In vitro and in silico prediction of drug-drug interactions with transport proteins

GUSTAV AHLIN
Dissertation presented at Uppsala University to be publicly examined in B21, Biomedical Center, BMC, Husargatan 3, Uppsala, Friday, October 2, 2009 at 13:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English.

Abstract

Drug transport across cells and cell membranes in the human body is crucial for the pharmacological effect of drugs. Active transport governed by transport proteins plays an important role in this process. A vast number of transport proteins with a wide tissue distribution have been identified during the last 15 years. Several important examples of their role in drug disposition and drug-drug interactions have been described to date. Investigation of drug-drug interactions at the transport protein level are therefore of increasing interest to the academic, industrial and regulatory research communities.

The gene expression of transport proteins involved in drug transport was investigated in the jejunum, liver, kidney and colon to better understand their influence on the ADMET properties of drugs. In addition, the gene and protein expression of transport proteins in cell lines, widely used for predictions of drug transport and metabolism, was examined.

The substrate and inhibitor heterogeneity of many transport proteins makes it difficult to foresee whether the transport proteins will cause drug-drug interactions. Therefore, *in vitro* assays for OCT1 and OATP1B1, among the highest expressed transport proteins in human liver, were developed to allow investigation of the inhibitory patterns of these proteins. These assays were used to investigate two data sets, consisting of 191 and 135 registered drugs and drug-like molecules for the inhibition of OCT1 and OATP1B1, respectively. Numerous new inhibitors of the transport proteins were identified in the data sets and the properties governing inhibition were determined. Further, antidepressant drugs and statins displayed strong inhibition of OCT1 and OATP1B1, respectively. The inhibition data was used to develop predictive *in silico* models for each of the two transport proteins.

The highly polymorphic nature of some transport proteins has been shown to affect drug response and may lead to an increased risk of drug-drug interactions, and therefore, the OCT1 *in vitro* assay was used to study the effect of common genetic variants of OCT1 on drug inhibition and drug-drug interactions. The results indicated that OCT1 variants with reduced function were more susceptible to inhibition. Further, a drug-drug interaction of potential clinical significance in the genetic OCT1 variant M420del was proposed.

In summary, gene expression of transport proteins was investigated in human tissues and cell lines. *In vitro* assays for two of the highest expressed liver transport proteins were used to identify previously unknown SLC transport protein inhibitors and to develop predictive *in silico* models, which may detect previously known drug-drug interactions and enable new ones to be identified at the transport protein level. In addition, the effect of genetic variation on inhibition of the OCT1 was investigated.

Keywords: Solute carrier, SLC transporter, OCT1, Organic cation transporter 1, SLC22A1, OATP1B1, SLCO1B1, Organic anion transporting peptide 1B1, Drug transport, Active transport, Genetic polymorphism, Cell lines, Gene expression, Multivariate data analysis, OPLS

Gustav Ahlin, Department of Pharmacy, Box 580, Uppsala University, SE-75123 Uppsala, Sweden

© Gustav Ahlin 2009

ISSN 1651-6192
urn:nbn:se:uu:diva-107492 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-107492)
List of Papers

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


II  **Ahlin G**, Hilgendorf C, Karlsson J, Al-Khalili Szigyarto C, Uhlén M and Artursson P. Endogenous gene and protein expression of drug transporting proteins in cell lines routinely used in drug discovery programs. *Accepted for publication in Drug Metabolism and Disposition*


V  **Ahlin G**, Karlgren M, Bergström CAS, Karlsson J and Artursson P. *In vitro* and *in silico* strategies to identify OATP1B1 inhibitors and properties governing OATP1B1 inhibition *In manuscript.*
Contents

Introduction ........................................................................................................... 9
Drug discovery and development ................................................................. 10
Drug transport through cellular barriers .................................................... 12
  Cellular transport mechanisms .......................................................... 12
Active transport .......................................................................................... 13
  Nomenclature ...................................................................................... 14
  The role of active transport for the ADMET properties of drugs .......... 14
  ATP-binding cassette (ABC) efflux transporters .................................. 14
  Solute carrier (SLC) uptake transporters ............................................ 16
    Distribution ..................................................................................... 16
    Structure ........................................................................................ 16
    Driving force .................................................................................. 17
Active transporters in the liver .............................................................. 18
  Organic cation transporters (OCT; SLC22) .................................... 18
  Organic anion transporting peptides (OATP; SLCO) ..................... 18
Genetic variation in transporters ............................................................. 20
Role of transporters in drug-drug interactions ...................................... 21
Development and validation of in vitro based experimental assays for
  studying transporters .............................................................................. 21
  Gene and protein expression ............................................................. 22
  In silico prediction of transporters ....................................................... 23
    Development of in silico models ..................................................... 23
      Determining the purpose of the model ......................................... 23
      Generating a data set .................................................................... 24
        Training and test set .................................................................... 24
      Generating molecular descriptors ................................................. 25
      Generation of experimental data ................................................... 25
    Model development ........................................................................ 26
      Principal component analysis (PCA) ........................................... 26
      Projections to latent structures by means of partial least squares
        (PLS) ....................................................................................... 26
    Model generation process .............................................................. 27
    Validation of the model ................................................................... 27
Aim of the thesis ............................................................................................ 28
Abbreviations

ABC  ATP-binding cassette
ADMET  Absorption, distribution, metabolism, elimination/excretion, toxicity
ASP+  4-(4-(dimethylamino)styryl)-N-methylpyridinium
BCRP  Breast cancer resistance protein
Caco-2  Human colon adenocarcinoma cell line
Caco-2 TC7  Human colon adenocarcinoma cell line clone TC7
Caki-1  Human renal carcinoma cell line
cDNA  Complementary DNA
ClogP  Calculated octanol-water partition coefficient
CYP  Cytochrome P450
E17βG  Estradiol-17β-glucuronide
FDA  Food and drug administration
HBSS  Hank’s balanced salt solution
HEK293  Human embryonic kidney cell line
HeLa  Human cervical cancer cell line
HepG2  Human hepatocellular carcinoma cell line
HL-60  Human promyelocytic leukemia cell line
HPR  Human proteome resource project
ID  Investigational drug
K562  Human myelogenous leukaemia cell line
logP  Octanol-water partition coefficient
MCT  Monocarboxylate transporter
MDR  Multi-drug resistance protein
MLR  Multiple linear regression
mRNA  Messenger ribonucleic acid
MRP  Multidrug-resistance associated protein
MVP  Major vault protein
MW  Molecular weight
MVDA  Multivariate data analysis
OATP  Organic anion transporting peptide
OCT  Organic cation transporter
OPLS  Orthogonal PLS
OPLS-DA  Orthogonal PLS discriminant analysis
PBS  Phosphate buffer saline solution
PCA  Principal component analysis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>P-gp</td>
<td>P glycoprotein</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares projection to latent structures</td>
</tr>
<tr>
<td>PrEST</td>
<td>Protein epitope signature tag</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>Research and development</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Human osteosarcoma cell line</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carriers</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
</tbody>
</table>
Introduction

Drugs need to pass a number of cellular barriers to reach the site at which they are to act. Orally administered drugs have to overcome the intestinal epithelial barrier before they are able to enter into the portal vein. Since the majority of drugs are administered orally, the intestinal barrier is an important determinant of the fraction of a drug that is absorbed. In addition, to be distributed to the entire systemic circulation the drugs also have to avoid being eliminated via the first-pass effect in the liver. The fraction of the administered dose of an unchanged drug that reaches the systemic circulation is defined as the bioavailability of the drug\(^1\). The intestinal barrier and the first-pass effect in the liver may be avoided by administering the drug intravenously directly to the systemic circulation. However, for reasons such as safety, economy and ease of use, the oral route is usually preferred.

In general, when the drug has reached the systemic circulation it still has to reach the site of action. Additional barriers/cell membranes may need to be overcome before the drug can be distributed to its site of action. For example, drugs targeting the central nervous system need to cross the blood-brain barrier\(^2\). In addition to its importance for drug uptake and distribution, cellular transport is also crucial for the elimination of drugs from the human body. When eliminated, drugs need to pass through at least two of the cell membranes in the liver and/or the kidney cells. To summarise, transmembrane transport of drugs is crucial for the uptake, distribution and elimination of drugs in the human body. These transport processes are also involved in other important mechanisms throughout the body, e.g. metabolism, drug-drug interactions and toxicity. Drug transport through cellular membranes is governed by a number of different mechanisms, displayed in Figure 1.

Drug discovery and development is a lengthy and expensive process with a high incidence of investigational drugs never reaching the market\(^3\). High attrition rates can, however, be avoided, especially during late stages in the drug development process, and for this, access to high quality \textit{in silico} models and \textit{in vitro} methods is of crucial importance. The implementation of such models to predict solubility, membrane transport and metabolism etc., in the drug development process was probably one of the major reasons for the clear reduction in the drug candidate attrition rate associated with inadequate bioavailability and pharmacokinetics\(^4\), \(^5\). The relatively new research field of active transport has increased the attention paid to and enhanced the importance of membrane transport proteins (transporters) in drug develop-
ment. Therefore, the demand for high quality in vitro and in silico models of transporters has increased. However, high quality models are scarce which highlights the importance of further research being dedicated to the development of these models, to allow fast and inexpensive data generation in early drug discovery.

The major focus of this thesis was to identify important transporters and to develop in vitro and in silico transporter models of high quality. This was performed by i) studying the distribution and expression levels of membrane transporters in human tissues involved in ADMET properties of drugs and also in cell lines widely used in pharmaceutical research ii) developing of simple in vitro assays for uptake and inhibition studies of transporters iii) investigating of the inhibition patterns and properties governing the inhibition of the important liver transporters OCT1 (SLC22A1) and OATP1B1 (SLCO1B1) iv) developing high quality in silico models for the inhibition of transporters and v) studying of the effect of genetic variation in the gene coding for OCT1 on drug inhibition.

Drug discovery and development

The discovery and development of modern drugs is a complex, lengthy and expensive process which needs to deal with problems such as potency, toxicity and drugability of a compound. In general, the process is divided into two major phases, discovery and development, with the development phase being further subdivided as illustrated in Figure 2. Decisions are made, whether or not to transfer the molecule into the next phase, throughout the process.
The goal of the discovery process is to generate new chemical entities for introduction into the development process. During the discovery process, drug candidates are identified and then synthesized. The therapeutic efficacy of the synthesized compounds is then characterized using in vitro screening assays. The identified effective drugs are entitled investigational drug (ID) and are transferred into the drug development process.

The development process, for an ID is divided into preclinical development, clinical development (itself split into Phases I-III) and ends with the finalized product being approved and thereby reaching the market (Figure 2). In the preclinical development phase, numerous in silico, in vitro and in vivo (animal trials) methods are used. The clinical development includes clinical trials involving thousands of individuals.

The whole drug discovery and development process takes approximately 12 years and costs on average $US868 million per approved drug. There was a substantial increase in R&D costs during the 1990s, which, if maintained, would result in a drug approved in year 2013 costing approximately $US1.9 billion. These costs are so high because for each successful drug, 75-80% of the IDs are terminated at different stages during the drug development process, leading to extremely high costs without any revenue. Because of this, it is crucial to reduce drug development costs by improving the attrition rate of IDs, and by ensuring that work on non-marketable drugs is terminated at as early a stage of the drug discovery and development process as possible. In the early 1990s, the primary reason for IDs failing to reach the market (accounting for about 40% of the fallout) was poor bioavailability and pharmacokinetics. However, implementation of new in vitro, in silico and in vivo methods to increase the knowledge in these areas has proven successful, with the attrition rate attributable to poor bioavailability and pharmacokinetics having been decreased to less than 10% in 2000. The development of new and the improvement of existing in vitro and in silico models is pivotal to the speed up of the drug discovery and development process, and should also make it more cost-effective and further reduce the attrition rate. The availability of improved models will allow for earlier and more reliable decisions to be made concerning the IDs, as well as reducing the time to an eventual market introduction.

