Chapter 3
In Vitro Characterization of Interactions with Drug Transporting Proteins

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Abstract In vivo, drugs that are substrates of transport proteins often interact with more than one transporter and may also be substrates of drug metabolizing enzymes. This complexity makes in vivo observations of potential transporter interactions difficult to interpret, and, as a result of this, most transporter interactions have been identified using controlled conditions in in vitro models. In this chapter, we review in vitro characterization of DDIs with transport proteins and, more specifically, for transporters that have been shown to have significant clinical effects. The focus will be on interactions taking place at the organ barriers known to influence pharmacokinetics in man. We will cover in vitro models used, methods for predicting DDIs with transport proteins, and the substrates and inhibitors recommended for use in such studies. We also exemplify how in vitro studies have been used to identify, predict, or explain transporter-mediated DDIs and comment upon how recent findings, e.g., quantitative proteomics, improve the in vitro predictions of the interactions.

Abbreviations

ABC	ATP-binding cassette
AMP	Adenosine monophosphate
AR	Absorption ratio
ASP+	4-(4-(Dimethylamino)styryl)-*N*-methylpyridinium

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ATP	Adenosine triphosphate
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
C_{max}	The maximum plasma concentration of the drug
CNS	Central nervous system
DDI	Drug–drug interaction
ER	Efflux ratio
F_a	Fraction absorbed
F_u	Fraction unbound
HEK293	Human embryonic kidney 293 cells
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
$I_{in,max}$	The maximum inhibitor concentration at the inlet of the liver
I_{max}	The maximum systemic plasma concentration of the inhibitor
k_a	Absorption constant
MATE	Multidrug and toxin extrusion
MDCK	Madin–Darby canine kidney
MRP	Multidrug resistance-associated protein
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
P_{dif}	Passive diffusion constant
PEPT	Peptide transporter
Pgp	P-glycoprotein
Q_h	Hepatic blood flow
SLC	Solute carrier
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry

3.1 Introduction

In total there are approximately 400 membrane transporters. More than 30 of these, most of which belong to the solute carrier (SLC) and ATP-binding cassette (ABC) protein superfamilies, have been covered in reviews on membrane transporters and drug disposition. So far, approximately a dozen of these are known to significantly influence the pharmacokinetics of drugs by altering the drug disposition through transport across the epithelial and endothelial cell barriers in the liver, kidney, intestine, and blood–brain barrier (BBB) (Zolk and Fromm 2011; Endres et al. 2006; Shitara et al. 2006). Both absorptive and exsorptive transporters have these effects. These include the liver-specific organic anion polypeptide transporters OATP1B1 and OATP1B3, the organic anion transporters OAT1 and OAT3 in the kidney, the

organic cation transporters OCT1 (in the liver) and OCT2 (in the kidney), multidrug and toxin extrusion 1 (MATE1) (in the liver and kidney) and MATE2-K (in the kidney), the intestinal oligopeptide transporter peptide transporter 1 (PEPT1), and ABC transporters such as P-glycoprotein (Pgp), several multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP), and bile salt export pump (BSEP).

An international transporter consortium, formed by representatives from academic institutions, the drug industry, and regulatory agencies, recently prioritized seven transport proteins as the ones that are particularly important to consider in drug–drug interaction (DDI) studies "based on practical considerations and on clinical evidence that these transport proteins influence, to a varying degree, drug disposition and/or side effects" (Giacomini et al. 2010). These were the liver-specific absorptive transporters OATP1B1 and OATP1B3 and three transporters of relevance for DDIs in the kidney, OAT1, OAT3, and OCT2; two widely expressed exsorptive ABC transporters, Pgp and BCRP, were also included. In addition to these, the European Medicines Agency identified the organic cation transporter OCT1 as one of the "transporters known to be involved in clinically relevant in vivo drug interactions" in their draft *Guideline on the investigation of drug interactions*, issued in 2010 (European Medicines 2010). In this document, it is also argued that the bile acid exsorptive transporter BSEP should be studied to detect pharmacodynamic interactions and for safety reasons. In the following presentation, we review in vitro characterization of DDIs with transport proteins, in particular those that have been shown to have significant clinical effects. Further, owing to species differences, the focus will be on human transport proteins.

3.2 Methods Used to Study Transporter Interactions

3.2.1 Membrane Vesicles

Transport proteins are integral membrane proteins and can only be maintained in their native conformation in the presence of cell membranes. As a consequence, there are no high throughput screening assays available for investigating the activity of purified transport proteins. The "purest" methodology to study membrane transport is to use isolated membrane vesicles from cells transfected with the transporter of interest, usually an exsorptive transporter (as illustrated in Fig. 3.1a). Various cell types in which the transport protein of interest has been overexpressed, such as insect (Sf9) cells and eukaryotic cell lines like human embryonic kidney 293 (HEK293) cells, are used to this end (Glavinas et al. 2008; Karlsson et al. 2010). The insect cell line has less cholesterol in its membranes which may influence the results, as has been shown for BSEP (Kis et al. 2009).

The preparation of membrane vesicles is a demanding exercise, and, therefore, most investigators purchase membrane vesicles from commercial sources.

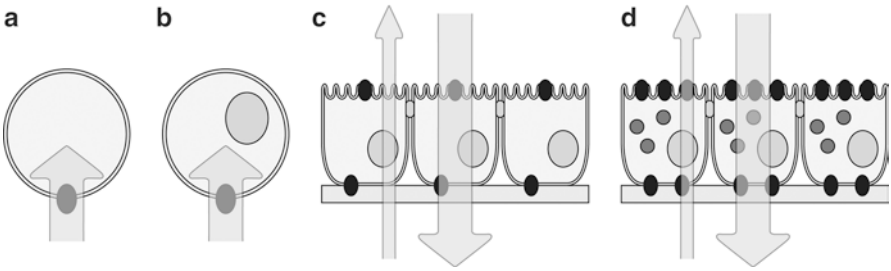

Fig. 3.1 Drug transport in different kinds of in vitro models. (**a**) Drug transport in inverted membrane vesicles. This model is primarily suitable for exsorptive ABC transporters since the ATP-binding domain, which in intact cells is facing the cytosol, will here be facing outwards and give free access to ATP when it is added to the incubation medium. (**b**) Uptake of drugs in suspended or adherent cells overexpressing one transporter. This type of model is primarily used for transiently or stably expressed absorptive transporters. (**c**) Transport of drugs in monolayer-forming cells (e.g., MDCK and Caco-2) grown on permeable support. During conventional growth conditions, these cells form tight junctions leading to a cellular barrier over which drug transport can be studied in both the absorptive and exsorptive directions. (**d**) Drug transport in monolayers of primary cells. The intention with primary cells is that they should maintain the organ-specific cell phenotype; hence, the endogenous expression of both transporters and metabolizing enzymes makes these models complex

During the preparation, a significant fraction of the vesicles are inverted, so that the inner leaflet of the membrane is facing outwards, exposing the cytosolic side of the cell membrane to the medium (Shilling et al. 2006). This makes this model suitable for studies of exsorptive ABC transporters, since they are primary transporters that require adenosine triphosphate (ATP). ATP is bound to conserved sites of ABC transporters localized within the cytosolic side of the plasma membrane. In inverted membrane vesicles, the ATP-binding domains will be facing outwards, giving free access to ATP when ATP is added to the incubation medium. Importantly, membrane vesicles that are not inverted (which accounts for up to half of the vesicles) will remain inactive since the ATP-binding domains will be trapped inside the vesicles without access to the membrane-impermeable ATP molecules in the incubation medium.

