Review

Biochemical and Molecular Pharmacological Aspects of Transporters as Determinants of Drug Disposition

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Summary: Membrane transporters are integral membrane proteins typically having 12 transmembrane domains. Most of the SLC family transporters consist of 300–800 amino acid residues with a molecular mass of 40–90 kDa, while the corresponding values of ABC family transporters are 1,200–1,500 residues and 140–180 kDa, respectively. Each transporter has a characteristic tissue distribution and subcellular localization. I have isolated cDNAs of various transporters, including oligopeptide transporter PEPT1, monocarboxylic acid transporter MCT1 and organic cation/carnitine transporters (OCTNs), and determined their tissue distribution and subcellular localization. I have also determined the absolute expression levels of transporters to evaluate their relative contributions to drug transport in various tissues. It is important to note that expression levels of transporters can be changed under various physiological conditions and by administration of drugs. Changes in expression level, subcellular localization and functional properties can all be involved in inter-individual differences in drug pharmacokinetics. Transporters are among the key determinants of drug disposition.

Key words: membrane transport; oligopeptide; small intestine; quantitative-PCR; adenovirus; heterologous expression

Introduction

After oral administration, drugs pass through the intestinal membranes and are distributed throughout the body according to their physicochemical properties. However, specific membrane transporters are expressed in the luminal and/or basolateral membranes of intestinal absorptive epithelial cells, hepatocytes, renal tubular epithelial cells and other important barrier tissues, including the blood-brain barrier, blood-testis barrier and the placental barrier. Some transporters accept as substrates not only physiological or endogenous compounds, but also xenobiotics, including drugs, and they therefore influence the concentrations of these drugs in blood and peripheral tissues. In other words, transporters are one of the determinants of drug disposition. Recently, their clinical significance has attracted great attention. Differences in transporter expression levels, functional properties and single nucleotide polymorphisms (SNPs) can contribute to inter-individual differences in pharmacokinetics.1)

Drug transporters are integral membrane proteins typically having 12 transmembrane domains (TMDs), although there are some exceptions (i.e., 10, 11, 13 or 17 TMDs). Many SLC family transporters consist of 300–800 amino acid residues with a molecular mass in the range of 40–90 kDa, while the corresponding values for ABC family transporters are 1,200–1,500 residues and 140–180 kDa, respectively. In the early days, obtaining direct evidence that a protein is a transporter usually involved purification of the protein and reconstitution of the activity on artificial lipid membranes. This was extremely difficult. Biochemical analysis of transporter proteins has been very challenging compared with that of soluble proteins because of their hydrophobic nature, relatively high molecular weight and low abundance among cellular proteins. However, the development of the innovative technique of expression
cloning using *Xenopus laevis* oocytes led to enormous progress, including the cDNA cloning of PEPT1, OAT1, OCT1, OATP and so on. These pioneering studies opened the way to cloning of homologous genes by using PCR or blot hybridization techniques. Extensive studies on functional expression of transporters have been performed by many scientists using heterologous gene expression systems, including Xenopus oocytes and transfected mammalian cell lines such as 293, CHO, HeLa and COS cells. Those studies have revealed that, in general, the substrate specificities of transporters are lower than those of physiological receptors. Transporters recognize many structurally related and unrelated compounds with Km or Ki values at submicromolar to millimolar levels.

It is very important to clarify the tissue distribution and subcellular localization of transporters, because they can greatly influence the absorption, distribution, metabolism and excretion (ADME) characteristics of drugs. Trans-cellular transport of drugs involves two distinctive trans-membrane transport processes; entry into the cell across the apical or luminal plasma membranes and discharge through the opposite cell membrane of epithelial or endothelial cells. A comprehensive description of trans-cellular transport requires characterization of the transport systems in both membranes of the cell. Membrane transporters have been classified into the solute carrier (SLC) and the ATP-binding cassette (ABC) transporter families according to the guidelines of the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/). A comprehensive list of transporters has become available in the past decade owing to extensive membrane physiological analysis of transport phenomena and biochemical analysis of transporter proteins, as well as progress in the molecular biological analysis of transporter genes. Having deduced the amino acid sequence of a transporter, one can raise a specific antibody against it, which is essential for immunohistochemical analysis. Gene databases mean that purification of the intact transporter protein is no longer necessary for immunization. I have placed stronger emphasis on raising many polyclonal antibodies against synthetic peptides corresponding to fragments of the deduced amino acid sequences of transporters.