Even though transporters have been proven to be important for the ADMET properties of drugs, high quality in vitro and in silico models for membrane transporters are scarce. Therefore, this thesis is focused on identifying the highly expressed membrane transporters that are important for the ADMET properties of drugs. Further, the suitability of cell lines widely used in in vitro assays for the study of transporters has been investigated. In addition, new in vitro assays for the important liver transporters OCT1 and OATP1B1 was developed. These assays were used to identify transporter inhibition patterns and used to develop in silico models for inhibition.
Drug transport through cellular barriers

Translocation over cellular membranes throughout the human body plays a pivotal role for the properties of endogenous and exogenous compounds. For drugs, transport through cellular barriers is important in a large number of tissues. After oral administration of a drug, transport through the intestinal epithelium and the hepatocytes determine the unchanged amount of the drug reaching the systemic circulation. To reach the site of action, the drug usually needs to be distributed through additional cellular membranes. Membrane transport is also involved in the distribution of drugs into and out of the organs primarily responsible for metabolism and elimination, namely the liver and kidneys. Further, membrane transport may affect the risk for toxicity of drug compounds.

Cellular transport mechanisms

The cellular transport of endogenous and exogenous compounds can be subdivided into five types of processes (Figure 1). Transcellular passive diffusion (1), which translocates small, neutrally charged and lipophilic drugs, is the process with the highest capacity. This process neither involves any carrier protein, nor does it require an energy input as it is driven by the concentration gradient over the cell membrane. Transcytosis is the low capacity transcellular transport of large hydrophilic compounds, where the compounds are engulfed in vesicles in the cytosol, transported through the cell and released outside the cell at the opposite membrane. Paracellular transport translocates small, hydrophilic and charged compounds through the intercellular space. However, it is an inefficient process owing to the small surface area of the tight junctions in comparison to that of the cell membranes (3). Transcellular passive diffusion and paracellular transport are energy inde-
dependent and driven by the concentration gradient, as a result of which, these processes are only able to translocate compounds with the concentration gradient. However, active processes governed by membrane transporters and driven by, e.g. energy, co-transport and membrane potential (4 and 5) are capable of translocating drugs with and against the concentration gradient. The active processes are mainly responsible for transporting hydrophilic and charged compounds with low transcellular passive diffusion. In contrast to transcellular passive diffusion, active transport is a saturable process which can, consequently, lead to drug interactions. The active process, which is discussed in the next section, is subdivided into efflux transporters (governed by ABC-transporters; 4) and uptake transporters (governed by SLC-transporters; 5).

Active transport

Active transport is mediated by transporter proteins located in the plasma membrane (4 and 5 in Figure 1). These transporters are involved in translocation of endogenous and exogenous compounds over cellular membranes throughout the entire human body. In contrast to transcellular passive diffusion, the heterogeneous tissue distributions of transporters lead to differential membrane transport patterns in different tissues.

At least 5% (1000-2000) of the approximately 20,500 human genes coding for human proteins are generally assumed to be transport related\(^{10,11}\). To date, a few hundred human genes have been identified as membrane transporters. These are subdivided into two major classes, the efflux (ABC; denoted 4 in Figure 1) and uptake (SLC; denoted 5 in Figure 1) proteins, with approximately 50 ABC and 360 SLC transporters, respectively, having been identified to date\(^{10,12,13}\). Both of the two families are further subdivided into groups depending on the amino acid homology between the proteins.

The ABC transporters are energy (ATP) dependent and they have a structure that tends to be comprised of two nucleotide binding domains and a number of transmembrane domains (TMD). The SLC transporters consist of a number of TMDs but lack ATP-binding sites, since they rely on processes such as co-transport and membrane potential to provide the driving force instead of ATP hydrolysis.

The ABC and SLC proteins interact with a vast number of compounds (substrates, inhibitors and inducers). Compared to passive diffusion, which is independent of membrane proteins, active transport is carried out by a finite number of proteins in the cell membrane, with the result that the active transport process is saturable in contrast to passive diffusion.
Nomenclature

The nomenclature within the transporter field is, as often in new and emerging fields of research, somewhat confused. A good example of this is provided by the human OATP1B1 protein, a liver-specific uptake transporter. This protein was identified by two different groups in 1999 and was first called LST1 (liver specific transporter 1) and OATP2 (organic anion transporting peptide 2) respectively by its respective discoverers\textsuperscript{14, 15}. Later, this transporter came to be called OATP-C, and the current official name is now OATP1B1. Further, the name for the gene coding for OATP1B1 has been altered from SLC21A6 to SLCO1B1. To complicate matters even more, the gene and protein names are often being used arbitrarily with the gene name, SLCO1B1, often being used when discussing the protein OATP1B1 and vice versa. It is advisable to avoid misunderstandings by using official protein names when discussing the protein\textsuperscript{13}. The same approach should be adopted for the gene names. Table 1 addresses the problems associated with nomenclature by presenting the old and new names of proteins alongside their aliases.

The role of active transport for the ADMET properties of drugs

The importance of transporters for the ADMET properties of drugs is indicated by the vast number and wide tissue distribution of transporters throughout the human body. Since transporters are a relatively new research field, their impact on drug treatment is not yet fully understood. However, they have been shown to be involved in a various number of drug related processes. As an example, efflux transporters are partially responsible for the build up of a resistance to drugs by patients with different forms of cancer\textsuperscript{16}. In addition, targeting to the intestinal peptide transporter, PEPT1, has been used to enhance the bioavailability of several drugs including the antiviral drug acyclovir\textsuperscript{17} and polymorphisms in the gene coding for the statin-transporter OATP1B1 have been shown to increase the risk for statin-induced myopathy\textsuperscript{18}.

ATP-binding cassette (ABC) efflux transporters

The ABC transporters are efflux transporters that translocate compounds from the inside to the outside of the cells. Approximately 50 human ABC transporters have been identified to date\textsuperscript{12}. Overall, their structure consists of two nucleotide binding domains, which provide energy via ATP hydrolysis to drive the transporter, and a number of TMDs, which form a pathway through the membrane for the transporter substrates\textsuperscript{19}. ABC transporters are expressed in many tissues but also highly expressed in important protective barriers, like the intestine and the blood-brain barrier\textsuperscript{20}. Further, ABCs are also overexpressed in cancer cells, thereby explaining one of the reasons for drug resistance in cancer treatment\textsuperscript{16}.
Table 1. Past and present nomenclature of the investigated transport proteins. The indicated official gene and protein name should be used when discussing the genes and proteins respectively.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
<th>Other aliases</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>MDR1</td>
<td>P-gp, CLCS, PGY1, ABC20, CD243, GP170</td>
</tr>
<tr>
<td>ABCB4</td>
<td>MDR3</td>
<td>PGY3, ABC21, GBD1, MDR2, MDR2/3, PFIC-3, Pgp3</td>
</tr>
<tr>
<td>ABCB11</td>
<td>BSEP</td>
<td>PGY4, SPGP, ABC16, PFIC2, BRIC2</td>
</tr>
<tr>
<td>ABCC1</td>
<td>MRP1</td>
<td>GS-X, ABC29, ABCC</td>
</tr>
<tr>
<td>ABCC2</td>
<td>MRP2</td>
<td>DJS, cMRP, ABC30, cMOAT</td>
</tr>
<tr>
<td>ABCC3</td>
<td>MRP3</td>
<td>MLP2, ABC31, MOAT-D, cMOAT2</td>
</tr>
<tr>
<td>ABCC4</td>
<td>MRP4</td>
<td>MOATB, MOAT-B</td>
</tr>
<tr>
<td>ABCC5</td>
<td>MRP5</td>
<td>SMRP, ABC33, MOATC, MOAT-C, pABC11</td>
</tr>
<tr>
<td>ABCC6</td>
<td>MRP6</td>
<td>ARA, PXE, MLP1, ABC34, MOATE</td>
</tr>
<tr>
<td>ABCC8</td>
<td>MRP8</td>
<td>MRX, MXR, ABCP, BMDP, MXR1, ABC15, BCRP1</td>
</tr>
<tr>
<td>CDH17</td>
<td>HPT1</td>
<td>Cadherin</td>
</tr>
<tr>
<td>SLC10A1</td>
<td>NTCP</td>
<td>NTCP1, LBAT</td>
</tr>
<tr>
<td>SLC10A2</td>
<td>ASBT</td>
<td>IBAT, ISBT, NTCP2</td>
</tr>
<tr>
<td>SLC15A1</td>
<td>PEPT1</td>
<td>HPECT1, Oligopeptide transporter 1, H+/peptide transporter 1</td>
</tr>
<tr>
<td>SLC15A2</td>
<td>PEPT2</td>
<td>Oligopeptide transporter 2, H+/peptide transporter 2</td>
</tr>
<tr>
<td>SLC16A1</td>
<td>MCT1</td>
<td>HHF7</td>
</tr>
<tr>
<td>SLC16A4</td>
<td>MCT5</td>
<td>MCT4</td>
</tr>
<tr>
<td>SLC22A1</td>
<td>OCT1</td>
<td>LST1</td>
</tr>
<tr>
<td>SLC22A2</td>
<td>OCT2</td>
<td>hEMT</td>
</tr>
<tr>
<td>SLC22A3</td>
<td>OCT3</td>
<td></td>
</tr>
<tr>
<td>SLC22A4</td>
<td>OCTN1</td>
<td></td>
</tr>
<tr>
<td>SLC22A5</td>
<td>OCTN2</td>
<td>CT1, CDSP, SCD, OCTN2VT</td>
</tr>
<tr>
<td>SLC22A6</td>
<td>OAT1</td>
<td>PAHT</td>
</tr>
<tr>
<td>SLC22A7</td>
<td>OAT2</td>
<td>NLT</td>
</tr>
<tr>
<td>SLC22A8</td>
<td>OAT3</td>
<td></td>
</tr>
<tr>
<td>SLC22A9</td>
<td>UST3</td>
<td>OAT4, OAT7, UST3H</td>
</tr>
<tr>
<td>SLC22A11</td>
<td>OAT4</td>
<td></td>
</tr>
<tr>
<td>SLC28A3</td>
<td>CNT3</td>
<td></td>
</tr>
<tr>
<td>SLC01A2</td>
<td>OATP1A2</td>
<td>OATP-A, OATP</td>
</tr>
<tr>
<td>SLC01B1</td>
<td>OATP1B1</td>
<td>LST-1, OATP2, OATP-C</td>
</tr>
<tr>
<td>SLC01B3</td>
<td>OATP1B3</td>
<td>OATP8, LST-3</td>
</tr>
<tr>
<td>SLC01C1</td>
<td>OATP1C1</td>
<td>OATP-F, OATP-RP5, BSAT1</td>
</tr>
<tr>
<td>SLC02B1</td>
<td>OATP2B1</td>
<td>OATP-B, OATP-RP2</td>
</tr>
<tr>
<td>SLC03A1</td>
<td>OATP3A1</td>
<td>OATP-D, OATP-RP3, MJAM</td>
</tr>
<tr>
<td>SLC04A1</td>
<td>OATP4A1</td>
<td>OATP-E, OATP-RP1, POAT, OATPRP1</td>
</tr>
<tr>
<td>SLC04C1</td>
<td>OATP4C1</td>
<td>OATP-H, OATP-M1, OATPX</td>
</tr>
</tbody>
</table>


a HPT1 (CDH17) transporter is a member of the cadherin superfamily.
Solute carrier (SLC) uptake transporters

The solute carrier (SLC) proteins are uptake transporters that generally translocate compounds from the outside to the inside of the cells. About 360 human SLC proteins have been identified so far, and these are subdivided into some 50 families. A transporter is assigned to a specific SLC family if it has an amino acid sequence overlap of at least 20–25% with other members of that family. The SLC proteins are involved in the transport of a vast number of substrates, with transporters having heterogeneous substrate acceptance, e.g. copper (SLC31), urea (SLC14) and peptides (SLC15). Despite the vast number of SLC transporters identified, relatively few are involved in the transport of xenobiotic compounds and drugs. SLC transporters interacting with drugs includes the bile acid (SLC10), peptide (SLC15), monocarboxylic acid (SLC16), nucleoside (SLC28 and SLC29), anion (SLCO and SLC22) and cation (SLC22 and SLC47) transporter families. However, the diverse substrate specificity and heterogeneous tissue distribution of these groups contribute to a complex drug transport pattern.