Inverted membrane vesicles are used in two different assay formats. In the first and simplest of these, an indirect approach is used where substrate-dependent ATP hydrolysis is measured as inorganic phosphate release during substrate transport (Ishikawa et al. 2005; Keppler et al. 1998). Drugs that inhibit substrate transport will reduce ATP consumption and, hence, the formation of inorganic phosphate will also be reduced. The method can be configured in a high throughput format but has a significant incidence of false positives and negatives in comparison to direct studies of substrate transport and inhibition (Polli et al. 2001). Therefore, results obtained with this methodology often need to be confirmed by measurements using the second type of assay.

In the second type of assay, the transport of a substrate into the vesicle is measured directly. This assay is restricted to hydrophilic substrates with low membrane

permeability since more lipophilic substrates with a higher membrane permeability will not be trapped inside the vesicles but will quickly diffuse out into the medium again and/or (if the substrate is very lipophilic) will accumulate in the membranes of the vesicles, potentially obscuring the active transport process. To allow the use of substrates with low but significant passive membrane permeability, control vesicles are used that are exposed to 5′-adenosine monophosphate (AMP) instead of ATP. By subtracting the passive transport observed in the control vesicles from that in vesicles where the transport is fueled by ATP, a better estimate of the active transport component is obtained (e.g., Pedersen et al. 2008). While hydrophilic substrates should be used in this assay, inhibitors can be both hydrophilic and lipophilic. In this context, the inverted membrane vesicles have an advantage over assays based on intact cells. In the latter, substrates and competitive inhibitors need to be distributed into the cell membrane and cell interior before they can interact with the substrate binding sites of ABC transporters such as Pgp and BCRP. Thus, hydrophilic substrates and inhibitors will not be distributed effectively from the extracellular compartment to the substrate binding site without the aid of, e.g., an uptake transporter, leading to false negative results. However, this problem is circumvented with inverted membrane vesicles, and hence, studies of DDIs with hydrophilic (membrane-impermeable) substrates are possible for this system. For studies comparing DDIs assay formats, see Polli et al. (2001) and Szeremy et al. (2011).

3.2.2 Cell Lines Expressing One or More Drug Transporting Proteins

3.2.2.1 Simple Adherent and Suspension Cell Lines

Eukaryotic cell lines transiently or stably transfected with the transporter of interest are commonly used for DDI studies with absorptive transporters (as illustrated in Fig. 3.1b). In these cell lines, which are often but not always of human origin, the transport protein is presented in its natural lipid environment, which is advantageous for its activity (Rajendran et al. 2010; dos Santos et al. 2011). Common simple cell lines used for such studies include HEK293, HeLa, HepG2, and CHO cells, of which the three first are of human origin, whereas the latter is derived from hamster ovary. Cellular content of adherent cells is easily measured after washing the culture plates with attached cells with cold medium; assays in the suspension format require an additional separation step, e.g., filtration or centrifugation (Pedersen et al. 2008).

It should not be taken for granted that a cell line is capable of transporting a transfected protein to the cell surface. For example, in HEK293 cells transfected with the ABC transporter MRP2, the protein is predominantly retained in intracellular domains rather than being localized to the plasma membrane (Keitel et al. 2003). This is a normal mechanism of short-term regulation of MRP2 as well as

several other transport proteins and may therefore occur also in other cell lines (Sekine et al. 2008). As a result, most of the MRP2 protein will be nonfunctional in a cell-based transporter assay. Common cell lines, such as those listed above, were isolated decades ago and have therefore been exposed to different selection pressures during cultivation in different laboratories over extended periods of time. As a result, a single cell line will display a somewhat different phenotype, depending on the conditions under which it has been maintained. It follows, therefore, that the expression of specific proteins, including endogenous transporters, may differ for the same cell line maintained in different laboratories. The background expression and function will also vary from one cell line to another (Ahlin et al. 2009). If a drug transporting protein displays a high endogenous expression that translates into function in a certain cell line and if this is not accounted for, this background activity may affect the outcome and interpretation of the results of drug interaction studies. One way to account for this is to use mock-transfected control cells, the underlying assumption being that these cells have the same endogenous background as the corresponding cells transfected with the transport protein (Ahlin et al. 2009).

As for membrane vesicles, a sufficiently hydrophilic model substrate of the overexpressed transport protein is usually chosen to reduce the contribution of passive uptake and to trap the substrate inside the cells. The interacting compound is added and the change in substrate uptake is monitored. Easily cultivated cell lines, such as those listed above, have also been used to express single exsorptive transporters, such as Pgp and BCRP. The assay design for exsorptive transporters is more demanding than for absorptive ones in that the cells first have to be loaded with the substrate, usually in the presence of an inhibitor. After loading, the inhibitor is removed and the efflux of the compound from the inside of the cell out into the extracellular medium is observed (e.g., Matsson et al. 2007). Alternatively, the effect of an added inhibitor on steady-state intracellular levels of a model substrate can be used as a surrogate measure of exsorptive transport inhibition.

3.2.2.2 Monolayer-Forming Cell Lines

The most commonly applied monolayer-forming cell line used to overexpress one or more transport proteins is Madin–Darby canine kidney (MDCK) cells (e.g., Evers et al. 1998). This cell line forms cell monolayers sealed by tight junctions between the cells under conventional growth conditions. The tight junctions have predominantly narrow pores, and in common with all monolayer-forming cell lines that have found practical application, the solute transport via these pores is inefficient and often considered to be insignificant for compounds of comparable size to drugs (Linnankoski et al. 2010). It is notable that the permeability of the tight junctions (i.e., the paracellular permeability) may increase as a result of the sometimes harsh transfection procedure using routine methodologies, and, therefore, newer and milder transfection protocols should be considered. When grown on permeable supports, such as polycarbonate filters in single-use Ussing chambers (typically Transwells or similar products), MDCK cells form a cellular barrier over which

drug transport can be studied in both the absorptive and exsorptive directions. The barrier properties are always checked with either a hydrophilic probe that is not subject to active transport, such as ^{14}C-labeled mannitol, or by measuring the transepithelial electrical resistance. For a general description of culture conditions and quality control of monolayer cultures, see Hubatsch et al. (2007). MDCK cells have been extensively used in basic cell biology and are known to have a protein sorting machinery that, in most cases, sorts transport proteins correctly to either the apical or basolateral plasma membrane. An illustration of monolayer-forming cells grown on filters can be seen in Fig. 3.1c. MDCK cells often have a significant background transport activity mediated by endogenous canine transporters, in particular Pgp, whose impact varies between different MDCK clones and from one laboratory to another. Because of this, untransfected or—preferably—mock-transfected MDCK cells are generally used as controls for the MDCK cells transfected with the transporter of interest. Recently, a procedure for selection of MDCK cells with low efflux activity based on iterative fluorescence-activated cell sorting with calcein-AM as an efflux substrate was presented (Di et al. 2011).

