Yuichi Sugiyama
Bente Steffansen

Editors

Transporters in Drug Development

Discovery, Optimization, Clinical Study and Regulation

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Editors

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Clinical Study and Regulation

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Preface

This volume of *AAPS Advances in Pharmaceutical Sciences Series* presents a review of the principles and progress concerning membrane transporters and how evolved understanding of membrane transporters has influenced drug development and regulatory assessment. The objective of the book is to present current knowledge in the research field from an industrial, regulatory, and academic perspective.

Membrane transporters are expressed all over the human body and it is well accepted that they play a major role in absorption, distribution, metabolism, and elimination (ADME) of a wide range of drug substances/candidates.

Transporters in Drug Development: Discovery, *Optimization*, *Clinical Study and Regulation* discusses how transporters may alter delivery of their drug substrates and how such alteration may influence the pharmacokinetics and give rise to possible drug–drug interactions on transporters. In vitro characterization of transporters is described related to the organs of most importance for ADME, i.e., the transporters' role in the intestine, liver, and kidney. The book also describes how in vitro characterization may be translated into clinical relevance by in vitro–in vivo correlation and/or by simulation. Identified biomarkers and probes for transporters are described and discussed as well as how targeted proteomics may improve our understanding of the abundance of transporters, not only in a qualitative but also in a quantitative manner. ENJOY!

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Acknowledgements

Yuichi and Bente want to thank Daan Crommelin for asking and for having faith in us to coedit the book *Transporters in Drug Development: Discovery*, *Optimization*, *Clinical Study and Regulation* although at the same time making us aware of how impact of transporters may be black swans (Nassim Nicholas Taleb, 2010. The Black Swan: The Impact of the Highly Improbable) in ADME and thus of how too simplistic explanations for their roles we have been given.

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Chapter 1
Membrane Transporters in ADME

Bente Steffansen, Carsten Uhd Nielsen, and Birger Brodin

Abstract Transporter-mediated absorption or efflux of drug compounds across tissue barriers may affect drug ADME properties. This is exemplified in the present chapter, where we demonstrate how the intestinal proton-coupled amino acid transporter PAT1 may act as a mediator of intestinal gaboxadol absorption. We also discuss how organic anions may be substrates for multiple intestinal transporters. The role of the apical proton co-transporter OATP2B1 and the basolateral facilitative OSTα/β transporter in absorptive and exsorptive transport of the organic anion model substrate E_1S is treated in detail.

Distribution of drug compounds across the blood–brain barrier does rely on transporters to a large extent, and we describe the challenges of developing in vitro methods which may predict drug distribution to the CNS.

Abbreviations

δ-ALA	δ-Aminolevulinic acid
ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism, and excretion
ASBT	Apical sodium-dependent bile acid transporter
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
Caco-2	Colorectal adenocarcinoma cells
DDI	Drug–drug interactions

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DHEAS	Dihydroepiandrosterone-3-sulfate
E_1S	Estrone-1-sulfate
EMA	European Medicines Agency
FDA	US Food and Drug Administration
K_m	Michaelis constant
L-Pro	L-Proline
L-Trp	L-Tryptophan
MATE	Multidrug and toxin extrusion
MCT	Monocarboxylate transporter
MRP	Multidrug resistance protein
NCE	New chemical entity
NTCP	Na^+ taurocholate cotransporting polypeptide (human)
NVU	Neurovascular unit
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OST	Organic solute transporter
P_{APP}	Apparent permeability
PAT	Proton-coupled amino acid transporter
PEPT	Proton-coupled di-/tripeptide transporter
P_{UP}	Uptake/influx permeability
SLC	Solute carrier
SLCO	Solute carrier organic anion
TCA	Taurocholic acid
TJ	Tight junctions
V_{max}	Maximal transport rate

1.1 Introduction

Membrane transport proteins, also named membrane transporters, are now generally accepted to play an important role in absorption, distribution, metabolism, and excretion (ADME) of many drug substances. Subsequently, the research field of drug transporters in ADME has evolved rapidly over the last decades.

To avoid confusion about the transporters and their molecular identity membrane transporters are classified into two super families, i.e., the ATP-binding cassette (ABC) family and the solute carrier family (solute carrier (SLC) and solute carrier organic anion (SLCO)). Recommended overviews of the two families are found in the following two databases http://bts.ucsf.edu/fdatransportal/index/ and http://www.bioparadigms.org/slc/intro.htm (Morrissey et al. 2012).

To understand how transporters define ADME properties of drug substances and new chemical entities (NCEs), it is necessary to gather detailed knowledge of which transporters are expressed in especially the primary organs, i.e., intestine, liver, kidney, blood–brain barrier (BBB), and placenta. Furthermore, the polarization of transporters in the cell membranes is important, as this defines if the transport

direction is in the absorptive or exsorptive direction. Consequently, extensive research related to identification of tissue-specific expression, including expression level, functional characterizing of single transporters, as well as to identification of substrate and/or inhibitors, probes, and biomarkers for single transporters is ongoing. The underlying issue is to investigate for possible transporter-related nonlinear kinetics of the NCE/drug substances and/or for possible drug–drug and/or drug–food interactions on transporters to elucidate when this has clinical relevance. The US Food and Drug Administration's (FDA) information page for drug developers is a valuable resource on drug interactions enclosing summarized FDA recommended substrates and inhibitors, for the majority of known human drug transporters (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm).

The FDA draft guidance for industry and the European Medicines Agency (EMA) final guidance on the investigation of drug interactions describe procedures for drug interaction studies—study design, data analysis, implications for dosing, and labeling recommendations (FDA 2012; EMA 2012).

However, even though it has become generally accepted that transporters are important in ADME of many drugs it is still a challenge for the field to translate in vitro results to the clinical setting. There are many reasons for this and one is that many drug substances are likely to be substrates/inhibitors for several transporters rather than being substrates for a single transporter and also that single transporters may be expressed in several organs. It can therefore be challenging to elucidate and describe the dynamic interplay between transporters in intestinal permeability/absorption and hepatic and/or renal clearance.

The objective of this chapter is to describe membrane transporters' role in ADME by exemplifying that (1) proton-coupled nutrient transporters may affect ADME properties of their drug substrates; (2) the proton-coupled amino acid transporter, PAT1, may act as a mediator of intestinal drug absorption; (3) overlap and interplay between intestinal transporters may influence absorption of organic anions; and (4) transporters in the BBB control drug distribution to CNS.

1.2 Proton-Coupled Nutrient Transporters Affect ADME Properties

Transporters may be functionally described by their (1) substrate specificity, (2) dependency on ions or other substrates in generating the total driving force for solute movement, and (3) their kinetic parameters for substrate translocation. One group of transporters, that are suggested to be important for intestinal absorption of drugs, is the diverse group of proton-coupled transporters (Thwaites and Anderson 2007). These transporters utilize the proton gradient established between the acidic microclimate (pH ~6.5–6.8) next to the intestinal brush border and the inside of the enterocyte (pH ~7.4) to move drug substances across the luminal cell membrane

(Daniel et al. 1989). The transporters have structurally very different substrates such as, e.g., amino acid for PAT1 (SLC36A1), di- and tri-peptides for PEPT1 (SLC15A1), organic ions such as the sulfate-conjugated steroids estrone-1-sulfate (E_1S) and dehydroepiandrosterone-3-sulfate (DHEAS) for the organic anion transporting polypeptide OATP2B1 (SLC02B1), short-chain fatty acids such as L-lactate, butyrate, propionate, and acetate for the monocarboxylate transporter MCT1 (SLC16A1), and folic acid (vitamin B9) for the proton-coupled folate transporter PCFT (SLC46A1). One of the most well studied absorptive intestinal proton-coupled transporters is probably PEPT1, which numerous reviews have dealt with in detail (see, e.g., Brandsch et al. 2008; Brandsch 2009; Brodin et al. 2002; Nielsen et al. 2002; Nielsen and Brodin 2003). However, PEPT1 is also expressed in the luminal membrane of the early proximal tubule, whereas the paralog PEPT2 (SLC36A2) is mainly expressed in the luminal membrane of the late proximal tubule where both the transporters mediate the reuptake of dipeptides from the ultrafiltrate (Daniel et al. 1991; Ganapathy et al. 1980; Ganapathy and Leibach 1983; Miyamoto et al. 1985; Smith et al. 1998). A few studies have suggested that PEPT2 could influence both distribution and the excretion of peptidomimetic drug substances. The clearance of Gly-Sar, L-carnosine, and cefadroxil is increased in *PepT2* (−/−) mice after intravenous injection (Ocheltree et al. 2005; Shen et al. 2007; Kamal et al. 2009), which is consistent with an involvement of PEPT2 in reabsorption of dipeptides and dipeptidomimetic drug substances, thereby affecting the overall pharmacokinetic profile of PEPT2 substrates. These studies also suggest that, for the investigated substances and at the renal concentrations achieved, PEPT2 seems more important than PEPT1 in reabsorbing substances in the kidney. At the same time studies in *Pept2* knockout mice have suggested a role of PEPT2 in drug disposition, since loss of PEPT2 results in substantially lower concentrations of δ-aminolevulinic acid (δ-ALA) in the choroid plexus epithelial cells, and substantially higher concentrations of δ-ALA in cerebrospinal fluid and interstitial fluid surrounding the parenchymal cells (Hu et al. 2008).

    As with many other transporters suggested to be important for ADME properties most experimental evidence is, as for PEPT1/2 based on in vitro studies and circumstantial. In the following section, however, we'll present the in vitro–in vivo evidence for the ADME importance, collected so far, of the proton-coupled amino acid transporter, PAT1.

1.3   The Proton-Coupled Amino Acid Transporter, PAT1, as a Mediator of Intestinal Drug Absorption

In Sect. 1.3 the main focus will be on the interaction between PAT1 and gaboxadol in vitro as well as the results supporting a role of PAT1 in mediating the oral bio-availability of gaboxadol. The proton-coupled amino acid transporter, PAT1, is an amino acid transporter expressed in the luminal membrane of the small intestine

(Chen et al. 2003; Thwaites and Anderson 2011). PAT1 is a member of the SLC36 family, consisting of three additional members (Boll et al. 2003). Whereas PAT1 has been suggested to be important for the absorption of drugs such as gaboxadol, vigabatrin, and δ-ALA (Abbot et al. 2005; Broberg et al. 2012; Frolund et al. 2010; Holm et al. 2012; Larsen et al. 2009, 2010), the remaining members of the family, i.e., PAT2–4 are apparently not affecting ADME properties. PAT1 and 2 are expressed in the renal epithelium, but experiments in mice suggest that the PAT1 protein is localized inside the cell (Broer et al. 2008; Vanslambrouck et al. 2010). PAT3 is an orphan transporter, with an expression limited to the testis, whereas PAT4 is a high-affinity proline and tryptophan transporter not coupled to proton transport with a ubiquitous expression pattern (Boll et al. 2003; Pillai and Meredith 2010). The role of PAT1 in mediating the intestinal absorption of a drug compound has been investigated using gaboxadol. Gaboxadol is a GABA-A receptor agonist that has been in development for several indications such as insomnia, depression, and pain. However, the drug has not yet met the market, and we have used it as a model compound to investigate various aspects of PAT1-mediated drug transport. We first identified gaboxadol as a ligand for PAT1 based on the fact that GABA is a substrate of PAT1, and as gaboxadol is a GABA mimetic we also found gaboxadol to inhibit PAT1-mediated uptake of proline in mature Caco-2 cell monolayers (Larsen et al. 2008). That gaboxadol indeed is a substrate of PAT1 was confirmed in PAT1 expressing *Xenopus laevis* oocytes using two-electrode voltage clamp (Broberg et al. 2012; Frolund et al. 2012). These results identified that gaboxadol binds to PAT1 and that the transporter is able to translocate gaboxadol from outside of the cellular membrane into the cytosol of the cell. However, this does not implicate that the interaction is important neither in vitro nor in vivo. The total transport across a biological barrier, e.g., the intestine, is a sum of carrier-mediated and passive transport, and if passive transport is much faster than the carrier-mediated, the influence of the carrier net transport will be minimal. Furthermore, it is important to consider if there are other carriers at play, to evaluate if the capacity of the carrier is high enough considering the dose (and resulting concentration) of the drug in question, and to assure that the carrier is actually expressed in the segment of the intestine where absorption take place. We therefore measured the bidirectional transport of gaboxadol across Caco-2 cell monolayers. The permeability was measured at several concentrations; one being the human dose (20 mg) divided 250 mL, i.e., 0.3 mM. The transport of gaboxadol was highly polarized in the absorptive direction with an absorptive ratio of 5–14 (Larsen et al. 2009; Frolund et al. 2012). Furthermore, the permeability coefficient decreased with increasing donor concentration, indicative of a slight saturation of the carrier. As PAT1 is proton-coupled the apical to basolateral transport was significantly reduced in the absence of a pH difference between the donor chamber and the receiver chamber (Larsen et al. 2009). Furthermore, the presence of the PAT1 inhibitor L-tryptophan (L-Trp) decreased absorptive transport, and the basolateral to apical transport was similar to the apical to basolateral transport when the proton gradient was removed (Larsen et al. 2009). Collectively, these in vitro results suggest that in intestinal Caco-2 cells the absorption of gaboxadol is mainly carrier-mediated and that the main carrier is PAT1.

This is consistent with gaboxadol being relatively hydrophilic with a $\log D_{7.4}$ of -2.4 (value from our lab). Gaboxadol is almost completely absorbed from the intestine in human, rat, and dog with an absorption fraction above 0.8. This indicates that PAT1 could be important in intestinal absorption of gaboxadol. We performed absorption studies in rat and dog in order to verify this hypothesis and to generate knowledge about the in vitro to in vivo correlation. When gaboxadol was given orally to beagle dogs with increasing concentrations of L-Trp, the absorption rate constant, k_a, was decreased in an L-Trp concentration-dependent manner (Larsen et al. 2009). This decrease was not related to altered gastric emptying and not related to altered clearance since the elimination rate constant, k_e, also remained cosnstant (Larsen et al. 2009). The total gaboxadol absorption fraction also remained constant in the dosing groups containing increasing doses of L-Trp. Since gaboxadol is a substrate for PAT1 and L-Trp an inhibitor (Metzner et al. 2005) these results are circumstantial evidence for a role of PAT1 in mediating the absorption of gaboxadol. But questions, related to why the absorption fraction wasn't reduced and what the absorption window was, prompted us to do further studies. We then performed an experiment in rats where catheters were operated into three different segments of the intestine; the duodenum, the ileum, and the colon (Broberg et al. 2012). Gaboxadol was administered directly into these segments in the absence or presence of a mixture of L-Trp and L-proline (L-Pro) (Broberg et al. 2012). Gaboxadol was well absorbed from the duodenum and the ileum with absorption fraction above 0.8, but there was hardly any absorption from the colon (F_a of 0.04) (Broberg et al. 2012). The expression of PAT1 mRNA along the length of the rat intestine was measured, and in segment with high gaboxadol absorption there was PAT1 expression, whereas limited expression was observed in the rat colon (Broberg et al. 2012). In the duodenum and ileum coadministration of L-Pro and L-Trp markedly decreased the initial plasma concentration of gaboxadol, and after oral administration and administration into the jejunum the maximal plasma concentration was lowered and the time to reach this concentration increased (Broberg et al. 2012). Using in vitro and in vivo methods it seems that gaboxadol is bioavailable due to its direct interaction with PAT1, which mediates the intestinal absorption across the luminal membrane of the small intestinal enterocytes.

1.3.1 Transporter Overlap and Interactions: Implications for ADME

Drug substances are in many cases substrates for more than one type of transporters, and this will influence the overall ADME properties of the compound depending on the expression pattern of the transporters in different tissues. As described above, cefadroxil is a substrate for PEPT1 which mediates the intestinal absorption while PEPT2 mediates the renal reabsorption. The two different transporters thus cooperate in defining the plasma concentration–time profile of cefadroxil (Shen et al. 2007;

Bretschneider et al. 1999), and PEPT2 seems to affect the disposition into the cerebrospinal fluid (Shen et al. 2007). As mentioned above δ-ALA has been identified as a substrate for PEPT1 and PEPT2 (Doring et al. 1998), and it has been shown that this has implications for excretion and disposition of the drug substance. Recently, we have shown that δ-ALA is a substrate for an additional intestinal transporter, i.e., PAT1 (Frolund et al. 2010). PEPT1 and PAT1 are both expressed in the small intestine and in Caco-2 cells. They seem to be the only two transporters in mediating the intestinal absorption of δ-ALA (Frolund et al. 2010). δ-ALA is thus a substrate for both a peptide and an amino acid transporter. In the case of gaboxadol it was possible to show that some dipeptides, besides being substrates for PEPT1, could act as inhibitors of PAT1-mediated transport (Frolund et al. 2012).

This illustrates how nutrients are able to interfere with transporter-/carrier-mediated drug absorption. Likewise, it is possible that drugs in multiple dosing regimens may, either positively or negatively, modify/alter carrier-mediated intestinal absorption, renal excretion, or even drug disposition.

1.4 Transporter Overlap May Influence Absorption of Organic Anions

Drug substances that are fully organic anions at physiological blood pH (pH $\approx$ 7.4) and intestinal fluid pH (pH $\approx$ 4–7.8) are more prone to rely on transporters for their cellular influx and/or efflux than neutral substances. Consequently, impact of transporters on ADME and possible drug–drug interactions (DDI) between anionic drug substances/NCE on transporters are noticeable. Furthermore, several anionic drug substances seem to rely on more than one transporter for their intestinal absorption as well as hepatic and renal excretions.

Although DDI on- and overlap between hepatic transporters (and also between transporters and enzymes) for organic anions are well accepted (see Chap. 9), less is known about the corresponding intestinal transporters' role on absorption (Estudante et al. 2012). In the present section we provide evidence for interplay between intestinal transporters for organic anions and speculate on how such interplay may influence absorption of anionic drug substrates.

1.4.1 Overlap Between Intestinal Transporters Carrying E_1S

The overlap between intestinal transporters has been studied in vitro in Caco-2 cells by using the organic anion E_1S as probe. The structure of E_1S is shown in Chap. 2, Fig. 2.1. E_1S is present as its organic anion (sulfonic acid; pK_a of approximately 2.2) at physiological blood- and intestinal fluid pH (Gram et al. 2009a). It is therefore assumed that pH partitioning of the neutral species of E_1S can be neglected.

Thus it is the anionic species of this endogenous estrogen metabolite which is prone to rely on transporters and which is substrate for many transporters in the body (see Table 2.1 in Chap. 2). Transporters, which seem to also carry many anionic drug substances, therefore may play an important role in ADME.

In the present context, transporters for organic anions are defined as those carrying the E_1S probe. In hepatocytes and enterocytes, these transporters encompass organic anion transporting polypeptides OATP(SLCO)1A2/2B1/1B1 /1B3/3A1/4A1; organic solute transporter OSTα/β; breast cancer resistant protein BCRP(ABCG2) and multidrug resistant proteins MRP(ABCC)1/2; Na^+ taurocholate cotransporting polypeptide NTCP(SLC10A1), organic anion transporter OAT(SLC22)2/7, and multidrug and toxin MATE1 (SLC47A1) (Kullak-Ublick et al. 2001; Suzuki et al. 2003; Sai et al. 2006; Tamai et al. 2000; Ballatori et al. 2005; Qian et al. 2001; Spears et al. 2005; Ho et al. 2004; Shin et al. 2007; Kobayashi et al. 2005; Tanihara et al. 2007). Tissue and subcellular localization and transport direction for these transporters in intestine and liver are shown in Fig. 1.1.

Apparent permeability (P_{APP}) of E_1S has been studied across filter-grown Caco-2 cell monolayer in both absorptive (A–B) and exsorptive (B–A) directions. Much larger exsorptive than absorptive P_{APP} of E_1S was observed with a net exsorption (efflux ratio/ER) in the range of 7–12 (Gram et al. 2009a; Grandvuinet and Steffansen 2011; Rolsted et al. 2011). This overall vectorial P_{APP} of E_1S indicates transporter-mediated P_{APP} across the cell monolayer. In order to study the transporters involved, we investigated both the apical and basolateral linear uptake/ influx permeabilities (P_{UP}) in filter-grown cells from either ATCC grown for 25–28 days or DSMZ grown for 11–14 days. The DSMZ Caco-2 has maximal transepithelial electrical resistance (TEER) at 11–14 days of cultivation and E_1S uptake is similar from 6 to 25 days of cultivation whereas ATCC has maximal TEER of 21–28 days at which E_1S uptake is stable. The apical E_1S P_{UP} in DSMZ was generally twofold the P_{UP} in ATCC, but otherwise the systems seemed similar when studying E_1S (Grandvuinet and Steffansen 2011; Gradvuinet et al. 2013).

From the apical site, linear E_1S influx permeability P_{UP} was measured to 5.46 (±0.60)•10^{-6} cm/s. The P_{UP} was inhibited by the OATP2B1 inhibitor fluvastatin while the OATP1A2 inhibitor dexamethasone unexpectedly increased E_1S P_{UP} by approximately twofold (Grandvuinet et al. 2013). A similar observation has recently been described by Koenen et al., who suggest that dexamethasone may have a strong stimulatory effect on OATP2B1 (Koenen et al. 2012). However, when the cells were grown on solid plastic dishes we saw no effect of dexamethasone on E_1S P_{UP}, which we apparently can't explain. Despite this, the apical influx studies imply that OATP2B1 and not OATP1A2 is mediating E_1S apical influx in Caco-2 cells. This is further confirmed by the K_m for apical E_1S influx, in Caco-2 cells, which is determined to 9.9 μM (6.41–15.3) and previously 23 μM (13–40) since these K_m values are, respectively, within the range and close to the K_m value range determined for the E_1S influx in OATP2B1-transfected systems, i.e., 1.6–21 μM. For references see Grandvuinet et al. (2012).
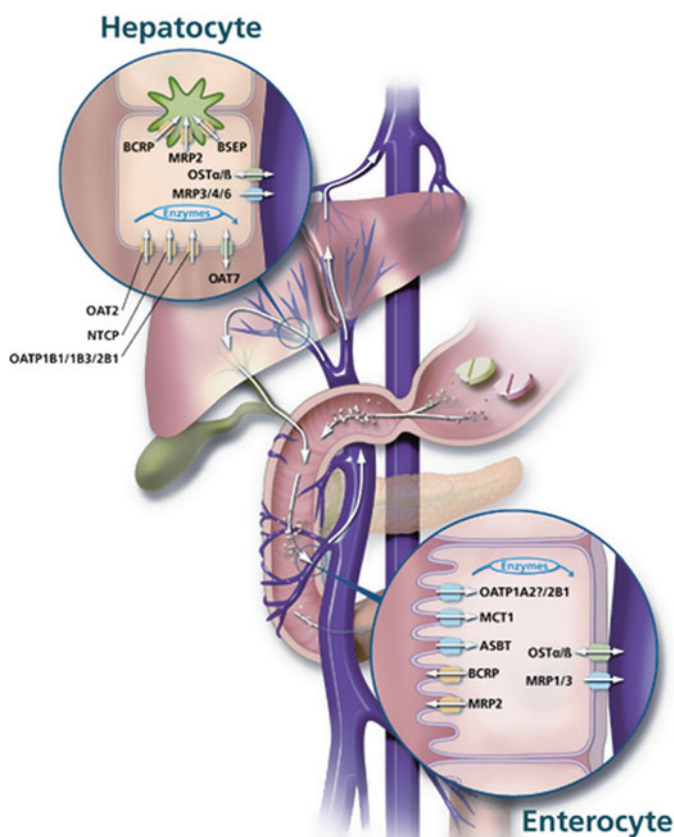
Fig. 1.1 Membrane transporters for organic anions, that in human intestine and liver, carry the probe E_1S. Only transporters that have been determined at protein level and where the subcellular localization and transport direction in the particular human tissue are known have been included in this figure. OATP1A2 has been marked with a question mark due to conflicting reports on its intestinal expression (Grandvuinet et al. 2012). Transporters depicted in *blue* are absorptive transporters. Transporters depicted in *yellow* are exsorptive/secretory transporters. Transporters in *green* are bidirectional transporters. *BCRP* breast cancer resistance protein, *MRP* multidrug resistance-associated protein, *OAT* organic anion transporter, *OATP* organic anion transporting polypeptide, *OSTα/β* organic solute transporter α/β. Redrawn with permission from illustrator Henning Dalhoff

Although inconsistent reports on the proton coupling of OATP2B1, it has been demonstrated by others that OATP2B1-mediated E_1S influx is pH-dependent. To confirm that OATP2B1 is responsible for the functional E_1S apical P_{UP}, we showed that the influx indeed was pH-dependent in the pH range 5–7.4 (Grandvuinet and Steffansen 2012). Thus these studies provide evidence that OATP2B1 is responsible for the apical influx of E_1S in filter-grown Caco-2 cells.

From the basolateral site the P_{UP} of E_1S influx was about three to fourfold higher (17.5 ± 3.1 in ATCC and 24.1 ± 1.77 in DSMZ 10^{-6} cm/s) than at the apical membrane (Rolsted et al. 2011; Grandvuinet et al. 2013). In enterocytes, OSTα/β is the only known basolateral transporter for organic anions and mRNA of this transporter have been identified in Caco-2 cells in several laboratories, including ours (Hayeshi et al. 2008; Ming et al. 2011; Li et al. 2012). Thus we studied the influence of known OSTα/β inhibitors, i.e., the bile acid taurocholic acid (TCA) and drug substance digoxin, on P_{UP} of E_1S and indeed showed that the P_{UP} was inhibited by these compounds (Grandvuinet et al. 2013). Basolateral P_{UP} of E_1S in Caco-2 cells has been shown to be saturable with K_m values determined to 44 μM (33–64) and 11.2 (6.60–18.9) in ATCC and DSMZ Caco-2, respectively. However these K_m values are lower than the K_m of 320 μM determined, by others, in OSTα/β-transfected oocytes (Seward et al. 2003). We are not able to explain this discrepancy and can thus not exclude that other unknown basolateral transporter(s) may be involved in E_1S influx at the basolateral membrane of Caco-2 cells.

In order to investigate involvement of efflux transporters we loaded the cells with E_1S followed by measuring its efflux to the apical and basolateral sites of the cells. Similar efflux of E_1S was observed at both sites; however, when adding the BCRP inhibitor fumitremorgin C or the MRP2 inhibitor PAH, after the E_1S loading, fumitremorgin C, but not PAH, reduced the apical efflux of E_1S. None of the inhibitors influenced the basolateral efflux but intracellular retention of E_1S was increased in the presence of fumitremorgin C. These studies imply that E_1S in Caco-2 cells is effluxed to the apical site by BCRP but not by MRP2. A possible explanation to this may be none or minor MRP2 expression in the cells. We then investigated if the OSTα/β inhibitor TCA would influence the basolateral efflux of E_1S; however, we were not able to show any effect of TCA. We therefore loaded the cells with TCA and investigated its efflux to both apical and basolateral sites and showed that basolateral efflux was significantly higher than apical which may indicate that TCA is effluxed by OSTα/β at the basolateral membrane. To further confirm this hypothesis we added spironolactone that is a known OSTα/β inhibitor and could show that it did increase the intracellular retention of TCA whereas this effect could not be confirmed by a decrease in basolateral efflux of TCA (Grandvuinet et al. 2013).

Based on these studies of E_1S in Caco-2 cells we imply that there is overlap between the apical influx transporter OATP2B1, the apical efflux transporter BCRP, as well as basolateral bidirectional transporter OSTα/β in E_1S P_{APP} across these cells.

1.4.2 Apical Influx Transport Is Implied to be Rate Limiting for Absorptive E_1S P_{APP} in Caco-2 Cells

As mentioned above, E_1S loading cells effluxed E_1S in similar amounts to apical and basolateral sites. Consequently it is not possible from these initial results to determine whether the apical (BCRP) or basolateral (possibly OSTα/β) is rate limiting. The rate limiting transporter for E_1S P_{APP} in both absorptive and exsorptive

Fig. 1.2 Illustration of the rate limiting transporter in permeability (*dashed arrows*) of the probe E_1S (in *red*) across Caco-2 cells
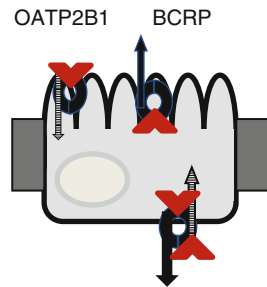
directions we have however simulated from the capacity (J_{max}), affinity (K_m), and intracellular retention of E_1S. Based on these studies, we suggest the apical influx transporter (OATP2B1) and the basolateral influx transporter (OSTα/β) to be rate limiting for E_1S P_{APP} in respective absorptive and exsorptive directions as illustrated in Fig. 1.2 (Rolsted et al. 2011). Thus by characterizing the overall transport direction, i.e., the polarized flux/P_{APP}, the K_m- and V_{max}-values, and intracellular retention we were able to simulate for each direction which transporter is rate limiting for E_1S.

1.4.3   A Number of (Drug) Substances/NCE Permeate Caco-2 Cells via Multiple Transporters

A number of substances were then investigated for their possible influence on E_1S permeability and intracellular retention in Caco-2 cells. Well known intestinal transporter overlap is between the apical sodium bile transporter (ASBT) and OSTα/β, both of which are transporting bile acids such as TCA in Caco-2 cells, resulting in much larger absorptive than exsorptive TCA P_{APP}. TCA and E_1S interact on basolateral influx transporter since TCA decreases basolateral influx intracellular retention and exsorptive permeability of E_1S, probably at OSTα/β (Grandvuinet and Steffansen 2011). Other transporter overlap in Caco-2 cells is seen for fluvastatin P_{APP}. Fluvastatin is a known BCRP and OATP2B1 inhibitor (Hirano et al. 2005; Noe et al. 2007). However, it also decreased basolateral P_{UP} intracellular retention, as well as decrease exsorptive P_{APP} of E_1S (Grandvuinet et al. 2013). Thus it is suggested that there may be overlap between BCRP, OATP2B1, and OSTα/β in fluvastatin absorption. Whether this has clinical relevance is yet to be investigated.

For drug substances/NCEs, whose absorption is dependent on OATP2B1 and which at the same time are substrate for BCRP and/or other apical efflux transporters, one would expect restricted absorption since the amount of the substance which enters the cell by OATP2B1 would be expected to be efficiently effluxed/exsorbed by BCRP, although dependent on the dose, K_m, and V_{max} for the substrate at the involved transporters.

Related to this hypothesis is the restricted intestinal absorption of the anionic drug substance sulfasalazine. Even though sulfasalazine is absorbed by OATP2B1, it is

exsorbed by both MRP2 and BCRP which is suggested to be the reason for its overall restricted intestinal absorption (Kusuhara et al. 2012; Dahan and Amidon 2009).

In contrast, if compound apart from being substrate for both BCRP and OATP2B1 is also substrate to basolateral OSTα/β-mediated efflux, one may speculate the overall absorption to be less restricted, since the efflux of intracellular amount of compound, in this case, is a competition between apical exsorption by BCRP and basolateral absorption mediated by OSTα/β efflux.

Interplay between OSTα/β and BCRP in rosuvastatin P_{APP} across Caco-2 cells and BCRP-MDCK has recently been suggested by Li et al., who showed net exsorption (efflux ratio/ER) of rosuvastatin at 83 and 5.8 in Caco-2 and BCRP-MDCK cells, respectively (Li et al. 2012). They suggested the reason for the large difference in ERs to be basolateral OSTα/β-mediated uptake of rosuvastatin in Caco-2 whereas absent in BCRP-MDCK. Rosuvastatin (acidic pK_a 4.2–4.6) is also substrate for OATP2B1 (Varma et al. 2011). Intestinal absorption of rosuvastatin in the in vivo situation may be different from in vitro (Caco-2) since the balance between the expression levels of the three involved transporters is likely to be different in vivo. Thus the authors suggest that rosuvastatin absorption to be mediated by both OATP2B1 in the apical membrane and OSTα/β in the basolateral. Due to efficient intestinal mesenteric blood flow one may expect the facilitated OSTα/β transporter to mediate its substrate permeability primarily in the absorptive direction in the in vivo situation even though the opposite may be observed in vitro.

Other example of overlap between intestinal transporters in Caco-2 cells is for the high permeability drug candidate A275 since increased retention of A275 was observed from both apical and basolateral sites when E_1S was co-administered, indicating that E_1S and A275 may compete for the same efflux transporters at apical (BCRP) and basolateral membranes (OSTα/β) (Gram et al. 2009b). When E_1S was co-administered with A275 to rats (i.v. and oral), E_1S clearly prolonged the absorption fraction (F_a) of A275. Whether this delay of A275 absorption was due to overlap/interplay between intestinal transporters in vivo or whether hepatic transporters were also involved could not be elucidated from the study (Gram et al. 2009b).

In conclusion transporter-mediated intestinal absorption of substrates seems to be dependent on the dose related to the kinetic parameters (K_m, J_{max}), transport direction, and substrate overlap between the involved transporters.

1.5 The Role of Blood–Brain Barrier Membrane Transporters in Determining Drug Distribution to the Brain

The BBB is one of the most restrictive barriers in the body, and a barrier where transporter function is determining CNS bioavailability of drug compounds. The BBB is the interface between the plasma and the brain interstitial fluid. The barrier controls the movement of solutes between the cells, in order to maintain a stable
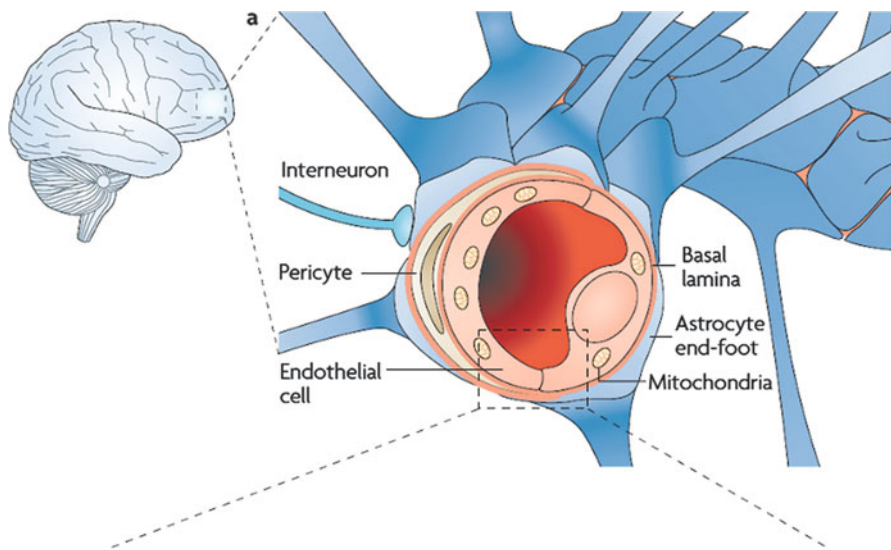
Fig. 1.3 Anatomy of the neurovascular unit (NVU). Endothelial cells form the walls of the capillaries. The endothelium is partly covered with pericytes embedded in the basement membrane at the abluminal side of the endothelium. The endothelium and pericytes are ensheathed in astrocytic endfeet. Neuronal contacts innervate the NVU. Reprinted from Begley (2004) by permission from Macmillan Publishers Ltd: Nature Reviews in Drug Discovery, Copyright 2007

environment in the brain allowing for optimal neuronal signalling and brain function. The BBB is a major hindrance for CNS drug delivery, since most drugs or drug candidates do not permeate the barrier to a significant extent.

The barrier function resides in the endothelial cells of the brain neurovascular unit (NVU). The endothelial cells of the brain capillaries are linked together via tight junctions (TJ). The capillary tubes are partly covered with pericytes, and surrounded by astrocytic endfeet as illustrated in Fig. 1.3. The capillaries may be innervated, and microglia may also be in direct contact with the NVU (Fig. 1.3).

The selectivity of the endothelial barrier is caused by a range of uptake and efflux transporters in the luminal and abluminal membranes (Fig. 1.4), by the tight junctions which are effectively controlling paracellular transport of even rather small hydrophilic molecules and by metabolizing/conjugating enzyme systems within the endothelial cells.

1.5.1 *Exsorptive Efflux Transporters in the Brain Endothelium*

The efflux transporter activity of the brain endothelial cells is a major player in determining CNS distribution of a number of drug compounds. P-glycoprotein (ABCB1) has traditionally been considered the most important; however, a large
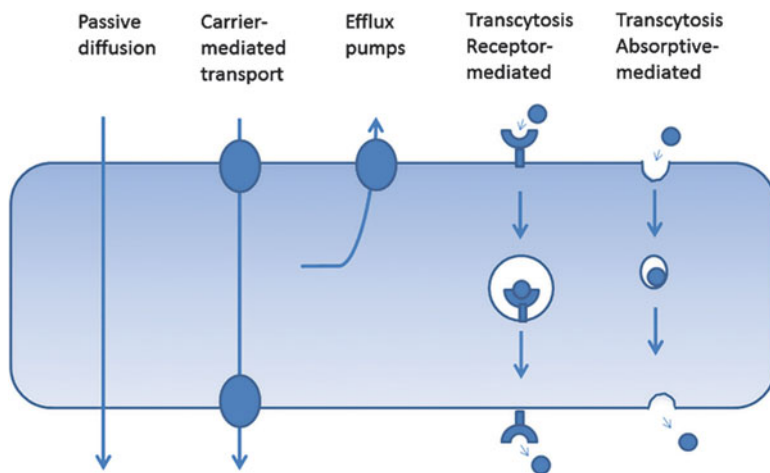
Fig. 1.4 A schematic overview of the transport pathways in the brain endothelial cells. Small (<400 Da) lipophilic compounds may permeate the endothelial barrier via passive diffusion. Nutrients and certain nutrient-like drug compounds may be transported from the blood to the brain interstitial fluid via carrier proteins in the luminal and the abluminal membranes. Efflux transporters of the ABC-type may hinder uptake of compounds from the blood, and may also participate in efflux of substances from the brain interstitial fluid. Compounds may also be transported via receptor-mediated transcytosis or adsorptive-mediated transcytosis (Begley 2004)

number of efflux transporter families have been identified in the endothelial cells, both on the luminal and the abluminal membrane.

Tanaka et al. showed that P-glycoprotein (P-gp) was expressed in brain endothelial cells (Tanaka et al. 1994). Schinkel et al. generated a mouse strain with a dysfunctional P-gp, and observed increased brain levels and toxicity of drugs after dosing, as compared to normal mice (Schinkel et al. 1994). P-gp is now recognized as a transporter of xenobiotics and has a major role in determining blood/brain distribution of drug substances by luminal efflux. Thus, P-gp knockout mice and cell lines transfected with the human P-gp are now standard tools in drug discovery and development.

Another important exsorptive transporter, breast cancer resistance protein (ABCG2), is also expressed in brain endothelial cells (Cooray et al. 2002). Although P-gp has been considered the dominant efflux transporter at the BBB, recent proteomics data indicate that in humans, the absolute expression levels of BCRP are larger than those of P-gp, implying that BCRP may play a larger role than previously anticipated (Uchida et al. 2011). It appears that the ratio between P-gp and BCRP expression differs from rodents to humans, a fact that needs to be taken into consideration when data from rodent models are used to predict drug distribution to the human brain.

An additional class of efflux transporters present in the endothelium is the MRP family of transporters. MRP1–5 (ABCC1–5) have been detected in the BBB (for references, see Abbott et al. 2010). The subcellular localization of the MRPs is still

debated; however, the presence of MRP4 at the luminal membrane has been clearly established (Roberts et al. 2008; Nies et al. 2004).

Overall, the efflux ABC transporters of the BBB endothelium contribute to the barrier function by the active, ATP-dependent efflux of a large range of xenobiotics, and to a large degree determine brain distribution of compounds which in theory possess the physicochemical properties necessary for passage of a lipophilic membrane environment. DDI may occur at the BBB, when two efflux transporter substrates are co-administered. It has been shown in mice that coadministration of, for example, the P-gp substrates, nortriptyline and verapamil, increased the brain-serum ratio of nortriptyline by 60 % (Ejsing et al. 2006). However, clinical evidence is still sparse and DDI at the transporter level can be hard to distinguish from interactions on metabolizing enzyme, due to the substrate overlap between P-gp and the CYP-enzymes (Linnet and Ejsing 2008).

1.5.2 Absorptive Transporters in the Brain Endothelium

The absorptive transporters of the brain endothelium play an important role in supplying essential nutrients and micronutrients to the brain. Due to their capacity they are also interesting from a drug delivery viewpoint; however, their drug delivery potential is limited to a certain degree by their substrate specificities. A large number of uptake transporters are expressed and some important examples will be listed below.

Glucose is the major energy source for the brain. Glucose is taken up from the blood and transported into the brain via Glut-1(SLC2A1), a facilitative hexose transporter. Glut-1 is situated both at the luminal and abluminal membranes of the endothelium and the transport will therefore be driven by the transendothelial glucose gradient. Glut-1 has furthermore been implicated in the absorption of L-dehydroascorbic acid, but overall has a rather restrictive substrate profile.

Ketone bodies may also be important energy sources for the brain, especially during periods of starvation or periods on high fat diets. Ketone bodies are taken up via the monocarboxylate transporters (MCTs) (Gjedde and Crone 1975). MCT1 (SLC16A1) has been found to localize both at luminal and abluminal membranes. MCT1 acts either as a proton-coupled uptake transporter or as an exchanger with monocarboxylates. MCT1 can also transport lactate and pyruvate and thus play other roles in brain energy metabolism than simply being an import system for ketone bodies (Uhernik et al. 2011).

Large neutral amino acids are transported across the luminal membrane via the large neutral amino acid transporter, LAT1 (Boado et al. 1999). LAT1 (SLC7A5) is an amino acid exchanger which associates with a heavy subunit, 4F2hc (SLC3A2). LAT1 has attracted a lot of attention due to the relatively broad substrate specificity of the transporter. The transporter mediates uptake of L-DOPA and thyroid hormones, as well as the uptake of baclofen and gabapentin has been attributed to this transport pathway (for references, see Del Amo et al. 2008).

A number of other absorptive transporters are expressed at the BBB, including a range of amino acid transporters, organic anion transporters such as OATP2B1, and nucleoside transporters (for references, see Abbott et al. 2010; Ohtsuki and Terasaki 2007).

The absorptive transporters may constitute uptake pathways for CNS drug substances, as exemplified above. The absorptive transporter pathways are however fairly restricted since drug substances must mimic endogenous- or nutrient substrates and transporters of the substrate must be present at both the luminal and the abluminal membrane, in order to facilitate drug distribution into the CNS. Furthermore, distribution of drug via BBB transporters may influence transport of endogenous substrates, and may thus demand careful preclinical evaluation. The absorptive transporters do, however, constitute a pathway for brain distribution which may be exploited in drug discovery programs.

1.5.3   Challenges in Investigating Transporter-Mediated BBB Distribution In Vitro

The distribution of new drug substances and NCEs to the brain has typically been evaluated in expensive and time demanding animal experiments. However, a number of cell culture approaches have been undertaken, in order to generate predictive in vitro models. MDCK-MDR1 cell lines as well as Caco-2 cells grown in confluent monolayers are used to screen drug candidates for interaction with P-gp using simple bidirectional transport experiments or inhibition of P-gp-mediated efflux of fluorescent probes (Hakkarainen et al. 2010; Eriksson et al. 2012). However, these cell lines do not express the absorptive transporters present in the brain capillary endothelium. A number of attempts have been made to generate immortalized cell lines of brain endothelial cells from a variety of species. These cell lines are, however, not able to generate the same tightness as observed in native tissue. Primary endothelial cell lines, cocultured with astrocytes, do however generate tight monolayers. We recently succeeded in generating an extremely tight in vitro coculture model, based on bovine endothelial cells, cocultured with rat astrocytes (Helms et al. 2010).

The model expresses the endothelial cell marker Von Willebrands factor as well as the tight junction protein Claudin-5 which is considered to be responsible for the tightness of the BBB for small molecules. The cultured endothelial cells furthermore expressed P-gp and BCRP, as well as a number of BBB marker proteins. The tightness of the in vitro model enables bidirectional transport studies of small molecular weight substances (<1,000 Da), as exemplified in a recent study where we analyzed bidirectional fluxes of the excitotoxic amino acid glutamate and were able to show that the BBB effluxes glutamate from brain to blood (Helms et al. 2012). Porcine in vitro coculture models have been used for a decade (Franke et al. 2000) and recent reports indicate that the porcine cocultures also can reach a high

resistance and express a number of the BBB-specific marker proteins (Patabendige et al. 2012). Although the bovine and porcine coculture models are robust and predictive, they are also work-demanding and one cannot exclude species differences in transporter expression and function. A lot of attention is therefore presently being devoted to the generation of in vitro cell culture models generated from human stem cells (Lippmann et al. 2012), and future characterizations of these types of models will show whether they may be useful in preclinical evaluation of brain distribution of drug substances.

1.5.4 Perspectives and Future Directions for Research in the Role of Blood–Brain Barrier Transporters in Drug Distribution to the Brain

The transporters of the BBB play a pivotal role in determining BBB permeability and thus CNS bioavailability of drug compounds. However, we still lack basic knowledge in the field. Although we are beginning to grasp a picture of the expression profile of transporters under physiological conditions, investigations are needed in order to analyze transporter expression and function under pathophysiological conditions, induction of transporters by therapeutic agents, as well as changes in functions during aging.

With regard to employing the absorptive transporters as drug delivery routes by means to CNS distribution, we need defined structure–translocation analysis of the transporter systems, in order to know the structural space for drug design that will allow barrier penetration. And regarding efflux transporters, we need more detailed knowledge in order to be able to predict drug–transporter interactions which may limit or enhance uptake. Hopefully, the advances within the field of in vitro models of the BBB will provide us with the experimental tools necessary for investigating structure–translocation relationships and DDI in a controlled setting.

1.6 Conclusion and Future Perspectives

In the present chapter we have described the role of membrane transporters in ADME by exemplifying how the proton-coupled transporters PEPT1, PAT1, and OATP2B1 expressed in the small intestine may influence intestinal absorption of drug substrates and also how intestinal and renal PEPT1/2 or PAT1/2 may retain central blood concentrations of their substrates by a combination of transporter-mediated intestinal absorption and renal reabsorption. We described the challenges of developing reliable in vitro models for especially the BBB. Transporter overlap is illustrated in the case of PAT1 and PEPT1, where δ-ALA is transported across the apical membrane of Caco-2 cells by both transporters. In the case of E_1S, it is

demonstrated that several compounds inhibit E_1S via multiple transporters such as OATP2B1, BCRP, and OSTα/β. Such dual or triple inhibition of E_1S implies that overlap is common on these transporters. Interpretation of overlap is however challenging, especially translation to the in vivo situation. In the future it seems relevant to further develop in vitro methods for the major organs/tissues in ADME to combine simulation and experimental methods by means to interpret the importance of transporters in ADME at an early state in development. Such combined approaches are under development by SimCyp and GastroPlus, not only at single tissue/organ level but at whole body level in which the major tissues/organs of importance to ADME are included in simulation programs. Such simulations are further described in Chap. 12.

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Chapter 2
In Vitro Kinetic Characterization of Transporter-Mediated Permeability

Bente Steffansen and Anne Sophie Grandvuinet

Abstract Permeability studies across cells or tissue are often applied to investigate for permeability being rate limiting in bioavailability. In addition permeability of drug substances/candidates is investigated in order to identify transport mechanism across the cells or tissues, i.e., by passive diffusion and/or by membrane transport proteins. Characterization of transport mechanism is important in order to elucidate if permeability could be altered/dose-dependent as drug substance/candidate is interacting on saturable transporters. In the present chapter is described how especially transporter-mediated permeability may be studied in vitro exemplified by using E_1S as probe. Furthermore, it is speculated upon if E_1S can be used as biomarker, in vivo, for the identification of possible clinical effects of drug substances/candidates interacting on transporter(s) which E_1S is substrate for and it is suggested that future studies should elucidate such possibilities.

Abbreviations

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ADME	Absorption, distribution metabolism and elimination
BCRP	Breast cancer resistance protein
C_0	Initial donor concentration
DDI	Drug–drug interactions
E_1	Estrone
E_1S	Estrone-3-sulfate
E_2	Estradiol
[I]	Inhibiting drug substance/candidate concentration

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IC_{50}	Drug substance/candidate concentration at which 50 % inhibition occurs
I_{max}	Maximal inhibitory effect of the drug substance/candidate
IVIVC	In vitro–in vivo correlation
J	Flux
J_{max}	Maximal carrier-mediated flux
K_i	Inhibitory affinity constant
K_m	Michaelis–Menten-derived substrate affinity constant
MATE 1/2-K	Multidrug and toxin extrusion 1/2-K
MRP 1/2	Multidrug resistance protein 1/2
NTCP	Na^+/taurocholate cotransporting polypeptide
OAT 3/4	Organic anion transporter 3/4
OATP 1A2/1B1/1B3/2B1/3A1/4A1	Organic anion transporting protein
OSTα/β	Organic solute transporter α/β
OSTα/β	Organic solute transporter α/β
P_{APP}	Apparent transcellular permeability
P_{Pas}	Passive permeability
P_{UP}	Uptake permeability
SLC	Solute carrier
SOAT	Sodium-dependent organic anion transporter

2.1 Introduction

In the present chapter, in vitro kinetic characterization of transporter-mediated permeability in cells is described and exemplified by estrone-3-sulfate (E_1S). Whether E_1S could be used as a biomarker in vivo for the identification of possible drug–drug interactions (DDI) on transporters, that E_1S is a substrate to, is also speculated upon.

E_1S is the endogen sulfonic acid ester of estrone ([1]E_1). The chemical structure of E_1S is shown in Fig. 2.1. E_1S is negatively charged at pH-values of the intestinal
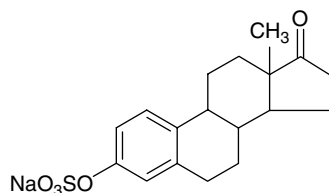
Fig. 2.1 Molecular structure of E_1S

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[1] Refers to the single hydroxyl functionality in the third position of the steroid molecule. Thus, the abbreviation E_1 is used to differentiate E_1 from the other estrogens, estradiol (E_2) and estriol (E_3), which contain 2 and 3 hydroxyl functionalities, respectively. The sulfonic acid ester of E_1 is named estrone-3-sulfate to indicate that the sulfonic acid ester is in the third position of the steroid molecule and for this reason E_1S is also sometimes abbreviated as E_3S. In the present chapter, however, the abbreviation E_1S is applied.

fluid and plasma due to its sulfonic acid functional group with pK_a 2.2 (Gram et al. 2009).

For this reason E$_1$S is believed to be primarily transported across cell membranes by membrane transport proteins. E$_1$S is shown to be substrate for several membrane transport proteins in the body including BCRP (ABCG2) (Hirano et al. 2005; Imai et al. 2003; Karlsson et al. 2010; Suzuki et al. 2003), MRP1 (ABCC1) (Qian et al. 2001; Nunoya et al. 2003), MRP2 (ABCC2) (Spears et al. 2005), OATP1A2 (SLCO1A2) (Spears et al. 2005; Bossuyt et al. 1996; Kullak-Ublick et al. 2001; Lee et al. 2005; Badagnani et al. 2006), OATP1B1 (SLCO1B1) (Noe et al. 2007; Gui et al. 2008; Hirano et al. 2004), OATP1B3 (SLCO1B3)(Gui et al. 2008), OATP2B1 (SLCO2B1) (Kullak-Ublick et al. 2001; Noe et al. 2007; Hirano et al. 2004, 2006; Nozawa et al. 2004; Pizzagalli et al. 2003; Sai et al. 2006; Tamai et al. 2001), OATP3A1 (SLCO3A1) (Tamai et al. 2000), OATP4A1 (SLCO4A1) (Tamai et al. 2000), OAT3 (SLC22A8) (Cha et al. 2001; Takeda et al. 2000, 2001; Ueo et al. 2005), OAT4 (SLC22A11) (Cha et al. 2000; Takeda et al. 2002), OSTα/β (OSTalpha, OSTbeta) (Cha et al. 2000; Takeda et al. 2002; Ballatori et al. 2005; Seward et al. 2003), SOAT (SLC10A6) (Geyer et al. 2007), NTCP (SLC10A1) (Ho et al. 2004), MATE1 (SLC47A1) (Tanihara et al. 2007), and MATE2K (SLC47A2) (Tanihara et al. 2007).

For this reason E$_1$S has been extensively used as a probe in the in vitro kinetic characterization of these transporters, i.e., especially in the determination of affinity constants (K_m) for the transporters that are generally in µM range. An overview of the affinity constants of E$_1$S to the transporters mentioned above is shown in Table 2.1.

E$_1$S is also used as probe to characterize inhibitor constants (K_i) for drug substances/candidates, which may interact on transporters that E$_1$S is a substrate for. This is done to investigate for possible DDI on these transporters in vitro and to evaluate if such observations may have clinical relevance (EMA 2012; US 2012).

2.2   Concepts of Flux and Permeability

In vitro kinetic characterization of membrane transporters is based on the concepts of flux and permeability. The number (S) per time (t) of a suitable probe transported across a defined area (A) of cellular/oocyte membrane is defined as the apparent flux (J) of the probe (2.1):

$$J = \frac{S}{t \cdot A} \tag{2.1}$$

Flux across a cell/oocyte membrane is typically given in units of, e.g., µmol s^{-1} cm^{-2}. Diffusional flux is generally described by Fick's first law (Fick 1855).

Table 2.1 K_m values of E_1S to the human transporters which E_1S is substrate for and transport direction for E_1S in vitro referred to as influx (in to cell) or efflux (out of the cell) or transport direction in vivo referred to as absorptive (A): direction into systemic blood circulation or exsorptive (E): direction out of systemic blood circulation

Transporter	K_m (μM)	Transport direction	References
BCRP (ABCG2) (MXR)	6.8–17	Efflux (E)	Hirano et al. (2005), Imai et al. (2003), Karlsson et al. (2010), Suzuki et al. (2003)
MRP1 (ABCC1)	0.73–2.1	Efflux (A)	Qian et al. (2001), Nunoya et al. (2001)
MRP2 (ABCC2)	ND	Efflux (E)	Spears et al. (2005)
OATP1A2 (SLCO1A2) (OATP-A)	14–59	Influx (A)	Spears et al. (2005), Bossuyt et al. (1996), Kullak-Ublick et al. (2001), Lee et al. (2005), Badagnani et al. (2006)
OATP1B1 (SLCO1B1) (OATP-C)	0.23–2.4	Influx (A)	Noe et al. (2007), Gui et al. (2008), Hirano et al. (2004)
OATP1B3 (SLCO1B3) (OATP8)	58	influx (A)	Gui et al. (2008)
OATP2B1 (SLCO2B1) (OATP-B)	1.6–21	Influx (A)	(Kullak-Ublick et al. (2001), Noe et al. (2007), Hirano et al. (2004), Hirano et al. (2006), Nozawa et al. (2004), Pizzagalli et al. (2003), Sai et al. (2006), Tamai et al. (2001)
OATP3A1 (SLCO3A1) (OATP-D)	ND	–	Tamai et al. (2000)
OATP4A1 (SLCO4A1) (OATP-E)	ND	–	Tamai et al. (2000)
OAT3 (SLC22A8)	2.18–7.5	Influx (E)	Cha et al. (2001), Takeda et al. (2000), Takeda et al. (2001), Ueo et al. (2005)
OAT4 (SLC22A11)	1.01–9.9	Bidirectional	Cha et al. (2000), Takeda et al. (2002)
OSTα/β (OSTalpha, OSTbeta)	320	Bidirectional	Cha et al. (2000), Takeda et al. (2002), Ballatori et al. (2005), Seward et al. (2003)
SOAT (SLC10A6)	12.0	–	Geyer et al. (2007)
NTCP (SLC10A1)	27	Influx (A)	Ho et al. (2004)
MATE1 (SLC47A1)	470	Efflux (E)	Tanihara et al. (2007)
MATE2K (SLC47A2)	850	Efflux (E)	Tanihara et al. (2007)

In order to translate flux into relevant in vivo parameters such as intestinal absorption, flux is often transformed into its corresponding apparent passive permeability (P_{Pas}). In this transformation the flux is *normalized* for initial donor concentration (C_0) of the probe according to (2.2):

$$P_{Pas} = \frac{J}{C_0} \qquad (2.2)$$

In the transformation it is assumed that the concentration gradient across the barrier is linear and constant, i.e., time-independent (sink conditions). If C_0 is given in units of μmol cm^{-3} and J in μmol s^{-1} cm^{-2}, then P_{Pas} is given in units of cm s^{-1}. Under

above-described circumstances P_{Pas} becomes constant and therefore dose-independent, i.e., independent of C_0 as illustrated in Fig. 2.3 (open symbols).

For this reason P_{Pas} determined in vitro, e.g., across filter-grown Caco-2 cells, is suggested for in vitro–in vivo correlation (IVIVC) of oral drug absorption. To circumvent variability between laboratories P_{Pas} of the investigated compound is related to internal standard of high- and low-permeability compounds such as propranolol (90 % absorption) and mannitol (16 % absorption), respectively (Artursson 1990; FDA 2000).

However, a probe may, in addition or as an alternative to passive diffusion, permeate by membrane transporter(s).

2.3 In Vitro Kinetic Characterization of Carrier-Mediated Permeability

In vitro kinetic characterization of membrane transporters is usually accomplished in cells or oocytes that express (naturally or due to transfection) the transporter(s) in question. Characterization of single membrane transporter/carrier is dependent on the carrier's preferred transport direction, i.e., whether it generally carriers the probe substrate into (influx) or out of (efflux) or both in and out of (bidirectional flux) the cell/oocyte.

Whereas passive diffusional-driven permeability (P_{Pas}) is characterized by being non-saturable and driven by electrochemical gradient(s) across the membrane(s), carrier-/transporter-mediated permeability (P_{Car}) is characterized by also being saturable. Thus P_{Pas} is generally described according to (2.1) whereas P_{Car} is described by Michaelis–Menten-like kinetics. P_{Car} is therefore characterized by the derived kinetic parameters K_m and J_{max} (Menten and Michaelis 1913) and thus described according to (2.3):

$$P_{Car} = \frac{J_{max}}{K_m + C_0} \tag{2.3}$$

in which K_m is the substrate affinity constant, J_{max} the maximal carrier-mediated flux, and C_0 the initial donor concentration of the substrate (Grandvuinet et al. 2012; Nielsen et al. 2010). When K_m is related to intestinal fluid concentration and/or maximal plasma concentration (C_{max}), the possible influence of saturable kinetics in the clinical situation, i.e., on absorption, distribution, metabolism, and elimination (ADME) and of possible DDI on the transporter(s) can be evaluated (Giacomini et al. 2010; see also Chap. 11).

2.3.1 Uptake (P_{UP}) Permeability

These kinetic parameters can be studied in vitro by the uptake permeability (P_{UP}) of a given drug probe from an extracellular donor solution into a cellular compartment. P_{UP} may depend on carrier(s) as well as on passive component(s). An influx

Fig. 2.2 Illustration of P_{UP} profile for E_1S in cells. From curve fitting K_m is estimated to 2.6 μM and P_{Pas} to $4.04 \pm 0.22 \times 10^{-6}$ cm s^{-1}. Figure with permission from Idris and El-Bajaj (2013)
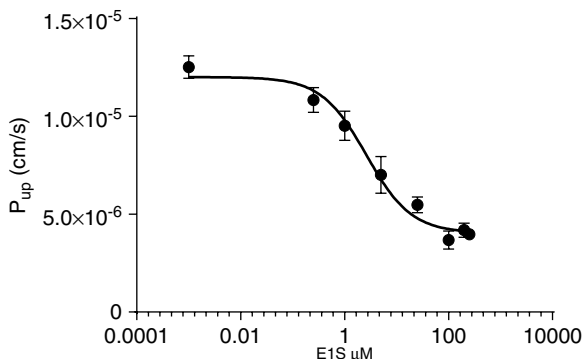
transporter whose binding site is assumed to be located in the extracellular donor environment may be studied in transfected models such as cells/oocytes that only express the human influx transporter protein of interest. Alternatively, in cells where other transporters than the probe is a substrate for, is fully inhibited or knocked down. Thus the initial linear uptake of a probe substrate is studied in a range of extracellular donor concentrations. The experimental assay can be considered as two-compartmental, i.e., the extracellular donor and the cellular receiver compartments. Since the cellular compared to extracellular (donor) compartment is small, the P_{UP} study at each donor concentration should be short (generally few minutes) in order to ensure that the concentration gradient of the probe across the membrane is maintained throughout the experiment. P_{UP} in such an assay is often composed of both a carrier-mediated component (P_{Car}) and a passive component (P_{Pas}) according to (2.4):

$$P_{UP} = P_{Car} + P_{Pas} = \frac{J_{max}}{K_m + C_0} + P_{Pas} \tag{2.4}$$

Equation 2.4 is modified from Grandvuinet et al. (2012). The passive component P_{Pas}, that may include nonspecific binding to the in vitro system, is usually determined in non-transfectants or alternatively in the presence of an inhibitor applied at a concentration that completely inhibits the translocation process of the transporter in question (Grandvuinet et al. 2012).

Figure 2.2 illustrates the profile of P_{UP} vs. the donor concentration for E_1S in which the profile is obtained by fitting experimental points to (2.4). The kinetic parameters K_m as well as P_{Pas} can then be directly estimated.

For efflux transporters it is not that straightforward to determine the kinetic parameters because the binding site is believed to be located inside the cell or in the cell membrane and the initial cellular donor concentration of the probe is difficult to measure. For probes with a low P_{Pas} it can also be a challenge to efficiently load cells if no or limited uptake transporter for the probe is expressed in the cells.

However carrier-mediated outward permeability (P_{Car}) has been estimated and the apparent $K_{m,app}$ deduced for some compounds. This is achieved by studying

probe accumulation in cells transfected with the efflux transporter and comparing accumulation in cells with the empty vector. Thus at each similar extracellular probe concentration (C_0), the difference in cellular accumulation in the transfected and the corresponding empty vector is determined. It is assumed that the concentration gradient across the cell membrane is proportional to the cellular probe concentration in the empty vector, whereas the cellular concentration in the cells transfected with the efflux transporter is kept constantly low, as long as the efflux transporter is not saturated. Consequently, the difference in cellular concentration between the two increases with C_0 until saturation at which cellular accumulation becomes proportional to C_0. In such an efflux assay, the derived apparent $K_{m,app}$ relates to the probe concentration placed on the extracellular side of the in vitro assay and not to the concentration at the cellular efflux transporter's binding site and the kinetic parameters can be derived according to (2.3).

To bypass the difficulties of the cellular binding site for efflux transporters described above, an inverted membrane vesicles assay is often employed. By this assay extracellular instead of cellular binding is obtained by opposite orientation of the efflux transporter and the donor concentration relates to the concentration at the binding site. K_m may consequently be deducted from (2.4). Many of the kinetic constants described in the literature for efflux transporters have been derived by this method. From above it becomes apparent that especially K_m values determined for efflux transporters are method-dependent and therefore variations between methods can be one explanation for differences in K_m values described in literature (for *Km-ranges* towards transporters for E_1S, see Table 2.1).

The inhibitory affinity constant K_i of a drug/candidate may be determined in a similar type of two-compartmental assay as described above. The initial uptake permeability of the probe is then measured in the absence (P_{UP}) or presence ($P_{UP,i}$) of increasing concentrations of the inhibiting drug/candidate [I] (Grandvuinet et al. 2012). When applied concentration of the probe is much lower than its K_m for the transporter, the following (2.5) modified from Grandvuinet et al. (2012), Gao et al. (2001), and Shitara et al. (2005) may be used for competitive inhibitors:

$$\frac{P_{UP,i} - P_{UP,Dif}}{P_{UP} - P_{UP,Dif}} = \frac{1}{1 + [I]/K_i} \tag{2.5}$$

2.3.2 Overall Apparent Permeability P_{APP}

The apparent transcellular permeability (P_{APP}) of a probe may be investigated in an assay, in which cells are grown to fully differentiated monolayers on Transwells®, and where the apical (A) and basolateral (B) membranes are differentiated by tight junctions (for illustration of the assay, see Fig. 9.2 column 2 row 2). The probe may then be analyzed in three compartments, i.e., the aqueous apical, the cellular, as well as the aqueous basolateral compartments. P_{APP} in this assay may be investigated in

Fig. 2.3 P_{APP} (B–A) concentration profile of E_1S across filter-grown Caco-2 cells (*filled symbols*) or in the additional presence of 0.26 mM glipizide (*open symbols*). Figure with permission from Gram et al. (2009)
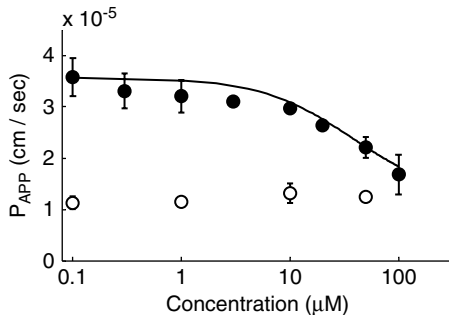
the absorptive (A–B) or exsorptive (B–A) directions in which the probe is placed in the apical or the basolateral compartments, respectively, and samples are then collected in the opposite compartment (basolateral and apical compartments, respectively) at sink conditions.

In Fig. 2.3 is illustrated the exsorptive permeability ($P_{APP, BA}$) of the probe E_1S measured at several concentrations (filled symbols). Similar assay has to be used to determine the K_m of an apically located efflux transporter. The K_m can then be derived for B–A permeability:

$$P_{APP,BA} = P_{Pas,BA} + P_{Car} = P_{Pas, BA} + \frac{J_{max}}{K_m + C_0} \qquad (2.6)$$

Similarly derived from absorptive permeability $P_{APP, AB}$:

$$P_{APP,AB} = P_{Pas,AB} - P_{Car} = P_{Pas,AB} - \frac{J_{max}}{K_m + C_0} \qquad (2.7)$$

In Fig. 2.3 is illustrated how the P_{Pas} can be derived from the fully inhibited transporter (open symbols).

The EMA draft guideline on investigation of drug interactions and the FDA draft guidance for industry on drug interaction studies recommend the permeability to be determined in both absorptive and exsorptive directions (EMA 2012; US 2012). For intestinal transport EMA suggests the study should be made at least in four different physiologically relevant concentrations, the range could be 0.1–1-fold the dose/250 mL unless solubility limits the concentration range (EMA 2012). The efflux ratio (R_E) of the exsorptive and absorptive transport of the probe substrate could then be calculated according to (2.8):

$$R_E = \frac{P_{APP,BA}}{P_{APP,AB}} \qquad (2.8)$$

For E_1S, R_E is described to be approximately 7–12 across Caco-2 cells, which shows net exsorptive permeability of E_1S across these cells (Gram et al. 2009;

Grandvuinet and Steffansen 2011; Rolsted et al. 2011). Whether net exsorption of E_1S is seen in vivo across intestinal membrane is to our knowledge not known.

The inhibitory affinity of a drug substance/candidate for transporter(s) may be determined by measuring either the A–B or the B–A permeability of probe after pre-incubation with increasing concentrations in both compartments of the drug/candidate to be tested for its inhibitory properties.

The K_i is then derived for B–A transport:

$$\frac{P_{BA,i} - P_{Pas,BA}}{P_{APP,BA} - P_{Pas,BA}} = \frac{1}{1 + [I] / K_i} \tag{2.9}$$

and A–B transport:

$$\frac{P_{Dif,AB} - P_{AB,i}}{P_{Dif,AB} - P_{AB}} = \frac{1}{1 + [I] / K_i} \tag{2.10}$$

A more sensitive method to determine the inhibitory affinity of a drug/candidate for transporter(s) is recommended by both FDA and EMA in which the IC_{50} is deducted from R_E of probe substrate in the presence of the inhibiting drug substance according to (2.11):

$$\frac{R_{E,i}}{R_E} = 1 - \frac{I_{max} \cdot [I]^n}{[I]^n + IC_{50}} \tag{2.11}$$

$R_{E,I}$ is the efflux ratio of the probe substrate in the presence of drug substance/candidate, I_{max} is the maximal inhibitory effect of the drug substance/candidate, and n is the Hill plot exponent. This method takes into account possible asymmetric permeability across apical and basolateral membranes of both probe and drug substance/candidate. In addition, the inhibitory effect is considered twice in the calculation, i.e., both in the absorptive and exsorptive directions, which gives rise to more potent IC_{50} values.

Altogether determining kinetic parameters in vitro such as K_m or K_i/IC_{50} for transporter(s) is valuable in the evaluation of possible dose-dependent pharmacokinetics of a drug substance/candidate due to transporters and in the evaluation of possible DDI on transporters. However, it is apparent from the above description that characterization of kinetic parameters is a challenge.