Distribution

The solute carriers have a heterogeneous distribution pattern in the plasma membranes throughout the human body. The diversity of the expression of transporters in human tissues has mainly been monitored using gene expression, but also, to some extent, with protein expression methodology. In contrast to the ABC transporters, many SLC transporters have more tissue specific members, e.g. OCT1 found in the liver, OAT1 in the kidneys and OATP1A2 in the central nervous system. There are also some SLC transporters distributed ubiquitously throughout the human body, e.g. MCT1 and OCTN2. The ubiquitous tissue expression may suggest that these transporters have an essential physiological role. In fact MCT1 transports lactic and pyruvic acid and, hence, is of importance in glycolysis and gluconeogenesis whereas OCTN2 is involved in the uptake of carnitine, an essential factor in long-chain fatty acid oxidation.

At the onset of this thesis, data on the gene expression patterns of transporters in human tissues involved in drug transport were incomplete and scattered in the literature. Therefore, the tissue distribution of 36 drug transporters in the human colon, jejunum, liver and kidney was investigated in Paper I. This allowed identification of specifically and ubiquitously expressed transporters in these tissues.

Structure

The structure of SLC transporters differs slightly from one subgroup to another. The SLC transporters consist of a number of transmembrane domains (TMD) and large intra- and extracellular loops. Since no crystal structures of the human SLC transporters have been published so far, the suggested struc-
tural configuration of the transporters is based on homology modelling using structurally similar template proteins\textsuperscript{31, 32}. The suggested three-dimensional structure of the transporters resembles a tube through the cell membrane with the TMD aligning to form a circle (Figure 3b). The binding site of the SLC transporters is thought to be located within the membrane, with specific TMDs forming the substrate binding cleft\textsuperscript{33, 34}. The large extracellular loops, present in some SLC transporters, contain consensus sites for N-glycosylation\textsuperscript{35}. Glycosylation at these sites is important for the regulation of transporter function and/or the trafficking of the transporter to the plasma membrane\textsuperscript{36}.

**Driving force**

In contrast to the homogeneous ATP driving force of the ABC transporters, the SLC transporters are driven by a number of processes. These include, but are not limited to, the co-transport of ions (e.g. $\text{H}^+$ and $\text{Na}^+$), facilitative transport (concentration dependent) and membrane potential\textsuperscript{31, 37, 38}. The driving force for some SLC transporters is still unknown\textsuperscript{39}. Given the known driving forces, in contrast to the primary active transport of the ABC, the SLC are largely driven by secondary active means (such as co-transport or membrane potential driven transport) or not energy dependent (facilitative transport). Despite the SLCs being generally considered to be uptake transporters, translocating compounds into the cell, some of them have been shown to transport compounds in both directions\textsuperscript{31, 40}.

![Figure 3. (a) The suggested general structure of OCT and OATP transporters, depicted by the human OCT1\textsuperscript{41}. The amino acids in dark grey indicate polymorphic sites leading to amino acid changes and deletions. (b) A simplified sketch showing the suggested tertiary structure of the OCT and OATP transporters, were the TMDs are aligned to form a tube through the cell membrane. This sketch depicts human OCT1.](image-url)
Active transporters in the liver

The transporters expressed in human liver play an important role in several drug related processes (Figure 4). Both the sinusoidal uptake and/or the canicular efflux transporters are involved in the transport of a large number of drugs and drug metabolites from the portal vein to the bile. Further, the bile acid transporters, OATP1B1, OATP1B3, NTCP, MRP2 and BSEP, are responsible for the final part of the enterohepatic recirculation of bile acids. The sinusoidal uptake transporters are also responsible for presenting many drugs to their respective metabolising enzyme in the hepatocytes and hence, determines the clearance of drugs with limited passive permeability.

Organic cation transporters (OCT; SLC22)

The group of human organic cation transporters consists of OCT1-3, OCTN1-2 and OCT6. The first member of the OCT family to be cloned was rat OCT1, and the first human OCT, OCT1, was simultaneously cloned by two groups in 1997. The proteins consist of 12 TMDs with one large glycosylated extracellular loop, between TMDs 1 and 2, and one large intracellular loop, between TMDs 6 and 7, (see the schematic in Figure 3b). The OCT transport is driven by concentration gradient and membrane potential, and is considered to be bidirectional. The OCTs display differing tissue distribution and are multispecific transporters with partly overlapping substrate patterns.

The OCT1 (SLC22A1) is significantly expressed in the sinusoidal membrane of the hepatocytes, and has a very low expression in other tissues (Table 2 and Figure 4). It is responsible for the uptake of drugs (such as metformin, imatinib and oxaliplatin) and of endogenous compounds (e.g., acetylcholine) from the portal vein into the hepatocytes. Studies of OCT1 function and inhibition often utilize different fluorescent (ASP+) or radiolabelled (TEA, MPP+ and metformin) substrates. OCT1 transport is considered to be of relevance for metformin uptake in the liver, and for imatinib and oxaliplatin uptake into cancer cells. OCT1 is a highly polymorphic protein (Figure 3a) with a number of variants affecting function in the human population. It has been suggested that these polymorphisms alter the access of metformin to the liver and, subsequently, reduce the glucose lowering effect.

In Paper III, the inhibition of OCT1 for 191 compounds, mainly drugs, was investigated using an in-house developed in vitro assay. The data obtained were used to identify properties governing OCT1 inhibition and to generate discriminant in silico models of OCT1 inhibition.

Organic anion transporting peptides (OATP; SLCO)

The human OATP transporter family consists of 11 proteins that are widely distributed throughout the human body (Table 2). In 1994 the first member of the OATP family, Oatp1a1, was cloned from rat liver and the first human member, OATP1A2, was cloned in 1995. Like the OCTs, the
OATPs consist of 12 TMDs with a suggested three-dimensional structure similar to that in the Figure 3b schematic. The OATPs mediate sodium-independent transport of a variety of structurally diverse compounds, including both drugs and endogenous compounds. Although the driving force of the OATPs has not been fully established, pH dependence has been suggested for the OATP2B1.

The OATP1B1 (SLCO1B1) was cloned in 1999 and is together with the OATP1B3 (SLCO1B3) the highest expressed and most important anion uptake transporters in the human liver. It is located to the sinusoidal membrane of the hepatocytes (Figure 4) and transports drugs (e.g. statins and rifampicin) and endogenous compounds (e.g. bile acids) from the portal vein into the hepatocytes. The OATP1B1 have been shown to be involved in clinically relevant interactions between statins and cyclosporin A and gemfibrozil. Estradiol-17β-glucuronide, estrone-3-sulphate and statins are often used as model substrates for the OATP1B1. OATP1B1 is a highly polymorphic transporter with variants displaying different function, leading to a lower statin-related effect and a higher risk of statin-induced myopathy.

Figure 4. The major drug interacting transporters expressed in human hepatocytes. The transporters can be subdivided into sinusoidal uptake (OATP1B1, OATP1B3, NTCP, OCT1 and OAT2) and efflux (MRP1 and 3) transporters as well as canicular efflux transporters (MDR, MDR3, MRP2 and BSEP).
In Paper V in this thesis, the properties governing OATP1B1 inhibition were studied. These data were used to develop an in silico model for prediction of OATP1B1 inhibition.

Table 2. The expression pattern of OCTs and OATPs in tissues throughout the human body. Distribution data was compiled from www.bioparadigms.org\textsuperscript{13}, Bleasby et al. 2006\textsuperscript{24} and Nishimura et al. 2005\textsuperscript{22}.

<table>
<thead>
<tr>
<th>OCT</th>
<th>SLC22</th>
<th>Strong expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT1</td>
<td>SLC22A1</td>
<td>Liver</td>
</tr>
<tr>
<td>OCT2</td>
<td>SLC22A2</td>
<td>Kidney</td>
</tr>
<tr>
<td>OCT3</td>
<td>SLC22A3</td>
<td>Ubiquitous, highest in skeletal muscle, prostate and salivary gland</td>
</tr>
<tr>
<td>OCTN1</td>
<td>SLC22A4</td>
<td>Ubiquitous, highest in trachea, kidney and bone marrow</td>
</tr>
<tr>
<td>OCTN2</td>
<td>SLC22A5</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>OCT6</td>
<td>SLC22A16</td>
<td>Testis and bone marrow</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OATP</th>
<th>SLCO</th>
<th>Strong expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1A2</td>
<td>SLC01A2</td>
<td>Brain and spinal cord</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>SLC01B1</td>
<td>Liver</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>SLC01B3</td>
<td>Liver</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>SLC02B1</td>
<td>Ubiquitous, highest in liver</td>
</tr>
<tr>
<td>OATP4A1</td>
<td>SLC04A1</td>
<td>Ubiquitous, highest in lung and placenta</td>
</tr>
<tr>
<td>OATP4C1</td>
<td>SLC04C1</td>
<td>Liver, kidney, blood cells</td>
</tr>
<tr>
<td>OATP1C1</td>
<td>SLC01C1</td>
<td>Brain and spinal cord</td>
</tr>
<tr>
<td>OATP3A1</td>
<td>SLC03A1</td>
<td>Ubiquitous, highest in testis</td>
</tr>
</tbody>
</table>

Genetic variation in transporters
Variations in genes coding for proteins are common within the human population and display different distribution patterns and frequencies in various subpopulations\textsuperscript{67,69,70}. These genomic variations can lead to unaltered (synonymous) or altered (non-synonymous) amino acid sequences in the proteome. Since only a small part (1.5%) of the human genome is comprised of regions coding for proteins\textsuperscript{71} most of the polymorphisms are located in non-coding regions of the genome. However, both synonymous polymorphisms within coding regions and polymorphisms in non-coding regions (e.g. promoter regions or introns) have been shown to alter the expression and function of transporters, despite not giving rise to any amino acid changes in the protein\textsuperscript{72-75}. The potential impact of polymorphism in transporters can be compared to the thoroughly investigated genetic variation in the genes coding for cytochrome P450 (CYP) enzymes, for which a vast number of clinically relevant drug-drug interactions have been identified\textsuperscript{69}.

In contrast to the CYPs, genetic variation in the genes coding for transporters has only recently started to attract the attention of the research community and, therefore, remain largely unexplored. Many transporters are highly polymorphic with a large number of non-synonymous mutations leading to amino acid changes/deletions. These amino acid alterations may affect membrane localization, function and capacity of the transporter\textsuperscript{41,76}. Polymorphisms in transporters have been shown to cause disease\textsuperscript{77,78} but they
may also be responsible for clinically relevant inter-individual differences in the response to drugs\textsuperscript{18, 48, 68, 79, 80}. The differences in amino acid sequence may also alter the ADMET properties of drugs and, consequently bring about clinically relevant drug-drug interactions\textsuperscript{81}. The importance of genetic polymorphism is indicated by the recommended genotyping of patients for some specific genetic non-transporter polymorphisms\textsuperscript{82}.

The impact of genetic variation on the substrate uptake patterns of different transporters has been investigated, but studies of the impact of transporter polymorphism on the inhibitory effect of drugs are scarce. With the intention of addressing this, the effect of genetic variation in OCT1 on drug inhibition was investigated in Paper IV.

Role of transporters in drug-drug interactions

The role of CYP enzymes in drug-drug interactions is well known\textsuperscript{83}. However, with many of the transporters being identified during the last 15 years, the research effort to identify their respective roles in drug-drug interactions have been compiled in data bases but are still relatively scattered\textsuperscript{84, 85}. Even so, a number of drug-drug interactions of significance at the transporter level have been described in the literature\textsuperscript{86, 87}. Furthermore, \textit{in vitro} evidence has suggested that transporters play a role in known drug-drug interactions\textsuperscript{88}. This implies that transporter-induced drug-drug interactions may play an important role for the ADMET properties of drugs and, consequently, the investigation of transporter drug-drug interactions are of increasing interest in the academic, industrial and regulatory research community\textsuperscript{6}. Since the transporters are widely distributed in the human body, there is a risk of drug-drug interactions at multiple sites and in different tissues. The drug-drug interactions are also difficult to foresee, since the transporters are often multispecific, accepting many substrates and even a larger number of inhibitors\textsuperscript{89-93}. In addition, the highly polymorphic nature of some transporters may increase the risk of drug-drug interactions\textsuperscript{81}.