The most common application has been to apply MDCK cells overexpressing Pgp (often named (MDCK-MDR1) for investigations of drug transport and DDIs, e.g., Rautio et al. 2006). In this configuration, MDCK cells have often been used as a BBB substitute, since Pgp is known to limit the penetration of many drugs into the brain. Recently, however, BCRP have been shown to have comparable protein expression to Pgp in the human BBB, indicating that further model development is required to better mimic the human BBB (Shawahna et al. 2011). MDCK cells can be transfected with two or more transport proteins that are sorted into the apical and basolateral cell membrane, respectively (see Fig. 3.1c). Thus, in this cell line, the interplay between two transport proteins situated in the opposing plasma membranes can be studied. Using such systems, an absorptive transport protein in the basolateral cell membrane may transport a membrane-impermeable substrate into the cell interior, where it is distributed to the opposite plasma membrane and then presented for an apically located exsorptive transport protein for efflux to the other side of the MDCK-monolayer barrier. In such double-transfected models, it is possible to reveal hydrophilic substrates for efflux transporters that would normally give false negative results in cell models expressing a single exsorptive transport protein (e.g., Alfaras et al. 2010; Liu et al. 2006). A drawback associated with transfection of multiple transport proteins is that they are difficult to express in the proportions observed in vivo (Sakamoto et al. 2011). Examples of transporter pairs expressed in the apical and basolateral membranes of MDCK cells include various combinations of OATP transporters and ABC transporters (Ishiguro et al. 2008), as well as combinations of OCTs and MATEs (Konig et al. 2011). Most recently, models that also include metabolic enzymes have been developed (e.g., Fahrmayr et al. 2011). These models will allow better controlled studies of the effects of DDIs on the interplay between drug transport proteins and metabolism.

Drug transport and transporter-mediated DDIs can also be studied in more tissue-specific cell lines with the capacity to form cell monolayers. The most prominent example is provided by Caco-2 cells (derived from a human colon cancer), which

express many of the transport proteins present in various parts of the human small intestine, and in some clones, this is achieved in proportions comparable to those observed in the human jejunum (Englund et al. 2006; Hilgendorf et al. 2007). As a result, these cells have been extensively used as a model of the small intestinal epithelial barrier to drug absorption (Hubatsch et al. 2007). Among the absorptive transporters, the oligopeptide transporter PEPT1 has been thoroughly studied in Caco-2 cells (Knutter et al. 2009). In addition, OATP2B1, an absorptive transporter that may be involved in the absorption of drugs such as fexofenadine and montelukast, has been studied in Caco-2 cells (Tamai 2011). As for exsorptive transporters, Caco-2 monolayers express Pgp and also functional MRPs and BCRP to varying degrees. They have, therefore, found wide application as a screening tool for "all-in-one" interactions with exsorptive ABC transporters (e.g., Lin et al. 2011). While this may speed up the search for compounds that do not interact with any of these ABC transporters, it becomes a liability when a specific interaction is of interest. To resolve this issue, Caco-2 cells have been transfected with transporter-specific siRNA in order to knock down the contribution of specific transporters (e.g., Darnell et al. 2010). It should be noted that Caco-2 cells and many other transformed cell lines are chromosomally unstable (Thompson and Compton 2008) and therefore have to be maintained under controlled conditions, including a limited number of passages, if they are to provide reproducible results, e.g., with regard to transporter expression (Hubatsch et al. 2007). As discussed for the other cell lines above, large variations in transporter expression and function can be observed when these cells have been maintained under different conditions in different laboratories (Hayeshi et al. 2008). Caco-2 cells have also been used to study the interplay between apical uptake and basolateral efflux transporters (e.g., Ming et al. 2011), as well as between transporters and metabolism (e.g., Raeissi et al. 1999). Most other monolayer-forming cell lines have found limited application because of the demanding cell culture procedures required, the lack of reproducibility, or poor resemblance to the primary cell type they are supposed to mimic. They are, therefore, not covered in this short chapter.

3.2.2.3 Primary Cells

Another type of cells used in DDI studies are primary cells isolated from the tissue of interest. The intention is to obtain cell cultures that maintain the organ-specific cell phenotype better than available immortalized cell lines (see Fig. 3.2d). As a rule, a huge amount of effort is invested in the development of such techniques, and a gradual refinement of the techniques and improvements in the culture performance can be observed in the literature over time, sometimes over decades. Hepatocytes, tubular kidney epithelium, and BBB endothelium have all been isolated and used in drug transport studies (Hewitt et al. 2007; Brown et al. 2008; Cecchelli et al. 2007). In contrast, human intestinal villus cells, representing the absorptive cells in the small intestine, have been difficult to maintain in culture with a differentiated phenotype. This is probably a result of their short life-span in vivo.

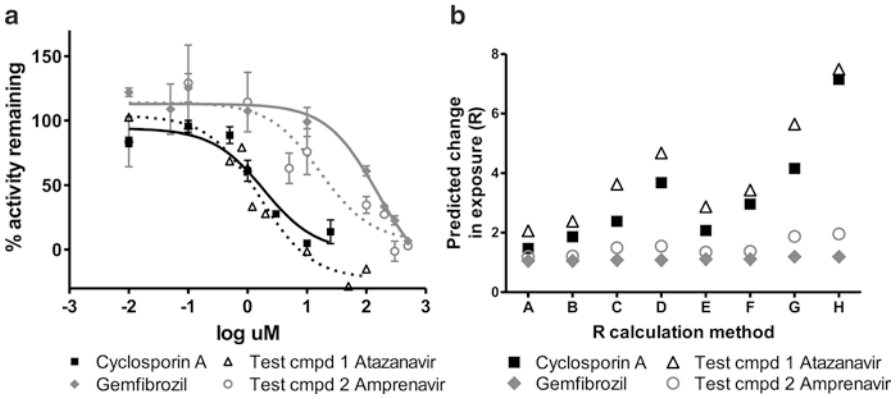

Fig. 3.2 In vitro to in vivo extrapolation for DDIs with the hepatic transporter OATP1B1. (**a**) Inhibition of OATP1B1-mediated atorvastatin uptake in stably transfected HEK293 cells for the two reference drugs cyclosporin A (*black-filled squares*, *solid line*) and gemfibrozil (*gray-filled diamonds*, *solid line*) and for the two test drugs atazanavir (*black open triangles*, *dashed line*) and amprenavir (*gray open circles*, *dashed line*). (**b**) Variation in the range of predicted change in exposure (*R*-values) by applying (3.3) and (3.4) with different k_a and different F_a (in total, eight calculations for each compound) according to Karlgren et al. (2011). For all four compounds, the predicted *R*-values are shown using the same symbols and colors as in (**a**). The figure was adopted from Karlgren et al. (2011) with kind permission from the publisher

The tissue removal and the isolation procedure used to obtain primary epithelial and endothelial cells impose stress on the cells. Their native differentiated phenotype is therefore difficult to maintain. For instance, transporters in the plasma membrane may be endocytosed and/or degraded as a result of this stress. When human hepatocytes are isolated from liver resections, large batch to batch variation, caused by interindividual differences, degree of hypoxia during surgery, disease background, etc., is also observed. Consequently, in primary hepatocytes, the gene expression and protein function varies; they also change with variations in isolation procedure and the subsequent cell culture conditions (Hallifax and Houston 2009). Nevertheless, human hepatocytes have found broad application, in particular in the drug industry, and are available from commercial sources as freshly isolated or cryopreserved cells. Naturally, hepatocytes isolated from experimental animals, and in particular from rodents, perform more consistently than their human counterparts and have also found widespread application. Drug interaction studies in plated or suspended hepatocytes are performed as described for plated or suspended cell lines such as HEK293 cells.

