ATP-Binding Cassette Efflux Transporters and Passive Membrane Permeability in Drug Absorption and Disposition

PÄR MATSSON
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Abstract

Transport into and across the cells of the human body is a prerequisite for the pharmacological action of drugs. Passive membrane permeability and active transport mechanisms are major determinants of the intestinal absorption of drugs, as well as of the distribution to target tissues and the subsequent metabolism and excretion from the body. In this thesis, the role of ATP-binding cassette (ABC) transporters and passive permeability on drug absorption and disposition was investigated. Particular emphasis was placed on defining the molecular properties important for these transport mechanisms.

The influence of different transport pathways on predictions of intestinal drug absorption was investigated using experimental models of different complexity. Experimental models that include the paracellular pathway gave improved predictions of intestinal drug absorption, especially for incompletely absorbed drugs. Further, the inhibition of the ABC transporters breast cancer resistance protein (BCRP/ABCG2) and multidrug-resistance associated protein 2 (MRP2/ABCC2) was experimentally investigated using structurally diverse datasets that were representative of orally administered drugs. A large number of previously unknown inhibitors were identified among registered drugs, but their clinical relevance for drug-drug interactions and drug-induced toxicity remains to be determined. The majority of the inhibitors affected all three major ABC transporters BCRP, MRP2 and P-glycoprotein (Pgp/ABCB1), and these multi-specific inhibitors were found to be enriched in highly lipophilic weak bases.

To summarize, the present work has led to an increased knowledge of the molecular features of importance for ABC transporter inhibition and passive membrane permeability. Previously unknown ABC transporter inhibitors were identified and predictive computational models were developed for the different drug transport mechanisms. These could be valuable tools to assist in the prioritization of experimental efforts in early drug discovery.

Keywords: ATP-binding cassette, ABC transporter, P-gp, P-glycoprotein, ABCB1, BCRP, Breast cancer resistance protein, ABCG2, MRP2, Multidrug-resistance associated protein 2, ABCC2, Membrane permeability, Drug transport, Active transport, Passive diffusion, Multivariate data analysis, PLS, OPLS, QSAR

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Papers discussed

This thesis is based on the following papers, which will be referred to by the Roman numerals assigned below:


IV  **Matsson, P.;** Pedersen, J.; Norinder, U.; Bergström, C. A. S. and Artursson, P. Comparison and Prediction of Inhibitors of the Three Major Human ATP-Binding Cassette Transporters P-gp, BCRP and MRP2. *In manuscript.*
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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1D</td>
<td>One-dimensional</td>
</tr>
<tr>
<td>2/4/A1</td>
<td>Immortalized rat intestinal epithelium cell line</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADMET</td>
<td>Absorption, distribution, metabolism, elimination/excretion, toxicity</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANN</td>
<td>Artificial neural network</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein (ABCG2)</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human colon adenocarcinoma cell line</td>
</tr>
<tr>
<td>ChemGPS</td>
<td>Chemical global positioning system</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CoMFA</td>
<td>Comparative molecular field analysis</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>FA</td>
<td>Fraction of an orally administered dose that is absorbed</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HDM</td>
<td>Filter-immobilized hexadecane membrane</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell line</td>
</tr>
<tr>
<td>hERG</td>
<td>Human ether-a-go-go-related ion-channel</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>Pig kidney epithelial cell line</td>
</tr>
<tr>
<td>logD</td>
<td>Octanol-buffer partition coefficient</td>
</tr>
<tr>
<td>logP</td>
<td>Octanol-water partition coefficient</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine-Darby canine kidney cell line</td>
</tr>
<tr>
<td>MLR</td>
<td>Multiple linear regression</td>
</tr>
<tr>
<td>MMFF</td>
<td>Merck molecular force field</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>MNLR</td>
<td>Multiple non-linear regression</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug-resistance associated protein (ABCC)</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>(n)</td>
<td>Number of observations</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>OPLS</td>
<td>Orthogonal PLS</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal PLS discriminant analysis</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein (ABCB1)</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares projection to latent structures</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>PLS discriminant analysis</td>
</tr>
<tr>
<td>Q²</td>
<td>Cross-validated (R^2)</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>(R^2)</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>RMSE</td>
<td>Root mean square error</td>
</tr>
<tr>
<td>(r_s)</td>
<td>Spearman rank order correlation coefficient</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>SVM</td>
<td>Support vector machine</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-epithelial electrical resistance</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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1. Introduction

Transport into and across the cells of the human body is a prerequisite for the pharmacological action of drugs. An orally administered drug needs to cross the intestinal epithelial cell layer if it is to reach the blood circulation (Figure 1). Many target tissues, such as the central nervous system (CNS), are protected by additional cellular barriers, and for intracellular pharmacological targets an even greater number of cell membranes need to be crossed before the pharmacological action can be elicited. An equally important role of membrane transport is evident in the elimination of drugs from the body. Transport across cellular membranes determines the access to intracellular drug-metabolizing enzymes in the liver and other metabolizing organs, and cellular transport processes have a pronounced effect on the renal and biliary excretion of the drug.

In 1991, inadequate bioavailability and poor pharmacokinetic profiles were the major causes for the termination of candidate drugs.¹ This prompted the inclusion of experimental and computational assays for predicting the absorption and metabolism of drugs in early discovery settings, in parallel with the pharmacological screening.² A decade later, the proportion of clinical drug failures that could be related to pharmacokinetic and bioavailability issues had been significantly reduced.³ Experimental and computational models of intestinal solubility and passive membrane permeability, as well as of hepatic metabolism, probably contributed to this development. However, the reduction of the clinical drug failures was in part counterweighed by an increase in the number of failures attributable to toxicological reasons, underlining the need for further characterization of discovery drugs.

During the past decade, the complexity of membrane transport has become increasingly apparent. The characterization of the human genome revealed a plethora of proteins facilitating the transmembrane transport of various solutes. Many of these transport proteins have the capacity to transport drug molecules, and significantly contribute to the pharmacological and toxicological activity of drugs.

In this thesis, the impact of passive permeability and active transport on the intestinal absorption, tissue distribution and elimination of drugs was examined with the aim of developing predictive computational models for important drug transport mechanisms. A particular emphasis was placed on drug efflux transporters from the ATP-binding cassette (ABC) transporter family.
Figure 1. The sequence of events following the administration of a solid dosage form to the gastrointestinal tract. The dissolution and the permeability across the epithelium are the most important determinants of intestinal drug absorption. Other factors that can limit the fraction of an orally administered dose reaching the systemic blood circulation include: enzymatic and chemical degradation and complexation in the gut lumen; enzymatic degradation in the intestinal epithelium; and enzymatic degradation and biliary excretion in the liver.

1.1. Transport of drugs across cellular barriers

Several distinct pathways affect the transport of drugs across tissue barriers such as the intestinal epithelium and the blood-brain barrier (BBB) (Figure 2). Although the relative importance of the pathways varies between one tissue and another and for different drugs, the general transport mechanisms are applicable to tissues throughout the human body.

The pathways can be divided in two types: the transcellular pathway, the use of which requires that a drug can cross the lipophilic cell membranes, and the paracellular pathway, in which diffusion occurs through the water-filled pores of the tight junctions between the cells. In addition, both passive and active (energy-dependent) transport can contribute to the permeability of drugs via the transcellular route. The four principal pathways of drug transport are presented in Figure 2. All of the transport processes may occur in both directions, depending on local drug concentrations (in the case of passive transport) and the directionality of the relevant transporter (in the case of active transport). The various transport mechanisms are discussed below, using the intestinal epithelium as an example. Examples of in vitro methods used in studies of membrane permeability and active drug transport are given in Box 1.
Figure 2. The drug-relevant transport pathways in the intestinal epithelium: 1, passive transcellular transport; 2, paracellular transport; 3, active efflux; 4, active uptake. Passive permeability can occur in absorptive (A) and secretory (B) directions, depending on local drug concentrations. Correspondingly, drug transport proteins are expressed in both the apical and the basolateral membranes, and mediate active transport in absorptive and secretory directions.

1.1.1. Passive transcellular transport

The passive transcellular pathway is the most important absorption pathway for the vast majority of orally administered drugs, and also significantly affects the distribution of drugs to other tissues. The first requirement for drug absorption by this route is that the solute permeates the apical cell membrane of the intestinal enterocyte. Enterocytes have a polarized cell membrane with distinct differences in the membrane composition between the apical and basolateral membranes.\(^4,5\) It is generally believed that the apical membrane has a lower permeability than the basolateral membrane, and therefore the former is considered to be the rate-limiting barrier to passive transcellular drug absorption.\(^6\) In addition, the composition of the phospholipids and proteins of the cell membranes varies from cell type to cell type and may, theoretically, give rise to different permeabilities in different cell types.

Early models of the transcellular pathway regarded the cell membrane as a homogenous barrier and drug permeability was, therefore, considered to be a two-stage process involving partitioning into the lipid bilayer followed by transmembrane diffusion.\(^7\) This ‘solubility-diffusion’ model was later extended with the so-called pH-partitioning theory, which states that only the uncharged form of an electrolytic molecule will partition into the cell membrane.\(^8\) In these early models, the cell membrane was considered to be an isotropic system. This assumption explains why drug partitioning in simple isotropic solvent systems, such as octanol-water, has been extensively used to predict passive membrane permeability.\(^9-11\) However, the lipid bilayer that constitutes the cell membrane is a highly complex system.\(^12\) Thus multiple-
step permeability models have been proposed to account for the anisotropic nature of the cell membrane. Molecular dynamics simulations have shown that drug molecules entering the cell membrane experience different diffusion rates in different parts of the membrane, since different inter-molecular forces affect the permeating substances depending on the local environment in the lipid bilayer.