Both the clinical effect and disposition of metformin are altered by genetic variation in the gene coding for OCT1\textsuperscript{47}, therefore the combined importance of OCT1 polymorphism and drug-drug interactions on the OCT1 mediated uptake of metformin were investigated in Paper IV.

Development and validation of \textit{in vitro} based experimental assays for studying transporters

The use of \textit{in vitro} methods in drug development has increased in recent years, reflecting the improvement in the quality and robustness of these \textit{in vitro} methods. To date, the only transporter for which the American regula-
tory authority FDA requires in vitro interaction testing is MDR1. However, action has been taken by the FDA to establish standard in vitro assays for a number of other transporters\textsuperscript{6}.

In vitro methods are based on tissues, part of tissues, cell lines or membrane vesicles being kept in an artificial physiological atmosphere to mimic the real physiological environment of the tissue in question. In vitro assays are used as models for specific tissues/organs or used to study specific cell mechanisms or proteins, and allow faster and less expensive data generation than in vivo methods. However, to ensure high quality and predictability, these assays need to be compared with and validated against their in vivo counterpart, e.g. Caco-2 cells as a model of the human intestine\textsuperscript{94-96}.

In this thesis, cell line based in vitro methods were used. The gene expression of a number of human cell lines, commonly used in in vitro assays, was investigated in Papers I and II. In Paper I, the expression of transporters in the Caco-2, HepG2 and Caki-1 cell lines was compared to that in the human jejunum, liver and kidney, respectively. Then, in Paper II, the endogenous transporter expression in common human cell lines was investigated. In Papers III-V new in vitro assays, allowing investigation of transporter interactions, were developed. The data obtained was used to identify transporter inhibitors, properties driving transporter inhibition and to generate predictive in silico models of transporter inhibition.

Gene and protein expression

The invention of the polymerase chain reaction (PCR) by Kary Mullis in 1984, for which he was awarded the Nobel prize in chemistry in 1993, allowed for easy, fast and cheap investigation of the human genome. Among the large number of techniques spawned from PCR, Real-Time PCR allows for rapid investigation of the gene expression of a large number of genes, relative to one or more reference genes. One of the major drawbacks with relative gene expression data is that the reference genes must be thoroughly validated to ensure that they are evenly expressed, and thereby suitable as reference genes in all samples investigated.

Further, although gene expression data measures the mRNA levels, which may give an indication of protein expression, the posttranscriptional regulatory mechanisms and variations in mRNA and protein stability may result in discrepancies between the gene and protein expression\textsuperscript{97, 98}. The drawbacks of gene expression have resulted in much more research being focused on proteomics, the study of proteins, in recent years. To facilitate this paradigm shift, new techniques allowing faster and simpler protein data generation are now becoming available\textsuperscript{99-101}. For instance, a large protein mapping project, the human proteome resource project (HPR), aims at mapping the majority of all human proteins in a large number of tissues, cancers and cell lines\textsuperscript{102}. 
So far, approximately 5000 human genes, corresponding to approximately 25% of the human genome, have been mapped in this project\textsuperscript{103}.

Gene expression of human tissues and cell lines was investigated in Papers I and II. In Paper II the gene and protein, using antibodies from the HPR, expression in six cell lines was compared.

\textit{In silico} prediction of transporters

The world around us is complex, so if we are to be able to understand and explain it we need to consider many different variables. Many problems in science, including interactions between a transporter and its substrates and/or inhibitors, are of a multivariate nature and therefore univariate statistical methods, which investigate simple correlations between two variables, will often not be sufficient to fully explain and/or solve these problems. The multivariate nature of the compound-transporter interaction is further indicated by the physicochemical heterogeneity often seen for the compounds interacting with human transporters. Therefore, \textit{in silico} based multivariate data analysis (MVDA) and structural modelling approaches are powerful and invaluable techniques which describe compound-transporter interactions. For human transporters, homology modelling\textsuperscript{31, 104}, pharmacophore models\textsuperscript{39, 52} and MVDA based models\textsuperscript{91, 92} have been described earlier. In this thesis MVDA modelling methodology were the main approach used for \textit{in silico} modelling of compound-transporter interactions. The obvious benefits of MVDA compared to traditional statistics have assigned it an important role in modern drug discovery and development. Further, once \textit{in silico} models have been developed, they allow fast and easy data generation without laboratory experiments.

Development of \textit{in silico} models

The development of a high quality predictive \textit{in silico} model using MVDA can be divided into a number of separate steps (Figure 5a). Each of these steps is important to assure high robustness, and to ensure the predictability and quality of the model.

Determining the purpose of the model

When developing \textit{in silico} models it is crucial to decide what to predict and what kind of information you want to be able to extract from the model (Figure 5a). This is important since the data used to generate the model determines the range and type of data that can be predicted with it. In this process it is also important to decide which type of assay and MVDA methodology to use.
Generating a data set

The importance of the data set generation process is often underestimated. However, it is the nature and quality of the compounds selected for model training that determines the quality and range of applicability of the analysed data and/or the models generated. In general, the model cannot be used to draw conclusions for properties outside the range of the data set used to train the model.

Two general approaches can be used when designing the data set (Figure 5b). In the first of these, a local data set is used to study a small subgroup within a larger population as shown by the grey area in Figure 5b. This data set includes only members of the subpopulation being studied, e.g., a library or series of homologous drugs, or a population with a specific genetic polymorphism. With this approach, the resulting local model will describe the subgroup in detail but it cannot be used for predictions in the larger population outside the subgroup. The second approach, which has the endpoint of studying and analysing a larger population, a more global data set, exemplified by the large outer circle in Figure 5b, has to be used. The global data set includes members that are evenly distributed throughout the whole population, e.g. a set of drugs covering the entire structural space of oral drugs. Thus, the resulting global model describes the whole population and allows for predictions to be made within the entire population. Since the global model has to describe a more diverse and often larger data set than a local one it will generally result in less specific predictions. Thus, the predictions of a global model will often be of lower quality than those of a local model.

Training and test set

Before generating a model it is crucial to divide the entire data set into a training set and a test set. Failure to do this will result in a model with unknown predictability and validity. Normally at least one third of the data set should be assigned to the test set. When assigning members to the training and test set, respectively, it is important to ensure that both sets cover the range of the whole data set adequately. This is done to ensure that the model can be applied in the range of the entire data set. The training set is used by the MVDA software to find correlations in the data and to generate and define the model. The test set, which will not have been involved in the model generation process, is used to validate the quality and predictability of the in silico model.
Figure 5. (a) A schematic diagram of the different steps of the model development process. (b) Two different modeling approaches. The small grey area depicts the small subpopulation used for the local model. In contrast, the entire population is used in the global modeling approach.

**Generating molecular descriptors**

A molecular descriptor is a parameter that describes a property related to the chemical structure of a compound in the data set, e.g. the molecular weight, lipophilicity or charge. The collection of descriptors constitutes the independent variables in the model \( (n=2-\infty \text{ for MVDA}) \) and should, therefore, be chosen on the basis of which information is to be correlated to the response variable. To date, there are a large number of commercially available or free software programs that can be used to generate molecular descriptors for drugs.

**Generation of experimental data**

It is crucial to generate experimental data of high quality. This is especially important when using the data for model development since the experimental data is used to fit the coefficients in the generated models. Poor experimental data will result in poor model performance or, even worse, models...
for which correlations are found by chance in the experimental data. Conversely, using high quality data will increase the chance of generating a model of high quality\textsuperscript{105}.

**Model development**

*In silico* models of protein interactions can be generated using various techniques. Pharmacophore models, describing the spatial arrangement of the structural features that determine the biological effect in a set of molecules, and molecular interaction fields, that describe the interaction between a molecule and its target, are examples of techniques that use the distances between features in the molecule to describe the interaction with the protein. In contrast, descriptor-based models use statistical regression techniques such as multiple linear regression (MLR), artificial neural networks and projections to latent structures by means of partial least squares (PLS) to relate the structure and physicochemical properties of the drugs (i.e., molecular descriptors) to the studied effect (e.g., inhibition of transport). In this thesis, PLS techniques implemented in the SIMCA-P+ (Umetrics, Umeå, Sweden) software package were used.

*Principal component analysis (PCA)*

PCA is a method using MVDA\textsuperscript{106} to find correlations, trends and outliers in a matrix (X) of data with N rows (observations) and K columns (variables). In structure-activity modeling, each observation typically corresponds to one of the drugs studied, but in other settings the observations could correspond to tissues, batches etc. Variables, in contrast describe the properties of the observations, molecular weight, lipophilicity, expression levels, etc. The PCA allows the identification of groups, trends and outliers in the data where compounds with similar properties are located close together\textsuperscript{107}.

In this thesis, PCA methodology was used to investigate grouping and positioning of tissues (Paper I) and compounds (Papers III and V) with regard to their transporter gene expression and physicochemical properties, respectively. Further, PCA was used in Papers III-V to ensure that the whole data set, training and test sets covered the structural space of oral drugs thoroughly.

*Projections to latent structures by means of partial least squares (PLS)*

PLS is a continuation of PCA that relates two data matrices to each other. The X matrix consists of variables describing the properties of the observations similar to those used in PCA modelling. In contrast to PCA, however, an additional matrix, the Y matrix, is introduced, which consists of one or more dependent variables (responses). Unlike MLR, PLS is an augmented linear regression method where the molecular descriptors are projected to a limited number of supervariables. This makes PLS useful for analyzing data with many, noisy and incomplete variables\textsuperscript{107, 108}.
Orthogonal PLS (OPLS) is an extension of PLS, where the molecular descriptor information related to the Y matrix is accumulated in predictive principal components. The remaining information is described by components orthogonal to the predictive component. OPLS is more transparent, and thereby easier to interpret, than PLS\textsuperscript{109}.

Discriminant analysis (DA) can be used if the measured data is qualitative, i.e., subdivided into different classes. Further, quantitative data can be transformed into qualitative data by introducing one or more cut-offs in the data range and thereby dividing the data set into different classes. This approach can be used to make in silico modelling possible when modelling with quantitative data fails.

In Papers III and V, OPLS and OPLS-DA were used to investigate the properties governing inhibition of the transporters OCT1 and OATP1B1. OPLS-DA was also utilised to generate predictive in silico models for these transporters.

Model generation process

In silico modelling using OPLS and OPLS-DA as described above is an iterative process. Initially, all variables in the X block are used to investigate relationships with the dependent variables, the Y block. However, the X block almost certainly will contain variables that do not contain information relevant to the problem (i.e., noise). These variables are removed from the model in a stepwise manner to optimize model performance. When removal of an additional X-variable results in poorer discrimination between inhibitors and non-inhibitors in the training set the model performance has been maximized.

Validation of the model

Proper model validation is important to ensure that the model developed is able to predict, correctly, an external data set that is not used in the model generation process. This external data set should cover the same range of molecular descriptors as the training set. The model is predictable and can be used if it correctly predicts a large part of the test set.
Aim of the thesis

The general objective of this thesis was to investigate the expression and distribution of drug transport proteins in human tissues and cell lines influencing in ADMET properties of drugs and to investigate inhibition patterns for liver specific uptake transport proteins.

The specific aims were:

- To investigate the gene expression of important drug transport proteins in human tissues (Paper I).
- To compare the expression of transport proteins, in the Caco-2, HepG2 and Caki-1 cell lines to human jejunum, liver and kidney, respectively (Paper I).
- To investigate and compare gene and protein expression patterns of drug transport proteins in human cell lines commonly used for in vitro studies of drug transport in drug discovery (Paper II).
- To develop in vitro methods and use these to study the inhibition pattern and identify properties governing inhibition of the highly expressed liver uptake transport proteins, OCT1 and OATP1B1 (Paper III and V).
- To develop in silico models for prediction of inhibition of the liver specific uptake transport proteins OCT1 and OATP1B1 (Paper III and V).
- To investigate the effect of common genetic variations in the OCT1 protein on the inhibition pattern and drug-drug interactions in vitro (Paper IV).
Methods

Data set selection
It is crucial to select the data set carefully to allow identification of drug properties important for transporter inhibition and to generate predictive in silico models for transporter inhibition.