Nevertheless, E_1S is suggested as probe in the determination of K_i/IC_{50} values of drug substances/candidates that possibly may interact on transporters which E_1S is substrate for (the transporters are listed in Table 2.1). Based on that, it is relevant to speculate if E_1S may be used as biomarker in vivo to evaluate if interactions on transporters that E_1S is substrate for would have any effect on the in vivo levels of E_1S and if that may give rise to possible clinical side effects such as altered ADME properties and DDI on transporters.

2.4   Speculations of Applying E_1S as a Biomarker for the Identification of Drug–Drug Interactions on Transporters

Plasma levels of E_1S have been studied by several groups and are generally consistent to the levels found by Remy-Martin et al. (1983). They found E_1S plasma levels in normal men to be relatively stable at 2.62 ± 0.79 nmol/L, whereas the plasma levels in normal women are cycle-dependent, i.e., 2.51 ± 0.90 nmol/L in the follicular phase and 5.33 ± 1.55 nmol/L in the luteal phase. In postmenopausal women the plasma level of E_1S is much lower at 0.89 ± 0.60 nmol/L (Remy-Martin et al. 1983). The plasma half-life ($t_{1/2}$) of E_1S is relatively long, 6 ± 1.1 h (male) and 4.3 ± 0.83 h (female), compared to the short $t_{1/2}$ of 20–30 min for E_1 and E_2 (Ruder et al. 1972). The long $t_{1/2}$ of E_1S is suggested to be explained by strong protein binding to serum albumin in human plasma, i.e., 98.46 ± 0.11 and 98.44 ± 0.13 in male and female, respectively (Rosenthal et al. 1972). Since the plasma level of E_1S is in nM range and the affinity constants to the transporters are in μM range (Table 2.1), one may speculate that plasma level of E_1S is relatively robust towards drug interacting on these transporters, i.e., for having any effect on E_1S plasma level the inhibiting drug substance/candidate should be a very potent inhibitor. Furthermore, E_1S is substrate to many transporters and for this reason inhibition of a single transporter by a drug substance/candidate may not necessarily influence overall plasma level of E_1S since other transporters may take over for the inhibited once. The question is therefore if E_1S would be a sufficient sensitive biomarker.

On the other hand, in diseased patients such as cirrhotic men plasma level of E_1S is found to be reduced to 1.43 ± 0.95 nmol/L compared to the normal levels in men of 2.62 ± 0.79 nmol/L. In contrast, plasma levels of E_1S in postmenopausal breast cancer patients were not significantly different from normal postmenopausal women, but increased E_1S level was found in the breast cancer tissue (Remy-Martin et al. 1983). Such increased E_1S level in breast cancer tissue can be due to increased secretion or altered distribution in the tissue. One can therefore not exclude that tissue/cellular levels of E_1S may be more sensitive biomarker than plasma levels. Analyzing for E_1S in tissue in order to investigate for possible drug interacting on the transporters that E_1S is a substrate for may be a challenge or even an impossible task in clinical situation. Nevertheless such studies can be interesting mechanistic studies in animals. We have observed that cellular concentrations of E_1S in Caco-2 cells are altered significantly compared to controls when the inhibitors, such as fluvastatin, were added to E_1S-treated cells (Gram et al. 2009; Grandvuinet and Steffansen 2011; Rolsted et al. 2011; Grandvuinet et al. 2013). Possible influence of drug substances/candidates on E_1S levels (plasma/tissue) in vivo could be interesting future studies.

2.5 Conclusions

Kinetic parameter of drug substances/candidates for membrane transporters is an important issue in drug development in order to characterize if ADME can be altered due to transporter-mediated saturable delivery. Thus, identification of possible transporters and in vitro kinetics characterization of these is an evolving discipline in drug development (see also Chap. 12). E_1S is substrate for many transporters and is therefore suggested as a probe in characterization of possible DDI on transporters listed in Table 2.1. Future investigations may show if E_1S is also an important in vivo biomarker when evaluating if drug substances/candidates interact on transporters that E_1S is a substrate to.

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Chapter 3
In Vitro Characterization of Interactions with Drug Transporting Proteins

Per Artursson, Pär Matsson, and Maria Karlgren

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.
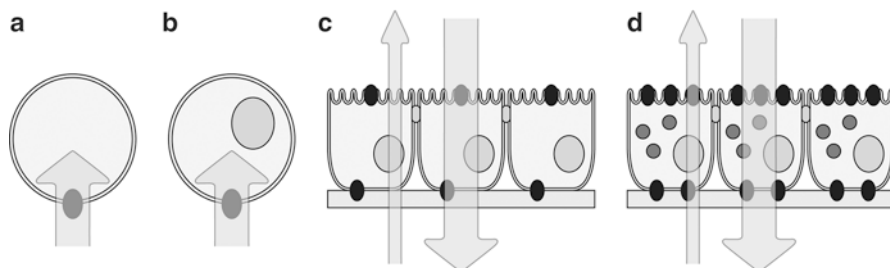
Fig. 3.1 Drug transport in different kinds of in vitro models. (**a**) Drug transport in inverted membrane vesicles. This model is primarily suitable for exsorptive ABC transporters since the ATP-binding domain, which in intact cells is facing the cytosol, will here be facing outwards and give free access 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 over-expressed 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.
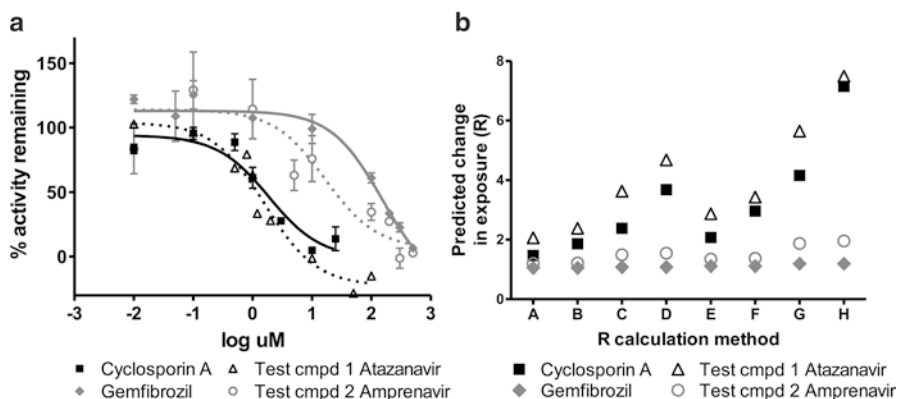
Fig. 3.2 In vitro to in vivo extrapolation for DDIs with the hepatic transporter OATP1B1. (**a**) Inhibition of OATP1B1-mediated atorvastatin uptake in stably transfected HEK293 cells for the two reference drugs cyclosporin A (*black-filled squares*, *solid line*) and gemfibrozil (*gray-filled diamonds*, *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).

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

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

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[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)

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

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, procain-amide, 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, furose-mide, bumetanide, methotrex-ate, 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, methotrex-ate, 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-tetraethylbenzimidazolcarbo-cyanine 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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Chapter 4
In Vivo Characterization of Interactions on Transporters

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Abstract Drug transporters play indispensable roles in the disposition of drugs in the body; e.g., in hepatic and renal eliminations, intestinal absorption, and in transport across active barriers, and consequently, in the response to drugs. Importance of the solute carrier family, such as OATP/*SLCO*, OCT/*SLC22*, OAT/*SLC22*, and MATE/*SLC47*, and the ATP-binding cassette transporters, such as P-glycoprotein/ABCB1, MRPs/*ABCC*, and BCRP/*ABCG2,* are well recognized in drug discovery and clinical situations. To date, it is however a great challenge to identify in vivo probe substrates and inhibitors applicable for investigating the impact of drug transporters in humans. This chapter has summarized the relevant clinical drug–drug interaction and pharmagenomic studies on drug transporters in humans, as well as some in vitro studies on transporters, in order to suggest applicable probe substrates and inhibitors for drug transporters. In addition to the drugs at the market, some endogenous and food-derived metabolites are probes for drug transporters. This chapter also highlights the impact of drug transporters on such compounds.

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Abbreviations

ABC	ATP-binding cassette
AUC	Area under the plasma concentration–time curve
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
$CL_{overall}$	Overall intrinsic clearance
CL_r	Renal clearance
CL_T	Total body clearance
CNS	Central nervous system
CR	Clearance ratio (ratio of renal clearance to the glomerular filtration)
DDI	Drug–drug interaction
F_h	Hepatic availability
f_p	Unbound fraction in plasma
GFR	Glomerular filtration rate
MATE	Mutlidrug and toxic compound extrusion
MRP	Multidrug-resistant associated protein
NMN	N-methylnicotinamide
NTCP	Sodium bile acid co-transporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PET	Positron emission tomography
P-gp	P-glycoprotein
PS_{eff}	The clearance for the back flux
PS_{inf}	The clearance for the influx
RAF	Relative activity factor
SLC	Solute carrier
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computed tomography
TEA	Tetraethyl ammonium
TIC	(15*R*)-16-*m*-tolyl-17, 18, 19, 20-tetranorisocarbacyclin

4.1 Introduction

Drug transporters are membrane proteins which may determine the elimination and tissue distribution of drugs. Their common characteristics are broad substrate specificity against drugs, natural, and synthetic compounds. The importance of drug transporters in drug disposition has been studied not only by in vitro and animal studies, but also by clinical drug–drug interaction (DDI) and pharmacogenomics studies. Transporters included in the present chapter are OATPs (OATP1B1/ *SLCO1B1*, OATP1B3/*SLCO1B3*, and OATP2B1/*SLCO2B1*), OAT (OAT1/*SLC22A6* and OAT3/*SLC22A8*), and OCT (OCT1/*SLC22A1* and OCT2/*SLC22A2*) as influx

transporters, and P-glycoprotein (P-gp)/*ABCB1*, BCRP/*ABCG2*, MRP2/*ABCC2*, and MATE (MATE1/*SLC47A1* and MATE2-K/*SLC47A2*) as efflux transporters (Giacomini and Sugiyama 2005; Giacomini et al. 2010). These transporters are involved in the absorption of drugs, elimination from systemic circulation, and/or general drug transport across biological barriers. The clinical evidence for the importance of drug transporters has prompted regulatory agencies to encourage pharmaceutical industries to evaluate the significance of drug transporters in disposition of drug candidates and also to evaluate the risk for DDI on known drug transporters. To achieve this, probe substrate and in vivo inhibitors are indispensable.

Clinical impact of the drug transporters depends on the pharmacokinetic properties of probe drugs and their route of administration besides the specificity to the target transporter. When probe substrates are given intravenously, its area under the plasma concentration–time curve (AUC_{iv}) is determined by the total body clearance (CL_T). Fraction of the hepatic and renal elimination to the systemic elimination affects the impact of inhibition of either hepatic or renal transporters on AUC_{iv}, respectively. In addition, since drugs undergo glomerular filtration in the kidney, contribution of the tubular secretion to the renal clearance (CL_R) is a factor affecting the impact of inhibition of kidney transporters on the CL_R. Furthermore, for probe drugs with high clearance compared with blood flow rate, the impact of inhibiting transporters becomes less pronounced compared with those with low clearance. Probe substrates are generally given by oral administration. In addition to its total body clearance, the effect of inhibitors on the probe bioavailability, which is a product fraction of absorbed amount, availability in the intestine, and hepatic availability, should be taken into consideration. Those probes with high bioavailability allow easy data analysis for interactions in the liver and kidney than those with low bioavailability.

This chapter summarizes the recent knowledge on probe substrates and inhibitors for drug transporters in the liver, kidney, small intestine, and blood–brain barrier (BBB).

4.2 Probe Substrates and Inhibitors for Hepatic Drug Transporters

Hepatocytes are the parenchyma cells in the liver, which are responsible for drug metabolism and excretion into the bile. Organic anion transporting polypeptides (OATPs) and OCT1 are expressed on the sinusoidal membrane of hepatocytes (Giacomini et al. 2010). OATPs accept both endogenous and exogenous compounds, including bile acids, thyroid hormones, eicosanoids, antineoplastics, antidiabetes, and cardiovascular drugs (Maeda and Sugiyama 2008; Niemi et al. 2011). In the liver, three OATP isoforms have been identified; OATP1B1, OATP1B3, and OATP2B1. OATP1B1 and OATP1B3 show overlapped substrate specificities. Their mutual contribution in transporting probe substrates into hepatocytes, a critical

factor for phenotyping OATP1B1 and OATP1B3 activities, has been studied in vitro (using their cDNA) in transfected cells and cryopreserved hepatocytes. These studies show that OATP1B1 is mainly responsible for the hepatic uptake of statins, i.e., pitavastatin, pravastatin, and rosuvastatin, whereas OATP1B3 is mainly responsible for the hepatic uptake of telmisartan and its glucuronide (Maeda and Sugiyama 2008). Hepatic uptake of olmesartan and valsartan is mediated by both OATP1B1 and OATP1B3. Expression of OATP2B1 protein was similar to those for OATP1B1 and OATP1B3 in liver (Ji et al. 2012). However, based on the relative activity factor (RAF) method where OATP2B1-mediated transport in the hepatocytes was estimated by the comparison of the transport activity normalized by protein expression of OATP2B1 in the hepatocytes and OATP2B1 expressing HEK293 cells, the contribution of OATP2B1 to the known OATP1B1 substrates is negligible (Maeda and Sugiyama 2008). Statins, such as rosuvastatin, pravastatin, pitavastatin, atrovastatin, and fluvastatin, were also found to be substrate of sodium bile acid co-transporting polypeptide (NTCP), NTCP/*SLC10A1* (Choi et al. 2011). However, the significance of NTCP remains controversial since cryopreserved human hepatocytes did not show any sodium-dependence in the uptake of pravastatin and pitavastatin (Nakai et al. 2001; Hirano et al. 2004).

Clinical DDI studies showed that cyclosporine A, rifampicin, and some protease inhibitors cause a marked increase in the AUC_{po} of OATP substrates (Yoshida et al. 2012). Substrate drug for which DDIs on OATPs are described are listed in Table 4.1 together with the drug metabolism enzymes and transporters involved in their disposition. Cyclosporin A was the first drug which was reported to cause a pharmacokinetic interaction with cerivastatin via OATP1B1 in patients with kidney transplantation (Shitara et al. 2003). Inhibition mechanism of cyclosporin A against OATP1B1 appears to be complex since it involves long-lasting effect; preincubation with cyclosporin A potentiates its inhibitory effect by unknown mechanism (Shitara et al. 2009). Subsequent studies also reported a pharmacokinetic interaction with other OATP1B1 substrates (Fig. 4.1). Cyclosporine A and protease inhibitors are not specific inhibitor of OATPs. Based on the clinical dose and K_i values, they can also inhibit other transporters and metabolic enzymes such as breast cancer resistance protein (BCRP), P-gp, and/or CYP3A4 at their clinical doses (Tachibana et al. 2009; Yoshida et al. 2012). A single administration of rifampicin (600 mg, iv) is considered to inhibit OATPs (Lau et al. 2007), although its repeated administration causes the induction of CYP3A4 or P-gp (Backman et al. 2005). Notably, the inhibitors applicable to clinical studies are not specific inhibitors either for OATP1B1 or OATP1B3. They inhibit both transporters with similar potencies (Matsushima et al. 2008; Yoshida et al. 2012).

Pharmacogenomic studies suggest probe substrates for OATP1B1 and OATP1B3. There are two important single nucleotide polymorphisms (SNPs) of OATP1B1, i.e., 388A>G and 521T>C, in which amino acid substitution at position 130 from Asn to Asp and position 174 from Val to Ala, respectively, are referred to as haplotype *1b and *5, respectively (Maeda and Sugiyama 2008; Ieiri et al. 2009; Niemi et al. 2011; Nakanishi and Tamai 2012). These two SNPs are in the linkage disequilibrium, forming haplotype *15 in Japanese population. Subjects with *5 or *15

Table 4.1 Summary of transporters and metabolic enzymes involved in the pharmacokinetics of OATP substrates

Drugs	Transporters			Metabolic enzymes		References
	Liver	Intestine	Kidney	CYP	Non-CYP	
Atorvastatin	OATP1B1/NTCP/**P-gp**/MRP2/**BCRP**	OATP2B1/**P-gp**/MRP2/**BCRP**		3A4[a]	**UGT1A3** (lactonization)	Chen et al. (2005), Lau et al. (2006), Shitara et al. (2006), Keskitalo et al. (2008), He et al. (2009), Kalliokoski and Niemi (2009), Keskitalo et al. (2009b), Riedmaier et al. (2010), Choi et al. (2011)
Cerivastatin	OATP1B1/1B3/P-gp/MRP2/BCRP	P-gp/MRP2/BCRP		2C8/3A4	UGT (lactonization)	Shitara et al. (2006), Kalliokoski and Niemi (2009)
Fluvastatin	OATP1B1/1B3/NTCP/**BCRP**	OATP2B1/**BCRP**	OAT3	**2C9**		Kirchheiner et al. (2003), Hirano et al. (2005), Shitara et al. (2006), Kalliokoski and Niemi (2009), Choi et al. (2011)
Pitavastatin	**OATP1B1**/1B3[b]/NTCP/P-gp/MRP2/BCRP	OATP2B1[b]/P-gp/MRP2/BCRP		2C9[a, b]	UGT (lactonization)	Hirano et al. (2004), Fujino et al. (2005), Shitara et al. (2006), Kalliokoski and Niemi (2009), Choi et al. (2011)
Pravastatin	**OATP1B1**/1B3//NTCP/P-gp/MRP2/BCRP/BSEP	OATP2B1/P-gp/MRP2/BCRP	OAT3	–[a]		Kitazawa et al. (1993), Shitara et al. (2006), Kalliokoski and Niemi (2009), Choi et al. (2011)
Rosuvastatin	**OATP1B1**/1B3[b]/NTCP/P-gp/MRP2/**BCRP**	OATP2B1/P-gp/MRP2/**BCRP**	OAT3	2C9[a, b]/2C19[a, b]		Martin et al. (2003), Ho et al. (2006), Shitara et al. (2006), Kitamura et al. (2008), Kalliokoski and Niemi (2009), Choi et al. (2011)
Glibenclamide	OATP1B1[c]/1B3[c]/BCRP	OATP2B1/BCRP	OAT3[c]	**2C9**[a]/2C19/3A4		Niemi et al. (2002), Naritomi et al. (2004), Gedeon et al. (2006), Kalliokoski and Niemi (2009)

(continued)

Table 4.1 (continued)

Drugs	Transporters			Metabolic enzymes		References
	Liver	Intestine	Kidney	CYP	Non-CYP	
Glimepiride	OATP1B3[c]			**2C9**[a]		Niemi et al. (2002)
Nateglinide	**OATP1B1**/1B3			**2C9**[a]/3A4		Weaver et al. (2001), Kirchheiner et al. (2004), Kalliokoski and Niemi (2009)
Repaglinide	**OATP1B1**[c]/1B3[c]			**2C8**/3A4		Kajosaari et al. (2005), Kalliokoski and Niemi (2009), Tomalik-Scharte et al. (2011)
Irbesartan	OATP1B1[c]			2C9[a]/3A4[a]	UGT[a]	
Telmisartan	OATP1B3				**UGT1A3**	Kalliokoski and Niemi (2009), Yamada et al. (2011)

For the transporter/metabolic enzymes indicated in bold letters, genetic polymorphisms significantly altered the pharmacokinetics of each substrate or its metabolites in clinical studies

UGT UDP-glucuronosyltransferase

[a]Data obtained from manufacturer's information (interview form)

[b]Minor contribution to the total hepatic clearance

[c]In-house data
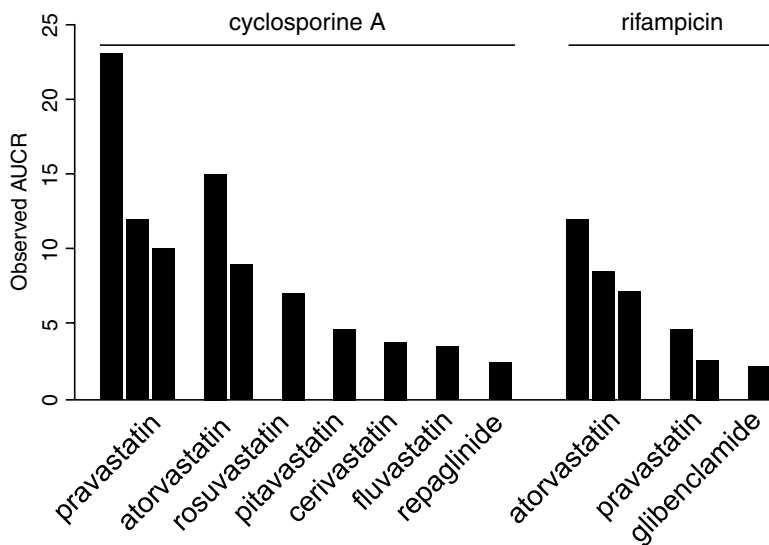
Fig. 4.1 Effect of inhibitors on the AUCR of OATP substrates. AUCR represents ratio of the mean value of AUC_{po} of OATP substrates in the inhibitor treated and control groups. Each bar represents the individually reported value

show higher systemic exposure of OATP1B1 substrate drugs compared with those with reference allele (Fig. 4.2). On the other hand, subjects with OATP1B1*1b allele rather show lower systemic exposure of pravastatin (Maeda et al. 2006). Taken together, pitavastatin has desirable properties as in vivo probe of OATP1B1, i.e., high F_h and no renal elimination. Actually, the systemic exposure of pitavastatin is highly sensitive to OATP activities in both DDI and pharmacogenetic studies (Figs. 4.1 and 4.2). Rosuvastatin and pravastatin are also good OATP probe substrates; however, since they undergo substantial urinary elimination, impact of OATPs inhibition is lower than that of pitavastatin. Other compounds with high F_h values might be used as good OATPs substrates, but the number of clinical studies suggesting the involvement of OATPs in their pharmacokinetic is limited and the contributions of OATPs are still unknown. For OATP1B3, telmisartan is considered as in vivo probe for OATP1B3 since an intronic SNP of OATP1B3 (rs11045585), which is associated with docetaxel-induced neutropenia (Kiyotani et al. 2008; Chew et al. 2011), shows a tendency of association with the systemic exposure of telmisartan in healthy subjects; heterozygotes show 1.4-fold higher than those with homozygotes of reference OATP1B3 (Yamada et al. 2011).

The canalicular membrane expresses ABC transporters, such as P-gp, MRP2, BCRP, and MATE1, mediating the efflux of drugs into the bile. According to studies in rats and mouse, MRP2 and BCRP are major transporters mediating the canalicular efflux of anionic drugs. Unlike the influx process, there is limited information regarding identified probe drugs for phenotyping these transporters in human liver. This can be attributed to the rate-determining process in the hepatic elimination of
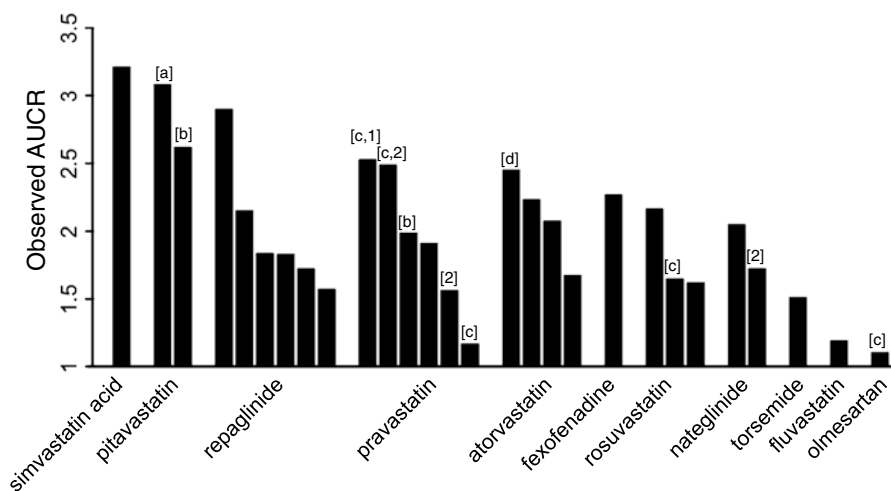
Fig. 4.2 Effect of OATP1B1 SNP (*5 or *15) on the AUCR of OATP substrates. AUCR represents ratio of the mean value of AUC_{po} of OATP substrates in the homozygotes of the OATP1B1 SNP and those of reference OATP1B1. Each bar represents individual data. a *15/*15 versus *1b/*1b (ABCG2 421C/C and 421C/A versus 421C/C), b *15/*15 versus *1a/*1a, c *15/*15 versus *1b/*1b, and d *15/*15 versus *1a/*1a, *1a/*1b, and *1b/*1b. The number in the parenthesis represents the number of subjects; [1] $n = 1$ in *15/*15 or 521C/C group. [2] $n = 2$ in *15/*15 or 521C/C group. If not indicated, the number of subject is 3 or more

drugs (see Sect. 4.5.2). Therefore, suitable probes and inhibitors for in vivo studies have not yet been recommended. A synonymous SNP in the coding region (1446C>G) of MRP2 is associated with 3.1-fold lower AUC_{po} of pravastatin and 1.95-fold higher mRNA expression in the liver in the heterozygotes compared with homozygotes of reference MRP2 (Niemi et al. 2006). A SNP (1249G>A), substituting Val for Ile at position of 417 in MRP2, is associated with larger non-renal clearance and lower bioavailability of talinolol in homozygotes than those in heterozygotes/reference allele (Haenisch et al. 2008). Lower bioavailability in the homozygotes of the mutant allele to the heterozygotes and homozygotes of the reference allele can be attributed to lower fraction absorbed and intestinal availability considering its high hepatic availability (>0.8). In addition, a SNP located in 5′ region of MRP2 (−24T>C) is associated with higher dose normalized trough level of mycophenolic acid after day 42 in renal alograft patients who received mycophenolic acid twice a day (Naesens et al. 2006), and with higher incidence of diclofenac toxicity with Odds ratio of 5.0 (Daly et al. 2007). Another SNP −1019A>G in MRP2 is associated with 12 % lower clearance of irinotecan, and its adverse reaction (diarrhea) with Odds ratio of 0.15 (de Jong et al. 2007). Gender difference has been suggested for BCRP; male liver shows higher expression than female liver (Merino et al. 2005). As described below, there is a SNP (421C>A) associated with lower BCRP protein expression, and disposition of BCRP substrate drugs. Although

the canalicular efflux of pitavastatin is markedly reduced in Bcrp knockout mice (Hirano et al. 2005), the SNP did not influence the systemic exposure of pitavastatin in healthy subjects (Ieiri et al. 2007).

Hepatocytes express renal type organic cation transporters (OCTs), OCT1 and MATE1 in the sinusoidal and canalicular membranes, respectively. OCT1 accepts hydrophilic organic cations, tetraethylammonium (TEA), and metformin are proto-typical substrates. The typical substrate drugs of OCT1, such as metformin, are mainly eliminated by the kidney, and thus, importance of OCT1 as clearance mech-anism remains unknown. OCT1 plays a significant role in the pharmacological effect of metformin in the liver (Wang et al. 2003; Shu et al. 2007). Subjects with OCT1 SNP show 1.2-fold higher AUC_{po} of metformin following oral administration (Shu et al. 2008). MATE1 is a proton/organic exchanger which shows an over-lapped substrate specificity with OCT1 (Tanihara et al. 2007) (see Sect. 4.3.2). Its protein expression was confirmed by Western blot and immunohistochemical stain-ing in human liver (Otsuka et al. 2005; Ito et al. 2010). Since human canalicular membrane vesicles show an accumulation of metformin, which was inhibited by a MATE inhibitor, pyrimethamine, and mouse treated with pyrimethamine show higher accumulation of metformin caused by an inhibition of canalicular efflux into the bile, MATE1 may mediate the efflux of metformin into the bile in human liver (Ito et al. 2010).

4.3   Probe Substrates and Inhibitors for Renal Drug Transporters

The renal elimination of drugs is determined by their glomerular filtration rate (GFR) which depends on drug charge, size, and unbound fraction in the blood, as well as tubular secretion of the drugs to- and reabsorption from the urine. The tubu-lar secretion occurs in the proximal tubules where many transporters are expressed in the epithelial cells and at which interaction with drugs may take place. The impact of the pharmacokinetic interaction with drug transporters on the renal clearance depends on the contribution of tubular secretion, i.e., the renal clearance has to be greater than the GFR, but below the renal blood flow rate.

4.3.1   Organic Anion Transport System

Organic anion transport system in the kidney is comprised of two multispecific OATs; OAT1/*SLC22A6* and OAT3/*SLC22A8* on the basolateral membrane. OAT2/*SLC22A7* and OATP4C1/*SLCO4C1* are also expressed on the basolateral membrane, however, their importance in the drug transport remains unclear.

OAT1 mainly mediates the small and hydrophilic organic anions, such as *p-aminohippurate*. OAT3 shows an overlap in the substrate specificity with OAT1,

but it also shows overlap with OATPs. The substrates of OAT3 are also characterized by including some cationic drugs, cimetidine, ranitidine, and famotidine (Kusuhara and Sugiyama 2009; Masereeuw and Russel 2010; Burckhardt and Burckhardt 2011). Probenecid has been used as an inhibitor of renal organic anion transport system; it inhibits the renal elimination of a variety of anionic drugs, zwitterions such as fexofenadine and Ro64-0802 (pharmacologically active form of oseltamivir), and a cationic drug famotidine (Table 4.2). Actually, it is a potent inhibitor of OAT1 and OAT3 in vitro with K_i of 7.0 and 1.3 μM, respectively (Ho et al. 2000; Tahara et al. 2005), and therapeutic dose of probenecid provides the concentration of unbound form sufficient to inhibit OAT1 and OAT3 almost completely. Since the inhibition potency of probenecid against OAT1 and OAT3 is similar, the contribution of OAT1 and OAT3 cannot be evaluated only based on a pharmacokinetic interaction study with probenecid. In vitro transport studies using human kidney slices is required to characterize the uptake mechanism; where OAT1 and OAT3-mediated uptake is preferably inhibited by PAH and benzylpenicillin (Nozaki et al. 2007).

Table 4.2 lists OAT drug substrates and their renal clearance (CL_r) when inhibited by probenecid. Assuming that reabsorption is negligible, clearance ratio (CR) was defined as ratio of renal clearance to the glomerular filtration. Drugs with large CR is suitable probes. Furosemide showed the highest CR due to high plasma protein binding followed by bumetanide and rosuvastatin. Furosemide is transported by both OAT1 and OAT3 (Hasannejad et al. 2004), and impaired either Oat1 or Oat3 blunted the diuretic effect in mice (Eraly et al. 2006; Vallon et al. 2008). Adefovir, cidofovir, tenofovir, and acyclovir are solely transported by OAT1 in cDNA transfected cells (Uwai et al. 2007; Windass et al. 2007). Consistent with low CR, the effect of probenecid on the renal clearance of cidofovir and acyclovir is moderate (Masereeuw and Russel 2010). CR of bumetanide and rosuvastatin, which show higher transport activities by OAT3 than OAT1, in vitro in cDNA transfected cells is high (Hasannejad et al. 2004; Windass et al. 2007). The renal clearance of bumetanide is highly sensitive to probenecid (Burckhardt and Burckhardt 2011), whereas pharmacokinetic interaction study with probenecid has not been conducted for rousvastatin. Benzylpenicillin, ciprofloxacin, and famotidine are good OAT3 substrates (Tahara et al. 2005; Nozaki et al. 2007; Vanwert et al. 2008) with high magnitude of interaction with probenecid (Tahara et al. 2005).

The transporter responsible for the luminal efflux has not been fully elucidated. Mrp4 knockout mice showed higher accumulation of hydrochlorothiazide, adefovir and tenofovir, and ceftizoxime and cefazolin (Ci et al. 2007; Hasegawa et al. 2007; Imaoka et al. 2007). Besides Mrp4, MRP2 is considered to mediate the luminal efflux of anionic drugs in human kidney based on the association of MRP2 SNP or mutation causing MRP2 defect with the renal clearance of methotrexate; subjects with a SNP or mutation in MRP2 exhibited delayed elimination of methotrexate via systemic elimination (Rau et al. 2006; Ranganathan et al. 2008).

Table 4.2 Summary of probe substrates of OAT1 and OAT3, and their interaction with probenecid

Drug	CL_r (mL/min/kg)	f_p	CR	In vitro cDNA transfectants	Kidney slice	Animal study	Clinical DDI inhibitor (CL_r, % of inhibition)
Furosemide	1.70	0.01	79.5	OAT1, OAT3	–	Oat1KO/Oat3KO	72
Bumetanide	0.85	0.03	15.4	OAT3	–	–	85[a]
Rosuvastatin	3.15	0.12	14.6	OAT3	–	–	–
Captopril	12.00	0.72	9.24	OAT1, OAT3	–	–	44
Benzylpenicillin	5.53	0.4	7.68	OAT3	OAT3	Oat3KO	61
Pravastatin	6.35	0.55	6.47	OAT3	–	–	–
Sitagliptin	4.74	0.61	4.30	OAT3	–	–	–
Ciprofloxacin	3.80	0.60	3.52	OAT3	–	Oat3KO	64
Acyclovir	4.64	0.85	3.03	OAT1	–	–	32
Famotidine	4.42	0.83	2.96	OAT3/OCT2	–	–	89
Ro64-0802	4.46	0.96	2.59	OAT3	–	–	53
Adefovir	3.33	0.95	1.95	OAT1	–	–	–
Fexofenadine	1.13	0.35	1.79	OAT3	OAT3	–	27, 70
	1.13	0.35	2.25	OAT3	OAT3	–	74
	3.78	0.35	5.99	OAT3	OAT3	–	70
Methotrexate	1.70	0.54	1.75	OAT3	OAT3	Oat3KO	56
Ganciclovir	3.09	0.99	1.75	OAT1	–	–	19
Cidofovir	2.15	1	1.55	OAT1	–	–	32
Tenofovir	2.13	0.99	1.20	OAT1	–	–	–

Pharmacokinetic data of drugs are cited from Goodman and Gilman (Eds by Brunton, L.L., Lazo, J.S., Parker, K.L., Goodman, L.S., Gilman, A.G., McGraw-Hill Professional Publishing, 2005). Clinical DDI data are cited from reference Masereeuw and Russel (2010), except for bumetanide Hasannejad et al. (2004), captopril Sinhvi et al. (1982), benzylpenicillin Nierenerg (1987), and Ro64-0802 Hill et al. (2002)

CL_r renal clearance, f_p unbound fraction in the plasma, *CR* clearance ratio

[a]Probenecid was given intravenously (1 g)

4.3.2 Organic Cation Transport System

OCT2/*SLC22A2* is the predominant multispecific OCT in the basolateral membrane. The typical substrates include metformin and platinum agents (Giacomini et al. 2010). MATEs are now considered to mediate the luminal efflux of cationic drugs. MATEs are comprised of two isoforms in MATE1 and MATE2-K in human, whereas their rodent counterpart is Mate1 (Otsuka et al. 2005; Masuda et al. 2006). Both MATE1 and MATE2-K are expressed on the brush border membrane of the proximal tubules in the kidney (Otsuka et al. 2005; Masuda et al. 2006). The substrates of MATE1 and MATE2-K are overlapped (Tanihara et al. 2007). Metformin is a typical substrate for OCT2 and MATE1, and MATE2-K. Importance of MATEs has been investigated in mice. Defect of Mate1 elevated systemic exposure of metformin, accompanied with the elevation in the kidney concentrations.

Cimetidine has been used as inhibitor of renal OCTs. After repeated administration of cimetidine, it reduced the renal clearance of a variety of cationic compounds such as amiloride and metformin, by 16–61 % in humans. The magnitude of the interaction is summarized in Table 4.3. It is generally believed that this interaction is caused by an inhibition of OCT2. According to published data, cimetidines inhibition constant for OCT2 showed large variation depending on the reports, ranging from 11 to 1,650 μM. When the lowest K_i value is considered, the maximum unbound concentration of cimetidine in the blood (4.9–7.6 μM) can be high enough to inhibit OCT2 significantly (Ito et al. 2012a). However, a comprehensive analysis using five test substrates for OCT2 reported K_i values of cimetidine for OCT2, ranged from 95 to 146 μM, producing at most 10 % inhibition at its clinically reported plasma unbound concentrations (3.6–7.8 μM) (Ito et al. 2012a). Therefore, at least, direct inhibition of OCT2 by cimetidine can be excluded. Instead, it is turned out that cimetidine is an inhibitor of MATEs with K_i being similar to the unbound concentration in the blood, suggesting in vivo relevance of MATE inhibition at clinical dose (Matsushima et al. 2009; Tsuda et al. 2009; Ito et al. 2012a). Actually, when cimetidine was given to mouse to achieve clinically relevant unbound plasma concentrations, the kidney accumulates metformin, TEA, and cephalexin, supporting the inhibition of luminal efflux by cimetidine (Ito et al. 2012a). In addition, we reported pyrimethamine, anti-malaria drug, to be a potent and specific inhibitor of MATEs. It is an inhibitor of OCT2 in vitro; however, the K_i value (10 μM) was 100- to 200-fold higher than those for MATE1 and MATE2-K (77 and 46 nM, respectively) (Ito et al. 2010). Its systemic elimination half-life is 96 h. A single oral dose of pyrimethamine (50 mg), which achieves unbound concentration of approximately 2–300 nM, allows long-lasting inhibition of MATEs. It inhibits the H^+-coupled transport of metformin in brush border membrane vesicles from mouse and human kidneys and increased the kidney-to-plasma concentration ratio of TEA and metformin in mice without affectg the systemic exposure (Ito et al. 2010). Furthermore, pyrimethamine moderately inhibited the renal elimination of metformin in healthy subjects (Kusuhara et al. 2011). Inhibition of efflux process

Table 4.3 Summary of probe substrates of OCT2 and MATEs, and their interaction with cimetidine

Drug	CL_r (mL/min/kg)	f_p	CR	cDNA transfectants	Animal study	Clinical DDI Inhibitor (CL_r, % of inhibition)
pilsicainide	3.08	0.35	8.03	-	-	28 (Shiga et al., 2000)
metformin	7.92	0.99[b]	4.45[c]	OCT2	Mate1 KO	28 (Somogyi et al., 1987)
	10.4	0.99[b]	6.52	MATE1/2-K		45 (Wang et al., 2008)
fexofenadine	3.78	0.35[b]	5.99[c]	MATE1/2-K	-	34 (Yasui-Furukori et al., 2005)
zidovudine	6.83	0.79[b]	5.72	-	-	56 (Fletcher et al., 1995)
ranitidine	4.72	0.64	5.48	OCT2	-	25 (van Crugten et al., 1986)
dofetilide	3.46	0.36[b]	5.35[c]	-	-	13 (Abel et al., 2000)
				-		33 (Abel et al., 2000)
amiloride	4.84	0.60(Spahn et al., 1987)	5.10	MATE2-K	-	16 (Somogyi et al., 1989)
S-(−)-pindolol	3.42	0.52	4.19	-	-	30 (Somogyi et al., 1992)
procainamide	4.95	0.82	3.36[c]	OCT2	-	43 (Somogyi et al., 1983)
	2.89	0.83[b]	1.93[c]	MATE1/2-K		36 (Christian et al., 1984)
	5.77	0.88	3.66[c]			36 (Rodvold et al., 1987)
	5.94[a]	0.83[b]	3.98[c]			31 (Lai et al., 1988)
	5.35[a]	0.83[b]	3.58[c]			40 (Lai et al., 1988)
R-(+)-pindolol	2.62	0.47	3.55	-	-	39 (Somogyi et al., 1992)
cephalexin	3.81	0.83	3.37	MATE1	Mate1 KO	21 (van Crugten et al., 1986)
temafloxacin	2.18	0.74(Sorgel and Kinzig, 1993)	1.63[c]	-	-	18 (van Crugten et al., 1986)
bisoprolol	1.81	0.65(Horikiri et al., 1998)	1.55[c]	-	-	45 (Kirch et al., 1986)
triamterene	0.96	0.41	1.45	-	-	62 (Muirhead et al., 1986)
levofloxacin	1.70[a]	0.69[b]	1.37[c]	MATE1	-	24 (Fish and Chow, 1997)
varenicline	1.71	0.85	1.27	OCT2 MATE1/2-K	Mate1 KO	25 (Feng et al., 2008)

[a] Calculated using GFR of 1.8 mL/min/kg
[b] Goodman & Gilman's The Pharmacological Basis of Therapeutics eleventh edition
[c] Calculated assuming body weight as 70 kg

accompanies the kidney accumulation of drugs, and consequently it could potentiate the drug effect. Actually, pyrimethamine sensitizes mouse to cisplatin (nephrotoxicity) (Nakamura et al. 2010).

4.4 Probe Substrates and Inhibitors for Drug Transporters in the Small Intestine

Drug transporters in the small intestine may influence the intestinal absorption of drugs. The epithelial cells express efflux transporters such as P-gp and BCRP to limit the intestinal absorption. Furthermore, recently, it is considered that the influx (absorption) of some drugs involves transporter to account for the pharmacokinetic interaction with fruit juice.

Repeated administration of rifampicin decreased the systemic exposure of digoxin in healthy subjects (Greiner et al. 1999). Since this treatment causes a significant increase in P-gp expression in the duodenum, it is considered that this interaction is caused by increased active intestinal efflux by P-gp. Tachibana et al. summarized the pharmacokinetic interaction of P-gp substrates digoxin, fexofenadine, and talinolol (Tachibana et al. 2009). Erythromycin, itraconazole, ketoconazole, ritonavir, and verapamil increased the AUC_{po} of fexofenadine, and erythromycin also increased that of talinolol (1.8-fold). The magnitude of the interaction between the inhibitors and the P-gp substrate drugs is shown in (Fig. 4.3). Since inhibition of the hepatic uptake of fexofenadine by itraconazole and ritonarvir is unlikely at this dose (50–200 mg for itraconazole, and 100 mg for ritonavir) (Yoshida et al. 2012), inhibition of P-gp in the small intestine is considered as likely DDI mechanism. Valspodar increased the AUC_{po} of digoxin 1.8-fold. Itraconazole also increased the AUC_{po} of celiprolol (1.8-fold) (Lilja et al. 2003). Since these inhibitors also are CYP3A4 inhibitors, and therefore may increase the AUC_{po} of CYP3A4 substrates, such as midazolame and triazolam (Tachibana et al. 2009), CYP3A4 inhibition should also be taken into consideration as the mechanism underlying the increase in the AUC_{po} of test compounds.

Importance of BCRP has been investigated by pharmacogenomic studies since there is a SNP (SNP number) in BCRP gene 421C>A, substituting an amino acid from Gln to Lys, which cause a marked reduction of BCRP protein on the plasma membrane (Kondo et al. 2004). According to HapMap project, this SNP is frequently observed in Asian population 29–31 %, but quite rare in Caucasian population. AUC_{po} of plasma sulfasalazine, rosuvastatin, and atorvastatin following oral administration is higher in the homozygotes with the mutant allele compared with subjects with reference allele, whereas there was no difference in the AUC_{po} of simavastatin, pravastatin, and pitavastatin (Zhang et al. 2006; Ieiri et al. 2007; Yamasaki et al. 2008; Keskitalo et al. 2009a, b) (Fig. 4.4). Particularly, sulfasalazine showed the largest difference, showing its usefulness as BCRP probe. DDI studies using these substrate drugs also would be helpful to identify in vivo inhibitors of BCRP. A pharmacokinetic interaction study with GF120918 has been performed
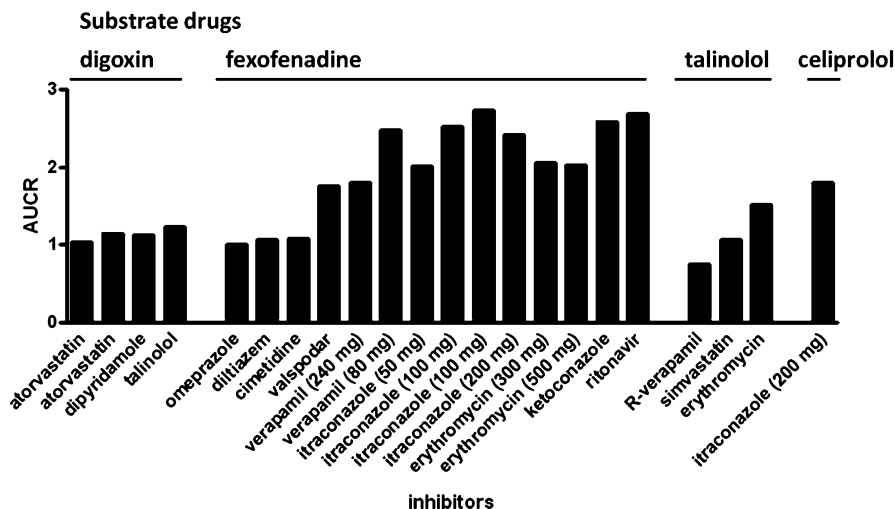
Fig. 4.3 Effect of inhibitors on the AUCR of P-gp substrates. AUCR represents ratio of mean value of AUC_{po} of the P-gp substrates (digoxin, fexofenadine, talinolol, and celiprolol) in the inhibitor treated and control groups. The information was cited from the summary by Tachibana et al. (2009)

Fig. 4.4 Effect of breast cancer resistance protein (BCRP) SNP (421C>A) on the AUCR of BCRP substrates. AUCR represents ratio of the mean value of AUC_{po} of BCRP substrates in the homozygotes of the BCRP SNP and those of reference BCRP
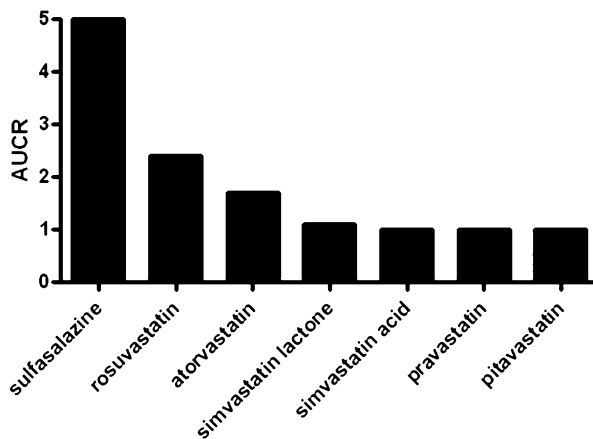
which increased the bioavailability of topotecan from 42 to 100 % at the dose of 100 mg in patients (Kuppens et al. 2007). Curcumin, the principal curcuminoid of turmeric, has an ability to inhibit BCRP in vivo in mice, and also in humans (Shukla et al. 2009; Kusuhara et al. 2012). Oral dose of curcumin (2 g) markedly enhanced the systemic exposure of sulfasalazine in healthy subjects. Notably, the impact of BCRP inhibition on the systemic exposure of sulfasalazine is different depending on the dose of sulfasalazine; 2.0-fold in microdose, and 3.7-fold in the therapeutic dose due to nonlinear pharmacokinetics of sulfasalazine (Kusuhara et al. 2012). Based on the fraction of absorbed (7 %) at therapeutic dose (Azadkhan et al. 1982),

Fig. 4.5 Effect of grapefruit juice on the AUCR of beta-blockers and fexofenadine. AUCR represents ratio of the mean value of AUC_{po} of beta-blockers and fexofenadine in the inhibitor treated and control groups
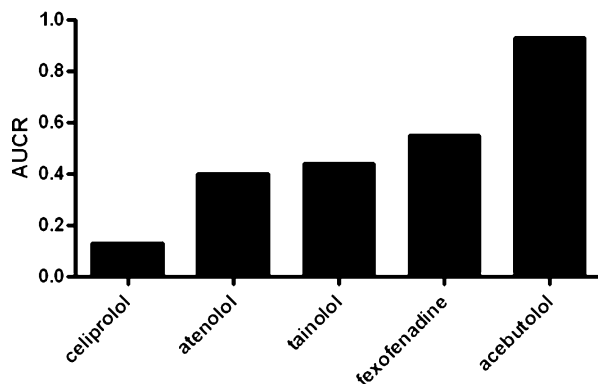
it is estimated to be 60 % at microdose. To use sulfasalazine as probe substrate of BCRP, therapeutic dose is recommended.

There are clinical reports on the effect of lowering intestinal absorption of fexofenadine and beta-blockers such as celiprolol (Lilja et al. 2003, 2005a, b; Schwarz et al. 2005; Bailey et al. 2007). Among these compounds, celiprolol showed the largest difference, 7.7-fold, between control and grapefruit juice-treated group (Fig. 4.5). This has been interpreted as inhibition of absorptive (luminal/apical influx) transporters for these drugs. OATP1A2 and OATP2B1 have been proposed as responsible transporter for this observed reduced drug absorption; the expression of OATP1A2 mRNA is pretty low compared with OATP2B1, whereas the substrate specificity and inhibiter profile of OATP1A2 is consistent with the clinical data (Bailey et al. 2007; Kato et al. 2009; Shirasaka et al. 2010). Pharmacogenomic studies, which focused on the SNP of OATP2B1 (935G>A and 1457C>T) substituting the amino acid from Arg to Gln and from Ser to Phe at position of 312 and 486, respectively, elucidated that the intersubject variation of the plasma concentrations of montelkast and fexofenadine is associated with the genotype of OATP2B1. The plasma concentrations of montelkast and fexofenadine were three- and twofold lower in subjects with 935G>A and 1457C>T, respectively, compared with those with reference OATP2B1 (Mougey et al. 2009; Imanaga et al. 2011), whereas there is another report where subjects with 1457C>T showed rather 1.2-fold higher AUC_{po} (Akamine et al. 2010). In addition, a nonlinear pharmacokinetics also suggests the expression of an influx transporter. The AUC of sulfasalazine normalized by the dose is rather lower in microdose study (100 μg) than that in therapeutic dose study (2 g) in healthy subjects (Kusuhara et al. 2012). It was confirmed that sulfasalazine is a substrate of OATP2B1 in HEK293 cells expressing OATP2B1. Its K_m value for OATP2B1 (1.7 μM) is higher than the concentration in the lumen (8–25 nM), microdose divided by the apparent volume in the lumen (10–30 L, Tachibana et al. 2009) which was reported based on the pharmacokinetic interaction with CYP3A4 and P-gp substrates, but lower than the concentration at therapeutic dose (160–500 μM). Saturation of OATP2B1 is considered as the mechanism underlying the nonlinearity. This also suggests a possibility that sulfasalazine is an in vivo OATP2B1 inhibitor. Taken together, significance of OATP2B1 in the small intestine remains debatable.

4.5   Imaging Probes for In Vivo Drug Transport Monitoring

4.5.1   BBB Transport

Brain capillary endothelial cells form tight monolayer by highly developed tight junction between adjacent cells and separate the central nervous system (CNS) from the blood. Furthermore, it expresses efflux transporters, such as P-gp, BCRP, and MRP4, on its luminal membrane (blood-facing membrane). These transporters extrude their substrate drugs into the blood circulation, and thereby blunting their CNS effects. Pharmacokinetic interaction at the BBB involving these transporters elevates CNS exposure of drugs, and consequently their effect. Actually, defect of P-gp potentiates the CNS effects of ivermectin and asimadoline in mice (Schinkel et al. 1994; Jonker et al. 1999), and quinidine potentiates the CNS effect of loperamide (respiratory depression) in healthy subjects in which the CNS effect of loperamide was significantly enhanced in quinidine-treated group when the plasma concentration–time profiles were similar (Sadeque et al. 2000). Because of low contribution of the drug transport at the BBB to the distribution volume in the brain, variation of the efflux transport activity at the BBB does not affect the plasma concentrations. Quantitative investigation of these transporters in human BBB requires direct measurement of brain concentrations. Positron emission tomography (PET) is a powerful noninvasive method for its high sensitivity, good spatial–temporal resolution, and enables to determine drug distribution in vivo using the drug labeled with a positron-emitting radionuclide. Several P-gp substrates applicable to the PET study have been reported. Of these test drugs, clinical PET data using ^{11}C-verapamil and [11]C-*N*-desmethyl-loperamide in healthy subjects and patients have been already reported where these PET probes can be available for the evaluation of DDI at the efflux transport at the BBB (Table 4.4) (Muzi et al. 2009; Wagner et al. 2009; Arakawa et al. 2010; Bauer et al. 2010; Eyal et al. 2010; Kreisl et al. 2010). The K_1, a rate constant for the influx across the BBB, of these ligands is susceptible to P-gp inhibition by cyclosporin A and tariquidar. Effect of disease conditions on the efflux activities by P-gp has been also examined (Table 4.4). Diseases such as drug resistant unilateral temporal lobe epilepsy (Langer et al. 2007), chronic paranoid schizophrenia (de Klerk et al. 2010), idiopathic Parkinson (Bartels et al. 2008b), Parkinson disease, progressive supranuclear palsy, and multisystem atrophy (Bartels et al. 2008a) did not affect the kinetic parameters, where distribution volume was decreased in the patients suffering from major depressive episode using anti-depressants (de Klerk et al. 2009). PET tracers for BCRP have been produced which include [^{11}C]gefitinib (Kawamura et al. 2009), [^{11}C]XR9576 (Kawamura et al. 2010), [^{11}C]GF120918 (Kawamura et al. 2011), [^{11}C]methyl 4-((4-(2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl)phenyl)amino-carbonyl)-2-(quinoline-2-carbonylamino)benzoate (Mairinger et al. 2010), befloxatone (Tournier et al. 2011), and [^{13}N]dantrolene (Kumata et al. 2012). The brain uptake of these PET probes, except for befloxatone and [^{13}N]dantrolene, has been examined in wild-type and P-gp/Bcrp knockout mice. The knockout mice show higher brain accumulation

Table 4.4 Summary of clinical pharmacokinetics at the blood–brain barrier

Probes	Inhibitor/disease state	Changes observed compared to control	References
Studies with P-gp inhibitors			
Verapamil	Cyclosporin A 2.5 mg/kg/h iv	$K_1 \times 1.7$ SUV $\times 1.3$ AUCR $\times 1.9$ $V_d \times 1.7$	Muzi et al. (2009)
Verapamil[a]	Cyclosporin A 2.5 mg/kg/h iv	$K_1 \times 1.8$	Eyal et al. (2010)
Verapamil[a]	Clarithromycin 400 mg/kg, 2 days	K_1 no change	Arakawa et al. (2010)
R-verapamil	Tariquidar 2 mg/kg, iv	V_d no change	Bauer et al. (2010)
R-verapamil	Tariquidar 2 mg/kg, iv	$K_1 \times 1.5$ $V_d \times 1.2$	Wagner et al. (2009)
N-desmethyl-loperamide	Tariquidar 2 mg/kg, 4 mg/kg, 6 mg/kg, iv	$K_1 \times 2$–3	Kreisl et al. (2010)
Studies with disease			
R-verapamil	Drug-resistant unilateral temporal lobe epilepsy	K_1 no change V_d no change	Langer et al. (2007)
Verapamil[a]	Chronic paranoid schizophrenia	K_1 no change V_d no change	de Klerk et al. (2010)
Verapamil[a]	Idiopathic Parkinson	K_1 no change	Bartels et al. (2008b)
Verapamil[a]	Parkinson Disease, progressive supranuclear palsy (PSP), multisystem atrophy (MSA)	V_d no change	Bartels et al. (2008a)
Verapamil	Major depressive episode using anti-depressants	$V_d \times 0.6$	de Klerk et al. (2009)

V_d distribution volume, K_1 influx rate constant, K_2 efflux rate constant, *SUV* standardized uptake value, *AUCR* brain-to-blood ratio of area under the radioactivity concentration curves
[a]Determined one or more ABCB1 genotypes (G1199A, C1236T, G2677T, C3435T) of patients

of PET ligands. In human BBB, expression of several influx transporters has been reported such as OATP1A2 and OATP2B1 on the luminal membrane of the endothelial cells (Bronger et al. 2005). These transporters may help drugs to penetrate into the CNS.

4.5.2 Separate Evaluation of the Influx and Efflux in Overall Hepatic and Renal Elimination

When the influx process to hepatocytes involves transporters, the intrinsic heptic organ clearance ($CL_{overall}$) is a complex parameter consisting of the influx clearance (PS_{inf}) and efflux clearance across the basolateral and canalicular membranes (PS_{eff}, and CL_{int}, respectively).

$$CL_{overall} = PS_{inf} \cdot CL_{int} / (PS_{eff} + CL_{int})$$

When PS_{eff} is negligible compared to CL_{int}, $CL_{overall}$ is approximated by PS_{inf}. Namely, the influx process is the rate-determining process in the overall elimination (uptake-limited) (Kusuhara and Sugiyama 2009; Watanabe et al. 2010b). Hepatic elimination of statins has been suggested uptake-limited in humans as well as in rodent (Watanabe et al. 2009a, b, 2010a). Under this condition, variation in CL_{int} hardly affects the $CL_{overall}$. Actually, inhibition of CYP3A4 by itraconazole hardly influenced the systemic exposure of atorvastatin, although the plasma concentrations of its metabolites were markedly reduced (Maeda et al. 2011). It is worth noting that this study was done by *so-called* cassette dosing in microdose study. Cocktails which include isoform-specific substrates have been proposed for simultaneous and comprehensive analysis of DDI involving CYPs (Breimer and Schellens 1990; Fuhr et al. 2007). In this study, beside a test drug (atorvastatin), OATP and CYP3A4 probes (pravastatin and midazolam, respectively) were administered in cocktails to assess the effect of rifampicin and itraconazole in the same subjects (Maeda et al. 2011).

In order to evaluate PS_{inf}, and CL_{int} separately, measurement of tissue concentration–time profile is indispensable. Following integration plot analysis, using early phase of plasma concentrations and tissue accumulation data, the uptake clearance with regard to the plasma concentration can be obtained. This uptake clearance represents organ clearance but not intrinsic influx clearance, i.e., a complex parameter consisting of unbound fraction in the blood, blood flow rate, and PS_{inf}. One can convert this parameter to PS_{inf} using the blood flow rate and unbound fraction in the blood assuming well-stirred, tube, or dispersion models. Dividing amount excreted into the bile or urine by AUC of tissue concentration provides the product of unbound fraction and intrinsic efflux clearance. To measure these kinetic parameters in humans, noninvasive method is required to measure the tissue concentration in humans. A PET ligand, N-[^{11}C]acetyl-leukotriene E4, is a Mrp2 substrate, and impaired canalicular efflux clearly delayed its elimination from the liver (Guhlmann et al. 1995). A pharmacokinetic analysis of a SPECT ligand, mebrofenin, which is a substrate of OATP1B1 and OATP1B3, MRP3 and MRP2, has been studied in healthy subjects and determined the biliary clearance of this drug in humans (Ghibellini et al. 2008). [^{11}C]TIC is a carboxy ester of TIC-A; the total radio activity is predominantly eliminated into the bile after intravenous injection of TIC (Takashima et al. 2010, 2012). A pharmacokinetic interaction study with rifampicin was conducted in humans (Takashima et al. 2012). Integration plot analysis shows that the hepatic uptake clearance of TIC-A is not blood flow limited. Consistent with the fact that TIC-A is a substrate of OATP1B1 and OATP1B3, rifampicin significantly decreased the tissue uptake clearance by 45 % in healthy subjects given 600 mg orally before the intravenous injection of [^{11}C]TIC. In rat study, [^{11}C]TIC undergoes extensive metabolism, and subsequently excreted into the bile by Mrp2 since Mrp2 deficiency caused a significant reduction in the biliary excretion. However, the major metabolite in the liver and bile is different, and thus, calculation of CL_{int} just based on PET data does not provide meaningful kinetic parameter. In human, the biliary excretion of the

total radioactivity of [^{11}C]TIC was also decreased by rifampin. This is speculated to be by MRP2 inhibition. In addition, telmisartan has been also developed as PET tracer (Shimizu et al. 2012). Following intravenous injection, most of the radioactivities were rapidly taken up by the liver followed by excretion into the biliary tract and intestine. Although the transporters responsible for the hepatobiliary transport remains unknown, a metabolite of cerecoxib (SC-62807) has been synthesized as PET probe, which is also predominantly excreted into the bile without metabolism (Takashima-Hirano et al. 2011). Application of this compound to characterize in vivo interaction with drug transporters is expected.

4.6 Endogenous Probes for Drug Transporters

Substrates of drug transporters include endogenous and food-derived ones, which can be used as endogenous probes of drug transporters. Such probe compounds have an advantage in their capability of evaluation of in vivo transporter function without administrating probe drugs, whereas there are several disadvantages, the use of endogenous compounds is based on empirical evidence and large interindividual difference, lack of information on metabolic pathway and their contributions. For instance, creatinine, a cyclic anhydride of creatine and an end product of muscle metabolism, is an endogenous substrate of OCT2, and MATEs (Urakami et al. 2004; Tanihara et al. 2007). Creatinine is eliminated into the urine, and its renal clearance is used as index of renal function. Its renal clearance appears to be similar to GFR; however, many clinical findings suggest that creatinine undergoes tubular secretion in the kidney. The secretory component of creatinine elimination could account for 10–40 % of urinary creatinine excretion in healthy individuals (Levey et al. 1988). The bias in renal function data estimated on the basis of creatinine clearance could be reduced by determining the clearance after the administration of cimetidine, bringing the GFR close to the value estimated by insulin clearance (81.8 ± 23.6 mL/ min) (Kabat-Koperska et al. 2004). Furthermore, inhibition of either or both OCT2 and MATEs by DX-619 (a fluoroquinolone) or pyrimethamine elevated the plasma concentration of creatinine and reduced its renal clearance without affecting the GFR in healthy subjects (Opravil et al. 1993; Sarapa et al. 2007; Imamura et al. 2011; Kusuhara et al. 2011). Drugs causing an elevation in serum creatinine will include in vivo inhibitors of either or both OCT2 and MATEs. In addition to creatinine, recently, N-methylnicotinamide (NMN), a metabolite of nicotinamide, was also found as an endogenous probe of OCT2 and MATEs (Ito et al. 2012b). Unlike creatinine, its plasma concentration was not sensitive to the inhibition of MATEs, but pyrimethamine reduced the renal clearance of NMN by 70 % in healthy subjects. The renal clearance of NMN in pyrimethamine-treated group was close to the GFR, indicating complete inhibition of the tubular secretion of NMN by clinical dose of pyrimethamine. It was found that complete OATP1B1 and OATP1B3 deficiency causes Rotor syndrome (OMIM #237450), an autosomal recessive disorder characterized by conjugated hyperbilirubinemia (van de Steeg et al. 2012). This could be

also observed in Oatp1a/1b knockout mice which also accumulate bile acids in the plasma (Krumpochova et al. 2012). A SNPs of OATP1B1 is associated with plasma bile acids, such as tauroursodeoxycholic acid and taurochenodeoxycholic acid (Xiang et al. 2009), and with unconjugated bilirubin, but not with bilirubin glucuronides (Ieiri et al. 2004). Bilirubin glucuronide and these bile acids may be biomarkers for phenotyping of OATPs. Bilirubin glucuronide is also an endogenous substrate of MRP2, and its impairment causes Dubin Johnson syndrome (OMIM #237500), an autosomal recessive disorder characterized by conjugated hyperbilirubinemia. A pharmacogenomic study in healthy subjects grouped according to the MRP2 genotypes (rs12762549), which is associated with docetaxel-induced neutropenia (Kiyotani et al. 2008), suggested that phase II metabolites of flavonoids, such as sulfoglucuronide of genistein, showed a significant difference in the plasma according to the MRP2 genotypes (Kato et al. 2012). Mrp2-deficient mutant rats and knockout mice also accumulate such phase II metabolites (Krumpochova et al. 2012; Kato et al. 2012). Although they are not endogenous metabolites, they may be biomarkers for MRP2 besides bilirubin glucuronides.

4.7   Conclusion

Inhibitors and probe substrates for drug transporters have contributed to establish the importance of drug transporters in the disposition of drugs by clinical DDI and pharmacogenomics studies. Such probes and inhibitors are yet not established for all the drug transporters. This is partly due to the limitation in the non-radiolabeled probes in terms of pharmacokinetics. However, PET probes overcome this problem, and they provide further insight into the importance of drug transporters in the tissue concentrations including brain. Furthermore, endogenous probes have an advantage in evaluation of transport activities without administrating probe substrates. This merits pharmaceutical industries in evaluation of risk of DDI in early phase of drug discovery. We expect that such endogenous biomarkers greatly contribute to elucidate the change in transport activities under diseases conditions and dig out drugs which can be in vivo inhibitors of drug transporters.

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Chapter 5
Applications of Targeted Proteomics in ADME for IVIVE

Larissa M. Balogh and Yurong Lai

Abstract Membrane transporters act as physiological "gatekeepers" that regulate the distribution of endogenous and exogenous compounds. It is therefore imperative that drug discovery/development research considers the function and expression of drug transporters, which can dictate drug concentration to pharmacological targets or may be the drug target themselves. Variation in transporter expression across species and in vitro models is recognized as a major complicating factor encountered during in vitro–in vivo extrapolations that can limit a model's predictive power. This is particularly problematic in scenarios such as biliary secretion that are dependent upon in vitro and preclinical data due to lack of clinical bile samples. Consequently, quantification of drug transport proteins becomes a fundamental element in establishing important correlations for pharmacokinetic predictions that are of significant interest during drug discovery. In this chapter we provide an overview of methodologies relevant to protein quantification and their important limitations, followed by a review of recent studies in which mass spectrometry-based targeted quantifications of drug transporters are applied in predictions of transporter-mediated drug clearance.

Abbreviations

ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism, elimination
AQUA	Absolute quantification
BCRP	Breast cancer resistance protein (human)
Bcrp	Breast cancer resistance protein (other species than human)

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BLAST   Basic local alignment search tool
BSEP   Bile salt export pump (human)
Bsep   Bile salt export pump (other species than human)
CHAPS   3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CYP   Cytochrome P450
ELISA   Enzyme-linked immunosorbent assays
ESI-Q-TOF   Electrospray ionization quadrupole time of flight
IS   Internal standard
IVIVE   In vitro–in vivo extrapolation
LC   Liquid chromatography
LC-MS/MS   Liquid chromatography tandem mass spectrometry
MDCK   Madin–Darby canine kidney
MDR1   Multidrug resistance protein (P-gp)
MRM   Multiple reaction monitoring
MRP2   Multidrug resistance-associated protein 2 (human)
Mrp2   Multidrug resistance-associated protein 2 (other species than human)
MS   Mass spectrometry
MSD   Membrane-spanning domain
NBD   Nucleotide-binding domain
OATP   Organic anion-transporting polypeptide
P-gp   Multidrug resistance protein (MDR1)
PK   Pharmacokinetics
PSAQ   Protein standard absolute quantification
PTM   Posttranslational modifications
RAF   Relative activity factor
RT-PCR   Reverse transcription polymerase chain reaction
SCH   Sandwich-cultured hepatocyte
SDS   Sodium dodecyl sulfate
SIL   Stable isotope-labeled
SILAC   Stable isotope labeling by amino acids in cell culture
SLC   Solute carrier
SNP   Single nucleotide polymorphisms
TOF   Time of flight
WT   Wild type

5.1   Introduction

Absorption, distribution, metabolism, elimination (ADME)-related proteins, such as cytochrome P450 (CYP) enzymes and drug transporters, represent versatile metabolism and transport systems that play a pivotal role in the disposition of xenobiotics as well as endogenous substrates such as vitamins, peptides, and hormones. The expression of these proteins can be influenced by a number of factors such as disease, genetics, and exposure to inducers. The subsequent impact can include a

change in the total expression of a given family of proteins and can also involve perturbations in the tissue expression of specific isoforms. Since ADME-related proteins regulate the disposition of drugs, it becomes imperative to determine protein expression in various organs under various pathophysiological conditions in order to enable the prediction of disposition and adverse interactions with co-administered drugs. In this regard, the prediction of human pharmacokinetics (PK) remains an active and challenging area in drug discovery and development and consequently in vitro and in vivo preclinical ADME models have been investigated for their capability to predict human PK parameters. In such models, the Michaelis–Menten equation is one of the best-known to describe enzyme kinetics and it has been applied for in vitro–in vivo extrapolations (IVIVE) related to enzyme catalyzed clearance (Iwatsubo et al. 1997; Shimada et al. 1994), wherein V_{max} and K_m are the two determinants of clearance. While K_m is a unique parameter of a designated substrate for a given protein, V_{max} is derived from the catalytic rate constant and protein expression level in a given system. Accordingly, the application of a known amount of CYP enzyme, in an in vitro incubation study, is a key component for successful IVIVE when CYP-mediated metabolism is to be predicted (Obach 2001). While IVIVE is fairly well-established for the prediction of CYP clearance, similar approaches applied to predict drug transporter-mediated clearance are not straightforward. One of the main factors complicating prediction of transporter-mediated clearance is the large difference encountered in the clearance rates/routes across species and in vitro models (Lai 2009). Thus, a more comprehensive understanding of the mechanisms underlying the interspecies differences encountered in these models could increase confidence in human PK predictions. For instance, multiple in vitro systems, including transporter-over-expressed membrane vesicles and immortalized cell lines, have been widely used to determine transporter-involved drug disposition, but obtaining protein expression levels in these models is a recognized obstacle for translating the activity to in vivo. IVIVE is also not well-established from preclinical species to human, particularly in elimination routes such as biliary secretion that involve transporter-mediated clearance mechanisms yet to be fully characterized. This is further complicated by biliary secretion models that are highly dependent upon in vitro and preclinical data due to lack of clinical bile samples (Ghibellini et al. 2004). When coupled with kinetic parameter determinations, the quantification of drug transporters can facilitate scaling for extrapolation from preclinical models to human to further promote drug discovery and development. However, the hydrophobic nature of integral membrane protein domains, low expression levels, disconnects between protein and surrogate measures, and lack of reliable protein standards collectively underlie challenges with respect to protein quantification methods.