Regardless of whether the ‘solubility-diffusion’ model or a more complex description of the cell membrane is used, passive transcellular drug permeability can usually be relatively well described using rather simple molecular properties such as size, charge, polarity and hydrogen bonding. Many experimental and computational models of intestinal drug permeability have therefore been based on descriptors of these basic molecular properties.

An important general assumption made in these models is that the passive transcellular route dominates the drug permeation. However, when other transport routes, such as the paracellular or active transcellular routes are involved to a significant extent, they will introduce a bias that reduces the predictivity of the model. The influence of the passive transcellular, paracellular, and active drug transport routes on intestinal permeability predictions were investigated in Paper I in this thesis.

1.1.2. Paracellular transport

Drugs of small to moderate molecular weight can permeate the intestinal epithelium through pores between the cells, a process known as paracellular transport. The narrow, water-filled pores are created by extracellular tight junction proteins that connect adjacent cells. In the intestine, the paracellular pores only correspond to around 0.01–0.1% of the absorptive surface area, and the average pore sizes have been estimated to be 8–13 Å in the human small intestine, decreasing to only about 4 Å in the colon. This route is, therefore, mainly accessible to smaller molecules (MW<350 g mol\(^{-1}\)) and is of significance only if these small drugs are too hydrophilic to distribute into the cell membranes at appreciable rates. Similar to the intestinal epithelium, endothelial cells in the brain microvasculature are also connected by tight junctions. This results in a much tighter endothelial layer than in capillaries in other parts of the body, and contributes to the protective role of the blood-brain barrier.

Paracellularly permeating drugs are usually incompletely absorbed since absorption only occurs in the small intestine where the pores are sufficiently large to allow the passage of small solutes. The paracellular pathway of the small intestine has been described as a dual-pore system, with small pores at the villus tips and larger ones further down the crypt-villus axis (Figure 3). Given this, it can be hypothesized that a hydrophilic drug that is partitioning slowly into the intestinal cell membrane will have time to diffuse further down the crypt-villus axis and eventually be absorbed via paracellular pores.
that are significantly larger than those available at the tips of the villi. Thus, such compounds may exhibit higher *in vivo* absorption than would be expected from their passive transcellular permeability.

**Figure 3.** Permeability properties of different parts of the crypt-villus axis. A. Schematic representation of the crypt-villus axis, divided into upper villus, basal villus and crypt. B. The effective intestinal surface area that the drug encounters will be determined by the epithelial permeability to the drug. Compounds with poor permeability (open circles) will have time to diffuse further down the crypt-villus axis, and can therefore be absorbed across a large surface area (dashed line). In contrast, high-permeability compounds (closed circles) are rapidly absorbed at the villus tips and therefore utilize a smaller absorptive surface area (solid line). The average size of the paracellular pores varies along the crypt-villus axis, with the pores getting narrower as the cells mature and move towards the villi tips.33
1.1.3. Active transport

In addition to the passive diffusion processes described above, transport proteins situated in the membranes of the cell significantly affect the movement of substrate drugs across cellular barriers. Of the more than 20,000 genes in the human genome, around 500 encode transport proteins and ion channels. Although the majority of these have specific physiological roles in, e.g., nutrient uptake or neuronal recirculation of transmitter substances, an increasing number have been found to have more general transport functions. These transporters, which have broader substrate specificity, are often referred to as polyspecific transport proteins, and have the capacity to transport drugs.

The ATP-binding cassette (ABC) transporters and the solute carriers (SLC) are the two dominating gene families among the plasma membrane transporters. ABC transporters utilize the energy from ATP hydrolysis to translocate their substrates across cellular membranes. Some members of the SLC family mediate facilitated diffusion of their substrates along a concentration gradient, whereas others are secondary active transporters that use ion gradients to mediate concentrative substrate transport. ABC and SLC transporters with the capacity to transport drugs are expressed in tissues throughout the body, and have been shown to significantly affect pharmacokinetic processes such as intestinal absorption, distribution to the CNS, and uptake into, and subsequent excretion from the hepatocyte.

In this thesis, particular emphasis was placed on three major drug-transporting members of the ABC transporter family: P-glycoprotein (P-gp; ABCB1), breast cancer resistance protein (BCRP; ABCG2), and multidrug-resistance associated protein 2 (MRP2; ABCC2).

1.2. ABC transporters in drug disposition

ABC transporters constitute one of the largest superfamilies of membrane proteins, with members having been found in all species studied so far. In humans, 48 members have been identified, and these have been phylogenetically divided into seven subfamilies (labeled ABCA through ABCG). Although the exact physiological roles are still unknown for many of the human ABC transporters, several are implicated in genetic disorders such as Tangier disease, Stargardt disease, Dubin-Johnson syndrome, and cystic fibrosis. The human ABC transporters with affinity for drugs are mainly found in the ABCB, ABCC and ABCG subfamilies. The following sections will briefly review the structure, mechanism, and the roles of the major drug efflux transporters P-gp, BCRP, and the MRPs in drug disposition.
Box 1. *In vitro* methods for studying permeability and active transport

**Experimental models of intestinal permeability**

The most widely used model for determining the intestinal permeability to drugs is the Caco-2 cell line, which is derived from a colon carcinoma and has been shown to differentiate to a phenotype similar to the mature small intestinal enterocyte.\(^{50, 51}\) Alternative cell lines with slightly different properties include the Caco-2 cell clone TC7 which, in contrast to the parental Caco-2 cells, expresses some drug-metabolizing enzymes,\(^ {52, 53}\) and the mucus-producing HT29-MTX clone.\(^ {54}\) The 2/4/A1 cell line is an immortalized cell line derived from rat intestinal epithelium.\(^ {55, 56}\) It has a more leaky, small intestine-like paracellular pathway than that of Caco-2 monolayers, and does not express important drug transporters from the SLC and ABC transporter families.

As an alternative to cultured cells, artificial membranes can be used to study passive drug permeability. These systems are either based on simple organic solvents such as hexadecane,\(^ {57}\) that mimic the hydrophobic interior of the plasma membrane, or on different mixtures of membrane phospholipids.\(^ {58, 59}\)

Details of the methodology of permeability assessment using Caco-2 and 2/4/A1 cells and artificial hexadecane membranes are given in Reference 51 and in Section 3.3.1.

**Experimental models for studying active drug transport**

Caco-2 cells express many drug-relevant transport proteins, and can thus be used to determine the overall influence of active transport on the permeability. For mechanistic studies of specific transporters, cell lines transfected with one or more transporters are often preferred. Madine-Darby Canine Kidney (MDCK) cells\(^ {60}\) and the pig kidney cell line LLC-PK1\(^ {61}\) are often used to this end, and since they form monolayers they can be used to study active transport processes in Caco-2-like permeability assays.

Cell lines such as HEK293, CHO, K562 and Saos-2 that do not form confluent monolayers are generally used to assess the intracellular accumulation of fluorescent or radiolabeled transporter substrates, thereby giving information about active transport mechanisms.\(^ {62-65}\) Efflux transporters are often studied using inside-out membrane vesicles.\(^ {66-68}\) The inverted nature of these systems enables direct interaction of investigated drugs with the transporter, without the need for adequate membrane permeability to reach intracellular binding sites. As an alternative to following the transport of a fluorescent or radiolabeled probe, ATP consumption is often used as a surrogate measure of drug transport in cell-based systems and in membrane vesicles.\(^ {67, 69}\)
1.2.1. The structure of ABC transporters

ABC transporters share a common domain organization consisting of two transmembrane domains (TMDs) that form the passageway for the transport of the substrate, and two nucleotide-binding domains (NBD) that provide the energy for the transport by catalyzing ATP hydrolysis. In prokaryotes, both ABC importers and ABC exporters have been identified, whereas in eukaryotic cells the transport is exclusively in the exporting (efflux) direction. Because of the difficulty of crystallizing membrane proteins, still only four high-resolution crystal structures of complete ABC transporter proteins are available, three of which are ABC importers. The only available structure of an ABC efflux transporter is that of Sav1866 from Staphylococcus Aureus (Figure 4A). Recent biochemical evidence suggest that the domain arrangement is similar in Sav1866 and human P-gp, and consequently, the structure has received much attention as a template for homology models of human ABC transporters.

ABC efflux transporters contain a conserved core of 12 transmembrane (TM) helices, which are believed to form the transport pathway. For P-gp, the secondary structure can be divided into two similar halves, each containing 6 TM helices and one NBD (Figure 4B). This basic ABC efflux transporter structure is shared by MRP4 and MRP5, whereas MRP1-3 and MRP6 contain an additional aminoterminal domain consisting of 5 TM helices.

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Figure 4. Structure and membrane topology of ABC efflux transporters. A. Crystal structure of the bacterial ABC exporter Sav1866 (pdb id 2HYD). The transmembrane (TM) helices and the intracellularly located nucleotide binding domains (NBD) are indicated in the picture; the gray box shows the position of the plasma membrane. B. Predicted membrane topology for the human ABC transporters P-gp, MRP1-6 and BCRP. P-gp, MRP4 and MRP5 consist of the conserved 12 TMs and two NBDs, whereas MRP1-3 and MRP6 have an additional aminoterminal domain consisting of 5 TMs. BCRP is a half-transporter, and as such only consists of 6 TMs and one NBD. The position of the membrane is indicated by the gray boxes, and all transporters are depicted with the same orientation, down being towards the inside of the cell.
In contrast, BCRP is a so-called *half transporter* that consists of only 6 TM helices and one NBD. However, biochemical and low-resolution crystallographic data have demonstrated that the functional BCRP unit is formed by two protein molecules in a homodimeric structure, and thereby BCRP fulfills the conserved domain architecture.