Through an elegant approach, where hepatocytes are sandwiched between two layers of extracellular matrix, the hepatocytes can differentiate into polarized cells with a basolateral surface facing the cell culture medium and an apical surface facing reconstructed bile canaliculi (Lecluyse et al. 1994). In this configuration, the cells partly regain transport functions, and the transport of compounds from the basolateral (i.e., the medium) side into the bile canaliculi can be studied. DDIs involving interplay between basolateral and apical transport proteins can be

investigated, and their effects on intrinsic drug clearance can be estimated (Swift et al. 2010). There are significant species differences in transport protein expression between, e.g., rat and human hepatocytes (Li et al. 2009) as well as in function (Yamazaki et al. 2001; Zimmermann et al. 2008) which may influence the interpretation of DDI studies.

Human kidney tubular epithelium, containing mainly proximal tubular cells, can be isolated from nephrectomies performed for oncological reasons, using demanding preparation methods (Brown et al. 2008). The cells form monolayers that are quite leaky, but despite this, rather impressive results have been obtained in studies with single drugs (Verhulst et al. 2008). These cultures are only maintained by a few laboratories and, therefore, have yet to find wide-scale applications in drug interaction studies with transport proteins.

While human hepatocytes can be isolated from liver resections obtained from patients undergoing surgery, primary BBB endothelium cultures are for obvious reasons obtained from animal brain tissues. Isolated endothelial cells from the BBB form quite leaky monolayers when maintained under standard cell culture conditions (Gumbleton and Audus 2001). Co-cultures with other brain cells or addition of factors secreted by brain cells is required to improve the tight junction integrity, making this a very special cell culture model (Cecchelli et al. 2007). Significant interspecies differences have recently been observed for transport proteins expressed in the BBB of various mammals, including humans (Shawahna et al. 2011).

3.3 Methodologies for In Vitro Investigations and In Vivo Extrapolation of Transporter DDIs

In this section, we describe the principles for performing in vitro investigations of transporter DDIs. Such studies can be performed either prospectively, in order to identify DDIs that may have clinical impact and to prioritize in vivo interaction studies, or retrospectively, to delineate the transport mechanisms involved in an observed clinical DDI.

Investigations of DDIs in inverted membrane vesicles and simple cell lines overexpressing a single transport protein (referred to here as simple models) are performed with similar methodologies. Similarly, DDI studies in cell lines and primary cells forming cell monolayers are performed using comparable methodologies. The principles for performing such studies are briefly outlined in the paragraphs below, as are criteria for extrapolating the results to the in vivo situation. For details, the reader is recommended to reference (Giacomini et al. 2010) and references therein.

In the simple models, where the transporter of interest is overexpressed, the transport protein should first be characterized by transport kinetics. Importantly, the transport kinetics are model dependent and have to be determined for each experimental system. The traditional Michaelis–Menten kinetic parameters K_m and V_{max} were developed for enzymes in solution rather than for proteins integrated in a

membrane handling concentration gradients across membrane barriers. This means that these parameters are not true constants; instead, they will vary with intracellular drug concentration, which, in an uptake experiment, will increase over time (Korjamo et al. 2007). Thus, short incubation times are an advantage, since intracellular accumulation will be lower. Great care should be exercised to maintain the conditions under which the transport kinetics are determined. The linearity of the transport of a substrate drug is first tested at a low concentration before a typical interaction study is conducted where the effects on the uptake of the substrate drug, sometimes referred to as the victim drug,[1] are examined. This is followed by a determination of the transport kinetics, usually obtaining K_m and V_{max} values from curves of concentration-dependent uptake. The apparent K_m value is usually in the μM range for drug transporting proteins, with the exception of the intestinal oligopeptide transporter PEPT1, which is characterized as a low affinity/high capacity transporter with K_m values in the mM range. Therefore, DDIs with PEPT1 are generally not significant. Typically, the uptake kinetics are assessed by plotting the initial uptake rate (e.g., by determining the uptake after 1 min or just a few minutes) against the substrate concentration [S], and the apparent K_m and V_{max} are determined by nonlinear regression fitted to (3.1).

$$v = \frac{v_{max}[S]}{K_m + [S]} + P_{dif} \times [S] \tag{3.1}$$

where P_{dif} is the passive permeability of the substrate.

From these studies, a substrate concentration that is at or below the observed K_m value is chosen for the inhibition studies. This is to assure that the interaction will be studied in the linear range of the concentration vs. transport rate curves. When the substrate (victim) drug is well characterized in vivo, it is sometimes possible to make a rough prediction of the potential for in vivo interactions. Then, a substrate concentration similar to the unbound concentration at the site of the interaction is used (usually, this means the unbound concentration in the blood at C_{max} or in the steady state, where C_{max} is the maximum plasma concentration of the drug). Alternatively, in the case of absorption studies, the total concentration in the intestine is applied (assuming that the highest therapeutic dose is administered and that the intestinal liquid volume is 250 mL).

In the next step, a suitable concentration (interval) is chosen for the interacting drug (sometimes called the perpetrator[2] drug). The choice of concentration will be dependent on the purpose of the study. When a large number of inhibitors are to be screened, a single concentration is sometimes used initially, and then followed by more detailed examinations. If the purpose of the study is to find a sufficiently large data set of hits, e.g., because it is the intention to perform structure-inhibition

[1] Victim drug: a drug affected by a drug–drug interaction, leading to a change in its pharmacokinetics or pharmacodynamics.

[2] Perpetrator drug: a drug which alters the pharmacokinetics or pharmacodynamics of another drug.

analysis, then a higher concentration than that found in vivo for the perpetrator drug can be chosen as exemplified in references (Pedersen et al. 2008; Ahlin et al. 2008). When in vivo interactions are to be predicted, then relevant in vivo unbound concentrations should be investigated as described for the victim drugs above. The time at which the inhibitor is added may influence the results. Preincubation with the inhibitor before addition of the substrate usually results in inhibition at lower perpetrator concentrations than the simultaneous addition of substrate and inhibitor. This effect is powerful when exsorptive transporters are investigated in cell-based assays, in which the inhibitor may need time to reach intramembranous or intracellular binding sites.

After the identification of hits in a screen of the putative perpetrator at a single concentration, or when a limited number of perpetrator drugs are being investigated, concentration-dependent inhibition should be studied. The IC_{50} values (i.e., the concentration at which the substrate transport is inhibited to 50 %) are determined from the sigmoidal inhibition curves using nonlinear curve fitting available in standard statistics software. Often, the apparent inhibition constant, K_i, is calculated from the IC_{50} values (and from K_m and substrate concentration [S]), for example, using the following equation which assumes that the inhibition is competitive:

$$K_i = IC_{50} / \left(\frac{[S]}{K_m} + 1 \right) \tag{3.2}$$

Note that the equations for calculating K_i from IC_{50} differ depending on the mechanisms of inhibition. Typically, if the selected substrate (victim) concentration is much lower than its K_m, the equations simplify so that K_i equals IC_{50}. A wide concentration range should be used, preferably spanning at least four orders of magnitude, to capture the IC_{50} values accurately. However, owing to solubility limitations of the interacting drug, incomplete inhibition curves are not uncommon.