The data sets included in this thesis were based on drugs and drug-like compounds to allow investigation drug-transporter interactions. Further, to allow investigation of the structurally diverse oral drug space, drugs from various therapeutic classes were included in the data sets. In addition, the data sets were compiled to cover a wide range of important physicochemical descriptors, e.g. molecular weight, lipophilicity, flexibility, polarity and charge. The data sets also incorporated compounds known to interact with the investigated transporter or suspected of so doing. This allowed the properties driving inhibition of transporters to be identified, and new inhibitors and groups of inhibitors to be identified.

When generating in silico models, two data sets are required, one for training, which is used for model development, and one for testing, used to validate the model. The large data sets investigated in this thesis were divided into training and test sets by listing the compounds in alphabetic order and then assigning every other compound to the training set and the remainder to the test set.

Experimental methods
Relative gene expression analysis
Quantitative PCR also known as real-time PCR (RT-PCR) was carried out using an ABI Prism 7900HT Sequence Detection System with custom designed 384-well cards loaded with Assay-on-Demand Gene Expression assays (Applied Biosystems, Foster City, CA). The cycling conditions were 2 minutes at 50°C, 10 minutes of polymerase activation at 95°C, and 40 cycles alternating at 95°C for 15 seconds and 60°C for 1 minute.

The amplification curves obtained were analyzed using SDS2.1 software (Applied Biosystems), setting baseline and threshold values for all samples, and the cycle time value, when the fluorescence is higher than a defined threshold level, was extracted for each sample.
Relative gene expression measures expression levels of the gene of interest relative to one or more endogenous reference genes. Using this methodology, it is crucial that the endogenous reference genes reflect all variables in the sample handling (e.g., loading variability, RNA integrity, primer and enzyme performance in the assay). Therefore, more than one of the endogenous reference genes present in all stages of the preparation and analysis procedure are often included. An Excel-based tool, BestKeeper, was used to determine the optimal endogenous reference genes for the comparison of all samples. For the relative quantification of the transporter genes in Paper I and II, a geometric mean was calculated for cyclophilin A and the major vault protein (MVP) and used as endogenous reference. Relative gene expression levels were calculated using $2^{-\Delta\Delta C_t}$ (Applied Biosystems, 1997).

Protein expression using immunohistochemistry

The transporter expression data was generated as a part of the large Human Proteome Resource Project. Briefly, two Protein Epitope Signature Tags (PrEST), consisting of a 50-150 amino acid sequence that is unique to the specific protein, were identified and expressed as recombinant proteins as described previously. Each PrEST was injected subcutaneously in New Zealand rabbits to produce an immune response. The resulting antibodies were affinity purified from serum by depletion of tag specific antibodies, followed by purification of monospecific antibodies using affinity columns loaded with the protein specific PrESTs. Quality assurance was performed by i) sequence verification of the PrEST clone ii) analysing the size of the resulting recombinant protein to assure that the correct antigen has been produced and purified iii) and checking the antibodies for cross-reactivity to PrESTs spotted on protein arrays. A thorough internal validation of the antibodies was performed.

When performing immunohistochemistry (IHC), high-throughput staining was achieved by ensuring that the cell lines were subcultured and agarose cell gels were prepared and used to produce tissue microarrays containing approximately 450 cells each. The cell microarrays were IHC stained in duplicates and the resulting images were annotated using an automated image-analysis application. The staining patterns used in Paper II were divided into five groups (labelled not representative, and negative, weak, moderate and strong expression) depending on the intensity of the staining and the number of cells stained.

Methods based on fluorescence detection

A fluorescent compound emits light of a specific wavelength (emission wavelength) when illuminated with light of a lower specific wavelength (excitation wavelength), e.g. the OCT1 substrate ASP has an excitation
wavelength of 475 nm and an emission wavelength of 605 nm. Fluorescence based methods are sensitive, selective and fast, and are therefore widely used as an integral part of a vast number of different methods and in many research fields. However, the use of fluorescence is limited by the fact that only a small fraction of all chemical compounds are fluorescent.

A number of fluorescence based techniques were used in the Papers of this thesis. Real-Time PCR, which uses fluorescent probes to track the gene amplification, was used for the gene expression studies. Further, confocal microscopy was used to visualize the uptake of the fluorescent OCT1 substrate ASP+ and a fluorescence plate reader was used to investigate the uptake of ASP+.

Transport assays based on radioactivity detection

Compounds labelled with radioactive tracer atoms (e.g. ³H and ¹⁴C) are widely used to track compounds, by measuring the decay of the radioactive atom, in in vitro experiments. The methods using radiolabelled compounds are selective and sensitive, but in general more expensive and time consuming than fluorescence based methods. Further, the use of radiolabelled compounds imposes safety restrictions and involves special procedures. However, more drugs are available with radioactive labels than for fluorescence studies, enabling the investigation of more compounds with the former.

Radiolabelled compounds (¹⁴C-lactic acid, ¹⁴C-metformin and ³H-estradiol-17β-glucuronide) were used for uptake and inhibition experiments in Papers II, IV and V respectively.

Investigation of OCT1 and OATP1B1 inhibition

The assays used for investigation of OCT1 and OATP1B1 inhibition were developed using the flow-chart in Figure 6.

HEK293-OCT1 and empty vector cells were seeded in black 96-well plates two days prior to the experiment and cultured at 37°C in 5% CO₂. On the day of the study, the cells were washed twice with 37°C PBS to remove the cell cultivation medium.

In Paper III and IV, the cell layers were incubated for five minutes in triplicates with solutions containing 1 μM of the OCT1 substrate ASP+ and the test compounds. Wash and incubation steps were carried out in a Freedom EVO200 liquid-handling station (Tecan, Männedorf, Switzerland). Since ASP+ exhibits negligible fluorescence outside the cells, no post-incubation wash was needed. The plates were analyzed in a Saphire² plate reader (Tecan, Männedorf, Switzerland), adjusted to read fluorescence inside the cells at the ASP+-specific excitation (475 nm) and emission wavelength (605 nm). All investigated compounds were analyzed at the ASP+-specific wavelengths to ensure that the intrinsic fluorescence of each compound did not
disturb the assay. Compounds showing more than 50% inhibition of ASP$^+$ uptake were defined as OCT1 inhibitors.

In Paper IV, an additional assay setup was used to study the uptake of $^{14}$C-metformin by OCT1. In contrast to the ASP$^+$ assay, the cells were washed five times after the incubation to remove extracellular and/or membrane bound substrate. Thereafter, trypsin was added to detach the cells from the well surface and the cells were lysed and neutralized. 100 μl of the cell solution was transferred to a new plate, supplemented with 150 μl scintillation cocktail (PerkinElmer, Waltham, MA) and analysed in a Topcount NXT (PerkinElmer, Waltham, MA). The scintillation data was normalized to the protein content of each well using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL).

In Paper V, HEK293-OATP1B1 and empty vector cells were seeded in 96-well plates two-three days hours prior to the experiment and cultured at 37°C in 5% CO$_2$. Before the experiment the cells were washed twice with 37°C HBSS to remove the cell culture medium. Triplicate samples were incubated with solutions containing the model substrate estradiol-17β-glucuronide (E17βG) and test compounds (20 μM) for five minutes. The incubation was terminated by adding 200 μl ice-cold HBSS after exactly five minutes and washing the wells additionally four times to remove extracellular and/or membrane-bound substrate. The wells were incubated with 50 μl trypsin solution for 30 minutes to detach the cells from the well surface and thereafter the cells were lysed for two hours using 200 μl 1 M NaOH per well. 100 μl of the lysed solutions was added to scintillation vials, neutralised with HCl and 3 ml of Ultima Gold scintillation cocktail (PerkinElmer, Shelton, CT) was added to the vial. The samples were analysed using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter, Fullerton, CA). The protein content of each well was measured using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL) to correct the scintillation data to the protein amount per well. A compound was defined as an OATP1B1 inhibitor if it showed more than 50% inhibition of the E17βG uptake.

Investigation of MCT1 function

In Paper II, the function of the ubiquitously expressed MCT1$^{26, 28}$ in six human cell lines was investigated using the MCT specific substrate $^{14}$C-lactic acid (15.3 μM), and the MCT1 inhibitor, quercetin (100 μM)$^{116, 117}$. MCT1 was defined as functional in the cell lines if incubation with quercetin led to a significant decrease in the lactic acid uptake.
Figure 6. A schematic picture of the assay development process. This is applicable for assays using both fluorescent and radiolabelled methods. All steps are essential for generating an assay of high quality. A large number of variables may be included in the optimization phase. Experimental design was used during the optimization phase.

Confocal microscopy

Confocal microscopy was used to visualise both the uptake of the fluorescent OCT1 substrate ASP\(^{+}\) and the inhibition of ASP\(^{+}\) uptake in Paper III. HEK293-OCT1 and empty vector cells were seeded on cover glasses 48 h before the experiment. The medium was removed, and the cells were washed once with PBS before being incubated for 5 minutes with 1 \(\mu\)M ASP\(^{+}\), with 1 \(\mu\)M ASP\(^{+}\) + 3 mM TEA (a known OCT1 inhibitor) or with HBSS only. After the incubation, fluorescence micrographs were obtained at ASP\(^{+}\)-specific wavelengths; these were analyzed with the help of Leica confocal software (Leica, Wetzlar, Germany).

In Paper IV, the GFP-tagged variants of OCT1 were used to visualise the localization of the transporter in the genetic variants expressed in HEK293 cells. The stably transfected cells were seeded on 12-mm poly-d-lysine-coated glass cover slips (BD Biosciences, Franklin Lakes, NJ) in 24-well plates. The cells were stained using the Image-IT LIVE labelling kit (Invi-
trogen, Carlsbad, CA) and fixed in 4% paraformaldehyde according to the manufacturer’s protocol. Cover slips were mounted in VECTASHIELD antifade solution (Vector Laboratories, Burlingame, CA) on glass microscope slides and visualized by confocal microscopy using a Zeiss 510 laser scanning microscope.

Generation of physicochemical descriptors
The SciFinder Scholar software (American Chemical Society, Columbus, Ohio) was used to generate 2D structural sdf files for the compounds in the data sets. Corina 3.0 (Molecular Networks, Erlangen, Germany) was used to convert the 2D to 3D structure files. The software SELMA (AstraZeneca R&D, Mölndal, Sweden), in Paper III, and DragonX 5.4 (Talete, Milano, Italy), in Papers IV and V, used these structural files to generate physicochemical descriptors used for the \textit{in silico} modelling.

Statistical analysis
Spearman rank order correlation coefficients were used in Paper I to determine the correlations of transporter gene expression between human tissues and corresponding cell lines and between human and rat tissues.

Two-sided t-test was used, in Papers II, III and IV to determine the significance levels between groups.

PCA methodology, as implemented in the SIMCA-P+ software (Umetrics, Umeå, Sweden) was used to visualise the structural coverage of the data sets in Papers III-V. PCA was further applied to investigate the relationship between the different tissues and cell lines investigated in Paper I.

In Papers III and V, \textit{in silico} model development was conducted by using the OPLS and OPLS-DA methods implemented in the SIMCA-P+ software (Umetrics, Umeå, Sweden). A discriminant analysis approach was used to generate the models, where compounds displaying more than 50% inhibition were defined as inhibitors and the remainder were labelled as non-inhibitors.

In Paper V, an experimental design approach was used for assay development. This methodology is based on MVDA techniques.
Results and discussion

Gene expression in human tissues

In Paper I, the gene expression of 36 transporters in human jejunum, kidney, liver and colon were investigated. The transporters were selected on the basis of their importance in drug transport over biological membranes, whereas the tissues were chosen due to their involvement in the ADMET properties of drugs.

The jejunum displayed high expression of peptide (HPT1 and PEPT1) and efflux (BCRP, MRP2 and MDR1) transporters (Figure 7a). The high expression of peptide transporters was not surprising since they are responsible for the uptake of peptides over the enterocytes\(^\text{37, 118}\). Further, peptide transporters play a role in the uptake of β-lactam antibiotics, angiotensin-converting enzyme inhibitors, and antiviral and anti-cancer agents\(^\text{118-120}\). The high expression of the jejunal efflux transporters had been expected as these are involved in protecting the human body from toxic exogenous compounds\(^\text{121}\). The colon displayed a gene expression pattern similar to that of the jejunum.

