Part Three
Role of Transporters and Metabolism in Oral Absorption

10
Transporters in the Gastrointestinal Tract

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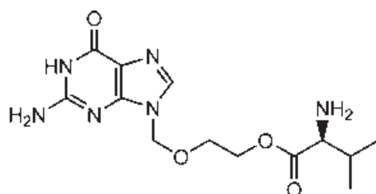
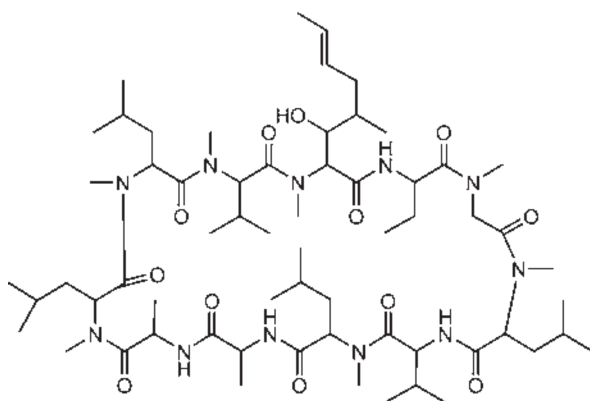
Abbreviations

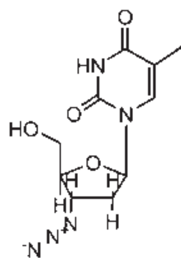
ABC	ATP-binding cassette
ATP	Adenosine 5′-triphosphate
CDX	Caudal-type homeobox transcription factor
CNT	Concentrative nucleoside transporter
ENT	Equilibrative nucleoside transporter
EMT	Epithelial–mesenchymal transition
GIT	Gastrointestinal tract
GO	Gene ontology
NCE	New chemical entity
PEPT1	Di/tri-peptide transporter 1
PMT	Pharmacogenetics of Membrane Transporters Project
QSAR	Quantitative structure–activity relationship
SAR	Structure–activity relationship
SLC	Solute carriers (SLCs)

10.1
Introduction

In 1992, Beauchamp *et al*. published a paper on the synthesis of 18 amino acid esters of the antiherpetic drug, acyclovir [1]. These esters were synthesized as potential prodrugs intended for oral administration, and were less potent than the parent compound with respect to *in vitro* antiviral activity against herpes simplex virus type 1 (HSV-1). However, 10 of the prodrugs produced greater amounts of the parent drug in the urine of rats used in the study. The L-amino acid esters were better prodrugs than the corresponding D- or D,L-isomers, suggesting the involvement of a stereo-







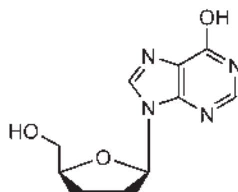
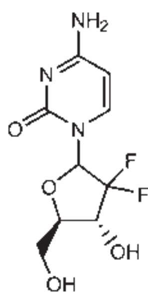


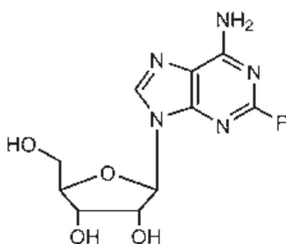
Figure 10.1 Chemical structures of some selected compounds involved in active drug absorption.

selective transporter. The L-valyl ester was the best prodrug (cf. Figure 10.1 (**I**)),[1] and in 1998 de Vrueh and coworkers showed that the responsible transporter was the di/tripeptide transporter also termed PEPT1 (SLC15A1) [2]. This illustrated the impact of targeting transporters, although the discovery of valaciclovir as a PEPT prodrug

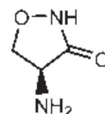
1) In the following text, roman numbers refer to the chemical structures throughout, as illustrated in Figure 10.1.



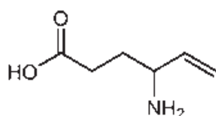
V. Gemcitabine



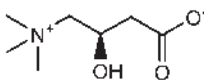
VI. Fludarabine



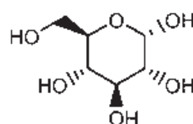
VII. D-cycloserine



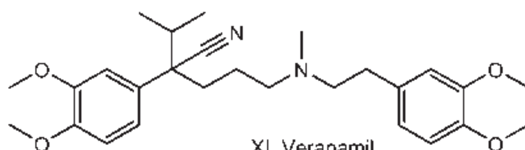
VIII. Vigabatrin



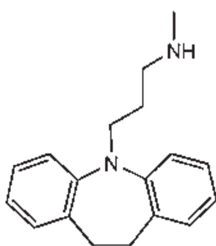
IX. L-carnitine



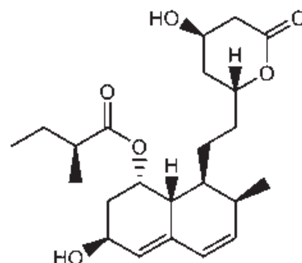
X. Glucose



XI. Verapamil



XII. Desipramine



XIII. Pravastatin

Figure 10.1 (*Continued*)

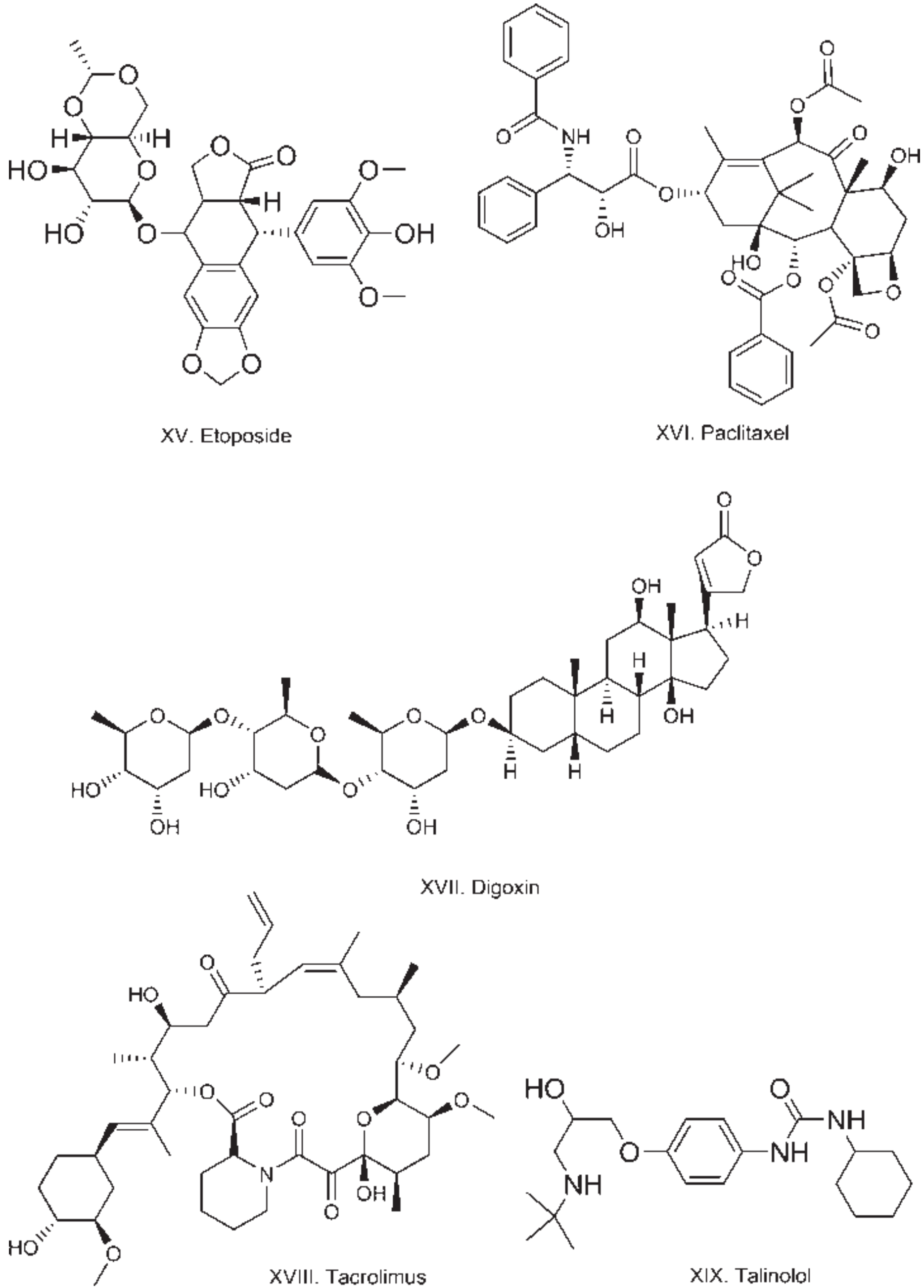
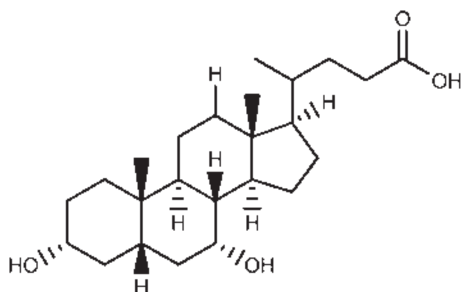
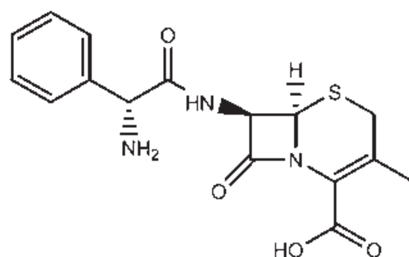