Among the protein quantification methods, mass spectrometry (MS)-based targeted quantification can readily provide the relative amounts of transporters expressed in different systems that are necessary for scaling transporter-mediated clearance from in vitro to in vivo or from preclinical to clinical. Quantification of transporter proteins may increase the understanding of the variability of transporter-mediated clearance across species or specific populations, although differential

binding affinities must still be taken into account by other means. In this chapter we highlight some of the unique advantages of MS-based protein quantification and discuss important experimental methods, applications, and limitations with an emphasis on the recent targeted quantifications reported for transporters of particular relevance to drug disposition.

5.2 Protein Quantification Approaches

Attempts to quantify proteins of interest have incorporated a variety of approaches. Gene quantification methods including Northern blot, quantitative RT-PCR, and DNA array analyses have all been applied as measurements of protein expression (Vander Borght et al. 2006; Sun et al. 2002; Tanaka et al. 2005; Figge et al. 2004; Bleasby et al. 2006). The outcomes have indeed captured organ gene expression patterns and transcriptional regulation of genes via nuclear receptors (Teng and Piquette-Miller 2005; Nishimura and Naito 2005). Despite this success, these methods are ultimately a surrogate assessment of protein expression levels, which can be complicated by differences in stabilities and expression rates, as well as lack of information regarding posttranslation modifications (Haynes et al. 1998). Noteworthy examples of such oppositions between mRNA transcript and protein levels have been reported (Bleasby et al. 2006; Belinsky et al. 2005; Diao et al. 2010; Haimeur et al. 2004). Immunoblotting techniques, such as western blot analysis or enzyme-linked immunosorbent assays (ELISA), have also been popular approaches to directly characterize protein expression. While these assays can be sensitive and robust, the applications can be limited by lack of pure protein standards or the cross-reactivity and availability of suitable antibodies (Michaud et al. 2003).

Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS)-based quantitative proteomics has been increasingly employed for targeted protein quantification within the enzyme and transporter disciplines (Kamiie et al. 2008; Li et al. 2008, 2009a, b, c, 2010; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a, b; Zhang et al. 2011; Kawakami et al. 2011; Seibert et al. 2009). Generally, intact proteins are digested into peptides with subsequent separation and detection by LC-MS/MS to measure peptide ion intensities as a surrogate measurement of protein levels. In contrast to surrogate transcripts, surrogate peptides are derived directly from proteins and hence are decoupled from posttranslation disconnects. These advantages render MS-based quantification a useful tool to help elucidate remaining gaps not addressed by previous approaches. Furthermore, unlike immunochemical methods, which can be limited by restricted access to an appropriate antibody, MS-based quantifications use peptides unique to the protein of interest. Such unique peptides can be readily obtained from commercial sources and serve as surrogate standards for the protein of interest and consequently overcome the absence of protein standards. Despite these advantages, significant analytical challenges in MS-based quantifications remain as certain ADME proteins, in

particular drug transporters, are present at relatively low expression levels and encompass multiple hydrophobic domains. Improvements in sample preparation methods (including a combination of immunoaffinity protein enrichment as well as anti-peptide antibodies (Anderson et al. 2009)), MS instrumentation, and ultra performance LC have emerged as a means to overcome low expression levels. It is however important to note that structural differences among proteins can result in varying levels of proteolysis. Since proteolysis has a significant impact on accuracy at the protein level, it may thereby limit *absolute protein* applications, especially in the absence of protein standards as further discussed in Sect. 5.4.2.

5.3 Targeted Proteomics

5.3.1 Overview of Mass Spectrometry-Based Targeted Protein Quantification

Quantitative targeted proteomics represents a subset of proteomic analyses in which highly sensitive and reproducible multiple reaction monitoring (MRM) MS methodologies are commonly used to detect specific peptides in a complex mixture (Yocum and Chinnaiyan 2009; Elschenbroich and Kislinger 2011). These peptides are generated through the digestion of intact proteins with subsequent separation and detection by LC-MS/MS (Fig. 5.1). The detection of intact proteins by MS-based analyses, beyond the scope of this chapter, is also used to study membrane proteins (Whitelegge et al. 2006). However, the drug transporter quantifications reviewed here all utilize a *bottom-up* approach in which peptides produced from the digestion of proteins are used as a surrogate measure of the original protein. While the low abundance of a given transporter protein may still underlie detection sensitivity challenges, with respect to LC column capacity, the targeted nature of these analysis
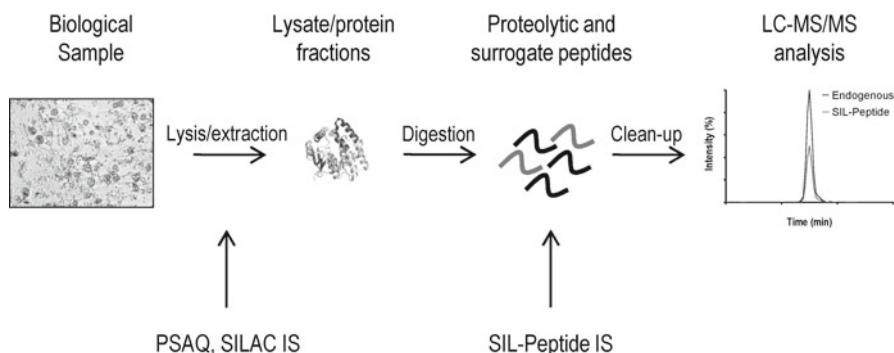
Fig. 5.1 Overview of experimental workflow and different methods for internal standard introduction

provides the means to detect specific peptides present in a complex mixture using routine triple quadrupole instrumentation (Chalkley 2010).

The first step of a targeted quantification consists of selecting candidate peptides that can serve as a surrogate measure for the protein of interest. This can be accomplished through the use of in silico predictive tools (Kamiie et al. 2008; Zhang et al. 2011), which can be complemented with experimental tools discussed below (Li et al. 2008). Although there are several proteases available (as well as chemical cleavage methods), trypsin can be an ideal initial choice as it often produces fragments amenable to detection by MS in terms of size and amino acid composition. As a general guide, the m/z value of a doubly charged precursor will need to lie within the detectable range of the mass spectrometer being used, although triply charged precursors may be detected in some cases. The vast majority of the theoretical peptides produced by an in silico digestion (http://prospector.ucsf.edu/prospector/mshome.htm) can be excluded on the basis of size, stability, and sequence identity. Resources such as the PeptideAtlas can also assist in identifying proteotypic peptides (Deutsch et al. 2008). In terms of sequence, peptides containing cysteine/methionine residues or N-terminal glutamine residues, with the potential for chemical modifications and spontaneous cyclizations, respectively, should be excluded during selection. In the interest of optimizing digestion efficiency, the adjacent sequence can also be screened to avoid any continuous segments of arginine and lysine, which could potentially hinder trypsin digestion. Given the complexity of sample digests, a surrogate peptide with a sequence unique to the target protein is necessary. Among the unique peptides that are identified using protein BLAST/homology searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome; http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology) against a pertinent species database, those not known to contain posttranslational modifications (PTM) or mutations derived from single nucleotide polymorphisms (SNP) make optimal candidates unless a particular PTM or SNP is purposely being targeted. It is also important to keep in mind that not all modifications have undoubtedly been reported. Although the detection of peptides derived from the transmembrane domain can be of interest (Eichacker et al. 2004), an ideal target peptide will be located in the more exposed and soluble domains of the protein. In cases where explicit information regarding soluble domains is not available, the consensus of common topology predictions algorithms (Punta et al. 2007; Nam et al. 2009) can assist in discriminating between soluble and transmembrane segments during candidate peptide selection.

Peptide stability is the secondary criteria for the peptides that are filtered through above criteria. The selection process can generally be complemented by target peptide verification in sample digests analyzed by a high-resolution instrument such as a quadrupole time of flight (TOF) MS or linear ion-trap (Chalkley 2010; Prakash et al. 2009). The high-resolution instrumentation can take advantage of high mass accuracy that is needed to confidently identify a peptide in the absence of standards and known fragmentation patterns. The peptide with the best apparent detection sensitivity can then be selected as the quantification probe and the corresponding synthetic peptides can be used for analytical optimization of the

precursor-to-product transitions. Typical quantifications are conducted on a triple quadrupole mass spectrometer by utilizing MRM analyses, which offer two stages of mass filtering for each targeted transition (Elschenbroich and Kislinger 2011; Lange et al. 2008). Although these are directed toward unique peptides, due to the resolution of typical instrumentation, adequate resolution at the LC level is also important to separate interfering factors. Since matrix complexity represents such a significant obstacle for quantification of endogenous material in which a true blank is not available, in addition to LC separation, multichannel MRM analyses should be conducted to verify results with at least three transitions per peptide. The stable isotope-labeled (SIL) peptides that will serve as the co-eluting internal standard (IS) are also monitored in this manner. These isotopes can be incorporated into MS-based quantifications at various stages of the workflow as further outlined in Sect. 5.3.2.

Once validated, the resulting surrogate peptide and MRM method can be used for the digestion and targeted quantification of samples derived from whole cell lysates, membrane extractions, or other relevant preparations that are generally reduced and alkylated to help prevent the reformation of higher-order structure and thereby facilitate protease access. Due in part to the variable composition of integral membrane proteins that reside in a given location, different membrane compartments have their own characteristic properties. The concentration of active transporters present at the cell surface plasma membrane is presumably the most relevant with respect to predictions; however, it should be noted that total membrane protein fractions may include protein beyond the functional transporter on the cell surface. The plasma membrane can be difficult to completely isolate but procedures such as cell surface biotinylation can be incorporated into the process to enrich this fraction (Elschenbroich et al. 2010; Qiu and Wang 2008).

Although membrane protein analyses have also included techniques involving gel-based protein resolution (Rabilloud 2009; Wu and Yates 2003), many recent drug transporter quantifications focused upon herein exploit the targeted detection of hydrophilic peptides produced upon the in-solution digestion of complex samples prior to LC-MS/MS. But as reported with gel-based techniques, hydrophobic protein segments resist exposure to aqueous environments, which can lead to aggregation and sample loss. Therefore, solubilization and denaturation limitations still remain with respect to facilitating protease access and digestion efficiency, the implications of which are discussed in Sect. 5.4.2. When reliable standards are not readily available, which is often the case for the integral membrane drug transporters of interest, it is important to appreciate the effect of each portion of the experimental design as primary and secondary structural differences among proteins can amount to different levels of proteolysis. Standard protein solubilization and denaturation tactics involve the use of chaotropes (urea and guanidine), detergents (triton, SDS, CHAPS, and more recently, MS-compatible detergents such as RapiGest™ SF surfactant (Waters, Milford, MA)), bile acids (deoxycholate), organic solvents, and organic acids (Speers and Wu 2007; Helenius et al. 1979; Lin et al. 2008; Proc et al. 2010). Since these reagents can interfere with proteolysis, their initial concentrations are reduced to a compatible level during the digestion. Evaluations including an assessment of the time course for a digestion are valuable to ensure the

digestion is complete with respect to what is achievable under a given set of conditions. After the digestion is quenched (this is often the point of IS introduction), various peptide enrichment and separation strategies can be used, which at the very least include LC separation prior to MS detection. As alluded to above, several IS options exist. Regardless of which option is incorporated, the IS will be distinguishable by mass spectrometry and can be used to normalize the results (the extent of normalization depends on what type of IS is used), while unlabeled synthetic peptides can be used to construct a standard curve to determine quantification values for each sample.

5.3.2   Internal Standard Strategies

Different *bottom-up* techniques related to the isotope dilution concept have been described for MS-based protein quantification (Gerber et al. 2003; Barr et al. 1996; Zhang et al. 2010; Brun et al. 2009), wherein the identity and the timing of IS introduction offer different advantages (Fig. 5.1). Among the most commonly employed are SIL methods where a SIL synthetic peptide with an identical sequence to the proteotypic peptide is used as the IS during analyte peak area normalization. Other IS approaches have been described, some of which include chemical derivatization, metabolic incorporation of heavy-labeled amino acids, and protein standard absolute quantification (PSAQ) (Elliott et al. 2009). Because peptides can only serve as a surrogate of protein levels, quantitative studies with more precise and accurate methodologies represent advancements with respect to errors derived from variability in native membrane protein extraction, denaturation, and digestion. The stable isotope labeling by amino acids in cell culture (SILAC) approach is one such method which offers a metabolic-labeling strategy for label incorporation during culture (Ong et al. 2002). In this situation, both heavy and light proteins can be combined at the beginning of the experiment and digested together, after which the heavy isotope-labeled peptide serves as the co-eluting IS. Thus, SILAC is recognized for the ability to normalize for losses/enrichments derived from any portion of the workflow and increase the precision across different measurements (Ong et al. 2002; Geiger et al. 2011; Hanke et al. 2008; Harsha et al. 2008; Ong and Mann 2006, 2007). PSAQ methods are similar in concept to SILAC in that the IS is derived from a labeled protein; however, in the case of PSAQ (or "absolute SILAC"), the concentration of the IS is known, usually by quantitative amino acid analysis of purified material, thereby allowing for better accuracy at the protein level (Hanke et al. 2008; Brun et al. 2007; Ishihama et al. 2005; Lebert et al. 2011). In contrast, due to different biochemical properties, SIL peptides must be added either during or post-digestion to serve as the IS for the remainder of the experiment. Consequently, this is the least accurate approach at the *protein* level, as variability prior to digestion cannot be accounted for. Nonetheless, SIL methods such as the absolute quantification (AQUA) method (Gerber et al. 2003) are usually the most feasible and rapid methods that may be perfectly suited to extract scaling factors from relative

quantification studies. Indeed, SIL peptides are among the most common IS techniques used to evaluate the expression of drug transporters thus far (Kamiie et al. 2008; Li et al. 2008, 2009a, b, c, 2010; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a, b; Zhang et al. 2011).

5.4 Applications

Given that the expression of ADME-related proteins is known to vary with species as well as developmental and pathological characteristics (age, sex, disease state) (Meier et al. 2006; Renton 2001, 2004), it is necessary to monitor fluctuations in protein expression in order to understand the in vivo disposition of many drugs and improve the predictability of in vitro models (Li et al. 2010). In 1997, Crespi and Penman developed the relative activity factor (RAF) method to characterize specific CYP isoform contributions (Crespi and Penman 1997), which has also been implemented for the characterization of transporter-mediated drug disposition (Kitamura et al. 2008; Maeda et al. 2010). While the RAF approach provides a useful tool to characterize the contribution of enzyme/transporter isoforms, routinely observed overlaps in substrate specificities still underlie major hurdles with respect to identifying "clean" reference substrates. In turn, the quantification of ADME proteins becomes a key factor in establishing the correlations between in vitro models, preclinical species, and human, which could ultimately accelerate the early stages of drug discovery. The potential applications of targeted proteomics can span several stages of drug discovery and development (recently reviewed by Ohtsuki et al. 2011) and encompass other areas of research such as protein drugs and protein drug targets, biomarker validation, and PTM investigations. In addition to the obvious applications, other intriguing avenues within the scope of MS-based proteomics have emerged in protein topology and interaction research (Wu and Yates 2003; Wu et al. 2003). This additional research may provide underlying mechanistic information that can increase our understanding of transporters, which can ultimately be applied to advance predictions. However, to date, the vast majority of quantification studies within the drug transporter discipline have emphasized the targeted determination of transporter levels in species, tissues, and in vitro models in the interest of understanding expression differences which are among the confounding factors encountered in IVIVE.

5.4.1 LC-MS/MS-Based Quantification of Drug Transporters

The application of targeted LC-MS/MS-based proteomics has facilitated the detection and quantification of numerous proteins with relevance to drug disposition. Our focus here is devoted to transporter proteins; however, other notable ADME-related proteins such as the CYP enzymes are an active area of targeted quantification

analyses (Kawakami et al. 2011; Seibert et al. 2009; Langenfeld et al. 2008). Although the challenges derived from low abundance and a proteolytically resistant nature presumably contributed to an initial lag in integral membrane protein quantifications as compared with soluble protein studies, SIL peptide-based methods have been increasingly incorporated to evaluate drug transporters. Two popular approaches have emerged, the first of which involves the characterization of individual transporter proteins that is often coupled with method optimization. The second approach utilizes a higher-throughput strategy that can rapidly quantify numerous proteins, although individual secondary characterizations may eventually be required for certain applications, as this approach does not include time-consuming sample preparation optimizations that may be necessary with respect to detection sensitivity for some proteins.

5.4.1.1   Individual Characterizations

Over the course of a series of studies by (Li et al. 2008, 2009a, b, c, 2010), SIL peptides were implemented to examine individual ATP-binding cassette (ABC) transporters in order to characterize the different cell models that are routinely used to assess transporter involvement with potential drug candidates. These analyses also aimed to determine the relative transporter expression across species and tissues. In the earliest of these studies (Li et al. 2008), multidrug resistance-associated protein 2 (MRP2) immunoprecipitation-enriched samples were initially digested and detected with high resolution using nanospray ESI-Q-TOF analyses in order to select the proteotypic peptide with the best apparent detection sensitivity. The resulting peptide was used to quantify MRP2-transfected Madin–Darby canine kidney (MDCK) cells as well as the endogenous canine Mrp2, which was notably undetected by immunoblotting. Since the proteotypic peptide identified was able to selectively recognize the MRP2/Mrp2 protein in multiple species (Fig. 5.2), they subsequently utilized this method to examine the hepatobiliary transporter in liver tissues and hepatocytes from human, dog, rat, and monkey (Li et al. 2009c). In total, the amount of MRP2/Mrp2 in livers was found to rank rat ≫ monkey > dog ≈ human, wherein Mrp2 was approximately tenfold higher in rat than MRP2 in human. Interestingly, a 40 % loss of Mrp2 was also observed in cryopreserved hepatocytes, which provides insight regarding the potential differences between in vivo circumstances and important models of transport even within a single species. Li et al. 2009b also extended their quantifications to include measurements of bile salt export pump (BSEP) and breast cancer resistance protein (BCRP) in livers and hepatocytes. The proteotypic peptides identified in this study determined the amount of BCRP/Bcrp and BSEP/Bsep ranked dog > rat > monkey ≈ human and rat ≈ monkey > dog ≈ human, respectively.

   To the extent that interspecies differences can limit a model's predictive power, these results represent key findings with respect to translational gaps in IVIVE for substrates undergoing transporter-mediated elimination. The sandwich-cultured hepatocyte (SCH) model, which provides the proper orientation and localization of

Fig. 5.2 Schematic representation of membrane topology of BCRP/Bcrp and BSEP/Bsep and protein alignment across species. The proteotypic peptide for BCRP/Bcrp was selected from the intracellular *N*-terminal (**a**) or intracellular nucleotide-binding domain for BSEP/Bsep. (**b**) The stable isotope-labeled (SIL) internal standard was indicated with a single residue substitution of $[^{13}C_6, ^{15}N_1]$ Leu. Genebank number: human BCRP (NP_004818); rat Bcrp (NP_852046); dog Bcrp (NP_001041486); monkey Bcrp (AAX56948); human BSEP (NP_003733); rat Bsep (NP_113948) (with permission from Li et al. 2009b)
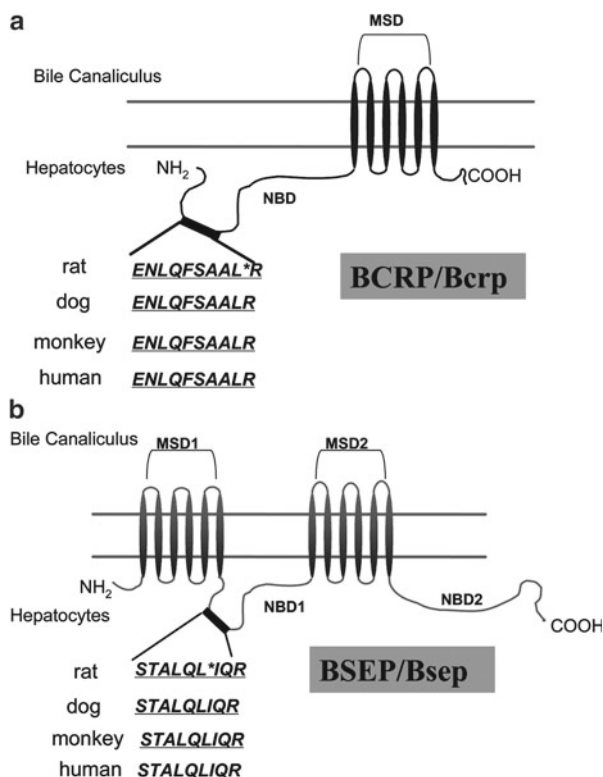
transporters along with the development of intact bile canaliculi, has become an important tool for investigating vectorial transport that cannot be assessed in hepatocyte models lacking cell polarity. However, the absence of quantitative information regarding transporter expression in SCH (which can also fluctuate during cell culture periods) complicates IVIVE. Collectively, the methods described for MRP2, BSEP, and BCRP were used to characterize SCH to compare levels across the cell culture periods and with in vivo findings (Li et al. 2009a). A number of alterations were observed and the results nicely illustrated that the amounts of MRP2/Mrp2 and BCRP/Bcrp could be correlated with the intrinsic clearance of test compounds in SCH (Fig. 5.3). Furthermore, by integrating a scaling factor to reflect the mass recovery of hepatobiliary transporters between in vitro SCH and in vivo and the respective contribution when multiple transporters are involved, the prediction of biliary secretion in rats was improved (Li et al. 2010). In total, the applications targeting the efflux transporters increase confidence for IVIVE of human biliary clearance and provide insight regarding the hepatocyte lot- and culture time-dependent expression of transporters that can underlie inter-experimental variation. The hepatic uptake transporters, which can perform the rate limiting step of clearance for specific compounds (Giacomini et al. 2010), also need to be addressed. As of this writing, to our knowledge efflux transporters have been the focus of
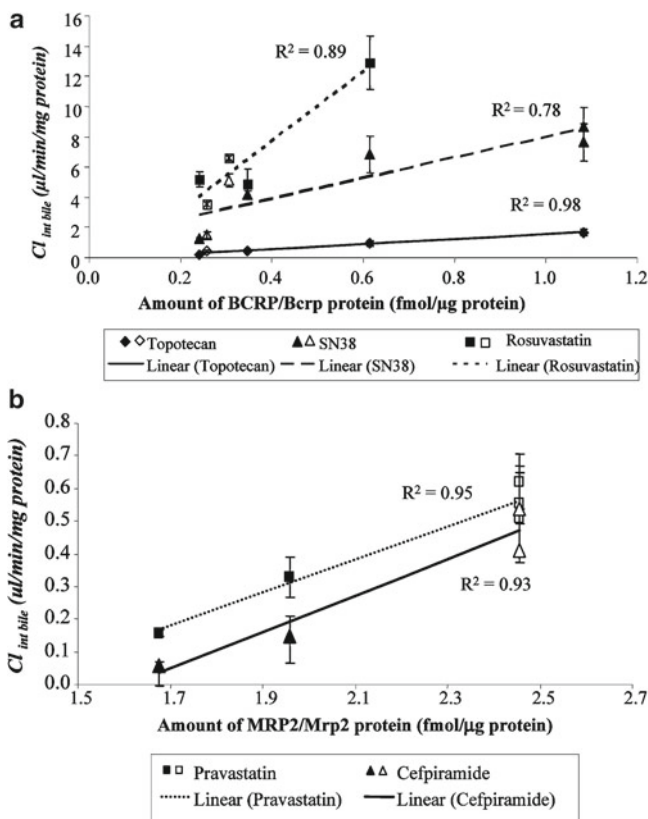
Fig. 5.3 Correlation of protein amount and intrinsic biliary secretion in SC hepatocytes. (**a**) The intrinsic biliary secretion of SN38, topotecan, and rosuvastatin was plotted against the protein level of BCRP/Bcrp in difference lots of human or rat hepatocytes. *Solid symbols* represent freshly isolated human or rat hepatocytes, while the *open symbols* represent cryopreserved human hepatocytes. (**b**) The intrinsic biliary clearance of cefpriamide and pravastatin was plotted against the amount of MRP2/Mrp2 protein in the corresponding lot of SC hepatocytes. *Solid symbols* represent cryopreserved human hepatocytes, while *open symbols* represent fresh rat hepatocytes (with permission from Li et al. 2009a)

published studies correlating LC-MS/MS protein quantification with functional activity; however, organic anion-transporting polypeptides (OATPs) have also been examined by targeted protein quantification (Sakamoto et al. 2011; Uchida et al. 2011a; Niessen et al. 2009; Balogh et al. 2012) and will presumably be incorporated for IVIVE in future studies.

P-glycoprotein/multidrug resistance protein 1 (P-gp/MDR1) is also among the efflux transporters characterized by targeted protein quantification. Using a similar methodological approach as described above, Zhang et al. (2011) developed a LC-MS/MS method applied to quantify human P-gp in gene-transfected MDCK cells. The selected peptide was also able to provide a comparison with the

endogenous canine P-gp present in MDCK cell lines. In addition to the anticipated uses of this quantification method to enable more accurate predictions of in vivo drug disposition, the MS-based quantification of canine P-gp was recently used to assist in characterizing a new cell line, MDCKII-LE (low efflux), that was found to exhibit fivefold lower P-gp levels than MDCKII-WT (Di et al. 2011). This cell line is expected to offer an advantage over existing WT cells currently used for passive permeability measurements by diminishing the interference derived from endogenous canine P-gp activity. Most recently, P-gp was also examined by targeted quantification in a study aimed at assessing the brain distribution of P-gp substrates (Uchida et al. 2011b). P-gp-mediated efflux can ultimately affect brain distribution and hence pharmacological action. Accordingly, brain-to-plasma ratios can be used as an indicator in this regard during drug identification/selection processes. To this end, the level of P-gp quantified in both transfected cells and in mouse brain capillaries was integrated with in vitro transport activity (derived from efflux rates) and drug unbound fraction in order to reconstruct brain-to-plasma concentration ratios (K_p), which were found to provide a reasonable estimate (within threefold) of in vivo values for nine of the 11 compounds tested. As the level of transporter will vary from in vitro systems to brain endothelial cells, the results of this study demonstrate the utility of protein quantification information to further support IVIVE for prediction of drug penetration into the brain, which can then assist in identifying effective central nervous system drugs.

5.4.1.2 High-Throughput Characterizations

A series of publications reporting the quantification of an extensive number of membrane proteins have utilized a single set of sample preparation conditions combined with multiplexed selected reaction monitoring to conduct higher-throughput quantifications on brain, liver, kidney, and platelet samples (Kamiie et al. 2008; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a; Ito et al. 2011; Niessen et al. 2010), the precision and accuracy of which (at least at the peptide level) has been demonstrated to be reliable for various drug transporters and drug metabolizing enzymes (Sakamoto et al. 2011). Among the major studies, a paper by Kamiie et al. (2008) combined in silico peptide selections with the simultaneous analysis of 36 membrane proteins, including ABC and solute carrier (SLC) transport proteins, 26 of which were reported to be adequately detected/quantified in at least one of the tissue types analyzed (mouse brain capillaries, liver membranes, renal cortex membranes, and renal medulla membranes). As noted by the authors, since a major bottleneck encountered in drug discovery and development is the time elapsed between preclinical and clinical studies, this approach could serve as a useful tool for rapidly comparing a given protein's level across biological samples and species to help reduce the delay for advancement. Follow-up studies extended upon the surrogate peptide library to target an extensive number of membrane proteins in human and monkey brain microvessels (Shawahna et al. 2011; Uchida et al. 2011a; Ito et al. 2011). The detailed results of two of these studies were recently

highlighted in a review dedicated to targeted proteomics in ADME research (Ohtsuki et al. 2011). Collectively, these aforementioned studies provide vital information with respect to understanding species differences in transporter expression at the blood–brain barrier. However, it should also be emphasized that a large percentage of the targeted proteins in these studies could not be adequately detected/ quantified. Among these presumably reside some transporters that are in fact present at significant levels, but are simply not detectable under the chosen sample preparation conditions. Due to the protein- and denaturant-dependent solubilization and digestion efficiency of these analyses, future method optimizations for individual proteins could result in a drastic increase of surrogate peptide production (Proc et al. 2010; Balogh et al. 2012) and reveal the relative levels of the undetected proteins reported in the species comparisons. The protein- and denaturant-dependent nature of these analyses also underscores a second limitation (expanded upon in Sect. 5.4.2) inherent to both individual and high-throughput characterizations with SIL peptides, which generally complicates reliable comparisons between the levels of *different* proteins.

5.4.2 Important Limitations of Targeted Quantifications in the Absences of Reliable Standards

In addition to the determination of scaling factors for individual proteins, there is significant interest in characterizing the contribution of co-localized transporters with overlapping substrate specificity in order to better understand the key determinants of drug disposition and predict drug–drug interactions. Due to the absence of reliable membrane protein standards of known concentration, the studies reviewed above ultimately use the AQUA of a surrogate *peptide* to analyze a *protein*, where the peptide levels, which can be determined with a high level of accuracy (Sakamoto et al. 2011), will reflect the relative amounts of the specific protein in different samples. However, despite the popular usage of phrases such as *absolute/accurate protein quantification*, the true accuracy at the protein level is generally not known and can vary to a significant extent from method to method and from protein to protein (Proc et al. 2010; Brun et al. 2007, 2009; Lebert et al. 2011; Balogh et al. 2012; Klammer and MacCoss 2006). Consequently, caution should be exercised during interpretation when a clear stoichiometrical relationship between the amount of proteolytic peptide detected and corresponding protein is not explicitly established. This issue derives from the fact that primary and secondary structural differences among proteins can amount to varying levels of proteolysis under the sample preparation conditions that are compatible with typical studies using trypsin digestion and LC-MS/MS-based peptide detection. Solubilization and denaturation limitations have been well-recognized within the general proteomic literature, particularly in studies and reviews tackling membrane proteomics that note the optimum conditions will vary across proteins (Rabilloud 2009; Wu and Yates 2003; Speers and Wu 2007; Helenius et al. 1979; Proc et al. 2010; Brun et al. 2007, 2009;

Lebert et al. 2011; Balogh et al. 2012; Klammer and MacCoss 2006; Agarwal et al. 2010; Arsene et al. 2008; Grant and Wu 2007; Zhang et al. 2009). However, the magnitude of impact on conclusions drawn from a subset of transporter-related quantifications has yet to be addressed in this respect. In particular, two of the studies highlighted above, which initially targeted >100 proteins, directly equate peptide quantification to protein levels in order to compare the abundance of different transporters in human brain microvessels (Shawahna et al. 2011; Uchida et al. 2011a). In this application, conclusions such as *BCRP was expressed 1.6-fold more than MDR1* (Shawahna et al. 2011), or *BCRP was the most abundant followed by MDR1* (Uchida et al. 2011a), rely on the assumption that the sample preparation conditions do not have a significant impact on the digestion efficiencies for each protein. Although differences in solubility are assumed to be negligible based on auxiliary studies with MDR1 and BCRP (Kamiie et al. 2008), tests with alternate preparations were not reported. Moreover, research surrounding the effect of multiple digestion schemes for several plasma proteins (Proc et al. 2010; Brun et al. 2007; Klammer and MacCoss 2006), as well as our in-house studies with OATP transporters (Balogh et al. 2012), indicates this assumption cannot be extrapolated to compare numerous proteins since surrogate-based results will generally not reflect the underlying endogenous ratios between different proteins. This is particularly concerning under the typical enzymatic digestions employed in these types of studies, which requires a balance between conditions that enhance proteolysis through denaturation of the target protein and conditions that will not significantly inhibit the proteolytic activity of the digestion enzyme.

Since solubility remains one of the major hurdles in identification and quantification, optimizations tailored to a specific protein often encompass membrane solubilization strategies that typically examine organic solvents, detergents, and chaotropic agents, provided they are compatible with the route of digestion and subsequent MS analysis. The production of target peptide can also be monitored over the course of the digestion in order to ensure samples are taken at an optimal time point. This is an important evaluation to perform during method development; however, it is also important to understand that each method-protein combination can appear to reach completion at its own plateau, although the digestion may not be complete with respect to the maximum that is theoretically possible (Fig. 5.4) (Proc et al. 2010; Balogh et al. 2012). Incomplete digestion can at least be partially addressed by utilizing a combination of proteases and additional cleavage methods (Wu and Yates 2003). For example, the combination of Lys-C and trypsin can be advantageous to increase digestion efficiency. Due to the importance of membrane protein characterization, new tools are continually being explored. For example, lipid-based protein immobilization provides immobilization and digestion of bilayer-embedded native membrane proteins to rapidly probe the solvent-exposed domains in a flow cell format (Sui et al. 2011). But despite extensive research that will presumably be advantageous in the future, the efficient digestion of proteins is still limited under the routine conditions employed to obtain the majority of drug transporter levels reported thus far. Targeted method optimization can of course improve detection and accuracy by increasing the detection signal and closing the gap between the
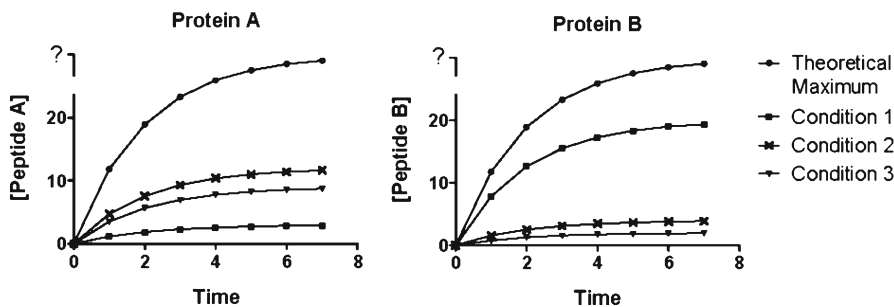
Fig. 5.4 Digestion profile evaluation. The progress curves derived from a sample that is processed by different solubilization/denaturation methods may appear to reach completion, although the digestion may not be complete with respect to the maximum peptide production expected for the initial protein amount (which is not known in the absence of quality standards). The effect of sample preparation conditions with respect to digestion efficiency can also vary between proteins (Protein A vs. Protein B). The optimization of native protein processing for a targeted protein can significantly improve digestion efficiency and thereby improve detection and accuracy by closing the gap between the theoretical maximum and the observed peptide production under a given set of conditions

observed peptide production and the theoretical maximum. This approach can be valuable for relative quantification studies when sensitivity is a limiting factor, but it is emphasized that an unknown gap in accuracy still remains (Fig. 5.4).

In addition to screening numerous detergents and solvents, alternative attempts have examined digestion efficiency with extended surrogate peptides that contain a few amino acids from the surrounding sequence on either side. However, the proteolytic accessibility of a short peptide is unlikely to reflect that of a large integral membrane protein under protease-compatible conditions and hence can significantly overestimate digestion efficiency. Pursuit of purified protein, which structurally mimics endogenous material throughout the entire experiment, would offer the ideal IS to control for levels of protein loss/enrichment, extraction, denaturation, and digestion, as illustrated by PSAQ approaches (Hanke et al. 2008; Brun et al. 2007; Ishihama et al. 2005; Lebert et al. 2011). In this situation the accuracy would only be limited by the initial assessment of the standard material rather than the preparation-dependent nature of relative analyses. However, obtaining reliable membrane protein standards is not a trivial task. Therefore, to the extent that ratios determined under different sets of sample preparation conditions can underlie completely different conclusions, surrogate peptide-based quantifications are generally limited to relative determinations until further advancements are achieved.

5.5 Summary and Perspectives

Changes in ADME protein expression and associated function are realized as important components to understand in vivo drug disposition and improve the predictability of in vitro models. Many protein expression characterization options

exist, each with their own advantages and disadvantages such that there is no recognized "one-size-fits-all" approach that can accomplish every quantification task. For example, in situ immunohistochemical staining offers a unique opportunity to investigate the differential distribution of proteins in different parts of biological tissues. But in contrast to the aforementioned challenges and disconnects highlighted for immunochemical as well as mRNA transcript approaches, targeted protein quantification offers the opportunity to assess protein expression in various biological matrices by way of a sensitive and selective method amenable to high-throughput formats. The information obtained has the potential to fill gaps in understanding for transporter-mediated clearance across species, IVIVE, and populational distributions underlying variations in drug disposition. Reliable membrane protein standards are not yet readily available and caution is still warranted in relative peptide quantifications that may not directly reflect the relative abundance of different proteins. However, significant technological advances and individual method optimizations can indeed improve detection and accuracy by addressing the sample handling, digestion efficiency, and separation challenges that affect quantification in *bottom-up* proteomic workflows. Once established, these methods will undoubtedly continue to make LC-MS/MS-based quantifications more reliable and accessible in the ADME community.

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Chapter 6
Prediction of Hepatic Transporter-Mediated Drug–Drug Interaction from In Vitro Data

Kazuya Maeda and Yuichi Sugiyama

Abstract The importance of transporter-mediated drug–drug interaction (TP-DDI) has been rapidly recognized by the recent publication of its clinical evidences and subsequent updated regulatory guidance (guideline). The methods of TP-DDI prediction are roughly divided into two approaches; static model and dynamic model. Static model with theoretically maximum unbound concentration is useful to sensitively catch the signal of DDIs, but predicted DDI risk should always be overestimated. Dynamic model fully considers the time courses of the plasma and tissue concentrations of both substrate and inhibitor drugs by the physiologically based pharmacokinetic (PBPK) model, thus accurate estimation of DDI risk can be achieved. However, the universal methods to set up model parameters based on the in vitro results with scaling factors remain to be discussed. This chapter is mainly focused on the basic theory and recent progress of the methods for TP-DDI predictions.

Abbreviations

AUC	Area under the concentration-time curve
BCRP	Breast cancer resistance protein
BSP	Bromosulfophthalein
CYP	Cytochrome P450

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DDI   Drug–drug interaction
$E_2 17\beta G$ Estradiol-17β-D-glucuronide
EMA   European Medicines Agency
FDA   Food and Drug Administration
MRP   Multidrug resistance-associated protein
NTCP   Sodium taurocholate cotransporting polypeptide
OATP   Organic anion transporting polypeptide
PBPK   Physiologically based pharmacokinetic
PET   Positron emission tomography
P-gp   P-glycoprotein
TP-DDI   Transporter-mediated drug–drug interaction

6.1 Introduction

The number of clinical drug–drug interaction (DDI) studies involving drug transporters has increased rapidly in recent years, and the ability to predict transporter-mediated DDIs (TP-DDIs) is needed in the process of drug development. The liver is one of the most important organs for the detoxification of drugs. The liver expresses many kinds of metabolic enzymes and uptake/efflux transporters, and DDIs with hepatic enzymes or transporters often lead to a change in the plasma concentration and subsequently the pharmacological and toxicological effects of drugs. The US Food and Drug Administration (FDA) DDI draft guidance and European Medicines Agency (EMA) DDI guideline note that organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 in the liver are important transporters for the hepatic uptake of various organic anions and that pharmaceutical companies must investigate whether new chemical entities are substrates or inhibitors of OATP1B1 and OATP1B3. In previous clinical reports, the plasma concentrations of several OATP substrates were increased significantly by coadministration of OATP-inhibitor drugs such as cyclosporine A and rifampicin (Fig. 6.1). By contrast, several biliary efflux transporters such as P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP) can recognize a broad range of compounds as substrates. The inhibition of these efflux transporters may lead to an increase in the hepatic concentration of substrate drugs, but not their plasma concentration, suggesting that such DDIs may be difficult to be detected.

This chapter briefly reviews the theoretical background and experimental methods for DDI prediction and recent progress in DDI prediction strategies.

6.2 Basic Theory of the Quantitative Prediction of Transporter-Mediated DDIs

When the kinetic property of transporter function follows traditional Michaelis–Menten kinetics, the intrinsic transport clearance of substrates via target transporters (CL_{int}) can be described as follows:

$$CL_{int} = \frac{V_{max}}{K_m + C_u} \tag{6.1}$$
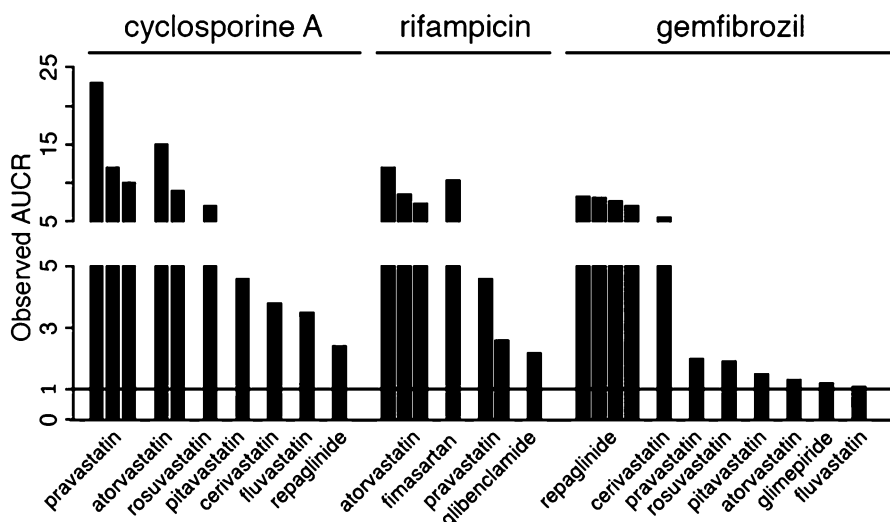
Fig. 6.1 Effects of coadministration of cyclosporine A, rifampicin, and gemfibrozil on the plasma AUCs of OATP substrate drugs (statins, sartans, and antidiabetic drugs) (cited from Yoshida et al. 2013). *Y*-axis indicates the ratio of plasma AUC of substrates in the presence of inhibitors to that in their absence

V_{max}, K_m, and C_u represent the maximum transport velocity, Michaelis–Menten constant, and concentration of protein-unbound compounds, respectively, which are thought to be recognized by transporters as substrates. If the unbound concentration of a substrate is far below the K_m value, (6.1) can be converted into (6.2).

$$CL_{int} = \frac{V_{max}}{K_m} \qquad (6.2)$$

Assuming that an inhibitor drug inhibits the transporter in a competitive or noncompetitive manner, the intrinsic transport clearance of a substrate in the presence of an inhibitor drug can be described by the following equation:

$$CL_{int}(+\text{inhibitor}) = \frac{V_{max}}{K_m \bullet \left(1 + \dfrac{I_u}{K_i}\right)} \qquad (6.3)$$

$$\frac{CL_{int}(+\text{inhibitor})}{CL_{int}(-\text{inhibitor})} = \frac{1}{1 + \dfrac{I_u}{K_i}} \qquad (6.4)$$

Thus, according to (6.4), the decrease in the transport function of a target transporter can be estimated quantitatively by two parameters, I_u and K_i, which are defined as the protein-unbound concentration of an inhibitor at the vicinity of the target transporter and the inhibition constant, respectively. Because the $1 + I_u/K_i$ value is key to predicting the change in intrinsic clearance, we sometimes call it the "R value."

To consider the impact of decreased function of a single target transporter by DDIs on the in vivo pharmacokinetics of substrate drugs, one must consider the following points based on the pharmacokinetic theory.

6.2.1 Relative Contribution of a Target Transporter to the Overall Membrane Transport

Several transporters are expressed on the same side (basal or apical) of the plasma membrane of polarized cells, and their substrate specificities often overlap each other (e.g., OATP1B1 and OATP1B3 in the liver). Thus, multiple transporters can often mediate the membrane transport of a single compound in the same direction (uptake or efflux). If a compound is lipophilic enough to pass partially through the plasma membrane by passive diffusion, intrinsic membrane transport clearance ($PS_{transport}$) is defined as the sum of the intrinsic clearance of passive permeation through the plasma membrane ($CL_{passive}$) and active transport mediated by transporter i ($CL_{TP,i}$) as follows:

$$PS_{transport} = CL_{passive} + CL_{TP,1} + CL_{TP,2} + \cdots + CL_{TP,i} \tag{6.5}$$

If the function of transporter 1 is inhibited only by inhibitor drugs, the fold-change in the $PS_{transport}$ depends largely on the fraction of intrinsic transport clearance mediated by transporter 1 in the $PS_{transport}$ ($f_{m,1}$).

$$\frac{PS_{transport}(+\text{inhibitor})}{PS_{transport}(-\text{inhibitor})} = \frac{\dfrac{CL_{TP,1}}{1+\dfrac{I_u}{K_i}} + (CL_{passive} + CL_{TP,2} + \cdots + CL_{TP,i})}{CL_{TP,1} + (CL_{passive} + CL_{TP,2} + \cdots + CL_{TP,i})} = \frac{f_{m,1}}{1+\dfrac{I_u}{K_i}} + (1 - f_{m,1})$$

$$\tag{6.6}$$

When a target transporter is inhibited completely by an inhibitor drug, $PS_{transport}$ decreases to $(1 - f_{m,1})$ at a maximum, and thus estimation of the relative contribution of each transporter to the overall membrane transport of a substrate drug in the normal condition requires knowing the lower limit of the decreased intrinsic clearance in the presence of potent inhibitors of the target transporter. Moreover, inhibitor drugs

sometimes simultaneously inhibit multiple transporters with different inhibition potencies. In this case, (6.6) is modified as follows:

$$\frac{\text{PS}_{\text{transport}}(+\text{inhibitor})}{\text{PS}_{\text{transport}}(-\text{inhibitor})} = \sum_{p=1}^{k} \frac{f_{\text{m},p}}{1+\dfrac{I_{\text{u}}}{K_{\text{i},p}}} + \left(1 - \sum_{p=1}^{k} f_{\text{m},p}\right) \tag{6.7}$$

Thus, knowing the f_{m} and K_{i} values of inhibitor drugs for each target transporter is needed for the precise prediction of the change in $\text{PS}_{\text{transport}}$.

6.2.2   Rate-Limiting Step of the Overall Intrinsic Organ Clearance

In the "traditional" assumption, if a drug is metabolized extensively, its intrinsic organ clearance is thought to be determined by metabolic clearance. Several reports have indicated that the hepatic intrinsic clearance of a drug can be predicted by a simple scale-up of in vitro intrinsic metabolic clearance with human liver microsomes. However, there are several new drugs that are substrates of both metabolic enzymes and transporters. For example, atorvastatin is eliminated in the liver by extensive metabolism by cytochrome P450 (CYP) 3A4, whereas fluvastatin, torsemide, glibenclamide, and nateglinide are metabolized mainly by CYP2C9. On the other hand, these drugs are also substrates of hepatic uptake transporters, the OATPs. In this case, the detoxification efficacy of these drugs in the liver is determined by the functions of the uptake and efflux transporters as well as the metabolic enzymes, and the traditional assumption can no longer be applied for the prediction of the intrinsic clearance of transporter substrates (Shitara et al. 2006; 2013; Yoshida et al. 2013). In the "extended" pharmacokinetic theory, hepatic intrinsic clearance of transporter substrates ($\text{CL}_{\text{int,all}}$) should be determined by the metabolic intrinsic clearance (CL_{met}), intrinsic clearance of hepatic uptake (PS_{inf}), sinusoidal efflux (PS_{eff}), and biliary excretion in an unchanged form (PS_{ex}), as in the following equation (Fig. 6.2):

$$\text{CL}_{\text{int,all}} = \text{PS}_{\text{inf}} \; \frac{\text{PS}_{\text{ex}} + \text{CL}_{\text{met}}}{\text{PS}_{\text{eff}} + \text{PS}_{\text{ex}} + \text{CL}_{\text{met}}} \tag{6.8}$$

According to (6.8), if the PS_{eff} is much smaller than the sum of PS_{ex} and CL_{met}, $\text{CL}_{\text{int,all}}$ can approximate the PS_{inf} value.

$$\text{CL}_{\text{int,all}} \sim \text{PS}_{\text{inf}} \tag{6.9}$$

On the other hand, if the PS_{eff} is much larger than the sum of PS_{ex} and CL_{met}, $\text{CL}_{\text{int,all}}$ can be described by (6.10).
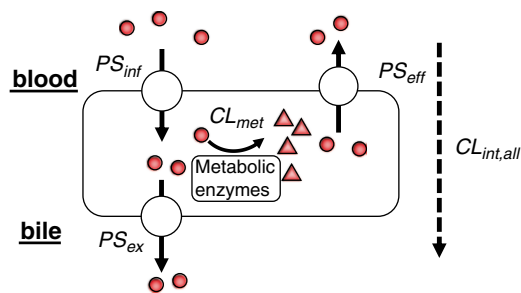
Fig. 6.2 The intrinsic processes making up overall hepatic intrinsic clearance ($CL_{int,all}$). PS_{inf} intrinsic clearance for hepatic influx transport, PS_{eff} intrinsic clearance for sinusoidal efflux transport, PS_{ex} intrinsic clearance for biliary efflux transport in an unchanged form, CL_{met} intrinsic clearance for metabolism

$$CL_{int,all} \sim PS_{inf} \cdot \frac{PS_{ex} + CL_{met}}{PS_{eff}} \qquad (6.10)$$

If a drug can pass rapidly through the plasma membrane mainly by passive diffusion and PS_{ex} is negligible, PS_{eff} is very large compared with PS_{ex} and CL_{met} and is equal to PS_{inf}, and (6.6) can be converted as follows:

$$CL_{int,all} \sim CL_{met} \qquad (6.11)$$

Thus, under such conditions, the aforementioned "traditional" assumption, in which metabolic intrinsic clearance solely dominates the overall intrinsic hepatic clearance, can be applied for the prediction of intrinsic clearance of drugs from in vitro metabolism assay using liver microsome.

Watanabe et al. showed that the in vivo intrinsic hepatic clearance of four kinds of statins (pravastatin, pitavastatin, atorvastatin, and fluvastatin), two of which are eliminated from the body by extensive CYP-mediated metabolism, is similar to the uptake intrinsic clearance estimated using the multiple-indicator dilution method in rats and an in vitro uptake assay using cryopreserved human hepatocytes (Watanabe et al. 2010). By contrast, the metabolic intrinsic clearance obtained from an in vitro metabolism assay using rat or human liver microsomes considerably underestimated the in vivo intrinsic hepatic clearance. A clinical microdosing study also indicated that the rate-limiting step of the hepatic clearance of atorvastatin is the hepatic uptake process mediated by OATP transporters in vivo in humans (Maeda et al. 2011). This was based on the observations that the plasma area under the concentration-time curve (AUC) of atorvastatin was increased markedly by orally administered rifampicin, a typical OATP-selective inhibitor, but not by intravenously administered itraconazole, a typical CYP3A4-selective inhibitor, although the AUC of the major hydroxy metabolites of atorvastatin decreased significantly.

We sometimes define "β value" as an indicator representing whether the rate-limiting step of hepatic intrinsic clearance is likely to be an uptake process according to the following equations:

$$\beta = \frac{PS_{ex} + CL_{met}}{PS_{eff} + PS_{ex} + CL_{met}} \tag{6.12}$$

$$CL_{int,all} = PS_{inf} \cdot \beta \tag{6.13}$$

If the β value is close to 1, $CL_{int,all}$ can approximate PS_{inf} as in (6.9), whereas if the β value is far less than 1, $CL_{int,all}$ can be described as in (6.10).

Let us consider the impact of a DDI at each transport process on the overall intrinsic hepatic clearance based on the "extended" pharmacokinetic concept. If uptake transporters are inhibited by a coadministered drug, the reduction in the uptake intrinsic clearance (PS_{inf}) always directly affects the decrease in the overall intrinsic hepatic clearance ($CL_{int,all}$) regardless of the β value. On the other hand, if biliary excretion transporters or metabolic enzymes and uptake transporters are inhibited simultaneously by coadministered drugs, when the β value is close to 1, even in the presence of an inhibitor drug, $CL_{int,all}$ should not be changed according to (6.14).

$$\frac{CL_{int,all}(+inhibitor)}{CL_{int,all}(-inhibitor)} \sim \frac{PS_{inf} \bigg/ \left(1 + \dfrac{I_u}{K_{i,inf}}\right)}{PS_{inf}} = \frac{1}{R_{inf}} \tag{6.14}$$

However, when the β value is much smaller than 1, the ratio of $CL_{int,all}$ in the presence of an inhibitor relative to that in its absence is described by (6.15) or (6.16) if the compound is eliminated from the body by extensive metabolism or by biliary excretion in an unchanged form, respectively.

$$\frac{CL_{int,all}(+inhibitor)}{CL_{int,all}(-inhibitor)} \sim \frac{PS_{inf} \bigg/ \left(1 + \dfrac{I_u}{K_{i,inf}}\right) \dfrac{CL_{met} \Big/ \left(1 + \dfrac{I_u}{K_{i,met}}\right)}{PS_{eff}}}{PS_{inf} \dfrac{CL_{met}}{PS_{eff}}} = \frac{1}{R_{inf}} \frac{1}{R_{met}} \tag{6.15}$$

$$\frac{CL_{int,all}(+inhibitor)}{CL_{int,all}(-inhibitor)} \sim \frac{PS_{inf} \bigg/ \left(1 + \dfrac{I_u}{K_{i,inf}}\right) \dfrac{PS_{ex} \Big/ \left(1 + \dfrac{I_u}{K_{i,ex}}\right)}{PS_{eff}}}{PS_{inf} \dfrac{PS_{ex}}{PS_{eff}}} = \frac{1}{R_{inf}} \frac{1}{R_{ex}} \tag{6.16}$$

Thus, the reduction in the overall intrinsic hepatic clearance is estimated by the product of the decreased fraction of uptake clearance and that of metabolic clearance or biliary excretion clearance.

6.2.3 Impact of the Change in the Intrinsic Clearance on Organ Clearance and In Vivo Pharmacokinetics of Substrate Drugs

Based on the pharmacokinetic theory, after oral administration of a drug, the blood AUC (AUC_B) is calculated by the following equation:

$$AUC_B = \frac{F_a F_g \cdot F_h \cdot \text{Dose}}{CL_{tot}} \tag{6.17}$$

$F_a F_g$, F_h, and CL_{tot} represent the fraction of a drug reaching the portal vein from the intestinal lumen while avoiding intestinal metabolism for an orally administered dose (intestinal availability), the fraction of a drug reaching the systemic circulation while avoiding first-pass hepatic metabolism (hepatic availability), and total clearance, respectively. Total clearance is described simply as the sum of organ clearance (in the liver, kidney, etc.). Organ clearance, defined as the rate of elimination of a drug divided by its blood concentration, is dominated by the tissue intrinsic clearance, blood flow rate in tissues, and protein-unbound fraction of a drug in the blood. Several models have been created to relate the intrinsic clearance to organ clearance. Among them, the "well-stirred" model is used most frequently because of its simple mathematical handling. In this model, rapid and complete mixing (hence its name) of a drug coming from the blood circulation and blood in the tissue occurs, and the blood concentration of a drug at the exit of tissue is assumed to be equal to that in the tissue. Under such an assumption, hepatic clearance (CL_h) can be expressed as in (6.18).

$$CL_h = \frac{Q_h \cdot f_B \cdot CL_{int,h}}{Q_h + f_B \cdot CL_{int,h}} \tag{6.18}$$

Q_h, f_B, and $CL_{int,h}$ represent the hepatic blood flow rate, protein-unbound fraction of a drug in the blood, and the hepatic intrinsic clearance of a drug, respectively.

When Q_h is much smaller than $f_B CL_{int,h}$, (6.18) is approximated by (6.19), and hepatic clearance is determined solely by hepatic blood flow rate.

$$CL_h \sim Q_h \tag{6.19}$$

In this case, when the intrinsic hepatic clearance is decreased by DDIs, hepatic clearance is not changed if $Q_h \ll f_B CL_{int,h}$ is still maintained in the presence of inhibitor drugs. On the other hand, when Q_h is much larger than $f_B CL_{int,h}$, (6.18) is

approximated by (6.20), and hepatic clearance is affected by the change in intrinsic hepatic clearance.

$$CL_h \sim f_B \cdot CL_{int,h} \tag{6.20}$$

When a drug is administered orally and eliminated from the liver alone, the blood AUC can be converted into (6.21) based on (6.17) and (6.18).

$$AUC_B = \frac{F_a F_g \cdot F_h \cdot Dose}{CL_{tot}} = \frac{F_a F_g \cdot \dfrac{Q_h}{Q_h + f_B\,CL_{int,h}} \cdot Dose}{\dfrac{Q_h \cdot f_B \cdot CL_{int,h}}{Q_h + f_B \cdot CL_{int,h}}} = \frac{F_a F_g \cdot Dose}{f_B \cdot CL_{int,h}} \tag{6.21}$$

Thus, regardless of the rate-limiting step of hepatic clearance (Q_h or $f_B CL_{int,h}$), the AUC ratio (+inhibitor/−inhibitor) is inversely proportional to the ratio of hepatic intrinsic clearance (6.22).

$$\frac{AUC_B(+inhibitor)}{AUC_B(-inhibitor)} = \frac{CL_{int}(-inhibitor)}{CL_{int}(+inhibitor)} \tag{6.22}$$

6.3 In Vitro Experimental Methods to Estimate the Kinetic Parameters Used for the Prediction of Transporter-Mediated DDIs

To predict precisely the extent of transporter-mediated DDIs, several key parameters such as the K_i value of an inhibitor for the target transporter and the relative contribution of each transporter to the overall membrane permeation of a substrate (f_m value) should be estimated. A wide variety of in vitro experimental tools are now available to estimate the kinetic parameters describing the transport properties of drugs. This section briefly reviews the current in vitro experimental systems and methods.

6.3.1 Determination of K_i Values of Inhibitors for Uptake and Efflux Transporters

As described above, the K_i value is one of the most critical parameters to quantitatively estimate the alteration of intrinsic clearance by transporter-mediated DDIs. In general, the K_i value can be obtained by observing the uptake clearance of substrates mediated by a single isoform of transporters in the presence of various

concentrations of inhibitors and fitting the theoretical curve calculated from (6.4) to the observed data. The IC_{50} value, which is defined as the inhibitor concentration that decreases the function of a transporter by half, is sometimes used in the literature instead of the K_i value. The relationship between the IC_{50} and K_i is expressed in (6.23), assuming competitive inhibition.

$$K_i = \frac{IC_{50}}{1 + \dfrac{S}{K_m}} \tag{6.23}$$

S and K_m represent the substrate concentration used in the inhibition assay and the Michaelis–Menten constant of a substrate, respectively. Because the IC_{50} value becomes higher as the substrate concentration increases, the risk of a clinical DDI is possibly underestimated by the calculation of the R value using IC_{50} instead of K_i when the IC_{50} value is determined with a higher concentration of substrates compared with the clinical unbound concentration of a substrate drug at the target site. From (6.23), if the substrate concentration is much lower than the K_m value, the IC_{50} value is regarded as equal to the K_i value.

Several experimental systems, such as immortalized cell lines that stably express the transporter and transporter cRNA-injected *Xenopus* oocytes, can be used to characterize an uptake transporter. Human cryopreserved hepatocytes can be purchased from various commercial sources, and suspended hepatocytes are also used in the characterization of hepatic uptake of compounds, but the apparent K_i value can be obtained only from an in vitro inhibition assay with hepatocytes because inhibitor drugs sometimes inhibit multiple transporters that can also recognize typical substrates with different K_i values. The function of efflux transporters is usually evaluated by measuring the ATP-dependent uptake of compounds into inside-out membrane vesicles that overexpress efflux transporters or canalicular membrane vesicles (CMVs) obtained from liver samples, or the directional transcellular transport of substrates in single- or double-transfected polarized cell lines, or in sandwich-cultured hepatocytes. When using cell lines, the K_i value for an efflux transporter should be measured with regard to the intracellular unbound concentration of an inhibitor. In practical applications, the apparent K_i value is often estimated based on the medium concentration in the compartment to which an inhibitor is added initially. However, if the intracellular protein-unbound concentration of an inhibitor is not the same as its (protein-unbound) medium concentration because of its active transport, the apparent K_i value with regard to the medium concentration is not identical to the real K_i value for efflux transporters, and the ratio of these K_i values should correspond to the ratio of the unbound concentration of an inhibitor inside and outside the cells ($K_{p,uu}$ value) (Shitara et al. 2013).

In the routine high-throughput assay in the process of drug development, the K_i values of inhibitors are sometimes estimated using the same typical substrate for the target transporter, and the K_i values are used to predict the risk of DDIs with other substrate drugs. However, previous reports indicated that some transporters have

more than two substrate-binding sites, and thus inhibition potency of an inhibitor sometimes largely depends on the substrates used. For example, Noe et al. demonstrated that 200 μM gemfibrozil can potently inhibit the OATP1B1-mediated uptake of taurocholate and statins, but not that of estrone-3-sulfate and troglitazone sulfate (Noe et al. 2007). Soars et al. compared the IC_{50} values of eight drugs for OATP1B1-mediated uptake of three typical substrates, pitavastatin, estradiol-17β-glucuronide ($E_2$17βG), and estrone-3-sulfate (Soars et al. 2012). The overall trend in the rank order of IC_{50} values was $E_2$17βG ≤ pitavastatin < estrone-3-sulfate. Thus, it is recommended to use the real combination of substrate and inhibitor to estimate the K_i value when predicting a specific case of DDI, although for the first high-throughput screening, $E_2$17βG might be useful as a sensitive substrate for OATP1B1 inhibition. Moreover, some drugs have been reported to increase the transporter-mediated transport, possibly because of their binding to the allosteric site of the transporters. Several compounds have been shown to simulate the uptake into MRP2-expressing Sf9 membrane vesicles and the transcellular transport of an MDCKII monolayer expressing MRP2 (Zelcer et al. 2003). In particular, 1 mM sulfanitran increased the MRP2-mediated transport of $E_2$17βG almost 30 times. For OATP1B1 and OATP1B3, several nonsteroidal anti-inflammatory drugs, such as diclofenac and ibuprofen, significantly increased the uptake of pravastatin, but not that of bromosulfophthalein (BSP) (Kindla et al. 2011). At present, the significance of these phenomena in in vivo DDIs has not been characterized.

Interestingly, the time-dependent inhibition of OATP transporters has been observed in in vitro experiments. Shitara et al. showed that in vivo hepatic uptake of BSP determined by the liver uptake index method was significantly decreased 3 days after administration of cyclosporine A in rats and that the uptake of BSP in rat hepatocytes was also decreased after preincubation with cyclosporine A, despite its removal from the medium during the BSP uptake assay (Fig. 6.3a) (Shitara et al. 2009). Amundsen et al. also confirmed this preincubation effect in OATP1B1-expressing HEK293 cells and have shown that the apparent K_i value of cyclosporine A for the uptake of atorvastatin after a 1 h preincubation with cyclosporine A was 1/22 of that after its coincubation (Fig. 6.3b) (Amundsen et al. 2010). We note that such a phenomenon can also be applied to all the OATP-inhibitor drugs because the K_i value obtained from a conventional inhibition assay may be overestimated, which leads to the underestimation of the risk of DDIs.

6.3.2 Determination of the Relative Contribution of Each Transporter to the Overall Membrane Permeation of a Substrate (f_m Value)

As mentioned above, the f_m value is important for determining the lower limit of the decreased intrinsic clearance of membrane permeation when a target transporter is potently inhibited by inhibitors. As for CYP-mediated metabolism, the methods to
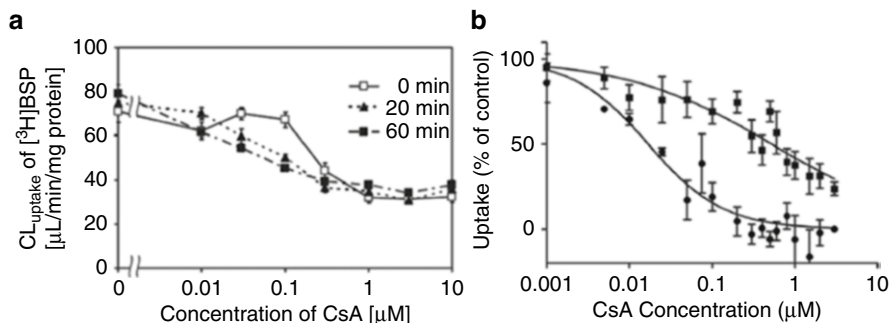
Fig. 6.3 Effect of preincubation of cyclosporine A on its K_i value for the transport of OATP substrates. (**a**) The inhibitory effect of cyclosporine A on the uptake of BSP in rat hepatocytes after preincubation with cyclosporine A (cited from Shitara et al. 2009). Hepatocytes were exposed to different concentrations of cyclosporine A for 0 (*open squares*), 20 (*closed triangles*), or 60 min (*closed squares*), subsequently followed by inhibition studies with the same concentrations of cyclosporine A. (**b**) Inhibition of OATP1B1-mediated uptake of atorvastatin acid (0.5 μM) into OATP1B1-expressing HEK293 cells by preincubation (*closed circles*) or coincubation (*closed squares*) of cyclosporine A (cited from Amundsen et al. 2010)

determine the contribution of each CYP isoform to the overall hepatic metabolism of substrates have been established by the use of isoform-specific metabolism of substrates and a specific inhibitor for each CYP isoform used with human liver microsomes or human hepatocytes. Similar methods can also be applied to transporter-mediated membrane permeation. The first approach is to use the relative activity factor (RAF) method, which was established originally in the field of metabolic enzymes by Crespi and Penman (1997). In this method, the uptake clearances of specific substrates for each transporter are measured in transporter expression systems and hepatocytes, and their ratio (hepatocytes/expression systems) for transporter i is defined as "$R_{act,i}$." Once the uptake clearance of a test compound in cells expressing transporter i ($CL_{test,i}$) is determined, the uptake clearance of a test compound mediated by transporter i in hepatocytes can be estimated by the product of the $R_{act,i}$ and $CL_{test,i}$ values. Assuming that the hepatic uptake clearance of a test compound ($CL_{hep,test}$) can be explained by the functions of transporter $1 - i$, the following equation should be true:

$$CL_{hep,test} = \sum_i R_{act,i} \times CL_{test,i} \qquad (6.24)$$

Kouzuki et al. originally proposed a method using reference compounds of rat Oatp1a1 ($E_2 17\beta G$) and sodium taurocholate cotransporting polypeptide (Ntcp) (taurocholate) to determine their contributions to the hepatic uptake of bile acids and organic anions in rats, although these compounds are no longer used for selective substrates of these transporters because many other transporters have been identified since the original publication (Kouzuki et al. 1998; Kouzuki et al. 1999). Hirano et al. applied this concept to human hepatocytes to estimate the relative contribution

of OATP1B1 and OATP1B3 to the hepatic uptake of $E_2$17βG and pitavastatin in cryopreserved human hepatocytes by the use of estrone-3-sulfate as an OATP1B1-selective substrate and cholecystokinin octapeptide as an OATP1B3-selective substrate (Hirano et al. 2004). They showed that the hepatic uptake of both compounds is mediated mainly by OATP1B1. Their observed uptake clearances in human hepatocytes were similar to the sum of their estimated clearances mediated by OATP1B1 and OATP1B3. They also confirmed their results using two different approaches. One was the direct estimation of the ratio of the expression levels of OATP1B1, 1B3, and 2B1 in human hepatocytes to that in the expression systems by comparing the band density in Western blot analysis and then estimating their contributions using the ratio instead of the $R_{act,i}$ value (Hirano et al. 2006). The absolute protein amounts of transporters in human liver samples can now be estimated directly by the quantification of peptide fragments digested with trypsin. This method provides a more accurate estimation of the relative expression levels compared with that from the band density in Western blot analysis (Li et al. 2009; Ohtsuki et al. 2011).