Investigation of the inhibition mechanism is a demanding exercise requiring experimental precision. In this case, a range of inhibitor concentrations needs to be studied for each of a range of substrate concentrations. The ratio of the substrate concentrations to the uptake rates is plotted against the inhibitor concentration (to obtain Cornish-Bowden plots), and the reciprocal uptake rates are plotted as a function of the inhibitor concentration (these are known as Dixon plots). Information on the inhibition mechanism can then be extracted from the shapes of the curves in these graphs (Ahlin et al. 2008).

The IC_{50} and/or the K_i values can be used to predict whether an interaction may be clinically relevant (Hirano et al. 2006a; Giacomini et al. 2010). In predictions of in vivo drug interactions from in vitro data, part of the theory is borrowed from the more established recommendation for predictions of metabolic inhibition. Accordingly, an interaction with the exsorptive ABC transporters Pgp or BCRP may be possible in vivo, when the mean steady-state unbound C_{max} (in blood) at the highest clinical dose of the perpetrator drug divided by the IC_{50} (or K_i) obtained in vitro results in a ratio greater than or equal to 0.1, giving a tenfold safety margin for the

IC_{50} or K_i. Alternatively, for an orally administered drug, if the maximal gastrointestinal concentration of the perpetrator drug (estimated as the concentration obtained when dissolving the highest clinical dose in a volume of 250 mL) when divided by the IC_{50} (or K_i) obtained in vitro results in a value equal to or larger than 10, there is a risk of an in vivo interaction. It has been observed that, for drugs in clinical use, clinically relevant examples of inhibition of Pgp during the absorption phase are rare, even when a low dose drug such as digoxin is used as victim drug (Fenner et al. 2009). However, in clinical practice, where interaction with more than one drug is a reality, significant effects on digoxin plasma concentrations can be observed (Englund et al. 2004).

In predictions of interactions with drug transport via absorptive transporters, similar ratios between unbound plasma concentrations and IC_{50} or K_i values are used for simple vesicle or cell line models. For predictions of interactions in the liver, additional calculations to those discussed above have been recommended to improve the predictions of the changes in drug exposure. The in vitro–in vivo extrapolation is typically conducted by calculating the so-called *R*-values, according to

$$R = 1 + \frac{F_u \times I_{in,max}}{IC_{50}} \tag{3.3}$$

$$R = 1 + \frac{F_u \times I_{in,max}}{K_i} \tag{3.4}$$

in which F_u is the fraction unbound. $I_{in,max}$ is the maximal inhibitor concentration at the inlet of the liver, which is calculated using (3.5) (Giacomini et al. 2010; Hirano et al. 2006a)

$$I_{in,max} = I_{max} + \frac{F_a \times \text{Dose} \times k_a}{Q_h} \tag{3.5}$$

where F_a is defined as the fraction of the intact drug that is absorbed across the intestinal epithelium, dose is the maximum oral dose given, I_{max} is the maximum total systemic plasma concentration, k_a is the absorption constant, and Q_h is the hepatic blood flow. Equation (3.4), using K_i for *R*-extrapolation, was used by Hirano et al. (2006a); these authors recommended setting the F_a equal to 1 and using a value of $k_a = 0.1$ to estimate the maximum $I_{in,max}$. This is also recommended in the EMA draft guideline as a worst-case scenario (European Medicines 2010). In contrast, a recent paper from the International Transporter Consortium used (3.3) that incorporates IC_{50} instead of K_i and uses a value of $k_a = 0.03$ for the *R*-extrapolation (Giacomini et al. 2010). In the latter publication, no recommendation is made regarding F_a, although $F_a = 1$ is used for the examples provided by the authors. Thus, by combining the different ranges of values for the fraction absorbed (F_a) and the absorption rate constant (k_a) suggested in the literature cited above, quite different *R*-values will

be obtained for each interacting compound (Karlgren et al. 2011). The effects on the predictions are visualized in Fig. 3.2. Since the IC_{50}, and hence the K_i values, will vary with the model system (Glavinas et al. 2011) and the experimental conditions (e.g., time for addition of inhibitor), the *R*-value also becomes model- and method-dependent. Despite this variability, it has somewhat surprisingly been suggested that a fixed *R*-value equal to or larger than 2 should be indicative of a significant in vivo interaction (Giacomini et al. 2010). In our experience, it is better to use an *R*-value calibrated from the applied in vitro system. Determination of model-specific IC_{50} values of reference drugs, with known clinical interactions with the transporter of interest, should, therefore, always be performed to get a better appreciation of the model-specific *R*-value that provides the border between a potentially significant and a nonsignificant interaction (Giacomini et al. 2010; Karlgren et al. 2011).

As mentioned above, drug interaction studies in monolayer cultures of cell lines and primary cells are performed using similar approaches. The transporter interaction studies in the monolayer cultures investigate the interference on the transport *across* the entire cell, rather than uptake into or efflux from the cell (which is studied in the simple models). This is possible since the tight junction-sealed cell monolayers are grown on filters that allow almost unrestricted access to the monolayers both from the absorptive and exsorptive sides, thereby making it possible to compare the transport rates of a transporter substrate in both directions across the monolayers. Typically, the transport of a reference or victim drug that is a substrate for the transporter of interest is studied initially in, for example, MDCK cells overexpressing Pgp or in Caco-2 cells, which have significant endogenous expression of various transporters.

In investigations and documentation of drug interactions with candidate drugs in monolayer models, the so-called efflux ratio (ER) or absorption ratio (AR) is usually one of the first experiments performed. By taking the ratios between the transport rates (often expressed as permeability coefficients) obtained in each of the two directions across the cell monolayers, either the ER or, when an absorptive transporter is studied, the AR will be obtained. Based on experience and comparison with in vivo data, obtaining an $ER > 2$ is often taken as an indication of significant transporter-mediated transport (Giacomini et al. 2010). Thus, as a first step it is secured that the intended victim/probe drug displays a significant ER or AR ratio. As discussed for the *R*-value above, the optimal threshold value will vary with model system and method used.

After establishing the baseline flux ratio in the absence of a perpetrator drug, the next step is to investigate the effect of increased concentrations of the perpetrator on the net flux ratio of the victim drug. In its simplest form, the ER in the presence of an inhibitor is calculated from the ratio of the transport rate in the presence and absence of the perpetrator drug for inhibition in the exsorptive direction and as the ratio in the absence and presence of the perpetrator drug in case of inhibition in the absorptive direction. Then, the ratios between the ERs in the presence and absence of the inhibitor (at different concentrations) are calculated. The IC_{50} values can then be determined from these ratios (Balimane et al. 2008). This is followed by analysis of the ratios between the IC_{50} values and the clinical blood or gastrointestinal

concentrations, as described in (3.3–3.5) and in the text above. Critical analysis of various ER calculations has resulted in refined alternatives, in particular for exsorptive transporters, for example, in references (Kalvass and Pollack 2007; Troutman and Thakker 2003a; Lin et al. 2011). A thorough analysis of the differences between IC_{50} and K_i values obtained using the various ER equations was recently published (Lumen et al. 2010). The same paper also included an analysis of factors contributing to that the IC_{50}/K_i ratios as a rule are considerably larger than unity (when inhibition of exsorptive transporters (here Pgp) is investigated). These factors included Pgp concentration in the plasma membrane, membrane partitioning coefficients, and elementary rate constants.