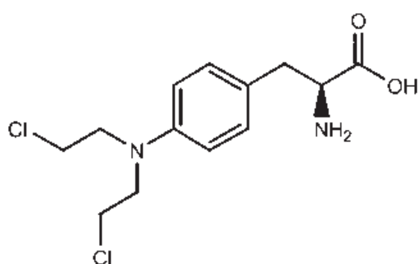
Figure 10.1 (*Continued*)



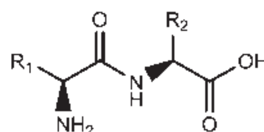
XX. Chenodeoxycholic acid



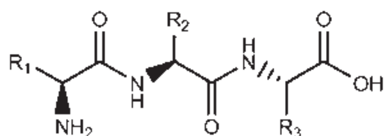
XXI. Cephalexin



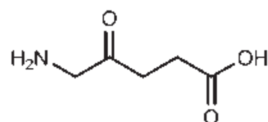
XXII. Melphalan



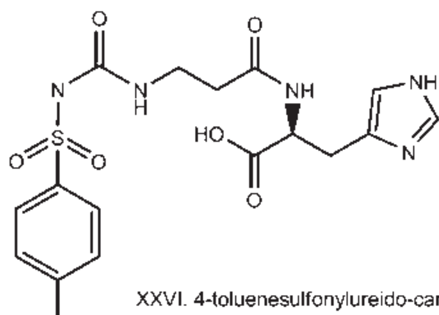
XXIII. Dipeptide backbone



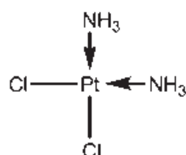
XXIV. Tripeptide Backbone



XXV. δ-amino levulinic acid



XXVI. 4-toluenesulfonylureido-carnosine



XXVII: Cisplatin

Figure 10.1 (*Continued*)

seems to have been serendipitous. Nevertheless, over the past two decades, an increased focus has been on transporters – absorptive as well as efflux – in relation to drug absorption.

The aim of this chapter is to provide an overview of transporters present in the gastrointestinal tract, their role in intestinal drug absorption, and their relevance as a drug delivery target to the medicinal chemist. Drug discovery is a multidisciplinary process, which we would like to illustrate in this chapter. We will cover elements of prodrug design, structure–activity relations, expression profiles, transporter genomics, and regulation and briefly mention methods for studying these elements all in specific relation to the relevance of active transport in the gastrointestinal tract (GIT) for drug absorption or pathophysiological conditions. In addition, in the context of pathophysiological states of the intestine, we will discuss the role of transporters not only as drug carriers but also as direct drug targets.

In the previous edition of this book, the chapter on transporters in the gastrointestinal tract described the various transporters and their families in general. In this edition, we will focus on selected transporters that have proven relevant to the medicinal chemist or pharmaceutical scientist in general. The chapter is structured in the following manner: Section 10.2 provides an overview of active transporters present along the intestine and their influence on drug absorption, Section 10.3 deals with transporters and their genomics, Section 10.4 discusses structural features for targeting intestinal transporters, and Section 10.5 illustrates the altered expression of transporters in diseased staten of the intestine. We will outline at the end of each section, the impact on medical chemistry and how these various aspects affect the work of the medical chemists.

10.2 Active Transport Along the Intestine and Influence on Drug Absorption

Transporters in the intestine generally fall into two distinct groups. One group consists of absorptive transporters belonging to the solute carrier (SLC) family and a second group consists of drug efflux transporters of the ATP-binding cassette (ABC) family (ABC transporters). In general, active transporters are transporters where the translocation of substrate is linked to the ATP consumption, whereas transporters where the translocation of substrate is down the concentration gradient of the substrate or coupled to the influx of ion such as Na^+ or H^+ are termed carriers. Transporters are often referred to as drug transporters, which in the context of drug discovery or development is certainly an appropriate term. However, it is important to remember that the normal function of transporters is to move nutrients or xenobiotic waste products across the cell membrane. In Table 10.1, a number of relevant intestinal drug transporters are listed. In Figure 10.2, a few examples of the localization of transporters in the apical and basolateral membranes are shown. The distribution of different transporters is part of the cellular polarization of cellular components, and the asymmetric distribution gives rise to polarized transport across the intestine. Certain drug compounds and xenobiotic natural products are exported

Table 10.1 List of relevant transporters for intestinal absorption and/or diseased states of the intestine.

Transporter family	Transporter[a]	Source[b]: Expression	Source[c]: SNP	Xenobiotic substrates[d]
Peptides				
SLC15A	*SLC15A/PEPT1*	Small intestine, all three segments [3, 4]	[61, 62]	β-Lactam antibiotics (e.g., cephalexin), various amino acid and dipeptide prodrugs (e.g., valaciclovir) [2, 8–10]
	CDH17/HPT1	Small intestine, colon [3, 4]	N/A	Valaciclovir [6, 12, 204]
Nucleosides				
SLC28A	*SLC28A1/CNT1*	Small intestine, all three segments [3, 4]	[64]	Azidothymidine, cemcitabine [20–22]
	SLC28A2/CNT2	Small intestine, all three segments [3, 4]	[65]	2′,3′-dideoxyinosine [23]
	SLC28A3/CNT3	Small intestine, duodenum, and jejunum [3, 4]	[66]	Gemcitabine, cladribine, and fludarabine [17]
SLC29A	*SLC29A1/ENT1*	Small intestine, duodenum, and jejunum [3, 4]	PMT	Cladribine, cytarabine, and gemcitabine [205]
	SLC29A2/ENT2	Colon [3, 4, 16]	[67]	AZT, gemcitabine [205]
	SLC29A3/ENT3	Small intestine, duodenum, and jejunum [3, 4]	N/A	cf. ENT2 [206]
Amino acids				
SLC3A	SLC3A1/rBAT	Small intestine [207]	[208–210]	Gabapentin [28]
	SLC3A2/4F2HC	Intestine [4]	[68]	
SLC6A	*SLC6A14/ATB*$^{0,+}$	Colon [29]	[211]	D-Serine, L-carnitine [29]
SLC7A	SLC7A5/LAT1	Duodenum [58]	[68]	Gabapentin [205]
	SLC7A9/b0,+AT	cf. SLC3A1	[212, 213]	Gabapentin [205]
SLC1A	SLC1A1/EAAT3	Duodenum [4]	[214, 215]	N/A

(*Continued*)

Table 10.1 (*Continued*)

Transporter family	Transporter[a]	Source[b]: Expression	Source[c]: SNP	Xenobiotic substrates[d]
	SLC1A5/ATB0	Ileum [216]	[217]	N/A
SLC36A	*SLC36A1/PAT1*	Whole GIT [25]	N/A	GABA, vigabatrin, D-cycloserine [26, 27]
Monosaccharides				
SLC5A	*SLC5A1/SGLT1*	Small intestine [4]	[218]	Phloridzin[(I)] [95]
SLC2A	*SLC2A5/GLUT5*	Small intestine [4]	N/A	N/A
Organic cations				
SLC22A	SLC22A4/OCTN1	Small and large intestines [3, 4, 16]	[219, 220]	Quinidine, verapamil [30]
	SLC22A5/OCTN2	Small and large intestines [3, 4, 16]	[221]	Cephaloridine [30]
Organic anions				
SLCO2B1	*OATP2B1*	Jejunum, colon [3]	[222]	Pravastatin [32]
Monocarboxylates				
SLC16A	*SLC16A1/MCT1*	Jejunum, colon [3, 4]	N/A	Cefdinir, pravastatin, and valproic acid [35, 36]
	SLC16A8/MCT3	Duodenum [3, 4]	N/A	N/A
	SLC16A3/MCT5	Duodenum, jejunum [3, 4]	N/A	N/A
MRP/MDR (efflux) transporters				
ABC	*ABCB1/MDR1*	Jejunum, colon [3]	[69–71]	Vinblastine, doxorubicin, etoposide, paclitaxel, and dioxine [38]
	ABCC1/MRP1	Jejunum, colon [3]	[73]	Doxorubicin, etoposide, vincristine, and methotrexate [152]
	ABCC2/MRP2	Jejunum [3]	[72, 73]	Methotrexate, etoposide, doxorubicin, cisplatin, and vincristine [152]

	ABCC3/MRP3	Jejunum, colon [3]	[72]	Methotrexate, etoposide, doxorubicin, cisplatin, and vincristine
	ABCC5/MOAT-C	Jejunum, colon [3]	[72, 73]	6-Mercaptopurine, 6-thioguanine [152]
	ABCG2/BCRP	Jejunum, colon [3]	[70]	Doxorubicin, daunorubicin, mitoxantrone, and topotecan [152]
Miscellaneous				
SLC19A	*SLC19A1/RFC1*	Small and large intestines [223]	[224]	Methotrexate [205]
SLC10A	*SLC10A2/ASBT*	Duodenum, ileum [16]	[225]	Bumetanide [205]
Additional, relevant transporters				
SLC5A	SLC5A8	Tumor suppression [161]	N/A	N/A
SLC26A	SLC26A3	Tumor suppression [160, 165]	N/A	N/A
SLC16	SLC16A3/MCT4	Invasion, migration [168]	N/A	N/A

[a] In italics are transporters even more relevant and/or more is known about their involvement in drug and nutrient absorption.
[b] Nonexhaustive list of references regarding expression of the gene in the gut or its specific role in cancer.
[c] Nonexhaustive list of studies on single-nucleotide polymorphisms of the gene; N/A, no relevant reference could be found; PMT, pharmacogenetics of membrane transporters; http://www.pharmgkb.org.
[d] Nonexhaustive list of xenobiotic compounds transported by the protein.