The other approach is to estimate the inhibitable portion of the uptake of test compounds in human hepatocytes in the presence of a specific inhibitor for each transporter (Ishiguro et al. 2006). Estrone-3-sulfate can be used as an inhibitor of OATP1B1, but not OATP1B3. A previous report indicated that the uptake of pitavastatin and $E_2$17βG was potently inhibited by estrone-3-sulfate in human hepatocytes, whereas the uptake of telmisartan was not inhibited, which suggests that telmisartan is a selective substrate for OATP1B3 in human liver (Ishiguro et al. 2006). Some specific inhibitors of the efflux transporters have also been proposed. For example, Ko143 preferentially inhibits BCRP-mediated transport, whereas PSC833 and LY335979 inhibit P-gp-mediated transport more potently than they inhibit transport via other efflux transporters (Allen et al. 2002; Dantzig et al. 1996; Kusunoki et al. 1998). However, when applied to cell systems, most of these selective inhibitors also inhibit the cellular uptake process, and the efflux clearance must be investigated separately to evaluate the inhibitory effects of inhibitors on efflux transporters accurately (Oostendorp et al. 2009).

Gene-silencing techniques such as antisense, ribozyme, and RNA interference are also powerful tools to determine the transport activity of a specific protein. Hagenbuch et al. investigated the effect of coinjection of transporter (Ntcp or Oatp1a1)-specific antisense oligonucleotide on the uptake of BSP and taurocholate in *Xenopus* oocytes injected with total rat liver mRNA (Hagenbuch et al. 1996). The expression level of a target transporter was reduced specifically, and the authors concluded that Na$^+$-dependent and Na$^+$-independent uptakes of taurocholate were almost fully accounted for by Ntcp and Oatp1a1, respectively, whereas only half of the BSP uptake could be explained by Oatp1a1. Nakai et al. took the different approach to estimate the contribution of OATP1B1 to the hepatic uptake of pravastatin and $E_2$17βG in humans (Nakai et al. 2001). Oocytes microinjected with human liver poly (A) mRNA showed Na$^+$-independent uptake of pravastatin and $E_2$17βG, and the simultaneous injection of OATP1B1 antisense oligonucleotides completely abolished this uptake, suggesting that OATP1B1 is a major transporter for their uptake. However, one should also consider their underlying assumption that the

relative expression level of each uptake transporter in *Xenopus* oocytes is similar to that in rat liver. This approach has also been applied to human sandwich-cultured hepatocytes in a study (Liao et al. 2010) that found that the expression of uptake transporters did not change much in human sandwich-cultured hepatocytes, but decreased rapidly with time in rat sandwich-cultured hepatocytes (Kotani et al. 2011). A recent report showed that the relative contributions of OATP1B1 to the uptake of several substrates estimated with OATP1B1 siRNA were similar to those obtained from RAF methods (Williamson et al. 2013).

Regarding the biliary efflux transporters, Tian et al. succeeded in the application of siRNAs targeted to Mrp2 and Mrp3 in sandwich-cultured rat hepatocytes (Tian et al. 2004). The knockdown of Mrp2 resulted in a decrease in the biliary excretion index (BEI) of carboxy dichlorofluorescein (CDF), whereas knockdown of Mrp3 caused an increase in BEI; the extent of the decrease in protein expression was similar to the change in CDF uptake. Yue et al. also established an adenoviral vector expressing Bcrp siRNA; its infection into rat sandwich-cultured hepatocytes led to a decrease in the BEI for nitrofurantoin, a typical substrate of Bcrp, but not for digoxin, a typical P-gp substrate (Yue et al. 2009).

6.3.3 Determination of the Intrinsic Clearance of Each Transport Process of a Substrate

As mentioned in Sect. 6.2.2, overall intrinsic clearance is determined by several intrinsic processes; thus, the intrinsic clearance of uptake, backflux, biliary excretion, and metabolism of a substrate should be determined separately in the accurate prediction of DDIs. Hepatic uptake intrinsic clearance can be determined by an uptake assay with isolated hepatocytes. As is often the case with transporter substrates, if the uptake is the rate-determining process in the overall intrinsic hepatic clearance, the uptake intrinsic clearance can approximate the intrinsic hepatic clearance. Watanabe et al. demonstrated that the predicted hepatic clearances of several transporter substrates obtained from in vitro uptake assays with rat isolated hepatocytes correlated strongly with their observed biliary excretion clearances in rats (Watanabe et al. 2009a). The intrinsic hepatic clearance calculated from nonrenal clearance of compounds was also predicted well in humans from in vitro uptake assays with human cryopreserved hepatocytes (Watanabe et al. 2011). Soars et al. reported that the initial disappearance rates of parent compounds from the incubation buffer ("media-loss assay") predicted the in vivo intrinsic clearances better than did their clearances calculated from the AUCs of the parent compounds in the whole suspension, suggesting that hepatic uptake clearance mainly determines the overall hepatic intrinsic clearance and that the β value is thought to be nearly equal to 1 for these compounds (Soars et al. 2007).

The intrinsic clearance for each process can be determined by several methods. One approach is to evaluate each intrinsic clearance separately with different experimental systems and some assumptions. Watanabe et al. evaluated the uptake

intrinsic clearance from uptake assays with isolated human hepatocytes. They used an empirical scaling factor calculated from the ratio of in vivo uptake clearance to in vitro uptake clearance in rats, the sinusoidal efflux clearance from the nonsaturable uptake intrinsic clearance, biliary excretion clearance from transport in rat CMVs, and metabolic clearance from the S9 fraction to predict the whole-body pharmacokinetics of pravastatin in humans (Watanabe et al. 2009b). Umehara et al. determined the uptake clearance in isolated rat hepatocytes, sinusoidal efflux clearance, and biliary excretion clearance in sandwich-cultured rat hepatocytes, and metabolic clearance in rat microsomes for 13 compounds (Umehara and Camenisch 2012). They succeeded in finding a good prediction of in vivo hepatic clearances from the predicted clearances calculated based on (6.8) with in vitro parameters.

Another approach is to fit the in vitro time profile of the uptake of compounds in human hepatocytes to a mechanistic compartmental model that considers multiple intrinsic processes to determine simultaneously several intrinsic clearances from a single experimental system. Menochet et al. determined the kinetic parameters for uptake, sinusoidal efflux (considering only nonsaturable diffusion), and metabolism of repaglinide and telmisartan at the same time by fitting their time- and concentration-dependent uptake in human short-term (2 h) cultured rat hepatocytes to an extended mechanistic two-compartment model that included saturable and nonsaturable uptake clearance, sinusoidal efflux clearance, and metabolic clearance (Menochet et al. 2012). Jones et al. determined the multiple intrinsic clearances of uptake, sinusoidal efflux, and biliary excretion of various OATP substrates by the fitting their time profiles of uptake into sandwich-cultured human hepatocytes to a mechanistic two-compartment model including uptake, sinusoidal efflux, and biliary excretion (Jones et al. 2012). However, further empirical scaling factors are needed for the accurate prediction of pharmacokinetics of multiple drugs.

6.3.4 Estimation of the $K_{p,uu}$ Value of an Inhibitor in Hepatocytes

As discussed above, when considering the inhibition of intracellular targets such as metabolic enzymes and efflux transporters, the intracellular unbound concentration of inhibitor should be estimated for the accurate DDI prediction. It is impossible to measure the unbound intracellular concentration of inhibitor drugs in humans, and appropriate in vitro methods to estimate the $K_{p,uu}$ value are needed. The $K_{p,uu}$ value is theoretically described as follows (Shitara et al. 2013):

$$K_{p,uu} = \frac{PS_{inf,act} + PS_{dif}}{PS_{dif} + PS_{ex} + CL_{int}} \tag{6.25}$$

$PS_{inf,act}$, PS_{dif}, PS_{ex}, and CL_{int} represent the intrinsic clearances for active influx transport, diffusional membrane transport, biliary efflux transport, and metabolism, respectively, assuming that sinusoidal efflux comprises only passive diffusion.

A traditional uptake study with hepatocytes can measure only the uptake amount of total (unbound + bound) compounds. Thus, some appropriate methods to estimate separately the unbound intracellular concentration of compounds in hepatocytes are needed. Yabe et al. reported $K_{p,uu}$ values of anionic compounds determined by using the results of an in vitro uptake assay in human hepatocytes with a nonspecific CYP inhibitor and calculated by the following equation (Yabe et al. 2011):

$$K_{p,uu} = 1 + \frac{V_{max,inf} \ / \ K_{m,inf}}{PS_{dif}} \tag{6.26}$$

$V_{max,inf}$ and $K_{m,inf}$ represent the maximum transport rate and Michaelis–Menten constant for active influx transport, respectively. The $K_{p,uu}$ value calculated by (6.26) may include the membrane transport by facilitated diffusion and active transport, and this method does not consider the metabolic intrinsic clearance, which results in the overestimation of the true $K_{p,uu}$ value. Another strategy to estimate the $K_{p,uu}$ value is to stop the active transport without changing any processes (passive diffusion, intracellular binding, etc.). For this purpose, several strategies can be proposed including (1) decreasing the temperature (on ice), (2) adding a potent inhibitor of uptake transporters (e.g., rifampicin for anionic compounds), or (3) using high concentrations of substrates to saturate the uptake transporters. Application of ATP depletors is theoretically possible, but previous studies suggested that moderate concentrations of ATP depletors, which did not cause cell toxicity, could not completely inhibit the function of active transport. Although in vitro uptake experiments with isolated hepatocytes are easy, previous reports indicated that isolated hepatocytes lose their cell polarity and the functions of biliary efflux transporters decrease, thus the predicted $K_{p,uu}$ value may overestimate the real value. By contrast, sandwich-cultured hepatocytes can reproduce all the transport and metabolic functions of hepatocytes and is suitable for the estimation of the $K_{p,uu}$ value. However, we note that expression/function of some transporters and metabolic enzymes decrease rapidly with culture time, especially in cultures of rodent cells (Ishigami et al. 1995; Jigorel et al. 2005; Rippin et al. 2001; Kotani et al. 2011). Further validation of the methods is needed.

6.4 Quantitative Prediction of the Risk of TP-DDIs in the Liver

6.4.1 Prediction Strategies of TP-DDIs: A Static Model and Dynamic Model

The I_u value is essential for estimating the inhibition potency of inhibitors (R value), thus the precise estimation of the I_u value should be needed for the accurate prediction of DDIs. Considering the definition of I_u, which is the protein-unbound

concentration of an inhibitor in the vicinity of a target transporter, one must estimate the unbound inhibitor concentration in the blood facing the hepatocytes when determining DDIs involving hepatic uptake transporters. By contrast, for DDIs involving hepatic efflux transporters, one must estimate the unbound inhibitor concentration in the hepatocytes. However, it is difficult to estimate the I_u value accurately in humans because it cannot be directly measured. In addition, in a real situation, the I_u value always changes over time. To predict accurately the ratio of plasma AUC in the presence of inhibitor drugs to that in their absence (AUCR; $AUC_{+inhibitor}/AUC_{control}$), the time profiles of plasma and tissue concentrations of both the substrate and inhibitor drugs need to be reproduced. One possible approach is to construct an appropriate mathematical model to explain the real-time whole-body disposition of drugs. This approach is referred to as a "dynamic model" in the US FDA draft guidance (US Food and Drug Administration (FDA) 2012). The physiologically based pharmacokinetic (PBPK) model comprises multiple compartments corresponding to the tissues and blood pool, which are connected by blood flow to mimic the blood circulation in the body. Thus, the PBPK model enables one to observe the itinerary of a drug inside the body after its administration simply by in silico simulation. However, it is essential, but difficult, to set up the kinetic parameters in such a model because only the time profiles of the plasma concentration and urinary excretion of drugs can be obtained from humans, and multiple sets of parameters may be able to apparently reproduce the limited clinical data.

In vitro experimental data can be used to provide a clue to the optimized model parameters; good methods for in vitro–in vivo scale-up have been proposed by several researchers, but their universality has not been confirmed yet (Barton et al. 2013; Houston et al. 2012; Zamek-Gliszczynski et al. 2013). Particularly in the early stage of drug development, the pharmacokinetic properties of drug candidates in humans have not been well characterized in humans, and it is difficult to apply a dynamic model approach to DDI prediction. Instead, assuming that the inhibitor concentration is maintained at the same level, one can simply calculate the R value and subsequent AUC changes for substrates if one can identify an appropriate I_u value. This approach is referred to as the "static model" in the US FDA draft guidance (US Food and Drug Administration (FDA) 2012). To avoid false-negative predictions (clinically significant DDIs cannot be forecasted by the DDI prediction method), the I_u value can be set as a constant at the theoretical maximum concentration at the target site. Recent progress for this detailed prediction method is discussed below.

6.4.2   Prediction of TP-DDIs in the Liver with a Static Model

In the prediction of the risk of transporter-mediated DDIs with a static model, one key factor is to determine what kind of inhibitor drug concentration should be used to estimate the inhibition potencies of transporters in the liver. According to the traditional pharmacokinetic theory, the inhibitor concentration must be defined as that at the vicinity of the transporters, and only the protein-unbound form of
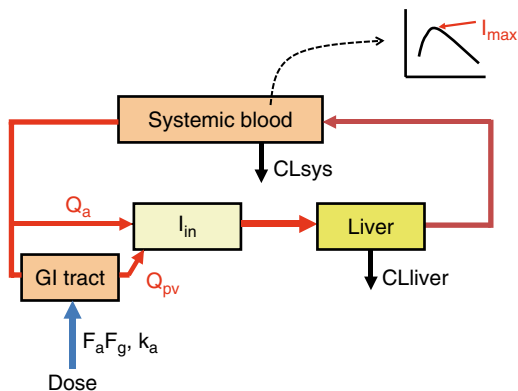
inhibitors can be involved in the inhibition of transporter function. Thus, the unbound blood concentration of inhibitors on the basal side of hepatocytes must be used for the prediction of inhibition potency against hepatic uptake transporters, whereas the unbound concentration in the hepatocytes must be used against hepatic efflux transporters. A static model is often applied for the sensitive detection of the signal of DDI risk, especially in the early stage of drug development when the detailed pharmacokinetic properties of a newly developed drug have not been well characterized in humans. Thus, it is essential to avoid the false-negative prediction, in which significant DDI actually occurs in the clinical situation, even though the DDI risk has been predicted to be negative. However, we also need to reduce the number of false-positive predictions because the number of DDI-positive cases increases as the inhibitor concentrations used for DDI prediction increase. Thus, one rational solution for this issue is to use the theoretically achievable maximum unbound concentration of inhibitors at the target site without considering their unrealistically excessive concentrations.

In the field of CYP-mediated metabolism, Ito et al. proposed a method to calculate the theoretically maximum unbound concentration of inhibitors at the inlet to the liver (Ito et al. 1998). The mathematical model for considering this method is shown in Fig. 6.4. For orally administered inhibitor drugs, the drugs flowing into the liver comprise those from the circulating blood supplied via the hepatic artery and those absorbed from the intestine supplied via the portal vein. Thus, it is possible that the inhibitor concentration is higher at the inlet to the liver than in the systemic blood. Based on this model, the amount (X_{abs}) and velocity (v_{abs}) of the drugs absorbed from the intestine into the portal vein can be described by the following equations:

$$X_{abs} = F_a F_g \ \text{Dose} \ \left(1 - e^{-k_a t}\right) \tag{6.27}$$

$$v_{abs} = \frac{dX_{abs}}{dt} = k_a \ F_a F_g \ \text{Dose} \ e^{-k_a t} \tag{6.28}$$

F_aF_g, k_a, and t represent the intestinal availability (fraction of drugs absorbed into the portal vein from the intestinal lumen), absorption rate constant, and time after oral administration, respectively. Thus, the flow rate of a drug into the liver (v_{in}) is calculated as follows:

$$v_{in} = Q_a \ I + Q_{pv} \ I + k_a \ F_aF_g \ \text{Dose} \ e^{-k_a t} \qquad (6.29)$$

Q_a, Q_{pv}, and I represent the blood flow rate at the hepatic artery, blood flow rate at the portal vein, and the concentration of inhibitors in the circulating blood. When considering the maximum v_{in} value based on (6.29), the observed maximum inhibitor concentration in the blood (I_{max}) can be used as the I value, and t should be 0 to maximize the v_{abs} value. Therefore, considering that the hepatic blood flow rate (Q_h) is the sum of Q_a and Q_{pv}, the protein-unbound maximum inhibitor concentration at the inlet to the liver ($I_{u,in,max}$) can be described as indicated in (6.30).

$$I_{u,in,max} = f_B \ \frac{v_{in}}{Q_h} = f_B \ \left(I_{max} + \frac{k_a \ F_aF_g \ \text{Dose}}{Q_h} \right) \qquad (6.30)$$

f_B indicates the protein-unbound fraction in blood. Because the F_aF_g and k_a values are not obtained easily, especially in the early stage of drug development, these values must be set as 1 and 0.1 min^{-1} (maximum gastric emptying time), respectively, because the maximum inhibitor concentration must be estimated for this calculation. When interpreting the results obtained from this approach, one should keep in mind that if the calculated R value ($=1+I/K_i$) with $I_{u,in,max}$ is close to 1, one can neglect the risk of DDIs, whereas even if the R value is estimated to be more than 1, one can never say that DDIs could occur in the clinical situation because the $I_{u,in,max}$ value always overestimates the real inhibitor concentration. This method is useful for sensitively screening out the possible DDI cases but is not suitable for the accurate prediction of the extent of the change in the AUC by DDIs; instead, prediction with a dynamic model should be considered for such purpose.

Ito et al. demonstrated the effectiveness of the prediction of DDI risk mediated by CYP enzymes using $I_{u,in,max}$ values (Ito et al. 2002). They analyzed the literature for CYP-mediated DDI cases and showed that the number of false-negative predictions was best minimized by the use of $I_{u,in,max}$ values rather than the unbound concentration of inhibitors in the blood ($I_{u,max}$) or total (bound + unbound) concentration of inhibitors in the blood (I_{max}) and that the number of true positive predictions was also highest using $I_{u,in,max}$ values. As shown for OATP transporters, Hirano et al. and Matsushima et al. investigated the inhibitory effects of several inhibitor drugs on the OATP1B1-mediated pitavastatin uptake and OATP1B3-mediated fexofenadine uptake in transporter-expressing HEK293 cells (Hirano et al. 2006; Matsushima et al. 2008). In their analyses, drugs with estimated R values at a therapeutic dose of more than two include cyclosporine A, rifampicin, rifamycin SV, clarithromycin, indinavir, and ritonavir for OATP1B1, and cyclosporine A and rifampicin for

OATP1B3. For most of these drugs, clinical DDI cases have also been reported, suggesting the validity of this prediction using a static model with $I_{u,in,max}$ value. Examples of the results calculated for marketed drugs using a static model with $I_{u,in,max}$ values are summarized in Table 6.1. This table shows that even if the K_i values of drugs for OATP transporters are relatively small, clinically relevant DDIs should not be seen in most cases in a static model prediction with $I_{u,in,max}$ values because of the low clinical dose and extensive protein binding of drugs. OATP1B1 can transport drugs and bilirubin, the breakdown product of normal heme catabolism (Cui et al. 2001). Thus, the potent inhibition of OATP1B1 by drugs results in the decreased hepatic clearance of bilirubin and subsequent drug-induced hyperbilirubinemia. Campbell et al. examined the inhibitory effects of several drugs on the OATP1B1-mediated uptake of $E_2 17\beta G$ to screen out the drugs that can induce hyperbilirubinemia (Campbell et al. 2004). Even though they used the $I_{u,max}$ value, which should be lower than $I_{u,in,max}$, when calculating their R values, indinavir, cyclosporine A, and rifamycin SV were estimated to inhibit OATP1B1-mediated transport by 41 %, 43 %, and 96 %, respectively. This is in good agreement with the clinical reports in which indinavir and cyclosporine A caused unconjugated hyperbilirubinemia in some, but not all, human subjects, whereas rifamycin SV caused hyperbilirubinemia in all subjects. By contrast, saquinavir inhibited OATP1B1 function by only 7 % in their estimation, which is also supported by a clinical report showing that saquinavir is not known to cause hyperbilirubinemia. Thus, the method for DDI prediction with a static model may also be used for the detection of the risk of drug-induced hyperbilirubinemia in the early stage of drug development.

Yoshida et al. recently investigated the predictability of the risk of DDIs involving OATP transporters using a static model (Yoshida et al. 2012). They systematically collected the 58 DDI studies involving 12 substrates of OATP transporters from the literature. They estimated the R value for each case and compared the predicted AUC increases with those observed and evaluated the effects of three assumptions on the number of false-negative predictions. In the first assumption, the maximum inhibitory effects of CYP3A and efflux transporters (P-gp, MRP2, BCRP) were considered if they were judged to be inhibited by drugs in the small intestine when using the drug interaction number (DIN) proposed by Tachibana et al. (2009). They defined DIN as the dose divided by the K_i value and set up the threshold values as 2.8 L for CYP3A and 10.8 L for P-gp, above which inhibition of CYP3A or P-gp may occur in the human intestine. Thus, if the DINs of inhibitor drugs exceeded the threshold values, $F_a F_g$ values were assumed to be 1 when inhibitor drugs were coadministered. When evaluating the impact of this assumption, the number of false-negative predictions was decreased slightly from 19 to 16 studies with this assumption for $F_a F_g$ values.

In the second assumption, Tachibana et al. compared the number of false-negative predictions when an inhibitor concentration was set to the following three choices: theoretically maximum unbound concentration at the inlet to the liver ($I_{u,in,max}$) (calculated from (6.30)), maximum unbound concentration in the blood circulation ($I_{u,max}$), and maximum total (unbound + bound) concentration in the blood circulation (I_{max}) (Fig. 6.5). As expected from the previous results of a static prediction of

Table 6.1 Inhibitory effects of selected drugs on the transport function of OATP1B1 and OATP1B3

Pharmacological action	Name	K_i or IC_{50} value for OATP1B1 (μM)	K_i or IC_{50} value for OATP1B3 (μM)	Dose (mg)	f_B	$I_{u,max}$ (μM)	$I_{u,in,max}$ (μM)	R value for OATP1B1	R value for OATP1B3
HIV protease inhibitors	Amprenavir	13	13	700	0.1	0.69	7.7	1.6	1.6
	Atazanavir	1.8	2.0	300	0.096	0.83	3.6	**3.0**	**2.8**
	Darunavir	3.1	3.3	600	0.05	0.54	4.0	**2.3**	**2.2**
	Lopinavir	0.50	5.1	400	0.015	0.23	0.89	**2.8**	1.2
	Nelfinavir	0.93	–	750	0.01	0.086	0.91	**2.0**	–
	Ritonavir	1.0	3.8	800	0.02	0.57	2.1	**3.1**	1.6
	Saquinavir	1.8	2.8	400	0.02	0.087	0.91	1.5	1.3
	Tipranavir	1.1	3.2	500	0.0042[a]	1.2	1.8	**2.6**	1.6
Macrolides	Clarithromycin	28	54	400	0.54	1.0	21	1.8	1.4
	Erythromycin	11	38	200	0.36	0.4	6.5	1.6	1.2
	Telithromycin	120	11	800	0.47	0.65	33	1.3	**4.0**
Immunosuppressants	Cyclosporin A	0.15	0.68	200	0.1	0.18	1.3	**9.7**	**2.9**
	Tacrolimus	0.61	–	9.6	0.01	0.00055	0.0080	1.0	–
Fibrates	Bezafibrate	45	–	200	0.06	0.58	2.6	1.1	–
	Fenofibrate	110	–	106.6	0.01	0.25	0.43	1.0	–
	Gemfibrozil	20	10	600	0.0065[a]	1.5	3.2	1.2	1.3
	(GEM-glu)[b]	14	74	–	0.12	2.3	–	1.2	1.0
Antidiabetic drugs	Glibenclamide	0.75	–	2.5	<0.01[a]	<0.0017	<0.0048	1.0	–
	Repaglinide	2.2	5.6	0.5	0.02	0.0003	0.00099	1.0	1.0
	Rosiglitazone	6.0	11	8	0.002[a]	0.017	0.031	1.0	1.0
Ca²⁺ channel blockers	Diltiazem	>100	190	60	0.32	0.046	2.9	1.0	1.0
	Mibefradil	95	180	100	0.01	0.019	0.16	1.0	1.0
	Nisoldipine	7.4	>100	10	0.08	0.00098	0.14	1.0	1.0
Azoles	Fluconazole	>100	>100	100	0.89	7.7	28	<1.3	<1.3
	Itraconazole	>100	>30	100	0.002[a]	0.0008	0.20	1.0	1.0
	Ketoconazole	19	19	200	0.01	0.066	0.30	1.0	1.0

(continued)

Table 6.1 (continued)

Pharmacological action	Name	K_i or IC_{50} value for OATP1B1 (μM)	K_i or IC_{50} value for OATP1B3 (μM)	Dose (mg)	f_B	$I_{u,max}$ (μM)	$I_{u,in,max}$ (μM)	R value for OATP1B1	R value for OATP1B3
Antibiotics	Rifampicin	1.3	1.2	600	0.11	0.87	6.4	**5.9**	**6.3**
	Trimethoprim	>100	>100	160	0.42	4.2	20	<1.2	<1.2
Uricosuric drug	Probenecid	76	130	1,000	0.11	12	35	1.5	1.3
	Sulfinpyrazone	32	47	200	0.02	0.96	1.6	1.1	1.0
Thrombopoietin receptor agonist	Eltrombopag	2.7	ND	75	<0.01[a]	<0.18	<0.30	1.1	–
Choleretic drug	Ursodeoxycholic acid	10	80	400	<0.3	<6.8	<28	**<3.8**	<1.4
Angiotensin II receptor antagonists	Telmisartan	0.44	0.81[c]	40	<0.01[a]	0.0015	0.050	1.1	1.1
	Valsartan	9.0	18[c]	40	0.06	0.19	0.53	1.1	1.0
Anti-hepatitis C virus drugs	Boceprevir	18	4.9	800	0.25	0.79	8.9	1.5	**2.8**
	Telaprevir	2.2	6.8	750	0.37	1.0	9.8	**5.5**	**2.4**

These parameters are cited from Yoshida et al. 2012, "Transportal" Web Database and package insert of each drug
[a]If f_B is less than 0.01, according to the reguratory guidances, f_B is regarded as 0.01 for the calculation of R values
[b]Gemfibrozil glucuronide; a major metabolite of gemfibrozil
[c]Reported K_m value
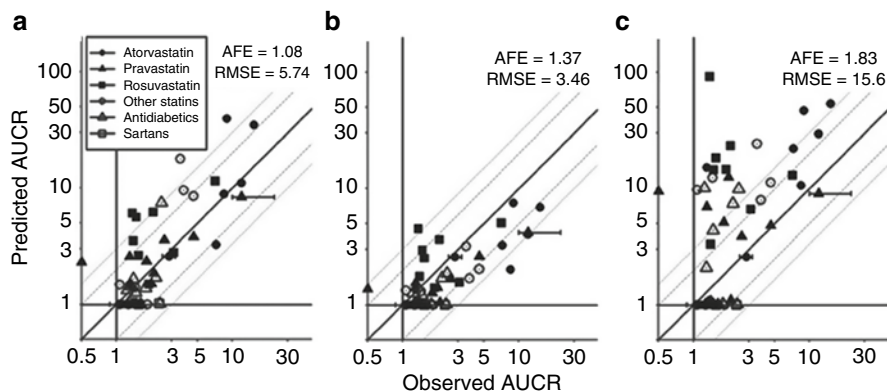
Fig. 6.5 Impact of the selection of inhibitor concentration used in a static model on the predictability of TP-DDIs involving OATP transporters (cited from Yoshida et al. 2012). Predictability of DDIs was compared when $I_{u,in,max}$ (**a**), $I_{u,max}$ (**b**), or I_{max} (**c**) was used in a static model. Each *point* and *horizontal bar* represents the median, maximum, and minimum values of the observed AUC ratios for the same combination of drugs. The *solid*, *dashed*, and *dotted lines* represent the line of unity, the 50–200 % range, and the 33–300 % range of the observed AUC ratios, respectively. *AFE* average fold error, *RMSE* root mean squared error

CYP-mediated DDIs (Ito et al. 2002), the use of $I_{u,in,max}$ minimized the number of false-negative predictions. Moreover, when I_{max} was used, the number of false-positive predictions increased because several clinically relevant OATP inhibitors showed a very low protein-unbound fraction and the discrepancy between the total and unbound concentrations of such inhibitors was large.

In the third assumption, the maximum inhibitory effects of biliary efflux transporters and/or metabolic enzymes as well as uptake transporters on the decreased hepatic intrinsic clearance were considered. As mentioned above, if the hepatic uptake clearance alone determines the overall intrinsic hepatic clearance, the ratio of the overall intrinsic clearance in the presence of inhibitor to that in its absence can be described by (6.14). On the other hand, when the β value is much smaller than 1, the ratio is described by (6.15) or (6.16) if compounds are eliminated from the body by extensive metabolism or biliary excretion in an unchanged form, respectively. Thus, to consider the maximum inhibitory effects on multiple processes when substrate drugs are recognized by uptake transporters, efflux transporters, and/or metabolic enzymes, the product of the R values for uptake and excretion and/or metabolism using the $I_{u,in,max}$ value should be considered (6.31) (Ueda et al. 2001).

$$\frac{CL_{int,all}(+inhibitor)}{CL_{int,all}(-inhibitor)} \sim \frac{1}{R_{inf}} \; \frac{1}{R_{ex/met}} < \frac{1}{1+\dfrac{I_{u,in,max}}{K_{i,inf}}} \; \frac{1}{1+\dfrac{I_{u,in,max}}{K_{i,ex/met}}} \qquad (6.31)$$

The number of false-negative predictions can be minimized when the product of R values is used for the DDI prediction rather than just the R value for uptake ($16 \rightarrow 11$ studies). In particular, DDI cases between fluconazole and CYP2C9 substrates (glimepiride, nateglinide, irbesartan) are predicted correctly when we consider the maximum inhibitory effects of both uptake and metabolism. This is reasonable because the clinical dose of fluconazole does not cause the inhibition of OATP-mediated uptake, but may inhibit CYP2C9-mediated metabolism. This may imply that the β values of these substrates are not close to 1, although this needs to be confirmed. However, the prediction accuracy was the highest when only the R value for uptake was used (76 and 90 % for predictions within two- to threefold of the observed the AUCR values, respectively). We note that the concentrated uptake of inhibitor drugs is not assumed in this equation because it is possible that the intracellular unbound concentration may be higher than the unbound concentration in the blood. If the $K_{p,uu}$ value in the liver is estimated from in vitro experiments, $I_{u,in,max} \times K_{p,uu}$ should be used instead of $I_{u,in,max}$ for the calculation of the maximum $R_{ex/met}$ value.

In the current FDA draft guidance on DDIs (US Food and Drug Administration (FDA) 2012), when the drug candidates are inhibitors of hepatic OATPs, in the first step, if the I_{max}/K_i value is more than 0.1, the R value should be calculated using the $I_{u,in,max}$ value. If the R value is more than 1.25, in vivo clinical DDI studies with OATP substrate drugs (e.g., rosuvastatin) are recommended. On the other hand, in the EMA guideline on DDIs (European Medicines Agency (EMA) 2010), the threshold for the R value calculated using the $I_{u,in,max}$ value is 1.04, which is stricter than that contained in the FDA draft guidance. The performance of the current DDI prediction method may need to be evaluated and optimized in future.

6.4.3 Prediction of TP-DDIs in the Liver with a Dynamic Model

Differing from a static model, a dynamic model enables one to predict the extent of the change in the AUC accurately because it considers the time-dependent change in the inhibition potency of inhibitors on the intrinsic hepatic clearance of substrates. The parameter settings are key to constructing a good model for describing the pharmacokinetic character of drugs appropriately (Shitara et al. 2013; Yoshida et al. 2013; Barton et al. 2013; Zamek-Gliszczynski et al. 2013; Rowland et al. 2011). Even a simple compartment model, which partly omits the physiological structure and machinery of detoxification of xenobiotics, can roughly reproduce the time profile of the plasma concentration of a drug. However, this is no longer useful if one wishes to consider the influence of the functional change of a single molecule on the pharmacokinetics of drugs because of the lack of parameters. Conversely, recent findings on the molecular mechanisms of pharmacokinetics allow one to create a very complex mechanistic model with many model parameters. However, it is difficult to set all the model parameters rationally, and there is a kind of trade-off for issues that have no clear answer.

The methods for setting the parameters are classified into two approaches. One approach is to determine the parameters using a simple scale-up method from only the in vitro experimental results; this is named the "bottom-up approach." For example, based on the traditional strategy of in vitro–in vivo extrapolation, in vivo metabolic intrinsic clearance can be predicted from in vitro metabolic clearance in liver microsomes by multiplying the in vitro clearance by the liver weight per unit body weight (e.g., 24.3 g liver/kg in humans) and the microsomal protein amount per unit liver weight (e.g., 40 mg protein/g liver in humans). However, some researchers have noted that a simple scale-up method cannot be effective for accurately predicting the in vivo parameters. For example, Houston et al. collected in vitro intrinsic clearances of 89 compounds estimated with human cryopreserved hepatocytes and carefully scaled up the in vitro data to predict the vivo hepatic intrinsic clearance based on physiologically based scaling factors (Houston et al. 2012). They observed systematic underprediction of the intrinsic hepatic clearance. Naritomi et al. showed that introduction of drug-specific empirical scaling factors, which are defined as the ratio of intrinsic clearance in vivo to that predicted from in vitro experiments in rats, can be useful for improving the prediction of hepatic intrinsic clearance of different species (Naritomi et al. 2001).

For the uptake transporters, Menochet et al. compared the predicted intrinsic hepatic clearances of six OATP substrates from in vitro uptake assays with cultured human hepatocytes and the observed clearance (Menochet et al. 2012). The predicted clearances of these compounds was 17-fold smaller than that observed on average, indicating that compound- and donor-specific empirical scaling factors are needed to predict the in vivo hepatic intrinsic clearances better. One strategy to determining an empirical scaling factor for each process is to estimate the ratio of in vivo intrinsic clearance to the in vitro predicted clearance in animal experiments and then to apply the same ratio to the prediction of human pharmacokinetics. Watanabe et al. succeeded in the prediction of the pharmacokinetics of pravastatin in rats using a PBPK model, in which the liver was divided into five subcompartments to mimic the dispersion model and in its prediction in humans by using the same scaling factors for influx (3.7) and biliary excretion (21) clearances (Watanabe et al. 2009b). Poirier et al. constructed a PBPK model of valsartan in humans, which included the relative contribution of OATP1B1 and OATP1B3 to the overall hepatic uptake of valsartan. A good prediction of the human pharmacokinetics of valsartan was achieved when they used an empirical scaling factor for hepatic uptake of 5, which was optimized to match the plasma concentration and biliary excretion profile of valsartan in rats (Poirier et al. 2009). Several possible reasons for the differences between in vitro and in vivo systems can be considered such as the difference in the expression/function of several transporters/enzymes between cell systems and intact tissues. However, no one has presented a definitive hypothesis about the cause of the apparent discrepancy between in vitro and in vivo systems.

Another approach is to determine the parameters by fitting in vivo clinical data to an appropriate pharmacokinetic model, which is named the "top-down approach." Johns et al. tried to confirm the predictability of the human pharmacokinetics of seven OATP substrates from the in vitro parameters obtained with

sandwich-cultured human hepatocytes and a PBPK model. The simple use of in vitro parameters for the PBPK model led to an overprediction of the exposure for all compounds (Jones et al. 2012). The authors concluded that the drug-dependent scaling factors for each process that best explained the time profile of plasma concentration after the intravenous administration in humans were 12–161 for uptake clearance and 0.024–0.12 for biliary excretion clearance. Because this approach requires human clinical data, the model parameters cannot be set for drug candidates whose clinical data are not available. Thus, at the moment, because a universal method to determine the empirical scaling factors for each parameter is not available, a combination of "bottom-up" and "top-down" approaches should be applied to predict the pharmacokinetics of drugs.

Several studies have used PBPK modeling to predict complex DDI cases quantitatively. Kudo et al. tried to use PBPK modeling to explain the complex DDI of repaglinide with gemfibrozil and itraconazole (Kudo et al. 2013). In this clinical case, the plasma AUC of repaglinide was increased by 1.4- and 8.1-fold by the coadministration of itraconazole and gemfibrozil, respectively (Niemi et al. 2003). By contrast, the plasma AUC of repaglinide increased by 19.4-fold when coadministered with both itraconazole and gemfibrozil (Niemi et al. 2003). Because repaglinide is a substrate of CYP2C8, CYP3A4, and OATP1B1, the cause of this DDI relates to the potent inhibition of CYP3A4 by itraconazole, the mechanism-based inhibition of CYP2C8 by gemfibrozil glucuronide, and the inhibition of OATP1B1 by gemfibrozil and its glucuronide. The cause of such observations is sometimes recognized as a "synergistic" inhibitory effect by the combined use of itraconazole and gemfibrozil. Kudo et al. constructed simple PBPK models for itraconazole, gemfibrozil and its glucuronide, and repaglinide (as shown Fig. 6.6a), and they optimized multiple model parameters by fitting the time profiles of the plasma concentrations of these drugs to the PBPK models (Fig. 6.6b) (Kudo et al. 2013). The optimized K_i values of itraconazole for CYP3A4, gemfibrozil and its glucuronide for OATP1B1, and the fraction of repaglinide metabolized by CYP2C8 were similar to the in vitro experimental results. Thus, the PBPK model analyses showed that this complex TP-DDI case can be explained simply by the multiple inhibitions of the clearance pathways of repaglinide without having to consider any empirical "synergistic" effect.

Varma et al. also used PBPK modeling of repaglinide to explain the multiple clinical DDI cases based on the available in vitro information. They initially tried to use the in vitro kinetic parameters of metabolism and transport of repaglinide obtained from experiments with hepatic microsomes and sandwich-cultured hepatocytes as model parameters. However, the use of in vitro intrinsic clearance for sinusoidal active uptake resulted in the overestimation of the systemic exposure of repaglinide, and a scaling factor of 16.9 was finally applied to explain the pharmacokinetics of repaglinide. Using this PBPK model, they succeeded in accurately predicting the AUC ratio of repaglinide after coadministration of ketoconazole, itraconazole, cyclosporine A, or gemfibrozil in previous clinical reports. They successfully predicted the dosing–time-dependent pharmacokinetic interaction of repaglinide with rifampicin by using a similar PBPK model incorporating the induction of CYP3A4 and reversible inhibition of OATP1B1 (Varma et al. 2013). Varma
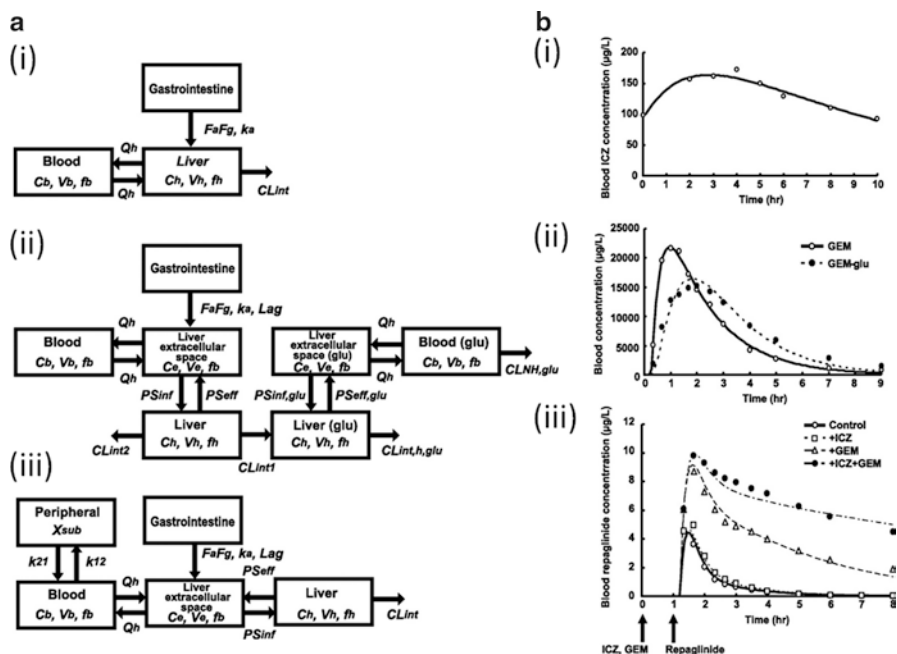
Fig. 6.6 Prediction of DDI of repaglinide with itraconazole and gemfibrozil by a dynamic PBPK model (cited from Kudo et al. 2013). (**a**) Structure of simple PBPK models for (*i*) itraconazole, (*ii*) gemfibrozil and its glucuronide, and (*iii*) repaglinide. (**b**) Predicted and observed time profiles of the blood concentration of (*i*) itraconazole, (*ii*) gemfibrozil and its glucuronide, and (*iii*) repaglinide with the coadminstration of itraconazole (*open squares*), gemfibrozil (*open triangles*), and combination use of itraconazole and gemfibrozil (*closed circles*) or without any coadministered drugs (*open circles*). The overlaid lines in each figure represent the predicted time profiles of blood concentration of drugs based on PBPK model

et al. also used a PBPK model to perform a similar type of DDI prediction for pravastatin (Varma et al. 2012). The time profiles of pravastatin were reproduced by the PBPK model with scaling factors of sinusoidal active uptake and canalicular efflux of 31 and 0.17, respectively, which related the model parameters to in vitro parameters in sandwich-cultured hepatocytes. Interestingly, they were successful at predicting the TP-DDIs with gemfibrozil and rifampicin, but the DDI with cyclosporine A was underpredicted by the PBPK model, suggesting that the in vivo K_i value might be smaller than the in vitro K_i value obtained from the in vitro inhibition assay (Varma et al. 2012). This may reflect the decreased K_i value by preincubation with cyclosporine A.

Gertz et al. also tried to predict the TP-DDI of repaglinide with cyclosporine A using a PBPK model. The prediction was successful when considering the preincubation effect of cyclosporine A on the K_i value and almost complete inhibition of P-gp and CYP3A4 in the intestine, whereas the effects of OATP1B1 inhibition by AM1, a major metabolite of cyclosporine A, played a minor role in the accurate DDI prediction (Gertz et al. 2013).
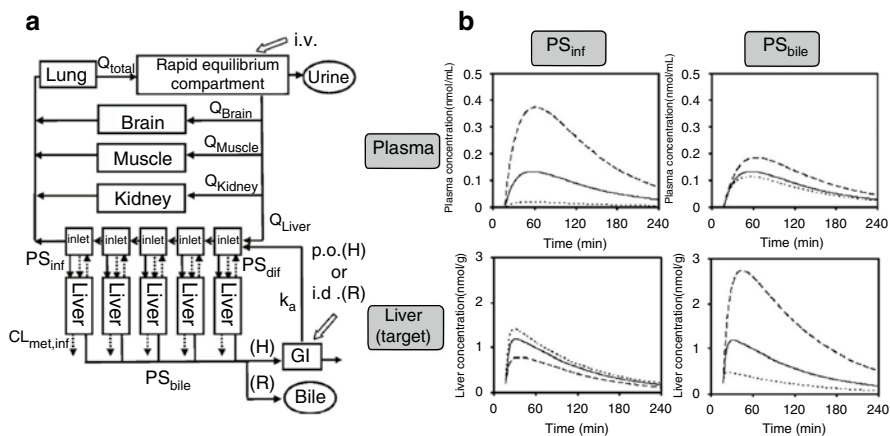
Fig. 6.7 Impact of the change in the hepatic influx and biliary excretion on the plasma and hepatic concentration of pravastatin based on the results of PBPK modeling (cited from Watanabe et al. 2009b). (**a**) Schematic diagram of the PBPK model predicting the concentration-time profiles of pravastatin. The liver compartment was divided into five compartments to mimic the dispersion model. (*H*) humans, (*R*) Rats, The enterohepatic circulation was incorporated in the case of humans. (**b**) Effects of changes in the transporter activity of hepatic influx and biliary efflux on the time profiles of plasma and liver (target organ) concentrations of pravastatin in humans. Plasma and liver concentrations after oral administration (40 mg) were simulated using the PBPK model with varying hepatic transport activities over a 1/3- to 3-fold range of the optimized values (*solid line*, initial; *dashed line*, ×1/3; *dotted line*, ×3)

One of the great advantages of the dynamic model approach is that one may simulate the time profiles of plasma and tissue concentrations of the substrates and inhibitor drugs when some model parameters are changed artificially once an appropriate PBPK model is obtained. This helps decrease the need for clinical studies. We can also perform a sensitivity analysis to search for the important parameters that markedly modify the plasma and tissue concentrations of drugs in the PBPK model. Watanabe et al. showed the impact of influx clearance and biliary efflux clearance on the plasma and hepatic concentrations of pravastatin by sensitivity analysis (Fig. 6.7) (Watanabe et al. 2009b). As observed for statins, the plasma concentration determines muscle toxicity, whereas the hepatic concentration is related to the pharmacological effect. In their analyses, decreasing the influx clearance markedly increased the plasma concentration of pravastatin, whereas its hepatic concentration did not change much. These simulation results are similar to the clinical observations that the pharmacological effects of statins are not affected much in subjects with the *SLCO1B1* 521T>C allele (Martin et al. 2012), which was reported to decrease the transport function, whereas the risk of muscle toxicity increases significantly in those with this allele (Link et al. 2008). By contrast, decreasing the biliary efflux transport did not change the plasma concentration, but markedly increased the hepatic concentration, suggesting the increased pharmacological effects of pravastatin. These kinds of findings can be realized only by using the PBPK-modeling approach.

6.5   Conclusions

This chapter has discussed novel methods for hepatic TP-DDI prediction and their applications. Selection of the prediction methods depends on the situation. In the early stage of drug development, because information about the pharmacokinetic properties of drugs is not readily available, a static model using the $I_{u,in,max}$ value is useful for sensitively identifying DDI risk of new chemical entities. After obtaining the pharmacokinetics of a new chemical entity in humans, the kinetic parameters for the PBPK model are optimized by using both in vitro experimental results ("bottom–up" approach) and clinical pharmacokinetic information ("top–down" approach). At present, although a complete bottom–up approach is ideal because the prediction of pharmacokinetics is realized only by using in vitro data without any clinical data, it is difficult to say whether the model parameters can be estimated by a simple scale-up of in vitro results. Several reports have shown the need for compound-dependent nonphysiological empirical scaling factors for each intrinsic clearance of drugs (Barton et al. 2013; Houston et al. 2012; Zamek-Gliszczynski et al. 2013). The mechanisms underlying discrepancies between in vivo and in vitro systems are not understood fully. In the validation of the PBPK model, only the plasma and urine concentrations, but not the tissue concentrations, of drugs are available in humans. Thus, the validity of multiple model parameters cannot be guaranteed by the limited information of drug concentration, and many sets of parameters might explain the time profiles of drug concentrations in plasma and urine. To overcome this problem, noninvasive imaging techniques such as positron emission tomography (PET) are powerful approaches to measure directly the tissue concentration of drugs. Takashima et al. characterized the transporter-mediated hepatobiliary transport of [^{11}C]-15R-TIC and found that the coadministration of rifampicin decreased the intrinsic clearance of both the hepatic uptake and biliary efflux of radioactivity (Takashima et al. 2012). Our group has proposed several kinds of PET ligands, which are substrates of selective transporters and can be used to characterize their hepatic transport properties in humans. This kind of information will provide clues to identify a set of optimized model parameters. In future, development of a pharmacodynamic model that incorporates the dynamics of the molecular machinery of the pharmacodynamic/toxicological actions of drugs based on the experimental approach combined with the dynamic PBPK model will provide the opportunity to predict quantitatively the real-time pharmacological actions of drugs under specific conditions.

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Chapter 7
Accounting for Transporters in Renal Clearance: Towards a Mechanistic Kidney Model (Mech KiM)

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Abstract The impact of transporters in modulating the disposition of drugs in the liver and their passage across the gut wall has received much more attention than their role in renal excretion, despite the fact that 25–30 % of drugs are cleared predominantly by renal clearance and renal transporters contribute significantly to this process. Thus there is a need to improve the ability to predict changes in renal clearance arising from genetic variability, the impact of disease and interactions related to renal transporters. Such changes may also influence the accumulation of xenobiotics within the kidney cell leading to nephrotoxicity. Attempts to develop mechanistic, physiologically based models of renal drug elimination have been limited. This chapter outlines the features and application of a new model (Mech KiM) that links drug characteristics to knowledge of renal physiology in predicting the contributions of glomerular filtration, active and passive secretion, active and passive reabsorption and metabolism to renal elimination.

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Abbreviations

CL_{PD}	Passive diffusion clearance
CYP450	Cytochrome P450
GFR	Glomerular filtration rate
ISEF-T	Inter-system extrapolation factor for transporters
IVIVE	In vitro–in vivo extrapolation
MATE	Multidrug and toxin extruder
MDRD	Modification of diet in renal disease
PBPK	Physiologically based pharmacokinetics
PTCPGK	Proximal tubular cells per gram of kidney
QSAR	Quantitative structure activity relationship
RAF	Relative activity factor
REF	Relative expression factor
tDDI	Transporter-mediated drug–drug interaction
UGT	Uridine glucuronosyltransferase

7.1 Introduction

Renal clearance is the largest (>50 %) component of the net systemic clearance of 25 % of the top 200 drugs prescribed in the USA (www.rxlist.com), and a similar figure has been reported for a database of 391 compounds by Varma et al. (2009).

In this chapter the modelling of renal drug elimination, with a particular emphasis on the role of transporters, is discussed, and a mechanistic framework (Mech KiM) is outlined that links the physicochemical characteristics of a molecule and in vitro data with prior knowledge of renal physiology to predict the integrated effects of glomerular filtration, active and passive secretion, intra-renal drug metabolism, active and passive tubular reabsorption, and the impact of covariates such as age, gender, genetics, race, diet, concomitant drugs and disease. The need for such a quantitative approach is underscored by concern about accumulation of drugs and endogenous compounds within the kidney cell and an associated risk of nephrotoxicity as a consequence of drug–drug interactions mediated by inhibition of efflux transporters (tDDI) (Table 7.1; Anzai and Endou 2007). Some preliminary examples of the application of Mech KiM are presented.

7.2 Determinants of Renal Clearance

The major processes contributing to the net excretion of drugs in the kidney are filtration in the glomerulus, active and passive transfer from blood to tubular fluid in the proximal tubules and passive and active reabsorption from the distal tubules. Active secretion is considered to be the dominant process in movement of drug from blood to the tubular fluid, while passive diffusion is primarily considered to

Table 7.1 Renal drug–drug interactions linked to renal transporter inhibition

Substrate	Inhibitor	Possible transporters involved	CL_R change (%)	Renal tubular secretion (%)	AUC change (%)	References
Acyclovir	Probenecid	OATs/MRPs	32 ↓			Laskin et al. (1982)
Cefmetazole	Probenecid	OATs/MRPs	40 ↓			Ko et al. (1989)
Cidofovir	Probenecid	OATs/MRPs	38 ↓			Cundy et al. (1995)
Famotidine	Probenecid	OATs/MRPs		89 ↓		Inotsume et al. (1990)
Fexofenadine	Probenecid	OATs/MRPs	68 ↓			Yasui-Furukori et al. (2005)
Furosemid	Probenecid	OATs/MRPs	50 ↓			Honari et al. (1977) and Vallon et al. (2008)
Oseltamivir	Probenecid	OATs/MRPs	52 ↓			Hill et al. (2002)
Penicillin	Probenecid	OATs/MRPs			240 ↑	Burnell and Kirby (1951)
Dofetilide	Cimetidine	OCT2/MATE	13–33 ↓			Abel et al. (2000)
Metformin	Cephalexin	OCT2/MATE	14 ↓			Jayasagar et al. (2002)
Metformin	Cimetidine	OCT2/MATE	27 ↓			Somogyi et al. (1987)
Metformin	Pyrimethamine	OCT2/MATE	23–35 ↓			Kusuhara et al. (2011)
Pilsicainide	Cimetidine	OCT2/MATE	28 ↓			Shiga et al. (2000)
Pilsicainide	Cetirizine	OCT2/MATE	41 ↓			Tsuruoka et al. (2006)
Pindolol	Cimetidine	OCT2/MATE	34 ↓			Somogyi et al. (1992)
Procainamide	Cimetidine	OCT2/MATE	44 ↓			Somogyi et al. (1983)
Procainamide	Ciprofloxacin	OCT2/MATE	15 ↓			Bauer et al. (2005)
Procainamide	Levofloxacin	OCT2/MATE	26 ↓			Bauer et al. (2005)
Procainamide	Ofloxacin	OCT2/MATE	30 ↓			Martin et al. (1996)
Procainamide	Trimethoprim	OCT2/MATE	47 ↓			Kosoglou et al. (1988) and Muller et al. (2011)
Digoxin	Ritonavir	P-gp	35 ↓			Ding et al. (2004)
Digoxin	Quinidine	P-gp	34 ↓			Hager et al. (1979) and De Lannoy et al. (1992)
Digoxin	Verapamil	P-gp	35 ↓			Pedersen et al. (1982)
Digoxin	Itraconazole	P-gp	20 ↓			Jalava et al. (1997)
Cimetidine	Itraconazole	P-gp	24 ↓			Karyekar et al. (2004)
Methotrexate	NSAIDs	MRP2/MRP4	20 ↓			Kremer and Hamilton (1995) and El-Sheikh et al. (2007)
Tenofovir	Lopinavir/ritonavir	MRP2/MRP4	18 ↓			Kiser et al. (2008)

determine the extent of reabsorption from tubular fluid to blood. In the case when the value of renal clearance equals fu×GFR, where fu is the free fraction in plasma and GFR is glomerular filtration rate, it may be concluded that either the compound is only cleared by filtration or the extents of subsequent secretion and reabsorption are equal. When the value exceeds fu×GFR, then net secretion by a transporter or transporters may be inferred, and when it is less than fu×GFR net reabsorption is indicated. For some drugs intra-renal drug metabolism may also have to be considered when assessing their renal elimination.

7.2.1 Glomerular Filtration

In a healthy young adult approximately 120 mL of water is filtered per min (GFR) through the glomerulus. This is accompanied by free drug presented in the plasma. The filtration process does not cause dissociation of any bound drug in plasma such that renal excretion by filtration is given by fu×GFR. Thus, the extent of plasma binding is the key drug parameter needed to predict the process.

7.2.2 Active Secretion

Active uptake of drugs from blood across the basal membrane of the tubular cell is known to be mediated by a number of transporters (see Sect. 7.2.3, Fig. 7.1), and the process is not restricted to free drug in the blood such that renal clearance by secretion can approach renal blood flow (1 L/min) irrespective of the extent of blood binding. Once inside the tubular cell, drug is then secreted across the apical membrane into the tubular fluid by one or more of a further array of transporters (Fig. 7.1). The activity of some of these transporters, such as the multidrug and toxin extrusion proteins MATE1 and MATE-2K, is proton-dependent and, therefore, can influence the pH of tubular fluid.

7.2.3 Passive Reabsorption

Passive reabsorption in the distal tubule can occur as a result of reabsorption of water down the tubule leading to a high concentration gradient across the cell between drug in tubular fluid relative to that in the blood perfusing the distal tubule. Consequently, highly lipid-soluble compounds can be reabsorbed to equilibrium, whereas more polar compounds may be only partly reabsorbed or not at all, allowing efficient excretion in the urine. Apart from lipid solubility, the degree of ionisation is an important drug parameter affecting the extent of reabsorption, since only the un-ionised form can easily diffuse back across the tubular membrane. The pH of tubular fluid (which can vary from about 5 to just under 8) will also affect the degree
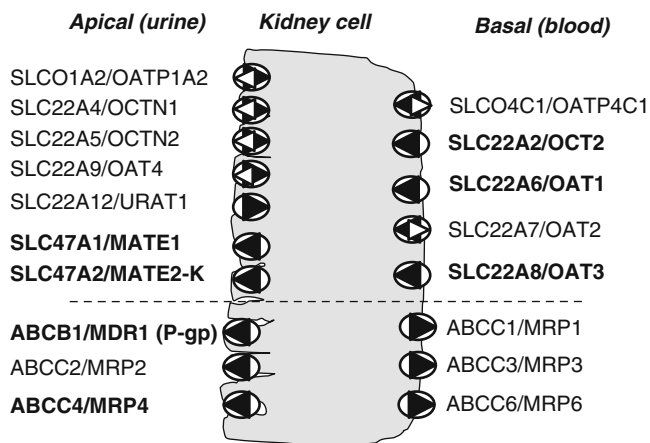
Fig. 7.1 Renal transporters (*OATP* organic anion transporting polypeptide, *OCT* organic cation transporter, *OAT* organic anion transporter, *OCTN1* organic cation/ergothioneine transporter, *OCTN2* organic cation/carnitine transporter, *MATE* multidrug and toxin extrusion protein, *MDR* multi drug resistance, *P-gp* permeability-limiting glycoprotein, *MRP* multidrug resistant-associated protein, *URAT* urate transporter, *ABC* adenosine triphosphate-binding cassette, *SLC* solute carrier)

of ionisation and, hence the extent of passive reabsorption. For drugs that are reabsorbed to equilibrium, increases in urine flow will increase their net recovery in urine. Any impact of active reabsorptive transport of drugs is poorly documented relative to passive reabsorption.

7.2.4 Renal Drug Metabolism

The expression of the major cytochromes P450 (CYPs) in the kidney is negligible (Lash et al. 2008), and any significant metabolism is likely to be due to glucuronidation, notably by UGT2B7 and UGT1A9 (Howe et al. 1992; Baldelli et al. 2007; Harbourt et al. 2012; Ma et al. 2012).

7.3 Models of Renal Elimination

7.3.1 QSAR Models and Allometric Scaling

Examples of Quantitative Structure Activity Relationship (QSAR) models for prediction of net renal clearance during candidate drug selection include those described by Doddareddy et al. (2006) and Manga et al. (2003). The former developed 94

Volsurf descriptors based on a training set of 130 diverse compounds and used them to predict the renal clearance of a further 20 compounds [$r^2 = 0.844$; $q^2 = 0.768$]. The latter applied 72 physicochemical and structural descriptors to a set of 160 compounds to allow classification of the extent of renal elimination. In general, allometric equations based on animal data for the prediction of renal drug clearance in the human are much more accurate than those developed to predict metabolic clearance (Mahmood 1998, 2009; Tang et al. 2007; Lavé et al. 2009; Paine et al. 2011; Ring et al. 2011). However, both allometry and QSAR models lack a mechanistic basis that would allow assessment of the effects of demographic, genetic and other differences on the variability of net renal clearance, or the impact of alterations in the specific processes involved in renal elimination on intra-renal drug exposure.

7.3.2   Mechanistic Models

There are a limited number of examples of the development of more mechanistic physiologically based kidney models incorporating some or all of the different components of renal drug clearance. These include models for predicting the influence of urine flow on the renal clearance of compounds neither secreted nor absorbed by active or pH-sensitive mechanisms, without (Tang-Liu et al. 1983; Hall and Rowland 1984) or with (Komiya 1986, 1987; Mayer et al. 1988) a glomerular filtration component, and models that also incorporate a Michaelis-Menten function to describe active secretion (Russel et al. 1987a, b, c; Katayama et al. 1990). Brightman et al. (2006a, b) incorporated elements of the Katayama renal model into general physiologically based models for predicting pharmacokinetics in rats and humans. While accounting for glomerular filtration, active reabsorption and passive reabsorption, the latter models did not include renal metabolism, passive secretion, active reabsorption, transporter scaling factors or population variability.

7.3.2.1   Mech KiM

Model Structure

Mech KiM (Fig. 7.2) is a new physiologically based model for the prediction of renal elimination that has been incorporated in version 12 (release 1) of the Simcyp Simulator (Jamei et al. 2009).

The nephron is divided into eight segments, beginning with the glomerulus followed by three segments representing the proximal tubule (in keeping with cellular morphology), segments representing the Loop of Henle, the distal tubule, and then collecting ducts, which are divided into cortex and medullary segments. Each segment has three compartments representing tubular fluid, cell mass and blood space.
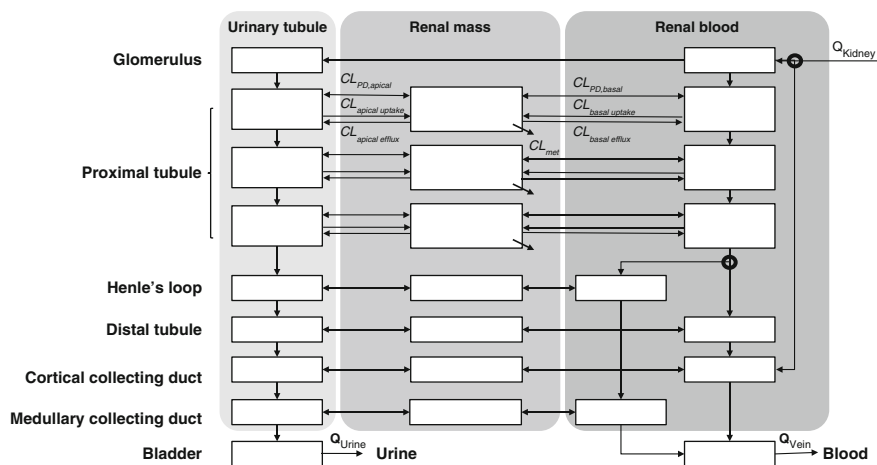
Fig. 7.2 Schematic representation of Mech KiM (equations are defined for drug as well as fluid flow according to this scheme)

Movement of drug between compartments and along segments is represented by ordinary differential equations that conserve mass balance for drug. The model has provision for the following elements:

- Bypass of some of the renal blood flow before entering the glomerulus and before the Loop of Henle and subsequent segments. A further aspect of 'shunting' not considered in the model is 'plasma skimming', whereby some erythrocytes are separated and delivered directly to the renal vein without contacting the renal tubule (Milne et al. 1958).
- Passive permeability across the basal and apical membranes of each cell compartment (passive components of secretion and reabsorption).
- Uptake and efflux transport across the basal and apical membranes of each proximal tubular cell compartment (active components of secretion and reabsorption).
- Metabolic clearance in each proximal tubular cell compartment.

Mech KiM is nested within the kidney compartment of the whole body PBPK model in the Simcyp Simulator (Fig. 7.3), such that drug input comes from the renal artery and output is through the urine and the renal vein.

System Data

The anatomical and physiological information included in Mech KiM includes nephron size and number, the number of proximal tubular cells per gram of kidney (PTCPGK), the volumes of cortex, medulla and renal blood vessels, the flow rates
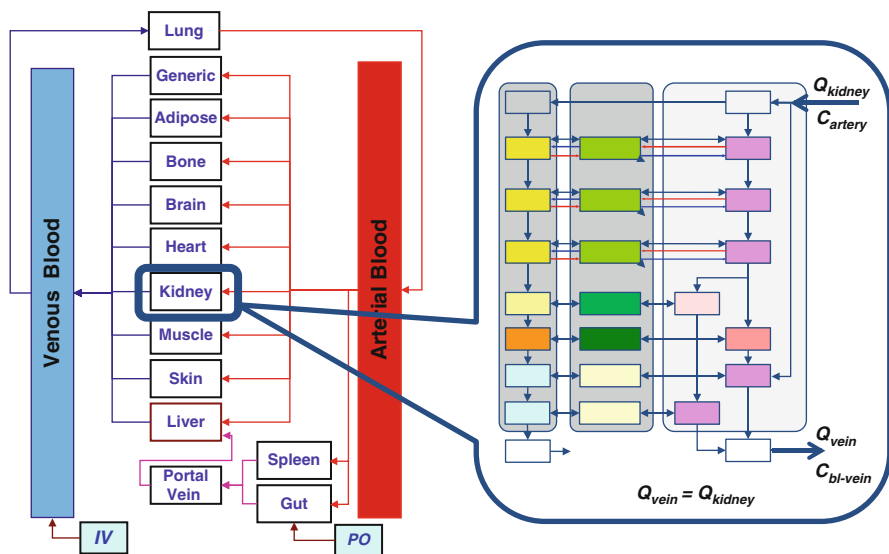
Fig. 7.3 Mech KiM nested in a whole body-PBPK model

of tubular fluid and urine, the extent of blood bypass (before the glomerulus and after the proximal tubule) and pH values in tubular cells and of tubular fluid.

The Size of the Nephron

The dimensions of human renal tubular cells in man are recorded by Pitts (1974). The total length of the average nephron is 30.5 mm (range 20–44 mm), comprising 18 mm (12–24 mm) for proximal segments, 7 mm (0–18 mm) for the Loop of Henle and 5.5 mm (2–9 mm) for the distal convoluted segment. The average length of the collecting duct is 22 mm. The external diameters of the structures are: proximal tubules 0.06 mm (range 0.05–0.065), loop of Henle 0.018 mm (0.014–0.022), distal tubules 0.05 mm (0.02–0.05) and collecting ducts (terminal portion) 0.2 mm.

The Number of Nephrons

There is a correlation between the number of nephrons and kidney weight but not with the volume of the glomerulus (Nyengaard and Bendtsen 1992). Therefore, except for glomerular filtration, inter-individual variability in kidney function is assigned according to kidney weight, which is a function of total body weight and height. Variability in GFR is simulated independently from creatinine clearance which, in turn, is estimated from age, gender and weight using either the Cockcroft-Gault method (Cockcroft and Gault 1976) or the Modification of Diet in Renal Disease (MDRD) method (Spruill et al. 2007).

Proximal Tubular Cells per Gram of Kidney (PTCPGK)

Values of $50–70 \times 10^6$ cells per cortical tissue (Trifillis 1999; Cummings et al. 2000) and 2×10^6 cells per renal tissue (Qi et al. 2007) have been reported. Unfortunately, cell recoveries were not reported and, in the second case, there was no information on the weight of tissue used. A default value of 60×10^6 cell per gram of kidney was chosen with the recommendation of performing a sensitivity analysis of the parameter in simulations.

Segmental and Compartmental Volumes

The volumes of cortex and medulla are based on total kidney volume and an estimate of 0.92 for the ratio of cortical to medullary tissue (Kojima et al. 2001). Peritubular capillary volume is estimated at 7 % of total kidney volume based on measurements in rat and supported by the fact that, in humans, renal vascular volume is a little greater than the volume of the interstitial space, which is 4 % of total cortical volume (Eaton and Pooler 2009). The volumes of tubular compartments are calculated from the length, diameter and number of nephrons, and the associated pertitubular blood volumes are assigned in proportion to the size of each compartment.

Fluid Dynamics

The difference between GFR (120 mL/min) and urine flow rate (1–2 mL/min) indicates the extent of water reabsorption down the tubule. Therefore, based on the volume reabsorbed in each segment (Pitts 1974; Guyton 1992; Knauf and Mutschler 1992) the fluid flow leaving each segment is assigned (proximal tubule 45 mL/min, the Loop of Henle 25 mL/min and the distal tubule 12 mL/min).

Tubular Fluid pH

Net tubular pH can be changed to reflect the physiological variation in urine pH (5–7.8). The capability of changing segmental pH is provided but no data on this gradient are available.

Transporter Abundances

Currently, no data are available on the absolute abundance of renal transporters, although information on mRNA and relative protein levels has been reported for some (Motohashi et al. 2002; Sakurai et al. 2004; Nozaki et al. 2007; Ogasawara et al. 2008; Cheng et al. 2012). Without absolute abundance values for specific transporters in the tubule, modelling of active renal transport and its variability relies on relative expression or activity factors obtained in vitro with cells expressing specific transporters and existing knowledge of phenotypic activity (Bhatnagar et al. 2006; Yee et al. 2010) (see section 'Transporter Kinetics' and Appendix). It will also be important to allow for the segmental distribution of transporters along the tubule (Smith et al. 1998; Broer 2008).

Drug Data

Binding and Ionisation

The un-ionised fraction of unbound drug concentration in each kidney compartments is defined based on the compound charge type and the fluid and cell pH values. The free fraction in kidney tissue can be derived from in vitro experiments or predicted based on kidney tissue composition and drug physicochemical properties using the Rodgers and Rowland equations (Rodgers et al. 2005; Rodgers and Rowland 2006). A default assumption is that all drug in the tubular fluid and urine is unbound, but this can be modified to reflect any degree of proteinuria.

Passive Permeability

Currently, there are no simple models to predict values of drug clearance by passive diffusion specifically across basal and apical tubular membranes. Passive permeation into isolated human proximal tubule cells may be used to estimate passive clearances. Otherwise, the values have to be derived by iteration using parameter estimation when employing the full model.