1.2.2. P-glycoprotein (P-gp; ABCB1)

P-gp was the first eukaryotic ABC transporter discovered, and was identified as the cause of a multidrug-resistant phenotype observed in drug-selected cells from Chinese hamster ovaries (CHO). Since then, a vast body of literature has been published on the structure, mechanism and physiological and pharmacological roles of P-gp. More than 12 000 papers concerning P-gp had been published by November 2007, making this the by far most studied drug transporter.

In spite of this, the physiological role of P-gp is still not completely understood. Suggested endogenous substrates include membrane components such as phosphatidylcholine and cholesterol, as well as steroid hormones and cytokines. The expression pattern suggests that P-gp plays a role in tissue protection and detoxification, with high expression in the apical membrane of intestinal enterocytes, in the bile canalicular membrane of the liver and in protective tissue barriers such as the BBB, the blood-testis barrier and in the placental syncytiotrophoblasts that connect maternal and fetal blood circulation.

P-gp distinguishes itself from most other transporters by the structural diversity of its substrates. Most P-gp substrates are lipophilic and uncharged or weakly basic, although acidic and hydrophilic substrates such as methotrexate have also been reported. The common denominator of P-gp substrates is their amphipathic character. It is generally accepted that P-gp binds its substrates from the inner leaflet of the plasma membrane, thereby explaining this preference for surface-active compounds. This model for drug binding to P-gp was recently corroborated by molecular dynamics simulations of surface active P-gp substrates. The compounds first partition into the lipid membrane, and subsequently exchange their interactions with water or the polar lipid head groups in the membrane-cytosol interface for interactions with polar and charged amino acids in the lipid-water interface region of P-gp.

1.2.3. Multidrug-resistance associated proteins (MRPs; ABCCs)

The ABCC subfamily consists of 12 distinct transporters, of which MRP1-6 are generally considered as drug transporters. MRP1 was first identified in a drug-resistant cell line, and it was subsequently shown to confer resistance to a similar, although not identical panel of cytotoxic drugs as that
observed for P-gp. In contrast to P-gp, MRP1 is localized to the basolateral membranes of intestinal enterocytes and hepatocytes. Typical MRP1 substrates are organic anions, although substrates lacking negative charges have also been reported. Such transport is however dependent on intracellular glutathione, and it is generally accepted that non-anionic MRP1 substrates are co-transported with reduced glutathione.

The MRP2 gene was identified on the basis of its similarity to MRP1, but it differs from its sibling by co-localizing with P-gp to the apical membrane of intestinal epithelium and the hepatocyte. The physiological role of hepatic MRP2 is to mediate biliary excretion of conjugated bilirubin and bile salts; genetically impaired MRP2 function results in a conjugated hyperbilirubinemia known as Dubin-Johnson syndrome. The substrate spectrum of MRP2 is distinct but similar to that of MRP1, with a preference for organic anions and reported co-transport of weakly basic substrates with reduced glutathione.

Several studies have demonstrated that transport mediated by MRP1 and MRP2 can be stimulated by the addition of other compounds. For MRP1, this is exemplified by the marked increase in etoposide and vincristine transport observed on the addition of glutathione. Conversely, etoposide and vincristine stimulate the transport of glutathione, indicating that these weakly basic drugs are co-transported with reduced glutathione. For MRP2, transport stimulation is readily apparent from the sigmoidal concentration-dependency observed for estradiol-17β-glucuronide. This is indicative of positive cooperative transport, i.e., estradiol-17β-glucuronide stimulates its own transport at higher concentrations. However, the fact that not all MRP2 stimulators are transported themselves led to the proposal that there are two distinct binding sites in MRP2: one transporting site and one stimulating site. With this explanatory model, transported stimulators have affinity for both sites, whereas other compounds, with an affinity for only one of the sites, act either as substrates or stimulators.

Immunolocalization experiments have demonstrated the expression of MRP3 and MRP6 in the basolateral membrane of hepatocytes, and MRP4 and MRP5 have been detected in the luminal membrane of BBB endothelial cells, co-localized with MRP1. Although these members of the ABCC family have not been extensively studied, reports indicate a similar substrate specificity of MRP3 and MRP6 to that of MRP1, and MRP4 and MRP5 have been shown to transport antiviral nucleotide analogs.

1.2.4. Breast cancer resistance protein (BCRP; ABCG2)

BCRP was first cloned in the late 1990s from a doxorubicin-resistant breast cancer cell line. Since then, the transporter has been detected in a variety of healthy as well as malignant tissues. Physiological expression of BCRP is
found e.g. in intestinal epithelium, in bile canaliculare membranes of hepatocytes, in placental syncytiotrophoblasts and in the ducts and lobules of the breast. Unlike most other half transporters, which are expressed in intracellular membranes, BCRP is localized to the plasma membrane. Similar to P-gp the physiological role of BCRP is still unclear, but the localization to apical membranes of tissue barriers suggests a role in tissue protection.

Since its discovery, BCRP has received considerable attention for its role in resistance to various cytotoxic agents. More recently, it has been shown to transport a diverse set of drugs from other therapeutic classes. The substrate specificity of BCRP overlaps with both the MRPs and with P-gp, and includes intrinsically anionic drugs such as methotrexate and pitavastatin, anionic conjugates such as estrone-3-sulfate and cationic compounds such as mitoxantrone.

It is becoming increasingly clear that BCRP is one of the major ABC transporters affecting drug disposition. This role was recently exemplified by a 111 times higher systemic exposure to the antiinflammatory drug sulfasalazine after oral administration to Bcrp1-knockout mice compared to wild-type mice. In addition, the human oral bioavailability of the BCRP substrate topotecan was more than doubled after coadministration with the potent inhibitor GF120918, highlighting the risk of significantly altered drug exposure as a result of BCRP inhibition.

1.3. Computational modeling of drug transport processes

The impact of transport processes on the pharmacological and toxicological action of drugs has become increasingly apparent during the past two decades. As a consequence, the application of methods for predicting the transport mechanisms of new drugs has become an integral part of the workflow in early drug discovery. Using mathematical and statistical tools it is possible to relate the transport characteristics to the chemical structure in a computational model, and to use these models to predict the properties of unknown, possibly not even synthesized compounds. Such models can then aid in selecting the compounds most suitable for further development.

1.3.1. Model development

The procedure of developing predictive computational models, e.g. describing membrane permeability or the binding to a transport protein, can be divided into five general steps: i) selecting a representative compound dataset; ii) generating or collecting experimental data for the property of interest; iii)
describing the chemical structure of the compounds in the dataset; iv) relating the structural description to the experimental data using a mathematical relationship; and v) validating the predictivity of the model using a test set of compounds that were withheld from the model development (Figure 5). Important aspects of these five steps are discussed in the following sections, and examples of computational models of drug transport are discussed.

**Figure 5.** The steps in the development of a computational model. i) Selecting the dataset, ii) determining experimental data, iii) describing the molecular structures, iv) connecting the experimental data to the chemical structures by statistical or mathematical tools and v) validating the model. Adapted from Reference 123.

1.3.2. Selecting the dataset

The dataset used to generate the model will determine its *applicability domain*, i.e., for which compounds the model can be expected to give reliable predictions. Models can be divided into *global* models, that are applicable to widely differing chemical structures, and *local* models, that are confined to a much more limited chemical space, e.g., specific series of homologous compounds. In general, there is a tradeoff between the applicability domain and the quality of the predictions. Consequently, global models are often used to provide initial, qualitative predictions (with yes/no answers or outcomes categorized in groups such as high, intermediate or low), whereas local models are used to provide more detailed, quantitative predictions. Hence, global models can be used to guide experimental efforts to compound series with favorable characteristics, for which local models providing more exact, quantitative predictions can be developed and applied.

Regardless of whether global or local models are developed, the dataset compounds should be structurally diverse within the chemical space of the model. A number of techniques available for determining structural diversity are reviewed in Reference 124. Furthermore, to ensure accurate predictions, it is important that the dataset is not skewed with regard to the modeled experimental parameter.
1.3.3. Describing the chemical structure

The chemical structures of the modeled compounds need to be translated into numerical representations before they can be related to the experimental parameter. The approaches adopted for obtaining numerical descriptions of the chemical structures can be divided into four main categories, although this distinction is somewhat arbitrary: i) molecular descriptors that encode structural information about the molecule as a whole or parts of the molecule; ii) molecular fingerprints that describe the presence or absence of a list of structural features in the molecule; iii) molecular interaction fields that encode the interaction of the molecule with probes in the surrounding space; and iv) pharmacophore models that describe how well the molecule can be superimposed on a three-dimensional arrangement of important functional groups. A brief overview of these four approaches is given below.

1.3.3.1. Molecular descriptors

Molecular descriptors encode properties related to the structure of the molecule, such as its size or polarity, or the connectivity between atoms in the molecule. Descriptors can be classified as one-dimensional (1D), two-dimensional (2D) or three-dimensional (3D), depending on the complexity of the structural representation used to calculate them (Figure 6). According to this scheme, 1D descriptors refer to bulk properties of the molecule, such as atom counts or the molecular weight. 2D descriptors are typically related to size, flexibility/rigidity or hydrophilicity/lipophilicity and can be calculated from a two-dimensional representation of the molecule. 3D descriptors are dependent on the conformation of the molecule and encode information about the arrangement of atoms in three-dimensional space, e.g., solvent accessible molecular surface areas and molecular volumes. Structural representations in the form of wave functions constitute a fourth level of complexity, and are used in quantum mechanics calculations of properties related to electron densities.