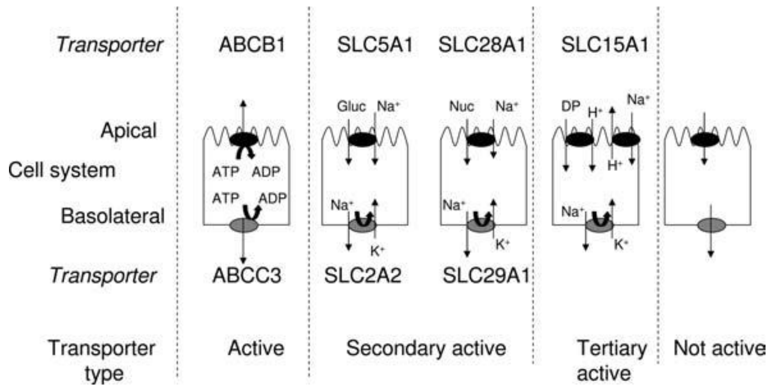


Figure 10.2 Illustration of the various forms of transporters and carriers working in an intestinal epithelial cell. Gluc, glucose; Nuc, nucleotide; DP, dipeptides; and ABC, ATP-binding cassette family transporter; SLC5A1, sodium-dependent glucose transporter; SLC2A2, glucose transporter 2; SLC28A1, concentrative nucleotide transporter 1; SLC29A1, equilibrative transporter 1; and SLC15A1, di/tripeptide transporter.

or effluxed from the epithelial cell by the apical localized ABCB1 (also termed P-glycoprotein (P-gp), and MDR1) and the basolateral localized ABCC3. Glucose is transported across the apical membrane by SLC5A1 (SGLT1) in a sodium-dependent manner, and the cellular efflux is mediated by SLC2A2 (GLUT2), which is not coupled to any other driving force. Nucleoside or nucleoside drugs are transported into the epithelial cell by SLC28A1 (CNT1) and effluxed by SLC29A1 (ENT1). Di/tripeptides and some peptidomimetic drugs are transported into the cell by SLC15A1 (PEPT1) and transported from the cell to the blood circulation by a transporter, which has as yet not been cloned.

In the following section, selected transporters relevant to drug absorption will be discussed along with known drug–drug interactions caused by transporters. The transporters will be discussed according to their natural substrates as indicated in Table 10.1.

10.2.1 Peptide Transporters

In the human intestinal cells, two di/tripeptide transporter genes have been detected by quantitative PCR methods (see Table 10.1): *SLC15A1* (*PEPT1*) (see Figure 10.2) and liver–intestine cadherin *CDH17* (*HPT1*). *PEPT1* is highly expressed in the small intestine, that is, duodenum, jejunum, and ileum and in healthy tissue absent in the colon, whereas *HPT1* is highly expressed in both small and large intestines [3, 4]. So far, the consensus in the literature has been that PEPT1 was the major peptide transporter for dietary uptake of amino acids in the form of di/tripeptides and also in terms of intestinal drug absorption [5–7]. PEPT1 has been shown to mediate the oral bioavailability of some β-lactam antibiotics and a number of amino acid and dipeptide

prodrugs [2, 8–10]. PEPT1 is discussed thoroughly in this chapter, since it remains one of most studied intestinal absorptive transporters in drug delivery and oral bioavailability. The intestinal expression of *HPT1* is higher than that of *PEPT1* [3, 4], and *HPT1* expression has in a study been correlated with the bioavailability of valaciclovir [6, 7, 11, 12]. However, since HPT1 belongs to the cadherin superfamily of calcium-dependent, cell–cell adhesion proteins and only has one transmembrane segment [13], its role as a transporter has not been fully resolved. Although not normally referred to a as peptide transporter, ABCB1 has been shown to transport peptides containing a varying number of amino acids from 3 to at least 11 (cyclosporine A (**II**) [14, 15]), thereby *de facto* acting as a peptide transporter. In the apical membrane, the transport direction is out of the cell, as opposed to PEPT1, which is inward directed and coupled to influx of protons, as illustrated in Figure 10.2. However, at the basolateral membrane, the transport direction of both an ABC transporter and a peptide transporter would be from the cell into the circulation.

10.2.2 Nucleoside Transporters

Transporters for nucleosides are present both in the apical (concentrative nucleoside transporters (CNTs)) and in the basolateral membrane of epithelial cells (equilibrative nucleoside transporters (ENTs)). *CNT2* is expressed primarily in the human duodenum and jejunum and to a much higher degree than *CNT1*, *CNT3*, *ENT2*, and *ENT2* [3, 4]. In the ileum, both *CNT1* and *CNT2* are expressed, whereas *ENT2* is expressed in the colon [16]. It has to be noted that human samples are often obtained from patients, and their medications may to some extent alter the expression profile of the investigated transporters, as exemplified in Section 5.4, where PEPT1 is expressed in the colon due to inflammation of the tissue. In the human intestinal epithelium, nucleoside transporters are responsible for the uptake of dietary nucleosides from the intestinal lumen and the regulation of adenosine concentrations on cells' surface. The nucleoside transporters accept a variety of drug molecules structurally related to nucleosides and they may thus be highly relevant from a drug delivery and drug discovery point of view. ENT1 and ENT2 transport both purine and pyrimidine nucleosides, although the substrate affinities in general are lower than those for the CNTs. The concentrative nucleoside transporters CNT1, CNT2, and CNT3 transport uridine and certain uridine analogues, but are otherwise in general (except for a modest transport of adenosine by CNT1) selective for pyrimidine (CNT1) and purine (CNT2) nucleosides, with CNT3 being nonselective for purine and pyrimidine nucleosides [17, 18]. The differences in purine and pyrimidine substrate recognition are also observed with pyrimidine and purine antiviral and anticancer nucleoside drugs. For example, CNT1 transports 3′-azido-3′-deoxythymidine (AZT) (**III**), 2′,3′-dideoxycytidine, and cemcitabine, whereas CNT2 carries 2′,3′-dideoxyinosine (**IV**) [19–23]. CNT3 translocates pyrimidine anticancer nucleoside structures such as 5-fluorouridine, 5-fluoro-2′-deoxyuridine, zebularine, gemcitabine (**V**), and purine nucleoside struc-

tures such as cladribine and fludarabine (**VI**) [17]. Although the concentrative nucleoside transporters have micromolar affinities for the proteotypical substrate uridine, they are interesting for targeting intestinal cells.

10.2.3
Amino Acid Transporters

Several amino acid transporters are present at both apical and basolateral membranes. They have overlapping substrate specificities, different affinities, and capacities and utilize different ions such as Na^+, H^+, and Cl^- for driving force (for a review on the physiology of intestinal amino acid, see Ref. [24]). Moreover, amino acid transporters are affected by a number of diseases such as Hartnup disorder and other absorptive pathological states. Recently, Kim *et al.* detected 12 genes for amino acid transport in the human intestine, where *EAAT3*, *LAT3*, *4F2HC*, and *PROT* were highest expressed [4]. The gene of a proton-coupled amino acid transporter, *PAT1*, has recently been cloned [25]. On the basis of Northern blot, this transporter is expressed in segments from the stomach to the latter parts of the colon, and the PAT1 protein localizes in the apical membrane [25]. PAT1 has millimolar affinity for its substrates (Pro, Gly, Ala, and GABA) and has been shown in *in vitro* studies to be involved in intestinal absorption of D-cycloserine (**VII**) and vigabatrin (**VIII**) [26, 27]. Thus, PAT1 seems to be a drug delivery target for some clinically relevant GABA-related drugs. However, gabapentin is not transported by PAT1 but may be transported by system b (rBAT/$b^{0,+}$AT, SLC3A1/SLC7A9), which transports neutral and dibasic amino acids in a sodium-independent manner [28]. Another amino acid transporter that seems to be interesting from a pharmaceutical point of view is SLC6A14/$ATB^{0,+}$ [29]. This transporter is located in the apical membrane of colonocytes but is not expressed in the small intestine. The transport of amino acid is coupled to Na^+ and Cl^-, and SLC6A14 has been suggested to be relevant to the intestinal absorption of D-serine, carnitine (**IX**), and nitric oxide synthase inhibitors as well as the γ-glutamyl ester of acyclovir [29].

Amino acid transporters seem to hold a great potential for intestinal absorption of drugs and drug candidates. However, overall, the information on the impact of these transporters in terms of mediating oral bioavailability of amino acid mimetics is rather limited, and systematic investigations using both *in vitro* and *in vivo* data are highly anticipated.

10.2.4
Monosaccharide Transporters

In the intestine, primarily two glucose (**X**) transporter genes are detected: *SGLT1* and *GLUT5* [4]. SGLT1 is located in the apical membrane of the small intestinal enterocytes and operates in a sodium-coupled way, whereas GLUT5 is located in the basolateral membrane (cf. Figure 10.2). SLGT1 has been suggested as a target not only for inhibiting the uptake of glucose but also for intestinal transport of glycoside-bond-containing substrate mimics.

10.2.5 Organic Cation Transporters

The organic cation transporters *OCNT1* (*SLC22A4*) and *OCNT2* (*SLC22A5*) are widely expressed in both small and large intestines [3, 4, 16]. *OCT1* (*SLC22A1*) is expressed in the jejunum and colon, whereas *OCT3* (*SLC22A3*) is expressed only in the jejunum [3]. OCNT1 seems to be located in the luminal membrane and transports compounds such as L-carnitine (**IX**), stachydrine, and the organic cations tetraethylammonuim (TEA), quinidine, pyrilamine, and verapamil (**XI**) [30]. OCNT2 is a Na^+-dependent, high-affinity transporter for L-carnitine, acetyl-L-carnitine, and the zwitterionic β-lactam antibiotic cephaloridine, but can also function alternatively as a polyspecific and Na^+-independent cation transporter [30]. OCT1 is localized in the basolateral membrane and transports tetraethylammonium as well as drugs such as desipramine (**XII**), acyclovir, and ganciclovir [31].