Transporter Kinetics

In vitro data on the kinetics of drug transport by renal systems can be obtained using kidney slices (Watanabe et al. 2011), immortalised human proximal tubule cells (Wilmer et al. 2010) or transporter-transfected cells, such as CHO-OCT2 cells and HEK293-OAT1 cells. As with hepatic and intestinal transport (Tachibana et al. 2010), care should be taken in obtaining accurate K_m values for efflux transport based on an estimate of intracellular drug concentration. Scaling of in vitro J_{max}/K_m values follows a similar procedure as for intestinal and hepatic transport, requiring multiplication by a relative expression factor (REF) or relative activity factor (RAF), the number of PTCPGK and kidney weight (Fig. 7.4; Table 7.2). Ideally, inter-system extrapolation factors (ISEF, Proctor et al. 2004) should be derived that, as mentioned previously, take account of activity per unit of transporter (absolute abundances) and can reflect any variation in activity that relates to the cell environment (Appendix). The model can be extended to predict renal transporter-mediated single pair or multiple drug–drug interactions, using in vitro estimates of inhibition constants. Currently, within Mech KiM, this can be done assuming a common Michaelis-Menten competitive inhibition for all inhibitors at each transporter.

Enzyme Kinetics

In vitro–in vivo extrapolation of renal metabolic clearance by UGTs can be implemented using data obtained with kidney slices, human proximal tubular cells or recombinant enzymes. Currently, the latter data requires REF or RAF scaling since, as indicated previously, absolute abundance values for UGTs in the kidney are not yet available for all UGTs (Milne et al. 2011; Harbourt et al. 2012).
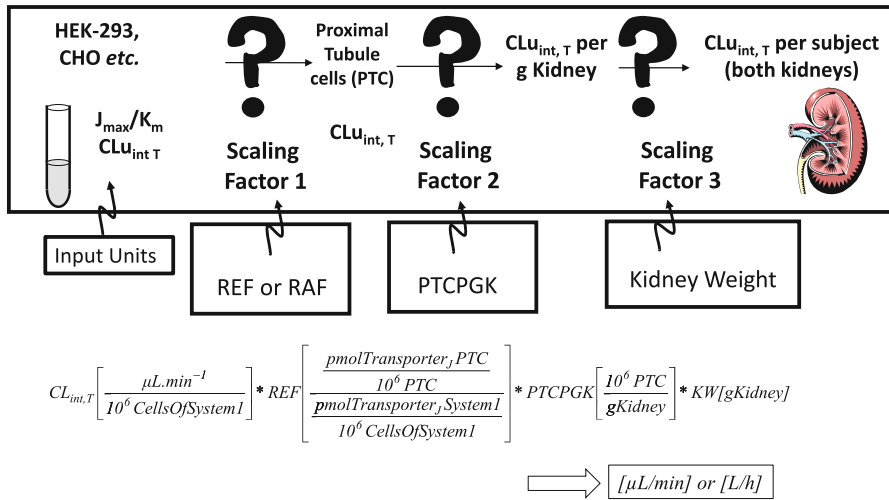
Fig. 7.4   Scaling of renal transporter data

Table 7.2   Scaling factors for IVIVE of transporter data

	Description
In vitro–in vivo scalars	
Relative mRNA/ protein expression factors (REF)	Dimensionless *system-based* scalars accounting for the in vitro–in vivo difference in transporter expression based on relative mRNA and/or protein measurement
Relative activity factors (RAF)	*Drug-based* scalars accounting for any in vitro–in vivo difference in activity based on $CL_{int,t}$ or V_{max} (Proctor et al. 2008)
Between species scalars	*Drug-based* scalars accounting for differences in transporter clearance between species, typically between rat and human (Watanabe et al. 2010). Often also called 'RAF' or 'Rat scalar'
Absolute protein expression and/or activity scalars	*System-based* scalars describing accounting for in vitro–in vivo difference in expression and/or a combination of expression and activity of a transporter based on absolute protein and/or activity measurements (Proctor et al. 2004). These scalars allow for the use of inter-system extrapolation factors for transporters (ISEF-T)
Organ scalars	
Membrane protein scalars	Scalars that allow conversion of the in vitro derived transporter $CL_{int,t}$ to organ $CL_{int,t}$. These could be scalars for the whole organ (e.g., membrane protein per kidney) or for segments/parts of an organ (i.e., proximal tubule, distal tubule)
Cellular scalars	Scalars that allow conversion of in vitro-derived transporter $CL_{int,t}$ to organ clearance $CL_{int,t}$ (e.g., HPGL, PTCPGK)

7.4   Applications of Mech KiM

The most common requirement with regard to understanding the renal handling of drugs in drug development is to predict renal clearance. While this can be done on a mechanistic basis using Mech KiM, the model also allows output of the total

amount of unchanged drug excreted in the urine, the relative contributions of glomerular filtration, secretion, reabsorption and intra-renal metabolism to net renal elimination, and prediction of the extent of renal transporter-mediated drug–drug interactions (tDDI) at the basolateral and/or apical membranes. Population variability is built into the model such that it might also be used, for example, to assess statistical power to establish the impact on drug exposure of specific genotypes of renal transporters against the background of other variables. The following applications of Mech KiM provide performance verification of some of its functionality and capability based on model compounds.

7.4.1 The Impact of Passive Permeability and Concentration of Tubular Fluid

The kinetics of a virtual neutral compound (100 mg intravenous dose) were simulated in an average male subject with the assumptions that renal excretion is the only route of elimination, that the compound undergoes no active transport, and that its passive permeability is the same at the basolateral and apical kidney tubular membranes.

As would be expected, there is a progressive increase in drug concentration moving down from the proximal tubular segments of the kidney out to the urine (Fig. 7.5a). The impact of varying passive permeability in the tubules on the systemic plasma drug concentration—time profile and on its rate of appearance in the urine is shown in Fig. 7.5b, c, respectively. When passive permeability clearance is relatively high, the tubules act as a single well-stirred unit; there is extensive reabsorption, the renal clearance approximates to urine flow and the compound is slowly eliminated from the body. As the passive permeability approaches zero, there is negligible reabsorption, the renal clearance approaches fu×GFR and the urinary recovery increases towards the dose more rapidly.

7.4.2 The Impact of Tubular Fluid (Urine) pH

The impact of changing tubular (urine) pH on the renal handling of a 100 mg intravenous dose of a monoprotic basic drug ($pKa = 10$) was simulated. In this case, the compound was assumed to undergo both passive absorption/reabsorption ($CL_{PD} = 0.05$ mL/min/million cells in both directions) as well as active tubular secretion (50 μL/min/million cells for both basal and apical transport), and 20 % hepatic metabolism/80 % renal excretion. The rate and extent of urinary recovery of unchanged drug is shown to decrease as tubular (urine) pH is raised from 5.0 to 7.4 to 8.0 (Fig. 7.6a), reflecting the impact of change in ionisation and, hence, tubular reabsorption. There is a corresponding increase in tubular cell concentration (Fig. 7.6b) and decrease in systemic exposure (Fig. 7.6c) as the pH increases.
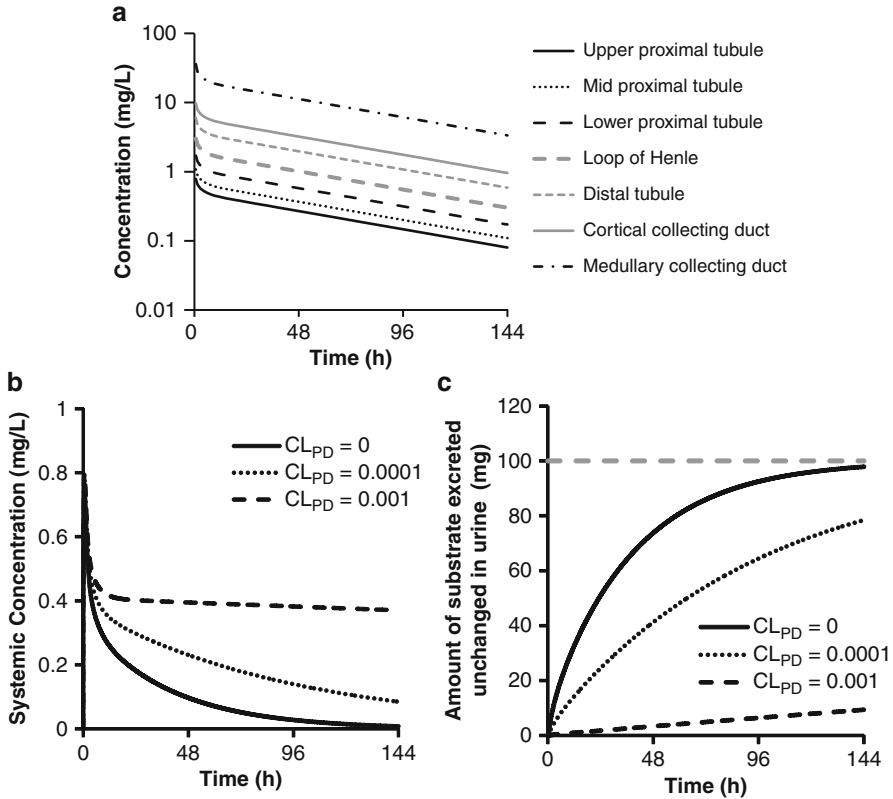
Fig. 7.5 Simulation of the kinetics of a neutral drug (100 mg iv) that is eliminated entirely unchanged in the urine and undergoes only passive diffusion within kidney tubular membranes (equal basal and apical clearances). (**a**) Drug concentration—time profiles in different regions of the nephron using a CL_{PD} of 0.0001 mL/min/million cells. (**b**) Impact on systemic drug concentrations of changing the value of passive clearances in the tubules. (**c**) Impact on the amount of drug excreted in the urine over time of changing the value passive drug clearances in the tubules

7.4.3 The Impact of the Relative Efficiency of Renal Uptake and Efflux Transporters

In this case, the model assumptions described in Sect. 7.4.1 were changed from passive permeability only to active transport only and the behaviour of a monoprotic acid with a pKa of 6.9 was simulated. The impact of different combinations of the values of uptake and efflux transporter-mediated clearances on the time courses of drug excretion in the urine and its concentration in kidney cells (Fig. 7.7) was investigated.
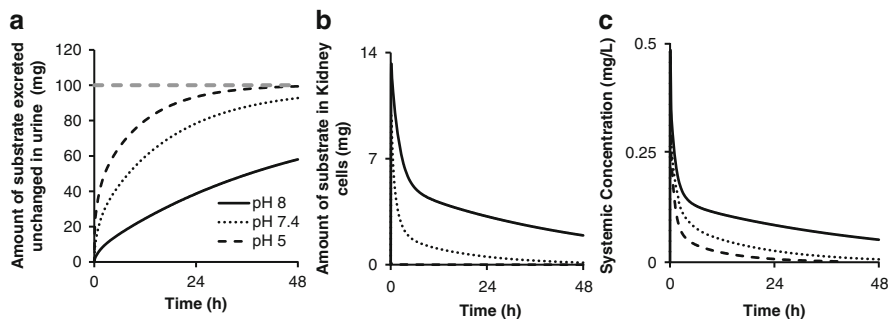
Fig. 7.6 Simulation of the time-courses of (**a**) urinary excretion, (**b**) tubular cell concentration and (**c**) plasma concentration of a weak base (pKa 10) (100 mg iv) that is 80 % renally cleared and 20 % metabolised in the liver as a function of tubular (urine) pH. Passive clearance across the tubular cells was assumed to be the same in both directions (CL_{PD} = 0.05 mL/min/million cells), and active basal uptake and apical efflux clearances were assumed to be the same (50 μL/min/million cells)

In the case where basal and apical transport clearances are identical, a decrease in the clearance value is associated with a delay in the appearance of drug in the urine and increase in the cellular concentration (Fig. 7.7a, b). In the limit, when there is no active transport at all, there will clearly be no cellular uptake. When basal clearance exceeds apical clearance (which would also represent saturation of efflux or its differential inhibition), there is little impact on the profile of urinary recovery (or systemic exposure—not shown), while the intracellular kidney drug concentration rises as it becomes rate limited by efflux (Fig. 7.7c, d). A decrease in basal relative to apical clearance (which would also represent saturation of influx or its differential inhibition) tends to delay urinary excretion as cellular uptake and accumulation decreases, while systemic exposure is not affected significantly. In the last case, where there is only active uptake of drug and no efflux from the kidney cell (representing complete and selective inhibition of efflux transport), the latter accumulates more and more drug as the value of basal clearance increases such that no drug appears in the urine other than that derived from glomerular filtration (Fig. 7.7g, h). Any increase in basal secretion will clearly decrease systemic exposure and availability for excretion by glomerular filtration since drug cannot leave the kidney cell. In reality, this irreversible accumulation would be offset by passive back-diffusion into the blood with or without augmentation by active apical uptake into the cell from the tubular fluid and/or active basal efflux into the blood. Nevertheless, the scenarios illustrated by the second and fourth cases described above clearly amplify the risk of excessive intra-renal drug accumulation and nephrotoxicity.

Most nephrotoxins provoke injury to cells in the first two segments of the proximal tubules, reflecting enrichment of their cellular concentration by transporters in the basal membrane of the cells and a relative low efficiency of apical efflux in this
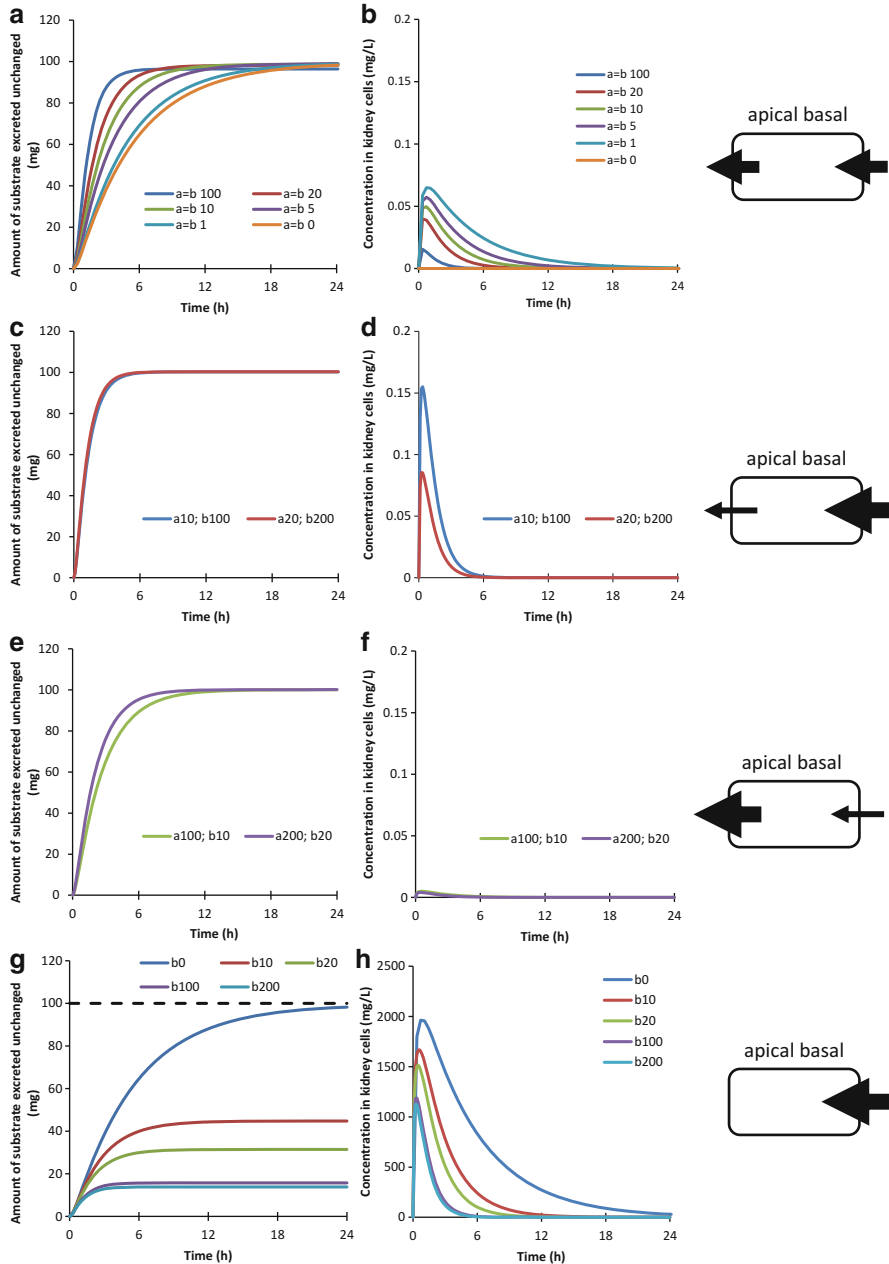
Fig. 7.7 Simulation of the kinetics in urine (**a**, **c**, **e**, **g**) and kidney cells (**b**, **d**, **f**, **h**) of a weak acid (pKa 6.9) drug that is entirely eliminated unchanged in the urine and which undergoes basal and apical tubular transport with no passive component. The impact of different permutations of basal vs. apical transport clearance values is shown (**a** and **b** through **g** and **h**)

region of the nephron (Berndt 1989). Furthermore, intra-renal drug metabolism to toxic products is most likely to occur within the cells in the second proximal tubule segment (Kaloyanides 1991). The functionality of Mech KiM is capable of exploring the implications of interplay at this locus between drug metabolism and basal and apical transporters.

7.4.4 The Impact of Inhibition of Renal Drug Transporters

The theoretical effects of inhibition of basal and apical transporters have been considered in the previous section. In this section, the results of simulations based on a real example are discussed. The clinical observation that probenecid decreases the renal clearance of famotidine in humans but not in the rat is explained by inhibition of OAT3, the major renal transporter for famotidine in humans and the fact that rat orthologue, oat3, is less effective in the transport of famotidine in that species (Shitara et al. 2006). Using Mech KiM, the impact of compound Y on the systemic exposure to compound X and its renal clearance was simulated for the conditions where Y inhibits a major or a minor basal uptake transporter of X in the kidney. For this purpose, it was assumed: (1) that there is no passive permeability at either basal or apical interfaces, (2) that X is taken up into the kidney cells by one major basal membrane transporter (intrinsic clearance = 100 μL/min/million PTC) and one minor basal membrane transporter (intrinsic clearance = 1 μL/min/million PTC), both of which can be inhibited by Y, and (3) that X is effluxed into tubular fluid by an apical transporter (intrinsic clearance = 100 μL/min/million PTC), which is not affected by Y.

Clearly, the simulation confirms intuitive expectation and the experimental findings with probenecid and famotidine in showing that only inhibition of the major uptake transporter (Fig. 7.8a) will have a significant impact on the systemic exposure of X (Fig. 7.8b). Inhibition of the major transporter causes an 80 % decrease in the renal clearance of X, while inhibition of the minor transporter decreases it by 0.4 %. The impact of major and minor inhibition on fluid, cellular and blood concentrations of X in the first segment of the proximal tubule is shown in Fig. 7.8c–e.

7.4.5 Inter-Individual Variability in Renal Elimination

The incorporation of variability of individual system and drug parameters (transporter and enzyme abundances/activities, PTCPGK, nephron number, GFR, kidney weight) in Mech KiM allows evaluation of the distributions of renal elimination in populations which, in turn, can be propagated into the whole body-PKPB model
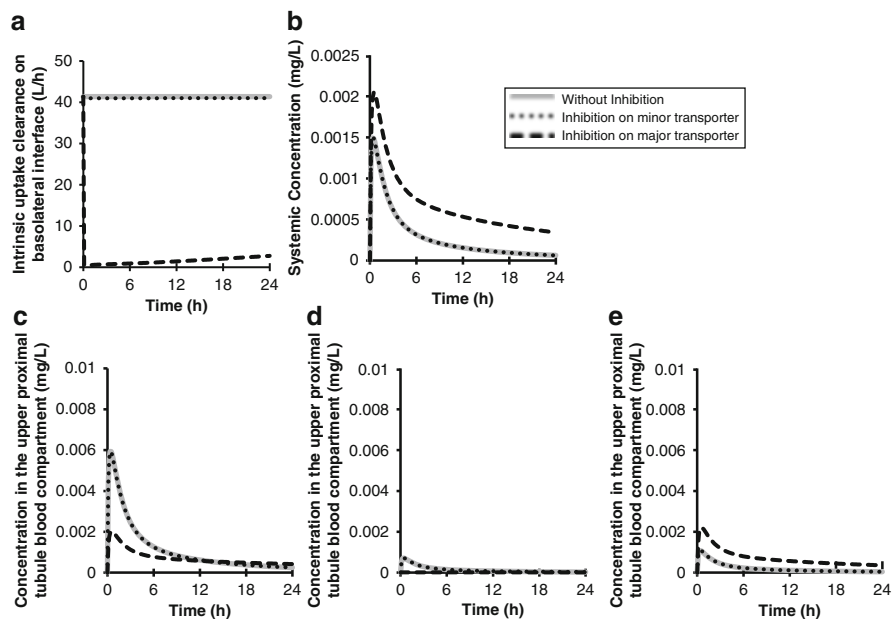
Fig. 7.8 Simulation of the kinetics of a virtual drug (X) which undergoes efflux transport into urinary tubular fluid mediated to a major extent by one transporter and to a minor extent by another. The impact of co-administration of a second drug (Y) that inhibits either the major or minor transporter (**a**) on the systemic plasma concentrations of X (**b**) and its concentrations in the fluid (**c**), blood (**e**) and cells (**d**) of the upper proximal tubule is indicated

within the Simcyp Simulator. This allows estimation of the extremes of drug exposure with respect to both systemic drug concentrations and local concentrations within kidney tissue. As shown in the previous examples of the application of Mech KiM, variability in transporter abundance/activity (e.g., due to genetic differences and drug–drug interactions), may not always be manifest in plasma drug concentration–time profiles, only becoming apparent when predicting intra-renal drug concentrations.

Using Mech KiM nested within the whole body-PBPK model, urine concentrations of a virtual drug were simulated in a virtual population of 100 healthy subjects (19–45 years old) (Fig. 7.9). A large inter-individual variability is apparent, with two subjects having particularly high urine concentrations, despite the fact that their systemic concentrations were similar to those in the other subjects. Exploration of these individuals revealed that they had relatively high GFR values and a low number of PTCPGK compared to the other subjects. Thus, much of the between-subject variability in the urine data can be assigned to local variability within the kidney rather than in the rest of the body. This illustrates the capability of Mech KiM to identify outlier individuals and the likely reasons for such extreme behaviour.

Fig. 7.9 Simulation of individual urinary concentration—time profiles of a virtual drug in a population of 100 healthy subjects considering known inter-individual variability of various physiological and biological elements making up the renal clearance (the *white line* indicates the population mean profile)
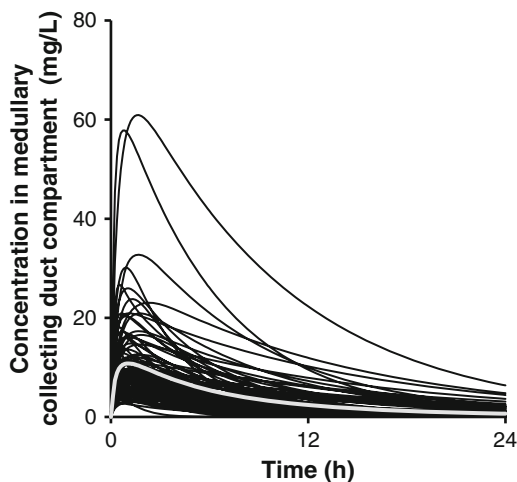
7.5 Prospect

In this chapter the elements of a mechanistic model of the handling of drugs by the kidney, including active transport, have been described together with some indication of its capabilities particularly in 'quantitative assessment' of the perturbations to various elements and in providing an integrated view when multiple parameters are involved in an interplay. Clearly, while these are early days in this development, definition of the model at least serves to emphasise the existing limitations and deficiencies in the availability of relevant systems and drug information necessary for its robust application and, pending these data, the model does allow important 'what if' questions to be explored during drug development. With respect to the transporter elements of the model, there is clearly a need to obtain more information on PTCPGK and its covariates and the absolute abundances of the different renal transporters and their distributions along the nephron. Obtaining these experimental data is not trivial and requires due attention to sample quality, assay specifications, the turnover and stability of protein and the impact, for example, of pH on transporter activity.

Appendix

IVIVE for renal transporters using rhTransporter cell systems such as CHO-OAT1, HEK293-OAT3, etc.
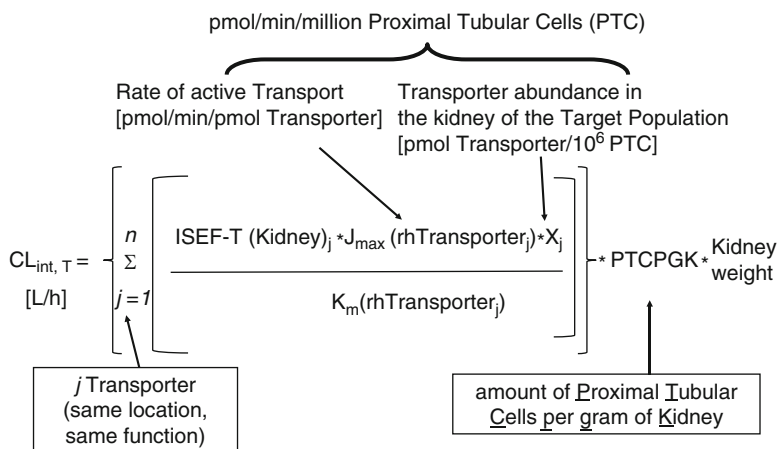
pmol/min/million Proximal Tubular Cells (PTC)

Rate of active Transport
[pmol/min/pmol Transporter]

Transporter abundance in
the kidney of the Target Population
[pmol Transporter/10^6 PTC]

$$CL_{int,\,T} = \left[\sum_{j=1}^{n} \frac{ISEF\text{-}T\,(Kidney)_j * J_{max}\,(rhTransporter_j) * X_j}{K_m(rhTransporter_j)} \right] * PTCPGK * \begin{matrix} Kidney \\ weight \end{matrix}$$

[L/h]

j Transporter
(same location,
same function)

amount of <u>P</u>roximal <u>T</u>ubular
<u>C</u>ells per <u>g</u>ram of <u>K</u>idney

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Chapter 8
Analysis of Intestinal Transporters

Ikumi Tamai and Takeo Nakanishi

Abstract Intestinal transporters are involved in both influx (absorption) and efflux (exsorption) of various drugs and thereby affect the bioavailability of those drugs. Intestinal tissues are heterogeneous, exhibiting regional variations in physiology and transporter expression, as well as having highly variable intestinal luminal contents. Furthermore, intestinal absorption may proceed via plural mechanisms, such as simple diffusion, carrier-mediated transport, and paracellular transport, in parallel. Accordingly, it is not necessarily easy to identify the mechanism(s) involved in absorption of particular drugs. However, by employing combinations of several experimental methods, some transporters involved in drug absorption and exsorption have been found. P-glycoprotein and BCRP are key efflux transporters that serve to limit absorption of various drugs. As for influx transporters, the picture has not yet been fully clarified, but PEPT1 and OATP have been demonstrated to contribute to drug absorption, and they are expected to be available as target molecules for improving the absorption of orally administered drugs. This chapter focuses on the current understanding of intestinal drug transporters, especially the less-studied absorptive transporters, as well as methods to analyze intestinal absorption and transport processes.

Abbreviations

BBB   Blood–brain barrier
BCRP   Breast cancer resistance protein
HEK   Human embryo kidney

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MDR   Multidrug resistance
OATP   Organic anion-transporting polypeptide
PEPT   Peptide transporter
Reb   Rebamipide

8.1 Intestinal Transporters and Drug Absorption

Intestinal absorption is a key issue in the development of new drugs, and compounds that are pharmacologically active in vitro are sometimes dropped if they show poor oral bioavailability. Bioavailability is affected by multiple factors, including solubility, intestinal permeability, and intestinal and hepatic metabolism. Among these factors, intestinal permeability is influenced by the distribution and activity of influx and efflux transporters (Fig. 8.1). Intestinal influx transporters expressed at the apical membrane of enterocytes are physiologically essential for the absorption of nutrients such as amino acids, oligopeptides, bile acids, water-soluble vitamins, nucleosides, hexose, and other nutrients. On the other hand, efflux transporters such as P-glycoprotein (encoded by *MDR1*/*ABCB1*) and BCRP (*ABCG2*) function as an absorption barrier, thereby protecting organisms from xenobiotic (toxic) compounds. Most drugs are potentially recognized as xenobiotics, i.e., they are recognized as substrates of efflux transporters, but not influx transporters. However, some orally administered drugs are actively absorbed from the intestinal lumen, probably because they are misrecognized by influx transporter(s) due to their structural similarity to endogenous substrates; in other words, some transporter(s) show a rather
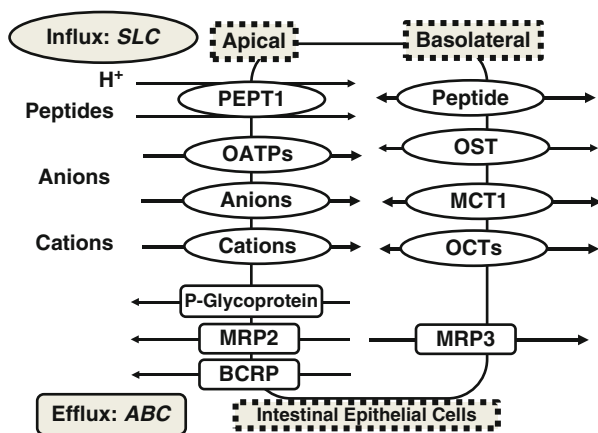
Fig. 8.1 Intestinal transporters affecting drug absorption. *Ovals* and *squares* indicate SLC and ABC transporters, respectively. Apical membrane transporters have been well studied, whereas information on basolateral transporters involved in drug transport is limited

broad substrate specificity. Influx transporters for nutrients expressed at the apical membrane usually translocate their substrates by utilizing a concentration gradient of sodium ions, protons, or other ions. So far, peptide transporter 1 (PEPT1) and organic anion-transporting polypeptides (OATPs) (especially OATP2B1) have been demonstrated to participate in drug transport in the absorptive direction. Accordingly, it may be feasible to utilize these transporters for oral delivery of certain drugs. But, drugs that are substrates of these transporters are sometimes also substrates of efflux transporters. In that case, their intestinal disposition could be influenced by both influx and efflux transporters, resulting in complex absorption characteristics. In this chapter, we will review and summarize (a) the current understanding of the role of transporter molecules in intestinal absorption (PEPT1, OATP2B1) and exsorption (P-glycoprotein and BCRP), (b) methods to analyze intestinal absorption and transport and problems associated with transporter studies, and (c) the application of influx transporters to oral delivery of drugs.

8.2 Oligopeptide Transporter PEPT1

8.2.1 Current Understanding of PEPT1-Mediated Intestinal Absorption

The oligopeptide transporter PEPT1 (*SLC15A1*) is the most extensively studied absorptive transporter. It is a member of the proton-dependent oligopeptide transporter (POT) family, which consists of four members: PEPT1, PEPT2 (*SLC15A2*), PHT2 (also termed PTR3/*SLC15A3*), and PHT1 (PTR4/*SLC15A4*). Among them, PEPT1 is expressed mainly at the apical membrane of intestinal epithelial cells and to a lesser extent in renal tubular epithelial cells and mediates intestinal absorption and/or renal reabsorption of di- and tripeptides as native substrates. PEPT2 expression is confined to the apical membrane of renal epithelial cells, and PEPT2 plays a key role in reabsorption of these oligopeptides from urine.

There are several reports on regional differences in the expression of PEPT1 along the intestine. In humans, relatively high mRNA expression of PEPT1 is maintained from duodenum through ileum, while the expression is much lower, though still significant, in colon (Meier et al. 2007). Another study showed that PEPT1 mRNA and protein expression decreases in the order of duodenum > jejunum > ileum; PEPT1 is also expressed in the stomach in some individuals, but not in colon (Terada et al. 2005). In rats, Pept1 mRNA expression was shown to be higher in the lower small intestine in the fed state, while its expression in the upper small intestine was increased in the starved state, becoming comparable to that in the lower small intestine (Naruhashi et al. 2002). Accordingly, PEPT1 is essentially expressed throughout the small intestine, though with some regional differences, and its expression level is affected by food and other factors, showing considerable inter- and intraindividual variability.

Although PEPT1 and PEPT2 accept di- and tripeptides as endogenous substrates, they also accept several peptide-mimetic drugs as substrates and contribute to their intestinal absorption and renal reabsorption (Matthew 1991; Daniel and Kottra 2004). PEPT1 is thought to exhibit broader selectivity for drugs than PEPT2 (Tsuji et al. 1987; Tamai et al. 1988; Terada et al. 2000). The structural requirements of di- and tripeptide substrates include the presence of one or two peptide bonds, an amino terminal, and a carboxyl terminal. Oligopeptides meeting these requirements are high-affinity substrates of PEPT1. However, several drugs that are transported by PEPT1 do not meet these criteria, so PEPT1 seems to possess a broader substrate selectivity. As for endogenous substrates of PEPT1, some 400 dipeptides and 8,000 tripeptides may be formed from ingested proteins, which may contain 20 different amino acids, though some of these peptides may have negligible affinity for PEPT1. This is in marked contrast to the amino acid transporters, which show high selectivity for substrate amino acids according to size and charge. In other words, the substrate selectivity of PEPT1 is strict in terms of molecular size, but not as regards the amino acid residues that constitute the di- and tripeptides. Accordingly, it seems likely that PEPT1 could mediate the intestinal absorption of various peptide-mimetic drugs.

The driving force for PEPT1-mediated transport is an inwardly directed proton gradient. Microclimate pH in the close vicinity of intestinal epithelial cells is maintained at a weakly acidic level by sodium/proton exchange, so that the environment is proton-rich. An interesting point is that the optimal pH of transport by PEPT1 is variable among substrates. Electrically neutral peptides containing an amino moiety and a carboxyl moiety show optimal transport at about pH 6. However, transport of acidic peptides (with a predominance of anionic moieties) is greater at lower pH, while transport of basic peptides (with a predominance of cationic moieties) is greater at neutral pH (Wenzel et al. 1996; Steel et al. 1997). Figure 8.2 shows the pH dependence of transport of peptides and peptide mimetics in Caco-2 cells. The transport activity for glycylsarcosine (a neutral peptide) was highest at pH of 5.5 or 6, while that for carnosine (β-alanylhistidine, a cationic peptide) was highest at neutral pH. In the case of acidic beta-lactam antibiotics, cefixime and FK089, higher transport activity was observed at lower pH, while neutral cefadroxil exhibited optimal transport at pH 6. Although the mechanism that determines the optimal pH of PEPT1-mediated transport is not clear, the ionization state of substrates clearly influences the apparent transport.

8.2.2 Potential Contribution of PEPT1 to Drug Absorption

As described above, PEPT1 exhibits relatively broad substrate selectivity and likely contributes to intestinal absorption of clinically important substrate drugs, though other transporters may also be involved (Tsuji and Tamai 1996; Brandsch et al. 2008). Interaction of PEPT1 with drugs was first established by characterizing PEPT1-mediated transport of orally active beta-lactam antibiotics, such as
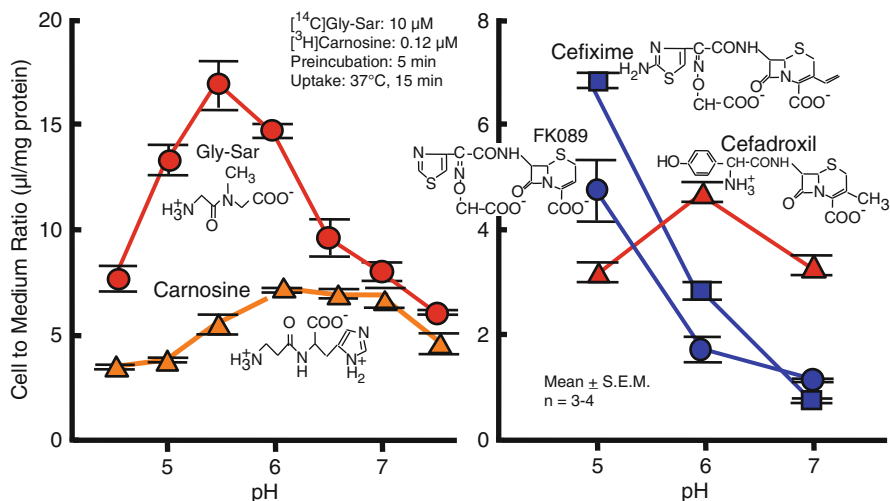
Fig. 8.2 Effect of pH on PEPT1-mediated transport of peptides and peptide mimetics. Uptake of test compounds by Caco-2 cells at various pH values is shown. Gly-Sar (glycylsarcosine) and cefadroxil are neutral, carnosine (beta-alanylhistidine) is cationic because of the histidine residue, and cefixime and FK089 are anionic. Neutral compounds showed optimal pH for transport of around pH 6, while anionic compounds show an increase of permeability with decrease of pH. Cationic carnosine shows an optimal pH value close to neutral. Optimal pH for transport by PEPT1 is substrate dependent

cefaclor (neutral), cephalexin (neutral), cefadroxil (neutral), cefixime (anionic), and ceftibuten (anionic). Anticancer agent ubenimex and antihypotensive midodrine have dipeptide-like structures and are transported by PEPT1. Several angiotensin-converting enzyme inhibitors, such as captopril and enalapril, have been suggested to be substrates of PEPT1, though a more recent study suggested a negligible contribution of PEPT1 to the membrane permeability of those angiotensin-converting enzymes inhibitors (Knütter et al. 2008). Some prodrugs lacking peptide bond(s) in their structure are accepted as substrates of PEPT1. Antiviral drugs such as valacyclovir (valine ester of acyclovir) and valganciclovir (valine ester of ganciclovir) are transported by PEPT1 (Balimane et al. 1998; Sugawara et al. 2000). The amino acid delta-aminolevulinic acid is also a substrate of PEPT1 (Döring et al. 1998). Considering the broad substrate selectivity of PEPT1, other drugs in clinical use could also be substrates.

To date, there are no clinically available drugs that were previously designed to be recognized by PEPT1 in the expectation of higher intestinal membrane permeability. Although PEPT1 targeting seems to be an attractive strategy for oral drug delivery, it faces many challenges (Ezra et al. 2000; Eriksson et al. 2005; Steffansen et al. 2005). In the next section, studies performed by the present authors to improve intestinal drug absorption via PEPT1 are described.
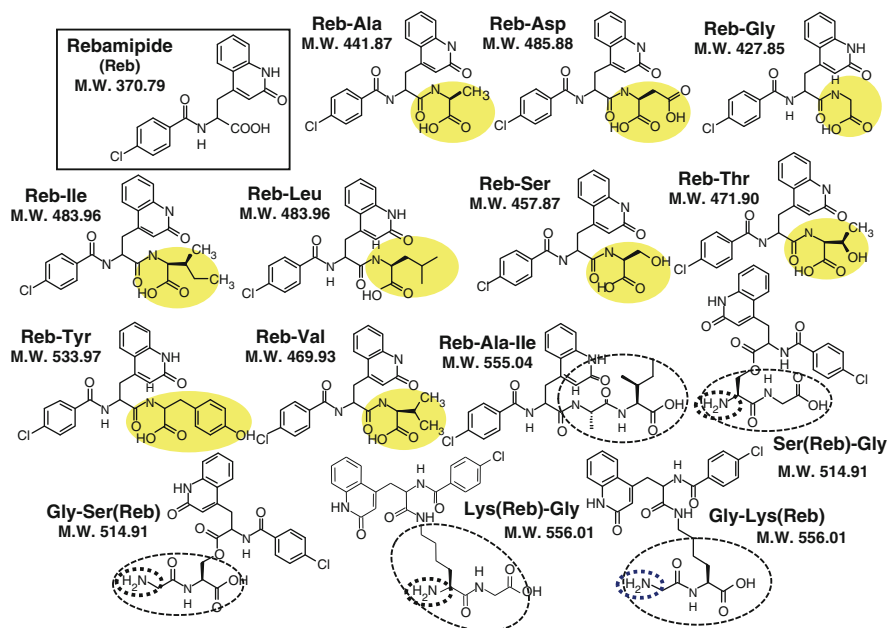
Fig. 8.3 Structures of synthesized amino acid and peptide derivatives of rebamipide. Derivatives shown by *dotted circles* have significant affinity for PEPT1. Four compounds (Ser(Reb)-Gly, Gly-Ser(Reb), Lys(Reb)-Gly, and Gly-Lys(Reb)) retain a free amino group as shown by the *dotted oval*. This figure is cited from Kikuchi et al. (2009) with modifications

8.2.3 Application of PEPT1 to Oral Drug Delivery

Strategies to utilize PEPT1 for oral drug delivery can be classified into two broad types: derivation of non-PEPT1 substrate drugs to peptide-mimetic compounds that can be recognized by PEPT1 and activation of PEPT1 function to increase PEPT1-mediated absorption of weakly recognized substrate drugs without structural modification of the compounds.

In the case of the first strategy, one option is to design compounds that mimic di- or tripeptides. Such compounds need not necessarily contain a peptide bond, as in the case of the antiviral agents described in the previous section. In this approach, the compounds are usually designed as prodrugs that can be transported by PEPT1 and subsequently cleaved to generate the active form after having been absorbed (Tamai et al. 1998).

Another option is to conjugate the candidate compound to a peptide structure that is recognized and transported by PEPT1. For example, rebamipide, an antiulcer drug that exhibits very low membrane permeability after oral administration, was molecularly modified by attaching amino acid or dipeptide moieties to it (Fig. 8.3), and the transport of these compounds was evaluated in vitro in PEPT1-expressing

cultured cells and Caco-2 cells (Kikuchi et al. 2009). The amino acid derivatives exhibited low or negligible affinity for PEPT1 and/or low solubility and were considered inadequate as prodrugs. However, several dipeptide derivatives of rebamipide (Reb), including Ser(Reb)-Gly, Gly-Ser(Reb), Lys(Reb)-Gly, and Gly-Lys(Reb), showed moderate affinity for PEPT1 in in vitro inhibition studies. These four peptide derivatives retain the intact amino and carboxyl terminals of the peptide moiety, since the hydroxyl group of serine or ε-amino group of lysine was used to carry the Reb moiety. Serine has a smaller molecular weight than lysine and may be more suitable for prodrugs. Further study on the transport of these peptide derivatives of rebamipide showed that Ser(Reb)-Gly and Gly-Ser(Reb) are PEPT1 substrates as well as PEPT1 inhibitors, whereas Lys(Reb)-Gly and Gly-Lys(Reb) are only inhibitors. Thus, it is possible to improve the membrane permeability by linking a suitable peptide moiety to a poorly permeable drug, which can then be carried across the intestinal membrane via PEPT1. There are several issues still to be solved, including adequate stability of the peptide derivatives in the intestinal lumen and efficient cleavage to generate the active compound after absorption, in order to obtain the pharmacological effect. However, it was established that PEPT1 can accept rebamipide–peptide derivatives with molecular weight larger than 500, since the molecular weight of rebamipide is 370 and that of the conjugated peptide part in this case is about 150. So, further modifications could be possible to improve the stability and affinity of the peptide moieties in order to optimize the delivery of various low-molecular-weight drugs.

The alternative strategy of activating/optimizing PEPT1 activity has also been proven effective (Nozawa et al. 2003). PEPT1 is an active transporter utilizing a proton gradient as the driving force, and so the transport is affected by luminal pH. As described above, the optimal pH for PEPT1-mediated transport can vary depending on the substrate. For example, the anionic beta-lactam antibiotic cefixime is a substrate of PEPT1, but its bioavailability is not high (about 30 % in human and rat). Since it is not metabolized and is excreted almost wholly in the unchanged form in urine, intestinal membrane permeability is considered to be the limiting factor, even though the compound is a substrate of PEPT1. In an in vitro experiment, higher permeability was observed at more acidic pH than at physiological pH (Fig. 8.2). At pH 5, cefixime exhibited comparable PEPT1-mediated permeability to cefadroxil, which is hydrophilic, but is absorbed almost completely. Accordingly, if the pH at the surface of the intestinal lumen can be modified to be more acidic, intestinal absorption of cefixime via PEPT1 may be considerably increased. When an acidic polymer, Eudragit L100-55, is added to a solution, the pH is maintained at an acidic level, depending on the concentration of the polymer, since the polymer releases protons. Therefore, if the polymer is coadministered with cefixime, it is expected that PEPT1-mediated intestinal absorption of cefixime would be enhanced due to the lowered intestinal luminal pH. Indeed, the strategy of administering cefixime as a 5 % Eudragit L100-55 solution was successful in increasing the bioavailability of cefixime from 27 to 62 % (Nozawa et al. 2003). In this experiment, the pH in the intestinal lumen was estimated to have been modified to about pH 5, based on the results of in vitro experiments. This strategy is unique, since it requires only an

appropriate formulation technology without any need for chemical modification of the active pharmaceutical ingredient, and so it should be readily applicable to other weak PEPT1 substrates.

8.3 Organic Anion-Transporting Polypeptide (OATP)

8.3.1 Current Understanding of OATP2B1-Mediated Intestinal Absorption

OATP2B1 (OATP-B, *SLCO2B1*) is expressed in various tissues, including the small intestine, liver, lung, and ovary (Tamai et al. 2000). This broad tissue expression profile is a distinctive feature of OATP2B1; in contrast, OATP1B1 and OATP1B3 are expressed exclusively in the liver (Abe et al. 1999; König et al. 2000). OATP2B1, OATP1B1, and OATP1B3 are commonly expressed at the sinusoidal membrane of hepatocytes (Kullak-Ublick et al. 2001). However, due to their specific and abundant expression in the liver and broad substrate selectivity, OATP1B1 and OATP1B3 are thought to be key transporters for hepatic uptake of drugs, as well as certain endogenous compounds, such as bile acids, bilirubin, and conjugated metabolites of steroid hormones (Mikkaichi et al. 2004; Kusuhara and Sugiyama 2009). OATP2B1 has been suggested to be involved in hepatic uptake of drugs in clinical use and may also play a significant role in drug disposition in other tissues, including intestine (Tamai 2012). The difference between the tissue expression profiles of these liver-specific OATPs (OATP1B1 and OATP1B3) and the more ubiquitous OATP2B1 could be partly explained by the difference in their regulatory transcription factors, HNF1alpha for liver-specific OATPs and general transcription factor Sp1 for OATP2B1 (Maeda et al. 2006).

Functionally, OATP2B1 is characterized by the pH dependence of its substrate transport. When OATP2B1 is expressed in HEK293 cells, uptake of estrone-3-sulfate by the cells is higher at acidic pH 5.0 than at pH 7.4. The increase is due to an increase of V_{max} (sevenfold), with only a slight increase of K_m (1.5-fold) at pH 5.0 compared with pH 7.4 (Kobayashi et al. 2003; Nozawa et al. 2004). Although the mechanism of the increase of transport activity at acidic pH remains unclear, FCCP, a protonophore, caused a significant decrease of uptake at acidic pH to 42 % of the control, with a smaller decrease at neutral pH (to 81 % of the control). In addition to estrone-3-sulfate, dehydroepiandrosterone sulfate, fexofenadine, and pravastatin were taken up to a greater extent at acidic pH (pH 5.0) than at neutral pH (pH 7.4) via OATP2B1 (Nozawa et al. 2004). Furthermore, pravastatin has been shown to exhibit proton-gradient-dependent transport in brush-border membrane vesicles prepared from rabbit small intestine, based on the observation of overshoot uptake in the presence of a proton gradient (Tamai et al. 1995; Shirasaka et al. 2011). Similar pH dependence was reported in OATP2B1-transfected cells and Caco-2

cells (Kis et al. 2010). Accordingly, these studies strongly support the idea that proton-coupled cotransport or exchange transport with hydroxyl ion contributes to the pH dependence of OATP2B1 transport activity. Since the physiological microclimate pH in the intestinal lumen is weakly acidic, as mentioned above, it may be important to characterize OATP2B1-mediated transport of drugs at acidic pH, but not neutral pH, in order to understand the physiological and pharmacological relevance of OATP2B1.

The broad substrate selectivity and some conflicting data regarding the effect of pH on OATP2B1 activity might be explained by the presence of multiple binding sites with differential substrate/inhibitor affinity and pH sensitivity (Satoh et al. 2005; Shirasaka et al. 2012). Other transporters may also have more than one substrate binding site. Accordingly, further studies are required to clarify the structural requirements for substrates of OATP2B1 and to establish optimum conditions for the application of OATP2B1 for oral drug delivery.

8.3.2 Pharmacogenomics of OATP2B1

Table 8.1 shows non-synonymous mutations found in the *SLCO2B1* gene. Among these genetic variants, *SLCO2B1*3*, which contains the mutation c.1457C>T (causing the amino acid change Ser486Phe), resulted in a decrease of transport activity for estrone-3-sulfate in HEK293 cells expressing the variant compared with that of wild-type *SLCO2B1*1*, after correction for expressed protein amount (Nozawa et al. 2002). This change was explained by a decrease of V_{max} to 43 % of that of the wild-type enzyme, with a negligible change of affinity, i.e., K_m 2.97 μM (*1) and 2.31 μM (*3). Individuals carrying the *SLCO2B1*3* allele showed decreased intestinal absorption of fexofenadine, in accordance with the difference of in vitro activity (Imanaga et al. 2011). Plasma concentration of celiprolol was similarly affected by the genotype *SLCO2B1*3* (Ieiri et al. 2012). Wild-type homozygotes of CC showed the highest plasma concentration, followed by heterozygotes CT and mutant homozygotes TT at the therapeutic dose of 100 mg. These results indicate involvement of OATP2B1 in celiprolol absorption. Interestingly, such an effect of genetic mutation was not detected at microdose levels (37.5 μg). The dose-dependent effect of the genotype of *SLCO2B1* gene was explained in terms of the contribution of P-glycoprotein to exsorption of celiprolol. At therapeutic doses, P-glycoprotein is saturated and has no apparent effect on celiprolol efflux, leaving OATP2B1 as the predominant determinant of intestinal absorption of celiprolol, while at microdose levels the effect of P-glycoprotein is predominant.

The allele frequency of *SLCO2B1*3* (c.1457C>T) was 30.9 % in Japanese. This frequency is relatively high, and individuals with genotype(s) associated with impaired transport activity may show decreased efficacy of substrate drugs. There is an ethnic difference in this SNP, because its frequency is low in Finns (2.8 %). On the other hand, OATP2B1*3 has higher activity for the transport of rosuvastatin

Table 8.1 Major single-nucleotide polymorphisms of the *OATP2B1* gene

rs number	Exon	Nucleotide variation	Amino acid variation	Allelic frequency		
				Japanese	Europeans	Africans
rs56837383	3	c.43C>T	P15S			
rs148248368	4	c.343C>T	P115S			
rs35199625	6	c.601G>A	V201M		2.1 (Laitinen and Niemi 2011)	
rs12422149	8	c.935G>A	R312Q		13.6 (Laitinen and Niemi 2011) 8.2 (Mougey et al. 2009)	13 (Mougey et al. 2009)
rs1621378	10	c.1175C>T	T392I	0 (Nozawa et al. 2002)		
rs111782322	10	c.1240G>A	G414S			
rs2306168	11	c.1457C>T	S486F	31 (Nozawa et al. 2002)	2.8 (Laitinen and Niemi 2011)	
rs140407559	11	c.1526G>A	R509H			
rs143480565	12	c.1624G>A	V542M			
rs145875125	13	c.1638C>A	N546K			
rs149242910	12	c.1642G>A	V548M			
rs149765874	15	c.2071G>A	V691I			

than wild-type OATP2B1, when expressed in HeLa cells (Ho et al. 2006). Accordingly, the effect of genetic polymorphisms on transport activity might be variable among substrates. Further studies on the mechanisms of alteration of apparent activity caused by mutation seem necessary. Another variant, c.1175C>T, which causes the amino acid substitution of Thr at codon 392 with Ile (*SLCO2B1*2*), resulted in a slight decrease in uptake of estrone-3-sulfate in HEK293 cells compared with the wild-type (Nozawa et al. 2002). Other variants of OATP2B1, such as c.43C>T (Pro15Ser), c.601G>A (Val201Met), and the three-amino-acid deletion (26–28, Gln-Asn-Thr), were preliminarily reported to have lowered transport activity for rosuvastatin (Ho et al. 2006). The variant c.935G>A, which causes a nonsynonymous mutation of OATP2B1 (Arg312Gln), has also been reported, though its effect on activity is poorly understood. The relationship between the *SLCO2B1* genotype and the pharmacological effect of the leukotriene receptor antagonist montelukast was examined in patients with asthma (Mougey et al. 2009). Compared to the wild-type allele 935G, individuals with 935A heterozygously exhibited a weaker response to treatment and a lower plasma concentration of montelukast. Based on these pharmacogenomic studies, it is clear that OATP2B1 contributes to drug absorption in vivo in humans.

8.3.3   Drug–Fruit Juice Interaction at OATP2B1

OATP transporters may be involved in drug–fruit juice interaction during intestinal absorption. Previously known pharmacokinetic interactions with fruit juices were explained mainly in terms of the inhibitory effects of ingredients of fruit juice on intestinal drug-metabolizing enzymes and efflux transporters (e.g., P-glycoprotein), resulting in increased plasma concentration of the affected drugs (Bailey 2010). However, in 2002, it was reported that fruit juices such as grapefruit, orange, and apple juices reduced the plasma concentration of fexofenadine after oral administration (Bailey et al. 2007). Since the observed effect could not be explained by previously reported drug–juice interactions at drug-metabolizing enzymes and/or exsorptive transporters, interaction may also occur at intestinal absorptive transporters. It was hypothesized that OATP transporters, especially OATP1A2, were involved in this interaction based on the results of in vitro transport studies. In another study, a species difference between rat and human in the effect of fruit juice on the plasma concentration of talinolol after oral administration was observed, i.e., the plasma concentration of talinolol was increased and decreased in rat and human, respectively, upon ingestion with grapefruit juice (Spahn-Langguth and Langguth 2001; Schwarz et al. 2005). Because talinolol is not metabolized and is a substrate of P-glycoprotein, a decrease in its plasma concentration in human cannot be explained by interaction at P-glycoprotein, though the increase of plasma concentration in rat could be explained by inhibition of P-glycoprotein. We found that talinolol is a substrate of human OATP and rat intestinal Oatps, and naringin in grapefruit juice inhibits both human and rat OATPs/Oatps at a concentration that is achievable following ingestion of grapefruit juice (Shirasaka et al. 2009, 2010). Further, rat but not human P-glycoprotein was inhibited by a juice ingredient at the same concentration as in juice. Accordingly, the effect of grapefruit juice on talinolol absorption exhibited species difference due to the difference in the affinity of grapefruit juice ingredient(s) for P-glycoprotein between human and rat, whereas the juice inhibited intestinal OATPs similarly in human and rat. Fexofenadine absorption was reduced by ingestion with grapefruit juice or apple juice due to inhibition of OATP1A2 (Bailey et al. 2007) or OATP2B1 (Imanaga et al. 2011). Furthermore, reduction of intestinal absorption of statins (Shirasaka et al. 2011), montelukast (Mougey et al. 2009), and aliskiren (Tapaninen et al. 2010) by grapefruit juice was explained in terms of inhibition of OATP2B1 and/or OATP1A2. Other fruit juices and beverages also affect drug absorption by interacting with OATPs. Absorption of fexofenadine was reduced by orange juice and apple juice (Dresser et al. 2002; Imanaga et al. 2011). Green tea catechins including epicatechin gallate and epigallocatechin gallate inhibited OATP2B1 and OATP1A2 (Roth et al. 2011). Accordingly, OATPs are likely involved in a variety of drug–beverage interactions during the intestinal absorption process. The results of these studies on drug–juice interaction in humans, as well as in vitro studies with transporter-expressing cells, represent strong evidence that these transporters contribute to drug absorption.

8.3.4 Potential Contribution of OATP1A2 to Drug Absorption

OATP1A2 protein is expressed at the apical membrane of human enterocytes, like OATP2B1 (Glaeser et al. 2007), and accepts as substrates various drugs that are mostly also substrates of OATP2B1. Accordingly, it is not easy to distinguish the contributions of OATP1A2 and OATP2B1 to the absorption of common substrate drugs. However, several reports show that OATP1A2 is expressed at a much lower level than OATP2B1 in human intestine or even at a negligible level, and *SLCO2B1* was reported to be more abundant in enterocytes (Tamai et al. 2000; Meier et al. 2007). In addition, there is convincing evidence that the in vivo effects of genetic polymorphisms of OATP2B1 parallel the in vitro transport activity of the mutated OATP2B1, which is again consistent with a significant contribution of OATP2B1 to drug absorption. Thus, although OATP1A2 may also contribute to drug absorption, this remains to be established. It seems likely that marked interindividual variability in expression level and/or changes in expression level of OATP1A2 in response to various factors may account for the conflicting observations regarding its expression in intestinal tissues.

8.4 Intestinal Efflux Transporters

8.4.1 P-Glycoprotein-Mediated Exsorption as an Absorption Barrier

It is well understood that efflux transporters such as P-glycoprotein and breast cancer resistance protein (BCRP) affect drug absorption by transporting drugs into the intestinal lumen. As shown in Fig. 8.4, there appears to be a hyperbolic relationship between intestinal permeability and lipophilicity of drugs, though there is an upper limit of the permeability (Terao et al. 1996). However, several drugs exhibited lower permeability than would be expected from this correlation, and many of these drugs were substrates of P-glycoprotein. When cyclosporin A was added as a P-glycoprotein inhibitor, an increase in permeability was observed for many of these drugs. These results suggest that P-glycoprotein is a major component of the absorption barrier. Indeed, studies with P-glycoprotein-expressing cells, Caco-2 cells, and intestinal tissues have indicated that P-glycoprotein-mediated intestinal exsorptive transport is a molecular mechanism of poor intestinal absorption of drugs (Fromm 2003; Lin and Yamazaki 2003). However, some P-glycoprotein substrate drugs show good intestinal absorption in clinical use. One of the reasons for such apparently inconsistent observations, despite active exsorption mediated by P-glycoprotein, might be because the intestinal luminal concentration in the clinical setting is high enough to saturate P-glycoprotein-mediated transport. Another possible reason is regional difference in the expression of P-glycoprotein, with higher expression at the lower part of the small intestine. Several P-glycoprotein
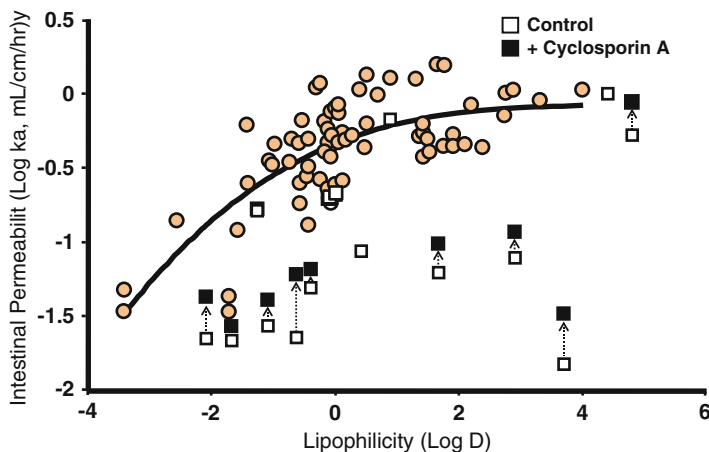
Fig. 8.4 Relationship between intestinal permeability and lipophilicity of various drugs. *Open and closed squares* show the absorption rate constants in the absence and presence of cyclosporine A as a P-glycoprotein inhibitor. The absorption rate constant is increased in the presence of cyclosporine A, showing that P-glycoprotein limits drug absorption to some extent. This figure is cited from Terao et al. (1996)

substrate drug in clinical use, such as quinidine, bepotastine, and azasetron, exhibit high intestinal permeability. As suggested above, this can be explained in terms of the high concentration of these drugs in the intestinal lumen after oral administration and/or higher absorptive permeability at the upper part of the small intestine, thereby avoiding exsorption mediated by P-glycoprotein due to its low expression in this region. However, these drugs are often not delivered into the brain across the blood–brain barrier (BBB) due to the efficient efflux transport via P-glycoprotein, since the plasma concentration may not be high enough to saturate P-glycoprotein at the BBB. If the pharmacological target of a drug is peripheral tissue, it is a good strategy to have high affinity for P-glycoprotein, since high intestinal permeability and low blood–brain barrier permeability can be expected due to the saturation of P-glycoprotein in gut but not in brain. Accordingly, care is needed in estimating the effect of efflux transporters on drug absorption, since it is not rare that drugs at clinically used doses are present at a high enough concentration in the intestinal lumen to saturate P-glycoprotein there. This is just one of the difficulties involved in predicting in vivo effects of transporters on drug absorption from in vitro studies.

8.4.2   BCRP-Mediated Exsorption as an Absorption Barrier

BCRP was named after its function in breast cancer. Ironically, it is frequently not expressed in breast tumor tissues, but it is expressed in several normal tissues, such as the intestine, liver, and placenta, which are involved in determining drug

pharmacokinetics. There is a species difference in the expression level in kidney, with high expression in murine kidney and low expression in human kidney, but common expression in intestinal tissues of both species (Maliepaard et al. 2001; Tanaka et al. 2004; Borght et al. 2006; Huls et al. 2007). It is known that BCRP is a characteristic marker of certain stem cell-like and progenitor cell populations in normal tissues; these cells show little or no staining with Hoechst33342, because of active BCRP-mediated efflux of the dye (Zhou et al. 2001). This reflects the physiological role of BCRP in protecting organisms from xenobiotics and toxins (Ross and Nakanishi 2010). More recently, a pharmacogenomic study of BCRP revealed that reduced activity of BCRP function due to genetic polymorphisms is associated with increased risk of hyperuricemia/gout (Matsuo et al. 2009). Since uric acid is a uremic toxin, BCRP may be one of the transporters that limits uric acid concentration in serum (Hosomi et al. 2012). BCRP is expressed in small intestine at relatively high levels, and since it serves as an efflux transporter for a wide variety of drugs, particularly chemotherapeutics, it is likely to function as an absorption barrier to drugs (Polgar et al. 2008; Nakanishi and Ross 2012). Sulfasalazine is used to treat ulcerative colitis and its target is the colon. Sulfasalazine is metabolized to sulfapyridine and 5-aminosalicylate by colonic bacteria, and the formed 5-aminosalicylate is the active agent. Accordingly, it is important that sulfasalazine is delivered to the colon without absorption in the small intestine. Since sulfasalazine is a substrate of BCRP, intestinal absorption of the drug is indeed low. However, individuals with a genetic variant of BCRP (c.421C>A in *ABCG2* gene) exhibited higher absorption of sulfasalazine due to decreased BCRP-mediated exsorptive transport in small intestine (Yamasaki et al. 2008). In the case of sulfasalazine, low, BCRP-limited absorption at the small intestine is clinically advantageous, but for most of substrate drugs, intestinal BCRP may reduce their bioavailability.

8.5   Evaluation of Absorption Mechanisms

As described above, absorptive (influx) and exsorptive (efflux) intestinal transporters may significantly influence the absorption of orally administered drugs. Although there is considerable evidence that this is the case for efflux transporters such as P-glycoprotein and BCRP, the contributions of influx transporters to drug absorption generally remain to be clarified. The reason for this is because multiple pathways exist for drug absorption, including simple diffusion, paracellular transport and carrier-mediated transport. Absorption via these mechanisms proceeds in parallel, and consequently elucidation of the relative contribution of each mechanism is not easy.

Several methods are available to evaluate intestinal transport and absorption (Table 8.2). For in vitro studies, isolated brush-border membrane vesicles, intestinal tissue-derived cultured cells such as Caco-2 cells, transporter-gene-transfected cultured cells, isolated enterocytes, and isolated intestinal tissues are widely used. These in vitro methods are useful for obtaining a mechanistic understanding of

Table 8.2 Analysis methods of intestinal transporters and absorption

Methods	Characteristics
In vitro	Good for mechanistic analysis
• Membrane vesicles:	Very artificial; easy to control experimental conditions
Brush-border and basolateral membranes from intestinal epithelial and cultured cells	Suitable for evaluation of driving force, affinity, inhibitors
	Limited applicability due to adsorption of some drugs on membrane
• Isolated epithelial cells	Cells are viable and intracellular conditions are well maintained
	No polarity
• Intestinal epithelial cell-derived cultured cells	
Caco-2	Maintained polarity; both influx and efflux can be measured
LS180	Expressional regulation can be evaluated
• Isolated intestinal tissue	Uptake and permeation can be measured in real tissue
Everted intestine	Maintaining viability is difficult
• Transporter-gene-transfected cultured cells	Responsible transporter molecule can be determined
HEK293, MDCK, LLCPK1, HeLa	Once established, easy to study with good reproducibility
	Influx and efflux can be evaluated (MDCK, LLCPK1)
Xenopus oocytes	Usually shows low background activity
	Handling is cumbersome compared with cultured cells
In situ	Intermediate between in vitro and in vivo; good for confirmation of in vitro hypotheses and as a bridge to in vivo study
• Intestinal perfusion	Intestinal permeability can be measured with blood circulation (viable)
	Low throughput
• Intestinal closed loop	Absorption mechanism can be estimated
	Luminal condition can be partly (but not completely) controlled
In vivo	Oral availability and absorption mechanism can be evaluated
• Gene knockout animal	Transporter effect on absorption can be assessed
	Limited availability
	Species difference between murine and human
• Clinical human study	Human absorption is directly evaluated
Effect of genetic polymorphism	Identification of responsible transporter is possible
Effect of interaction with drug and food (beverage)	Responsible transporter can be estimated

membrane transport, including identification of transporter molecules and their functional roles, driving forces for the transport, inhibitor selectivity, and affinity of particular drugs for the transporters. However, these methods do not necessarily throw light on the in vivo contribution of each mechanism. As in situ methods, intestinal perfusion and the intestinal closed loop method are useful for evaluation of apparent permeability and for rough estimating the roles of various transporter molecules and can form a bridge between in vitro and in vivo analyses. Although it is possible to estimate the involvement of certain mechanisms in drug absorption by means of in vitro and in situ intestinal transport studies, precise evaluation of the contributions of transporters to drug absorption in animals may require the use of transporter-gene knockout animals. At present, mice in which *Mdr1a/1b* (Schinkel et al. 1994, 1997), *Bcrp1* (Jonker et al. 2002; Krishnamurthy et al. 2004), *Pept1* (Hu et al. 2008), and some *Oatps* (Lu et al. 2008; Zaher et al. 2008; van de Steeg et al. 2010; Gong et al. 2011) have been knocked out are available. However, species difference is significant, especially in the case of Oatps, and that the correspondence between some human OATPs and murine Oatps remains controversial (Nakakariya et al. 2008). As for clinical studies in humans, transporters involved in drug absorption can be analyzed by evaluating the effects of genetic polymorphisms in the transporter gene on apparent functional activity. Another approach is to observe the alteration in pharmacokinetics of substrate drugs due to drug–drug and/or drug–food (or beverage) interactions. Indeed, findings on the modification of drug absorption by fruit juices and the effects of genetic polymorphisms that alter transporter function have already been described in this chapter. It is important to remember that, although transporter function can be analyzed relatively easily by means of the methods summarized in Table 8.2, it may still be difficult to fully understand the overall outcome of intestinal absorption processes in the complex in vivo environment, where the many variables may include transporter polymorphism, site-specific expression of transporters in the intestinal tract, microenvironmental changes of physiological pH or ion concentrations, variations in intestinal motility and contents, and modification of transporter function by dietary components.

8.6   Conclusion

Analysis of the mechanisms of intestinal drug absorption is extremely challenging, but there is now overwhelming evidence that PEPT1 and OATPs as influx transporters and P-glycoprotein and BCRP as efflux transporters play significant roles in the intestinal absorption of various drugs in clinical use. PEPT1 has broad substrate specificity and its expression is limited to small intestine, so it can potentially be utilized to enhance the absorbed fraction of appropriately designed prodrugs of soluble and poorly absorbable drugs. OATPs appear to be involved in absorption of many clinically used drugs, although the extent of their contribution varies from case to case. OATPs are often sites of drug–drug or drug–food (or beverage) interactions that influence the intestinal absorption of their substrate drugs.

Since P-glycoprotein and BCRP are exsorptive transporters, it is important that drugs should not be substrates of these transporters if they are to be efficiently absorbed. These four transporters seem to be the key players to interpreting alterations in intestinal absorption of drugs due to drug–drug or drug–food interactions. They are likely to be the main focus of future work to improve the bioavailability of new drugs, either by appropriate molecular modification of drug candidates or by modulation of the transporter function.