The gene expression in the liver was found to be dominated by the uptake transporters (OCT1, OATP1B1, NTCP and OATP1B3) and a number of efflux transporters (Figure 7b). These sinusoidal uptake transporters play an important role in the translocation of many drugs from the blood into the hepatocytes and in the metabolism and effect of some drugs by introducing alternative transport routes to that of passive diffusion\(^\text{42, 88}\). The four highest expressed efflux transporters (MRP2, MDR3, MDR1 and BSEP) were all located at the canicular membrane of the hepatocytes and are involved in drug transport into the bile\(^\text{42, 121}\). Sinusoidal and canicular transporters are believed to be involved in vectorial transport of drugs from the blood to the bile\(^\text{31, 122}\). Further, bile acids are vectorially transported into the bile via OATP1B1, OATP1B3, NTCP, MRP2 and BSEP\(^\text{43}\).

Organic anion transporters dominated the gene expression profile of the kidney, as has been described earlier in literature\(^\text{123}\) with OAT1, OAT3 and OAT4 among the four highest expressed transporters (Figure 7c). OAT1 and OAT3 were expressed at the basolateral membrane; they are responsible for the uptake of compounds from the blood into the renal tubuli cells. In contrast, OAT4 was expressed at the apical membrane and is responsible for the reabsorption of compounds from the renal tubuli. Further, MDR1 and MRP2
were highly expressed at the apical membrane of kidney cells where they are involved in transport from the blood to the proximal tubuli\textsuperscript{124}.

The MDR1 and MCT1 transporters exhibited significant levels of expression, ubiquitously in all tissues investigated, which suggests involvement in essential physiological processes. Such ubiquitous expression of MDR1 may be explained by its protective role in a number of tissues\textsuperscript{121}, whereas it has been suggested that MCT1 plays an important role in the processes of glycolysis and gluconeogenesis\textsuperscript{29}.

Animal studies, in particular, rodents are widely used as models for the in vivo situation\textsuperscript{125-127}. However, the scaling between animal data and the human situation may be problematic. Previously published transporter gene expression data for the rat ileum, liver and kidney\textsuperscript{128} was compared to the gene expression of the human jejunum, liver and kidney in Paper I. There was an obvious difference between the human and rat transporter expression patterns in all of the tissues investigated. These differences may, partly, explain frequently observed species differences between human and rat. Therefore, caution should be taken when using rat transporter data as a predictor of the importance of transporters in humans.

Figure 7. Relative gene expression levels of ABC (upper panel) and SLC (lower panel) transporters in the human jejunum (a), liver (b) and kidney (c) are plotted as dark bars on the right side of each graph. The gene expression in these tissues is compared to the gene expression in the tissue specific cell lines Caco-2 (a), HepG2 (b) and Caki-1 (c), light grey bars to the left. The bars represent the mean relative expression levels; error bars indicate the standard deviation from three to six samples analyzed in duplicate. * Absence of gene expression
Correlation between tissues and tissue specific cell lines

Cell lines are often used as in vitro models of human tissues\textsuperscript{129-131}, however, the quality of these in vitro models needs to be validated. Therefore, the correlations between the transporter expression in the jejunum, liver and kidney and Caco-2, HepG2 and Caki-1, respectively, were investigated in Paper I (Figure 7).

The Caco-2 cell line is used as an intestinal in vitro model to study the uptake process over the intestinal barrier\textsuperscript{94}. In Paper I, a more comprehensive study with 36 transporters showed that the gene expression of Caco-2 cells was in agreement with earlier investigations\textsuperscript{132, 133}. Further, the overall correlation between the gene expression of transporters in the human jejunum and Caco-2 was excellent (Figure 7a) as shown previously for smaller sets of transporters\textsuperscript{96, 134}.

The HepG2, derived from human liver, is used for its hepatocyte-like characteristics\textsuperscript{135, 136}. The gene expression of efflux transporters in HepG2 cells was similar to that of the hepatocytes, whereas the low expression of uptake transporters showed poor correlation to the human liver (Figure 7b).

The transporter expression in the kidney cell line Caki-1, used as an in vitro model for human proximal tubuli\textsuperscript{130}, was poorly correlated to the corresponding expression levels of the human kidney cells, for both efflux and uptake transporters (Figure 7c).

Thus, to summarise, the investigation in Paper I showed that the Caco-2 cells offer a suitable model for the human jejunum with regard to the expression of transporters. Further, the HepG2 and Caki-1 cell lines, derived from liver and kidney, respectively, were poorly correlated with their respective tissues of origin. Because of this, these two cell lines should not be used as in vitro models for liver and kidney transport.

Gene and protein expression and function in human cell lines

In Papers I and II, the gene expression profiles were investigated in eight human cell lines for 36 transporters. Further, in Paper II, monospecific antibodies were used to assess the protein expression for a subset of the transporters in six of the cell lines. The protein data was compared with the gene expression. In addition, the function of the ubiquitously expressed MCT1 was investigated and compared to the gene and protein expression.

The gene expression levels in the cell lines were generally low in comparison to the expression levels in the human tissues. The generally low endogenous transporter expression in HEK293, HeLa, K562 and Saos-2 supports the suitability of these cell lines for overexpression of transporters (Figure 8)\textsuperscript{137, 138}.
Figure 8. Relative gene expression of ABC (a) and SLC+HPT1 (b) transporters in HeLa, HEK293, HL-60, K562, Saos-2, Caco-2 and HepG2. The bars represent the mean relative expression levels for each transporter, coded as shown in the key.

The monospecific antibodies and protein expression images were generated, within the HPR, for six of the cell lines. The antibodies were validated both as a part of the HPR and through comparison of the IHC staining pattern with data published in the literature in Paper II. Antibodies for five transporters (MDR1, MRP1, MRP2, MCT1 and OATP1B3) passed the validation process and the protein expression displayed a surprisingly good correlation (67% overlap) to the gene expression data. In concert with the data in Paper I, the MCT1 gene and protein was ubiquitously expressed in all of the cell lines investigated. The MCT1 was also functional in most of the cell lines which further indicates the importance of MCT1 in essential physiological processes.

The HepG2 cell line exhibited significant expression of most ABC-transporters with high expression of MRP2 (Figure 7 and Figure 8). However, the IHC data in Figure 9 clearly demonstrate that MRPs are localized intracellularly, in contrast to MCT1, which is located in the cell membrane.
These results are in agreement with earlier studies where MRP2 remained intracellular in HEK293 cells due to normal short-term regulation\textsuperscript{77, 139}.

In conclusion, the gene expression of transporters was generally lower in human cell lines than in tissues. A reasonable correlation between the gene expression and protein expression was found, which suggests that gene expression data of transporters may be used to give an indication of the corresponding protein expression. Moreover, MCT1 was expressed and functional in most of the cell lines, further indicating its importance in physiological processes.

Figure 9. IHC staining patterns of the five transporters, with good validation scores, in HEK293 and Caco-2 cells. The arrows indicate intracellular staining in MRP1 and 2. Brown-black staining is antibody-specific, and the tissue section is counterstained with hematoxylin (blue staining) to enable visualization of microscopic features. The images were annotated using an automated image-analysis application. Staining levels: Red = strong, Orange = moderate, Yellow = weak and White = absent.

Development of cellular based \textit{in vitro} assays for transport

A thorough optimization and validation process is important when setting up new \textit{in vitro} assays. The assay development process needs to assure both good accuracy, which is the proximity of the measured or calculated value to the true one, and precision, which is the reproducibility of a number of measured or calculated values, of the assay. Since there are a large number of variables that may be important for the assay performance the assay development process can be a lengthy one.

MVDA based experimental design methodology, which identifies correlations and trends in the data, may be used in assay development. Experimental design allows the combined effect of two or more variables to be investigated and may, therefore, detect differences that ordinary development processes
overlook. Using this technique, the assay performance can be maximized and, at the same time the assay development process may be less time consuming.

Table 3. Examples of variables considered in the assay development in Paper V.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Investigated range</th>
<th>Assay settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of plates</td>
<td>Coated or non-coated</td>
<td>Coated</td>
</tr>
<tr>
<td>Cell passage influence</td>
<td>Different passages</td>
<td>No influence</td>
</tr>
<tr>
<td>Seeding density (cells/well)</td>
<td>30 000-70 000</td>
<td>30 000 or 60 000</td>
</tr>
<tr>
<td>Growth time</td>
<td>1-3 days</td>
<td>2 or 3 days</td>
</tr>
<tr>
<td>Substrate concentration (μCi)</td>
<td>0.5-2 μCi/ml</td>
<td>1 μCi/ml</td>
</tr>
<tr>
<td>Substrate concentration (μM)</td>
<td>0.5-1 μM</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>Inhibitor concentration</td>
<td>20 or 50 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>Incubation time</td>
<td>1-15 minutes</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Post-incubation wash</td>
<td>1-5</td>
<td>5</td>
</tr>
<tr>
<td>Inclusion of trypsin</td>
<td>Yes/No</td>
<td>Yes</td>
</tr>
<tr>
<td>Trypsinization time</td>
<td>5-30 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Protein measurement</td>
<td>Yes/No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The assays used in this thesis went through a thorough optimization and validation process. More than twenty variables were investigated in the assay set-up using both ordinary assay development, in Papers III-V, and experimental design, in Paper V. Examples of optimized variables are given in Table 3. A number of examples of the variables for which optimization has been conducted and the results that were obtained are given below.

- A well-characterized cell line is one of the foundations for a high quality *in vitro* assay. The cell lines used in this thesis were stably transfected to allow studies to be made of a specific transporter. Since background activity of the endogenous transporters could be a problem, the endogenous expression of transporters in cell lines was investigated in Paper II (Figure 8). In general, the HEK293 cells, used in this thesis for studying stable transfected proteins, showed a very low endogenous transporter expression.

- In this thesis, the cell lines used for uptake and inhibition studies were grown in 96-well plates. When using cell culture plates, it is important to determine how many cells to seed and the time in culture that allows the cells to form a monolayer. These variables were optimized during development of the assays (Table 3). Uneven cell growth between the inner and outer wells on a plate, or the edge effect, may cause variation in the samples, leading to a spread in the data obtained. Incubating the plates at room temperature for one hour before placing them at the correct growth temperature minimizes the edge effect and, thereby, the risk of data variation. Using this simple approach, the intra-plate variability was reduced in the assays.
• The apparent affinity ($K_m$) of the substrate should be known to allow selection of the substrate concentration used in the assay. In addition, $K_m$ and $V_{max}$ (the maximal transport velocity of the transporter) can be used to draw conclusions about the substrate kinetics and the function of the transporter. Because of this, Michaelis-Menten kinetics were used to determine $K_m$ and $V_{max}$ for all of the substrates investigated substrates in Papers III-V, e.g., Figure 10a. Consequently, the $K_m$ was partly used to determine the substrate concentration of the assays.

• Since active uptake/efflux is a relatively fast and saturable process, the incubation time should be at the linear part of the uptake curve to avoid saturation of the transporter. Time-dependent uptake, using the selected concentration of the substrates, was investigated until a clear saturation of the transporter was observed. Subsequently, the incubation time was chosen well within the linear part of the uptake curve.

• In the fluorescent inhibition assays used in Papers III-IV, a pipetting robot was used for all the washing and incubation steps. Using this equipment, the assay reproducibility was increased and the assay became less time consuming.

• Post-incubation treatment is also important for the assay performance. As an example, the washing procedure is different for different types of assay used. No washing step was required in the fluorescent assay used in Papers III and IV owing to the negligible fluorescence of ASP$^+$ in the extracellular medium, shown in Figure 10b. In contrast, five washes were required in the radioactivity based assays to remove all labelled compound from the outside of the cell and in the cell membrane, Table 3.

• Despite the thorough control of the number of seeded cells, uneven cell growth or other assay handling processes, e.g., post-incubation wash, may lead to inconsistency in the cell amount. This may affect the results of assays using the entire cell amount or fractions thereof. Therefore, the amount of protein in each well was measured and subsequently used to correct the compound uptake in Papers II, IV and V.