10.2.6 Organic Anion Transporters

OATP transporters, *SLCO1C1*, *SLCO2B1*, *SLCO3A1*, *SLCO4A1*, and *SLCO4C1* have been found in the jejunum, and also in the colon, with the exception of *SLCO3A1* [3]. OATP-B (SLCO2B1) accepts bile salts, as well as pravastatin (**XIII**) and sulfate conjugates of steroid hormones, and is localized in the apical membrane of enterocytes [32].

10.2.7 Monocarboxylate Transporters

Monocarboxylate cotransporters (MCTs) transport monocarboxylates such as acetate, propionate, lactate, and pyruvate in a proton-coupled manner or by exchanging one monocarboxylate for another [33]. In the intestine, *MCT1* and *MCT5* have been detected [3], and in another study *MCT3* was detected at a level similar to *MCT5* [4]. *MCT1* is expressed in both small and large intestines [3, 34], whereas *MCT5* was not found in the colon [3]. MCT1 is located in the apical membrane of epithelial cells, whereas MCT3 is located in the basolateral membrane [33]. MCT1 has been suggested to be involved in intestinal transport of the cephalosporin cefdinir, whereas pravastatin (**XIII**) and valproic acid (**XIV**) inhibit the cellular uptake of lactate *in vitro* [35, 36].

10.2.8 ABC Transporters

Some of the intestinal transporters that have received most attention in drug discovery and development are undoubtedly the ATP-binding cassette transport proteins and especially ABCB1 (for reviews see Refs [37–39]). There are numerous publications on these transporters, and we are, thus, able to give only a small glance

on the impact of efflux transporters on drug delivery, drug disposition, pharmacological therapy, metabolism, cancer therapy, and cancer development. Overall, in terms of drug bioavailability, efflux transporters in the intestine limit the intestinal absorption of a wide range of drugs. In the human jejunum and colon, *ABCB1*, *ABCC1* (*MRP1*), *ABCC3* (*MRP3*), and *ABCC4-6* (*MRP4-6*), as well as *ABCG2* (*BCRP*), are expressed [3]. *ABCB4* (*BSEP*) is expressed in the colon, and *ABCB4* and *ABCC2* (*MRP2*) are expressed in the jejunum [3]. The efflux transporters are located in different membranes of the enterocytes, with ABCB1, ABCC2, and ABCG2 located in the apical membrane and ABCC1 and ABCC3 located in the basolateral membrane [38].

ABCB1 has an extremely broad substrate specificity with a preference for lipophilic and cationic compounds; however, these general structural features are by no means restrictive. This is illustrated by the diversity in the substrates identified for ABCB1, including anticancer drug substances such as vinblastine, doxorubicin, etoposide (**XV**), and paclitaxel (**XVI**), cardiac drug substances such as digoxin (**XVII**) and some β-blockers, endogenous compounds such as steroid hormones and bile salts, HIV drugs such as indinavir and saquinavir, fluoroquinolones such as sparfloxacin, and immunosuppressive drugs such as cyclosporine A (**II**) and tacrolimus (**XVIII**) [38]. In general, ABCB1 substrates are thus amphipathic compounds with a molecular weight ranging from approximately 300 to 2000 Da. It has been suggested that ABCB1 binds substrates through an "induced-fit mechanism," where the shape and size of the substrate change the packing of the transmembrane segments to accommodate the substrate [40, 41]. There is some evidence that ABCB1 is functioning in conjunction with metabolizing enzymes. ABCB1 may thus play a dual role in limiting oral bioavailabilities of drug substrates, that is, by reducing their absorption and delivering them to metabolic enzymes or exporting metabolites. In the liver and intestine, this dual function is present in metabolic active cells. Phase-I enzymes such as cytochrome P450 and phase-II enzymes such as gluthathione-*S*-tranferases are key factors in limiting drug bioavailability. There is also some degree of overlapping substrate specificity between CYP3A4 and ABCB1 [42–44]. It is therefore important to consider both the efflux properties and the metabolic properties of drug candidates.

Digoxin (**XVII**) is a typical substrate for ABCB1, and it has been shown in P-gp knockout mice that intestinal efflux and plasma elimination of digoxin are decreased along with increased brain accumulation [45–47]. Coadministration of other ABCB1 substrates alters the pharmacokinetics of digoxin, as seen with an increased bioavailability after coadministration with talinolol (**XIX**) [48]. Other drug–drug interactions caused by intestinal efflux transporter are known for immunosuppressants such as tacrolimus and cyclosporin A, as well as talinolol [39]. It is important to remember that a large degree of overlapping substrate specificity exists between different ABC transporters, and an analysis of the impact of individual transporters on limiting intestinal absorption is perhaps less important than evaluating the combined impact on intestinal absorption of ABC transporters. Moreover, the oral bioavailability is in addition to intestinal absorption a result of many other processes involving ABC transporters. Nevertheless, efflux transporters are important for the

medicinal chemist, as they are involved in absorption, metabolism, and disposition of many diverse molecular structures.

10.2.9 Bile Acid Transporters

The apical localized sodium-dependent bile acid transporter (ASBT) is expressed in the human duodenum and ileum and is barely detectable in colon [16]. ASBT transports bile acids such as glycodeoxycholate and chenodeoxycholic acid (**XX**) [49, 50]. Few examples exist where the bile acid scaffold has been used as a promoiety for a prodrug approach. ASBT has micromolar affinities for the natural substrates, and the studies on ASBT are too few to make a general statement on the potential and role of this transporter in drug absorption [49, 50].

10.3 Transporters and Genomics

10.3.1 Introduction to Genomics Technologies

The analysis of the human genome sequence suggested the presence of 406 genes encoding ion channels and 883 genes encoding transporters, of which 350 are proposed intracellular transporters [51]. Most of the time, though, it is still unknown which transporters are involved in the uptake of a given drug. Therefore, knowing the expression of transporters along the intestine could help identify relevant drug carriers in the intestine.

The transport activity of drug carriers can be affected in many different ways. Thus, with respect to drug design, different questions have to be addressed: Which transporters can be utilized in the intestine for oral drug absorption? Can the expression of these transporters be affected by either disease states or type of nutrition, age, and sex? Is it possible that in a population, various genotypes exist with different transport activities and substrate specificities?

In recent years, various high-throughput technologies have been developed that are being used to address these questions. While expression arrays, such as Serial Analysis of Gene Expression (SAGE) and Massively Parallel Signature Sequencing (MPSS), are applied to measure the messenger RNA levels, genomic arrays are being used to look for potential amplifications and deletions in the genome, and the so-called single-nucleotide polymorphism (SNP) arrays are being used to perform genotyping of samples.

Using such a genome-wide approach allows not only the study of transporter genes but also can provide some insights into the regulation of transporter genes. Nowadays, commercially available gene chips contain the information of all currently annotated genes. Therefore, in one single experiment, changes of expression patterns of any gene can be analyzed and correlated to a reference gene.

It is important to note that RNA may not always correlate with the expression of encoded proteins. Use of proteomic techniques increasingly serves to resolve discrepancies between mRNA levels and expressed proteins. However, proteomics of integral membrane proteins still remains a challenge; moreover, comparing mRNA levels in similar tissues from different subjects is likely to provide an indication of protein levels. Any polymorphism that affects protein structure and function could also lead to false interpretations if the array fails to measure the functional polymorphism.

10.3.2 Gene Expression Profiling Along the Intestine and in Caco-2 Cells

Even though the transport activity can be influenced at various levels, the first step in elucidating the relevance of membrane transporters in drug absorption is studying the expression of these proteins. Various studies using a genome-wide approach have been done in recent years addressing this question. Some studies have been done using the mouse as a model system to assess the situation in humans. However, many times, *in vitro* systems are being used to perform high-throughput screening to assess the absorption rate of a given drug. The cell line of choice is usually Caco-2. As a consequence, different studies have been performed to characterize its genomic expression profile and to correlate it to the situation in humans. It is though important to note that the Caco-2 cells have significant higher transepithelial electrical resistances than the human intestinal mucosa. And as a consequence, a high correlation of the expression levels of transporters between the Caco-2 cells and the human intestinal mucosa may provide a reasonable prediction, but it may also overestimate the contribution of active transport in oral drug absorption due to the contribution of the paracellular route. In the following section, we will discuss some relevant examples of genomic profiling of the intestinal mucosa and the Caco-2 cell system.

10.3.2.1 Profiling of the Intestinal Mucosa

Bates *et al*. studied the expression profile of different gastrointestinal segments in mouse, that is, stomach, duodenum, jejunum, ileum, cecum, proximal colon, and distal colon using an 8.6 k cDNA chip [52]. Genes encoding transport proteins fell into three anatomical patterns: expression throughout the GIT (such as several ion channel and pumps and the polymeric immunoglobulin receptor), expression predominantly in the small intestine (such as intestinal fatty acid-binding protein and apolipoprotein A-IV, C-TI, and C-III), and expression in the colon (such as aquaporin 8, involved in water transport, and carbonic anhydrases). Moreover, when classifying all genes represented on the chip into functional groups, the authors concluded that genes encoding proteins, functioning in intermediary metabolism, transport, and cell–cell communication, have the most dynamically regulated expression profiles. However, the limited set of transporter genes investigated narrows the ability of this study for assessing global transport capacity. On the basis of their functional classification, 181 genes out of the 8638 mouse genes represented on the

chip were defined as genes encoding proteins with transport functions, whereas Venter *et al.* suggested that there are 1289 transporters/ion channels in the human genome [51]. Thus, only a fraction of membrane transporter genes has been analyzed in this study.