3.4 Interactions with Genetic Variants of Transport Proteins

DDIs in the presence of common genetic variants that cause reduced transport function may have more pronounced effects than the same DDI would have on a fully functional drug transporter. Polymorphisms have been identified for the majority of the important drug transport proteins, although their significance for DDIs varies considerably. For more information regarding transporter pharmacogenetics, we refer the reader to Chap. 7 on the pharmacogenomics of transporters, as well as to the references therein.

Most in vitro studies on genetic variants of transporters have focused on the altered transport function attributable to transporter polymorphisms and not on the potentially increased risk for transporter-mediated DDIs. Lately, some research on OCT transporters have also investigated this aspect. OCT1 is highly polymorphic, although most attention has been on the two common reduced function variants p.M420del and p.R61C. Both of these variants have been demonstrated to be more susceptible to drug inhibition, with IC_{50} values being up to a factor of more than 20 lower than the values for the reference OCT1 protein (Minematsu and Giacomini 2011; Ahlin et al. 2010). Furthermore, for OCT2, the OCT transporter primarily expressed in kidney, the variant p.A270S has proved to be more sensitive for drug inhibition suggesting an increased risk for DDIs for this variant (Kido et al. 2011).

How should the function and the risk of transporter interactions be investigated for genetic variants in vitro? Experiments using simple and monolayer-forming cell lines transiently or stably transfected with the different transporter variants as discussed above are the most common approaches. This is especially the case for genetic variants of uptake transporters where cell lines like HeLa, HEK293, and MDCK are frequently used (Tirona et al. 2001; Nozawa et al. 2002; Michalski et al. 2002), but similar approaches have been used also for efflux transporters, e.g., transient or stable expression in the HEK293 and kidney epithelial cell lines for Pgp (Crouthamel et al. 2006; Crouthamel et al. 2010; Salama et al. 2006; Yang et al. 2008). Other examples of systems used are isolated membrane fractions/vesicles from transfected insect cells (Sf9) or transfected eukaryotic cells (Sakurai et al. 2007; Hirouchi et al. 2004). In addition, several studies of Pgp genetic variants have

been based on peripheral blood cells isolated from whole blood, i.e., on primary cells that are much easier to isolate than those embedded in tissues such as the liver and BBB. (Storch et al. 2007; Oselin et al. 2003).

In addition to the stably transfected cell lines established using traditional methods, several studies of efflux as well as uptake transporters have during recent years utilized mammalian expression systems in which a single copy of the gene of interest can be integrated into the host genome in a controlled manner (Morita et al. 2003; Shu et al. 2007; Chen et al. 2009). In adopting this approach, the otherwise unavoidable clone variability, which is a result of the random integration and selection procedures that characterize traditional stable expression, is minimized. Thus, this approach is considered advantageous for elucidating the sometimes small differences between transporter genetic variants.

Why are not the more complex models described above, like primary cells, used when studying transporter interactions for genetic variants? First of all, the frequencies of transporter variant alleles are usually rather low, resulting in that the availability of primary cells for such studies will be very limited. Hence, extensive genotyping is needed to identify the rare primary cell batches having the desired allelic variant. Also, for endogenous expression of a variant allele in primary cells, it is important to keep in mind that the cells are diploid. Thus, they have two alleles and can therefore be heterozygous or homozygous for the variant allele. Lastly, interactions with other drug transporters and metabolizing enzymes active in the primary cells may make it difficult to draw conclusions without having a large number of samples. In conclusion, the controlled expression of a single variant transporter in a simple cell line with known background and with corresponding mock-transfected cells is recommended over the more complex models.

3.5 Recommended Substrates and Inhibitors

When drug interactions with enzymes such as those of the cytochrome P450 superfamily are studied, a large collection of well-characterized prototypic and rather specific substrates and inhibitors is at hand. In the less mature research field of investigating drug interactions with transport proteins, the experience of using various probe substrates and inhibitors is more limited. Numerous more or less specific transporter substrates and inhibitors have been presented in various reviews of transporter interactions, and, in Table 3.1, we present a limited collection of well-studied model substrates and inhibitors and examples of drug substrates. Many of the substrates and inhibitors have yet to be studied comprehensively for their specificity. This may be less of a problem in simple model systems, where only a single transporter or just a couple of transporters are overexpressed and where the endogenous expression of functional drug transporting and drug metabolizing enzymes is low. Nevertheless, more complex interaction patterns in these models should not be excluded; for example, active uptake of the prototypic Pgp substrate digoxin via an unknown sodium dependent mechanism was recently observed in HEK293 cells

Table 3.1 Selected model and drug substrates and model inhibitors of important drug transporters

Transporter	Aliases	Gene	Model substrates (selection)[a]	Drug substrates (selection)[b]	Model inhibitors (selection)
Pgp	MDR1	ABCB1	Digoxin, calcein-AM, *N*-methylquinidine, vinblastine, rhodamine 123	Digoxin, loperamide, irinotecan, doxorubicin, paclitaxel, fexofenadine, saquinavir, ritonavir	Cyclosporine-A, quinidine, tariquidar (XR9576), elacridar (GF120918), haloperidol, verapamil
MDR3		ABCB4	Phosphatidylcholine	Paclitaxel, digoxin, vinblastine	Verapamil, itraconazole, cyclosporine-A
BSEP	SPGP, cBAT	ABCB11	Taurocholic acid	Pravastatin, bosentan	Cyclosporine-A, rifampicin, glibenclamide
MRP2	cMOAT	ABCC2	Estradiol-17β-glucuronide, methotrexate	Methotrexate, etoposide, mitoxantrone, valsartan, olmesartan, glutathione and glucuronide conjugates	Verlukast (MK571), bromosulfophthalein, cyclosporine-A, delaviridine, efavirenz, emtricitabine
MRP3	MOAT-D, cMOAT2	ABCC3	Estradiol-17β-glucuronide, methotrexate	Methotrexate, fexofenadine, glucuronide conjugates	Verlukast (MK571), delaviridine, efavirenz, emtricitabine
MRP4	MOAT-B	ABCC4	Estradiol-17β-glucuronide, methotrexate	Adefovir, tenofovir, dehydroepiandrosterone sulfate, methotrexate, topotecan, furosemide	Verlukast (MK571), celecoxib, diclofenac
BCRP	MXR	ABCG2	Mitoxantrone, Hoechst 33342, estradiol-17β-glucuronide, methotrexate	Mitoxantrone, methotrexate, topotecan, imatinib, irinotecan, statins, sulfate conjugates	Fumitremorgin C, Ko143, Ko134, elacridar (GF120918), prazosin, estrone, 17β-estradiol
PEPT1		SLC15A1	Glycylsarcosine	Valaciclovir, cephalexin, cefadroxil, bestatin, enalapril, fosinopril, aminolevulinic acid, amoxicillin	Glycylproline, valine-lysine-fluorescein isothiocyanate
PEPT2		SLC15A2	Glycylsarcosine	Valaciclovir, cephalexin, cefadroxil, bestatin, enalapril, fosinopril, aminolevulinic acid, amoxicillin	Zofenopril, fosinopril