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Chapter 9
Analysis of Hepatic Transport Proteins

Kyunghee Yang, Kathleen Köck, and Kim L.R. Brouwer

Abstract The liver is the major organ responsible for the elimination of endogenous and exogenous compounds via metabolism and/or excretion. Hepatocytes, the predominant cell type in the liver, are polarized cells with discrete basolateral and apical membranes. In this chapter, localization and function of hepatic transport proteins responsible for hepatobiliary drug disposition in humans are introduced. Hepatic transport proteins on the basolateral membrane mediate influx of compounds from sinusoidal blood into hepatocytes (i.e., NTCP, OATPs, OATs, OCTs) or efflux from hepatocytes back to sinusoidal blood (i.e., MRP3-6, OSTα/β). Canalicular transport proteins such as BSEP, MDR3, P-gp, BCRP, MRP2, and MATE1 are responsible for biliary excretion of compounds. Furthermore, in vitro (i.e., membrane vesicles, transfected cell systems, hepatocytes, isolated perfused liver) and in vivo (i.e., biliary excretion studies, hepatobiliary imaging techniques) model systems and methods that are used to investigate hepatic transport proteins are discussed, and their applications, advantages, and disadvantages are considered.

Abbreviations

^{99m}Tc-HIDA	^{99m}Tc-N(2,6-dimethylphenyl carbamoylmethyl) iminodiacetic acid
ABC	ATP-binding cassette
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUC	Area under the concentration–time curve

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BCRP	Breast cancer resistance protein
bLPM	Basolateral liver plasma membrane
BSEP	Bile salt export pump
BSP	Bromosulfophthalein
CF-1 mice	Mdr1a-deficient mice
cLPM	Canalicular liver plasma membrane
CYP450	Cytochrome P450
DDI	Drug–drug interaction
DHEAS	Dehydroepiandrosterone sulfate
DILI	Drug-induced liver injury
DJS	Dubin–Johnson syndrome
E1S	Estrone 3-sulfate
$E_2 17G$	Estradiol-17β-D-glucuronide
EHBR	Eisai-hyperbilirubinemic Sprague–Dawley rats
FXR	Farnesoid X receptor
Gd-BOPTA	Gadobenate dimeglumine
Gd-EOB-DPTA	Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid
HBSS	Hanks-balanced salt solution
HCC	Hepatocellular carcinoma
HEK cells	Human embryonic kidney cells
HMG-CoA	3-Hydroxy-3-methyl-glutaryl coenzyme A
HPLC	High-performance liquid chromatography
IPL	Isolated perfused liver
iPS cells	Inducible pluripotent stem cells
K_m	Michaelis–Menten constant
LC/MS	Liquid chromatography mass spectrometry
LLC-PK1 cells	Porcine kidney epithelial cells
MATE	Multidrug and toxin extrusion
MDCK cells	Madin–Darby canine kidney cells
MDR	Multidrug resistance
MRI	Magnetic-resonance imaging
MRP	Multidrug resistance-associated protein
NCE	New chemical entity
NTCP	Sodium taurocholate cotransporting polypeptide (human)
Ntcp	Sodium taurocholate cotransporting polypeptide (other species than human)
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OST	Organic solute transporter
PBC	Primary biliary cirrhosis
PET	Positron emission tomography
PFIC2	Progressive familial intrahepatic cholestasis type 2
PFIC3	Progressive familial intrahepatic cholestasis type 3
Pgp	P-glycoprotein
RNA	Ribonucleic acid
RNAi	RNA interference

SCH	Sandwich-cultured hepatocytes
SD rat	Sprague–Dawley rat
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLC	Solute carrier
SLCO	Solute carrier organic anion
SPECT	Single-photon emission computed tomography
TR⁻ rat	Mrp2-deficient Wistar rat
UGT	Uridine diphosphate glucuronosyl transferase
V_{max}	Maximal transport velocity

9.1 Introduction

The liver is one of the major organs responsible for the metabolism and excretion of endogenous and exogenous compounds. Hepatocytes contain transport proteins that facilitate the influx of many compounds from sinusoidal blood. Once inside the hepatocyte, compounds may be biotransformed by metabolizing enzymes and/or excreted. Hepatocytes are polarized cells with distinct apical and basolateral domains (Fig. 9.1); transport proteins on the apical membrane are responsible for excretion of compounds into the bile canaliculus, whereas basolateral transport proteins mediate influx into hepatocytes and efflux back to sinusoidal blood. Biliary excretion of drugs and metabolites is an active process that requires energy, usually in the form of adenosine triphosphate (ATP); the multidrug and toxin extrusion (MATE) transporter is one exception that does not require ATP for drug transport into the bile canaliculus. ATP-dependent transport proteins also are located on the basolateral membrane and are able to efflux drugs and metabolites from hepatocytes into sinusoidal blood.
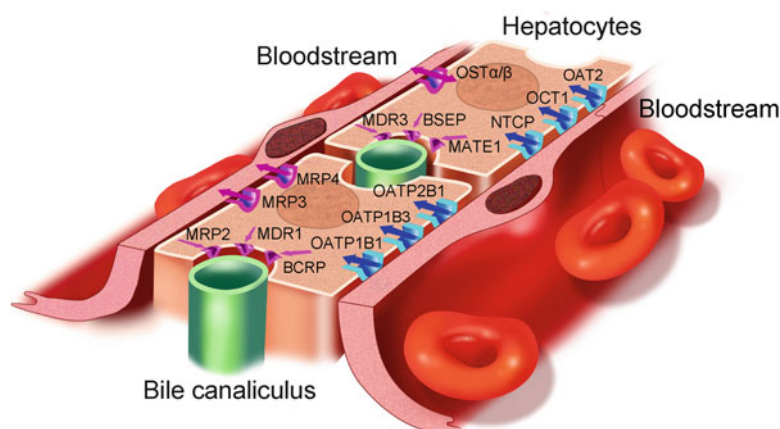
Fig. 9.1 In vivo architecture of polarized hepatocytes with distinct apical and basolateral domains facing respectively the bile canaliculus and bloodstream (Köck and Brouwer 2012)

In this chapter, the hepatic transport proteins important for drug disposition in humans are introduced based on their localization and function. The nomenclature and substrate specificity of hepatic transport proteins are summarized in Tables 9.1 and 9.2. In vitro and in vivo model systems and tools used to answer questions related to hepatic transport proteins are discussed, and more sophisticated approaches under development are introduced as future directions.

9.2 Hepatic Transport Proteins

9.2.1 Hepatic Influx Transport Proteins

The solute carrier (*SLC*) and solute carrier organic anion (*SLCO*) gene families are two representative families of transport proteins that mediate the hepatic influx of xenobiotics across the basolateral hepatocyte membrane (Fig. 9.1). The list of hepatic influx transporters and example substrates is included in Table 9.1.

NTCP *(SLC10A1)*. The sodium taurocholate cotransporting polypeptide (NTCP) is expressed exclusively in hepatocytes and plays a major role in the Na⁺-dependent hepatic influx of conjugated bile acids such as glycocholate and taurocholate (Stieger 2011). NTCP also is capable of transporting bromosulfophthalein (BSP), estrone 3-sulfate (E1S), and drugs such as pitavastatin and rosuvastatin, although the contribution of NTCP to the uptake of drug substrates into hepatocytes in vivo remains to be determined (Table 9.1). In rats, Ntcp also may be capable of transporting the thyroid hormones and the mushroom toxin α-amanitin (Gundala et al. 2004).

OATPs *(SLCO*, **previously** *SLC21A)*. The family of organic anion transporting polypeptides (OATPs) plays an essential role in sodium-independent influx of endogenous and exogenous compounds into hepatocytes and may be the rate-limiting step in the hepatobiliary clearance of some drugs, such as statins. OATPs exhibit broad and overlapping substrate specificity; the spectrum of OATP substrates includes organic anions, bulky organic cations (previously referred to as type II cations), and neutral steroids. Some OATP isoforms have been hypothesized to function as glutathione or bicarbonate antiporters (Briz et al. 2006; Li et al. 2000; Satlin et al. 1997), employing the high intracellular glutathione or bicarbonate concentrations as a driving force for hepatic influx of substrates with high efficiency.

Eleven human OATP isoforms have been identified so far; OATP1B1, OATP1B3, and OATP2B1 are the major human OATPs that play an important role in the hepatic influx of drugs across the basolateral membrane domain. OATP1B1 and 1B3 are liver-specific, whereas OATP2B1 is widely expressed (e.g., in intestine, brain, and kidney). OATP1B1 exhibits the largest diversity of substrates including bilirubin, BSP, bile salts, many antibiotics, angiotensin receptor antagonists, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), and anti-cancer drugs (Table 9.1). OATP1B1 is the major human liver transport protein that is involved in sodium-independent bile salt and bilirubin influx. Inhibition of

Table 9.1 Human hepatic influx transport proteins

Protein/trivial names	Gene	Substrate/references
NTCP	*SLC10A1*	Cholate; E1S; glycoursodeoxycholate; pitavastatin; rosuvastatin; taurocholate; tauroursodeoxycholate [U], BSP; glycocholate; taurochenodeoxycholate (Ho et al. 2006)
OATP1B1 OATP-C LST-1 OATP2	*SLCO1B1* (*SLC21A6*)	Atorvastatin; bilirubin; bisglucuronosyl bilirubin; bosentan; BSP; cholate; CGamF; DHEAS; E₂17G; E1S; fluo-3; fluvastatin; glycoursodeoxycholate; methotrexate; monoglucuronosyl bilirubin; olmesartan; pitavastatin; pravastatin; rifampicin; rosuvastatin; tauroursodeoxycholate; valsartan [U], benzylpenicillin (Tamai et al. 2000), BQ-123; DPDPE; LTC₄; PGE₂; T₃; T₄ (Kullak-Ublick et al. 2001), caspofungin (Sandhu et al. 2005), cerivastatin (Shitara et al. 2003b; Kameyama et al. 2005), SN-38 (Nozawa et al. 2005), microcystin-LR (Fischer et al. 2005), phalloidin (Meier-Abt et al. 2004), repaglinide (Kajosaari et al. 2005; Niemi et al. 2005), simvastatin (Kameyama et al. 2005), troglitazone sulfate (Nozawa et al. 2004)
OATP1B3 OATP-8 LST-2	*SLCO1B3* (*SLC21A8*)	Amantinin; bilirubin; bosentan; BSP; CCK-8; CGamF; digoxin; E₂17G; E1S; fexofenadine; fluo-3; fluvastatin; glycoursodeoxycholate; methotrexate; monoglucuronosyl bilirubin; olmesartan; paclitaxel; pitavastatin; rifampicin; rosuvastatin; taurocholate; tauroursodeoxycholate; telmisartan; telmisartan glucuronide; T₃; valsartan [U], BQ-123; deltorphin II; DHEAS; DPDPE; LTC₄; ouabain; T₄ (Kullak-Ublick et al. 2001), CCK-8 (Ismair et al. 2001), microcystin-LR (Fischer et al. 2005), phalloidin (Meier-Abt et al. 2004)
OATP2B1 OATP-B	*SLCO2B1* (*SLC21A9*)	Atorvastatin; bosentan; BSP; E1S; fexofenadine; fluvastatin; glyburide; pitavastatin; pravastatin; rosuvastatin; taurocholate; telmisartan glucuronide [U], DHEAS (Kullak-Ublick et al. 2001), pregnenolone sulfate (Grube et al. 2006)
OAT2	*SLC22A7*	2′-Deoxyguanosine; 5-fluorouracil; bumetanide; cyclic GMP; erythromycin; paclitaxel; PGE₂; PGF₂α; tetracycline; theophylline; zidovudine [U], allopurinol; L-ascorbic acid; DHEAS; E1S; glutarate (Kobayashi et al. 2005), methotrexate (Sun et al. 2001), ranitidine (Tahara et al. 2005)
OAT7	*SLC22A9*	DHEAS; E1S (Shin et al. 2007)
OCT1	*SLC22A1*	DASPMI; acyclovir; furamidine; ganciclovir; metformin; *N*-methylpyridinium; oxaliplatin; pentamidine; PGE₂; PGF₂α; ranitidine; tetraethylammonium; YM155 [U], ganciclovir (Takeda et al. 2002), azidoprocainamide methoiodide; *n*-methylquinidine; *n*-methylquinine; tributylmethylammonium (van Montfoort et al. 2001), choline (Grundemann et al. 1999), imatinib (Thomas et al. 2004), MPP⁺; *N*-methylnicotinamide (Gorboulev et al. 1997; Zhang et al. 1999), famotidine (Bourdet et al. 2005)

Table 9.1 (continued)

Protein/ trivial names	Gene	Substrate/references
OCT3 EMT	*SLC22A3*	Epinephrine; etilefrine; histamine; metformin; *N*-methylpyridinium; norepinephrine [U], adrenaline; noradrenaline; tyramine (Grundemann et al. 1998), agmatine; MPP^+; tetraethylammonium (Hayer-Zillgen et al. 2002; Grundemann et al. 2003; Wu et al. 2000), atropine (Muller et al. 2005), histamine (Grundemann et al. 1999)
OCTN2	*SLC22A5*	Acetyl-L-carnitine; D-carnitine; ipratropium; L-carnitine; quinidine; verapamil [U], cephaloridine; tetraethylammonium; choline; purilamine (Hagenbuch 2010)

[U] From UCSF-FDA TransPortal webpage (http://bts.ucsf.edu/fdatransportal/); the information about transporter substrates is listed by transporters or compounds under the "Transporter Data Index"

BSP bromosulphthalein, *BQ-123* cyclo(D-Trp-D-Asp-L-Pro-D-Val-L-Leu), *CCK-8* cholecystokinin 8, *CGamF* cholyl-glycyl amido-fluorescein, *DASPMI* 4-(4-dimethylamino)styryl-N-methylpyridinium, *DHEAS* dehydroepiandrosterone, *DPDPE* [D-penicillamine2,5]-enkephalin, *E1S* estrone 3-sulfate, *$E_2$17G* estradiol-17β-D-glucuronide, GMP guanosine monophosphate, *LTC_4* leukotriene C_4, *MPP^+* 1-methyl-4-phenylpyridinium, *PGE_2* prostaglandin E_2, *$PGF_{2\alpha}$* prostaglandin F_2, *T_3* triiodothyronine, *T_4* thyroxine

OATP1B1-mediated influx by drugs has been correlated with the incidence of hyperbilirubinemia (Campbell et al. 2004). OATP1B1 is important in the hepatic influx of statins; genetic polymorphisms in *SLCO1B1* have been shown to be associated with increased systemic exposure of statins and increased risk of statin-induced myopathy (Link et al. 2008). While OATP1B3 has overlapping substrate specificity with OATP1B1, distinct substrate specificity has been reported; repaglinide and troglitazone sulfate are more selective substrates for OATP1B1, while digoxin, cholecystokinin-8, and paclitaxel show selectivity for OATP1B3 (Table 9.1). Bile acids are known to be transported by OATP1A2, OATP1B1, and OATP1B3, but OATP2B1-mediated bile acid transport has not been investigated.

OATs *(SLC22A)*. Organic anion transporters (OATs) mediate transport of small anionic compounds in exchange for dicarboxylate ions. Among six human OATs that have been functionally characterized, OAT2 *(SLC22A7)* is expressed in the basolateral membrane of hepatocytes and renal proximal tubule cells, while OAT7 *(SLC22A9)* is expressed exclusively in the liver (Hagenbuch 2010). The known substrates for human OAT2 include prostaglandins, dehydroepiandrosterone sulfate (DHEAS), E1S, and anticancer drugs such as 5-fluorouracil and methotrexate (Table 9.1). OAT7, a recently characterized OAT, has been shown to transport sulfated hormones such as E1S and DHEAS when expressed in *X. laevis* oocytes (Shin et al. 2007). Interestingly, typical OAT substrates such as *para*-aminohippurate, α-ketoglutarate, prostaglandins, cyclic nucleotides, and salicylic acid were not transported by OAT7, while OAT7 could transport the short-chain fatty acid butyrate (Shin et al. 2007).

OCTs *(SLC22A)*. Organic cation transporters (OCTs) are electrogenic uniporters that primarily mediate the transport of small cations (previously referred to as type I cations) in a sodium-independent fashion. Human OCT1 *(SLC22A1)* is expressed exclusively at the basolateral membrane of the hepatocytes (Soroka et al. 2010). OCT1 is known to transport antiviral drugs such as acyclovir and ganciclovir, as well as the H_2-receptor antagonists famotidine and ranitidine (Table 9.1). OCT3 *(SLC22A3)* and OCTN2 *(SLC22A5)* also are expressed in the liver, but expression levels are relatively low compared to OCT1 and the functional role of these proteins in hepatic drug transport remains to be elucidated (Hagenbuch 2010).

OSTα/β *(SLC51A/51B)*. Organic solute transporter (OST) α/β is a heteromeric transporter that is expressed widely in the liver, small intestine, kidney, testis, and adrenal gland. In hepatocytes, OSTα/β is expressed in the basolateral membrane and is able to transport bile acids, E1S, and DHEAS. Since OSTα/β mediates substrate transport by facilitated diffusion, OSTα/β-mediated transport is bidirectional depending on the substrate's electrochemical gradient (Soroka et al. 2010). Gene expression levels of *SLC51A* and *SLC51B* are positively regulated by bile acids through farnesoid X receptor (FXR), and it has been shown that hepatic OSTα/β is up-regulated in patients with chronic cholestatic disease such as primary biliary cirrhosis (PBC) (Boyer et al. 2006).

9.2.2 Hepatic Canalicular Efflux Transport Proteins

Biliary excretion is an important elimination pathway for many endogenous and exogenous substances. Canalicular transport proteins responsible for biliary excretion of substances primarily belong to the ATP-binding cassette (ABC) family of proteins that mediate ATP-dependent transport of solutes.

P-glycoprotein *(MDR1, ABCB1)*. P-glycoprotein (P-gp) was first identified in multidrug-resistant (MDR) tumor cells (Juliano and Ling 1976) and is the most well-characterized ABC transport protein. P-gp is widely distributed in liver, intestine, kidney, and brain. In hepatocytes, P-gp is expressed in the canalicular membrane and is responsible for biliary excretion of bulky hydrophobic and cationic substrates including many chemotherapeutic agents (e.g., daunorubicin, doxorubicin, etoposide, paclitaxel, vinblastine, vincristine), cardiac glycosides (e.g., digoxin), rhodamine 123, cyclosporine A, and protease inhibitors (e.g., amprenavir, indinavir, nelfinavir, ritonavir, saquinavir). Substrate specificity of P-gp largely overlaps with that of CYP3A4, resulting in synergistic defense mechanisms against xenobiotics. The *ABCB1* gene is highly polymorphic, and hepatic expression levels of P-gp are highly variable between different individuals.

MDR3 *(ABCB4)*. MDR3, a phospholipid flippase, is involved in the biliary secretion of phospholipids and cholephilic compounds that form micelles with bile acids. Biliary excretion of phospholipids protects the lumen of the bile canaliculus

by solubilizing toxic bile acids (Oude Elferink and Paulusma 2007). A deficiency in the *ABCB4* gene leads to progressive familial intrahepatic cholestasis type 3 (PFIC3), a disease that is characterized by increased γ-glutamyltranspeptidase levels, ductular proliferation, and inflammatory infiltrate that can progress to biliary cirrhosis. Individuals with decreased MDR3 activity are susceptible to cholesterol gallstone formation, which is known as low-phospholipid-associated cholelithiasis (Rosmorduc et al. 2003). Inhibition of MDR3-mediated biliary phospholipid excretion is one proposed mechanism of hepatotoxicity induced by drugs such as itraconazole.

BSEP *(ABCB11)*. The bile salt export pump (BSEP) is the major transport protein that mediates the biliary excretion of conjugated and unconjugated bile acids. Some drugs such as pravastatin may be substrates for BSEP based on membrane vesicle studies (Hirano et al. 2005); however, the relative role of BSEP vs. other canalicular transport proteins in the biliary excretion of pravastatin in hepatocytes or the intact liver remains to be determined (Kullak-Ublick et al. 2000). PFIC2 patients do not express BSEP protein due to a genetic polymorphism in the *ABCB11* gene; this leads to hepatocellular injury and necrosis caused by increased intracellular concentrations of detergent-like bile acids (Kullak-Ublick et al. 2004). Inhibition of BSEP-mediated bile acid transport is purported to be one mechanism of drug-induced liver injury (DILI) associated with hepatotoxic drugs such as troglitazone, bosentan, and cyclosporine.

MRP2 *(ABCC2)*. Multidrug resistance-associated protein (MRP) 2 plays an important role in the biliary excretion of organic anions, including bilirubin-diglucuronide, glutathione conjugates, sulfated bile acids, and divalent bile acid conjugates, as well as numerous drugs such as sulfopyrazone, indomethacin, penicillin, vinblastine, methotrexate, and telmisartan (Table 9.2). The absence of functional MRP2 due to genetic mutations in *ABCC2* results in Dubin–Johnson syndrome (DJS), which is characterized by decreased biliary excretion of bilirubin conjugates and hyperbilirubinemia (Tsujii et al. 1999).

BCRP *(ABCG2)*. Breast cancer resistance protein (BCRP) is highly expressed in the canalicular membrane of hepatocytes as well as in the intestine, breast, and placenta. BCRP is a half-transport protein that forms a functional homodimer and is responsible for transport of glucuronide and sulfate conjugates (e.g., E1S, estradiol-17β-D-glucuronide ($E_2$17G), SN38-glucuronide), anticancer drugs (e.g., irinotecan, SN-38, methotrexate, daunorubicin, doxorubicin), and some statins (e.g., pitavastatin, rosuvastatin) (Table 9.2).

MATE1 *(SLC47A1)*. Human MATE1 is expressed predominantly in the canalicular membrane of hepatocytes and the luminal membrane of renal tubular cells. MATE1 has been shown to transport organic cations across the membrane in a bidirectional manner dependent on the proton gradient. Substrate specificity of MATE1 primarily overlaps with the OCTs; MATE1 substrates include acyclovir, *N*-methylpyridinium, and tetraethylammonium (Table 9.2).

Table 9.2 Human hepatic efflux transporter proteins

Protein/trivial names	Gene	Substrates/references
Hepatic canalicular efflux transport proteins		
MDR1 P-gp	*ABCB1*	Berberine; biotin; colchicine; dexamethasone; digoxin; doxorubicin; etoposide; fexofenadine; indinavir; irinotecan; loperamide; nicardipine; paclitaxel; rhodamine 123; ritonavir; saquinavir; topotecan; valinomycin; verapamil; vinblastine; vincristine [U], amprenavir; nelfinavir (Kim et al. 1998; Polli et al. 1999), aldosterone; corticosterone (Ueda et al. 1992), cyclosporin A; mitoxanthrone (Marie et al. 1992), debrisoquine; erythromycin; lovastatin; terfenadine (Cvetkovic et al. 1999), quinidine (Fromm et al. 1999), levofloxacin; grepafloxacin (Yamaguchi et al. 2000), losartan (Soldner et al. 1999), tacrolimus (Floren et al. 1997), talinolol (Spahn-Langguth et al. 1998), norverapamil (Pauli-Magnus et al. 2000)
MDR3 Phospholipid flippase MDR2/3	*ABCB4*	Digoxin; paclitaxel; verapamil; vinblastine [U], phospholipids (Smith et al. 2000)
BSEP Sister P-gp	*ABCB11*	Glycochenodeoxycholate; glycocholate; pravastatin; taurochenodeoxycholate; taurocholate [U]
MRP2 cMOAT cMRP	*ABCC2*	DHEAS; $E_2$17G; etoposide; irinotecan; methotrexate; olmesartan; *para*-aminohippurate; SN-38; SN-38 glucuronide; valsartan; vinblastine [U], LTC_4; bisglucuronosyl bilirubin; monoglucuronosyl bilirubin; ochratoxin A; cholecystokinin peptide; E1S; cholyl-L-lysyl-fluorescein (Keppler 2011) acetaminophen glucuronide; carboxydichlorofluorescein (Xiong et al. 2000), camptothecin; doxorubicin (Koike et al. 1997), cerivastatin (Matsushima et al. 2005), cisplatin; vincristine (Kawabe et al. 1999), glibenclamide; indomethacin; rifampin (Payen et al. 2000), pravastatin (Sasaki et al. 2002)
BCRP MXR ABCP	*ABCG2*	4-Methylumbelliferone sulfate; daunorubicin; doxorubicin; $E_2$17G; E1S; hematoporphyrin; imatinib; methotrexate; mitoxantrone; pitavastatin; rosuvastatin, SN-38; SN-38 glucuronide; sulfasalazine; topotecan [U], mitoxanthrone glucuronide and sulfate conjugates (Kawabata et al. 2001), irinotecan (Maliepaard et al. 1999), prazosin; rhodamine 123 (Ozvegy et al. 2001), testosterone; tamoxifen; estradiol (Janvilisri et al. 2003)
MATE1	*SLC47A1*	Acyclovir; cimetidine; E1S; ganciclovir; guanidine; metformin; *N*-methylpyridinium; paraquat; procainamide; tetraethylammonium; topotecan [U]

<div align="right">(continued)</div>

Table 9.2 (continued)

Protein/trivial names	Gene	Substrates/references
Hepatic basolateral efflux transport proteins		
MRP3 MOAT-D MLP1 cMOAT1	*ABCC3*	$E_2$17G; ethinylestradiol-glucuronide; fexofenadine; folic acid; glycocholate; hyocholate-glucuronide; hyodeoxycholate-glucuronide; leucovorin; LTC_4; methotrexate; *S*-(2,4-dinitrophenyl)-glutathione; taurocholate [U]; bisglucuronosyl bilirubin; monoglucuronosyl bilirubin; DHEAS (Keppler 2011), acetaminophen glucuronide (Tsujii et al. 1999), monovalent and sulfated bile salts (Hirohashi et al. 1999), etoposide (Stieger et al. 2000)
MRP4 MOAT-B	*ABCC4*	Adefovir; chenodeoxycholylglycine; chenodeoxycholyl-taurine; cholate; taurocholate; cyclic AMP; cyclic GMP; DHEAS; deoxycholylglycine; $E_2$17G; folic acid; methotrexate; olmesartan; *para*-aminohippurate; PGE1; PGE2; tenofovir; topotecan [U], cholylglycine; ursodeoxycholylglycine; ursodeoxy-cholyltaurine; urate; ADP; PMEA; fluo-cAMP (Keppler 2011), azidothymidine (Schuetz et al. 1999)

[U] From UCSF-FDA TransPortal webpage (http://bts.ucsf.edu/fdatransportal/); the information about transporter substrates is listed by transporters or compounds under the "Transporter Data Index"

BQ-123 cyclo(D-Trp-D-Asp-L-Pro-D-Val-L-Leu), *CCK-8* cholecystokinin 8, *DHEAS* dehydroepiandosterone, *E1S* estrone 3-sulfate, *E₂17G* estradiol-17β-D-glucuronide, *LTC₄* leukotriene C_4, *LTD₄* leukotriene D_4, *PGE₁* prostaglandin E_1, *PGE₂* prostaglandin E_2, *PMEA* 9-(2-phosphonomethoxyethyl) adenine

9.2.3   Hepatic Basolateral Efflux Transport Proteins

Xenobiotics in the liver also may be excreted across the basolateral membrane into sinusoidal blood. MRP1, 3, 4, 5, and 6 are involved in cellular transport of both hydrophobic uncharged molecules and hydrophilic anionic compounds. OATPs also may function as basolateral efflux transport proteins under certain conditions, although the in vivo role of OATPs in basolateral efflux remains to be elucidated (Li et al. 2000).

MRP3 *(ABCC3)*. MRP3 was first localized in human and rat hepatocytes and is also expressed widely in kidney, pancreas, enterocytes, cholangiocytes, and the gallbladder (Keppler 2011). The expression level of MRP3 in hepatocytes is low in normal liver, but markedly increased in patients with DJS who lack functional MRP2, and in patients with cholestatic liver disease, consistent with the important compensatory role of MRP3 when the function of biliary transport proteins is impaired (Konig et al. 1999; Wagner et al. 2009; Hirohashi et al. 1999). MRP3 is responsible for the basolateral efflux of glutathione and glucuronide conjugates (e.g., acetaminophen glucuronide), methotrexate, and $E_2$17G.

MRP4 *(ABCC4)*. MRP4 is localized in many different tissues including liver, kidney, brain, and prostate (Keppler 2011). The expression level of MPR4 in normal hepatocytes is low, but is markedly induced under cholestatic conditions. MRP4 is responsible for the basolateral efflux of bile acids when the normal vectorial transport of bile acids from the hepatocyte into bile is compromised (Wang et al. 2011). MRP4-mediated bile acid transport requires glutathione, because bile acids and glutathione are co-transported by MRP4 (Rius et al. 2003). MRP4 also transports cyclic nucleotides (e.g., cAMP and cGMP), nucleoside analogs (e.g., zidovudine, lamivudine, and stavudine), purine analogs (e.g., 6-mercaptopurine and 6-thioguanine), and non-nucleotide substrates such as methotrexate (Sampath et al. 2002).

Other MRPs. MRP1 (*ABCC1*) is expressed in several tissues including liver, lung, testis, kidney, skeletal and cardiac muscle, placenta, and macrophages (Keppler 2011). MRP1 is responsible for the efflux of various organic anions, such as glucuronide, glutathione, and sulfate conjugates of drugs. MRP5 (*ABCC5*) transports cyclic nucleotides (e.g., cAMP and cGMP) and purine analogs (e.g., 6-mercaptopurine and 6-thioguanine). The expression levels of MRP1 and MRP5 in healthy liver are relatively low, but protein levels of hepatic MRP1 and MRP5 were significantly increased in patients with PBC (Barnes et al. 2007). Protein expression of hepatic MRP5 also was increased in acetaminophen-induced liver failure, suggesting a protective role for this protein in hepatic injury (Barnes et al. 2007). MRP6 (*ABCC6*) is localized in the basolateral membrane of hepatocytes and transports glutathione conjugates and the endothelin receptor antagonist BQ-123. Expression of MRP6 was not altered in patients with PBC or acetaminophen-induced liver failure, and the functional roles of MRP6 remain to be explored (Barnes et al. 2007).

9.3   In Vitro Models and Methods to Study Hepatobiliary Drug Transport

9.3.1   Membrane Vesicle System

With the development of membrane vesicle assays, it became possible to perform functional studies to identify and characterize distinct efflux transport systems. Historically, vesicle transport assays were performed using membranes isolated from hepatic tissue from the relevant species. Functional studies of hepatic efflux transporters in either canalicular liver plasma membrane (cLPM) or basolateral liver plasma membrane (bLPM) vesicles were enabled by the development of a method to separate these two membrane leaflets in the early 1980s (Meier et al. 1984; Blitzer and Donovan 1984). This assay system was used to identify and characterize bile acid and bilirubin glucuronide transport across the canalicular membrane and led to the discovery of BSEP and MRP2 (Jedlitschky et al. 1997; Gerloff et al. 1999). The isolation of high-purity apical and basolateral membranes from tissue is labor-intensive and technically challenging. Since inside-out and right-side-out vesicles

coexist, ATP-dependent basolateral efflux data generated with bLPMs may be confounded by influx transporters. Naturally, these membranes contain multiple transport proteins; therefore, it is impossible to identify specific substrates for ABC efflux proteins.

Due to evolving molecular biology techniques and identification of individual transport proteins, this tissue-based assay system has been replaced by vector-transfected and virus-infected cell lines expressing a single ABC-transporter. In the early 1990s, baculovirus-infected insect cells (*Spodoptera frugiperda*, Sf9) were used widely to generate membrane vesicles containing the transport protein of interest because this system allowed high expression of transport proteins (Germann et al. 1990). Disadvantages of these membrane vesicles are the different glycosylation pattern and a lower cholesterol content in Sf9 cells compared to mammalian cell lines (Pal et al. 2007), which may affect the localization and function of transporters. For example, MRP2-mediated transport and ATPase activity were altered by membrane cholesterol content (Pal et al. 2007). Therefore, either transiently or stably transfected mammalian cell lines from human embryonic kidney (HEK) 293, Madin–Darby canine kidney (MDCK) II, or porcine kidney epithelial (LLC-PK1) cells are now used more frequently for preparation of membrane vesicles for transporter studies. These systems are suitable for high-throughput screening of substrates and inhibitors for a single transport protein.

The most commonly used membrane system for efflux transporters is the vesicular transport system that detects direct translocation of substrates into inside-out vesicles (Fig. 9.2). Substrates taken up into inside-out vesicles are separated from the incubation solution using rapid filtration and quantified by high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC/MS), scintillation counting, or fluorescence detection. ATP-dependent transport is calculated by subtracting the transport of substrate in the presence of AMP from that in the presence of ATP; endogenous transporter-mediated transport is excluded by subtracting ATP-dependent transport of substrate in control vesicles from that in transporter-expressing vesicles. This method detects direct transport of substrate, and kinetic parameters such as the Michaelis–Menten constant (K_m) and the maximal transport velocity (V_{max}) can be calculated. This method is ideal for the detection of drug–drug interactions (DDIs) or drug–endogenous compound interactions using a probe substrate. However, it is difficult to detect the transport of highly permeable compounds due to passive diffusion out of the membrane vesicles.

The ATPase method, which detects the hydrolysis of ATP in the presence of an interacting compound, is more suitable for determining the transport of highly permeable compounds. The ATPase method is based on the principle that ABC transporters utilize the chemical energy of ATP cleavage to mediate the transport of substrates across membranes. The inorganic phosphate produced during this process is directly proportional to the activity of the transporter and can, for example, be monitored by colorimetric detection. This method is most commonly used for high-throughput screening for P-gp and BCRP, although it also is available commercially for other ABC efflux transporters. However, ATPase systems are indirect
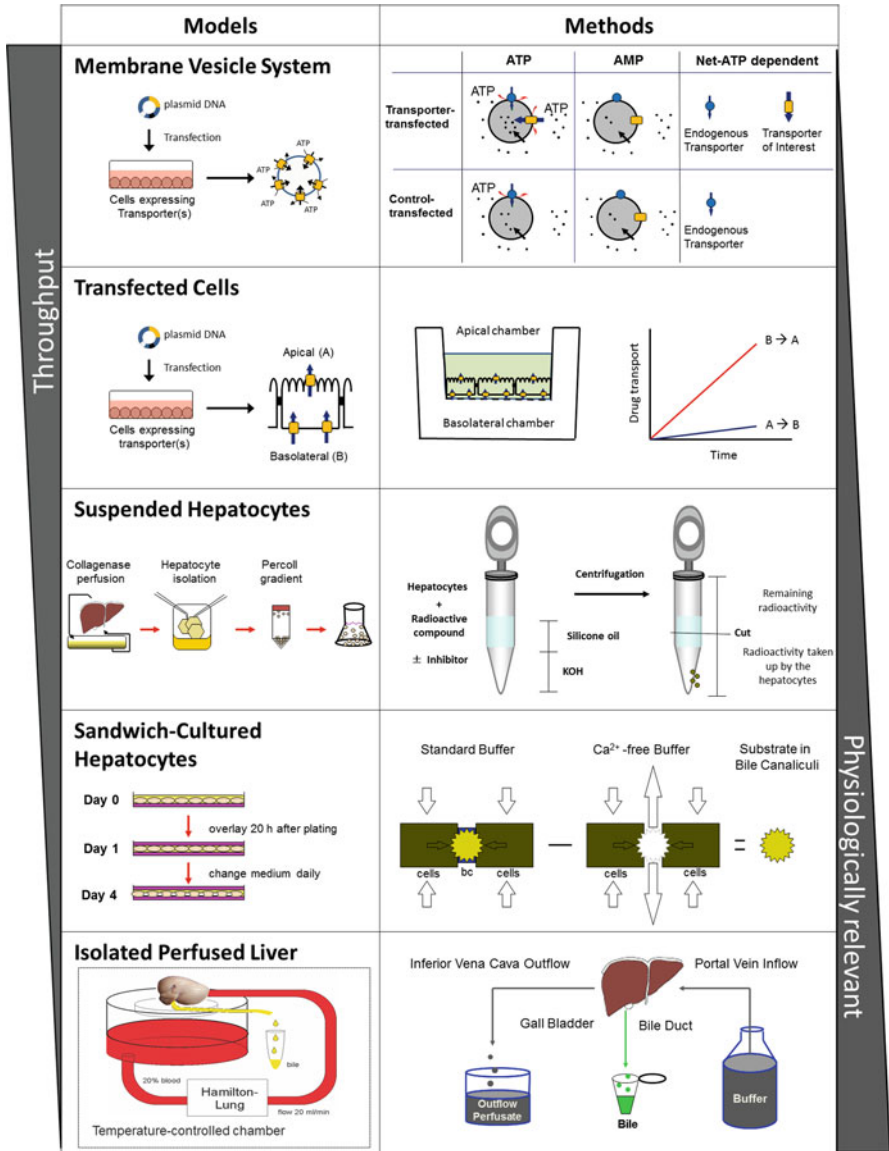
Fig. 9.2 In vitro models and the related methods to study hepatobiliary drug transport

measures of transport, and are not always suitable for distinguishing between potential substrates, inhibitors or modulators. Major applications of the membrane vesicle systems as well as their advantages and disadvantages when used to study hepatobiliary drug transport are summarized in Table 9.3.

Table 9.3 Summary of advantages and disadvantages of in vitro models used to study hepatobiliary drug transport

In vitro model	Major applications	Advantages	Disadvantages
Membrane vesicle system			
Plasma membrane vesicles from tissue	– Determine the transport of compounds at particular membrane domain	– All relevant transporters expressed – No interference from metabolism	– Isolation of high-purity membranes is labor-intensive and technically challenging – Difficult to determine the contribution of a single transporter – Activity of efflux transporters may be confounded by influx transporters
Membrane vesicles prepared from nonmammalian cells (Sf9)	– Functional characterization of new transporters for which a stable cell line has not been developed	– Low interference by background activity of endogenous transporters	– Lower levels of glycosylation or phosphorylation of target protein than in mammalian cell lines – Lower cholesterol content in lipid bilayer compared to mammalian cell lines
Membrane vesicles prepared from transfected mammalian cells	– High-throughput screening for substrates and inhibitors of a single transporter	– High-throughput	– Interference with background activity of endogenous transporters – Difficult to determine the transport of highly diffusable compounds – Difficult to assess the relative contribution of multiple transport proteins
Transfected cells			
Nonpolarized cells	– High-throughput screening for substrate and inhibitors	– High-throughput cell-based model	– Not optimal for study of efflux transport proteins – Species or cell type differences in protein trafficking or localization may exist
Polarized cells	– Vectorial transport across whole cells can be investigated	– Polarized phenotype mimics hepatocyte polarity – Multiple transfections can be conducted	– Difficult to standardize relative expression levels – Difficult to determine the relative contribution of a specific transport protein – Species or cell type differences in protein trafficking or localization may exist

Hepatocytes

		Advantages	Disadvantages
Suspended hepatocytes	– Investigate kinetics and mechanisms of hepatic influx	– Metabolic enzymes are intact – Applicable to hepatocytes from species of interest, including humans	– Only used for short-time influx studies because hepatocyte viability decreases over time – Canalicular (apical) transport proteins may be internalized – Not suitable for study of canalicular efflux transport
Sandwich-cultured hepatocytes	– Mechanistic studies on hepatobiliary disposition, drug interactions, and transporter regulation	– Sandwich culture allows for optimal transporter expression – Most hepatic transport proteins and drug-metabolizing enzymes are expressed and functional	– Static model with respect to blood flow – Inherent variability among donors in protein expression, function, and canalicular network formation

Isolated perfused liver

		Advantages	Disadvantages
Single-pass	– Study mechanisms of hepatic influx, metabolism, biliary excretion, and basolateral efflux	– Intact organ physiology (retains hepatic architecture and bile flow) – Rodents can be pretreated in vivo prior to liver isolation to examine effects of inducers or inhibitors on hepatobiliary clearance – Direct measurement of steady-state extraction ratio is possible	– Low throughput – Labor- and animal-intensive – Limited to ~3 h after liver isolation – Most amendable to rodents due to technical difficulties in perfusing livers from larger species
Recirculating	– Study mechanisms of hepatic influx, metabolism, and biliary excretion	– Intact organ physiology (retains hepatic architecture and bile flow) – Rodents can be pretreated in vivo prior to liver isolation to examine effects of inducers or inhibitors on hepatobiliary clearance – Metabolites accumulate in perfusate allowing mass-balance determination of metabolite formation and kinetic evaluation of hepatic influx of metabolites	– Low throughput – Labor- and animal-intensive – Limited to ~3 h after liver isolation – Most amendable to rodents due to technical difficulties in perfusing livers from larger species – Potential drug–metabolite interaction

9.3.2 Transfected Cells

Bacterial, insect, and mammalian cells have been transfected with vector constructs allowing over-expression of transport proteins for identification of transport processes. The transfected cell model is illustrated in Fig. 9.2. Nonpolarized cells such as Sf9 and HEK293 cells have been used for over-expression of a single transport protein, while polarized cells have been employed for the over-expression of one or more basolateral proteins in concert with apical protein(s). Transfected cell models can be used for high-throughput screening of substrates and inhibitors of a specific over-expressed transport protein. The major limitation of this model is that generally it is not suitable for the study of efflux transporters. Hypothetically, in transfected cells expressing a single efflux protein, transported substrates should demonstrate lower cellular accumulation, and inhibitors should increase drug accumulation compared to the parental cell line. However, substrates of drug efflux transporters are usually organic anions that do not easily penetrate into the cell in the absence of influx transport proteins. To overcome this limitation, polarized mammalian cells (e.g., MDCKII, LLC-PK1) have been used for transfection of one or more influx and/or efflux transport proteins. Depending on the transporter, the protein will be routed to the apical or basolateral membrane in polarized cells; expression of influx transporters allows import of the substrates transported by the ABC efflux transporters. Furthermore, the combined expression of influx and efflux proteins enabled the analysis of vectorial transport, which is a key step in hepatobiliary elimination (Sasaki et al. 2002; Cui et al. 2001). These double-transfected polarized cell lines were first developed by Cui et al. in the early 2000s (Cui et al. 2001) and are now valuable tools to study transcellular transport. Using MDCKII cells expressing both Oatp1b2 and Mrp2, Sasaki et al. demonstrated a good correlation between the clearance values obtained from in vitro transcellular transport and in vivo biliary clearance (Sasaki et al. 2004). Triple and quadruple transfected cell lines OATP1B1/MRP2/MRP3 or MRP4, as well as OATP1B1/OATP1B3/OATP2B1/MRP2 have been developed to better predict hepatobiliary processes (Kopplow et al. 2005; Hirouchi et al. 2009). Recently, a triple-transfected cell line expressing influx and efflux transporters as well as the drug-metabolizing enzyme uridine diphosphate glucuronosyl transferase (UGT) 1A1 has been described to study transporter-metabolism interplay (Fahrmayr et al. 2012). As our understanding of the role of influx and efflux transporters in facilitating the vectorial transport of xenobiotics across the hepatocyte has evolved, the use of polarized mammalian cells has become more popular to identify substrates and inhibitors of hepatic transport proteins. However, protein trafficking or localization in transfected cells may differ from human hepatocytes, depending on the species or type of transfected cells. Also, it is difficult to standardize the relative expression levels of transporters, and the relative contribution of a particular transport protein to overall transport of the substrate cannot be determined. The major applications of transfected cells together with their advantages and disadvantages when used to study hepatobiliary drug transport are summarized in Table 9.3.

9.3.3 Hepatocytes

Freshly isolated hepatocytes are the most comprehensive cell-based model to study hepatic drug transport and remain the gold standard. However, the scarcity of fresh, healthy human liver tissue suitable for hepatocyte isolation is a significant limitation, and isolation of human hepatocytes is technically challenging. Recent advances in technology have made good-quality cryopreserved human hepatocytes commercially available at the user's convenience. Cryopreserved human hepatocytes have been used widely to study drug metabolism (Lau et al. 2002) and substrate influx in suspension (Houle et al. 2003; Shitara et al. 2003a; De Bruyn et al. 2011); cryopreserved hepatocytes cultured in a sandwich configuration repolarize and form bile canalicular networks (Bi et al. 2006). Both the suspended and sandwich-cultured hepatocyte models are illustrated in Fig. 9.2. However, not all batches of cryopreserved hepatocytes are qualified for sandwich-culture due to limitations in cell attachment and the loss of expression, localization, and/or function of transport proteins and metabolizing enzymes. Thus, further research is needed to improve the cryopreservation process.

Suspended hepatocytes. Fresh or cryopreserved suspended hepatocytes are a useful tool to characterize hepatic influx and metabolism processes, and inhibition studies can be performed with this system. However, suspended hepatocytes cannot be used for induction studies because the viability of hepatocytes in suspension cannot be maintained longer than several hours. Additionally, hepatocytes lose their cellular polarity during isolation and internalization of canalicular transport proteins has been demonstrated, which precludes the use of suspended hepatocytes to predict biliary clearance (Bow et al. 2008).

Sandwich-cultured hepatocytes. In contrast to conventionally plated hepatocytes, hepatocytes cultured between two layers of gelled collagen ("sandwich-configuration") develop functional canalicular domains with proper localization of transport proteins and metabolic enzyme expression (Swift et al. 2010). Liu et al. demonstrated that rat sandwich-cultured hepatocytes (SCH) could be used to investigate the hepatobiliary disposition of substrates using Ca^{2+} depletion methods (Liu et al. 1999a, b). This method involves pre-incubation of SCH with Hanks' balanced salt solution (HBSS) containing Ca^{2+} (standard HBSS) or Ca^{2+}-free HBSS for 10 min. Ca^{2+}-free HBSS disrupts tight junctions and opens the bile canalicular networks, while incubation with standard HBSS maintains tight junction integrity. Subsequently, cells are rinsed and incubated with substrate in standard HBSS for a predetermined period of time. Accumulation of substrate in cells+bile vs. cells can be determined in standard and Ca^{2+}-free HBSS buffers, respectively. The amount of substrate excreted into the bile canaliculi can be estimated as the difference in accumulation in standard and Ca^{2+}-free HBSS buffers, and in vitro biliary clearance may be obtained by dividing the amount of drug in the bile compartment by the area under the concentration–time curve (AUC) in the dosing medium. This system has been applied to hepatocytes from many species and has been used extensively to assess biliary clearance as a measure to improve

hepatic clearance predictions (Swift et al. 2010). Biliary clearance values scaled from in vitro intrinsic biliary clearance measurements obtained in SCH have been shown to correlate well with in vivo biliary clearance data in rats (Abe et al. 2008; Fukuda et al. 2008; Liu et al. 1999c; Li et al. 2010, Nakakariya et al. 2012) and humans (Abe et al. 2009; Ghibellini et al. 2007).

To determine the contribution of a specific transport protein to the disposition of a substrate, a transporter-specific reference compound can be employed in hepatocytes as well as transport protein over-expressing cells (Kouzuki et al. 1999). Basically, this method compares the ratio of influx clearances of the test and the reference compound in both systems. However, the results are based on the assumption that the reference compound is specific for the respective transport protein, which—due to the overlapping substrate spectrum of transport proteins—is hardly ever the case.

SCH from naturally occurring, genetically deficient rodents lacking a specific transport protein, such as the Mrp2-deficient Wistar (TR⁻) and Eisai-hyperbilirubinemic Sprague–Dawley (SD) rats (EHBR), and the Mdr1a-deficient CF-1 mice, have been useful tools to evaluate the role of a transporter in the disposition of substrates (Abe et al. 2008). Also, genetically modified animals that lack specific transporter(s) can be used to assess the potential involvement of specific transport protein(s) in DDIs or polymorphisms that impair the function of drug transport proteins. However, care must be taken in interpreting the results of these studies because compensatory changes in drug-metabolizing enzymes and/or other transport proteins may exist. Also, species difference in the expression, localization, and function of transport proteins between humans and genetically modified animals may limit clinical applicability of the data.

RNA interference (RNAi) of single or multiple transport proteins is a powerful tool to explore the consequences of loss of transport protein function. Synthetic small interfering RNA (siRNA) was transfected into rat SCH to specifically knock down Mrp2 and Mrp3 (Tian et al. 2004). Infection of rat SCH with adenoviral vectors expressing short hairpin RNA (shRNA) targeting Bcrp showed a significant decrease in protein expression and activity of this canalicular transport protein (Yue et al. 2009). Recently, Liao et al. successfully knocked down OATP1B1, 1B3, and 2B1 in sandwich-cultured human hepatocytes using special delivery media containing siRNA (Liao et al. 2010). These studies have demonstrated the utility of knock down of specific transport proteins in SCH. However, careful optimization is required because knock down of one transport protein may alter the expression/function of metabolic enzymes and other transport proteins.

Other hepatocyte models. Limited exposure of liver tissue to collagenase results in **hepatocyte couplets** preserving closed canalicular vacuoles and hepatocyte polarity (Milkiewicz et al. 2002). Hepatocyte couplets have been used to study hepatobiliary transport mechanisms underlying bile secretion (Boyer 1997; Coleman et al. 1995). **HepaRG** cells, a human hepatoma cell line, maintain specific liver functions such as drug-metabolizing enzymes and transport proteins. In HepaRG cells, mRNA expression levels and functional activity of basolateral and canalicular transport

proteins were comparable to those of human hepatocytes (Le Vee et al. 2006). Also, expression levels of transport proteins were up-regulated by known inducers, indicating that HepaRG cells maintain transactivation pathways that regulate expression of transport proteins (Le Vee et al. 2006). Since HepaRG cells are readily available compared to human hepatocytes, they may be a useful system to study hepatobiliary transport of compounds. However, HepaRG cells differentiate into biliary epithelial cells as well as hepatocytes, and the fraction of cells that differentiate into hepatocytes varies among different cultures and plates. In addition, more characterization is warranted including cell polarity and polarized expression of relevant transport proteins. Human **inducible pluripotent stem (iPS) cells** have been successfully differentiated into hepatocyte-like cells that exhibit human hepatocyte function such as inducible CYP450 activity (Song et al. 2009). Although the expression and function of transport proteins still needs to be characterized, hepatocytes derived from individual-specific iPS cells may serve as a novel tool to study hepatobiliary transport of compounds in specific individuals. Newer three-dimensional microfluidic models (e.g., liverchip, Hμrel) that more closely resemble in vivo liver physiology are currently under development. Further investigations are needed to explore the utility of these more complex models. The major applications of hepatocytes as well as their advantages and disadvantages when used to study hepatobiliary drug transport are summarized in Table 9.3.

9.3.4 Isolated Perfused Liver Models

For decades, in situ or isolated perfused liver (IPL) studies have been used to investigate the physiology and pathophysiology of the liver. The model is illustrated in Fig. 9.2. Publications regarding the use of IPLs date back to the 1950s, when the metabolism of drugs and endogenous compounds was first studied using this approach. In contrast to in vitro models such as isolated hepatocytes and liver slices, the IPL preserves hepatic architecture, cell polarity, and bile flow. Furthermore, this model enables simultaneous sampling of bile as well as inflow and outflow perfusate; liver tissue may be obtained at the end of the study. Thus, the IPL provides a rich dataset amenable to pharmacokinetic modeling and makes this system useful for mechanistic studies of hepatobiliary transport.

In IPL studies, the liver may be perfused in a single-pass or recirculating mode. A single-pass perfusion system is used to determine directly the steady-state hepatic extraction ratio of a compound. In the single-pass system, outflow perfusate from the liver does not re-enter the system, and the perfusion medium is pumped into the liver at a constant rate. Thus, steady-state conditions can be achieved readily, and drug and metabolite disposition can be examined at different dose levels in a single preparation. Also, experiments can be designed so that each liver serves as its own control. Hemoglobin-free oxygenated perfusate often is used in the single-pass perfusion system because a large volume of perfusate is required. Furthermore, flow rates that are 2–3 times higher than physiologic blood flow are required to maintain

adequate oxygen delivery. In recirculating systems, blood-containing perfusate is oxygenated and recirculated through the liver at a constant flow rate that is similar to liver blood flow in vivo. The hepatic clearance of the drug can be determined from the dose introduced into the reservoir and the AUC in the reservoir. In recirculating systems, the only route of metabolite elimination is via biliary excretion, and metabolites usually accumulate in hepatocytes or in the perfusate, if they are able to flux across the hepatic basolateral membrane. Accumulation of metabolites may be advantageous in mass-balance determination of metabolite formation and kinetic evaluation of hepatic influx of metabolites. However, potential drug–metabolite interactions may be magnified in the recirculating system compared with the single-pass IPL.

The IPL model can be applied to transporter knock-out animals in combination with chemical inhibitors to investigate the contribution of specific hepatic drug efflux transporters (Hoffmaster et al. 2004; Zamek-Gliszczynski et al. 2005). However, these experiments are relatively expensive and low throughput. Furthermore, species-specific differences between human and rodent transport proteins may significantly limit the clinical applicability of information generated using this approach. Whether or not the results obtained from IPL analyses can be extrapolated to in vivo findings in humans remains compound-dependent. The major applications of the IPL model, as well as advantages and disadvantages when applied to studying hepatobiliary drug transport, are summarized in Table 9.3.

9.4 In Vivo Models and Methods to Study Hepatobiliary Drug Transport

In vivo pharmacokinetic/pharmacodynamic studies in humans are the gold standard for investigating the role of hepatic transporters. However, the complexity of the hepatobiliary system, and considerable substrate overlap for many of the transporters, makes it difficult to identify the function of specific transport proteins based on in vivo studies. Genetically modified animals and patients with polymorphisms in transporter genes are valuable in evaluating the function of transport proteins, but species differences in transport protein function, and compensatory up-regulation of other transport proteins, may confound the translation of in vivo data generated in preclinical species to humans.

9.4.1 In Vivo Biliary Excretion Studies

Biliary excretion is an important route of elimination for some drugs and a potential site of drug interactions that may alter hepatic and/or systemic drug exposure. Accurate measurement of biliary clearance and understanding the mechanism(s) of biliary excretion are very important in evaluating the contribution of biliary

clearance to total systemic clearance, predicting DDIs, identifying the contribution of enterohepatic recirculation to overall systemic and intestinal exposure, and elucidating potential mechanisms of hepatobiliary toxicity.

Bile duct-cannulation. Animals, primarily rodents, often are used to determine the extent and the mechanisms of biliary excretion in vivo. Complete collection of bile is possible in bile duct-cannulated animals, which generates information about the extent of biliary excretion and the potential involvement of enterohepatic recirculation in overall systemic exposure. Proper study design is critical to obtain useful information from bile duct-cannulated animals. For example, if bile flow is exteriorized for extended periods of time to obtain complete bile collection, intravenous or intestinal supplementation with bile acids should be considered to replenish the bile acid pool. In vivo biliary clearance data has been used to assess the accuracy of in vitro methods of estimating biliary clearance; reasonable in vitro-in vivo correlations have been obtained (Fukuda et al. 2008; Li et al. 2010; Abe et al. 2009; Nakakariya et al. 2012). Genetically modified animals that are deficient in specific transport proteins has improved our understanding of the complex molecular processes involved in excretion of endogenous and exogenous compounds into bile. However, significant interspecies differences in substrate specificity and regulation of transport proteins have been reported, which complicates the direct extrapolation of animal data to humans (Ishizuka et al. 1999).

Aspiration of duodenal fluids. Determining the biliary clearance of drugs in vivo in humans is challenging because it is difficult to access bile for sample collection from healthy human subjects. Bile samples can be collected in postsurgical patients with underlying hepatobiliary disease via a T-tube or nasobiliary tube (Brune et al. 1993; Verho et al. 1995). However, it is difficult to rule out the effects of underlying hepatobiliary disease (e.g., altered protein expression, function, localization, and/or bile flow) in these patients. In healthy subjects, feces often are used as a surrogate to quantify the amount of drug excreted via non-renal pathways. However, this method cannot distinguish between biliary excretion, intestinal secretion, and unabsorbed drug following oral administration. Moreover, unstable drugs may not be recovered in feces due to the long exposure to the intestinal contents and colonic flora. Furthermore, drugs that are reabsorbed in the intestine and undergo enterohepatic recycling will not be recovered completely in the feces.

Oroenteric tube. Sampling duodenal fluids in healthy volunteers using an oroenteric tube alleviates some of the above-mentioned problems. Duodenal bile is representative of gallbladder bile in terms of bile composition, and collecting bile upon discharge from the biliary tract into the small intestines excludes the contribution of intestinal excretion and minimizes loss associated with metabolism and/or reabsorption. Oroenteric tubes have been used commonly to withdraw pancreaticobiliary secretions from the duodenum in medical practice and have been used to study the biliary excretion of drugs (Galatola et al. 1991; Northfield and Hofmann 1975). Use of an occlusive balloon can facilitate more complete bile collection, and incomplete bile collection can be corrected by perfusing nonabsorbable markers.

The most challenging part of this method is incomplete and highly variable recovery of compounds excreted into bile. Cholecystokinin 8 (CCK-8) may be administered intravenously to pharmacologically stimulate gall bladder empting, but interindividual response is variable. Ghibellini et al. introduced a novel method to evaluate the degree of gallbladder contraction and to detect any leakage of bile due to partial occlusion of the intestine (Ghibellini et al. 2004). Subjects were administered a hepatobiliary imaging agent (e.g., ^{99m}Tc-mebrofenin), and the gall bladder ejection fraction was calculated from the abdominal gamma images of the study participants during gallbladder contraction. Incorporation of the ejection fraction as a correction factor in the calculation of the amount of drug excreted into the duodenum accounted for the variability in biliary excretion of the drug (Ghibellini et al. 2006). This type of study provides direct evidence for biliary excretion and more precise quantification of biliary clearance, but is not used widely due to requirements for a gamma camera and personnel with expertise in gamma scintigraphy. The major applications of the in vivo biliary excretion models, as well as advantages and disadvantages when used to study hepatobiliary drug transport, are summarized in Table 9.4.

9.4.2 Hepatobiliary Imaging Techniques

Although techniques are available to study genetic polymorphisms and the expression of drug transporters at the mRNA and protein level, these data do not necessarily correlate with transporter function. Thus, there continues to be considerable interest in studying transporter function noninvasively. Pharmacokinetic analyses based on plasma concentrations in clinical studies provide information on overall hepatic clearance; however, differentiation between influx and canalicular efflux is not possible. While variations in influx activity of transporters might have a profound influence on systemic concentrations, altered canalicular efflux might significantly affect liver concentrations without having measureable effects upon systemic exposure. This is especially relevant for drugs where the target site for effect or toxicity is within the hepatocyte. Therefore, quantitative estimations of tissue concentrations in vivo are necessary to investigate variations in efflux caused by DDIs or transporter polymorphisms. Furthermore, assessing the functional transport activity of P-gp, MRP2, or BCRP in the human liver might benefit the diagnosis of transporter deficiency-related diseases (e.g., PFIC3 and DJS). Several noninvasive imaging techniques such as magnetic-resonance imaging (MRI), single-photon emission computed tomography (SPECT) using ^{99m}Tc-labeled compounds, and positron emission tomography (PET) using short-lived ^{11}C, ^{13}N, ^{15}O or ^{18}F isotopes have been employed to visualize and measure hepatic transporter activity in vivo.

Magnetic-resonance imaging. The first MRI contrast agents were developed in the early 1980s (e.g., gadopentetate dimeglumine, gadodiamide, gadoteridol). These extremely hydrophilic compounds distributed primarily into the extracellular fluid and were excreted predominantly via the kidney. Because of this distribution

Table 9.4 Summary of advantages and disadvantages of in vivo models used to study hepatobiliary drug transport

In vivo model	Major applications	Advantages	Disadvantages
In vivo biliary excretion			
Bile duct-cannulation	– Direct measure of biliary excretion in animals	– Studies may be conducted in freely moving animals – Most physiologically relevant model	– Low throughput
Oroenteric tube	– Direct measure of biliary excretion in humans – DDI study using probe drug	– Direct measure of biliary excretion – Most physiologically relevant model	– Low throughput – Requires specialized personnel and equipment – Correction for gallbladder ejection fraction is required to accurately quantify biliary excretion
In vivo imaging			
MRI	– Anatomical imaging	– Safer compared to radiation-based imaging	– Parent drug and metabolites not differentiated
SPECT, PET	– Investigate the function of transporters or drug interactions at transporter level	– 3D image – Noninvasive, real-time quantitative estimation of tissue concentration of drugs in vivo	– Radionuclide-labeled probe – Parent drug and metabolites not differentiated

pattern, these contrast agents have been used primarily for angiography and to detect lesions in the brain. The development of gadobenate dimeglumine (Gd-BOPTA) and gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (GD-EOB-DPTA, gadoxetic acid) allowed liver imaging and facilitated the distinction between normal and pathological tissue. Using these imaging agents, most hepatic tumors appear as hypointense lesions because they do not possess functional hepatocytes, while positive hepatocyte-enhancement may be observed in patients with tumors of hepatocellular origin (e.g., hepatocellular carcinoma, HCC). Hepatocyte influx of gadolinium compounds is thought to be mediated by OATPs (Narita et al. 2009; Leonhardt et al. 2010), while MRP2 mediates biliary excretion (Pascolo et al. 2001). Indeed, studies indicated that the degree of expression and localization of OATP1B1/1B3 and MRP2 affect the degree of hepatocyte-specific enhancement in HCC (Narita et al. 2009; Tsuboyama et al. 2010).

SPECT and PET imaging. For quantitative determination of drug transporters, the radionucleotide-based molecular imaging techniques SPECT and PET hold great

promise. Initially, PET was used to quantify P-gp function in the blood–brain-barrier; several ^{11}C-labeled tracers have been developed for this purpose (verapamil, carvedilol, *N*-desmethyl-loperamide, daunorubicin, paclitaxel). However, PET imaging also can be employed to study influx and excretion in other tissues. In 1995, Guhlmann et al. determined the hepatobiliary and renal excretion of *N*-[^{11}C] acetyl-leukotriene E4 in rats and monkeys by PET analysis. In rats, cholestasis due to bile duct obstruction as well as Mrp2 deficiency (TR[−] rats) led to prolonged organ storage, metabolism, transport back into the blood, and subsequently enhanced renal elimination compared to wild-type rats (Guhlmann et al. 1995). Currently, compounds are being developed to evaluate hepatobiliary transport (Takashima et al. 2010). In order to be useful clinically, such probes will need to be metabolically stable in humans, and ideally, the probes should be a substrate for a specific hepatic influx and/or efflux transport protein.

SPECT tracers directly emit gamma radiation, whereas PET tracers emit positrons, which annihilate on contact with electrons, resulting in emission of gamma photons. Cholescintigraphy studies with [99m]Tc-*N*(2,6-dimethylphenyl carbamoylmethyl) iminodiacetic acid (^{99m}Tc-HIDA) were performed in patients with liver disease in the late 1970s/early 1980s for diagnostic imaging of hepatobiliary disorders (Stadalnik et al. 1981). Furthermore, ^{99m}Tc-mebrofenin has been used widely to diagnose cholestasis, gallbladder function, and bile duct leakage. In 2004, Hendrikse et al. proposed that both compounds were useful tools to evaluate the function of Mrp1, Mrp2, and P-gp in vivo (Hendrikse et al. 2004). Another compound, ^{99m}Tc-sestamibi, has been suggested to be a probe for P-gp function (Luker et al. 1997). This compound originally was developed for imaging of myocardial ischemia and is a positively charged, lipophilic compound that readily enters cells and accumulates in mitochondria. In vivo studies with ^{99m}Tc-sestamibi showed that this substance is retained in the liver and kidneys after P-gp inhibition with PSC833, suggesting that inhibition of P-gp transport in these organs can be imaged with ^{99m}Tc-sestamibi (Luker et al. 1997). However, ^{99m}Tc-sestamibi is also a substrate for MRP1 (Gomes et al. 2009). In vivo biliary clearance values for ^{99m}Tc-sestamibi and ^{99m}Tc-mebrofenin were determined in healthy volunteers using an oroenteric catheter, which was designed to aspirate pancreatico-biliary secretions from the duodenum (Ghibellini et al. 2004, Ghibellini et al. 2007). The DDI between ritonavir and ^{99m}Tc-mebrofenin was evaluated in humans using this technique combined with a semi-physiologically-based pharmacokinetic modeling approach (Pfeifer et al. 2013). The major applications of in vivo imaging together with advantages and disadvantages when used to study hepatobiliary drug transport are summarized in Table 9.4.

9.5   Conclusions

Hepatic transport proteins play important roles in the hepatic influx and biliary excretion of drugs and metabolites, thus affecting the therapeutic efficacy and toxicity of many drugs. Therefore, it is important to understand the roles of hepatic

Table 9.5 Preferred approaches to answer specific questions regarding hepatobiliary drug transport

	Membrane vesicles[a]	Transfected cells	Suspended hepatocytes	Sandwich-cultured hepatocytes	Isolated perfused liver	In vivo bile collection	In vivo imaging
Which influx transporters are capable of transporting the NCE?	NA	1	2[b]	2[b]	2[b]	NA	2[b,c]
Which efflux transporters are capable of transporting the NCE?	1	2	NA	2[b]	2[b]	NA	2[b,c]
What is the $Cl_{biliary}$ of the NCE?	NA	NA	NA	1	2	1	NA
Is the NCE subject to transporter-based DDIs in the liver?	2	2	2	1	1	2	1[c]
Is the NCE subject to DDIs at a specific transporter?	1	1	2[d]	2[d]	2[d]	NA	2[d]
Is the NCE likely to inhibit bile acid excretion?	1[d]	NA	NA	1[d]	1[d]	2	NA
Is the NCE likely to accumulate in the liver?	NA	NA	NA	1	1	NA	1
Is the NCE subject to transporter-enzyme interplay?	NA	2[e]	NA	1	1	2	NA

NCE new chemical entity
1 preferred, *2* possible, *NA* not applicable
[a] Membrane vesicles over-expressing transporter(s)
[b] If used with a specific inhibitor
[c] NCE needs to be labeled or fluorescent
[d] If used with a specific probe
[e] Fahrmayr et al. 2012

transport proteins in the disposition of drugs and metabolites during the drug development process. Table 9.5 summarizes preferred approaches that can be used to answer specific questions regarding hepatobiliary drug transport. It is relatively straightforward to determine which transport proteins are capable of transporting drugs and metabolites by using membrane vesicle systems or cell lines expressing a single transport protein. However, determining the contribution of each transport protein to the hepatic influx or efflux of a specific compound in the whole cell/intact organ is not as straightforward and may require the use of several model systems (e.g., transfected cell lines and SCH) and scaling factors (e.g., relative activity factor) (Hirano et al. 2004). Moreover, reference compounds used to obtain the scaling factor between different systems are often not specific to a single transporter, which makes it difficult to determine the precise contribution of a single transporter to overall disposition.

Accurate predictions of clinically relevant drug interactions in hepatobiliary transport [either DDIs, drug–endogenous compound interactions (e.g., competition with bilirubin for influx or excretion) or drug–transporter interactions] are critical in drug development. Direct competitive interactions with a single protein can be predicted from membrane vesicle or transfected cell assays; however, accurate extrapolation to the in vivo setting requires an understanding of the unbound concentration at the site of transport. The ability of other transport proteins or drug-metabolizing enzymes to compensate for drug interactions cannot be predicted accurately from these simplistic systems, and intact hepatocytes (suspended for influx studies; sandwich-cultured for hepatic efflux and overall hepatobiliary disposition) or whole organ and/or in vivo studies are required. More complex drug-transporter interactions involving signaling cascades and/or regulatory mechanisms or interactions that involve generated drug metabolites require the complex machinery of the intact cell.

DILI is one of the most common reasons for withdrawal of drugs from the market, or failure of new drugs in clinical trials. Inhibition of canalicular BSEP, which leads to elevated hepatic exposure of detergent-like bile acids, has been reported as one mechanism of DILI. Some hepatotoxic drugs also are potent inhibitors of NTCP and/or MRPs. Thus, determining whether the drug and/or generated metabolite(s) inhibit(s) bile acid transport would provide key information about the drug's potential for DILI. High-throughput screening is possible to determine the inhibitory effects of a specific compound on bile acid transport in membrane vesicles expressing a single transporter. However, model systems that enable the generation of metabolites and allow for direct measurement of bile acids accumulated in hepatocytes (e.g., SCH or IPL) provide more information to determine the potential for DILI. Hepatic exposure of the drug is important in predicting efficacy and toxicity, but this cannot be measured directly in vivo in humans nor predicted based on systemic exposure. Human SCH will provide invaluable information about the hepatic accumulation potential of drugs and generated metabolites, and how hepatic exposure changes when the function of transport proteins is altered due to disease states, drug interactions, or changes associated with genetic polymorphisms in transport proteins. Because only unbound drugs are available to interact with transporters, it is important to determine the intracellular unbound concentration. However, our

current knowledge about intracellular unbound concentrations is limited, and development of in vitro systems to characterize hepatocellular binding/sequestration and the unbound concentration in the intact cell is needed.