Similar to Bates *et al.*, we also investigated the expression of transporter genes along the mouse intestine [52, 53]. Using the Affymetrix technology, we could measure the genomic profile of 853 transporter genes. In contrast to Bates *et al.*, we could not observe that the class of transporters was significantly regulated compared to other GO classes; however, the subclass carriers, and in particular their daughter classes "antiporters" and "symporters," were significantly regulated [52, 53]. Both are classes in which typical transporters involved in drug delivery can be found. Figure 10.3 shows an overview of changes of expression of transporter groups in mice according to the GO system along the intestine [53]. Although some

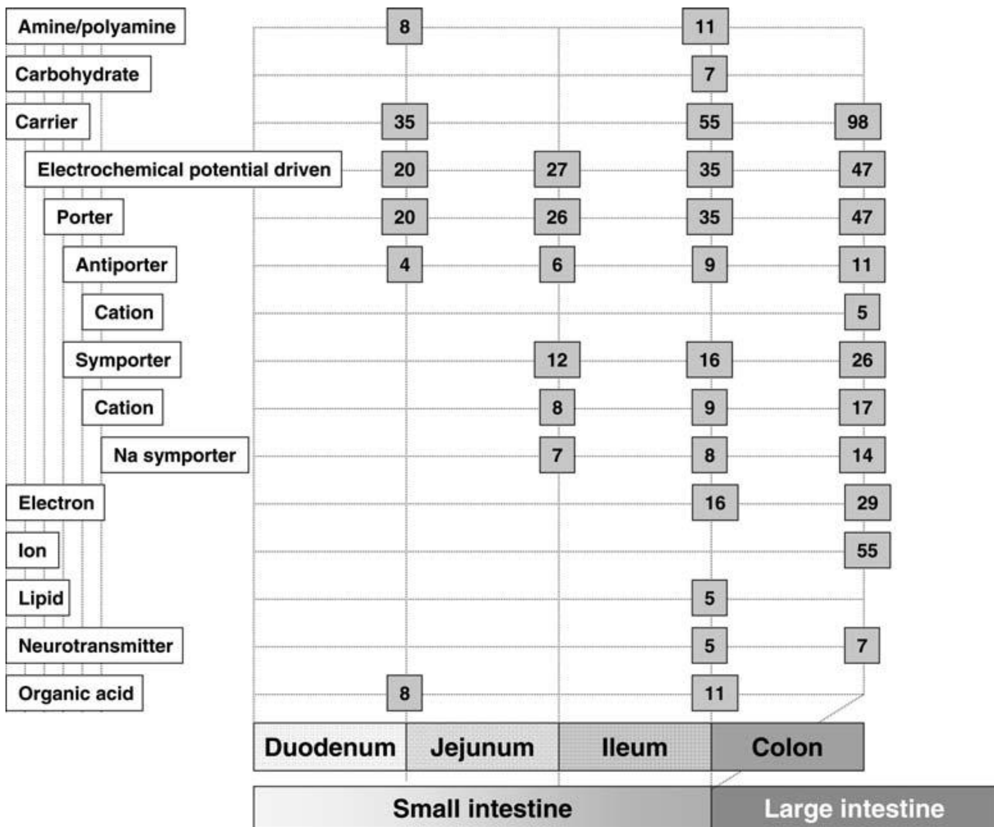


Figure 10.3 Overview of classes according to Gene Ontology (www.geneontology.org), which were significantly changed along the mouse intestine ($p \leq 0.05$). Gray boxes indicate the number of genes for each significant change between two adjoining regions. For a comparison with changes in humans see Ref. [53].

single transporters of a given group may be significantly different between the intestinal segments, the groups they are classified into may not be significantly altered.

Interestingly, the small intestine and the colon revealed that a similar number of genes were more highly expressed in either the small intestine or the large intestine. However, small intestinal transporters were clearly more overexpressed. The majority of differentially expressed transporters were members of the solute carrier super family. The most pronounced change (fold changes > 300), however, was observed for fatty acid-binding protein 1 (Fabp1). Among the solute carriers being more than 20 times overexpressed in the small intestine were the neurotransmitter transporter Slc6a19, the sulfate transporter pendrin-like protein 1 (Slc26a6), L-type amino acid transporter 2 (Slc7a8), facilitated glucose transporter 2 (Slc2a2), and the concentrative nucleoside transporter 2 (Slc28a2, CNT2). The Y(+)L-type amino acid transporter 1 (Slc7a7), the concentrative nucleoside transporter cotransporter (Slc28a3), and the B(0,+)-type amino acid transporter 1 (Slc7a9) were more than 10 times overexpressed. In the colon, the most overexpressed transporter was the neurotransmitter transporter Slc6a14, which was more than 200 times overexpressed in the colon than in the small intestine. The neurotransmitter transporter Slc6a14 and the facilitated glucose transporter 1 (Slc2a1) were more than 10 times overexpressed. By comparing our genomic data with the literature, we could confirm that most of the transporters were similarly expressed in humans. In conclusion, differences in absorption of drugs involving transporters along the intestine can be expected. Different intestinal segments need to be targeted depending on the structure of the compound when trying to imply active uptake systems.

10.3.2.2 **Profiling of Caco-2 Cells**

The first study analyzing the expression profiles of Caco-2 cells using microarrays was done by Mariadason *et al.*, in particular, investigating the influence of postconfluent differentiation in Caco-2 cells on mRNA expression profile [54]. They observed that consistent with cell differentiation, the expression profile reflected a more specialized phenotype. Specifically, Caco-2 cell differentiation was accompanied by coordinated downregulation of genes involved in cell cycle progression and DNA synthesis, which reflected the concomitant reduction in cell proliferation. This study investigated changes of expression profiles only in confluent cells. In any comparison with other studies, the degree of confluence must be considered as an important determinant of mRNA profiles. In a similar study, Fleet *et al.* analyzed the changes in genomic profiles upon differentiation in a subclone of the Caco-2 parental line, the so-called Caco-2 BBE cells [55]. In contrast to Mariadason *et al.*, they observed a significant number of transporter genes being regulated upon differentiation [54]. Even though a comparison between the expression changes observed by Fleet *et al.* and Mariadason *et al.* revealed a rather poor correlation, both studies identified overall the same major pathways that are likely to play a role in the differentiation process [54, 55]. Also, both concluded similarly that the Caco-2 cells express a very complex molecular signature that consists of features of a colonocyte, an enterocyte, and a tumor cell. Similar to these two studies, we analyzed with a home-made oligo array the expression of about

750 genes encoding transporter and channel proteins in differentiated and undifferentiated Caco-2 cells, human small intestine, and colon [56]. In summary, studying only transporter and channel genes, we observed nevertheless a similar trend, namely, that Caco-2 cells have the characteristics of colonocytes, small intestinal enterocytes, and tumor cells. Our results were in general in accordance with the study by Sun and coworkers [57, 58]. They compared the mRNA expression profile of the human duodenum and Caco-2 cells. In contrast to Bates *et al.*, they used the GeneChip U95A by Affymetrix, which contains 12 559 gene sequence tags including in total 443 transporters, ion channels, and metabolic enzymes [52]. The expressions of 26, 38, and 44% of these 443 genes, of which 170 are transporters or ion channels, were detectable in 4-day-old Caco-2 cells, 16-day-old Caco-2 cells, and human duodenum, respectively. Moreover, they compared permeability values in Caco-2 cells and human duodenum of various carrier-mediated and passively absorbed drugs. The *in vivo*/*in vitro* drug permeability measurements correlated well for passively absorbed drug, whereas the correlation coefficient decreased for carrier-mediated drugs. In general, the observed permeability of carrier-mediated drugs was higher in human duodenum than in Caco-2 cells. Most of the transporters expressed in the human duodenum were also expressed in Caco-2 cells. However, it has to be noted that a limited set of drugs has been tested, which are actively transported by only a fraction of transporters that are expressed in the intestine. In addition, the *in vitro* permeability rates in Caco-2 cells have been compared with the ones in the upper jejunum, whereas the expression levels have been compared with the ones in the duodenum.

The findings of each of the genomic studies discussed above contribute to a better assessment of the Caco-2 cells as a model system to study active transport in the human intestine. Having such a vast amount of data available certainly increases our understanding. However, it also requires new strategies to deal with such an immense amount of data to exploit them appropriately. Calcagno *et al.* developed an *in silico* approach to exploit publicly available data sets to assess the Caco-2 model system [59]. They combined genomic results from nine different labs that used different microarray platforms. Consequently, they could increase the robustness of their resulting findings. In their *in silico* approach, using principal component analysis, they showed that the Caco-2 cells express a transport protein profile that to some extent represents not only the absorptive enterocytes but also the colonocytes. However, the profile of the metastatic cell line SW620 differed significantly.

Clearly, genome-wide studies contribute to a better understanding why differences in absorption along the intestine, and between *in vitro* and *in vivo* situations, can be expected. These studies could on the one hand confirm that the most widely used *in vitro* system to study drug absorption, namely, the Caco-2 cells, may not always be very accurate in predicting uptake rates for a new compound. On the other hand, it has to be noted that compared to other *in vitro* systems, which are being used to study transport in liver and kidney, the Caco-2 cells correlate certainly the best with the *in vivo* system with respect to the mRNA expression of transporters [3].

From a medical chemistry point of view, genome-wide expression studies can be exploited to design drugs in such a way that highly expressed transporter proteins can

be better exploited. Moreover, these studies provide information not only about transporter systems but also about other groups of proteins, such as enzymes. Such information could be exploited when designing prodrugs, for instance. A very famous example is certainly valaciclovir (**I**) (cf. below).