Ideally, a molecular descriptor should be interpretable, so that conclusions about physicochemical interactions with the surroundings can be drawn, and so that models based on the descriptors can be used to guide medicinal chemistry efforts. In addition, the descriptors should be rapidly calculated, so that they can easily be applied to large chemical libraries. Rule-based methods have increased the speed of generating 3D conformations, facilitating the use of 3D descriptors in quantitative structure-activity relationships (QSARs). However, more sophisticated methods for generating molecular conformations, such as molecular mechanics calculations, are preferred for applications where the spatial orientation of functional groups is vital.
1.3.3.2. Pharmacophore models

A pharmacophore describes how the structural features that determine the biological effect of a set of molecules are arranged in 3D space (Figure 7A). A number of algorithms for developing pharmacophore models are available, with the common goal being to identify the optimal 3D arrangement of structural features that exist in all ligands.\textsuperscript{125-131} This is generally carried out in two steps: first the ligands are analyzed to find pharmacophore features that are common to all compounds. These structural features usually include hydrogen bond donors and acceptors, hydrophobic, aromatic, and positively and negatively ionizable features. In a second step, the ligand structures are superimposed onto each other, and different 3D arrangements of the features are scored according to how well they fit the ligands.

The algorithms available can be divided into two types: those that solely determine the overlapping features in a set of active ligands, such as the Catalyst HipHop algorithm,\textsuperscript{129} and those that also take the affinities of the ligands for the target structure into account, including among others the Phase and Catalyst HypoGen algorithms.\textsuperscript{128, 131} Since no assumptions about the relative potency of the active compounds are made in the former approach, these algorithms only need the structures of the ligands as an input. In addition to the structures, the latter approach requires the experimentally determined affinities for compounds ranging from completely inactive to highly active. This type of modeling has the advantage of not only classifying compounds as active or inactive, but also provide predictions of the ligand potency.
1.3.3.3. Molecular interaction fields

Molecular interaction fields are commonly used in 3D-QSAR models to describe the interactions between a molecule and its target structure in three-dimensional space. Various methods are available for determining the interaction fields, including the widely used CoMFA\textsuperscript{132} and GRID\textsuperscript{133} algorithms. With these methods, energies are calculated for the interaction between the molecule and probes placed at regularly spaced intervals in the surrounding space (Figure 7B). By selecting probes with different physicochemical properties, interaction fields related to steric effects, hydrogen bonding and electrostatic interactions can be obtained. Models relating interaction fields to activity can thereby define regions in a molecule in which structural modifications would result in an altered biological effect.

1.3.3.4. Molecular fingerprints

Molecular fingerprints can be thought of as a collection of substructural molecular descriptors that together provide a description of the structure of the molecule. The fingerprints are typically encoded using a string of binary values, where each bit position marks the presence or absence of a certain structural characteristic in the molecule (Figure 7C). This approach is often used to encode which of a large number of possible substructure fragments are present in a molecule. Similarly, pharmacophore fingerprints are used to describe which, out of all possible pharmacophore patterns, that exist in the molecule, thereby providing a picture of the potential interactions that a molecule can take part in.
1.3.4. Relating experimental parameters to chemical structure

Once the experimental data has been assembled and the chemical structures of the compounds in the dataset have been described numerically, the next step of the modeling procedure is to relate the experimental property to the structures using statistical methods. In general, single molecular descriptors are not enough to describe complex cellular processes such as drug transport, and multivariate statistics are therefore usually preferred. The available methods include both linear and nonlinear statistics, as well as different classification modeling techniques. The techniques differ in the types of problems they handle, and in the transparency of the resulting models.

1.3.4.1. Modeling continuous response variables

The most common methods used for modeling drug transport processes are the linear multivariate methods, including e.g. *multiple linear regression* (MLR) \(^{134-136}\) and *partial least squares projection to latent structures* (PLS).\(^{136, 137}\) Linear methods have the advantage of being relatively transparent and easy to comprehend. The regression coefficients obtained from linear methods provide easily interpreted information about the relative influence of the descriptors on the experimental parameter. A drawback of MLR and PLS is that they lack the ability to handle complex nonlinear problems in their standard forms, although nonlinear extensions are available to this end.\(^{138, 139}\)

Although MLR and PLS are both linear methods, they differ considerably in their handling of the multivariate problem. PLS can be viewed as an augmented linear regression method that does not display the general limitations of MLR, including the inability to handle inter-correlated descriptors or data matrices containing more descriptors than compounds.\(^{137}\) These advantages of the PLS methodology made it the statistical method of choice throughout this thesis. In PLS, the molecular descriptors are projected to a limited number of latent ‘supervariables’ or *principal components*. These latent variables contain the majority of the information originally described by a much larger number of molecular descriptors. *Orthogonal PLS* (OPLS) is an extension of PLS where the part of the information in the molecular descriptors that is related to the response parameter is collected in *predictive* principal components.\(^{140}\) The remaining information is described by components orthogonal to the predictive component.

Among the nonlinear methods used for modeling transport processes, *artificial neural networks* (ANN) are the most widely employed.\(^{136, 141-143}\) ANNs mimic the connections of neurons in the brain, and relate the molecular descriptors to the experimental parameter through a number of layers of neurons or *nodes*. This complex pattern of connections means that ANNs are able to describe complex nonlinear variations in the relation between the training set compounds and the experimental parameter. However, one
drawback is that the transparency is greatly reduced compared to the linear models, and it is generally not possible to deduce the influence of a specific descriptor on the experimental parameter. In addition, a particular problem associated with most ANN models is their tendency to become overtrained, i.e., the compounds used for model development are perfectly described, but the models lack the ability to predict new data.

1.3.4.2. Discriminant models

In some cases the aim of modeling is not to predict an exact binding affinity or transport rate, but rather, to classify compounds into groups according to different activities. For this purpose, various classification or discriminant modeling techniques can be used. These include decision trees, support vector machines (SVM) and PLS-discriminant analysis (PLS-DA).

Decision trees, or recursive partitioning techniques, are used to find branching points in the molecular descriptors that result in an optimal division of the compounds into different activity classes. The end result is a set of rules, such as ‘if the molecular weight is greater than 450 and the octanol-water partition coefficient is above 3.2, then the compound is active’.

SVMs divide the compounds into classes by identifying hyperplanes in the multidimensional descriptor space that result in the largest margin of separation between the activity groups. Since nonlinear functions can be used to define the separating hyperplanes SVMs can deal with complex nonlinear classification problems, although not without a considerable reduction in the transparency of the models.

Throughout this thesis, classification problems were handled using PLS-DA or OPLS-DA. These are variants of the standard PLS methods and, as such, displays the advantages of PLS, e.g., the ability to handle intercorrelated descriptors and large descriptor matrices. In PLS-DA and OPLS-DA, the continuous response variable in PLS is replaced with a matrix of dummy variables that describe the class memberships of the compounds using binary values (1/0 or 1/-1). Similar to continuous-variable PLS, the molecular descriptors are projected to latent variables, and these are used to divide the compounds into activity classes.

1.3.5. Validating the model

Before applying a model to the routine prediction of unknown compounds, the validity of the model must be assessed. The validation gives information about the predictivity of the model, and reveals whether the model is being overtrained on the studied compounds. Three approaches are commonly used for this purpose: cross-validation, permutation of the response parameter, and external test sets.

Cross-validation is a common technique for internal validation of a proposed model. The dataset is divided into groups (typically between four and
seven). One group is removed from the dataset, and a model is constructed from the remaining compounds. This model is used to predict the activities of the withheld group, and the procedure is repeated so that each cross-validation group remains outside the model once. The cross-validation coefficient of determination ($Q^2$) is then calculated as a measure of how much the predictions deviate from the true values of the response parameter.

Another technique for internal model validation is to randomly permute the order of the response variable. This results in the experimental values no longer being connected to the correct structural representation of the molecules. If the model based on the true order of the experimental parameter is valid, a clear difference in the predictivity compared to the random models will be apparent.

Finally, the most stringent test of a model’s predictivity is to apply it to a test set of compounds that were not included in the modeling procedure. This is usually done by keeping a representative subset outside the modeling, until the final model has been developed and internally validated using cross-validation and/or random permutation.

1.3.6. Models of drug transport processes

1.3.6.1. Intestinal drug absorption

Many different approaches have been adopted for the prediction of intestinal drug absorption in silico. In the most direct of these, the absorption of orally administered drugs is modeled directly from their molecular properties. However, the interpretation is complicated by the fact that several factors influence oral drug absorption, most importantly the solubility in the gastrointestinal tract and the intestinal drug permeability (Figure 1). In addition, oral absorption has only been characterized in detail for a small set of already approved drugs. Attempts to increase the dataset sizes require the inclusion of compounds with more complex pharmacokinetics, resulting in an increased uncertainty in the estimate of the fraction absorbed (FA).

An alternative approach is to instead model the discrete rate-limiting steps of oral drug absorption. This results in a greater transparency of the models, providing insight into whether solubility or permeability is rate-limiting for the absorption. Attempts to combine these two discrete rate-limiting steps in computational models of intestinal absorption have been proposed.