(continued)

Table 3.1 (continued)

Transporter	Aliases	Gene	Model substrates (selection)[a]	Drug substrates (selection)[b]	Model inhibitors (selection)
OCT1		SLC22A1	ASP+, tetraethylammonium, *N*-methylpyridinium	Metformin, oxaliplatin, lamivudine	Spironolactone, doxazosin, propafenone, quinine, quinidine, disopyramide
OCT2		SLC22A2	ASP+, tetraethylammonium, *N*-methylpyridinium	Metformin, pindolol, procainamide, ranitidine, amantadine, amiloride, oxaliplatin, varenicline, pramipexole	Disopyramide, imipramine, imatinib, rabeprazole, cimetidine, pilsicainide, cetirizine
OAT1		SLC22A6	Para-aminohippurate	Adefovir, cidofovir, zidovudine, lamivudine, zalcitabine, acyclovir, tenofovir, ciprofloxacin, methotrexate	Probenecid, novobiocin, adefovir, cidofovir, eosin Y
OAT3		SLC22A8	Estrone-3-sulfate, para-aminohippurate	Cefaclor, ceftizoxime, furosemide, bumetanide, methotrexate, nonsteroidal anti-inflammatory drugs	Probenecid, novobiocin, zidovudine, 6-carboxyfluorescein, eosin Y
MATE1		SLC47A1	ASP+, tetraethylammonium, *N*-methylpyridinium	Metformin, cisplatin, cimetidine, topotecan	Pyrimethamine, ondansetron, cimetidine, quinidine, procainamide
MATE2-K		SLC47A2	ASP+, tetraethylammonium, *N*-methylpyridinium	Metformin, cisplatin, cimetidine, topotecan	Cimetidine, quinidine, pramipexole
OATP1A2	OATP-A	SLCO1A2	Estrone-3-sulfate	Statins, fexofenadine, methotrexate, dehydroepiandrosterone sulfate, levofloxacin	Naringin, ritonavir, lopinavir, saquinavir, rifampicin

OATP1B1	OATP-C, OATP2, LST-1	SLCO1B1	Estradiol-17β-glucuronide, bromosulfophthalein, 8-fluorescein-cAMP, sodium fluorescein	Statins, repaglinide, valsartan, olmesartan	Saquinavir, ritonavir, lopinavir, rifampicin, cyclosporine-A
OATP1B3	OATP-8	SLCO1B3	Estradiol-17β-glucuronide, bromosulfophthalein, 8-fluorescein-cAMP, sodium fluorescein	Statins, digoxin, fexofenadine, telmisartan, valsartan, olmesartan	Rifampicin, cyclosporine-A, ritonavir, lopinavir
OATP2B1	OATP-B	SLCO2B1	Estrone-3-sulfate, bromosulfophthalein	Statins, fexofenadine, glyburide	Rifampicin, cyclosporine-A

Note that the listed compounds are not necessarily specific for a certain transporter and confounded results may thus be obtained in experimental models that express multiple transporters at significant levels

ASP+ 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium, *cAMP* cyclic adenosine monophosphate

[a]Selected substrates from the literature that typically give high signal-to-noise ratios in in vitro transporter studies

[b]Example drugs that have been reported to be substrates of transporters

(Taub et al. 2011). In contrast to the simple expression systems, cell models based on the cultures of primary cells present an entire cell-specific panel of drug transporting proteins and enzymes, making interpretation of interaction data considerably more difficult. For such models, the ideal well-studied specific substrate or inhibitor is hard to come by, but it is envisaged that, with time, the transporter field will mature and more specific probes will be identified, as has been the case in the cytochrome P450 field. As a part of this exercise, when more studies are performed, substrates and inhibitors previously considered to be specific for single transport proteins are revealed to be multi-specific because more thorough studies are performed. For example, MK571, an inhibitor of leukotriene transport by ABC transporters of the ABCC-family and extensively used as a specific inhibitor of in particular MRP2, was found to also inhibit Pgp and BCRP at overlapping concentrations, probably via binding to the conserved ATP-binding site in these proteins (Matsson et al. 2009). Similarly, a substrate that is specific for a certain transporter within the ABC transporter family may also interact with SLC uptake transporters. For instance, the anti-inflammatory drug sulfasalazine is a substrate with relatively high affinity for BCRP (Jani et al. 2009), but it is also an inhibitor of OATPs in the same concentration interval (Karlgren et al. 2010).

Many historical investigations of DDIs have relied on easily detected fluorescently or radioactively labeled substrates, in particular during the initial characterization of transport proteins. Through this approach, DDI studies can be performed without the requirement for compound-specific analysis methods. More recently, high throughput analytical equipment has become more generally available such as ultra performance liquid chromatography coupled to mass spectroscopy, and DDI studies based on compound-specific analysis are now more frequent in the literature. However, assays based on fluorescent substrates are quite sensitive and amiable to the use of higher throughput formats. Examples include calcein and rhodamine 123 efflux assays (Pgp and MRP efflux transporters) (Glavinas et al. 2011; Troutman and Thakker 2003b) ASP+ uptake assays (organic cation transporters) (Ahlin et al. 2008; Kido et al. 2011), assays using the fluorescent bile salt analog cholyl-glycylamido-fluorescein (several bile acid transporters) (Annaert et al. 2010), and mitoxantrone efflux assays for BCRP (Matsson et al. 2007). Recently introduced fluorescent model substrates include 8-fluorescein-AMP, which is a substrate for OATPs (Bednarczyk 2010), and D-luciferin which is a substrate for BCRP (Zhang et al. 2009). For radiolabeled substrates, the scintillation proximity assay provides a higher throughput format than the traditional scintillation counting assays (see Lohmann et al. 2007). The use of model substrates (that are not drugs) relies on the assumption that the results obtained can be extrapolated to real DDIs. This is only valid to a certain extent, though, since differences in substrate affinities and the sensitivity to certain inhibitors will result in different IC_{50} values, which may transform a potentially significant interaction into a nonsignificant one and vice versa. In summary, in vitro predictions of in vivo DDIs with transport proteins should, preferably, be performed with real drugs to avoid needing to make such extrapolations.

3.6 In Vitro Screening for Transporter DDIs

In several studies, hundreds or thousands of inhibitors have been screened for DDIs. Examples of such studies are listed in Table 3.2. In these screens, many previously unidentified interacting compounds have been found. As can be seen in Table 3.2, these studies are often performed at inhibitor concentrations that are too high to be of clinical relevance because the intention is to obtain a sufficient number of hits, e.g., to identify structural motifs or pharmacological groups that are overrepresented among compounds that bind to the transporter. The most interesting hits in such screens should, therefore, be followed up by studies of concentration-dependent inhibition as described above. Notably, the definition of an inhibitor varies between the extremes in the literature in that in some studies, compounds are defined as inhibitors at very high (mM) concentrations, while in other studies, inhibition at low μM concentrations is required for "significant" inhibition; see Table 3.2. Careful analysis of the original literature should therefore be performed before assuming that a compound will give significant inhibition in the model of interest.