This review will focus on the biochemical and molecular pharmacological aspects of drug transporters that have been studied mainly in my laboratory in the past decade, rather than describing detailed functional aspect of transporters, because those have been covered by many other excellent reviews. Instead, I will also discuss the prospects for future studies on transporter-mediated drug disposition.

cDNA Cloning and Determination of Subcellular Localization of Transporters

A cDNA of the rat orthologue of PEPT1 was isolated by cross-hybridization with a rabbit cDNA probe in collaboration with Miyamoto et al. The oligopeptide transporter PEPT1 (SLC15A1) was first isolated from rabbit in 1994 by means of an expression cloning strategy. Afterwards a human clone was isolated by Liang et al. The cDNA sequence of rat PEPT1 indicated that it is composed of 710 amino acids, having 77% and 83% amino acid sequence identity with rabbit and human PEPT1, respectively. Hydropathy analysis revealed 12 putative TMDs, with a long 204-amino-acid hydrophilic loop containing five N-glycosylation sites between TMD 9 and 10 in the predicted protein. PEPT1 mediates (re)absorption of various drugs, including orally active β-lactam antibiotics, angiotensin-converting enzyme inhibitors, the anticancer drug bestatin, and the antiviral agent acyclovir, as well as di- and tripeptides derived from nutrients. Previous studies of oligopeptide transport in the small intestine had been focused on functional characterization of transport activities at the brush-border membranes, but the presence of a basolateral transport activity had also been demonstrated. Therefore, I raised a polyclonal antibody against synthetic peptide corresponding to a fragment of the deduced amino acid sequence of PEPT1 and examined the distribution of PEPT1 protein between the brush-border and basolateral membranes. PEPT1 protein was shown to be localized in the brush-border membranes of absorptive epithelial cells by subcellular fractionation of the membranes on a sucrose density gradient and by immunohistochemistry using light and electron microscopy (Fig. 1).

cDNA of MCT1 (SLC16A1) has also been cloned from the rat small intestine. Rat MCT1 encoded a protein of 494 amino acids with an estimated core molecular mass of 53 kDa. Hydropathy analysis predicted 12 hydrophobic domains with a single large hydrophilic loop between TMD 6 and 7. The amino acid sequence was homologous with those of hamster and human, with 93.1% and 84.6% identity, respectively. Rat MCT1 transported [14C]lactate and [14C]benzoate. MCT1 was originally found as a pH-dependent lactate and pyruvate transporter in Chinese hamster ovary cells and has been shown to be present in a variety of tissues, including the intestine. I have examined the cellular and subcellular localization of MCT1 along the gastrointestinal tract (Fig. 2). MCT1 was detected throughout the intestinal tract from the stomach to the large intestine. In most immunopositive cells, the staining was localized at the cell membrane, with greater intensity on the basolateral membrane than on the apical membrane. The intensity of
Fig. 1. Distribution of PEPT1 protein between brush-border and basolateral membranes. Left panel: crude membrane fraction obtained from rabbit jejunum was separated on a 28–48% continuous sucrose density gradient. Ten fractions of similar volume were collected and analyzed for marker enzymes of brush-border and basolateral membranes, alkaline phosphatase and Na⁺/K⁺-ATPase, respectively (A), and subjected to Western blotting with anti-PEPT1 antiserum (B). Right panel: frozen sections were stained with anti-PEPT1 antiserum in the absence (A) or presence of blocking peptide antigen (B), and detected with horseradish peroxidase and DAB.

Fig. 2. MCT1 immunoreactivity in the small intestine. Paraformaldehyde-fixed frozen sections of rat duodenum were reacted with anti-MCT1 antibody with (B) or without (A) pre-absorption with peptide antigen. The immunoreactivity was observed over all the mucosal epithelial cells, with decreasing intensity from the crypts (c) to the tip of the villi (v). No immunoreaction was observed with absorbed antibody (B).