Structural diversity of the data sets

The applicability range of \textit{in silico} models is determined by the structural diversity of the data sets used to develop each model. The two large data sets in Papers III and V and the smaller one in Paper IV were selected to cover the structural space of oral drugs. A comparison with a data set including all oral drugs registered in Sweden showed that these data sets covered the structural space of oral drugs satisfactorily. Further, the data sets in Papers III and V were broad for a number of important compound properties, e.g. the molecular weight, lipophilicity and charge. The training and test sets in Papers III and V also covered the oral drug space, which allows the models
to be predictive throughout the entire structural space investigated and the test set to be suitable for validation purposes. The training and test sets were shown to display a similar distribution of inhibitors and non-inhibitors as found in the whole data sets.

Figure 10. (a) Kinetic characterization of ASP\textsuperscript{\textsuperscript{+}} uptake in HEK-OCT1 plotted according to the Michaelis-Menten equation, which allows the determination of apparent $K_m$ and $V_{\text{max}}$ values. (b) Fluorescence micrographs of HEK293-OCT1 cells incubated with 1 μM ASP\textsuperscript{+} (upper left) and 1 μM ASP\textsuperscript{+} + 3 mM of the OCT1 inhibitor TEA (upper right). HEK293-Vector transfected cells incubated with 1 μM ASP\textsuperscript{+} (lower panel).

Table 4. Summarized results from the inhibitory investigations of the liver specific uptake transporters OCT1 and OATP1B1.

<table>
<thead>
<tr>
<th></th>
<th>OCT1</th>
<th>OATP1B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of compounds investigated</td>
<td>191</td>
<td>135</td>
</tr>
<tr>
<td>Number of identified inhibitors</td>
<td>62</td>
<td>53</td>
</tr>
<tr>
<td>Properties governing inhibition</td>
<td>Positive charge, High lipophilicity, High hydrophobicity</td>
<td>Negative charge, Large size</td>
</tr>
<tr>
<td>Groups of compounds with inhibitory potential</td>
<td>Tricyclic antidepressants, Antipsychotic and antidepressive drugs, Antihistamines, Steroids, $\alpha$-receptor blocking agonists</td>
<td>Statins, Protease inhibitors, Bile acids</td>
</tr>
</tbody>
</table>
Inhibitors and inhibitor properties of OCT1 and OATP1B1

In Papers III and V the liver specific uptake transporters OCT1\(^{45, 46}\) and OATP1B1\(^{15}\) were investigated. The reason for this was to identify new inhibitors of and properties governing the inhibition of these transporters. With this aim, the inhibition potential of a large number of compounds was investigated at a fixed concentration (100 \(\mu\)M for OCT1 and 20 \(\mu\)M for OATP1B1).

For human OCT1, the inhibitory potential of 191 compounds, thoroughly covering the entire structural space of oral drugs, was investigated (Figure 11). This resulted in the identification of 62 inhibitors (32\%), including 47 new ones (Table 4). Tricyclic antidepressants, and other drugs used for the treatment of psychosis and depression that exercise their antidepressant actions by inhibiting different neurotransmitter receptors\(^{141, 142}\), showed enrichment in OCT1 inhibitors. In addition, neurotransmitters are substrates of OCTs which suggests a possible role for OCT1 in neurotransmitter uptake and drug-neurotransmitter interactions in the brain\(^{24, 143-145}\). Using the inhibition data and the descriptors in the \textit{in silico} models, physicochemical properties important for OCT1 inhibition were identified. In concordance with the literature, a positive net charge, seen for 66\% of the inhibitors, was important for OCT1 inhibition\(^{31, 146}\). Other important features governing the inhibition of OCT1 were hydrophobicity and lipophilicity. In contrast, high polarity and the existence of many H-bond donor and acceptor moieties were high among compounds not inhibiting OCT1.

The data set investigated for inhibition of OATP1B1 consisted of 135 compounds that covered the oral drug space. Among these compounds, 53 were being classified as inhibitors (39\%), with 28 of these not having been reported previously (Table 4). For the OATP1B1, a clear enrichment of inhibitors was seen among HMG-CoA inhibitors (statins), protease inhibitors and bile acids. Several drugs, including statins, had already been identified as OATP1B1 substrates\(^{63}\). The drug-drug interactions at OATP1B1 that had been described earlier further indicate that OATP1B1 has a potential role in drug-drug interactions\(^{61, 63, 147}\). In addition, the inhibitory overlap already suggested between OATP1B1 and MRP2\(^{148}\) was not obvious in our study, with 48\% of the investigated MRP2 inhibitors being OATP1B1 inhibitors. The experimental and \textit{in silico} data was used to identify important properties for OATP1B1 inhibition. In concert with the literature\(^{149, 150}\), most of the inhibitors (62\%) carried a negative charge at pH 7.4. A large molecular weight and high polarizability were further important properties governing OATP1B1 inhibition.

In summary, a large number of new inhibitors for the OCT1 and OATP1B1 transporters were identified in this thesis. In general, antidepressant drugs inhibit OCT1, whereas statins, protease inhibitors and bile acids
inhibit OATP1B1. In addition, the physicochemical properties driving OCT1 and OATP1B1 inhibition have also been identified.

Figure 11. Overview of the results from the screening of OCT1 inhibition. Each bar represents one point in the data set (n = 191). At least 50% inhibition (dashed line) was required for a drug to be classified as an inhibitor.

**In silico models of liver uptake transporters**

In Papers III and V, MVDA was used to generate *in silico* models predicting inhibition of the human liver transporters OCT1 and OATP1B1. The models were generated using data sets that were custom-made to ensure that the entire structural oral drug space for inhibition of the specific transporters was investigated. After generating the experimental data, OPLS-DA was used to develop and validate the models.

The OPLS-DA approach was used to generate two models of high quality for the OCT1. An easily interpretable model, which correctly predicted 75% of the inhibitors and 78% of the non-inhibitors in the test set, was generated to ensure transparency. The easily interpretable model was based on only three molecular descriptors (the nonpolar count/MW, ClogP and positive ionizability), which all were positive correlated to OCT1 inhibition. This suggests that the presence of nonpolar moieties, high lipophilicity and positive charge of a compound governs the OCT1 inhibition, which was supported by the previously reported properties of OCT1 inhibitors. Further, a model that incorporated more molecular descriptors was developed to maximize the predictive performance. This model, correctly predicted 82 and 88% of the inhibitors and non-inhibitors in the test set, respectively,
(Figure 12a). However, the increase of molecular descriptors used to generate the model (ten), resulted in a less transparent model. The ten descriptors generally defined hydrophobicity and lipophilicity as important for OCT1 inhibition, while hydrogen bonding capacity and negative ionizability were negatively correlated to OCT1 inhibition.

An OATP1B1 model based on six descriptors and composed of one principal component was developed using OPLS-DA. The final model included size (the molecular weight, randic connectivity index and variation) and polarizability descriptors (the sum of atomic polarizabilities, molar refractivity and polarity number) all of which were positively correlated to OATP1B1 inhibition. The model correctly predicted 81 and 90% of the inhibitors and non-inhibitors in the training set, respectively, and when challenged with a test set, successfully sorted 77 and 83% of the inhibitors and non-inhibitors, respectively (Figure 12b).

These studies have shown that MVDA can be used to generate high quality in silico models describing transporter inhibition. This strategy has been used successfully previously for a number of ABC transporters. The MVDA models can be used for simple and fast prediction of the inhibitory potential of a drug or a candidate drug in both academia and the pharmaceutical industry.

Recently, the first crystal structure for a mammalian transporter has been described. However, the lack of crystal structures for human uptake transporters and the poor performance of homology models based on bacterial transporters further strengthen the importance of pharmacophore and MVDA modelling. Pharmacophores, describing OCT1 inhibition and OATP1B1 transport, have been generated previously for small data sets, and attempts were made to use pharmacophore modelling to identify inhibitor pharmacophores for OCT1 and OATP1B1. However, this approach failed for both transporters in this thesis, probably because of the large heterogeneous data set covering the entire oral drug space and multispecific inhibitory pattern. In addition, a quantitative PLS (using the inhibition percent and the \( K_i \)-values respectively) was attempted, where the percentage inhibition or \( K_i \)-values would have been the output values instead of the discriminate inhibitor or non-inhibitor data. However, this approached resulted in models with poor performance, probably because of the rather narrow span of \( K_i \)-values.
The effect of genetic variation in the OCT1 protein

In Paper IV, the inhibitory patterns of common genetic variants of the highly polymorphic transporter OCT1 (R81C, M408V, M420del and G465R) were investigated. Single point inhibition measurements at 50 μM and inhibition curves with a large concentration range, for compounds acting as substrates, inhibitors or not interacting with the OCT1, were generated using the fluorescent model substrate ASP+ and the anti-diabetic drug metformin.

The inhibition and substrate interaction patterns for the OCT1-variants, from Paper IV and the literature, were shown to be compound specific\textsuperscript{41, 47}. This suggests that the compounds interact with different parts of the OCT1 substrate binding cleft\textsuperscript{34, 152}. In the case of inhibition, different types of compound inhibition types (competitive, non-competitive and uncompetitive) may result in specific inhibition patterns. The heterogeneity of the interaction patterns indicates that it is risky to draw general conclusions based on single substrate and/or inhibitor data.
When compared with the OCT1-reference, the inhibition of ASP\(^+\) uptake was clearly more pronounced in OCT1 variants with reduced function. This trend was obvious both for the single-point measurements and the inhibition curves with stronger inhibition and lower IC\(_{50}\) values, respectively, in variants with reduced function. Differences in membrane localisation of the protein or structural alterations in the postulated large substrate binding cleft\(^{34, 47}\) may explain the more pronounced inhibition in OCT1 variants. The lower drug concentrations needed to inhibit OCT1 variants with reduced function may increase the risk of clinically relevant drug-drug interactions in individuals carrying these variants.

The glucose lowering effect of the anti-diabetic drug metformin, which is an OCT1 substrate\(^{153}\), had been investigated earlier in individuals carrying different OCT1 variants\(^{47, 154}\). These studies produced contradictory data with respect to the effect of the genetic variation in OCT1, on the glucose lowering effect of metformin. However, the combination of the increased inhibition potency shown in Paper IV and the lower uptake of metformin in variants with reduced function may increase the risk of drug-drug interactions occurring at the OCT1, thereby altering the effect of metformin. Therefore, the inhibitory effect on metformin uptake attributable to drugs being concomitantly administrated with metformin was investigated in OCT1-reference and M420del (Table 5). The calcium channel blocker verapamil, used for treatment of hypertension and cardiac arrhythmia, is used by approximately five percent of the patients on metformin. Verapamil, identified as a strong OCT1 inhibitor in Paper III, inhibited metformin with an IC\(_{50}\) value of 1.75 \(\mu\)M in OCT1-reference and 0.21 \(\mu\)M in OCT1-M420del. The latter was below the reported C\(_{\text{max}}\) (0.60 \(\mu\)M) for OCT1-M20del, which suggests an increased risk for drug-drug interactions in patients carrying OCT1 variants with reduced function, especially when metformin and verapamil are coadministered (Table 5).

Table 5. IC\(_{50}\) values and the IC\(_{50}\) ratios for the OCT1-reference and M420del.