10.3.3
Intestinal Transporters and the Influence of Genotypes

So far we have only discussed the fact that in-depth knowledge of the expression of membrane transporters provides crucial information so as to select new, putative targets for active uptake. However, many times the activity of these proteins can be altered in different individuals due to mutations in the genome. Single-nucleotide polymorphisms account many times for differences in activity in individuals. In recent years, many studies have focused on this aspect and have genotyped a variety of genes including transporter genes. For instance, the UCSF "Pharmacogenetics of Membrane Transporters Project" (PMT) focuses mainly on the identification of sequence variants in genes encoding selected membrane transport proteins and the functional characterization of these variants (http://pharmacogenetics.ucsf.edu/index.html). The resulting data of this project are regularly deposited in the PharmGKB database (http://www.pharmgkb.org/), a publicly available database that serves as a genotype–phenotype resource focused on pharmacogenetics and pharmacogenomics. The database contains not only the genotype information of single genes but also the results obtained with SNP arrays. Similar to the classical expression oligo arrays, these arrays identify differences between samples of a vast number of genes with respect to SNPs [60]. So far, no study has been published with the SNP array technology focusing on transporter genes.

As mentioned above, interindividual differences in absorption rate can be explained by SNPs in membrane transporters. However, SNPs in other genes, which are involved in the regulation of transporter genes, could also contribute to interindividual differences. Therefore, the exploitation of array data will be very important to assess the influence of the various genotypes in drug absorption.

In recent years, various studies have been published showing the effects of mutations in the DNA of transporters. Many times, such mutations can account for diseases (cf. Table 10.1). In the following section, however, we will focus on studies in which transporters relevant to oral drug absorption have been sequenced and their genetic variants phenotypically characterized.

In the context of the PMT Project, we have genotyped all 23 exons and adjoining intronic regions of *SLC15A1* in 247 individuals, the so-called Coriell collection (i.e., 100 Caucasians, 100 African–Americans, 30 Asian–Americans – equal numbers of Chinese, Japanese, and Southeast Asians–, 10 Mexican–Americans, and 7 Pacific Islanders) [61]. Overall, significant differences between ethnic populations could be observed. Of 38 SNPs detected, 21 occurred in the intronic and noncoding region and 17 in the exonic, coding regions, of which 9 were nonsynonymous. Eight nonsynonymous SNPs were functionally characterized in transient-transfected Cos7 ad CHO cells. None of the variants had altered transport activity, except the low-frequency

PEPT1-F28Y. It showed a significant reduction of cephalexin (**XXI**) uptake. Altered pH dependence of substrate transport suggested a role of F28Y in H^+-driven translocation. In a similar study, Zhang *et al*. sequenced *SLC15A1* in 44 different individuals and found 13 SNPs in the coding region confirming our findings [62]. In addition to our data, they found another low-frequency SNP with altered transport activity, the PEPT1-P586L. In conclusion, genetic factors could possibly introduce some systematic variability for drugs critically dependent on PEPT1 activity for intestinal absorption. However, other factors acting on PEPT1 transport activity are more likely to cause interindividual variability, recently studied with valaciclovir in human subjects [63]. Alternative splicing cannot yet be ruled out as a clinically relevant variable. Overall, however, PEPT1 displays remarkable low genetic variability. This finding is important for drug therapy with peptoid drugs and for exploiting PEPT1 in prodrug design for improved bioavailability.

Various members of the nucleoside transporter families SLC28 and SLC29 have also been genotyped and their variants functionally characterized. Regarding *CNT1*, a total of 58 coding region haplotypes were identified in the same collection of individuals as the one used for *SLC15A1* (e.g., Coriell collection) [64]. The translated protein of 44 haplotypes contained at least one amino acid variant. More than half of the coding region haplotypes were population specific. Phenotypic studies of 15 protein-altering *CNT1* variants in *Xenopus laevis* oocytes revealed that all variant transporters took up [3H]thymidine with the exception of CNT1-Ser546Pro, a rare variant, and CNT1– 1153del, a single bp deletion, found at a frequency of 3% in the African–American population. The anticancer nucleoside analogue gemcitabine (**VI**) had a reduced affinity for CNT1-Val189Ile (a common CNT1 variant found at a frequency of 26%) compared to reference CNT1. These data suggest that common genetic variants of *CNT1* may contribute to variation in systemic and intracellular levels of anticancer nucleoside analogues. The same group also analyzed the family member *CNT2* in a very similar setup [65]. Six nonsynonymous variants were identified, and all were able to transport guanosine. The four common variants were further characterized with the antiviral nucleoside analogue drug ribavirin. No differences were observed among the four common variants in the uptake kinetics. However, variant CNT2-F355S F355S (3% allele frequency in the African–American sample) exhibited a change in specificity for the naturally occurring nucleosides, which according to the authors may have implications for nucleoside homeostasis. The third member of the SLC28 family has been genotyped by another group. The authors of this study also used the Coriell collection but only the Caucasians [66]. In summary, they identified different variants; however, these variants do not seem to have either an altered transport activity or transport specificity.

In addition, in the context of PMT, members of the equilibrative nucleoside transporter family have been investigated. So far, only *ENT2* has been completely genotyped and its protein product functionally characterized. Similar to *CNT3*, different variants were observed; however, only five haplotypes were sufficient to describe the entire sample set. Thus, the authors suggested that the low overall genetic diversity in *SLC29A2* makes it unlikely that variation in the coding region contributes significantly to clinically observed differences in drug response [67].

Similarly, Kuhne *et al.* analyzed *LAT1* (*SLC7A5*), *LAT2* (*SLC7A8*), and *4F2HC* (*SLC3A2*) and concluded that even though various SNPs are found in these sequences, genetic variation in the genes *4F2HC*, *LAT1*, and *LAT2* does not appear to be a major cause of interindividual variability in pharmacokinetics and of adverse reactions to melphalan (**XXII**) [68].

One of the best-studied transporter proteins is certainly the ABCB1. Many groups have sequenced this gene and studied the impact of the mutations on the transport activity of its protein product [69–71]. While Tang *et al.* analyzed a collection of 261 individuals of three distinct Asian groups (i.e., 104 Chinese, 93 Malay, and 68 Indian), Kroetz *et al.* used the Coriell collection as described above [69, 71]. Tang *et al.* selected four sites in the 5′ noncoding region and six sites in the coding regions as possible sites for SNPs based on publicly available data and verified them in their population [71]. Only three in the coding region and one in the 5′-flanking region could be confirmed. All three coding SNPs (exon 12 1236C > T, exon 21 2677G > T/A, and exon 26 3435C > T) were present in high frequency in each ethnic group, and the derived haplotype profiles exhibited distinct differences between the groups. In summary, fewer haplotypes were observed in the Malays ($n = 6$) compared to the Chinese ($n = 10$) and Indians ($n = 9$). Three major haplotypes (>10% frequency) were observed in the Malays and Chinese; of these, two were observed in the Indians.

In contrast to this study, Kroetz *et al.* sequenced the whole exonic region and the adjoining intronic regions of the gene in a more heterogeneous sample [69]. Considering only the variants used in the previous haplotype analysis by Tang *et al.*, they could find 7 of the 10 haplotypes in their relatively small Asian–American sample [71]. Overall, they identified 48 variant sites, including 30 novel variants and 13 codings for amino acid changes, resulting in 64 statistically inferred haplotypes. As with respect to the impact of various haplotypes on the transport activity of the protein product, the results published so far remain inclusive. Thus, further functional studies are still needed.

As far as the ABCC family members are concerned, only genotype studies have mainly been done so far. Thus, functional data regarding the influence of the different variants found are still missing. Briefly, Saito *et al.* screened entire genomic regions of 48 Japanese individuals and identified 81 SNPs in the *ABCC1* gene, 41 in *ABCC2*, 30 in *ABCC3*, 230 in *ABCC4*, 76 in *ABCC5*, 58 in CFTR, 102 in *ABCC8*, and 70 in *ABCC9* [72]. In contrast, Leschziner *et al.* sequenced only the exon regions of *ABCC1*, *ABCC2*, and *ABCC5* in 47 Caucasion individuals finding 61 SNPs in *ABCC1*, 41 in *ABCC2*, and 34 in *ABCC5* [73]. Overall, the authors concluded that the patterns of linkage disequilibrium (LD) across these drug transporter genes demonstrated large regions of high LD and low haplotype diversity, an important information for the search of a functional variants.

In summary, various important intestinal drug carriers have been characterized regarding their genetic variability and the impact on transport activity. For a good part, the genetic variability does not seem to be the major player in the interindividual variability in drug absorption. However, many times the genetic variation can contribute to an altered transport activity, which in some ethnic populations might be more relevant than in others. Thus, having a catalog of all known SNPs and

relevant haplotypes and their functional characteristics is the first step in estimating the impact of genetic variability on interindividual differences in drug absorption. Many times mutations in the sequence lead to changes in the structure of transporters and as a consequence to an altered functionality. Thus, predicting the impact of such mutations on the structure will be one of the major challenges for medical chemistry. On the one hand, for some established drugs, individualized drug therapy should be applied to account for the differences in drug transport. Moreover, an in-depth knowledge of all mutations and prediction of their possible impact on the transport activity can be exploited to avoid targets drug delivery targets for which different genotypes are expected to show alterations in drug transport.

10.4 Structural Requirements for Targeting Absorptive Intestinal Transporters

The discovery process has traditionally had a preference for evaluating new chemical entities (NCEs) with respect to their pharmacologic potency and selectivity against the selected drug targets. Intestinal transporters are generally not drug targets but could be classified as drug delivery targets [7]. The aim of exploiting a drug delivery target is most often to increase the intestinal absorption of the drug candidate to increase the oral bioavailability or modify the metabolic or stability profile of the drug candidate. Another strategy could be to target a given segment in the intestine in which the transporter of choice is highest expressed.

A lot of structure–activity information has been accumulated over the last two decades for various intestinal transporters. However, the two most studied transporters in terms of quantitative structure–activity relationship (QSAR) are SLC15A1 [74–80] and ABCB1 (for references see Ref. [81]), which will be the topic of Chapter 18. In the following section, the focus will be on strategies for increasing intestinal absorption of drug candidates by targeting intestinal peptide transporters.