Many different statistical methods have been applied to the prediction of permeability. These range from simple linear correlations of single parameters, through multivariate linear and non-linear regression (MLR/MNLR) to more complex methodology including principal component analysis (PCA), partial least squares (PLS), genetic algorithms and neural networks.
The molecular descriptors tend to be selected to contain information about the basic physicochemical properties believed to control the permeability process, i.e., the lipophilicity (related to the partitioning to the cell membrane), size, shape and flexibility (related to steric hindrance of membrane permeation) and polarity (describing the limiting effect of desolvation when the drug partitions from the water phase to the membrane). Computational models of intestinal permeability and absorption are reviewed in greater detail elsewhere. A common limitation of most absorption and permeability models is that the datasets are skewed towards completely absorbed drugs. Models based on such data will, therefore, have limited predictive power when applied to discovery compounds with low to intermediate oral absorption. In addition, models generally rely on the assumption that passive transcellular permeability is the dominant transport pathway. In many cases, however, the permeability of compounds that were initially believed to be passively transported has later turned out to be significantly influenced by active drug transporters. Correspondingly, paracellular diffusion can contribute significantly to the permeability of low-molecular, hydrophilic compounds, further complicating the mechanistic analysis of the model results.

To enable unbiased investigations of the different drug transport pathways affecting intestinal drug permeability, the dataset used in Paper I in this thesis was selected to evenly cover the entire range of FA in humans (0-100%). In addition, actively transported drugs were included to better reflect the situation in drug discovery where transporter affinities are not always known.

1.3.6.2. Models of drug transporters

Similar to the models of intestinal drug permeability, a multitude of modeling approaches have been applied to the transporter field. Most modeling studies have focused on P-gp, with computational models for other ABC and SLC transporters having appeared only in recent years. Transporter models have been developed according to two general approaches: protein-based models, describing the structure of the transport proteins, and ligand-based models, describing the structural features of the drug molecules that determine their interaction with the transporter.

Models that describe the three-dimensional structure of the transporter can be used for ligand docking, in which the interactions involved in ligand-transporter binding are scored using different approaches. However, the lack of high-resolution crystal structures for human transporters so far limits the application of ligand docking procedures in transporter studies. As an alternative, homology modeling allows the prediction of transporter structures based on their sequence similarity with a known protein structure. This is exemplified by the use of the bacterial ABC exporter Sav1866 (Figure 4A) as a template for several recent homology models of human ABC transport-
ers. However, the sequence identity is primarily found in the ATP-binding domains, whereas the transmembrane domains that contain the ligand recognition sites exhibit larger variations, making high-precision modeling of these regions more difficult. Unfortunately, the only other structure that had been published for a bacterial ABC exporter, that of MsbA from *Vibrio Cholera*, was recently retracted because of erroneous interpretation of the structure and protein topology. As a result of this, homology models based on the MsbA structure need to be reevaluated.

In contrast to protein-based models, ligand-based models do not require knowledge of the three-dimensional structure of the transporter. Instead the drug molecules are examined, with the aim of finding structural similarities between ligands and thereby obtaining information about the transporter binding site. The approaches used to derive ligand-based transporter models differ both in the ways the structures are described and in which statistical methods are used to relate the structures to quantitative measures of transporter affinity. In addition, classification models have been developed that discriminate between transporter substrates and non-substrates or between inhibitors and non-inhibitors.

While a wealth of data is available on drug interactions with P-gp, the experimental datasets for other drug transporters are still relatively small. As a consequence, the validity of many transporter models is restricted to a limited part of the chemical space, and can therefore not be used to predict interactions with other classes of compounds. Furthermore, small datasets limit the possibility of conducting thorough model validation using external test sets.

To conclude, the membrane permeability and interactions with transporters are major factors influencing the absorption, distribution, metabolism, excretion and toxicity (ADMET) profile of new drugs. It is therefore of great importance to determine these properties at an early stage of the development process. However, datasets used for the prediction of permeability are often highly skewed towards completely absorbed compounds, resulting in poor precision when models are applied to incompletely absorbed discovery compounds. Studies of drug transporters have generally been focused on P-gp, while considerably less attention has been given to other drug-transporting members of the ABC transporter family.

The focus of this thesis was, therefore, placed on studies of passive and active membrane transport, with the purpose of determining the molecular characteristics which are important for these transport mechanisms. Special consideration was given to the dataset selection. Compounds were selected to avoid skewed datasets and to maximize the structural diversity, so that the models developed would be representative of the chemical space of orally administered drugs. Furthermore, a significant effort was made to increase the datasets of BCRP and MRP2 inhibitors, enabling the development of globally applicable models.
2. Aims of the thesis

The general objective of this thesis was to determine the influence of passive permeability and active ABC transporter-mediated efflux on the intestinal absorption and tissue distribution of drugs. The specific aims were:

- To investigate the influence of the passive transcellular, paracellular, and active transport routes in experimental models used for prediction of intestinal drug absorption (Paper I).

- To map the affinity of orally administered drugs for the ABC transporters BCRP and MRP2 (Papers II and III), using global compound datasets representing the chemical space of oral drugs.

- To find new inhibitors with specificity towards individual or multiple ABC transporters (Papers II-IV).

- To describe differences and similarities in the drug affinity patterns of BCRP, MRP2 and P-gp (Paper IV).

- To develop predictive computational models of intestinal drug permeability (Paper I) and of drug affinity for the ABC transporters BCRP (Papers II and IV), MRP2 (Papers III and IV) and P-gp (Paper IV).
3. Methods

3.1. Dataset selection

To enable development of globally predictive models, all datasets in the thesis were selected to be representative of orally administered drugs. Depending on the specific aims of each study, different additional inclusion criteria were used.

In Paper I, the aim was to predict intestinal absorption from experimental and computationally predicted permeability determinations, with a particular focus on poorly absorbed drugs. The dataset was, therefore, selected to evenly cover the entire range of FA in humans (0-100%), so that the models would not be biased towards completely absorbed drugs. In Paper II, one aim was to compare the affinity pattern of BCRP to other major ABC transporters. Thus, compounds with a reported affinity for other ABC transporters and compounds for which no such reports could be found were included. In Paper III a similar approach was used to study the affinity pattern of MRP2. A benchmark dataset for transporter studies was introduced that included a diverse set of compounds with affinity for a panel of important ABC and SLC transporters, as well as markers of Cytochrome P450 (CYP) metabolism and hepatic toxicity. The aim of Paper IV was to compare the inhibitor affinity patterns of P-gp, BCRP and MRP2. For this purpose, high-quality data on inhibition of P-gp was compiled from the literature, and was compared with in-house data for BCRP and MRP2 that were determined under comparable experimental conditions.

3.2. Structural diversity

The structural diversity of the datasets was assessed with the ChemGPS methodology, using 2D descriptors from the SELMA descriptor collection. ChemGPS provides a three-dimensional map of the drug-like chemical space onto which the compounds of interest can be projected. In ChemGPS, the principal components calculated from a reference set of drug-like molecules are used as the x, y, and z axes of a three-dimensional coordinate system. The three most important principal components are summarized from a large number of molecular descriptors and represent mainly the size, polarity and flexibility of the molecules. The datasets used in this thesis were projected...
onto the ChemGPS coordinate system, and their structural diversity was determined by comparing their coverage of ChemGPS chemical space with that of a reference set of 450 orally administered drugs from the *Physician's Desk Reference*.

3.3. Experimental methods

3.3.1. Preparation of filter-immobilized hexadecane membranes (HDMs)

HDMs were prepared using a protocol modified from Wohnsland and Faller. A mixture of hexadecane and hexane (5% v/v hexadecane) was added to polycarbonate filter inserts (Transwell Costar, Badhoevedorp, the Netherlands; diameter 12 mm; pore size 0.4μm), and the hexane was evaporated for at least one hour prior to conducting the permeability measurements. [14C]mannitol-flux was used to measure the plate-to-plate variation of the HDM membranes, using three filters on each 12-filter plate. The trans-epithelial electrical resistance (TEER) was measured repeatedly during the assay setup, and was ≥5000 Ohms for all filters prepared using the protocol presented above.

3.3.2. Permeability measurements

Permeability measurements were performed as described previously. Briefly, the drug dissolved in Hank’s balanced salt solution (HBSS) buffered to pH 7.4 was added to the donor side of the membrane or the cells, and buffer not containing the drug was added to the receiver side. The membranes were incubated in a humidified atmosphere at 37ºC. At regular time points (every 10–120 min.), samples were removed from the receiver chamber and the volume was replaced with fresh, preheated buffer of pH 7.4 without any drug. The filter plates were stirred at a high rate (500 rpm) to minimize the influence of the aqueous boundary layer. The integrity of the membranes was determined for each batch of filters by measuring the membrane permeability to [14C]mannitol. Radioactively labeled compounds were analyzed using liquid scintillation counting (1900CA TRICARB, Canberra Packard Instruments, Downers Grove, IL), and a reversed-phase gradient HPLC system was used for the unlabeled compounds.
Apparent permeability coefficients ($P_{\text{app}}$, cm/s) were calculated using the non-sink condition analysis, which is also applicable to highly permeable compounds that do not exhibit linear drug flux:

$$C_R(t) = \frac{M}{V_D + V_R} + \left( C_{R,0} - \frac{M}{V_D + V_R} \right) \times e^{-P_{\text{app}} \times A \left( \frac{1}{V_D} + \frac{1}{V_R} \right) \times t} \quad \text{Equation 1}$$

where $V_D$ is the volume in the donor compartment (0.5 ml), $V_R$ is the volume in the receiver compartment (1.5 ml), $A$ is the area of the filter (1.13 cm$^2$), $M$ is the total amount of drug in the system, $C_{R,0}$ is the drug concentration in the receiver compartment at the start of the time interval and $C_R(t)$ is the drug concentration in the receiver compartment at time $t$ from the start of the interval.