epithelial MCT1 immunoreactivity was greater in more proximal regions of the duodenum-ileum axis, and in lower regions of the crypt-villus axis. In the relatively immature epithelial cells of crypts and villus bases, the reactivity was localized primarily at the basal membrane and lower lateral membrane. In contrast, in the absorptive epithelial cells of most villus regions the immunoreactivity was stronger on the upper lateral membrane and strongest beneath the tight junctions, while the brush-border membranes had a distinct but lower-intensity reaction. Western blot analysis revealed that the brush-border membrane fraction from rat small intestinal tissues reacted with the antibody, showing that MCT1 protein is present at the brush-border membrane at least in part, which is consistent with the monocarboxylic acid uptake activity in the vesicle preparation. The goblet cell and submucosal gland cells were free from immunostaining.7) Although
Fig. 3. Immunofluorescence staining of OCTN1 and OCTN2 in the kidney. Paraformaldehyde-fixed frozen sections of mouse kidney were incubated with affinity-purified antiserum against either OCTN1 (A) or OCTN2 (B) and Alexa954-labeled anti-rabbit IgG, and examined under a fluorescence microscope. OCTN1 and OCTN2 proteins were localized at the apical membranes of the proximal tubular epithelial cells, but not in glomeruli (G).

Hadjiaogapiou et al.\textsuperscript{21) used an antisense technique to establish major participation of MCT1 in butyrate uptake by Caco-2 cells, the contribution of MCT1 in the intestine remain to be clarified.

OCTN1 (SLC22A4) is a proton/organic cation transporter, first cloned from human fetal liver in 1997.\textsuperscript{22) OCTN1 encodes a 551-amino-acid protein with 11 TMDs and one nucleotide binding motif. It is strongly expressed in the kidney, trachea, bone marrow, fetal liver and several cancer cell lines, and is also weakly expressed in many other tissues. It is a member of the organic cation/carnitine transporter OCTN family, which also includes OCTN2 (SLC22A5) and OCTN3 (Slc22a9).\textsuperscript{23) Even though these three transporters show high homology, each has unique transport characteristics for carnitine and organic cations. OCTN1 and OCTN2 transport carnitine in a sodium-dependent manner, whereas OCTN3 is a sodium-independent carnitine transporter.\textsuperscript{23) Further, OCTN1 and OCTN2 transport organic cations in a sodium-independent fashion, but OCTN3 barely shows organic cation transport activity. In fetal tissues, OCTN2 is expressed strongly in kidney and weakly in liver, lung and brain. In adult, it is expressed strongly in kidney, skeletal muscle, placenta, heart, prostate, and thyroid and weakly in pancreas, liver, lung, brain, small intestine, uterus, thymus, adrenal gland, trachea, spinal cord, and several other tissues. I have examined the subcellular localization of OCTN1 and OCTN2 in the kidney by immunohistochemical analysis (Fig. 3).\textsuperscript{5,10} Both OCTN1 and OCTN2 are expressed in renal cortex, whereas expression is negligible in medulla. OCTN1 and OCTN2 are localized on the apical membrane of proximal tubular epithelial cells. They were not found in glomeruli.\textsuperscript{20)}

Quantitative Evaluation of mRNA Expression Levels of Transporters and Induction of Transporters by Nutrients and Co-Administered Drugs

The absorptive function of the intestine is altered under various conditions, such as dietary regulation, starvation, and anticancer drug-induced intestinal injury, and by various agents, such as sigma-ligand, insulin, etc.\textsuperscript{24–29) Transcriptional activation of the rat PEPT1 gene by amino acids and dipeptides in the diet was reported to increase the transport activity,\textsuperscript{25) which should enhance uptake of peptide-mimetic drugs. Administration of certain drugs has also been reported to influence the bioavailability of other drugs that are substrates of the intestinal transporters. After oral administration of rifampin, the plasma concentration of orally administered digoxin was decreased in healthy volunteers as a result of induction of the expression of MDR1 in the intestine.\textsuperscript{30)} However, in general, it remains unclear what factors influence transporter gene expression. It is important to investigate in detail the changes in expression levels of transporters.

I have examined the influence of various drugs and nutrients on the expression levels of intestinal drug transporters PEPT1, MDR1, MRP2, MRP3, as well as a drug-metabolizing enzyme, CYP3A4, using an intestinal epithelial cell line, Caco-2, and LS180 cells (Table 1).\textsuperscript{31) In Caco-2 cells, the expression of MDR1
**Table 1.** Quantification of expression of transporter mRNAs in Caco-2 and LS180 cells cultured under various conditions