Many tools and model systems are available to analyze the role of hepatic transport proteins in drug development. Current efforts are focused on assessing which tools should be appropriately used at defined steps in the drug development process, as well as how the resulting information can be used most efficiently to answer the key questions before the compound reaches the clinic. Important work continues to focus on mathematical modeling and simulation based on data generated from the various in vitro and in vivo models to accurately predict the role of hepatic transport proteins in drug disposition, and how alterations in hepatic transport could alter efficacy and/or toxicity.

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Chapter 10
Analysis of Renal Transporters

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Abstract The rapid technological advances in the drug transporter field have also greatly enhanced our knowledge on the expression, localization, function, and genetic variation of renal transporters. It is now widely acknowledged that carrier-mediated transport processes in the kidney proximal tubule are an important determinant of drug disposition and the extent to which drugs are accumulated in renal tissue. The study of renal transport has traditionally benefited a lot from physiological studies in isolated membrane vesicles, tubules, tissue slices, perfused kidneys, and intact animals. Together with molecular cloning and over-expression systems we now have a fairly good picture of the individual characteristics of the most important renal transporters. The next challenge will be to reconstruct the complexity of the interplay between the various uptake and efflux transporters of the proximal tubule in experimental and in silico models, in order to accurately predict renal drug clearance, drug–drug interactions, and the risk of nephrotoxicity in different populations. This chapter will give a critical review of current methods available for the exploration of renal drug transport.

Abbreviations

ABC	ATP-binding cassette
BBM	Brush border membrane
BLM	Basolateral membrane
CHO	Chinese hamster ovary cell line
ciPTEC	Human conditionally immortalized proximal tubule epithelial cell line

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COS-7   African green monkey cells
HEK293   Human embryonic kidney cells
HK-2   Human immortalized proximal tubule epithelial cell line
LLC_PK1   Pig kidney epithelial cells
MDCK   Madin-Darby canine kidney cells
PAH   *para*-Amino hippuric acid
pMEG   9-(2-phosphonylmethoxyethyl)guanine
Sf9   Spodeptera frugiperda (moth)
SLC   Solute carrier

10.1   Introduction

The mechanisms that contribute to the renal excretion of drugs and their biotransformation products are closely related to the physiological processes that take place in the nephrons, i.e., glomerular filtration, passive back diffusion, and transporter-mediated secretion and reabsorption. The major transport proteins that are relevant for the renal handling of drugs are mainly located in the proximal tubular cells. The same transporter families that play critical roles in drug influx and efflux in liver and intestine can also be found in the kidney (Degorter et al. 2012). From the solute carrier (*SLC*) gene superfamily, these are the oligopeptide transporters (PEPTs/*SLC15*), the organic anion/cation/zwitter ion transporters (OATs/OCTs/OCTNs/*SLC22*), the organic anion transporting polypeptides (OATPs/*SLCO*), and the multidrug and toxin extrusion transporters (MATE/*SLC47*). Members belonging to the ATP-binding cassette (*ABC*) superfamily important for renal drug efflux include P-glycoprotein (MDR1/*ABCB1*), the multidrug resistance-associated protein (MRP/*ABCC*) family, and breast cancer resistance protein (BCRP/*ABCG2*). An overview of the transporters currently considered to have a well-defined influence on renal drug clearance is given below and in Fig. 10.1. It is important to recognize that the interplay between these transporters located on the basolateral and luminal membrane in proximal tubular cells is critical in determining the extent and net direction of drug movement. Transport across the proximal tubule could be impeded or facilitated by the asymmetrical membrane distribution of influx and efflux transporters, which ultimately influences the plasma clearance and urinary excretion of a drug substrate.

   The study of the mechanisms by which the kidney actively secretes compounds foreign to the body started with the pioneering paper in 1923 by Marshall and Vickers, who obtained the first conclusive evidence for this process with the anionic dye phenolsulphonphtalein (Marshall and Vickers 1923). Ever since, our understanding of the molecular and cellular mechanisms of renal drug excretion has been evolving by the advent of increasingly advanced techniques in the transporter field, including isolated renal cortical slices, isolated perfused tubules and kidneys, microperfusion, membrane vesicles, cell cultures and over-expression cell systems,
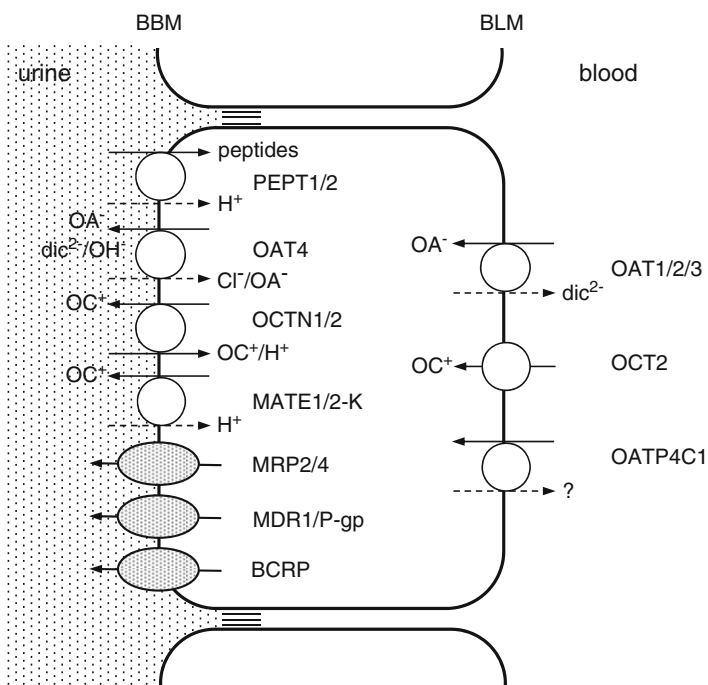
Fig. 10.1 Schematic model of the major drug transporters in human renal proximal tubular cells. SLC transporters are depicted by open circles and ABC transporters by shaded ovals. Solid arrows indicate the direction of drug transport. Dashed arrows depict the movement of driving ions. OCT2 is an electrogenic uniporter that transports organic cations (OC+) from blood into the cell driven by the inside-negative membrane potential. OCTN1 mediates luminal OC+ uptake as a H+/OC+ antiporter or can operate like OCTN2 as a bidirectional cation exchanger, mediating influx or efflux. MATE1 is a urinary OC+ efflux transporter that operates as a H+ antiporter. Peptidomimetic drugs are taken up by the H+/peptide symporters PEPT1 and PEPT2. Organic anions (OA-) are taken up by the antiporters OAT1, OAT2, and OAT3, which are driven by the exchange with dicarboxylates (dic^{2-}), and released at the luminal side by OAT4 in exchange for Cl-. OAT4 can also operate as a reabsorptive transporter coupled to cellular dicarboxylates or hydroxyl ions. A few amphipathic drugs are transported into the cell by the organic anion antiporter OATP4C1, for which the driving ion is unknown. The primary active ABC transporters MDR1/P-gp, MRP2, MRP4, and BCRP drive the efflux of a wide variety of amphipathic drugs and metabolites into urine

knockout mouse models, double transfected cell lines, physiologically based pharmacokinetic modeling, and simulation.

To date, no single method or model can accurately predict the contribution of renal transporters to overall drug clearance and disposition in humans in vivo. The purpose of this chapter is to discuss key technologies, including their strengths and limitations, and to examine some of the current challenges and future perspectives in studying renal drug transporters.

10.2   Important Human Transporters Involved in Renal Drug Handling

For most drugs that are handled by renal transporters elimination can be considered as a vectorial process, involving uptake from the blood across the basolateral membrane into proximal tubular cells, followed by efflux across the apical membrane into urine. At the basolateral membrane separate transporters are located for the influx of mainly hydrophilic, small molecular weight (MW < 400–500 Da) organic anions and cations (Masereeuw and Russel 2001). Because these systems are characterized by a high clearance capacity and wide substrate specificity, many drug substrates tend to accumulate in the cell sometimes causing kidney injury. To ensure the rapid efflux of potentially toxic compounds into urine, the apical membrane is equipped with a large number of efflux transporters belonging to different transporter families (Fig. 10.1, Table 10.1).

The organic anion transporters OAT1, OAT2, and OAT3 regulate the uptake of anionic drugs at the basolateral membrane of renal proximal tubule (Burckhardt 2012; Burckhardt and Burckhardt 2011). They operate as antiporters, actively driven by the inside>out concentration gradient of dicarboxylates. OAT1 and OAT3 have long been considered as the major uptake transporters, because of limited evidence for the expression of OAT2 and its unknown role in drug transport. OAT1 has highest affinity for hydrophilic organic anions with small molecular weights, like p-aminohippuric acid (PAH), adefovir, cidofovir, and tenofovir (Table 10.1). OAT3 also transports some larger amphipathic anions, including benzylpenicillin, pravastatin, and olmesartan, and even some cationic drugs, such as cimetidine and ranitidine (Table 10.1). A recent study showed that OAT2 probably mediates the active tubular secretion of the cGMP-like antiviral drugs acyclovir, ganciclovir, and penciclovir (Cheng et al. 2012). Whereas human OAT2 is expressed at the basolateral membrane, the mouse and rat orthologs are localized to the apical membrane of the proximal tubule (Burckhardt and Burckhardt 2011). The broader specificity of OAT3, as well as the relatively higher renal expression levels compared to OAT1 and OAT2 suggests a more pronounced role of OAT3 in human renal organic anion transport (El-Sheikh et al. 2008a; Masereeuw and Russel 2001, 2010). Serious drug–drug interactions have been reported between methotrexate and nonsteroidal anti-inflammatory drugs due to competition for OAT1- and OAT3-mediated uptake, although an interaction at the level of the apical efflux transporters MRP2 and MRP4 probably also contributes to this mechanism (El-Sheikh et al. 2007; Masereeuw and Russel 2010).

The first step in proximal tubular secretion of cationic drugs is mediated by OCT2, the predominant organic cation transporter in the basolateral membrane (Nies et al. 2011). OCT2 operates as a uniporter that facilitates the uptake of comparatively small monovalent cationic drugs by diffusion down the inside-negative electrochemical gradient of the proximal tubular cell (Table 10.1). In rodents, Oct1 is also expressed in the kidney, in addition to Oct2 (Grundemann et al. 1994). A clinically important OCT2 substrate is metformin, which is among the most widely prescribed

Table 10.1 Major human SLC and ABC drug transporters expressed in kidney

Gene	Protein	Mechanism	Membrane localization	Examples of drug substrates
SLC15 family				
SLC15A1	PEPT1	H$^+$/peptide symporter	Apical	Ampicillin, amoxicillin, bestatin, cefaclor, cefadroxil, cefixime, enalapril, temocapril, temocaprilat, midodrine, valacyclovir, valganciclovir (Brandsch et al. 2008; Dobson and Kell 2008; Rubio-Aliaga and Daniel 2008; Russel et al. 2002)
SLC15A2	PEPT2	H$^+$/peptide symporter	Apical	Amoxicillin, bestatin, cefaclor, cefadroxil, valganciclovir (Brandsch et al. 2008; Dobson and Kell 2008; Rubio-Aliaga and Daniel 2008; Russel et al. 2002)
SLC22 family				
SLC22A2	OCT2	OC uniporter	Basolateral	Mepiperphenidol, memantine, cimetidine, famotidine, ranitidine, metformin, propranolol, pancuronium, quinine, zidovudine, cisplatin (Ciarimboli 2008; Dobson and Kell 2008; Koepsell et al. 2007)
SLC22A4	OCTN1	H$^+$ or OC antiporter	Apical	Mepyramine, quinidine, verapamil, ergothioneine, gabapentin (Dobson and Kell 2008; Koepsell et al. 2007; Urban et al. 2008)
SLC22A5	OCTN2	OC antiporter Na$^+$ symporter (carnitine)	Apical	Mepyramine, quinidine, verapamil, valproate, cephaloridine, emetine (Dobson and Kell 2008; Koepsell et al. 2007)
SLC22A6	OAT1	DC/OA antiporter	Basolateral	Adefovir, cidofovir, acyclovir, didanosine, stavudine, trifluridine, ganciclovir, PMEG, PMEDAP, tenofovir, zalcitabine, zidovudine, tetracycline, methotrexate, bumetanide, furosemide, ibuprofen, indomethacin, ketoprofen, PAH, cimetidine, ranitidine (Burckhardt 2012; Burckhardt and Burckhardt 2011; Dobson and Kell 2008; Russel et al. 2002)
SLC22A7	OAT2	DC/OA antiporter	Basolateral	Acyclovir, ganciclovir, penciclovir, erythromycin, cimetidine, ranitidine, zidovudine, 6-fluorouracil, methotrexate, taxol, bumetanide, salicylate, PAH (Burckhardt 2012; Burckhardt and Burckhardt 2011; Cheng et al. 2012; Russel et al. 2002)

(continued)

Table 10.1 (continued)

Gene	Protein	Mechanism	Membrane localization	Examples of drug substrates
SLC22A8	OAT3	DC/OA antiporter	Basolateral	Benzylpenicillin, tetracycline, valacyclovir, zidovudine, cimetidine, ranitidine, methotrexate, furosemide, ibuprofen, indomethacin, ketoprofen, salicylate, PAH, pravastatin, olmesartan (Burckhardt 2012; Burckhardt and Burckhardt 2011; Dobson and Kell 2008; Kusuhara and Sugiyama 2009; Russel et al. 2002)
SLC22A9	OAT4	$Cl^-/OH^-/DC/OA$ antiporter	Apical	Tetracycline, zidovudine, methotrexate, bumetanide, ketoprofen, salicylate, PAH (Burckhardt 2012; Burckhardt and Burckhardt 2011; Dobson and Kell 2008; Russel et al. 2002)
SLC47 family				
SLC47A1	MATE1	H^+/OC antiporter	Apical	Cimetidine, procainamide, metformin, cephalexin, cephadrine, fexofenadine (Koepsell et al. 2007; Matsushima et al. 2009; Moriyama et al. 2008; Tanihara et al. 2007; Terada and Inui 2008)
SLC47A2	MATE2-K	H^+/OC antiporter	Apical	Cimetidine, procainamide, metformin, fexofenadine, oxaliplatin (Ho and Kim 2005; Koepsell et al. 2007; Matsushima et al. 2009; Moriyama et al. 2008; Terada and Inui 2008)
SLCO family				
SLCO4C1	OATP4C1	ND	Basolateral	Digoxin, ouabain, methotrexate (Dobson and Kell 2008; Hagenbuch and Gui 2008; Hu et al. 2008)
ABC family				
ABCB1	MDR1/P-glycoprotein	primary active	Apical	Vinblastine, vincristine, daunorubicin, doxorubicin, colchicine, docetaxel, paclitaxel, ortataxel, etoposide, imatinib, methotrexate, bisantrene, mitoxantrone, paclitaxel, topotecan, digoxin, digitoxin, celiprolol, talinolol, indinavir, nelfinavir, ritonavir, saquinavir, levofloxacin, grepafloxacin, sparfloxacin, erythromycin, ivermectin, chloroquine, amiodarone, lidocaine, losartan, lovastatin, mibefradil, fexofenadine, terfenadine, carbamazepine, desipramine, loperamide, methadone, morphine, sumatriptan, vecuronium, cyclosporin A, tacrolimus, sirolimus (Dietrich et al. 2003; Hu et al. 2008; Murakami and Takano 2008; Oostendorp et al. 2009; Russel et al. 2002; Sarkadi et al. 2006; Zhou 2008)

ABCC2	MRP2	primary active	Apical	Vinblastine, vincristine, doxorubicin, etoposide, cisplatin, methotrexate, indinavir, ritonavir, saquinavir, grepafloxacin, glutathione conjugates, PAH (Nies and Keppler 2007; Russel et al. 2002; van de Water et al. 2005; Zhou et al. 2008)
ABCC4	MRP4	primary active	Apical	Methotrexate, leucovorin, topotecan, 6-mercaptopurine, 6-thioguanine, adefovir, tenofovir, ceftrizoxime, cefazolin, cefotaxime, cefmetazole, hydrochlorothiazide, furosemide, olmesartan, edaravone glucuronide, PAH (Borst et al. 2007; Russel et al. 2002, 2008; van de Water et al. 2005; Zhou et al. 2008)
ABCG2	BCRP	primary active	Apical	Mitoxantrone, flavopiridol, topotecan, SN-38, camptothecin, methotrexate, imatinib, gefitinib, erlotinib, abacavir, lamivudine, zidovudine, nelfinavir, cerivastatin, pitavastatin, rosuvastatin, glibenclamide, olmesartan, dipyridamole, cimetidine, edaravone sulfate, albendazole sulfoxide, oxfendazole, ciprofloxacin, norfloxacin, ofloxacin, sulfasalazine, nitrofurantoin (Cusatis and Sparreboom 2008; Robey et al. 2009; Sarkadi et al. 2006; van Herwaarden and Schinkel 2006)

PMEG 9-(2-phosphonylmethoxyethyl)guanine, *PMEDAP* 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine, *DC* dicarboxylate, *PAH* p-aminohippuric acid, *SN-38* active metabolite of irinotecan, *ND* not determined

drugs for the treatment of type 2 diabetes. Genetic polymorphisms of OCT2 have been associated with a decrease in renal metformin clearance (Song et al. 2008). Using metabolomics, tryptophan was recently identified as a specific endogenous substrate of OCT2 related to metformin disposition, and consequently a potential biomarker of genetic variability in transporter activity (Song et al. 2012). Coadministration of cimetidine with metformin has been shown to reduce the renal clearance of metformin, leading to a clinically relevant increase in plasma concentrations (Wang et al. 2008). It has been suggested from in vitro studies that cimetidine is an inhibitor of OCT2; however, because of its relatively low inhibitory constant this seems unlikely at therapeutic plasma concentrations (Lepist and Ray 2012).

OATP4C1 is the only OATP family member expressed in human proximal tubular cells (Obaidat et al. 2012). The transporter is located in the basolateral membrane and substrate specificity is restricted to a few drugs that are mainly excreted by the kidney, i.e., methotrexate, the cardiac glycosides, digoxin, and ouabain, as well as thyroid hormones (Table 10.1). The mechanism by which OATP4C1 translocates drugs across the membrane and the counter ion it exchanges its substrates for are not yet identified. OATP1A2 expression was identified in the apical membrane of distal nephrons, but its role in renal drug handling is unclear (Lee et al. 2005). The renal expression of OATPs is remarkably different in rodents. Except for the ortholog Oatp4c1 in the basolateral membrane, at least three different Oatps are located in the brush border membrane of rodent kidney, none of which are expressed in humans (Sekine et al. 2006).

At the apical membrane of the proximal tubule, the ABC transporters P-glycoprotein, MRP2, MRP4, and BCRP mediate the primary active efflux of drugs. P-glycoprotein is likely involved in the urinary excretion of digoxin and a number of hydrophobic cationic drugs (Masereeuw and Russel 2001, 2012; Zhou 2008). The *ABCB1* gene encoding for P-glycoprotein is highly polymorphic, and a relationship has been suggested with calcineurin inhibitor efficacy and toxicity in renal transplant patients. However, data on the clinical relevance of these polymorphisms are not unequivocal (Cascorbi 2011). Anionic drugs, including glucuronide, glutathione, and sulfate conjugates, formed in the proximal tubular cells or taken up from the circulation, are pumped into urine via MRP2 and MRP4 (van de Water et al. 2005). As compared to MRP2, MRP4 appears to have a higher affinity for small organic anions and its protein expression is approximately fivefold higher (Russel et al. 2008; Smeets et al. 2004). BCRP was only recently identified in the apical membrane of the proximal tubule (Huls et al. 2008). The overlap in substrate specificity with P-glycoprotein and the MRPs suggests its potential involvement in renal drug excretion (Masereeuw and Russel 2012).

The organic cation transporters MATE1, MATE2-K, OCTN1, and OCTN2 mediate the secondary active efflux of cationic drugs across the luminal membrane. The steep outside>in transmembrane H^+ gradient provides a powerful driving force for the MATE transporters. MATE1 is expressed throughout the body, but predominantly in liver and kidneys, whereas MATE2-K is exclusively located in kidney proximal tubules (Nies et al. 2011). Genetic polymorphisms of MATE1 and MATE2-K have been linked to the variability in renal handling of cationic drugs

like metformin and to accumulation of oxaliplatin, causing drug-induced nephrotoxicity (Kajiwara et al. 2009). Cimetidine appears to be a potent inhibitor of both transporters and there is increasing evidence that the inhibition of MATEs rather than OCT2 is a likely mechanism underlying the renal drug–drug interaction of cimetidine with metformin and other cationic drugs (Ito et al. 2012; Lepist and Ray 2012). The carnitine/organic cation transporters, OCTN1 and OCTN2, are also driven by H^+/organic cation antiport or organic cation/organic cation antiport. Their substrate specificity is comparable to the MATEs, and because of their bidirectional mode, OCTNs could also be involved in organic cation reabsorption (Tamai 2013).

PEPT1 and PEPT2 are H^+-coupled peptide symporters that mediate the active reabsorption of antiviral drugs, beta-lactam antibiotics, and angiotensin-converting enzyme inhibitors from the primary urine. They are both expressed in a sequential order along the renal proximal tubule (Brandsch et al. 2008). PEPT2 has the highest affinity and appears to be the major player in the renal reabsorption of peptide-like drugs (Kamal et al. 2008).

OAT4 is only expressed in humans; there exists no ortholog in rodents or other species (Burckhardt and Burckhardt 2011). The transporter is able to operate as a bidirectional asymmetric antiporter mediating the influx and efflux of organic anions. As an influx transporter, OAT4 couples the luminal uptake of endogenous substrates like urate and estrone sulfate to the release of dicarboxylates or hydroxyl ions from the proximal tubular cell. In the efflux mode, anionic drugs are excreted into urine in exchange with luminal Cl^-. The number of drugs accepted by OAT4 seems somewhat smaller than for OAT1 and OAT3 (Burckhardt 2012; Rizwan and Burckhardt 2007).

10.3   Methods to Analyze Renal Drug Transport

This paragraph discusses key methods and new technical developments to study renal drug transport, including discussion of their advantages and disadvantages, which are summarized in Table 10.2.

10.3.1   Mechanistic Understanding Through Molecular Models

The need for robust in vitro assays in preclinical drug development to optimize the pharmacokinetic properties of drug candidates has led to numerous cell-based and membrane vesicle-based assays. Both approaches include transfection of yeast, insect, or mammalian cells with cDNA, using viral vectors, physical methods, or biochemical agents, leading to the functional over-expression of a specific transport protein.

For SLC transporters, uptake assays have been developed by incubating transporter-transfected cells in Petri dishes or multi-well plates with potential substrates.

Table 10.2 Summary of key technologies used to study renal drug transport

	Molecular models	Cell systems	Organotypic systems	Perfused kidney systems	Preclinical
Techniques used	(1) Membrane vesicles (2) Transfected nonpolarized cells (3) Oocyte expression system	(1) Freshly isolated cells (2) Cryopreserved cells (3) Cell cultures in 2-D (4) Transfected polarized cells in 2-D (5) 3-D cell cultures	(1) Killifish renal tubules (2) Kidney-on-chip (3) Kidney slices (4) Isolated (perfused) mammalian tubules	(1) Isolated perfused kidney (2) Nonfiltering perfused kidney (3) Single-pass perfused kidney (4) Stopped-flow capillary microperfusion	(1) Spontaneous mutants (2) Transgenic or knockout animals (3) Physiological-based models
Advantages	(1–3) Substrate specificities/molecular characterization	(1–5) Vectorial transport (1–3, 5) Suitable for regulatory processes and protein routing	(1, 2, 4) Suitable for vectorial transport, regulatory processes and protein routing (3) Uptake kinetics	(1–4) Accurate determination of overall renal drug clearance	(1–3) Overall drug clearance (3) Includes all functional characteristics

Disadvantages	(1–3) Regulation of transport proteins (almost) not possible	(1–5) Transport rates slow as compared to in vivo (1) Short viability, transporter retraction from plasma membrane (2) Dedifferentiation after immortalization, uptake activity insufficiently conserved during the isolation/cryopreservation process (4) Misrouting, regulatory pathways often absent	(1, 3, 4) Short viability (1) Comparative model (2) Selection of cell types (3) Efflux cannot be measured (3) Lumen often collapses	(1–4) Sophisticated equipment; difficult to distinguish between transport proteins	(3) Molecular data needed as input
Relevant references	Astorga et al. (2011), Cihlar and Ho (2000), El-Sheikh et al. (2007), El-Sheikh et al. (2008c), Hagmann et al. (1999), Ishikawa et al. (1999), Kuze et al. (2004), Mutsaers et al. (2011a), Robey et al. (2011), Shukla et al. (2012), and Soreq and Seidman (1992)	Brown et al. (2008), Jenkinson et al. (2012), Konig et al. (2011), Miller (1992), Mutsaers et al. (2011b), Ryan et al. (1994), Sato et al. (2008), Terlouw et al. (2001), Wilmer et al. (2010), and Windass et al. (2007)	Asthana and Kisaalita (2012), Long et al. (2011), Miller (2002), Oo et al. (2011), Wever et al. (2007), and Wright and Dantzler (2004)	De Kanter et al. (2002), Heemskerk et al. (2007, 2008), Hori et al. (1988), Huh et al. (2012), Jung et al. (2011), Maack (1980), Masereeuw et al. (2003), Tsuruoka et al. (2001), and Ullrich et al. (1984)	Johnson et al. (2010), Jonker et al. (2002), Rowland et al. (2011), Russel et al. (2011), Russel et al. (1987), and Zhao et al. (2011)

After termination of cellular uptake, cells should be washed to remove the substrate and after cell lysation the intracellular content can be analyzed. The mammalian vector technology by Invitrogen is often used to over-express SLC proteins for this purpose in nonpolarized cell lines, including African green monkey cells (COS-7) or the Chinese Hamster overay cell line (CHO) (e.g. Astorga et al. 2011; Cihlar and Ho 2000; Kuze et al. 1999). Furthermore, human embryonal kidney cells (HEK293) have proven validity in studying SLC transporters (Han et al. 2010), even in high-throughput optimization assays (Lohmann et al. 2007). However, cell lines will remain heterogeneous after transfection, and to obtain lines stably expressing the transporter of interest, clonal selection has to be performed usually by serial dilution of the clone mixes and followed by propagation of clonal cell lines. These cell lines are also widely commercially available. In addition, expression of SLC transporters in oocytes of *Xenopus laevis* by cRNA injection has shown to be a promising method to elucidate the molecular characteristics of transporters. Important requirements for this technique are that the endogenous transport activity of the oocytes must be low and the assay used to assess transport activity must be sensitive enough to monitor in a few oocytes at least a twofold increase in transport signal above background (Soreq and Seidman 1992). Oocyte systems expressing some transporters are commercially available as well.

Baculovirus-transduced cell lines have proven their suitability, especially for expression of ABC transporters in insect cells or in mammalian cells. Expression in insect cells, such as cells from the moth *Spodoptera frugiperda* (Sf9), is valuable for structural studies as large quantities of purified proteins can be obtained (e.g., Ishikawa et al. 2004; Radanovic et al. 2003). ABC transporter expression in mammalian cells, on the other hand, allows for functional characterization of the transporters (e.g., Hagmann et al. 1999), and evaluating drug interactions (e.g., in studying the effect of nonsteroidal anti-inflammatory drugs with MRP2 and MRP4-mediated methotrexate transport (El-Sheikh et al. 2007) or interactions of uremic toxins on MRP4 and BCRP-mediated transport (Mutsaers et al. 2011a), and for mutational analysis (e.g., of MRP4 El-Sheikh et al. 2008c; Wittgen et al. 2012b). Functional studies with these transporters are particularly well performed in vesicular assays using isolated inside-out crude membrane fractions or membrane vesicles derived from transduced cells. Major advantages of this method are that metabolism is eliminated and that the composition of solutions on both sides of the membrane can be controlled. But the transporter over-expressing cell lines can also be used in whole cell-based studies (Robey et al. 2011). These cell lines can be used for efflux assays as well as for drug accumulation assays in which the difference in absence and presence of a specific inhibitor of the ABC transporter reflects the activity of the efflux pump (Wittgen et al. 2012a). This approach is also valuable for studying kinetics and interactions of lipophilic substrates for which the vesicular transport assays are hampered by technological difficulties. The baculovirus system also proved to be suitable for studying SLC transporter function and interactions in cell-based systems (El-Sheikh et al. 2008b), although these transporters are generally more difficult to over-express and often stable transfections (as described earlier) are necessary to detect significant transport. Despite high transduction efficiencies and controllable batch-to-batch variations by applying the histone deacetylase

inhibitor butyrate to increase protein expression (Shukla et al. 2012), a drawback of the baculovirus system is that expression is transient which hampers studying regulatory aspects of the transporters and their function in disease models.

10.3.2   *Proximal Tubule Cell Systems for Transepithelial Transport Determinations*

Freshly isolated renal proximal tubule cells are useful in studying overall cellular uptake kinetics and accumulation, but uptake is a hybrid parameter determined by both influx and efflux rates. Overall transport characteristics of isolated cells in suspension seem to resemble basolateral to luminal flux as compared to cells on filters or isolated perfused kidneys, although primary active transporters, like MRP2, were found to be retracted from the plasma membrane (Terlouw et al. 2001).

Primary cultures of cells grown as monolayers on permeable supports (filters) have several technically important advantages and allow studying cellular kinetics (Brown et al. 2008; Windass et al. 2007), but a major obstacle is dedifferentiation resulting in a selective loss of transporter systems, as is shown for renal organic anion uptake (Miller 1992). To overcome these problems, carcinoma cell lines have been characterized and proven to be suitable for studying drug transport, such as the human conditionally immortalized proximal tubule epithelial cell line (ciPTEC) (Wilmer et al. 2010) and HK-2 (Ryan et al. 1994), although the use of the latter cell line in studying drug transport seems rather limited (Jenkinson et al. 2012; Mutsaers et al. 2011b). Furthermore, cell lines have been developed that over-express one or more transport proteins. Polarized cells used for transporter transfection are, among others, Madin-Darby Canine Kindey cells (MDCK) or pig kidney cells (LLC-PK1). For example the double transfected MDCK II cell line, which expresses both hOCT2 and hMATE1, provides a useful model for studying renal vectorial transport (Konig et al. 2011; Sato et al. 2008). Important advantages are that transport mechanisms remain functional upon culturing, allowing the study of vectorial transport and regulation of transport proteins, and the preparation can be maintained for long term use. A major disadvantage of all cell cultures described is that transport rates are rather low as compared to in vivo kinetics.

10.3.3   *An Optimal Microenvironment Allows Functional Transport*

As proximal tubule cells are highly polarized, maintenance of this polarity is critical for optimal functioning and responsiveness to environmental signals. This is dependent on communication between cells, which include features such as paracrine and autocrine signals but also biomechanic, haptotactic, and chemotactic processes, all influencing cell proliferation, migration, and differentiation.

With respect to transporter activities in more physiological models, signaling information can be important in transepithelial fluxes under normal but also under pathological circumstances. When cultured in 2-D, the functional polarity is only partially retained. Advances in 3-D platforms showed a benefit for tubular epithelial cells to grow in spheroids or tubule-like structures (Asthana and Kisaalita 2012). These platforms contain polymeric scaffolds or hydrogels, both without and with scaffolds to put some restraints on the size of the microtissue formed. One concern is, however, the threshold for oxygen diffusion into the tissue, as hypoxia can result in gene expression perturbation leading to a wide variety of changes in protein levels (Brooks et al. 2007). Most likely also drug transporters will be affected, as differences in expression levels were found in ischemic mouse kidneys (Huls et al. 2006).

More recently, bioreactors have been developed that allow proximal tubule cells to grow on hollow fibers in 3-D configuration under flow and oxygen-rich conditions. With these reactors, both uptake and secretion can be studied in one system. Although drug transport studies have, as yet, not been reported for hollow fiber cultures, clearly different transporter expression levels were determined when cells were cultured in a bioreactor under flow conditions as compared to static cultures (Oo et al. 2011). This emphasizes that the microenvironment indeed might influence proximal tubule cell transport function. The hollow fibers clearly have advantages over isolated renal tubules from different animal species, as these cell cultures are less fragile, can be of human origin, and potentially reduce the number of animals needed for drug testing. Moreover, in mammalian tubules the lumens collapse quickly after isolation, which makes this preparation unsuitable for investigating tubular secretion. Hence, techniques for perfusion of single, isolated tubules have been developed, exhibiting a high viability and allowing determining cellular uptake and tubular secretion rates with high accuracy (Wright and Dantzler 2004), processes which are in general faster in primary tissue as compared to cell cultures. Furthermore, nonmammalian vertebrates such as killifish (*Fundulus heteroclitus*) and zebrafish (*Danio rerio*) proximal tubules as a comparative models are very suitable for studying both uptake and efflux steps of renal tubular excretion (Long et al. 2011; Wever et al. 2007). By using fluorescent substrates and confocal microscopy it was shown that multiple drug transport mechanisms identified in mammalian models are present. With this transporter-based assay system not only substrate characteristics but also (hormonal) regulation of transporter proteins could be investigated (Miller 2002).

10.3.4   Multiple Cell Types for Overall Renal Drug Handling Assessments

The latest developments in 3-D culture technologies concern the microchips and microfluidics approaches to create cell-culture microenvironments for tissue differentiation and reconstitution of the microenvironments of living kidneys by using

two or more cell types (Jung et al. 2011). With these "organs-on-chips," human physiology can be studied in a tissue-specific context and potentially might replace animal studies in drug development (Huh et al. 2012), although a large number of (technical) hurdles need to be taken until a prototype kidney with its multiple cell systems can be mimicked on a chip.

Traditionally, tissue models used for drug transport studies or drug–drug interaction determinations include kidney slices (isolated) perfused kidneys. Kidney slices in studying drug transport was reintroduced in the last decade with new cryopreservation methods that allow an accurate in vitro tool for prediction of in vivo renal drug uptake and metabolism (De Kanter et al. 2002). Disadvantages are that rates of uptake are much lower than those observed in vivo and this tissue appeared to be unsuitable for studying drug efflux. In the 1980s, Ullrich and coworkers contributed significantly to the knowledge on structure-transport relation of renal organic anion and organic cation transport by using in vivo stopped-flow capillary microperfusion studies of rat kidney (Ullrich et al. 1984). The ex vivo isolated perfused kidney allows accurate determination of drug clearance under controlled conditions and in the absence of non-renal effects (Maack 1980). The viability of 3–4 h for both preparations is acceptable and the model is also suitable for studying transport under disease conditions (Heemskerk et al. 2007, 2008). A nonfiltering isolated perfused rat kidney model, with preserved renal perfusate flow and cellular integrity, also permits the study of proximal tubular transport independent of luminal events (Maack 1980). Furthermore, by using a single-pass perfusion system, the different membrane transport rates involved in excretion, viz. passive or facilitated diffusion, carrier-mediated uptake, intracellular accumulation, and secretion, can be determined by indicator dilution (Hori et al. 1988). Perfused kidney has shown its use in studying pharmacokinetics in transporter mutant animals as well (Masereeuw et al. 2003). In addition, an in situ mouse kidney perfusion model has been described, with a carotid artery cannula for measurement of blood pressure and for blood sampling, and cannulated bladder for urine sampling. In this way, blood pressure, renal plasma flow, and renal clearance of drugs can be determined in anesthetized mice (Tsuruoka et al. 2001). While technically challenging, the mouse perfusion model offers the great advantage of using the single and multiple transporter knockout models currently available.

10.3.5   Translational Models

In man, pharmacokinetic studies are usually limited to analysis of plasma disappearance curves and urinary excretion data due to obvious ethical reasons. Therefore, the majority of in vivo transport studies are performed in laboratory animals, such as rats and mice, but larger animals are used as well especially when metabolism has to be taken into account. Various animal models have been developed and/or evaluated to study drug transport in absence or malfunction of a transporter protein. These may be provided by spontaneous mutation of a gene

resulting in disturbances, but also transgenic and gene-knockout manipulations have provided new and potentially powerful approaches for studying the functional and pathologic roles of transporter proteins, as described for example in *Bcrp* knockout mice (Jonker et al. 2002).

Drug excretion in vivo is described best by a physiologically based pharmacokinetic model, which includes all functional characteristics of the kidney that determine the excretion of drugs. These include protein binding, renal plasma flow, urine flow, glomerular filtration, tubular secretion, and cellular retention (Russel et al. 1987). Integration of in vitro findings are required for a better insight in renal drug handling, drug interactions, (hormonal) regulation of drug transport, and interindividual variability. In addition, all individual compartments of biological systems can be incorporated into multi-compartment models by using data empirically obtained from in vitro and animal studies (Zhao et al. 2011).

Novel systems models such as the Simcyp simulator (www.simcyp.com) have been developed to simulate drug pharmacokinetics and pharmacodynamics in virtual populations, with which drug–drug interactions and pharmacokinetic outcomes in clinical populations can be predicted. This platform uses databases that contain human physiological, genetic, and epidemiological information, which can be integrated with in vitro and clinical data to allow predictions (Johnson et al. 2010; Rowland et al. 2011).

10.4 Conclusion

The last decade has witnessed rapid technological progress in the field of transporter research, which has also greatly accelerated the gain of knowledge on renal drug transporters. A wealth of information has been generated about individual transporters by using molecular cloning techniques and functional characterization in over-expression systems, but much remains to be resolved regarding the coordinated action and regulation of the influx and efflux transporters in a proximal tubular cell as an integrated system. Although knockout mice have provided valuable insight into the in vivo role of different renal transporters, these studies need to be interpreted with some caution because of compensatory mechanisms and species-related differences in transporter expression and substrate specificity.

There is still a long way to go before we will be able to make accurate predictions of the renal clearance and exposure of drugs on the basis of the kinetic characteristics of individual transport proteins. Quantitative information on activity, substrate specificity, interindividual variation and abundance of transport proteins, as already available for many drug metabolizing enzymes, is required for physiologically based pharmacokinetic modeling and simulation of drug handling by the kidney. An important step has been made by the recent development of proteomics-based LC-MS/MS methods that enable the successful determination of absolute protein concentration levels of transporters in over-expression systems, proximal tubular cells, and human kidney tissue, which are useful to feed into the computer models

as in vitro-in vivo scaling factors (Ohtsuki et al. 2011). Currently, the lack of availability of a comprehensive in vitro model system of the proximal tubule, as predictive as for example Caco-2 cells are for intestinal transport, is a major limitation. While technically challenging, there is a great deal of promise in 3-D renal proximal tubular cell-culture systems with the potential of reconstructing the complex dynamic interplay among all the different transporters.

Personalized medicine through individualization of drug therapy is an important challenge for the future. The activity of transporter proteins may be influenced by genetic variation, which can be investigated by over-expression of the variants in a cellular system. But to get a picture of the actual impact on renal drug clearance, in vivo studies are required in human subjects with genetic polymorphisms to define the clinical relevance of certain transporters and to refine and validate the in silico models. As compared to the redundancy in renal organic anion transporters, the transporter-mediated renal excretion of cationic drugs seems to be more susceptible to drug–drug interactions and genetic variation. Based on current insights, it is expected that combined genetic polymorphisms in OCT2 and MATE1/MATE2-K variants could have important implications for cationic drug clearance and renal toxicity (Nies et al. 2011).

In summary, important advances have been made in the study of renal drug transporters. Whereas the functional characteristics of individual transporters have been relatively well-defined, there is a great need for comprehensive proximal tubular cell models and improved extrapolation of in vitro data to the clinical situation. Technical developments in molecular biology, tissue engineering, and systems pharmacology will provide new approaches to reach the ultimate goal of accurately predicting renal drug clearance, toxicity, and drug–drug interactions in an individual patient before the drug is actually administered.

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Chapter 11
The Role of Transporters in Drug Development: Regulatory Science Perspectives from the FDA

Lei Zhang and Shiew-Mei Huang

Abstract Pharmacokinetic drug interactions can lead to altered systemic exposure and varied drug response. Evaluation of a new molecular entity's (NME's) drug–drug interaction (DDI) potential is therefore an integral part of drug development and regulatory review prior to its market approval. Transporters are expressed in varying abundance in all tissues in the body where they govern the access of molecules to cells or their exit from cells, thereby controlling the overall distribution of drugs to their intracellular site of action. Clinically relevant interactions mediated by transporters are of increasing interest in drug development. Research in this emerging area has revealed that drug transporters, acting alone or in concert with drug metabolizing enzymes, can play an important role in modulating drug absorption, distribution, metabolism, and excretion, thus affecting the pharmacokinetics and/or pharmacodynamics of a drug. The newly published draft drug interaction guidance by the Food and Drug Administration (FDA) in 2012 includes updated recommendations in addressing transporter-mediated drug interactions with various decision trees to help guide drug development and regulatory review. This chapter discusses, from a scientific perspective, role of transporters in drug development with a focus on transporter-mediated DDIs. First, transporter-related recommendations in the recent FDA's draft drug interaction guidance are discussed. Second, additional transporters that are emerging to be important in drug disposition are discussed. Third, recent review examples and transporter-related labelings are presented. Finally, future directions are discussed.

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Abbreviations

ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism, and excretion
AUC	Area under the plasma concentration-time curve
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
CL_r	Renal clearance
CL_{total}	Total clearance
C_{max}	Maximal plasma concentration
cMOAT	Canalicular multispecific organic anion transporter (also named MRP2)
DDI	Drug–drug interaction
F_aF_g	Fraction of dose of inhibitor which is absorbed
FDA	Food and Drug Administration
f_u	Fraction unbound
GFR	Glomerular filtration rate
$[I]_1$	Mean steady state total (free and bound) C_{max} following the highest proposed clinical dose
$[I]_2$	Dose of inhibitor (in mol)/250 mL
$I_{in,max}$	Estimated maximum inhibitor concentration at the inlet to the liver
ITC	International transporter consortium
K_a	Absorption rate constant
LST	Liver-specific transporter
MATE	Multidrug and toxic compound extrusion transporter
MRP	Multidrug resistance-associated protein
NDA	New drug application
NME	New molecular entity
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PD	Pharmacodynamics
PFiC2	Progressive familial intrahepatic cholestasis type 2
P-gp	P-glycoprotein
PK	Pharmacokinetics
PMC	Postmarketing commitment
PMR	Postmarketing requirement
Q_h	Estimated hepatic blood flow
SGLT2	Sodium-glucose cotransporter 2
SLC	Solute carrier
TEA	Tetraethyl ammonium

11.1 Introduction

Transporters are membrane-bound proteins that control the access of endogenous substances and xenobiotics (drugs) to various sites of the human body. In contrast to metabolizing enzymes, which are largely concentrated in the liver and intestine, transporters are present in all tissues in the body and play important roles in drug absorption, drug distribution, tissue-specific drug targeting, and elimination, thus influencing drug pharmacokinetics (PK) and pharmacodynamics (PD) (both benefit and risk). Transporters can also work in concert with metabolizing enzymes in affecting a drug's PK and PD. As discussed in previous chapters, similar to metabolizing enzymes, transporters have binding sites that are saturable and can be inhibited or induced.

Transporters are expressed in varying abundance in all tissues in the body where they govern the access of molecules to cells or their exit from cells, thereby controlling the overall distribution of drugs to their intracellular site of action. Changes in transporter expression or activity via either genetic factors or drug interactions can contribute to variability in drug exposure and response. Many drug–drug interactions (DDIs) that cannot be explained by interactions at the metabolizing enzyme level may be mediated by transporters (Giacomini et al. 2010; Zhang et al. 2010; Zhang et al. 2011). One example is the interaction between cyclosporine and rosuvastatin. Cyclosporine increased rosuvastatin exposure by sevenfold in heart transplantation patients as compared to healthy subjects (Simonson et al. 2004). This interaction was "unexpected" because rosuvastatin is not extensively metabolized by Cytochrome P450 enzymes and it is also not transported by P-glycoprotein (P-gp), both of which are inhibited by cyclosporine. Recent studies suggested that OATP1B1 (organic anion transporting polypeptide 1B1), OATP1B3, and BCRP (breast cancer resistance protein) are involved in rosuvastatin disposition and may have contributed to this interaction (Ho et al. 2006; Niemi 2010; Xia et al. 2007).

From the human genome project, more than 400 transporters are identified. These transporters belong to one of two superfamilies: ATP-binding cassette (ABC) and solute carrier (SLC). The advancement of molecular cloning of various transporters and in vitro cell overexpression system to study interaction of drugs at the transporter level has enabled researchers to examine the underlying mechanism of DDIs. This improved understanding has provided the foundation to potentially predict in vivo drug interaction based on in vitro assays. For example, it was found that many statin drugs are OATP1B1 substrates; their interactions with cyclosporine (an OATP1B1 inhibitor) are therefore "anticipated." In addition, recent findings that many HIV protease inhibitors are OATP1B1/OATP1B3 inhibitors (Annaert et al. 2010) are critical in the design of needed clinical drug interaction studies in order to manage myriad of potential drug interactions between HIV protease inhibitors and other concomitantly administered drugs, including statins (http://www.fda.gov/Drugs/DrugSafety/ucm293877.htm).

Transporters have been drug targets for novel treatments. For example, drugs have been developed to be selective inhibitors of sodium-glucose cotransporter 2 (SGLT2) as potential therapeutics for treatment of type 2 diabetes (Pfister et al. 2011). Drugs that are substrates for a highly concentrative, broad-specific amino acid transporter (SLC6A14) may be developed for cancer treatment, e.g., estrogen-receptor positive breast cancer (Karunakaran et al. 2011).

This chapter discusses, from a regulatory science perspective, role of transporters in drug development with a focus on transporter-mediated DDIs. Transporter-related recommendations in the recent Food and Drug Administration's (FDA's) revised draft drug interaction guidance are discussed. Additional transporters that are emerging to be important in drug development are also discussed. In addition, recent review examples and transporter-related labeling are presented. Finally, future directions are discussed.

11.2 Regulatory Perspectives with Regard to Studying of Transporters During Drug Development

11.2.1 FDA Guidance Development Regarding Evaluation of Transporters During Drug Development

As part of the drug development strategy, transporters have been studied and evaluated during drug development (Chap. 14). This evaluation has become an important part of the new drug submission and approval process because transporters can play a key role in safety by mediating DDIs. The importance of transporter-mediated drug interactions has been discussed at FDA advisory committee meetings in recent years (November 3–4, 2004, http://www.fda.gov/ohrms/dockets/ac/cder04. html#PharmScience and October 18–19, 2006, http://www.fda.gov/ohrms/dockets/ ac/cder06.html#PharmScienc). Based on these committee discussions, in the 2006 FDA draft drug interaction guidance, the FDA recommended that sponsors study certain types of transporter-mediated drug interactions (http://www.fda.gov/Drugs/ DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ ucm093606.htm). Particular attention was paid to P-gp, which was thought to be a key transporter affecting the pharmacokinetics of digoxin, a narrow therapeutic index drug. Studying a new molecular entity's (NME's) inhibition or induction potential with P-gp is clinically relevant to appropriate dosing with digoxin and other molecules with similar characteristics.

Within more than 400 transporters in human genome, only about 30 transporters are found to date to be involved in absorption, distribution, metabolism, and excretion (ADME) processes and can be subjected to drug interactions. Besides P-gp, a reasonable question is what other transporters may be important and should be studied during the course of drug development? In 2007, an International Transporter

Consortium (ITC), which includes members from academia, industry, and the FDA, was formed with the goal of determining transporters that are of emerging importance in clinical drug interactions, establishing standards for in vitro evaluation of transporter-based interactions that may reduce the need for in vivo studies, and achieving, where possible, a consensus on the current knowledge of transporters in drug development (Huang and Woodcock 2010; Huang et al. 2010). The ITC organized an FDA critical path initiative-funded transporter workshop in October 2008 and authored a transporter whitepaper that was published in Nature Reviews Drug Discovery in March 2010 (Giacomini et al. 2010). The publication shared experiences, stimulated further discussions, and provided strategic directions in the following scientific areas: key transporters with clinical implications, in vitro methodologies, and decision trees on key transporters as to when to conduct in vitro and in vivo drug interaction evaluations (Giacomini et al. 2010).

The 2010 ITC paper discussed the following transporters as established and may be appropriate to evaluate during drug development: P-gp, BCRP, OATP1B1, OATP1B3, Organic Cation Transporter 2 (OCT2), and organic anion transporters (OAT1 and OAT3) (Giacomini et al. 2010). This was based on compelling clinical evidence that these transporters are involved in drug absorption, disposition, and/or drug interactions. Recent research indicates the important role of various transporters in the absorption (e.g., intestinal P-gp and BCRP), distribution (e.g., P-gp at the blood–brain barrier, OATP1B1, and OATP1B3 in the liver), and excretion (e.g., organic anion and cation transporters, OAT1, OAT3, and OCT2 in the kidney) of drugs. Several drugs (e.g., quinidine, verapamil, itraconazole) can increase plasma levels of digoxin by inhibiting the efflux transporter, P-gp, at the intestinal level. Plasma levels of many statins, including simvastatin, atorvastatin, pravastatin, and rosuvastatin, can be increased by inhibitors of hepatic uptake transporters (OATP1B1), such as cyclosporine, lopinavir/ritonavir, or eltrombopag. In addition, probenecid, a general inhibitor of OAT1/3, has been shown to increase the plasma levels of furosemide, tenofovir, and ciprofloxacin, possibly by the inhibition of the active renal tubular secretion of these substrates. Table 11.1 lists selected clinically relevant transporter-based DDIs. Many of these interactions resulted in two- to threefold increases in systemic exposure of the substrates; some were more than ninefold (e.g., when comparing pravastatin levels in the presence of cyclosporine in transplant patients with those without cyclosporine in healthy subjects). Other interactions resulted in decreases in plasma levels (e.g., tipranavir/ritonavir and loperamide). It is therefore important to consider, during drug development, which transporters can affect the ADME of an investigational drug and how the investigational drug can affect other drug's ADME due to its effect on transporters.

Following the publication of the ITC whitepaper, FDA discussed transporter-mediated drug interactions at an advisory committee meeting in March 2010 (http://www.fda.gov/AdvisoryCommittees/Calendar/ucm201691.htm) to seek committee members' advice on whether the current science, technology, and clinical importance related to transporters would support the recommendation to evaluate the above-mentioned major transporters routinely during drug development. The seven transporters listed in the whitepaper were considered by the FDA

Table 11.1 Selected transporter-mediated clinically significant drug–drug interactions (modified from tables in Giacomini et al. 2010 and various literature sources)

Gene	Aliases[a]	Tissue	Function	Interacting drug	Substrate (affected drug)	Changes in substrate plasma AUC (AUC ratios)	References
ABC transporters of clinical importance in the absorption, disposition, and excretion of drugs							
ABCB1	P-gp, MDR1	Intestinal enterocyte, kidney proximal tubule, hepatocyte (canalicular), brain endothelia	Efflux	Dronedarone	Digoxin	2.6-fold	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm
				Quinidine	Digoxin	1.7-fold	Rameis (1985), Hager et al. (1979)
				Ranolazine	Digoxin	1.6-fold	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm, Jerling (2006)
				Tipranavir/ritonavir	Loperamide	0.5-fold	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm, Mukwaya et al. (2005)
				Tipranavir/ritonavir	Saquinavir/ritonavir	0.2-fold	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm
ABCG2	BCRP	Intestinal enterocyte, hepatocyte (canalicular), kidney proximal tubule, brain endothelia, placenta, stem cells, mammary gland (lactating)	Efflux	GF120918	Topotecan	2.4-fold	Kruijtzer et al. (2002)
				Curcumin	Sulfasalazine	3-fold	Kusuhara et al. (2012)
SLC transporters of clinical importance in the disposition and excretion of drugs							
SLCO1B1	OATP1B1	Hepatocyte (sinusoidal)	Uptake	Lopinavir/ritonavir	Bosentan	5–48-fold[b]	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm
	OATP-C			Cyclosporine	Pravastatin	9.9-fold	Neuvonen et al. (2006), Hedman et al. (2004)
	OATP2 LST-1			Rifampin (single dose)	Glyburide	2.3-fold	Zheng et al. (2009)

Gene	Transporter	Tissue	Direction	Inhibitor	Substrate	Fold change	References
SLCO1B3	OATP1B3, OATP-8	Hepatocyte (sinusoidal)	Uptake	Cyclosporine	Rosuvastatin	7.1-fold[c,d,e]	Simonson et al. (2004)
				Cyclosporine	Pitavastatin	4.6-fold[e]	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm
				Lopinavir/ritonavir	Rosuvastatin	2.1-fold[e]	http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm, Kiser et al. (2008)
SLC22A2	OCT2	Kidney proximal tubule	Uptake	Cimetidine	Dofetilide	1.5-fold	Abel et al. (2000)
				Cimetidine	Pindolol	1.5-fold	Somogyi et al. (1992)
				Cimetidine	Metformin	1.4-fold[f]	Somogyi et al. (1987), Somogyi and Muirhead (1987)
SLC22A6	OAT1	Kidney proximal tubule, placenta	Uptake	Probenecid	Cephradine	3.6-fold	Li et al. (2006), Mischler et al. (1974)
				Probenecid	Cidofovir	1.5-fold	Li et al. (2006), Cundy (1999)
				Probenecid	Acyclovir	1.4-fold	Li et al. (2006), Laskin et al. (1982)
SLC22A8	OAT3	Kidney proximal tubule, choroid plexus, brain endothelia	Uptake	Probenecid	Furosemide	2.9-fold[g]	Li et al. (2006)

BCRP breast cancer resistance protein, *P-gp* P-glycoprotein, *MDR* multidrug resistance, *LST* liver-specific transporters, *OATP* organic anion transporting polypeptide, *OCT* organic cation transporter, *OAT* organic anion transporter

[a] Implicated transporter refers to the likely transporter; however, because the studies are in vivo, it is not possible to assign definitively specific transporters to these interactions

[b] Minimum predose plasma level (Ctrough) data from day 4 (48-fold), day 10 (5-fold) after coadministration

[c] Interaction could be partly mediated by BCRP

[d] Based on cross-study comparisons

[e] Interaction could be partly mediated by OATP1B1

[f] Interaction could be partly mediated by MATE-1/MATE-2K

[g] Interaction could be partly mediated by OAT1

Advisory Committee as being well demonstrated to play a role in DDIs in humans and should be considered for routine evaluation during drug development. The FDA's 2012 draft drug interaction guidance (http://www.fda.gov/downloads/ Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362. pdf) included recommendations on when to evaluate transporter-based drug interactions.

11.2.2 Highlights of FDA's 2012 Draft DDI Guidance on Transporter Evaluation During Drug Development

The purpose for transporter-mediated DDI studies is to ensure patient safety for marketed drugs and NMEs during clinical trials. Two important questions should be addressed before determining whether a transporter study is to be conducted:

1. Will the new drug (NME) become unsafe if a marketed drug inhibits a transporter that affects NME's exposure levels, i.e., does the NME's level depend on a given transporter?
2. Will the NME make other marketed drugs unsafe by inhibiting a transporter that a marketed drug is a substrate for?

The first question can be addressed by assessing whether the NME is a substrate of major transporters and the second by studying whether the NME is an inhibitor of major transporters.

To study an NME as a substrate of transporters, the ADME properties (major route of elimination) of the NME and the location of major transporters in human organs are key points to consider (see Fig. 11.1). For example, if the NME is highly metabolized or mainly eliminated by biliary secretion, liver transporters (OATP1B1, OATP1B3, P-gp, and BCRP) may be important for its disposition. Conversely, if a drug is mainly eliminated by renal elimination, then renal transporters such as OAT and OCT should be considered. In addition to OAT and OCT, multidrug and toxin extrusion transporters (MATEs) in the kidney may be important as well (see more discussion below in Sect. 11.2.3). The NME's physicochemical properties and structure can also be important determinants for its being a substrate for certain transporters. For example, OCTs and OATs mainly recognize low molecular weight cationic drugs and anionic drugs, respectively. However, there are exceptions; for example, cimetidine is recognized as a substrate for both OCT2 and OAT3.

Decision models to determine if an NME is a substrate for various transporters are included in the 2012 FDA draft drug interaction guidance (http://www.fda.gov/ downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ UCM292362.pdf). In the FDA recommendations, an in vitro overexpression cell system that is transfected with a specific transporter tested with appropriate positive and negative control drugs can be one of systems to evaluate the NME as a substrate for that transporter. In vivo contribution of the transporter to the NME's disposition can be evaluated with either a specific in vivo inhibitor or in subjects with different
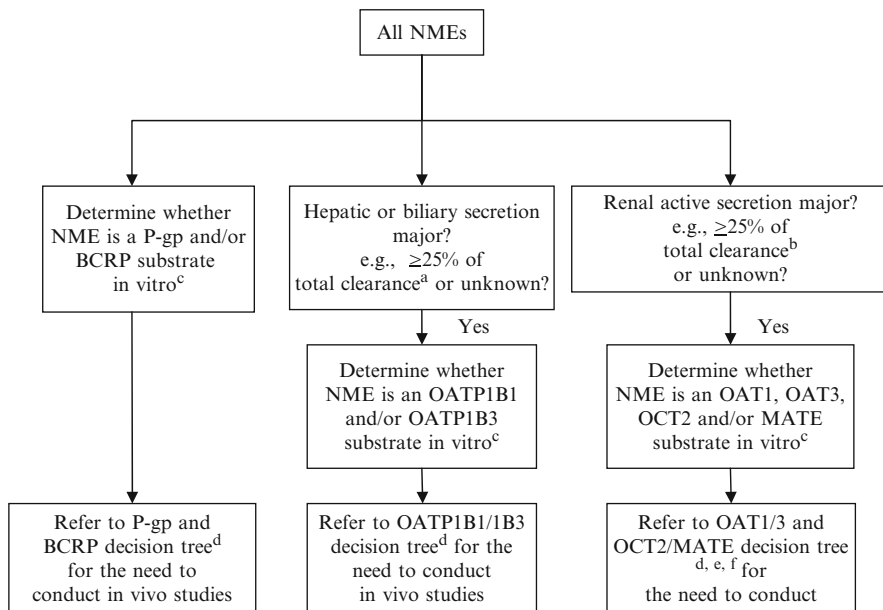
Fig. 11.1 Evaluation of new molecular entities (NMEs) as substrates for P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2/MATE transporters (modified from Giacomini et al. 2010; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf)

[a] Biliary secretion can be estimated from preclinical data, in vitro hepatocyte uptake data or radio-labeled ADME data, and non-renal clearance data

[b] % Active renal secretion was estimated from $(CL_r – fu*GFR)/CL_{Total}$

[c] The sponsor has the option to use in vitro tools first for the evaluation

[d] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf

[e] http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm

[f] Zamek-Gliszczynski, MJ et al. 2012

genotypes if the transporter has known polymorphism. A Biopharmaceutics Classification System (BCS) Class 1 drug that is highly soluble and highly permeable is not likely to be limited by an efflux transporter for its absorption even if it is recognized as a substrate in vitro. Whether this may apply for other BCS class drugs (i.e., Class 2 drugs) needs to be determined further.

To determine whether an NME is an inhibitor of major transporters, the 2012 FDA draft drug interaction guidance has also recommended decision trees to help determine when an in vivo clinical study is needed based on in vitro data (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf). The trees that were constructed included relatively conservative criteria for each decision point so as to avoid false negatives, i.e., a conclusion of no drug interaction when there may be drug interactions. The FDA's recommendations in the 2012 draft guidance differ from those from the ITC whitepaper in that the total maximum inhibitor concentration instead of unbound concentration
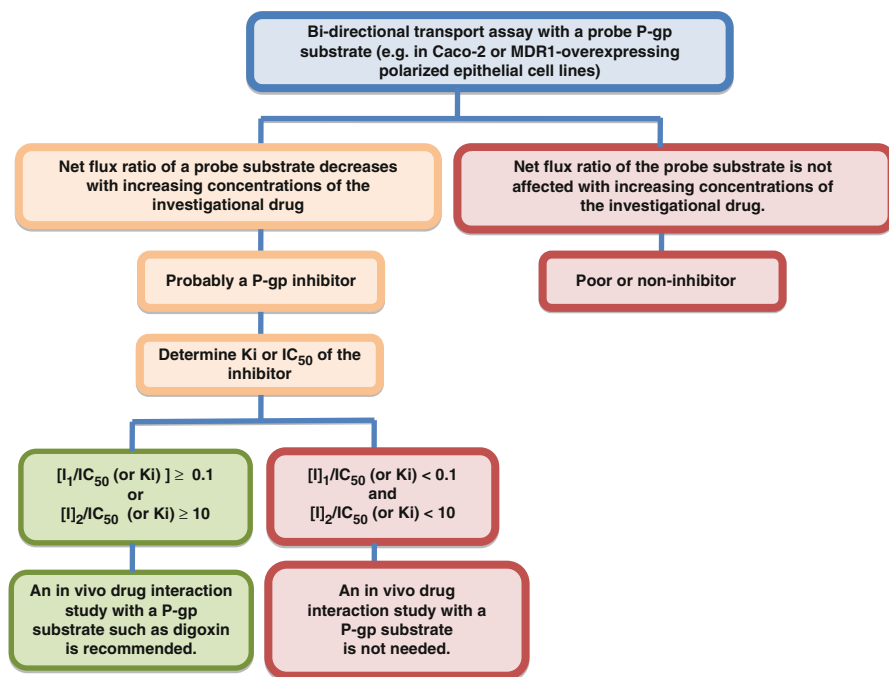
Fig. 11.2 P-gp/BCRP inhibition tree. Decision tree to determine whether an investigational drug is an inhibitor of P-gp and when an in vivo clinical study is needed. A similar model can be applied to a BCRP inhibitor (modified from figure in reference (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf)). $[I]_1$ represents the mean steady-state total (free and bound) C_{max} following administration of the highest proposed clinical dose. $[I]_2 =$ Dose of inhibitor (in mol)/250 mL (if IC_{50} is in a molar unit). For IC_{50} determination, a unidirectional assay (e.g., B to A) based on the probe substrate can also be considered

was used in the P-gp/BCRP and OATP decision trees (Figs. 11.2 and 11.3). In addition, in the OATP inhibition tree, a cut-off value of 1.25 for "*R*" instead of 2 was recommended. These modifications were based on limited data available to date for drugs that have both in vitro and in vivo interaction data (Agarwal et al. 2012; Vaidyanathan et al. 2012). Compared to the criteria proposed in the ITC whitepaper, the new criteria proposed in the FDA draft drug interaction guidance showed a lower number of false negative cases (Agarwal et al. 2012; Vaidyanathan et al. 2012). For example, in Table 11.2 we compared the criteria used in the ITC whitepaper and the FDA guidance for the OATP1B1 inhibition decision tree. Based on a dataset of 28 inhibitor–substrate pairs that have both in vitro OATP1B1 inhibition and in vivo interaction data, the ITC criteria (two steps) showed an overall 7 false negative cases and 2 false positive cases and the FDA criteria (two steps) showed an overall 4 false negative cases and 2 false positive cases. Interestingly, using the total C_{max}/IC_{50} in the first step as proposed in the FDA decision tree, there was 0 false negative and 2 false positives. By using an *R* of 1.25 in the second step in the two-step
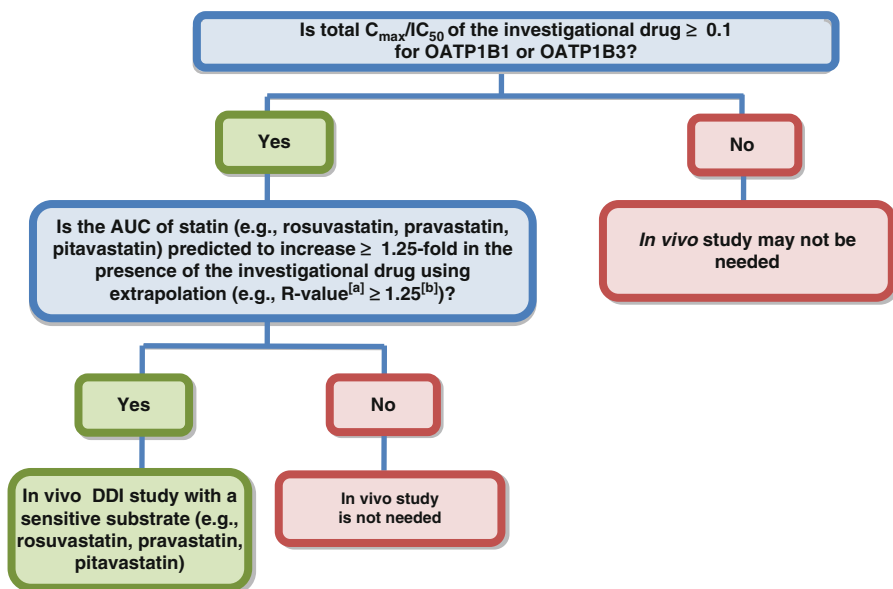
Fig. 11.3 OATP inhibition tree. Decision tree to determine whether an investigational drug is an inhibitor of OATP1B1 or OATP1B3 and when an in vivo clinical study is needed (modified from figures in reference (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf))

[a]R-value = 1+ (fu × $I_{in,max}$/IC_{50}), where, $I_{in,max}$ is the estimated maximum inhibitor concentration at the inlet to the liver and is equal to: C_{max} + (k_a × Dose × F_a F_g/Qh). C_{max} is the maximum systemic plasma concentration of inhibitor; Dose is the inhibitor dose; F_aF_g is the fraction of the dose of inhibitor which is absorbed; k_a is the absorption rate constant of the inhibitor and Qh is the estimated hepatic blood flow (e.g., 1500 mL/min). If Fa Fg values and ka values are unknown, use 1 and 0.1 min^{-1} (Ito et al. Pharmacol Rev. 50:387–412, 1998) for FaFg and ka, respectively because the use of theoretically maximum value can avoid false-negative prediction. For drugs whose fu values are less than 0.01 or fu cannot be accurately determined due to high protein-binding, then assume fu = 0.01, to err on the conservative side to avoid false negative predictions

[b]These are the suggested values according to the upper limit of equivalence range. We are open to discussion based on sponsors' interpretation

process, 2 false positives remained and there were 4 false negative cases. The available, limited data may suggest that one step instead of two steps may be sufficient as an initial screening to avoid false negative cases. However, the false negative cases based on two-step approaches may be attributed to mechanisms other than OATP1B1 inhibition for the interaction observed or to the variability in IC_{50} values determined in various laboratories as well as potential substrate-dependent IC_{50} values. For example, the cyclosporine–pitavastatin interaction represented one of the false negative cases based on R calculations. Using the FDA criteria, cyclosporine is classified as an inhibitor of OATP1B1 in vitro using total C_{max}/IC_{50} in the first step; however, it was not classified as an inhibitor of OATP1B1 in vitro based on R in the second step (i.e., $R < 1.25$). In vivo interaction data showed a significant increase in

Table 11.2 OATP1B1 inhibition potential prediction: comparison of total C_{max} vs. unbound C_{max} in the calculation of step 1 and R of step 2 vs. observed in vivo interactions based on ITC and FDA-proposed criteria

Inhibitor	Affected drug	In vivo AUC fold change[a]	IC$_{50}$ in μM	In vitro substrate used for IC$_{50}$ determination	ITC step 1: unbound C_{max}/IC$_{50}$ ≥ 0.1? (value)	FDA step 1: total C_{max}/IC$_{50}$ ≥ 0.1? (value)	Step 2: predicted R value[a]/ ≥2 (ITC)? or ≥1.25 (FDA)?	Category based on ITC two steps	Category based on FDA two steps	Category based on FDA first step only
Atazanavir	Pitavastatin	1.31	1.7	Cholyl-glycylamido-fluorescein (CGamF)[b]	Yes (0.6)	Yes (4.5)	2.1	TP	TP	TP
Atorvastatin	Repaglinide	1.14	0.7	Estradiol-17-β-glucuronide[b]	No (0.0006)	No (0.03)	1.0	TN	TN	TN
Cyclosporin	Atorvastatin	7.44–15.30	0.021	Atorvastatin	Yes (3.7)	Yes (53)	8.7	TP	TP	TP
Cyclosporin	Bosentan	1.97	0.3	Bosentan	Yes (0.22)	Yes (3.2)	1.5	FN	TP	TP
Cyclosporin	Cerivastatin[c]	3.80–4.75	0.238	Cerivastatin	Yes (0.26–0.46)	Yes (3.6–6.5)	1.5–1.7	FN	TP	TP
Cyclosporin	Fluvastatin	1.89–3.55	0.021	Atorvastatin[b]	Yes (1.5)	Yes (21)	4.5	TP	TP	TP
Cyclosporin	Pitavastatin	4.51	0.7	Pitavastatin	No (0.045)	Yes (0.64)	1.09	FN	FN	TP
Cyclosporin	Pravastatin	9.92–22.83	0.021	Atorvastatin[b]	Yes (1.5)	Yes (21)	4.5	TP	TP	TP
Cyclosporin	Rosuvastatin	7.08	0.31	Rosuvastatin	Yes (0.14)	Yes (2.1)	1.28	FN	TP	TP
Cyclosporin	Repaglinide	2.43	0.021	Atorvastatin[b]	Yes (1.8)	Yes (26)	4.0	TP	TP	TP
Cyclosporin	Simvastatin[c]	2.55–7.97	0.021	Atorvastatin[b]	Yes (2.7)	Yes (38)	6.6	TP	TP	TP
Darunavir/ritonavir	Pitavastatin	0.74	3.1	Cholyl-glycylamido-fluorescein (CGamF)[b]	Yes (0.14)[e]	Yes (2.9)[e]	2.4[e]	FP	FP	FP

Erythromycin	Bromocriptine	3.7[d]	Estradiol-17-β-glucuronide[b]	9.2	No (0.08)	Yes (0.52)	1.13	FN	FN	TP
Gemfibrozil	Atorvastatin	1.34	Atorvastatin	32	Yes (0.1)	Yes (3)	1.25	FN	TP	TP
Gemfibrozil	Pitavastatin	1.45	Pitavastatin	38	No (0.08)	Yes (2.7)	1.21	FN	FN	TP
Gemfibrozil	Repaglinide	7.42	Rosuvastatin[b]	4	Yes (0.77)	Yes (25)	2.96	TP	TP	TP
Gemfibrozil	Rosuvastatin	1.88	Rosuvastatin	4	Yes (0.77)	Yes (25)	2.96	TP	TP	TP
Lopinavir/ritonavir	Bosentan	5.22	CGamF[b]	0.5	Yes (0.7)[f]	Yes (47)[f]	2.97[f]	TP	TP	TP
Lopinavir/ritonavir	Pitavastatin	0.8	CGamF[b]	0.5	Yes (0.7)[f]	Yes (47)[f] (false positive)	2.97[f]	FP	FP	FP
Lopinavir/ritonavir	Rosuvastatin	1.6–2.07	CGamF[b]	0.5	Yes (0.7)[f]	Yes (47)[f]	2.97[f]	TP	TP	TP
Rifampin	Atorvastatin	4.30–9.33	Atorvastatin	3.25	Yes (0.96)	Yes (3.8)	2.32	TP	TP	TP
Rifampin	Bosentan	6.00	Bosenten	3.2	Yes (0.96)	Yes (3.9)	2.34	TP	TP	TP
Rifampin	Caspofungin	1.61	Estradiol-17-β-glucuronide[b]	0.6	Yes (5.2)	Yes (20.9)	8.14	TP	TP	TP
Rifampin	Pravastatin[c]	2.27	Estradiol-17-β-glucuronide[b]	0.6	Yes (5.2)	Yes (20.9)	8.14	TP	TP	TP
Sildenafil	Bosentan	1.33	Bosentan	1.5	No (0.02)	Yes (0.6)	1.05	FN	FN	TP
Simvastatin	Talinolol	0.99	Estradiol-17-β-glucuronide[b]	5	No (0.00002)	No (0.0004)	1.00	TN	TN	TN
Tiprinavir/ritonavir	Atorvastatin	9.36	Estradiol-17-β-glucuronide[b]	1.1	Yes (1.72)[g]	Yes (86)[g]	3.73[g]	TP	TP	TP
Tiprinavir/ritonavir	Rosuvastatin	1.37	Estradiol-17-β-glucuronide[b]	1.1	Yes (1.72)[g]	Yes (86)[g]	3.73[g]	TP	TP	TP

Table 11.2 (continued)

Summary

N=28		ITC 2-steps		FDA 2-steps		FDA 1 step (i.e., C_{max}/IC_{50})	
FN	TP	8	16	4	20	0	24
TN	FP	2	2	2	2	2	2
Accuracy = (TN+TP)/N*100 %		64 %		79 %		93 %	

TP true positive, *TN* true negative, *FP* false positive, *FN* false negative

[a] AUCR $\geq$ 1.25 is considered positive interaction

[b] IC_{50} values determined using a different substrate as the in vivo-affected drug

[c] Two different assays were used to determine cyclosporin concentrations (C_{max} following single dose and multiple dose were used)

[d] Interaction likely to be mediated by CYP3A

[e] Data shown are based on darunavir. R value from ritonavir was lower, 1.2. $F_a = 1$ and $K_a = 0.1$ min^{-1} were used in the calculation. If darunavir and ritonavir are combined, $R_{total} = 1 + (1.4+0.2) = 2.6$

[f] Data shown are based on lopinavir. R value from ritonavir was lower, 1.13. $F_a = 1$ and $K_a = 0.1$ min^{-1} were used in the calculation. If lopinavir and ritonavir are combined, $R_{total} = 1 + (1.97+0.13) = 3.1$

[g] Data shown are based on tipranavir. R value from ritonavir was lower, 1.27. $F_a = 1$ and $K_a = 0.1$ min^{-1} were used in the calculation. If tipranavir and ritonavir are combined, $R_{total} = 1 + (2.73+0.27) = 4$

pitavastatin plasma concentration when it was co-administered with cyclosporine that was thought to be mediated by OATP1B1 (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm) (see Table 11.2). One possible reason is that IC_{50} determined in vitro may be higher than in vivo potency. Alternatively, the interaction between these two drugs may be mainly due to BCRP inhibition in vivo rather than OATP1B1 inhibition. The BCRP decision tree, in this case, may be better in projecting the interaction potential between cyclosporine and pitavastatin. The I_1/IC_{50} and I_2/IC_{50} of cyclosporine for BCRP were estimated to be >0.1 and 10, respectively (Xia et al. 2007), indicating a positive interaction using the BCRP decision tree (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM292362.pdf). Therefore, multiple transporter decision trees may need to be considered for drugs that are substrates or inhibitors for multiple transporters to avoid false negatives. Mechanistic understanding of drug interaction is key when applying decision trees to predict drug interactions. In vitro assays will help provide information on a drug's potential as a substrate or inhibitor for certain transporters.