### 3.3.3. Measurements of BCRP efflux inhibition

Inhibition of BCRP-mediated mitoxantrone efflux was studied in Saos-2 cells transfected with wild-type (Arg$^{482}$) human BCRP (Saos-2/wtABCG2). The cells were analyzed for mRNA expression of other transporters, showing negligible expression levels for drug transporters from the ABC and SLC families. The cells were cultured under an atmosphere of 5% CO$_2$ at 37ºC, and were incubated with 1 μM mitoxantrone for 60 minutes, with or without the addition of 50 μM of the compound under study. The intracellular mitoxantrone fluorescence was analyzed using a BeckmanCoulter FC500 flow cytometer (BeckmanCoulter, Fullerton, CA). To determine if the observed increase in mitoxantrone accumulation was caused by specific inhibition of BCRP, the compounds were also studied in mock-vector transfected cells. The increase in intracellular accumulation of mitoxantrone on co-incubation with the compounds under study was used as a measure of the BCRP inhibition and was normalized to the value obtained using 0.5μM of the potent BCRP inhibitor Ko143 (100% inhibition).

### 3.3.4. Measurements of MRP2 efflux inhibition

Inhibition of [³H]-estradiol-17β-D-glucuronide (E$_{17}$G) transport into MRP2-overexpressing inverted Sf9 membrane vesicles was studied using a rapid filtration technique modified from Ishikawa et al. Briefly, membrane vesicles (SOLVO Biotechnology, Budapest, Hungary) were incubated with 50 μM E$_{17}$G for 10 minutes, with or without the addition of 80 μM of the compound under study. The transport was stopped by addition of ice-cold buffer, and the reaction mixture was immediately filtered through a 96-well glass filter plate with a pore size of 0.65 μm (Millipore Corp., Bedford,
MA). The radioactivity associated with the membrane vesicles was measured in a 1900CA TRICARB liquid scintillation counter (Canberra Packard Instruments, Downers Grove, IL). ATP-dependent transport rates were determined by subtracting the transport in vesicles incubated with AMP from the rate in ATP-incubated vesicles. The inhibitory effects of the test compounds were calculated as the ratio between the ATP-dependent E_{17G} transport rates in the presence and absence of the test compound.

3.4. Computational methods

3.4.1. Generation of molecular descriptors
Molecular structures were either entered manually into MacroModel\textsuperscript{207} (Paper I) or obtained from SciFinder Scholar\textsuperscript{208} (Papers II-IV). Corina\textsuperscript{209} was used to generate 3D structures (Papers II-IV). The structures were then energy minimized in vacuum using MacroModel,\textsuperscript{207} with a 2000-step Polak-Ribiere conjugate gradient procedure using the MMFF94s force field and a dielectric constant of 1. After performing a pre-minimization of the structures, a 500-step Monte Carlo conformational analysis (Paper I) or a 1000-step low-mode conformational analysis (Paper II) was used to identify low energy conformations. Unique conformations with energy levels lower than 50 kJ/mol above the minimum energy conformation were stored and used for further calculations of dynamic surface areas (Paper I) or for pharmacophore generation (Paper II).

A selection of 2D and 3D molecular descriptors related to the molecular size, polarity, flexibility, charge distribution, connectivity, and electrotopological state indices was calculated using the programs Molconn-Z\textsuperscript{210} (Paper I), SELMA\textsuperscript{201} (Papers I and II), DragonX\textsuperscript{211} (Papers III and IV), ADMET-Predictor\textsuperscript{212} (Papers II-IV), and HYBOT\textsuperscript{213} (Papers III and IV). The free molecular surface areas for each different atom type was calculated using the in-house software MAREA\textsuperscript{214} (Papers I-IV), as described previously.\textsuperscript{149}

3.4.2. Pharmacophore modeling
Low-energy conformations obtained from MacroModel were imported into Catalyst.\textsuperscript{215} Ten common feature pharmacophore hypotheses were developed for the BCRP inhibitors in the training set using the common features algorithm (HipHop) with the hydrogen bond acceptor, hydrogen bond donor, and positively ionizable, negatively ionizable, hydrophobic and ring aromatic features as possible pharmacophore features. The hypothesis that was most successful in distinguishing between BCRP inhibitors and non-inhibitors
was selected for visualization of the preferential orientation of the BCRP inhibitors.

### 3.4.3. Statistical analysis

In Paper I, Spearman’s rank order correlation coefficients were used to determine the ability of the experimental and computational permeability models to rank the dataset compounds according to their oral absorption. The rank order correlation coefficients were calculated as the linear correlation coefficient for two separate rankings of $n$ items, according to:

$$ r_s = \frac{\sum_{i=1}^{n} (R_i - \bar{R})(S_i - \bar{S})}{\sqrt{\sum_{i=1}^{n} (R_i - \bar{R})^2} \sqrt{\sum_{i=1}^{n} (S_i - \bar{S})^2}} $$

Equation 2

where $R_i$ and $S_i$ are the ranks associated with the x and y-values for compound $i$, $\bar{R}$ and $\bar{S}$ are the mean ranks for the x and y-values, respectively, and $n$ is the number of compounds.

PLS, PLS-DA or OPLS-DA, all implemented in Simca-P, were used to derive multivariate models in Paper I, IV, and II-III, respectively. All molecular descriptors were mean centered and scaled to unit variance, and any descriptors with a variance close to zero were excluded from the analysis. The models were evaluated using the $R^2$ and the leave-many-out cross-validated $Q^2$ with 7 cross-validation groups. For the discriminant models, the influence of differently sized groups was balanced by replication of the compounds belonging to the smaller classes in the datasets. To avoid bias in the models, all replicates of a molecule were removed simultaneously during the cross-validation procedure.

For the continuous PLS models, the root-mean-square error of prediction (RMSE) was used as a measure of the model predictivity:

$$ RMSE = \sqrt{\frac{1}{n-1-p} \sum_{i=1}^{n} (y_{i,predicted} - y_{i,measured})^2} $$

Equation 3

where $n$ is the number of observations, $p$ is the number of latent variables in the PLS model, and $y_{measured}$ and $y_{predicted}$ are the experimentally determined and predicted values of the response parameter, respectively.

The models were optimized using a variable selection procedure in which groups of molecular descriptors that did not contain information relevant to the problem (i.e., noise) were removed in a stepwise manner. Descriptors
were kept outside the model if removing them resulted in a statistically improved model based on the $Q^2$ (for continuous models) or the classification accuracy for the training set (for discriminant models).

The statistical validity of the models was tested using a random permutation test, in which the order of the response variable was randomly changed 100 times (Papers II-IV). Furthermore, test sets consisting of compounds not included in the model development were used to validate the models (Papers I-IV).
4. Results and discussion

4.1. Diversity of datasets for drug transport modeling

A computational model will only be predictive within the chemical space of the compounds on which it is based. Thus, to assess the quality of a prediction, it is essential to know the applicability domain of the model. In this thesis, the aim was to investigate the membrane permeability and ABC transporter inhibition of compounds representing the chemical space of drug-like compounds. The emphasis was placed on orally administered drugs since they comprise the majority of the pharmaceutical market.

The structural diversity of the compounds was assessed using the ChemGPS methodology (Section 3.2). The datasets applied in the investigations of ABC transporter inhibition (Papers II-IV; \( n = 123, 191 \) and 126, respectively) covered the volume of the oral drug space satisfactorily (Figure 8B-D). In Paper I, the compounds were selected to evenly cover the entire range in human FA (0-100%; \( n = 30 \); Figure 9D). The inclusion of a large proportion of hydrophilic, incompletely absorbed compounds resulted in a shift towards lower values in the polarity-related second ChemGPS component (Figure 8A).

The dataset in Paper I was selected to evenly cover the entire range of FA (D). In contrast, literature datasets are often skewed towards high FA (A-C).59,217-219
4.2. Influence of different transport pathways on intestinal permeability

In Paper I, the influence of different transport pathways on drug permeability predictions was studied in experimental permeability screening assays of differing complexity: the epithelial cell lines Caco-2 and 2/4/A1, and filter-immobilized artificial hexadecane membranes (HDM) (Box 1). The 2/4/A1 cells lack functional expression of several important drug transporters, and form monolayers with a more leaky, small intestine-like paracellular pathway than Caco-2 monolayers (Figure 10, top row). The HDMs completely lack active transport pathways, and, in addition, the paracellular pathway is absent in these models.

The $P_{\text{app}}$ in the HDM and Caco-2 models were comparable, especially for the highly permeable compounds, whereas $P_{\text{app}}$ in the small-intestine-like 2/4/A1 model was up to 100-fold higher, resulting in values comparable to those observed in the human jejunum.\textsuperscript{218, 220} This difference is most likely caused by the larger influence of the paracellular pathway in the 2/4/A1 cell line. The experimentally determined $P_{\text{app}}$ was used to classify compounds as being acceptably or poorly absorbed, and to rank them according to their intestinal absorption. The cell-based models gave better classification results and resulted in considerably stronger rank order correlations between $F_A$ and $P_{\text{app}}$ ($r_s = 0.74$ and 0.73 for 2/4/A1 and Caco-2) than the HDM model ($r_s = 0.47$; Figure 10A-C). This suggests an important role of the paracellular pathway in intestinal permeability screening. In line with the experimental results, the computational model based on 2/4/A1 permeability resulted in the best \textit{in silico} prediction of $F_A$, with a rank order correlation similar to the experimental 2/4/A1 data ($r_s = 0.85$; Figure 10D). The computational 2/4/A1 model also successfully predicted the intestinal absorption of an external test set, resulting in a rank order correlation coefficient of 0.74.