10.4.1 Strategies for Increasing Drug Absorption Targeting Transporters

Different strategies for targeting intestinal transporters to increase intestinal permeability have been described:

(a) design of prodrugs targeting the transporter,
(b) substrate mimicry, and
(c) the use of a formulation approach.

A prodrug is composed of an active drug and a promoiety (or transport moiety) and is by definition a pharmacologically inactive compound. As mentioned in the Section "Introduction," an example of a prodrug targeting an intestinal transporter is valaciclovir (**I**). The prodrug has a bioavailability of 63% in rats compared to 15–21% of the parent drug acyclovir due to the transport activity of PEPT1 [1, 2]. A quite similar example is valganciclovir, which is also a substrate for PEPT1 [82],

where the oral bioavailability in human is increased from approximately 6 to 61% [83]. It has to be noted that other transporters also have been suggested to participate in the intestinal absorption of valaciclovir and valganciclovir such as HPT1 and the Na^+/Cl^--coupled amino acid transporter $ATB^{0,+}$ [11, 84]. Several examples exist where the promoiety itself is not a substrate for the transporter and often valine is used [85–89]. Prodrugs designed to use the transport activity of PEPT1 have also been designed by using natural or modified di- or tripeptidomimetic substrate as a promoiety for transporter recognition [90–92]. Prodrugs may also be used to circumvent the transport of efflux transporters and instead utilize an absorptive transport system as in the case of the Val-Val-saquinavir prodrug [91]. Assessing the uptake of a prodrug by a given carrier is many times difficult due to the involvement of other mechanisms of transport.

Another way of exploiting transporters is to optimize intestinal absorption by using QSAR-based database mining to select an NCE, which could be a substrate for an appropriate absorptive transporter or alternatively not substrate for efflux mechanisms [93, 94]. Substrate mimicry is also a possibility in the case of inhibition of transport activity, which maybe relevant not only for efflux transporters. Phloridzin is an inhibitor of the sodium-coupled glucose transporter SGLT1 (SLC5A1), which is located in the apical membrane of enterocytes (cf. Figure 10.2). Poly(gamma-glutamic acid) (gamma-PGA) modified with phloridzin (PGA–PRZ) has been suggested as a novel oral antidiabetic drug [95]. PGA–PRZ was able to decrease glucose transport from the mucosal to serosal side of the everted rat small intestine, and its inhibitory effect was as strong as that of intact phloridzin [95]. After oral administration of PGA–PRZ to rats before glucose administration, the glucose-induced hyperglycemic effect was significantly suppressed, which was not observed in the case of phloridzin administration. These results suggest that the gamma-PGA–phloridzin conjugates have potential as oral antidiabetic drugs due to the interaction with SLC5A1.

As with respect to a formulation approach, the use of pharmaceutical excipient Eudragit L100-55 decreased the pH *in situ* in ileal, closed loops of rats and increased the disappearance of cefadroxil and cefixime from the loop. The plasma concentration after oral administration was increased significantly by coadministration of Eudragit L100-55, whereas a proton-nonreleasing analogous polymer, Eudragit RSPO, did not have any effect. These results indicate that improvement of intestinal absorption of peptide mimetics via PEPT1 is possible by increasing the driving force for PEPT1 transport activity through coadministration of a proton-releasing polymer [96]. As far as ABCB1 is concerned, lipid excipients and surfactants such as Cremophor, Tween 20 and 80, sucrose monolaurate, and Pluronic block copolymers or other ABCB1 substrates/inhibitors have been shown to inhibit ABCB1-mediated efflux of drugs such as cyclosporin A, paclitaxel, ciliprolol, digoxin, and HIV inhibitors [97, 98]. Such pharmaceutical excipients may increase the intestinal permeability (or decrease metabolism) by inhibiting the ABCB1-mediated intestinal apical efflux, but these may also be the cause of drug–drug interactions in a multidrug dosage regimen. Collectively, lipid-based drug delivery system may not only increase solubility of a given drug but also modulate the transport activity of efflux transporters

such as ABCB1. Another strategy may be to develop a formulation capable of releasing the drug in intestinal segments where transporter expression is high (for absorptive transporter) or low (for efflux transporter).

Various strategies for exploitation or limitation of the impact of transporters are available for the discovery and development of drug candidates. Most often, the impact of transporters has been realized retrospectively. However, as knowledge increases on transporter expression and function, genomics of transporter, SAR, and protein structure, the basis for a rational design of substrates or inhibitors of transporter activity is enhanced.

10.4.2
Changing the Substrate: SAR Established for PEPT1

The number and variation in the structure of possible combinations of natural dipeptides (**XXIII**) and tripeptides (**XXIV**) indicate that the structural requirements for binding to PEPT1 are relatively flexible in terms of charge, size, and chemical structure. In general, affinity of natural di/tripeptides for PEPT1 is stereochemically specific for peptides composed of L-enantiomers [99–101]. β-Lactam antibiotics also have a chiral center and therefore exist in both D- and L-forms, and stereoselective uptake of cephalexin (**XXI**) and loracarbef has been demonstrated, with the L-enantiomers displaying the highest affinity for PEPT1 [101]. However, it must be emphasized that di- or tripeptides containing a D-configured amino acid may have a high affinity for PEPT1 after side chain (R_1, R_2, or R_3) derivatization, especially using hydrophobic derivatives [102–104].

The presence of a peptide bond was originally thought to be a prerequisite for transport via PEPT1 [105]. However, Temple *et al*. demonstrated PEPT1-mediated transport of the peptide mimic 4-aminophenylacetic acid (4-APAA), and Doring *et al*. showed that ϖ-amino fatty acids, such as δ-amino levulinic acid (δ-ALA) (**XXV**), are substrates transported by PEPT1 [106–108]. δ-ALA is a Gly-Gly mimetic, in which the amide bond is replaced by a ketomethylene amide bond bioisoster. Vabeno and coworkers investigated the amide bond isosteres ketomethylene, (*R*)- and (*S*)-hydroxyethylidene, and (*R*)- and (*S*)-hydroxyethylene and found that the ketomethylene amide bond isoster had the highest potential as a bioisoster with respect to PEPT1 affinity [109, 110]. Valaciclovir (**I**) is another example of a PEPT1 substrate lacking an amide bond. As shown by Brandsch *et al*., the peptide bond oxygen atom may be replaced by sulfur in Ala-Ψ[CS-N]-Pro, a modification of Ala-Pro, without abolishing transport by PEPT1 [111]. Very interestingly, the study of Brandsch *et al*. showed that dipeptides in *cis* and *trans* conformations around the peptide bond have different affinities and that PEPT1 selectively transports dipeptides in the *trans* conformation. This was later confirmed using naturally occurring dipeptides [112]. The transporter does also accept some dipeptides with a *N*-methylated peptide bond exemplified by Gly-Sar and some tripeptides with *N*-methylated amide bond; however, *N*-methylation does not seem to be a general stabilization approach, since the affinity for PEPT1 is not retained for all the investigated compounds [113, 114].

In general, the highest affinity for PEPT1 is observed for compounds having free amino-terminal and carboxyl-terminal groups. Yet, acetylating the N-terminus of Phe-Tyr to give an amide highly decreased, but did not abolish, the affinity for PEPT1, and the N-terminal modification of L-carnosine to 4-toluenesulfonylureido-carnosine (**XXVI**) also retained a medium affinity for PEPT1 [6, 12, 115]. The C-terminus is not absolutely essential for affinity, since C-terminal amidation of Phe-Tyr retained an affinity (although lowered) for PEPT1 [6, 12]. Moreover, single amino acid modified in the C-terminus with piperidide, thiazolidide, anilide, 4-nitroanilide, 4-chloroanilide, or 4-phenylanilide has in some cases been shown to retain affinity for PEPT1 [116].

Absolute affinities (K_i-values) for natural di- and tripeptides are normally in the range of 0.1–3 mM. To evaluate relevant affinity values, Brandsch and coworkers have proposed the following classification: $K_i < 0.5$ mM is high affinity, $0.5 < K_i < 5$ is medium affinity, and $K_i > 5$ is low affinity, and values above 15 mm seem to be irrelevant [5]. The latter is probably based on the observation made with β-lactam antibiotics where the oral bioavailability seems to be at least partly mediated via PEPT1-mediated transport, and a threshold of 15 mm was proposed [8].

The impact of the above in terms of drug discovery and development suggests the seeming wide possibilities for substrate mimicry and prodrug design. Even though most dipeptides and tripeptides studied so far are substrates for PEPT1, a number of examples exist where affinity and translocation are absent, for example, observed for Lys-Lys, Trp-Trp, Arg-Arg, and Arg-Lys.

10.4.3
Methods for Investigating Affinity and Translocation

The initial evaluation of novel compounds synthesized for targeting a transporter will normally have to be based on the following assays, to get the desired information needed to verify if the compound indeed is a substrate for the transporter and if this results in an increased intestinal permeability:

(a) investigation of the affinity for the transporter,
(b) investigation of translocation of the compound by the transporter,
(c) transport across an *in vitro* culture model of the intestine or across animal intestinal tissues.

Subsequently, *in vivo* animal studies are necessary to investigate the oral bioavailability.

