Chapter 1
Membrane Transporters in ADME

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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 bioavailability 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 ≈ 7.4) and intestinal fluid pH (pH ≈ 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).

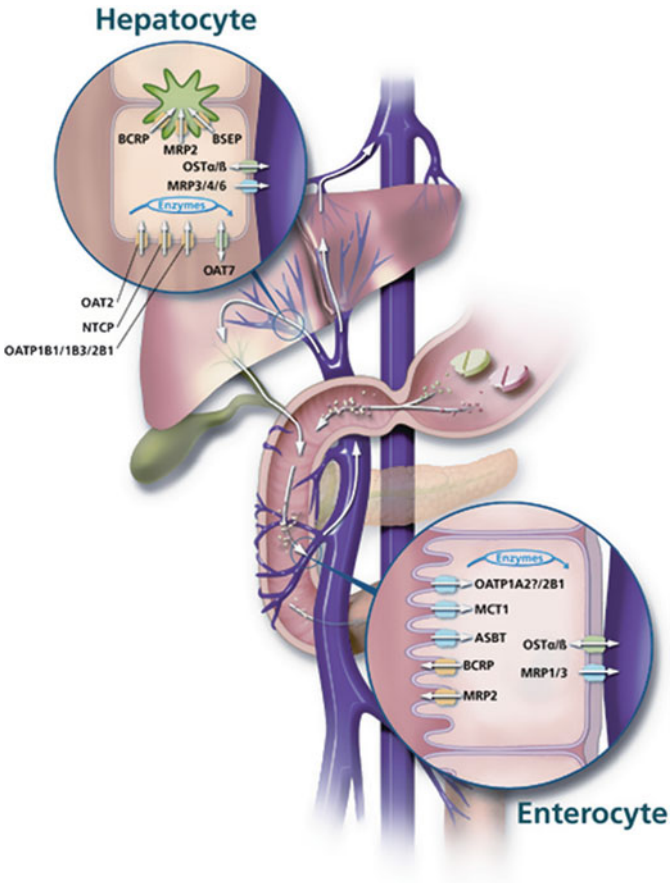

Fig. 1.1 Membrane transporters for organic anions, that in human intestine and liver, carry the probe E_1S. Only transporters that have been determined at protein level and where the subcellular localization and transport direction in the particular human tissue are known have been included in this figure. 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

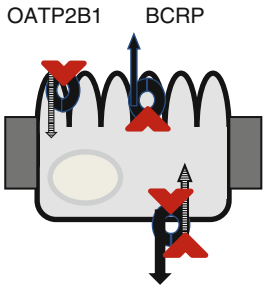

Fig. 1.2 Illustration of the rate limiting transporter in permeability (*dashed arrows*) of the probe E_1S (in *red*) across Caco-2 cells

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

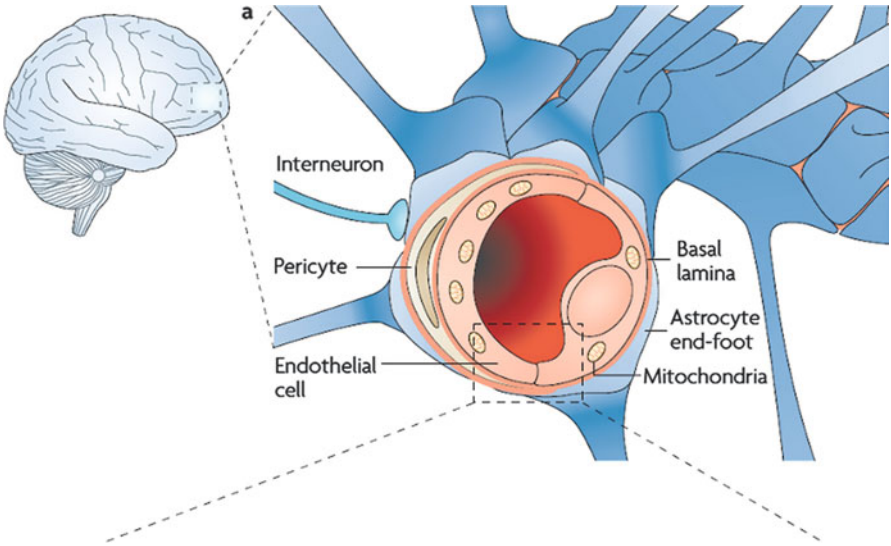

Fig. 1.3 Anatomy of the neurovascular unit (NVU). Endothelial cells form the walls of the capillaries. The endothelium is partly covered with pericytes embedded in the basement membrane at the abluminal side of the endothelium. The endothelium and pericytes are ensheathed in astrocytic endfeet. Neuronal 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

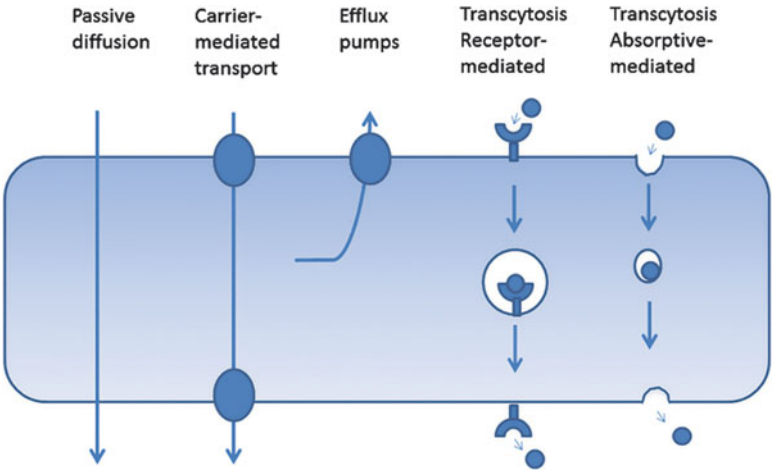

Fig. 1.4 A schematic overview of the transport pathways in the brain endothelial cells. Small (<400 Da) lipophilic compounds may permeate the endothelial barrier via passive diffusion. Nutrients and certain nutrient-like drug compounds may be transported from the blood to the brain interstitial fluid 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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