![Figure 10. Rank order correlation between $F_A$ and $P_{\text{app}}$ determined experimentally in the HDM (A), Caco-2 (B) and 2/4/A1 (C) models, or calculated using a computational model based on 2/4/A1 $P_{\text{app}}$ (D). The squares denote the training set compounds and the circles denote compounds in the test set. Closed symbols denote passively transported compounds, and open ones denote actively transported compounds. 1: Glycylsarcosine; 2: methotrexate; 3: digoxin. When these outliers were removed, the 2/4/A1 correlation (C) was excellent ($r_s = 0.95$).](image)
Permeability-limiting molecular descriptors related to polarity and hydrogen bond interactions dominated the models of Caco-2 and 2/4/A1 permeability, whereas the model based on HDM $P_{app}$ had a larger influence of permeability-driving descriptors related to nonpolar interactions. This difference is probably reflecting the relative simplicity of the HDM model, where the hexadecane describes the diffusion step across the hydrophobic membrane interior.\(^{17}\)

Notably, only three significant outliers (digoxin, glycylsarcosine, and methotrexate) were observed in the experimental 2/4/A1 correlation. Glycylsarcosine and methotrexate are substrates for uptake transporters,\(^{221-223}\) and the absence of the majority of the active transport pathways in the 2/4/A1 cell line probably explains these false predictions. The underestimation of digoxin FA could be related to the fact that compounds with a relatively low permeability coefficient may have time to encounter a larger absorptive surface area in the intestine. This would result in a higher absorption \textit{in vivo} than is expected from the permeability (see Figure 3B). When the three outliers were removed the experimental 2/4/A1 rank order correlation was excellent ($r_s = 0.95$).

Interestingly, FA was well predicted for all other compounds in the dataset, despite the fact that several of them are substrates for drug transporters. High intestinal drug concentrations are often observed after oral dosing,\(^{222}\) and the limited effect of active transport on the intestinal absorption of most drugs in Paper I might, therefore, be a result of saturation of the intestinal transporters. However, the results further show that none of the experimental models was able to predict FA for all compounds exhibiting significant active transport mechanisms \textit{in vivo}. The influence of active transporters is likely to be greater for compounds that are administered in low, sub-saturating doses, or that rely on uptake transporters to gain access to the enterocytes. For such compounds, inhibition of important drug transporters by co-administered drugs can markedly affect the intestinal absorption.\(^{39, 122, 224}\)

### 4.3. Identification of inhibitors of BCRP and MRP2

Papers II and III aimed to identify inhibitors of the ABC efflux transporters BCRP (ABCG2; Paper II) and MRP2 (ABCC2; Paper III) using datasets that were representative of orally administered drugs. Inhibition of BCRP was studied in Saos-2 osteosarcoma cells over-expressing human wild-type (Arg\(^ {482}\)) BCRP, whereas inverted membrane vesicles were selected as a model system for MRP2 inhibition.

Saturable transport was demonstrated for standard substrates of each transporter (Figure 11A and B). Saos-2/wtABCG2 cells effluxed mitoxantrone with an apparent $K_m$ of $18\pm9$ μM (Figure 11A), and the transport was completely inhibited by the potent BCRP inhibitor Ko143 (Figure 11C). For
MRP2, a $K_m$ of 94±7 μM was determined for the transport of E$_{17}$G (Figure 11B). The transport rate was sigmoidally related to the substrate concentration, which is in line with previous reports demonstrating positive cooperative transport for MRP2.$^{68, 102}$ MRP2-mediated transport of E$_{17}$G was completely inhibited by the MRP inhibitor MK571 (Figure 11D).

![Figure 11](image)

**Figure 11.** Characterization of the BCRP and MRP2 inhibition assays. A. Mitoxantrone efflux from BCRP-expressing Saos-2 cells was saturable and followed Michaelis-Menten kinetics. B. The sigmoidal concentration dependency of the E$_{17}$G transport in MRP2-expressing inside-out Sf9 membrane vesicles indicated positive cooperative transport. C. BCRP-mediated mitoxantrone transport was inhibited by the model BCRP inhibitor Ko143. D. MRP2-mediated E$_{17}$G transport was inhibited by the model MRP inhibitor MK571.

Of the 123 compounds studied for BCRP inhibition, a total of 46 resulted in significantly reduced mitoxantrone efflux (Figure 12A). For MRP2, a somewhat lower hit rate was observed, with 42 out of 191 compounds resulting in significantly reduced E$_{17}$G transport (Figure 12B). Several previously unknown inhibitors were discovered for BCRP ($n = 29$) and MRP2 ($n = 27$).

In Paper II, we wanted to study whether BCRP inhibition functions by a similar mechanism to that observed for P-gp, where lipophilic drugs bind to the transporter from within the plasma membrane.$^{87-90}$ For this purpose, com-
Pounds spanning a wide range of lipophilicity were included in the study. Notably, no compounds with a value for \( \log D_{7.4} \) below 0.5 were identified as inhibitors in the cell assay. The BCRP inhibitors had markedly higher \( \log D_{7.4} \) than the non-inhibitors (with a median of 4.0 and 0.7, respectively; Figure 13), indicating that partitioning to the plasma membrane significantly influences inhibitor binding to BCRP. This observation was supported by the fact that the hydrophilic BCRP substrates nitrofurantoin and cimetidine were unable to inhibit BCRP under the standard assay conditions, which is in line with their low membrane permeability. However, at increased concentrations, both compounds inhibited BCRP. These results show that a high lipo-

![Figure 12](image)

Figure 12. Inhibition of BCRP (A) and MRP2 (B) by drug-like compounds. A. The increase in cellular mitoxantrone accumulation on co-incubation with potential BCRP inhibitors was measured for 123 compounds, resulting in the identification of 46 inhibitors. B. The relative rates of E17G transport on co-incubation with potential MRP2 inhibitors were measured for 191 compounds. Forty-two compounds resulted in a reduction in the transport rates of at least 50% (i.e., transport inhibitors). In addition, 13 compounds significantly increased the E17G transport (i.e., transport stimulators). All data are presented as means ± SE.
philicity is not necessary for the binding of drugs to BCRP *per se*, but is rather determining whether the compound can reach the binding site in sufficient amounts to elicit an inhibitory effect.

Likewise, a higher lipophilicity was generally observed for MRP2 inhibitors compared to non-inhibitors (median logD_{7,4} = 3.4 and 0.8, respectively; Figure 13). However, MRP2 inhibitors that have also been reported to be substrates, and that likely bind competitively to the transport site, were found to be more hydrophilic than non-transported inhibitors (median logD_{7,4} = 0.5 and 3.6, respectively; Figure 13). This significant difference between transported and non-transported inhibitors suggests that several distinct inhibitory binding sites may exist in MRP2. Further, it is possible that the lipophilic non-transported inhibitors might bind to an intramembranous site similar to that proposed for drug binding to P-gp and BCRP.

![Figure 13](image.png)

*Figure 13.* Distribution of lipophilicity in the datasets in Papers II and III. The distribution in inhibitors of BCRP (Paper II) and MRP2 (Paper III) is shown in white, and the distribution in non-inhibitors is shown in black. The inhibitors differ from the non-inhibitors by a shift to higher lipophilicity. For MRP2, the inhibitors that are also reported as substrates are generally less lipophilic than those not reported as substrates. The boxes show the inter-quartile distances and the median values, and the whiskers show the span between the lowest and the highest value.

### 4.4. Overlapping inhibitor specificity of P-gp, BCRP and MRP2

The results presented in Papers II and III demonstrated a significant overlap in the inhibitor specificities of BCRP and MRP2. These transporters are co-localized with P-gp in protective tissues throughout the human body, with important roles in restricting the intestinal absorption of foreign compounds and mediating the secretion of drugs and metabolites to bile.\(^{84, 112, 225-227}\) Inhibition of P-gp, BCRP and MRP2 can, consequently, lead to serious adverse effects, for instance through the accumulation of toxic drugs and metabolites in the liver.\(^{228, 229}\) However, so far, knowledge of the overlap in the affinity of ABC transporters has been based on scattered observations for single compounds or small compound series.\(^{230-234}\)
In Paper IV, therefore, we examined the specificity of inhibitors towards these three major ABC efflux transporters using a common dataset of 126 structurally diverse drugs. Inhibition of BCRP was determined for 42 new compounds, and was complemented with data from Paper II. MRP2 data for the same compounds were obtained from Paper III, and P-gp data determined under comparable experimental conditions were collected from the literature.

Of the 126 compounds studied, 70 inhibited at least one of the transporters (Figure 14). As many as 46 of the identified inhibitors affected more than one of the transporters in the investigated concentration range. Of these, 22 compounds inhibited P-gp, BCRP and MRP2. These multi-specific inhibitors contributed the majority (76%) of the MRP2 inhibitors and a significant proportion of the P-gp and BCRP inhibitors (39% and 41%, respectively). Notably, only about half as many inhibitors were found for MRP2 ($n = 29$) as for P-gp ($n = 56$) and BCRP ($n = 53$). The largest overlap was seen between P-gp and BCRP, with 43 common inhibitors. In contrast, only two and one compounds were shared by P-gp and MRP2 or by BCRP and MRP2, respectively, while not affecting the third transporter. The results in Paper IV thus emphasize the significant affinity overlap previously indicated for these major drug efflux transporters.