Screening studies of transporter interactions that are repeated with matching compound collections on multiple proteins can give information on the degree of specificity of the inhibitors. When the same compound collection was investigated for inhibition of Pgp, BCRP, and MRP2, both specific and general inhibitors were identified (Matsson et al. 2009). Other transporters have been compared in similar investigations (Kido et al. 2011; Karlgren et al. 2010; Badolo et al. 2010). In the screening studies, perpetrator drugs that stimulate transport activity are sometimes also found. The stimulation usually occurs at low concentrations of the interacting compound, often turning into inhibition at higher concentrations, and its in vivo significance remains to be shown. For a recent review of stimulation of the MRP2 transporter, see Heredi-Szabo et al. (2009).

The primary focus of most transporter screening efforts so far has been to identify compounds that may interfere with the transport of other drugs (i.e., DDIs). However, inhibition of transporters for endogenous substrates is increasingly appreciated as a liability in drug development. Such interactions can result in severe adverse effects. For example, drug-mediated inhibition of BSEP, the ABC transporter that transports bile acids from hepatocytes into bile, can result in cholestasis and severe liver damage. In a recent screen using inverted membrane vesicles and radiolabeled taurocholate as the model substrate, a correlation between BSEP inhibition and cholestasis and other side effects in the liver was observed (Morgan et al. 2010). Inhibition of other transporters that predominantly accept endogenous substrates may have similar clinically important effects, and further developments in this field are envisaged.

A large number of detailed in vitro investigations of specific DDIs have been performed, aiming to elucidate the mechanism of clinically observed interactions or to determine the significance of in vitro findings. Examples include inhibited transport of the widely prescribed HMG-CoA reductase inhibitors (statins) which may lead to increased plasma concentrations and ensuing myotoxicities (Niemi 2010;

Table 3.2 Examples of screening studies to identify transporter inhibitors

Transporter	Model	Model substrate	Number of compounds studied	Inhibitor concentration (μM)	Number of hits (%)	References
Pgp	Transfected MDCKII cells	Calcein-AM	63	50–200	24 (38 %)	Polli et al. (2001)
Pgp	Transfected MDCKII cells	Calcein-AM	93	100	10 (11 %)	Mahar Doan et al. (2002)
Pgp	Drug resistance selected K562 cells	Calcein-AM	66	0.08–100 (IC50)	23 (35 %)	von Richter et al. (2009)
Pgp	Drug resistance selected Jurkat cells	JC1	880	5	8 (1 %)	Ivnitski-Steele et al. (2008)
BSEP	Inverted Sf9 membrane vesicles	Taurocholate	42	100	5 (12 %)	Hirano et al. (2006b)
BSEP	Inverted Sf9 membrane vesicles	Taurocholate	217	0–133 (IC50)	54 (25 %)	Morgan et al. (2010)
MRP2	Inverted Sf9 membrane vesicles	Estradiol-17β-glucuronide	191	80	42 (22 %)	Pedersen et al. (2008)
BCRP	Inverted Sf9 membrane vesicles	Methotrexate	47	200	8 (17 %)	Saito et al. (2006)
BCRP	Transfected Saos-2 cells	Mitoxantrone	123	50	46 (37 %)	Matsson et al. (2007)
BCRP	Drug resistance selected IgMXP3 cells	JC1	880	5	6 (1 %)	Ivnitski-Steele et al. (2008)
BCRP	Transfected HEK293 cells	D-Luciferin	3,273	17	219 (7 %)	Zhang et al. (2009)
OCT1	Transfected HEK293 cells	ASP+	191	100	62 (32 %)	Ahlin et al. (2008)
OCT1	Cryopreserved human hepatocytes	MPP+	84	20	3 (4 %)	Badolo et al. (2010)
OCT1	Cryopreserved rat hepatocytes	MPP+	176	20	34 (19 %)	Badolo et al. (2010)
OCT2	Transfected HEK293 cells	ASP+	910	20	244 (27 %)	Kido et al. (2011)
OATP1B1	Transfected HEK293 cells	Estradiol-17β-glucuronide	146	20	65 (45 %)	Karlgren et al. (2011)
OATP1B1/3	Cryopreserved human hepatocytes	Estradiol-17β-glucuronide	84	20	4 (5 %)	Badolo et al. (2010)
OATP1B1/3	Cryopreserved rat hepatocytes	Estradiol-17β-glucuronide	176	20	45 (26 %)	Badolo et al. (2010)

To facilitate comparisons, the number of hits was recalculated from the original reports using the same cutoff of either a 50 % reduction in transport activity at the screening concentration or an IC_{50} below 50 μM to classify compounds as inhibitors. Note that the inhibitor concentrations used in the different screening approaches range over two orders of magnitude, and careful analysis of the original references is thus necessary before comparing results between experimental systems
ASP+ 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium, *JC1* J-aggregate-forming lipophilic cation 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide, *MPP+* 1-methyl-4′-phenylpyridinium

Shitara et al. 2005); inhibition of BCRP-mediated efflux of the anticancer agents topotecan and SN-38 by the tyrosine kinase inhibitor imatinib mesylate, with the potential for co-treatment of drug-resistant tumors (Houghton et al. 2004; Robey et al. 2007); and inhibition of renal anion transporters by probenecid to reduce the risk for nephrotoxicities from the antiviral agent cidofovir (Lalezari and Kuppermann 1997; U.S. Food and Drug Administration 1999). It is outside the scope of this chapter to discuss this extensive body of literature in any detail, and the interested reader is therefore referred to the reviews cited throughout this text for additional examples and references (see, e.g., Chaps. 4 and 6).

3.7 Conclusions and Outlook

The study of DDIs at the level of transport proteins is a rapidly developing, but still rather immature, research field. Transporter DDIs can affect overall drug disposition by altering the absorption, distribution to target and off-target tissues, and excretion of the affected drug. Reports of transporter DDIs have been accumulating over the recent years, and, consequently, regulatory agencies worldwide are increasingly demanding documentation of transporter interactions for new drugs. A number of in vitro models have been essential for advancing our understanding of these interactions, since they provide tightly controlled experimental conditions, where a single or several transport proteins can be studied in isolation. Although the in vitro models and methodologies have given us a good appreciation of the interactions with several important transport proteins, a comprehensive understanding of the ways to handle complexities like the interplay between different transporters and intracellular drug metabolizing enzymes is still only starting to emerge. Guidelines on how to investigate transporter interactions are available, but the lack of properly characterized specific transporter substrates and inhibitors of a quality comparable to those available for studies of metabolic interactions remains a problem. Once the newly developed tools for global and quantitative proteomics have become more robust and have become broadly available, the relative capacities and hence the relative importance of the different transport proteins will be revealed. We know that the normal expression of transport proteins is significantly altered in various disease states, and once characterized, such alterations will be accounted for in models of drug transport, as will interindividual variability in transporter expression levels and function. Mechanistic computational models where the interplay between transport proteins and metabolic enzymes is considered will play a major role in the advancement of our understanding of DDIs with transport proteins and the effects of natural and disease-induced variability.

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