränkt användningsområde. En fördjupad analys visade att hämmand BSEP-transport kan fördubbla risken för läkemedelsrelaterade leverskador, och bland läkemedel med ett redan känt samband med leverskador
var andelen som hämmer BSEP-transport kraftigt förhöjd. I andra försök bestämdes mängden transportprotein i levern. Genom att kombinera mängden protein med proteinernas transportförmåga var det möjligt att förutsåga hur mycket av ett ämne som utsändrades till gallgången både i de experimentellt odlade levercellerna och i levern i kroppen.

Utöver de experimentella metoderna utvecklades även datamodeller med målet att kunna prediktera interaktioner med transportproteinerna från läkemedelssubstanternas struktur. De här modellerna kan användas i tidig läkemedelsframställning, redan innan ämnet syntetiserats kemiskt, för att styra utvecklingen av nya läkemedel bort från oönskade interaktioner med transportproteinerna. Enkla beskrivningsmodeller av molekyler visade sig vara tillräckligt för att utveckla modeller som korrekt förutsöger interaktioner mellan läkemedlet och proteinerna. Molekylstrukturer som ökar risken för interaktion med de fyra transportproteinerna kunde också identifieras.

Arbetet i den här avhandlingen har resulterat i nya experimentella metoder som kan användas för att studera transporten till gallan från levercellen samt nya datormodeller som snabbt kan förutsäga risk för interaktioner med transportproteiner. De modeller som utvecklats är viktiga för studier av läkemedelsbiverkningar och kommer att främja utvecklingen av nya, säkra läkemedel.
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