![Figure 14](image-url)

**Figure 14.** Overlapping inhibition of P-gp, BCRP and MRP2. In total, 70 (56%) of the compounds investigated inhibited one or more of the transporters. The number of specific inhibitors were 11, 9 and 4 for P-gp, BCRP and MRP2, respectively. A large overlap was observed between P-gp and BCRP, with 43 inhibitors in common.
4.5. Computational models of ABC transporter inhibition

To define molecular features important for drug-mediated inhibition of BCRP and MRP2, the experimental data in Papers II and III were used to develop computational models to discriminate between inhibitors and non-inhibitors. For BCRP, the inhibitors could be distinguished from the non-inhibitors using just two molecular descriptors: logD$_{7.4}$ and the molecular polarizability, the latter descriptor reflecting electron delocalization in conjugated systems (Figure 15A). Both of these descriptors are correlated to the passive membrane permeability, further emphasizing the importance of membrane partitioning for BCRP inhibition. Despite the simplicity of the developed model, it successfully classified the compounds of the training and the test sets (Table 1).

Table 1. Prediction of BCRP and MRP2 inhibition using computational models.

<table>
<thead>
<tr>
<th>Transporter model</th>
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<tr>
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<td>inhibitors</td>
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<td>BCRP (Paper II)</td>
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<td>MRP2 (Paper III)</td>
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<td>Test set</td>
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Figure 15. Computational models of BCRP and MRP2 inhibition (from Papers II and III, respectively). In both A and B, the compounds falling in the shaded area were predicted to be non-inhibitors. Closed symbols denote those compounds which were experimentally determined to be inhibitors, and open symbols denote the non-inhibitors. The training set compounds are shown as squares and the test set compounds as circles. C. The five molecular descriptors in the MRP2 inhibition model are related to the lipophilicity (calculated octanol-water partition coefficient), size (2nd largest dimension, total structure connectivity index), aromaticity (number of aromatic bonds) and hydrogen bonding potential. Descriptors with positive coefficients have higher values in inhibitors, and descriptors with negative coefficients have higher values in non-inhibitors.
Interestingly, the computational model of MRP2 inhibition developed in Paper III showed a similar importance of lipophilicity and aromaticity as the BCRP model. A larger number of molecular descriptors was needed to discriminate between inhibitors and non-inhibitors for MRP2 than for BCRP, possibly reflecting a more complex binding pattern with several drug binding sites in MRP2 (Figure 15C; Table 1).

The molecular features important for inhibition of ABC transporters were further investigated in Paper IV. First, individual models were developed for P-gp, BCRP and MRP2 (Figure 16A-C). The size, shape, lipophilicity and aromaticity were the dominating factors distinguishing inhibitors from non-inhibitors for the dataset in Paper IV, which only partly overlapped with the datasets used in Papers II and III. The similarities between the computational models for each transporter suggested that these molecular properties might be related to the large number of multi-specific inhibitors affecting all three transporters. We therefore modeled the multi-specific inhibitors in independent of the specific ones, confirming the importance of lipophilicity and aromaticity for the overlapping inhibitors (Figure 16D). The median logD$_{7.4}$ was 4.6 for the multi-specific inhibitors, indicating that they will accumulate in the plasma membrane. This suggests that this group of inhibitors may interact with binding sites located in the membrane-protein interface. The fact that a large number of the multi-specific inhibitors have been reported to be

![Figure 16. Prediction of ABC transporter inhibitors from: computational models of P-gp inhibitors (A), BCRP inhibitors (B), MRP2 inhibitors (C) and models of overlapping (D) and general (E) ABC transporter inhibition. Descriptors related to the size, shape, lipophilicity and aromaticity were important for all three models of individual transporters (A-C). This importance was mirrored in the models of overlapping and general ABC transporter inhibition (D and E), indicating that these properties might be related to the multi-specific inhibitors.](image-url)
P-gp substrates\textsuperscript{235} indicates that such a site may be part of the intramembranous substrate binding pocket in P-gp.

Finally, the large inhibitor overlap and the similarities between the individual transporter models suggested that it would be possible to derive a model describing general ABC inhibition. We therefore used the same approach as for the individual models to develop a model capable of discriminating between compounds inhibiting any of the three ABC transporters from compounds lacking an inhibitory effect. The resultant model mirrored the importance of lipophilicity and aromaticity revealed in the individual models. The classification accuracy was 82% of the inhibitors and 85% of the non-inhibitors in the test set (Figure 16), which is an improvement to most published models of drug-transporter interaction.\textsuperscript{196, 197, 200}
5. Conclusions and future outlook

In this thesis, the role of passive membrane permeability and ABC transport proteins on drug absorption and disposition was investigated. Particular emphasis was placed on defining the molecular properties of importance for these transport mechanisms. Experimental models that include the paracellular pathway were found to result in improved predictions of intestinal drug absorption, especially for incompletely absorbed drugs. Previously unknown inhibitors of BCRP and MRP2 were identified from datasets that were representative of orally administered drugs. Finally, the overlap in the inhibitor affinity between P-gp, BCRP and MRP2 was described and a majority of the inhibitors was found to affect more than one of these transporters. The work in this thesis demonstrates the usefulness of simplistic approaches, where different mechanisms are studied in isolation. Such data provide the basis for understanding the intricate interplay affecting cellular drug disposition. The specific conclusions were:

- The paracellular drug transport pathway plays a significant role in the prediction of the intestinal drug permeability of incompletely absorbed drugs.
- Permeability data from the 2/4/A1 cell model, which has a small intestine-like paracellular pathway, gave better classification and ranking of drugs according to their intestinal absorption than the artificial hexadecane membrane model which exclusively models the hydrophobic interior of the plasma membrane.
- A large number of structurally diverse drugs inhibited the ABC transporters BCRP and MRP2. The chemical space of BCRP inhibitors was similar to that of P-gp, whereas fewer drug-like compounds inhibited MRP2.
- The multi-specific ABC transporter inhibitors, i.e., those that affected P-gp, BCRP and MRP2, were, in general, highly lipophilic weak bases.  
- The passive membrane permeability to drugs and the inhibitor affinity for ABC transporters could be modeled using easily interpreted molecular descriptors. The models can be used as predictive filters in drug discovery to guide further experimental efforts.
The research described in this thesis has led to an increased knowledge of the mechanisms determining the intestinal permeability of incompletely absorbed drugs and of the molecular characteristics of importance for inhibition of the major ABC efflux transporters P-gp, BCRP and MRP2.

In human tissues, the transport mechanisms studied here act in concert with one another and with additional transporters from the ABC and SLC families. A drug can be a substrate of several different transporters and can also influence the disposition of coadministered drugs by inhibiting one or more transporters, resulting in clinically important drug-drug interactions. Furthermore, transporter inhibition can lead to accumulation of toxic drugs and metabolites and subsequent cellular toxicity. A major challenge for future investigations in this field is, therefore, to clarify the interplay between different transport mechanisms.

Much work also remains to be done in terms of characterizing the influence of membrane transport on intracellular processes. The transport into the cell will affect the presentation of the drug for intracellular pharmacological targets and metabolizing enzymes, thereby influencing both the pharmacokinetics and the pharmacodynamics of the drug. In addition, many drug transporters are expressed in the membranes of intracellular organelles. This can result in widely differing drug concentrations in different subcellular compartments, sometimes leading to subcellular toxicity.

Approaches to the study of complex interplay between different cellular mechanisms include the use of cells transfected with multiple drug transporters and knock-out animal models. Further, pharmacokinetic and systems biology modeling can be applied to cellular drug flux, thereby gaining an insight into the relative importance of different transport pathways. The data presented here for specific transport mechanisms will be useful for selecting suitable model drugs and identifying the optimal experimental conditions for such endeavors.
6. Svensk populärvetenskaplig sammanfattning

För att ett läkemedel ska kunna ge den önskade effekten krävs att läkemedelsmolekylerna når fram till målcellen. Kroppens organ skyddas av olika vävnadsbarriärer, och för att passera dessa måste läkemedlet ta sig in i och igenom kroppens celler. Transporten genom cellernas omgivande membran är därför avgörande för läkemedlets effekt.


När en patient tar flera läkemedel samtidigt, kan dessa påverka varandras effekt. Om ett läkemedel hämmar transporten av ett annat, kan det leda till att detta inte når sitt mål och att effekten uteblir. Hämning av transporten kan också göra att giftiga substanser anrikas i kroppens vävnader, vilket kan leda till allvarliga biverkningar. I avhandlingen undersöktes därför ett stort antal olika läkemedel, för att utreda hur de påverkar transportproteiner. Dessa transporterar bland annat medlemmar av den ABC-familjen, dual specificity mitochon- 
drial protein (MRP2). Ett avsevärt antal läkemedel som används i klinisk praxis visades hämma dessa transportproteiner. Ytterligare undersökningar krävs dock för att visa ifall detta kan leda till allvarliga biverkningar.

De experimentella resultaten användes vidare för att utveckla datorbase-rade modeller som beskrivs hur läkemedels kemiska struktur påverkar om de hämmar transportproteinet eller inte. Bland annat visades att läkemedel som påverkar flera ABC-transportörer är mer fettlösliga än andra läkemedel. Datorbaserade modeller av detta slag kan användas för att förutsäga ifall nya läkemedel kommer påverka transporten av andra läkemedel. På så vis kan
läkemedel som riskerar att ge allvarliga biverkningar sorteras bort i ett tidigt stadium av utvecklingsprocessen. Detta leder till att nya, säkrare läkemedel kan utvecklas effektivt och till en lägre kostnad, vilket i slutändan även gynnar patienterna.
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