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>PEPT1</th>
<th>MDR1</th>
<th>MRP2</th>
<th>MRP3</th>
<th>CYP3A4</th>
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<tr>
<td><strong>Control</strong></td>
<td>38.7 ± 2.6</td>
<td>71.7 ± 0.6</td>
<td>5.4 ± 0.9</td>
<td>34.5 ± 6.1</td>
<td>4.6 ± 0.0</td>
</tr>
<tr>
<td><strong>FBS (20%)</strong></td>
<td>5.4 ± 0.9</td>
<td>34.5 ± 6.1</td>
<td>4.6 ± 0.0</td>
<td>5.4 ± 0.9</td>
<td>34.5 ± 6.1</td>
</tr>
<tr>
<td><strong>L-Lysine (20 mM)</strong></td>
<td>5.4 ± 0.9</td>
<td>34.5 ± 6.1</td>
<td>4.6 ± 0.0</td>
<td>5.4 ± 0.9</td>
<td>34.5 ± 6.1</td>
</tr>
<tr>
<td><strong>Amitriptyline</strong></td>
<td>54.8 ± 7.8</td>
<td>0.2 ± 0.0</td>
<td>52.4 ± 3.9</td>
<td>0.0</td>
<td>52.4 ± 3.9</td>
</tr>
<tr>
<td><strong>Imipramine</strong></td>
<td>50.1 ± 4.6</td>
<td>0.2 ± 0.0</td>
<td>47.2 ± 2.6</td>
<td>0.0</td>
<td>47.2 ± 2.6</td>
</tr>
<tr>
<td><strong>Midazolam</strong></td>
<td>102.8 ± 11.4</td>
<td>0.5 ± 0.1</td>
<td>90.0 ± 6.7</td>
<td>0.1*</td>
<td>90.0 ± 6.7</td>
</tr>
<tr>
<td><strong>Nifedipine</strong></td>
<td>153.7 ± 29.5</td>
<td>0.7 ± 0.2</td>
<td>124.3 ± 4.3</td>
<td>0.0</td>
<td>124.3 ± 4.3</td>
</tr>
<tr>
<td><strong>Rifampin (10 µM)</strong></td>
<td>31.4 ± 2.2</td>
<td>237.6 ± 37.2</td>
<td>2.0 ± 0.1</td>
<td>200–1,000</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td><strong>ATRA (10 µM)</strong></td>
<td>66.8 ± 4.6</td>
<td>127.2 ± 25.0</td>
<td>4.8 ± 1.7</td>
<td>4.5 ± 0.3</td>
<td>4.5 ± 0.3</td>
</tr>
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</table>

Data are expressed as the absolute, normalized copy number of transcripts per nanogram of total RNA. Drugs shown in bold were dissolved in DMSO and used at a final concentration of 10 µM in 0.1% DMSO. Each column represents the mean ± SEM (n = 3). *p < 0.01 vs control. **ATRA, all-trans retinoic acid.

**Correlation between mRNA Expression Levels and Transport Activities**

Even if expression of transporters has been detected by RT-PCR, Northern blot, or Western blot analysis in certain organs, there is still a question that remains to be clarified: How many transporter molecules in the cell membranes are required for their function to be predominant over membrane permeation via nonsaturable and passive diffusion processes? This question arises because, in contrast to enzymatic reactions, nontransporter-mediated processes such as passive diffusion are often not negligible in the transport of many drugs.

I have determined the absolute value of PEPT1 mRNA expression along the intestinal tract to examine whether the expression level is well correlated with the transport activity of PEPT1 (Fig. 4). The absolute expression level was quantified by means of a real-time PCR method. I determined the distribution pattern of PEPT1 mRNA expression along the small intestine in absolute, not relative, terms per unit of length and examined its correlation with absorptive function for the PEPT1 substrate cefadroxil. The mRNA expression of PEPT1 in the small intestine was in the range of 200–1,000 × 10^6 copy/segment intestine. The values were highest in the lower ileum segment and lowest at the upper duodenum segment. The permeability coefficient was 3–10 × 10^6 cm/sec. A good correlation was found between PEPT1 mRNA expression levels and the permeability coefficient, suggesting that PEPT1 is the major determinant of the absorption of cefadroxil. Importantly, when PEPT1 mRNA expression level was extrapolated to zero value, there remained a permeability of about 2.5 × 10^6 cm/sec. This value was very close to that for non-specific permeation evaluated with inulin (Fig. 4). These results suggested that as little as 200 × 10^6
Fig. 4. Correlation between rat PEPT1 expression level and permeability coefficient of cefadroxil along the small intestinal tissues. A, Expression level of PEPT1 mRNA in each segment of same length from small intestine of starved (open column) and fed (closed column) rats. Each column represents means ± SEM of three rats. B, Values of the absorptive permeability coefficient of cefadroxil in fed (squares) and starved (circles) rats were examined using Ussing chambers. PEPT1 expression levels were also examined in each sample. Absorptive permeability of inulin (triangles) was evaluated as a measure of non-specific permeation. The small intestinal tissues were obtained from upper (open), middle (shaded) and lower (closed) segments. A significant correlation between absorptive permeability coefficient and PEPT1 mRNA expression was observed. Each point represents means ± SEM of 4–9 determinations.

copies/segment of intestine are sufficient for PEPT1 to mediate cefadroxil absorption in the small intestine.