Concerns were expressed that an abundance of false positives will have a detrimental effect on the development of new drugs because of conducting studies that do not need to be conducted (i.e., these drugs have no drug interaction risk). Thus, a data-driven balance between false positive and false negative in deriving the cut-off criteria in the decision trees is clearly needed. The evolution and appropriate application of these decision trees will require constant monitoring and they should be revised as the knowledge base increases over time (Giacomini et al. 2010; Agarwal et al. 2012; Tweedie et al. 2013; http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm).

Drug interactions can occur by various mechanisms. Understanding the roles of metabolizing enzymes as well as transporters in a drug's ADME will provide a starting point to evaluate drug interactions during drug development. In vitro models to predict drug interaction potential are well established for CYP enzyme-based mechanisms, and are evolving for transporter-based mechanisms. The decision trees as described (in Figs. 11.1, 11.2, and 11.3 and in FDA draft guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf)) provide directions to link the in vitro data (as a substrate or an inhibitor) or other drug characteristics (e.g., physicochemical properties, in vivo ADME data) with their interaction potential. Various technologies and standards developed for the P450 enzymes have enabled us to understand possible P450-mediated drug interaction during drug development to support strategies intended to manage drug interactions to optimize the benefit/risk ratio in patient populations. Similarly, with the advancement of our understanding of transporters and development of various tools and standards to study transporters, we will be able to better predict transporter-mediated interactions.

Finally, the clinical significance of transporter-mediated DDIs needs to be interpreted in the framework of exposure–response relationship. Understanding transporters and their interactions will provide a mechanistic approach to explain

variability in a drug's pharmacokinetics, pharmacodynamics, efficacy and safety in human subjects, and to identify subgroups of patients at risk of developing adverse events or being undertreated.

11.2.3 Emerging Transporters

In March 2012, the ITC organized a second transporter workshop that included discussions of emerging transporters such as MATEs, multidrug resistance-associated proteins (MRPs), and bile salt export pumps (BSEPs) (Zamek-Gliszczynski et al. 2012; Hillgren et al. 2013).

11.2.3.1 MATEs

The multidrug and toxin extrusion transporter MATE1 (*SLC47A1*) is expressed in both kidney and liver cells at the apical side of the cell membrane, whereas MATE2-K (*SLC47A2*) is mainly expressed in the kidney (Giacomini et al. 2010; Damme et al. 2011; Masuda et al. 2006; Otsuka et al. 2005). Two isoforms of MATE2 have been identified, one of which, MATE2-K, has been characterized as a membrane transporter in the kidney (Masuda et al. 2006). Various drugs, including metformin, as well as endogenous substances, such as guanidine, have been shown to be substrates of MATE1 (Otsuka et al. 2005). MATE2-K, like MATE1, appears to transport an array of structurally diverse compounds, including many cationic drugs and endogenous compounds (Tanihara et al. 2007). Komatsu et al. (2011) characterized isoform 1 of MATE2 and showed that both human MATE2 (isoform 1) and MATE2-K (isoform 2) (1) operate in the kidney as electroneutral H+/organic cation exchangers; (2) express and localize in the kidney, with MATE2-K being slightly more abundant than MATE2; (3) transport tetraethyl ammonium (TEA); and (4) have similar inhibitor specificities. Since some substrates (e.g., metformin) or inhibitors (e.g., cimetidine) recognized by OCT2 are also recognized by MATEs (Tanihara et al. 2007), MATEs may act in concert with OCT2 to mediate the excretion of some drugs (Choi et al. 2011; Kusuhara et al. 2011). More evidence has shown that MATEs may play a role in the elimination of organic cationic drugs or in DDIs that was thought to be mediated by OCT2 in the kidney (Zamek-Gliszczynski et al. 2012; Masuda et al. 2006). Therefore, when evaluating drugs for their interaction potential with OCT2, their potential as a MATE substrate or inhibitor needs to be considered. MATEs (*SLC47A*) may be considered for prospective investigation along with OCT2 in drug development (Zamek-Gliszczynski et al. 2012; Hillgren et al. 2013).

11.2.3.2 MRPs

The MRP (*ABCC*) family of transporters is closely related and structurally similar to the MDR family. MRP transporters constitute nine members of the

ATP-binding cassette C subfamily (*ABCC1–6*, *10–12*). Other transporters in the ABCC subfamily are the cystic fibrosis transmembrane conductance regulator (*ABCC7*) and two sulfonylurea receptor isoforms (*ABCC8 and -9*) (Keppler 2011; Klaassen and Aleksunes 2010). Cloning, functional characterization, and cellular localization of most MRP subfamily members have identified them as ATP-dependent efflux pumps that transport a broad spectrum of endogenous and xenobiotic anionic substances across cellular plasma membranes (Keppler 2011; Klaassen and Aleksunes 2010).

MRP1 (*ABCC1*), MRP2 (*ABCC2*), and MRP4 (*ABCC4*) have been the most widely studied members of the MRP family in the context of PK and drug response. MRP1 was initially identified in lung cells which were known to not express P-gp and pumps anionic compounds (Cole et al. 1992). Substrates for MRP1 include anionic endogenous products; glutathione, glucuronosyl, and sulfate conjugates; and, in some cases, neutral molecules coupled to glutathione transport without conjugation. MRP2 (*ABCC2*) is similar to MRP1 except in its tissue distribution and localization. It is expressed on the canalicular membrane of hepatocytes and was formerly known as the canalicular multispecific organic anion transporter (cMOAT). The hepatobiliary and renal elimination of many drugs and their metabolites is mediated by MRP2 in the hepatocyte canalicular membrane and by MRP4 as well as MRP2 in the luminal membrane of proximal renal tubules. Therefore, inhibition of these efflux pumps affects PK unless compensation is provided by other ATP-dependent efflux pumps with overlapping substrate specificities. Genetic mutations in MRP2 cause Dubin–Johnson syndrome, a disease characterized by hyperbilirubinemia resulting from reduced transport of conjugated bilirubin into bile (Paulusma et al. 1997). MRP3 has been recently shown to transport phenolic glucuronide conjugates of acetaminophen, etoposide, methotrexate, and morphine from the basolaterol surface of hepatocytes into blood (Zelcer et al. 2005). MRP4 (*ABCC4*) has been shown to transport a number of endogenous substrates, such as eicosanoids, urate, conjugated steroids, folate, bile acids, and glutathione, as well as many drug substrates including cephalosporines, methotrexate, and nucleotide analog reverse transcriptase inhibitors (Schuetz et al. 1999; Russel et al. 2008). Based on preclinical or clinical data, a drug or its conjugates as MRP2 substrates may need to be considered along with other efflux transporters in the liver. The determination for studying a drug's inhibition on MRP2 may be based on preclinical and clinical observations of liver toxicity (Zamek-Gliszczynski et al. 2012; Hillgren et al. 2013).

11.2.3.3 BSEP

Enterohepatic circulation of bile acids is mediated by specific transporters in the hepatocytes and enterocytes (Klaassen and Aleksunes 2010). BSEP (*ABCB11*) is a transporter that is expressed exclusively on the canalicular side of hepatocytes and is involved in the biliary efflux of monovalent bile acids whereas MRP2 exports divalent and sulfated and/or glucuronidated bile acids and other conjugated anions including Phase II drug metabolites. Although BSEP primarily transports bile acids,

it can also transport drugs such as pravastatin (Hirano et al. 2005). A number of BSEP inhibitors have been identified (e.g., cyclosporine A, rifampicin, gliben-clamide) (Byrne et al. 2002).

Altered expression or function of bile acid transporters can be either a cause or a consequence of cholestasis. Progressive familial intrahepatic cholestasis type 2 (PFIC2) is caused by mutations in the *ABCB11* gene, which encodes BSEP (Jansen et al. 1999; Strautnieks et al. 1998). Mutations in the ABCB11 gene can lead to a rapid progressive hepatic dysfunction in early infancy. In such patients, the biliary bile salt levels can be reduced to less than 1 % of that in normal subjects. These defects or inhibition of BSEP may contribute to certain types of drug-induced cho-lestasis or other liver injury (Noe et al. 2005; Ogimura et al. 2011). Further research is needed to determine how drugs can be studied early in their development to assess their BSEP-related safety liabilities (Morgan et al. 2010). The determination for studying a drug's inhibition on BSEP may be based on preclinical and clinical observations of liver toxicity such as cholestasis (Zamek-Gliszczynski et al. 2012; Hillgren et al. 2013).

11.3 Inclusion of Transporter Information in Labeling of Recently Approved NMEs by the FDA

Labeling is an important communication tool for health care practitioners to understand risk–benefit profile of a drug. Managing DDI is an important compo-nent for minimizing adverse events related to polypharmacy. Agarwal et al. recently conducted a survey on the inclusion of transporter-related information in the package inserts (also referred to as PIs or labeling) of 183 NMEs (excluding biologics) approved between 2003 and 2011 (Agarwal et al. 2011, 2013). These analyses indicate that with recent advancement in the transporter research area, the FDA's 2006 draft drug interaction guidance may have encouraged drug com-panies to evaluate the role of transporters in a drug's ADME and incorporate transporter-related information in new drug applications (NDAs) as suggested by the increased percentage of NMEs with transporter information in the PIs approved in 2007–2011 (56 %) as compared to those of 2003–2006 (23 %) (Table 11.3 and Fig. 11.4). In vivo drug interaction studies with digoxin (without prior in vitro assessments) were conducted less frequently in the 2007–2011 period (3 %, 3/95), as compared to those in 2003–2006 period (15 %, 13/88), as indicated in the labelings. This finding may indicate that the FDA-proposed in vitro P-gp decision tree in the 2006 draft drug interaction guidance may have influenced the decision-making and negative in vitro inhibition data have reduced the need for in vivo digoxin drug interaction studies during drug development (Agarwal et al. 2012).

Table 11.3 Summary of transporter information in the NME labeling (2003–2011)

	2003–2006	2007–2011	Total
Total number of all approved NMEs	88	95	183
Number (%) of NME labelings that have information with specific transporter name(s)	21 (24 %, 21/88)	53 (56 %, 53/95)	74 (40 %, 74/183)
(a) P-gp	15 (75 %, 15/20)	49 (92 %, 49/53)	64 (88 %, 64/73)
(b) Other transporters	7 (33 %, 7/20)	20 (38 %, 20/53)	27 (36 %, 27/73)
Number (%) of NME labelings with in vivo digoxin DDI study data (without mentioning the involvement of specific transporters)	14 (16 %, 14/88)	3 (3 %, 3/95)	17 (9 %, 17/183)
Number (%) of NME labelings that include transporter-related information in the "Highlights" section of PI	6 (7 %, 6/88)	14 (15 %, 14/95)	20 (11 %, 20/183)

Modified from Supplemental Table 1 in Agarwal et al. (2013)
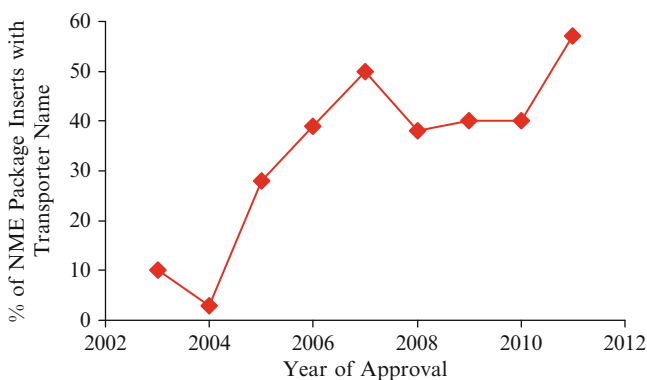
Fig. 11.4 Percentage of NME labelings with transporter information (2003–2011)

11.4 Transporter-Related Postmarketing Requirement/ Postmarketing Commitment (PMR/PMC)

In the past, FDA has used the word *postmarketing commitment* to cover both required and not required studies and clinical trials that sponsor conducted post drug approval. In 2007, a new section 505(o) of the Federal Food, Drug, and Cosmetic Act (the Act) (21 U.S.C. 355(o)) was added to section 901 of the Food and Drug Administration Amendments Act of 2007 (FDAAA) in 2007. Section 505(o)

(3) authorizes FDA to require certain postmarketing studies and clinical trials for prescription drugs and biological products. Under FDAAA, PMR studies are required by the FDA "if FDA becomes aware of new safety information," and PMC is agreed upon studies between FDA and the applicant but it is not required (Guidance for Industry 2011). The results from postmarketing studies or trials can help further refine the safety, efficacy, or optimal use of a product.

As the field of transporter research is evolving rapidly, transporter-related studies may not have been conducted during drug development or considered in the premarketing approval decision, especially for drugs in therapeutic areas for which there are major unmet medical needs, such as oncology. Therefore, the FDA has recently asked for postmarketing studies of potential transporter-mediated DDIs when appropriate. A review of recent PMC and PMR studies included in the NME approval letters (2007–2011) indicated that there were more than 20 PMC and PMR studies that were related to evaluation of transporter-based DDIs either in vitro or in vivo (Fan et al. 2012). Results from these studies, when completed, will provide helpful information in the label. Transporters could be an important determinant for safe and effective use of a drug. Because there is a lag time between drug approval and PMR/PMC fulfillment, the sponsors are encouraged to consider and collect relevant information related to transporters, when appropriate, during drug development and make it part of their drug development plan.

11.5   Recent Review Examples

We present some recent examples related to P-gp in NDA reviews in this section to illustrate how in vitro data may have helped to determine the need for in vivo DDI studies and be included in the labeling for guiding the drug usage.

Table 11.4 lists four NME examples (boceprevir, rilpivirine, ezogabine, and ticagrelor that were approved in 2011). All four NMEs contain in vitro inhibition data on P-gp (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm). Based on the P-gp decision tree (Fig. 11.2), i.e., using the ratio of the in vivo exposure (C_{max} or dose) and their corresponding in vitro parameters (IC_{50} values) as an indicator for their potential to inhibit P-gp in vivo, all four of them suggested positive inhibition. Among these four drugs, only ticagrelor had a follow-up in vivo study with digoxin (a P-gp substrate) during drug development and the information (positive in vivo data) was included in the "Highlights" of the labeling (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm). The other three drugs had a PMR study with digoxin in their approval letter. However, the in vitro P-gp inhibition data were presented variably in the labeling. Ezogabine's information is in the "Highlights" section on the metabolite inhibition on digoxin based on in vitro data, while boceprevir's in vitro P-gp inhibition information is in the "Drug Interactions" section and rilprivirine's labeling does not mention P-gp or digoxin. Considerations of each drug's therapeutic areas, other clinical pharmacology information (e.g., other significant drug interactions), and related clinical practice may have affected

Table 11.4 Selected examples of NME approved in 2011 with labeling information related to P-gp and/or with postmarketing requirement (PMR) studies

Drug name (brand name)	I_1/IC_{50} (>0.1?)	I_2/IC_{50} (>10?)	In vivo study with digoxin conducted at the time of approval?	Labeling (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm)	Regulatory outcome (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm)
Boceprevir (VICTRELIS)	0.13 (Yes)	246 (Yes)	No	Section 7.2: P-gp inhibitor based on in vitro "Boceprevir is a potential inhibitor of P-glycoprotein (P-gp) based on in vitro studies. The potential for a drug interaction with sensitive substrates of P-glycoprotein (e.g., digoxin) has not been evaluated in a clinical trial"	A PMR study with digoxin is recommended: "Conduct an in vivo drug-drug interaction trial between boceprevir and a sensitive substrate of P-glycoprotein (e.g. digoxin)"
Rilpivirine (EDURANT)	0.05 (No)	27 (Yes)	No	No mentioning of P-gp or digoxin	A PMR study with digoxin is recommended: "Conduct a clinical trial in healthy subjects to evaluate the effect of rilpivirine at steady state on the single dose pharmacokinetics of digoxin. The pharmacokinetics of digoxin when coadministered with rilpivirine (test arm) will be compared to the pharmacokinetics of digoxin by itself (reference arm). The primary digoxin pharmacokinetic parameters that will be evaluated are $AUC_{(0-\infty)}$, $AUC_{(0-t)}$, and C_{max}"

(continued)

Table 11.4 (continued)

Drug name (brand name)	I_1/IC_{50} (>0.1?)	I_2/IC_{50} (>10?)	In vivo study with digoxin conducted at the time of approval?	Labeling (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm)	Regulatory outcome (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm)
Ezogabine (POTIGA)	<0.06 (IC_{50} >100 μM) for the parent drug (no)	<53 (IC_{50} >100 μM) for the parent drug (indeterminant)	No	In Highlights: "N-acetyl metabolite of ezogabine may inhibit renal clearance of digoxin, a P-glycoprotein substrate. Monitor digoxin levels. (7.2)" Also in Sections 7.2 (Drug Interactions/Digoxin) and 12.3 (Clinical Pharmacology/Drug Interactions)	A PMR study with digoxin is recommended: "A clinical trial to evaluate the acetyl metabolite of ezogabine (NAMR) as an inhibitor of P-glycoprotein using digoxin as a probe substrate. Refer to the Agency's Guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072101.pdf) for more detailed recommendations regarding transporter-based drug-drug interactions"
	~0.6 for metabolite NAMR[a] (Yes)	Not Applicable for metabolite.			
Ticagrelor (BRILINTA)	0.17 (Yes)	88 (Yes)	Yes	In Highlights: "Monitor digoxin levels with initiation of or any change in BRILINTA. (7.4)" Also in Section 7.5 (Drug Interactions/Digoxin): "Digoxin: Because of inhibition of the P-glycoprotein transporter, monitor digoxin levels with initiation of or any change in BRILINTA therapy [see *Clinical Pharmacology (12.3)*]." And in Clinical Pharmacology Section of the labeling	N/A
			Positive interaction observed (AUC ↑ 28 %, C_{max} ↑75 %)		

PMR postmarketing requirement, *NAMR N*-acetyl metabolite of ezogabine
[a]The major metabolite (NAMR) was found to be an in vitro inhibitor of P-gp

these labelings. The publication of the new draft drug interaction guidance and the clinical pharmacology labeling guidance may help to have consistent labeling.

11.6   Conclusions and Future Directions

Currently, transporters are being studied to varying degrees during drug development across the industry. More research is needed to develop and optimize various technologies (e.g., in vitro, in silico, imaging) to better study transporters and drug interactions. Specific guidelines and decision trees have been provided in a white paper and FDA's draft DDI guidance for assessing transporter-mediated drug interactions for transporters with demonstrated clinical relevance: P-gp, BCRP, OCT2, OAT1, OAT3, OATP1B1, and OATP1B3 (Giacomini et al. 2010; http://www.fda. gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ UCM292362.pdf). With the advancement of molecular biology and availability of various cell lines expressing transporters, the in vitro systems have been increasingly used and will have a greater utility. However, many challenges remain despite recent progress. For example, in vitro assays have their physiological limitations in mimicking the in vivo situation, in particular, the interplay between enzymes and transporters and the possible compensatory increase in activities of one or more other transporters when the activity of a transporter is suppressed. Development of best practices of in vitro assays will facilitate greater utility of in vitro studies, minimize inter-laboratory variability, and increase data quality and data interpretation (Brouwer et al. 2013).

For in vivo studies, monitoring plasma concentrations in drug interaction studies may not reveal the interaction effect at the tissue level as interactions with transporters can affect a drug's tissue uptake and local concentrations leading to undesirable effects with no or little change in systemic exposure (Watanabe et al. 2010). Physiologically based pharmacokinetic (PBPK) modeling has been increasingly used during drug development and regulatory review in addressing DDIs (Zhao et al. 2011; Huang et al. 2013). PBPK models incorporating multiple processes involving both transporters and enzymes and other intrinsic and extrinsic patient factors may be developed to help address these complex questions (Huang and Rowland 2012). Furthermore, clear labeling and education efforts are needed in order to provide useful information to health care providers and patients about their individual risk of drug interactions related to transporters, and how to identify patients at risk.

Continual collaboration among academia, industry, and government agencies including regulatory agencies, such as the model set by ITC, is key to move the science forward in the spirit of the critical path initiative of FDA (Huang and Woodcock 2010).

Disclaimer The views presents in this chapter are those of authors and do not necessarily reflect the official view of the FDA.

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Chapter 12
Industrial Evaluation of Drug Transporters in ADME

Mitchell E. Taub, Naoki Ishiguro, Olaf Schaefer, and Donald J. Tweedie

Abstract To date, more than 400 absorptive and exsorptive membrane transporters have been identified in the human genome, many of which have been characterized and are known to be important from the perspective of pharmaceutical development. Determining the relative importance of these transporters and their influence on drug disposition, therapeutic efficacy and safety, e.g., drug–drug interactions, has been the focus of considerable research, both in academia and the pharmaceutical industry. The interaction of a drug with transporters can potentially lead to alterations in exposure, resulting in toxicity or in certain instances therapeutic failure. For scientists working in the pharmaceutical industry, the importance of understanding drug–transporter interactions is critical as evidenced by the inclusion of drug transporters in recent regulatory guidances. Transporter scientists at Boehringer Ingelheim provide experimental data and an expert interpretation of these data to project teams and work collaboratively with all supporting functions in efforts to determine the potential clinical impact.

Abbreviations

ABC   ATP-binding cassette
ADME   Absorption, distribution, metabolism, excretion
BBB   Blood–brain barrier
BCRP   Breast cancer resistance protein

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BCS	Biopharmaceutics Classification System
BSA	Bovine serum albumin
CLr	Renal clearance
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
CYP450	Cytochrome P450 enzyme
D	Dose
DDI	Drug–drug interaction
DIN	Drug interaction number
DME	Drug metabolizing enzyme
DMPK	Drug metabolism and pharmacokinetics
ENT	Equilibrative nucleoside transporter
EoPhII	End of Phase 2
FDA	US Food and Drug Administration
Fu	Fraction unbound
GFR	Glomerular filtration rate
hADME	Human absorption, distribution, metabolism, and excretion study
HCV	Hepatitis C virus
I	Inhibitor
$[I]$	Inhibitor concentration
$[I_2]$	Inhibitor concentration in the gastrointestinal tract
IC_{50}	Concentration at which 50 % inhibition occurs
ITC	International Transporter Consortium
K_i	Dissociation constant of the transporter-inhibitor complex for competitive inhibition
K_m	Michaelis-Menten constant, substrate concentration at which the rate is half maximal
KO	Knockout animal model
LC-MS/MS	Liquid chromatography, tandem mass spectrometry
M&S	Modeling and simulation
MATE	Multidrug and toxin extrusion transporter
MDR1	Multidrug resistance protein 1
MRP (1/2/3)	Multidrug resistance associate protein (1/2/3)
NCE	New chemical entity
NDA	New Drug Application
NTCP	Sodium taurocholate co-transporting polypeptide
OAT(1/3)	Organic anion transporter (1/3)
OATP(1B1/1B3)	Organic anion transporting polypeptide (1B1/1B3)
OCT2	Organic cation transporter 2
OCTN	Organic cation transporter, novel
Papp	Apparent membrane permeability
PBPK	Physiologically based pharmacokinetic modeling
P-gp	P-glycoprotein
PGx	Pharmacogenomics

PK   Pharmacokinetics
PoC   Proof of Concept
R&D   Research and development
S   Substrate
SGLT-2   Sodium-dependent glucose transporter 2
SLC   Solute carrier protein
SNP   Single nucleotide polymorphism
SoPD   Start of pre-development
SoPhI/II/III Start of clinical Phase I, II, or III
SUR1   Sulfone urea transporter 1
TR-M1/2   Transporter milestone meeting 1 and 2
V_g Volume of the gastrointestinal fluid

12.1   Introduction

Drug transporters recognize and transport a structurally diverse range of substrates, including drugs, metabolites, and endogenous compounds. Transporters are expressed in a wide variety of cell types and play a critical role in facilitating absorption, elimination, and distribution of drugs into various organs (Kusuhara and Sugiyama 2002; Shitara et al. 2006; Shugarts and Benet 2009). Additionally, the process by which absorptive and exsorptive transporters maintain intracellular drug concentrations ultimately affects overall tissue concentrations, which in turn directly affects rates of drug elimination via metabolism and excretion. Thus, understanding whether a development compound is a substrate or has the ability to modulate the function of membrane transporters is a critical factor to consider when characterizing the disposition of a drug or development compound. The field of drug transporters presents inherently difficult challenges, such as (a) very few selective substrates and inhibitors have been identified for clinical use; (b) compared to what is known about CYP450-mediated DDIs, relatively sparse conclusive clinical data exist describing transporter-mediated DDIs; and (c) substrates found to interact with one absorptive or exsorptive transporter typically interact with multiple absorptive and/or exsorptive transporters (Giacomini et al. 2010; Eyal et al. 2009). This latter point is very important as we attempt to extrapolate from in vitro findings to their importance in vivo. There is an obvious risk of drawing conclusions based on a limited in vitro perspective which can result in an inaccurate and overly conservative risk assessment.

The potential for DDIs has traditionally focused on interactions involving drug metabolizing enzymes (DMEs); however, it is now recognized that the interaction of a drug with transporters can also contribute to DDIs (Shugarts and Benet 2009; Wacher et al. 1995; Zhang and Benet 2001; Benet and Cummins 2001; Shitara et al. 2005). Additionally, the potential effects of genetic polymorphisms on transporter function can influence drug disposition (Chinn and Kroetz 2007; Cusatis and Sparreboom 2008; Song 2008; Tirona et al. 2001; Link 2008; Zhang and Wang 2008; Pasanen et al. 2008). Certain DDIs previously ascribed to interactions with

DMEs are now known to be due to interaction between both DMEs and transporters, and as such, the interplay of transporters with DMEs is a critical concern when investigating the potential impact of transporters on drug absorption and elimination (Giacomini et al. 2010; Niemi 2007; Fischer et al. 2005; Cummins et al. 2002).

Regulatory agencies worldwide are requesting information on drug–transporter interactions to support the registration of new drugs, including recommendations of how such interactions should be evaluated both in vitro and in vivo (www.fda.gov) (Huang et al. 2007; Huang 2008; Huang and Woodcock 2010; Zhang et al. 2008). Initial ideas and perspectives have been made public by regulatory agencies, including suggestions as to how the interaction of drugs with membrane transporters can be addressed to support development compounds. It has been acknowledged by the FDA that several transporters which were not originally thought to be important for drug development, e.g., BCRP, OATP1B1, OATP1B3, and the organic anion and cation transporters, are increasingly considered to be important, as they can potentially contribute to DDIs and variability in drug response (Huang and Woodcock 2010).

The International Transporter Consortium (ITC) white paper was recently published by a group of industrial, regulatory (FDA), and academic scientists with expertise in drug metabolism, transport, and pharmacokinetics (Giacomini et al. 2010). In this paper, recommendations are provided with the intent to support the clinical development of a drug through filing of an NDA. The authors focus on the following key issues: (a) which transporters are clinically important in drug absorption and disposition, (b) which in vitro methods are suitable for studying DDIs with these transporters, and (c) which clinical studies should be conducted. It is clearly emphasized in this paper that there are significant complex interdependencies and various potential difficulties concerning studies that can be done to evaluate drug–transporter interactions. The authors also emphasize the need for flexibility, as this rapidly evolving field presents realistic challenges for regulatory agencies as well as pharmaceutical companies. In this paper, the ITC has identified areas of immediate need and has provided decision trees for the evaluation of various transporters. Many of the ideas presented in the ITC white paper have been incorporated into the current strategy for evaluation of drug–transporter interactions at Boehringer Ingelheim. In this chapter, an example of strategies and procedures for assessing interactions of compounds with uptake and efflux transporters are described, including but not limited to the assessment of potential DDIs.

12.2 General Strategy for Assessment of Transporter Interactions at Boehringer Ingelheim

12.2.1 Timing of Transporter Studies in Development

Systematic assessment of the interaction potential of an investigational compound with transporters is generally conducted at Boehringer Ingelheim during the development stage, starting approximately 1 year prior to the Phase Ia clinical study.

However, certain in vitro and in vivo transporter studies may be conducted during the discovery stage. Such activities can include standard screening for interactions with absorptive and exsorptive transporters, such as P-gp, BCRP, and OATPs, and in certain instances, information on DDIs is required in order to enable candidate profiling and final development compound selection. For example, this strategy applies to drugs targeting the CNS or anticancer drugs and indications where an add-on therapy to administered drugs poses an increased likelihood of DDIs (e.g., statins and OATPs). These early studies are typically confined in scope, providing a basis for rank ordering multiple compounds by research teams during the candidate selection process.

The start of preclinical development (SoPD) milestone marks the transition from research to development and triggers a series of comprehensive nonclinical and clinical studies, aimed at appropriately assessing a development compound with regard to safety and efficacy. Pharmacokinetic studies to evaluate absorption, distribution, metabolism, and excretion (ADME) include the evaluation of DDI potential mediated by DMEs and transporters. Initial in vitro transporter profiling studies are conducted to determine the interaction of a development compound as a potential substrate and/or inhibitor of the most relevant human drug transporters. In accordance with the recent ITC recommendations (Giacomini et al. 2010), potential interactions with P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2 are currently evaluated during this initial profiling stage. Results from these studies are used to plan clinical studies, including potential in vivo DDI studies, which are completed ahead of start of clinical Phase II (SoPhII).

We have opted to focus on the use of radiolabeled substrates to determine whether a development compound interacts with membrane transporters as a substrate and/or inhibitor. In vitro inhibition studies, which do not require labeled test compound, can be prioritized in cases where ^{14}C-labeled compound is not available at SoPD (Fig. 12.1). Consolidated data obtained from the initial profiling campaign is presented to the multidisciplinary R&D project team at a milestone meeting (TR-M1) to thoroughly evaluate the impact on the overall development strategy and to determine if any further investigations are required at this point during early development.

Following the initial in vitro profiling experiments, more detailed in vitro or in vivo mechanistic studies are conducted, the timing of which is not as closely linked to a formal development milestone. Typical triggers for mechanistic studies are clinical or nonclinical PK data or regulatory feedback obtained after SoPhII that require further clarification of the role of one or more transporters with regard to safety and efficacy. Coincident with achievement of clinical proof-of-concept (PoC) milestone during development, indication-specific transporter studies can also be conducted, if appropriate (see Sect. 12.5). Following the human ^{14}C-ADME study, which is typically conducted during Phase II to quantitatively study absorption, distribution, metabolism, and excretion of the development compound in humans, the clearance and excretion routes of the development compound and its metabolites are elucidated. This information can then be used to design any clearance route-specific transporter studies, if appropriate (see Sect. 12.4). A second milestone meeting (TR-M2) is held among R&D project team members prior to the end
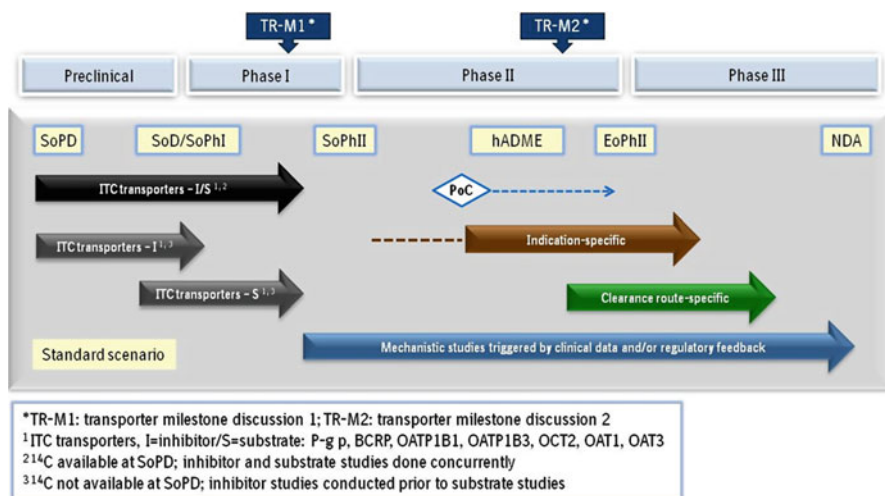
Fig. 12.1 Transporter studies and timeline for a compound in development. *SoPD* start of preclinical development, *SoD* start of development, *SoPh1* start of clinical Phase I, *SoPhII* start of clinical Phase II development, *hADME* human ADME clinical trial using ^{14}C-labeled compound, *EoPhII* end of clinical Phase II development, *NDA* new drug application filing, *I/S* inhibition/substrate studies, *PoC* clinical proof-of-concept

of Phase II (EoPhII) meeting with the regulatory agencies to coincide with availability of final data from the human ^{14}C-ADME study. Again, the impact on the overall development strategy is assessed and the conduct of any additional in vitro or in vivo studies required with regard to safety and efficacy of the development compound (mechanistic, clearance route specific, or indication specific) is evaluated.

12.2.2   Selection of Key Drug Transporters and Appropriate Endpoints

Transporters investigated during the initial profiling campaign are P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, and OCT2, reflecting the current recommendations of the ITC, based on what was known when the ITC white paper was published (Giacomini et al. 2010). However, as is clearly stated in the ITC white paper, other transporters can be clinically relevant depending on factors such as the route of administration, therapeutic area, and individual pharmacokinetic properties of a drug. As nonclinical and clinical data describing interactions with drug transporters is rapidly evolving and regulatory requirements are periodically revised, the selection of transporters studied on a standard basis will certainly change. Balancing effort with the benefit derived from comprehensive transporter screening early in

development remains a difficult proposition for transporter scientists across the pharmaceutical industry, and different researchers may place different emphasis on transporters other than those described above. Similarly, timing of studies will differ according to individual experience and the need to satisfy requests for information from clinical development teams.

Endpoints typically determined during the initial profiling comprise kinetic parameters such as K_m and inhibitory potential (IC_{50}). The obtained K_m and IC_{50} values are used to estimate the in vivo interaction potential if dose strength and therapeutic concentrations are known. In cases where a development compound is identified as an enzyme inducer, transporters could also be induced through the same receptor activation, e.g., PXR (Zhou 2008). Determination of mRNA is a straightforward approach to assess upregulation, appreciating that the levels of mRNA may not accurately reflect changes in protein expression. Recently, sensitive and specific methods for the quantification of multiple transporter proteins in plasma membrane preparations by LC-MS/MS have been developed (Kamiie et al. 2008; Sakamoto et al. 2011) which could offer a more reliable endpoint to study the expression and regulation of drug transporters.

12.2.3 Transporter Polymorphisms: Pharmacogenomics-Based Approaches, In Vitro and In Vivo

Several allelic variants in cytochrome P450 enzymes, e.g., CYP2D6, CYP2C9, and CYP2C19, have been reported (Winjnen et al. 2007). Although there has been an increasing appreciation for similar phenomena in the field of drug transporters, this area is relatively new and not nearly as well defined as for DMEs. However, various functional polymorphisms of OATP1B1 have been characterized (Pasanen et al. 2008; Niemi 2005), most notably the *SLCO1B1*5* haplotype (2 % frequency in Caucasians) and the *SLCO1B1*15* haplotype (16 % frequency in Caucasians, 2 % in sub-Saharan Africans, 9–12 % in Asians). Clinical pharmacokinetic studies have demonstrated that individuals possessing one of these OATP1B1 haplotypes demonstrate increased exposure of various widely prescribed statins, such as pravastatin (Nishizato 2003), atorvastatin (Pasanen et al. 2008), and rosuvastatin (Lee 2005). It is known that *SLCO1B1*5* or *SLCO1B1*15* genotypes can have a profound effect on pravastatin exposure. Notably, although the recognized allelic variants of OATP1B1 can be of concern with respect to interindividual differences in drug response, they also provide an opportunity to study their potential effect on the pharmacokinetic profile of a drug or development compound in a clinical setting (Niemi 2010; Pasanen et al. 2006). The single nucleotide polymorphism (SNP) *SLCO1B3* 334T>G for OATP1B3 was reported to be associated with higher transport activities to several substrates in vitro (Letschert et al. 2004) as well as increased in vivo clearance of afatinib (Yamakawa et al. 2011).

DDIs related to P-gp interactions have been reported and various SNPs in the MDR1 gene have been identified; however, the reports available to date have been

inconsistent, and a definitive assessment of the influence of MDR1 polymorphisms on ADME and pharmacological response has not yet been demonstrated (Giacomini et al. 2010; Chinn and Kroetz 2007; Williams 2008).

Pharmacogenomic (PGx) approaches have become an integral part of clinical development programs throughout the pharmaceutical industry. The initial identification of major human transporters involved in drug disposition prior to Phase II supports a PGx strategy as part of the clinical development plan with regard to drug transporter polymorphisms. PGx-based methodologies can be applied both prospectively and retrospectively. Patient stratification can be based on prior knowledge of genetic variations in drug transporters, pharmacological target, and DMEs. Collection of genetic data can be applied to phenotype–genotype correlation, e.g., linkage of variations in the pharmacokinetic and/or safety profile observed during a clinical trial to a specific genotype, including drug transporters. Complex interactions with absorptive and exsorptive transporters as well as DMEs almost demand that we collect these data, and as additional examples of PGx related to membrane transporters are discovered, there will an increased need to evaluate their potential impact on the safety and efficacy of compounds.

12.3   Assays Employed

12.3.1   Initial Profiling Studies

Understanding whether a drug or lead candidate is a substrate, inhibitor, or modulator (Taub et al. 2005) of membrane transporters is important from a safety perspective. Initial profiling studies are standardized work packages which are conducted in support of all development projects. Two cell lines are routinely used for initial profiling studies. The Caco-2 cell line, which is derived from a human colorectal adenocarcinoma, is one of the most commonly used in vitro models to evaluate the interaction of NCEs as substrates and inhibitors of exsorptive transporters (Burton et al. 1993; Artursson and Karlsson 1991; Taipalensuu et al. 2001; Sun et al. 2008). Similarly, HEK293 cells expressing SLC transporters are often routinely used. These studies are conducted for the ITC recommended transporters and provide us with substrate and inhibitor characteristics of development compounds (Figs. 12.2 and 12.3). Data from these studies are not only prerequisites for an initial risk assessment of transporter-mediated DDIs at the TR-M1 meeting but can also be important for selecting patients for Phase II clinical studies and also for choosing in vivo probe substrate and probe inhibitor(s) which can be used in clinical DDI studies (see Sect. 12.4). However, the data generated may not be sufficient to address specific questions arising during the initial preclinical or early clinical development phases. As such, more complex mechanistic experiments can be conducted.
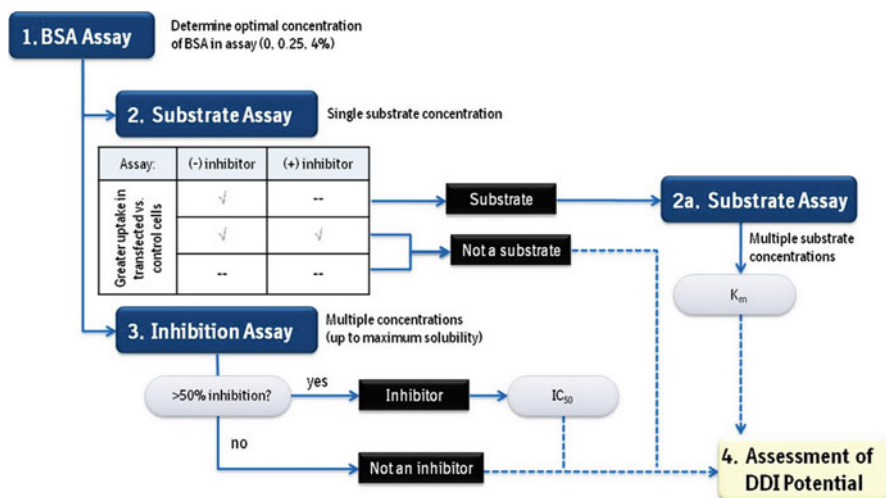
Fig. 12.2 Flow chart for assessment of a development compound with SLC transporters OATP1B1, OATP1B3, OAT1, OAT3, and OCT2 during initial profiling studies. *BSA* bovine serum albumin
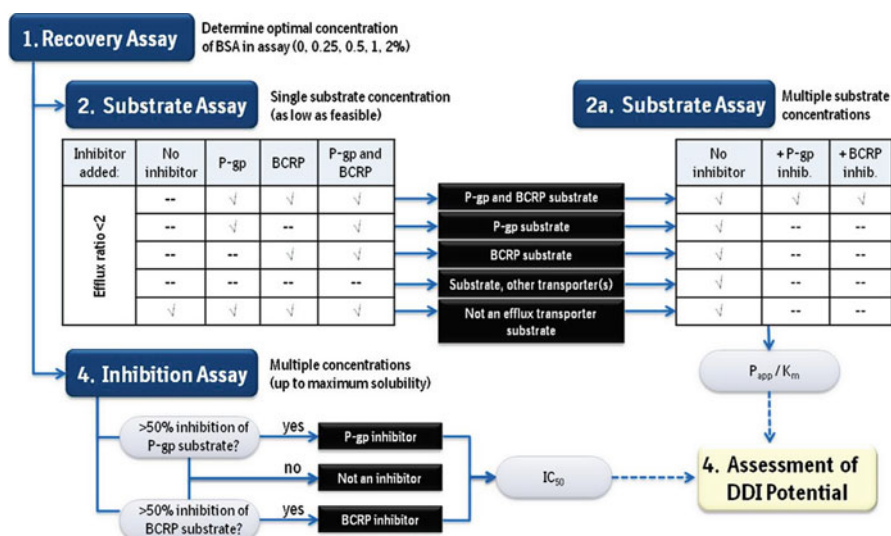
Fig. 12.3 Flow chart for assessment of a development compound with ABC transporters P-gp and BCRP during initial profiling studies

12.3.2   Mechanistic Studies

The execution of mechanistic studies is based on individual project needs. In certain instances, an evaluation of the interplay of transporters and DMEs can be conducted to supplement initial transporter profiling studies. Various in vitro tissue models can be used to study drug–transporter interactions, including but not limited to primary cultured proximal tubule cells, rat brush-border membrane vesicles, and, more recently, human kidney slices (Watanabe et al. 2011). Isolated hepatocytes are now routinely used to evaluate drug–transporter interactions and can also be used to investigate the interplay of absorptive/exsorptive transporters with DMEs. Currently, the most widely used in vitro hepatocyte model is the sandwich-cultured format, which allows for the estimation of biliary clearance and can maintain hepatic phenotype and transport activity for up to 10 days in the absence of serum (Liu et al. 1999; Tuschl et al. 2009). The sandwich-cultured hepatocyte format is considered more applicable to the evaluation of transporter interactions compared to suspended hepatocytes and simple monolayers, as this model promotes establishment of cell polarity, exhibits reasonable expression levels of both absorptive and exsorptive transporters, and offers the opportunity to study the effect of nuclear receptor activation (Liu et al. 1999; Tuschl et al. 2009).

Absorptive transporter expression in sandwich-cultured hepatocytes declines following several days in culture, thus affecting clearance prediction and possible DDI potential (Kotani et al. 2011). Similarly, internalization of exsorptive transporters after hepatocyte isolation has been shown to occur (Bow et al. 2008). Recent advances in hepatocyte culturing and engineering have resulted in a new generation of hepatocyte culture models that exhibit improved longevity and in vitro-like enzyme and transporter functions. For example, a micropatterned hepatocyte co-culture system has been developed, using a combination of cryopreserved hepatocytes and mouse fibroblasts that can maintain expression levels of DMEs over periods of up to 42 days (Khetani and Bhatia 2008). This hepatocyte co-culture has been effectively used to reflect in vivo oxidative and conjugative metabolism (Wang et al. 2010). There are certain advantages to in situ generation of metabolites subject to active efflux by transporters. The utility of micropatterned hepatocyte co-culture systems for evaluation of transporter interactions, and the interplay between metabolism and transport, is currently the focus of research in multiple laboratories.

Similarly, various in vivo and ex vivo models are available to study drug–transporter interactions, including but not limited to rat blood–brain barrier (BBB) penetration studies using knockout (KO) models, isolated perfused rat lung, transporter knockout mice, and whole-body autoradiography. Both KO mouse models, particularly mdr1a, mdr1b, and the combined mdr1a/mdr1b (Schinkel 1999; Doran et al. 2005; Xia et al., 2007), have been applied to gauge possible changes in brain exposure of NCEs. While there can be an overestimation of the potential for changes in brain levels in humans based on the mouse data (Ayrton and Morgan 2001; Sasongko 2005), use of these models can still provide valuable insights. In addition,

although the rat KO model is less advanced (Zamek-Gliszczynski et al. 2006), chemical knockout of P-gp using selective inhibitors can be used (Choo et al. 2000), and the rat provides an easier animal model compared to the mouse for multiple sampling for PK analysis.

12.4 Clearance Route-Specific Transporter Studies

12.4.1 Application of Human ADME Study Data Toward Nonclinical Study Design

Determination of the mechanism by which a drug is cleared may help project teams to decide whether or not evaluation of an interaction with drug transporters will add value to the clinical development program. Clinical data, such as the human ADME study, can provide critical information for consideration when designing clearance route-specific transporter studies.

The mechanism by which a drug is cleared may have an appreciable impact on whether or not evaluation of an interaction with drug transporters is warranted. Elucidating the relative contribution of a transporter-mediated pathway toward the clearance of a drug can help to determine if conducting a particular study will add valuable/actionable information to a clinical program. For example, our experience has been that the PK profile of a development compound demonstrating high solubility and high membrane permeability and/or that is highly metabolized is less likely to be affected by a co-administered drug that is a P-gp inhibitor. Alternatively, the PK profile of a drug that has poor solubility, limited membrane permeability, and is relatively metabolically stable (eliminated primarily as parent) is more likely to be affected by a co-administered drug that inhibits P-gp.

12.4.2 Examples of Clearance Route-Specific Studies

Case 1—Absorption: Was there a linear relationship between dose and exposure in the Phase Ia study, and is the expected therapeutic dose within this linear dose/ exposure range? If this is the case, it is less likely that exsorptive transporters expressed on intestinal enterocytes will affect the absorption of the development compound. However, the influence of absorptive and exsorptive transporters on the distribution of the development compound into tissues expressing membrane transporters may still be an important consideration.

Case 2—Distribution: Is it important that the development compound penetrates the BBB and reaches the CNS to hit its pharmacologic target? If this is the case, it may be important to evaluate the interaction with transporters expressed on the apical/

luminal membrane of brain capillary endothelial cells. For certain indications it may be desirable to ensure that the development compound does not reach the CNS; in such cases, evaluating the potential interaction with these transporters may also be necessary.

Case 3—Elimination/biliary clearance: From the human ADME study, were parent drug and metabolites identified in feces following acceptable plasma exposure of parent compound, thus suggesting that biliary excretion is involved in clearance of parent and metabolites? If this is the case, then it may be important to evaluate the interaction of the development compound with transporters expressed on the canalicular and sinusoidal membranes of hepatocytes.

Case 4—Elimination/renal clearance: Is the development compound or its metabolites subject to significant renal clearance (e.g., >50 % of total clearance)? If parent drug or its metabolites are identified in urine and CLr > 1.5 fu × GFR, it may be important to evaluate the interaction with transporters expressed on the luminal or abluminal membrane of proximal kidney tubular cells.

12.5   Indication-Specific Transporter Studies

12.5.1   *Distribution to Pharmacologic Target and the Impact of Transporter Interactions*

It is clear that transporters can affect the disposition of many drugs, in particular the ability of a drug to reach tissues in which the pharmacologic target is located. For example, the interaction of a development compound with transporters can be substantially influential when the target is cancer, as transformed cells typically overexpress exsorptive transporters such as P-gp and BCRP. P-gp is the product of the multidrug resistance (MDR) gene, which was named due to its ability to recognize and efflux many chemotherapeutic drugs out of transformed cells (Litman et al. 2001). The value of conducting indication-specific transporter interaction studies should also be considered in cases where the pharmacologic target resides in a cell or tissue that expresses membrane transporters other than those evaluated during the initial profiling studies. In the following subsections, the impact of transporter interactions in selected diseases will be summarized, including a description of which transporters may be worth evaluating.

12.5.1.1   Pulmonary Disease: OCT and OCTN

The lungs provide a unique absorption surface for drug delivery. The most common inhaled drugs are bronchodilators and anti-inflammatory agents used in the therapy of airway diseases such as asthma and chronic obstructive pulmonary disease.

Effective airway absorption of these drugs is a prerequisite for local pharmacologic effects. It has been reported that SLC transporters (OCT and OCTN, in particular) are expressed on the airway epithelium, smooth muscles cells, and bronchial blood vessels. These transporters have an important role on the duration of action and onset of action of inhalational drugs (Mendes et al. 2008; Horvath et al. 2007). For development compounds which are administered via inhalation, it is important to evaluate their potential interaction with SLC transporters expressed in pulmonary tissue.

12.5.1.2   Diabetes: Renal Transporters, Complex DDI Profile

To address the increasing need for treatment of glycemic control and cardiac disease, combination therapies are commonly utilized. Such combination therapies can be complex to develop due to the increased potential for DDIs (Koehn et al. 2008; Neumiller et al. 2010; Nakagomi-Hagihara et al. 2007). Metformin, a first-line treatment for patients diagnosed with diabetes mellitus, is eliminated by tubular secretion and is known to be a substrate of human OCT2 and MATE (Kimura et al. 2005; Tanihara et al. 2007). The OCT and MATE inhibitor cimetidine (Kimura et al. 2005; Matsushima et al. 2008) has been shown to substantially increase metformin exposure (Somogyi et al. 1987), although the exact mechanism of the DDI is not completely elucidated.

In addition to pharmacologic resistance to therapy, resistance to drugs used to treat diabetes mellitus can occur due to the function of ABC transporters expressed on target cells (Koehn et al. 2008). For example, ABCC8, or the sulfonylurea receptor 1 (SUR1), is expressed in human pancreatic beta cells and is the target of many antidiabetic drugs such as glimepiride, nateglinide, and tolbutamide (Koehn et al. 2008). In addition to the pancreas, the kidney plays a key role in glucose homeostasis, not only by contributing toward gluconeogenesis and glomerular filtration but also by facilitating the reabsorption of glucose from proximal tubules (Neumiller et al. 2010). A relatively new class of antidiabetic drugs that inhibit reabsorption of glucose from the proximal convoluted tubule by targeting a sodium-dependent glucose transporter (Scheepers et al. 2004), known as SGLT-2 inhibitors, offer promising new treatments to people afflicted with diabetes.

12.5.1.3   Cancer: Exsorptive Transporters Expressed on Transformed
Cells, ABC Transporters

Failure of cancer chemotherapy can occur through increased efflux transport of chemotherapeutic agents out of targeted cells, leading to a reduction of intracellular drug concentration and drug insensitivity (Juliano and Ling 1976). This phenomenon often compromises the efficacy of multiple chemotherapeutic agents and can include various drug classes and chemical structures. A well-established cause of MDR involves the increased expression of members of the ABC transporter family (Gottesman et al. 2002). The most extensively characterized transporters include P-gp, MRP1, and

BCRP (Doyle et al. 1998; Ambudkar et al. 2003; Szakacs et al. 2006; Munoz et al. 2007; Bunting 2002; Robey et al. 2007). In addition, a number of other ABC transporters also recognize many anticancer drugs and potentially can effectively extrude chemotherapeutic compounds from cells (Fletcher et al. 2010). In the case of solid tumors, the grade or degree of differentiation typically reflects relative aggressiveness of the tumor, with less-differentiated tumors possessing the greatest proliferative potential and a more aggressive phenotype (Fletcher et al. 2010). Various ABC transporters are expressed at higher levels in tumor subtypes or zones that are less differentiated (Oda et al. 2005; Weinstein et al. 1991; Oevermann et al. 2009; Vander et al. 2008; Zochbauer-Muller et al. 2001; Hanada et al. 2008; Ohtsuki et al. 2007). To efficiently identify and develop anticancer drugs, it is important to take into consideration the type of cancer and what may be required, from a physiological perspective, to successfully target a chemotherapeutic to the site of action. For example, there are many nucleoside-derived drugs that are frequently used for cancer therapy, and it has been demonstrated that concentrative nucleotide transporters (CNTs) and equilibrative nucleotide transporters (ENTs) facilitate the membrane transport of such nucleoside-derived cancer drugs (Hodge et al. 2011; Errasti-Murugarren and Pastor-Anglada 2010). Thus, as our knowledge of ENT and CNT transporters increases, in vitro studies to evaluate the interaction of nucleoside-derived chemotherapeutics can be used to predict cellular targeting and probability of clinical success.

12.5.1.4   CNS: Transporters at the Blood–Brain Barrier

The target organ for CNS drugs is the brain; however, the brain can be a challenging target to hit due to the presence of the BBB. The BBB is composed of microvessel endothelial cells sealed by tight junctions; is surrounded by pericytes, neuron endings, and astrocyte foot process; and effectively restricts the entry of many drugs into the CNS. The lipophilicity of a compound increases its ability to penetrate across the BBB (van Bree et al. 1988; Levin 1980; Shah et al. 1989). Binding to plasma proteins also strongly influences the entry of drugs into the CNS; it is generally accepted that only unbound drug can freely penetrate across the BBB due to restricted entry of large molecules such as albumin (Norrby 1985). In addition to the presence of tight junctions between cells that comprise the BBB, active transport plays a substantial role to enhance or restrict the brain distribution of CNS drugs. Cells comprising the BBB form an exceptionally tight monolayer, and exsorptive transporters such as P-gp, BCRP, and MRPs are densely expressed (Dauchy et al. 2008). On the other hand, essential nutrients, hormones, and certain drugs selectively enter the brain by way of influx transporters expressed at the BBB (Ohtsuki and Terasaki 2007). Currently, our understanding of transporter expression and function at the BBB is generally considered to be not well understood compared to what is known about transporter expression and function in the liver and kidney. However, research on the physiology and function of the BBB has made enormous progress over the past 2 decades, and recently, several astrocyte/endothelial cell co-culture models of the BBB and immortalized human endothelial cells are available for

pharmaceutical industry. The combined results from such in vitro systems and in vivo data, not only from human but also from animal species, will provide valuable clues to facilitate more accurate prediction of efficacy and potential CNS side effects.

12.5.1.5   HCV: Liver Transporters, Absorptive and Exsorptive, Transport into Bile, and Effect of Disease

For an indication such as human hepatitis C (HCV) where the target primarily resides in the liver, it is important to evaluate the potential interaction of a development compound with transporters expressed on the sinusoidal and/or canalicular membrane of hepatocytes. In such cases it may also be important to use in vitro hepatocyte models to determine the potential for a development compound to undergo active, i.e., transporter-mediated absorption into plated hepatocytes. Thus, such in vitro studies may be supplemented with complementary in vivo studies to obtain a more detailed understanding of the mechanism and better extrapolate the data to a potential clinical effect.

Presently, the standard of care therapy for HCV infection is a combination of pegylated interferon and ribavirin; however, this therapy does not completely eradicate HCV infection nor does it prevent relapse in 30–50 % of patients treated, for reasons that are not well understood (Kikuchi et al. 2010; Fukuchi et al. 2010). It has been shown that an equilibrative nucleoside transporter (ENT1) and an as yet unidentified sodium-dependent transporter facilitate the absorption of ribavirin into hepatocytes (Fukuchi et al. 2010; Bengsch and Thimme 2010). Interestingly, hepatic transporter and DME expression is known to be altered during active HCV infection, an effect that correlates with the elevated production of inflammatory cytokines in response to the disease. For example, HCV infection has been shown to elicit an increase in the expression of MDR1, MRP1, and MRP3 (Ros et al. 2003); however, HCV infection also elicits a downregulation in the expression of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP1B1, MRP2, and OCT1 (Nakai et al. 2008; Hinoshita et al. 2001).

As more information appears in the literature regarding alteration of levels of DMEs and transporters during HCV infection, it will become necessary to mechanistically evaluate the interplay of drugs used to treat HCV with DMEs and transporters using advanced mechanistic models that can be used to determine the relative contributions to clearance pathways and the potential for clinically relevant DDIs.

12.6   Use of In Vitro Data to Predict Potential Clinical Significance

The in vitro assessment of ADME properties is performed as a standard part of drug development programs across the pharmaceutical industry. Advanced test systems, including tissue and cell-based models, provide scientists with human in vitro data

that can be related or extrapolated to pharmacokinetic parameters, typically considered in conjunction with animal in vivo data. The challenge for nonclinical DMPK scientists, however, is the selection and consolidation of the relevant information from a plethora of data and to ultimately provide expert guidance to clinical scientists and drug development teams.

Alignment of the data generated according to certain critical milestones along the development timeline, which includes a balance of initial profiling, mechanistic, clearance route, and/or indication-specific studies, is our current strategy at Boehringer Ingelheim. Functional characterization and validation of the in vitro systems, preferably using marketed drugs, with reported clinical interaction potential as probe substrates and inhibitors is a prerequisite and provides key information concerning the capabilities and limits of a given employed system to answer a specific analytical question. For example, recombinant expression systems such as MDCK-MDR1 or LLCPK-BCRP are well suited to detect interactions of a development compound with ample sensitivity. On the other hand, expression of P-gp or BCRP can be 1–2 orders of magnitude higher in these recombinant cell systems when compared to the expression level in human intestine (unpublished data). Similarly, it is well established that there are marked differences in efflux ratios for compounds with Caco-2 vs. MDCK cell-based assays, which can be accounted for by differences in the expression level of transporter proteins (Taub et al. 2005; Luo et al. 2002). As the efflux ratio can significantly differ depending on the concentration of functional protein expressed per surface area of biological membrane, caution is advised when extrapolating from in vitro data to in vivo (Shirasaka et al. 2008). Since in vivo transporter expression varies not only qualitatively but also quantitatively between organs and tissues, different tissue-derived in vitro systems may be required to study the interaction potential in the intestine, liver, kidney, or brain (CNS). Newly available analytical technologies such as the use of LC-MS/MS to quantitative membrane-associated proteins can be employed to measure differences in tissue expression of various transporters and to identify the most suitable in vitro model.

Predicting potential clinical significance from in vitro data is challenging, as standard in vitro systems cannot yet simulate the complexity of the in vivo situation. Key parameters such as interindividual differences in metabolism and disposition, and the interplay of multiple enzymes and transporters at different sites of the body, are thus not represented when employing such tools. However, several approaches to estimate the potential impact of interaction with transporters have been proposed. For example, the authors of the ITC white paper recommend using the $[I_2]/IC_{50}$ ratio for assessing the in vivo interaction potential of a drug with intestinal P-gp, with a threshold of ≥ 10 given at which DDIs based on P-gp inhibition in the intestine are considered likely.

As $[I_2]$ is the predicted intestinal drug concentration calculated from the administered oral dose dissolved in a volume of 250 mL of intestinal fluid, this approach may overestimate the DDI potential for low solubility drugs that are inhibitors of P-gp. Any drug classified as a BCS II/IV class compound per definition will not completely dissolve at the given dose strength in a volume of 250 mL of water at

any pH present in the gastrointestinal tract. Alternative approaches for prediction of clinical DDI have been described which include physiochemical properties of development compounds. The drug interaction number (DIN) has also been proposed as a useful parameter for assessing in vivo DDI likelihood to account for solubility (Tachibana et al. 2009). The DIN is calculated for soluble inhibitors and drugs as D/K_i, with D being the administered oral dose. In the case of poor solubility drugs or inhibitors, the DIN is calculated as $[I] \cdot V_g/K_i$, where $[I]$ represents the maximum solubility and V_g the volume of intestinal fluid. Based on an analysis of clinical DDI data, Tachibana et al. suggested that a DIN value of <10.8 L indicates a low intestinal DDI risk when the inhibitor is co-administered with a P-gp substrate, while a DIN value >27.9 L is indicative of a high DDI risk (Tachibana et al. 2009). However, it is important to note that none of the currently described models has yet been validated to be free of false predictions, and as such, results obtained from applying these models should generally be regarded as approximations rather than definite criteria for planning of clinical DDI studies.

12.7   Need for Continuing Education and Rational Perspective on In Vitro and Clinical Data

Accurately ascribing the role of drug transporters in ADME adds a significant degree of difficulty to the overall drug development process. The ITC white paper (Giacomini et al. 2010) was intended to provide a basic understanding of current status of this evolving field; however, the authors admit that not all issues presented were representative of global opinions (Shi et al. 2011; Lee et al. 2011). Although the decision trees included in the ITC white paper will change as the science moves forward, their inclusion provided an important mechanism to drive discussion by acting as a focus for debate and dialog.

The common goals of the pharmaceutical industry and the FDA, i.e., to ensure safety for patients and volunteers in clinical studies, will be best served by having active discussions on this complex topic. This should be balanced with an appreciation that an overly conservative approach will impede the discovery and development of new drugs. A critical question then is, "How do we resolve this apparent dilemma?"

Modeling and simulation (M&S) can offer valuable insights into the relative importance of multiple processes such as the role of absorptive and exsorptive transporters and their interplay with DMEs. Various M&S approaches, including physiologically based pharmacokinetic (PBPK) modeling (Poirier et al. 2009) and PK predictive models, have been applied to identify transporters as the rate limiting step in these processes.

The dearth of selective substrates and inhibitors will not be resolved overnight, and as such, scientists in the field of transporters, drug development experts, and clinicians need to tackle the problem of conducting insightful, decision-making clinical studies in a significantly different manner than approaches taken with

DMEs. While an obvious solution would be to conduct multiple studies with partially selective probes (substrates or inhibitors), the added costs to clinical trials is not trivial.

12.8 Conclusions

Membrane transporters play an important role in drug absorption, distribution, metabolism, elimination, therapeutic efficacy, and safety. Drug transporter-mediated DDIs can occur, and in certain cases DDIs result from interactions involving transporters and DMEs. Transporter scientists at Boehringer Ingelheim provide experimental data and an expert interpretation of these data to development project teams, work collaboratively with all development project team supporting functions in efforts to determine the potential clinical impact, and optimize our nonclinical and clinical strategic approach in all development programs. Scientists with expertise in this field at the principal development sites at Boehringer Ingelheim are globally coordinated to maximize synergy, optimize available resources and technical capabilities, and streamline information flow to and from development project teams.

The field of transporters is rapidly advancing and provides complex challenges. Similarly, regulatory agencies worldwide are in the process of responding to the continuous evolution of this field, and more frequently challenge sponsors to provide detailed nonclinical and clinical data related to drug–transporter interactions from Phase Ia through submission.

12.9 Regulatory Guidelines

FDA Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft) http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf

EMA Guideline on the Investigation of Drug Interactions (Draft) http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf

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ERRATUM

Transporters in Drug Development

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