<table>
<thead>
<tr>
<th></th>
<th>Frequency (%)</th>
<th>Reported C(_{\text{max}}) ((\mu)M))</th>
<th>Predicted C(_{\text{max, portal}}) ((\mu)M))</th>
<th>Reference IC(_{50}) ((\mu)M)</th>
<th>M420del IC(_{50}) ((\mu)M)</th>
<th>Ratio Ref/M420del</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>3.2</td>
<td>0.72</td>
<td>33.0</td>
<td>11.3 (±1.66)</td>
<td>12.6 (±1.69)**</td>
<td>0.90</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>37.0</td>
<td>0.73</td>
<td>1.41</td>
<td>199 (±1.47)</td>
<td>85.8 (±1.97)**</td>
<td>2.32</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>21.3</td>
<td>4.49</td>
<td>12.3</td>
<td>185 (±1.46)</td>
<td>178 (±2.62)*</td>
<td>1.04</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>26.7</td>
<td>0.13</td>
<td>9.03</td>
<td>89.0 (±1.25)</td>
<td>26.5 (±1.87)**</td>
<td>3.36</td>
</tr>
<tr>
<td>Verapamil</td>
<td>4.9</td>
<td>0.60</td>
<td>15.05</td>
<td>1.75 (±1.62)</td>
<td>0.21 (±0.62)</td>
<td>8.33</td>
</tr>
</tbody>
</table>

1. The treatment frequency of the drugs were derived from 11319 US patients treated with the type 2 diabetes drug metformin.
2. The reported total C\(_{\text{max}}\) obtained from Goodman and Gillman’s and Clarke’s isolation and identification of drugs.
3. Predicted portal vein concentration as described by Its et al. 1998
4. IC\(_{50}\) ratios between OCT1-reference and M420del
Conclusions

In this thesis, the expression patterns of 36 transport proteins, that may influence the ADMET-properties of drugs, in the colon, liver, kidney and eight widely used human cell lines was investigated. Both ubiquitous and specifically expressed transport proteins were identified in these samples. Further, with the intention of studying the limitations of gene expression data, this was compared to protein data. *In vitro* assays for the liver specific uptake transport proteins OCT1 and OATP1B1 were developed to allow these transport proteins to be investigated. These assays were used to study the inhibition patterns of OCT1 and OATP1B1 for two custom-made data sets for each of the respective transport proteins. This resulted in the identification of a large number of new inhibitors. Further, the inhibition data was used to develop predictive *in silico* models for OCT1 and OATP1B1 inhibition. The influence of genetic variation in the gene coding for OCT1 on inhibition patterns was investigated. In general, OCT1 variants with reduced function were more susceptible to drug inhibition than variants displaying normal function.

The specific results and conclusions were:

- Tissue specific and ubiquitously expressed transport proteins in jejunum, colon, liver, kidney and eight cell lines were identified.

- The Caco-2 cell line correlated to the human jejunum with regard to the gene expression of transport proteins. However, the transport protein gene expression of the HepG2 and Caki-1 cell lines did not correspond to the expression in the human liver and kidney respectively.

- Antibodies with a good validation score resulted in a reasonable correlation between gene and protein expression for the investigated cell lines.

- MCT1 was ubiquitously expressed in all tissues and cell lines investigated. It was also functional in several of the cell lines studied.

- *In vitro* assays for the OCT1 and OATP1B1 transport proteins were developed.
- A large number of new inhibitors of the liver specific transport protein OCT1 (n=62) and OATP1B1 (n=53) were identified. Further, properties governing inhibition of these transport proteins were identified.

- Predictive *in silico* models for OCT1 and OATP1B1 inhibition were developed. These models are useful in predicting drug-transporter and drug-drug interactions at an early stage of the drug development process.

- Genetic OCT1 variants with decreased function were found to be more susceptible to inhibition than OCT1 with normal function. Using *in vitro* methodology, a drug-drug interaction between metformin and verapamil at the OCT1 was proposed. The potency of this interaction was further increased in genetic OCT1 variants with reduced function.
The research described in this thesis has expanded the knowledge of transport protein distribution in the human body and human cell lines. It has also provided new \textit{in vitro} assays and \textit{in silico} models for human transport proteins.

A large number of transport proteins (approximately 400) have been identified and it is therefore important to focus the research on those influencing the ADMET properties of drugs. In the next stage, these investigations should focus on transport protein expression in diseased tissues since regulation of transport proteins frequently occurs in various disease states. Further, much is still unknown about the substrate and inhibitor specificity and the binding sites of most transport proteins. One important step towards decoding the structure of transport protein binding sites, and thereby its interaction specificities was taken recently when the first crystal structure of mouse P-gp was published\cite{151}. However, crystal structures of human transport proteins are required urgently to advance the transport protein field. Nonetheless, further refinement of pharmacophore and MVDA \textit{in silico} models will also contribute to understanding transport protein interaction and the properties of interacting compounds. The findings of this thesis will hopefully stimulate the use and development of new predictive transport-interaction models.

If the investigations of OCT1 and OATP1B1 inhibitors in Papers III and V are combined with similar studies for other liver transport proteins, a drug inhibition map for hepatocyte uptake and efflux can be assembled. Such a map could be used to identify “transporter fingerprints” or biomarkers for liver transport proteins. This information would allow early prediction of drug-transporter interaction patterns. In addition, the “transporter fingerprints” could be expanded to include enzyme interactions and thereby obtain a more complete picture of drug interactions in the liver. Thus, a challenge for the future is to investigate the combined effects of transport and metabolism for ADMET properties of drugs in the liver. For such models to be useful, physiologically based cell kinetic studies will be required.

Clinical studies of drug interactions usually involve two drugs e.g., one substrate and one inhibitor. However, many patients are taking many more than just a couple of drugs and the study of such clinically relevant drug combinations would probably be unethical to study in health subjects. In contrast, clinically relevant polypharmacy can easily be studied \textit{in vitro}. Thus, an extension of the studies in this thesis would be to include combina-
tions of all clinically relevant drugs in certain disease states *in vitro*. In addition, *in vitro* consideration of clinically relevant transport protein polymorphisms may also contribute to refining the predictions of drug-drug interactions and, subsequently, promote advances towards personalized health care.

Most of the issues addressed above will increase the demand for further development of *in vitro* assays and *in silico* models. With drug discovery and development costs rising in the pharmaceutical industry, and increasing demands being imposed by the regulatory communities, use of more predictive *in vitro* and *in silico* methods will probably ensure lower attrition rates and costs. These methods will, hopefully, contribute to more efficient drug development. It is my conviction that improved prediction in the ADMET field will require the consideration of transport proteins as indicated by the results of this thesis.
Svensk populärvetenskaplig sammanfattning

Ett läkemedel måste, för att vara verksamt, kunna transporterat till den del av kroppen och till det organ där det skall utöva sin effekt. För att nå denna plats måste ett antal barriärer bl.a. i tarmen och levern samt i målcellerna passeras. Läkemedel måste även passera att antal barriärer, framför allt i levern och njurarna, för att ta sig ut ur kroppen. Transporten genom dessa barriärer kan ske på olika sätt (Figure 1). Ett viktigt transportsätt är via transportproteiner eller transportörer (nr 4 och 5, Figure 1) vilka bildar kaneler genom de olika barriärerna. Det är genom dessa kanaler som många näringsämnen, vitaminer etc. som kroppen behöver transporteras. Hittills har flera hundra olika mänskliga transportörer identifierats. Dessa finns i varierande mängd i olika organ och förflyttar en stor mängd olika ämnen.

I första delen av min avhandling (artikel I och II) undersökte jag hur mycket av läkemedelstransportörer som finns i människans tarmar, lever och njure. Dessa organ studerades därför att de spelar en viktig roll vid ett läkemedels upptag i och utsöndring ur kroppen. Jag har också undersökt vilka av dessa transportörer som finns i vissa cancerceller, vilka har en omfattande användning som modeller för olika organ och kroppsfunktioner. Genom att använda cancerceller vid läkemedelsforskning kan man minska behovet av djurförsök, vilket är fördelaktigt ur både etisk och ekonomisk synvinkel.

I andra delen av min avhandling (artikel III och V) har jag testat en rad olika läkemedels förmåga att minska eller blockera funktionen hos två viktiga transportörer i levern. Det är viktig kunskap eftersom kännetecknom hur ett läkemedel påverkar dessa transportörer har betydelse för ett läkemedels effekt och eventuella biverkningar. Ett läkemedel som minskar eller blockerar en transportörs funktion kan leda till att läkemedlet får sämre effekt, helt saknar effekt eller kanske ännu värre får ökad effekt vilket kan ge allvarliga biverkningar. Jag hittade många läkemedel som blockerar de två transportörerna jag undersökte och dessa resultat användes även för att utveckla datormodeller för transportörerna. Dessa datormodeller kan användas för att undersöka om och i så fall hur ett läkemedel påverkar transportörerna i kroppen och är mycket användbara då de är billiga och snabba.

Den tredje delen (artikel IV) har jag testat hur skillnader i transportörerna mellan olika människor påverkar transportörernas funktion. Vidare undersöktes hur dessa skillnader i en viktig levertransportör påverkar läkemedels förmåga att blockera dess funktion. Det visade sig att transportörer med lägre funktion effektivare blockeras så att funktionen hos transportören blir
ännu sämre, vilket kan leda till att en grupp av människor påverkas annorlunda av visst läkemedel.
Sammanfattningsvis har jag utvecklat laborativa och datorbaserade metoder för att studera och förutsäga hur läkemedel påverkas av transportörer. Dessa metoder kan användas innan ett läkemedel testas i djur och människor och därmed minska behovet av djur- och människoförsök.
Acknowledgements

The studies were carried out at the Department of Pharmacy, Faculty of Pharmacy, Uppsala University, Sweden.

I gratefully acknowledge the financial support from AstraZeneca, Swedish Research Council (Grant 9478), the Knut and Alice Wallenberg Foundation, the Swedish Foundation for Strategic Research, the Swedish Fund for Research Without Animal Experiments and the Animal Welfare Agency. My participation in research courses and conferences was made possible by travel scholarships from Uddeholms stipendiestiftelse, IF:s stiftelse, Leffmans resestipendium, Rektors resebidrag från Wallenbergsstiftelsen and AAPS travelship.

I wish to express my sincere gratitude to all of you who, in some way, contributed to this thesis:

Mina handledare: Professor Per Artursson, för att du är en visionär med ständigt nya idéer och infallsvinklar, även om detta ofta leder till jobb för mig. Jag slutar aldrig att förundras hur mycket du kan.  
Doktor Christel Bergström, för din hjälp och ditt stöd i tid och otid och för att du alltid har tid att diskutera forskning och färgkodning med mig.  
Doktor Johan Karlsson, för all hjälp genom åren och för att ha visat mig hur forskarlivet ter sig utanför akademin.

Professor Göran Alderborn och professor Martin Malmsten för tillhandahål-lande av trevliga lokaler samt en mycket god arbetsmiljö.

My co-authors, you are quite a few: Professor Kathleen M. Giacomini, Professor Mathias Uhlén, Professor Rolf Larsson, Professor Ulf Norinder, Assoc. Professor Anna-Lena Ungell, Dr. Constanze Hilgendorf, Dr. Pär Matsson, Dr. Maria Karlgren, Dr. Lena Gustavsson, Dr. Cristina Al-Khalili Szigyarto, Dr. Alexandra Ianculescu, Dr. Robert Davis, Jenny Pedersen, Annick Seithel and Ying Chen.

Befintliga, gamla och nya medlemmar i cell-, transport- och prediktionsgruppen för en mycket trevlig forsknings- och arbetsmiljö. Ett särskilt stort och hjärtligt tack till Dr. Lucia Lazorova för all hjälp, och det är inte lite,
med apparater, metoder, beställningar m.m. under åren. Du är en klippa! Dr. Pär Matsson som trots mitt tjat, tålmodigt lyssnade och svarade på mina ibland ogenomtänkta frågor. Jenny Pedersen och Dr. Maria Karlgren för alla diskussioner kring transportörer och annat.

My students Jenny Pedersen, Zhaosha Li, Anne Filppula and Sina Saidiakbarzadeh for all excellent work and the contribution to my research.


Eva Nises Ahlgren, Ulla Wästberg-Galik, Harriet Östlund, Christin Magnusson, Göran Ocklind, Eva Lide Johansson, Birgitta Rylén och Lotta Wahlberg för all hjälp med diverse saker.

Alla medarbetare på institutionen för farmaci, särskilt alla doktorander, för att ni skapar en trevlig stämning och gör det en fröjd att komma till jobbet. Ett särskilt tack till Peter som villigt delat med sig av sin minst sagt brokiga filmsmak.

Mina vänner i världen utanför för alla fantastiska rallyn, spelade golfrundor och annat skoj.

Britt-Marie, Sven och Johan för alla trevliga stunder tillsammans.

Mina systrar, Elin och Karin, för allt kul vi har haft genom åren. Storebror till trots, två är alltid fler än en.

Min älskade mamma och pappa för att ni alltid har funnits där och för att ni alltid stöttar och uppmuntrat mig.

Hanna, för att du betyder allt för mig. Jag älskar dig!


57. Hirano, M.; Maeda, K.; Shitara, Y.; Sugiyama, Y. Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *J Pharmacol Exp Ther* 2004, 311, 139-46.


82. FDA. http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 104

Editor: The Dean of the Faculty of Pharmacy

A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)