A comprehensive list of drug transporters would enable us to use transporters for drug delivery. However, in some cases, several transporters that share the same substrates are expressed in the same tissue. Then we have to establish which transporter is the major contributor. Expression of a transporter gene in a certain tissue does not necessarily mean that the transporter is functional. If the expression level of one transporter is low, it may make only a minor contribution to the overall transport process. Information about these matters is needed to establish efficient strategies for the use of transporters in the delivery of drugs.

In Vivo Heterologous Transduction of Transporters

I have examined the feasibility of heterologous transduction of a transporter gene into tissues including the blood-brain barrier (BBB) and the liver.33,34 Peptides have multiple biological actions in the brain; therefore, they are potentially valuable as neuropharmaceuticals in the treatment of various disorders, such as Alzheimer’s disease and depression. Delivery of peptide drugs to the brain, however, is a challenge because the distribution of peptides to the brain is generally very low owing to the BBB. I have constructed a recombinant adenovirus vector encoding PEPT1 and transduced the transporter into the BBB of rats by carotid artery injection of the vector (Fig. 5).33 Heterologous expression of PEPT1 at the BBB successfully increased the brain distribution of a model PEPT1 substrate (cefadroxil) (Fig. 5).33

Current treatment methods for brain cancer are still inadequate. Malignant gliomas are the most common primary neoplasm of the central nervous system, and the prognosis for patients diagnosed with high-grade glioma (glioblastoma multiforme) remains bleak; survival is less than 1 year.35 Therefore, new therapeutic strategies need to be developed for gliomas. It was reported that metalloprotease inhibitors, including puromycin and bestatin, induce apoptosis in glioma cells.36 Bestatin is an anticancer agent that is transported by PEPT1. Although PEPT1 is not expressed in brain tissues,12 heterologous expression of PEPT1 at the BBB may allow bestatin to enter the brain efficiently across the BBB. Furthermore, this system could also be applicable to a new melphalan prodrug designed for tumor-selective activation.37 It is not clear, however, whether heterologous expression of PEPT1 by adenovirus is safe for the brain tumors if less toxic and more effective vectors can be developed.

I think that the feasibility of this approach for human therapeutic interventions is dependent not only on the safety of transient expression of PEPT1 by adenovirus, but also on whether any alternative is available to the patient. Many pharmacologically effective peptide-mimetic drugs have been developed, but the BBB permeability of peptides is extremely low, even though expression of some peptide transporter genes at the BBB has been reported. Importantly, the substrate recognition spectrum of the oligopeptide transporter PEPT1 is extremely broad. As reported recently, PEPT1 accepts not only traditional peptide-mimetic agents, but also valacyclovir, 4-aminophenyl, γ-aminolevulinic acid, 6-aminohexanoic acid, and so on.15 These observations suggest that a peptide bond is not essential for substrates of this transporter.15 Accordingly, novel non-
Fig. 5. In vivo transduction of transporter by adenovirus vector. Heterologous expression systems can be used to manipulate expression levels of a transporter in a tissue. This technique is useful to evaluate the contribution of the transporter as a determinant of drug disposition. A, Schematic representation of adenovirus vector-mediated transduction of transporter. B, Kp value of cefadroxil in rat brain without transduction (open column) or with transduction with AdhPEPT1 (closed column) and AdGFP (gray column) for 3 days. Kp values were obtained 30 min after intravenous administration of cefadroxil in rat with or without transduction of adenovirus. Each value represents the mean±SEM of 3–5 rats.

peptide PEPT1 substrates with pharmacological activity in the brain could also be candidates for this approach.

Conclusion

We are now entering a new era of drug delivery. Although several drug transporters have been utilized as drug delivery systems, transport mechanisms for the majority of drugs are still unknown. Therefore, in order to establish the potential utility of transporter-mediated approaches, we have to identify all the participants, find a way to evaluate the relative contribution of each transporter, and determine the appropriate target for each drug.

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