When studying drug candidates or prodrugs of PEPT1, the affinity is often assessed by competition experiments using nonhydrolyzable peptides such as Gly-Sar (which has a methylated peptide bond) or L-carnosine (β-Ala-His). The affinity is expressed as the IC_{50} value (the concentration of compound, substrate or inhibitor, which causes a 50% inhibition of the uptake). The compounds can thus be described in terms of their IC_{50} value or, more meaningful, their K_i value, since the IC_{50} value depends on the Gly-Sar concentration used. When the concentration of the substrate is much lower than K_m, IC_{50} approaches K_i [117, 118]. The basic outline of an affinity study has been performed in different *in vitro* systems using epithelial cell

lines such as Caco-2 cells or in cell lines using transient and stable transfection with *PEPT1 cDNA* such as LLC-PK1 cells, HeLa cells, Chinese Hamster Ovary (CHO) cells, and Cos7 cells [11, 61]. Expression systems such as Xenopus laevis oocytes have also been applied extensively to study PEPT1 [119]. The methylotrophic yeast, *Pichia pastoris*, has also been used for heterogonous expression of the rabbit intestinal peptide transporter Pept1 [120]. However, care must be taken, as pointed out by Brandsch and coworkers, when comparing affinities obtained in different laboratories due to the different protocols used where pH, different *in vitro* systems, different isoforms, and different isotopes are employed [5].

It must be emphasized that compounds, which are characterized only by their K_i or IC_{50} affinity values, are not necessarily transported by PEPT1, and the only information this parameter yields is that the compound competes for binding to the transporter. The ability of a compound to be translocated by PEPT1 can be studied using radiolabeled compounds or by the two-electrode voltage clamp technique [121–123]. However, it is often expensive to get new compounds radiolabeled, and the two-electrode voltage clamp technique is not suitable for low or medium high-throughput screening. Recently, an assay based on the measurement of membrane depolarization resulting from the cotransport of protons with PEPT1 substrates in MDCK/PEPT1 has been described [124–126]. Membrane potential changes are tracked with a voltage-sensitive fluorescent indicator (FLIPR membrane potential assay kit from Molecular Devices). This allows the investigation of translocation via PEPT1 of a reasonable number of compounds per experimental day. The last step in the *in vitro* characterization is to assess the intestinal permeability, and this may be performed in cell culture models such as Caco-2 cells or MDCK/PEPT1 cells or in intestinal animal tissues [127, 128]. The compound is applied to one side of the epithelium, and samples are taken at the opposite side at various time points postaddition. Transepithelial flux across the intestinal epithelium is determined when the flux across the cells is in steady state; this occurs within 30–60 min, depending on the tissue.

During all investigations, stability and metabolism are an issue, especially for prodrugs, which in nature must be biolabile. Today's analytical tools allow a careful analysis of compounds present after the various types of experiments, thus leading to solid interpretations of transporter interactions before going into *in vivo* studies.

10.4.4
Quantitative Structure–Activity Relations for Binding of Drug to Transporters

In Part IV of this book, "Computational Approaches to Drug Absorption and Bioavailability," computational models for studying drug absorption and bioavailability are described in detail. Chapter deals with quantitative structure–activity relations for ABCB1. Computation-based QSAR models are pursued to gain insight into how steric, electrostatic, hydrophobic, and hydrogen-bonding interactions influence biological activity and to derive predictive 3D QSAR models for designing and forecasting the activity of drug candidates targeting intestinal transporters. The following section discusses some studies of absorptive transporters with a focus on PEPT1. Several

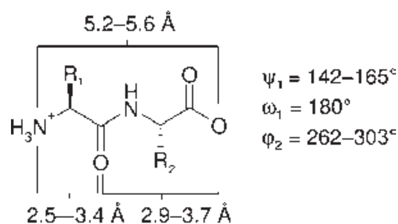


Figure 10.4 Geometrical data for the bioactive conformation of PEPT1 dipeptide substrates proposed by Gebauer *et al.* The dipeptides conformation is defined by backbone torsion angles $\psi_1 = 142°–165°$, $\omega_1 = 180°$, and $\varphi_2 = 262°–303°$ and the distance between the N-terminus and the C-terminus, $d_{N-C} = 5.2–5.6$ Å [77].

models for ligand binding to PEPT1 have been suggested [74–77, 79, 80, 107, 129, 130]. The term ligand is used here since the computational methods published for PEPT1 are based on affinity values and these may not reflect actual translocation, complicating the interpretation of the impact of proposed models on drug design. An extract of the work by Gebauer *et al.* and Biegel *et al.* using comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) studies is mentioned in the following section [76, 77]. Part of the bioactive conformation is indicated for dipeptides in Figure 10.4.

The model by Gebauer *et al.* describes two electrostatic fields (E_1 and E_2) that represented favorable interactions with the positive N-terminal charge (E_1) and the negative C-terminal charge (E_2) [77]. Derivatization of the N-terminus to give an amide functionality (neutral nitrogen) reduces the interaction in E_1, whereas the introduction of an N-terminal β-amino acid results in misplaced orientation of the positive charge (as in 4-toluenesulfonylureido-carnosine). The removal of the C-terminal carboxyl group results in a dramatic decrease in affinity, while its replacement with an electron-rich aromatic moiety is accepted. Two large cavities were described where L-configured R1 and R2 side chains, respectively, interact favorably. Three disfavored steric areas were also identified, mainly representing steric clash for (i) Pro-Xaa dipeptides, (ii) the R1 side chain/amino group (D-configuration), and (iii) the R2 side chain/hydrogen (D-configuration) . Two areas were described where lipophilicity contributes favorably, one of which partly coincided with the R2 steric cavity indicating that a bulky hydrophobic R2 side chain is favorable. There was no lipophilic contribution overlapping the R1 pocket, suggesting no specific preference in this position. Moreover, two areas where hydrophilicity contributes favorably were described, mainly representing the charged N- and C-termini, in agreement with the electrostatic fields. Three areas were suggested that represent the presence of H-bond acceptor sites in the "binding pocket." These areas represent favorable interactions with H-bond donors, that is, the positively charged α-amino group (L-configuration). Three areas were identified where interaction with H-bond donors were unfavorable, such as the imino hydrogens of Pro-Xaa, an N-terminal amide hydrogen, or the α-amino group

(D-configuration). One area represents the presence of H-bond donor sites in the "binding pocket," meaning that interactions with H-bond acceptors are favorable. It is usually the C-terminal carboxyl group (L-configuration) and the H-bond donor field that are closely related to the E_2 field. A CoMFA-based model highly depends on the initial chosen conformation for subsequent alignment. However, Andersen *et al.* used an approach based on VolSurf descriptors, which are alignment independent, and correlated the VolSurf descriptors of a set of tripeptides with their experimental binding affinity for PEPT [74]. Larsen *et al.* recently expanded this approach to integrate VolSurf, GRIND, and MOE descriptors into a hierarchical PLS model for prediction of affinity for PEPT1 [129].

Substrate-based QSAR models have been proposed for the following absorptive transporters: ENT1 [131], SERT [132], CNT1, and CNT2 [133] and others (see Ref. [93])

The use of QSAR models is of great potential to the work of medicinal chemists since it allows the virtual synthesis and screening of novel drug candidates targeting intestinal transporters. Furthermore, it allows QSAR-based data mining to identify putative ligands in publicly accessible databases or company databases. Moreover, potential drug–drug interactions caused by transporters may be identified [134]. However, the models need to be predictable and able to distinguish between inhibitors or substrates.

10.5 Transporters and Diseased States of the Intestine

10.5.1 Intestinal Diseases

Transporters can affect in various ways the well functioning of our body. Many times an altered transport rate of important nutrients due to, for instance, mutations in the sequence can provoke diseases (cf. Table 10.1). In addition, changes in our body state due to, for example, infection and inflammation can affect the regulation of transporters [135]. Edinger reviewed very extensively how the control of cell growth and cell survival is regulated by nutrient transporter expression, which is very important in carcinogenesis [136]. This shows that changes in expression of transporters need to be considered in drug design when targeting transporters as drug carriers. These findings also show clearly that in some specific cases, transporters can serve as direct drug targets. In the following section, we will mainly focus on the regulation and role of transporters in typical intestinal diseases such as colon cancer and chronic inflammatory diseases, that is, inflammatory bowel disease (IBD) and Crohn's disease. In the following paragraphs, we will give a brief introduction into carcinogenesis and especially colon carcinogenesis. We will show how chronic inflammation is linked to tumor development in the colon and what role transporters play in these diseases.

10.5.2 Basic Mechanisms in Cancer and Specifically in Colon Carcinogenesis

10.5.2.1 Basic Mechanisms

Tumor development is recognized as a multistep process. Carcinogenesis is generally considered to consist of three major stages: tumor initiation, promotion, and progression, which is certainly an oversimplification of the mechanisms involved. Tumor initiation is a rapid and irreversible process that includes exposure of a single cell or multiple cells to a carcinogen and involves DNA alterations. In contrast to the initiation phase, tumor promotion is considered to be a lengthier and reversible process, in which actively proliferating neoplastic cells accumulate. The final stage, tumor progression, involves the growth of tumor that has invasive and metastatic potential [137]. The initiation process can persist in otherwise normal tissue indefinitely until the occurrence of a second type of stimulation, which can result from the exposure of initiated cells to chemical irritants. The cancer cell genotype can be summarized as a manifestation of six essential alterations in cell physiology: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion, and metastasis [138]. Each of these changes is acquired during tumor development. Hanahan and Weinberg suggest in their work that most of the time all six characteristics are shared by most, if not all, types of cancers [138].

The causal relationship between inflammation, innate immunity, and cancer is now widely accepted. Normal inflammation is self-limiting because the production of proinflammatory cytokines is followed by that of anti-inflammatory cytokines. In tumors, the inflammatory process persists. Chronic inflammation seems to be due to persistence of the initiating factors or failure of mechanisms that are required to resolve the inflammatory response [139]. The strongest association of chronic inflammation with malignant diseases is in colon carcinogenesis in patients with inflammatory bowel diseases such as chronic ulcerative colitis and Crohn's disease.

Thus far, cancer research has focused mainly on understanding the biology of tumor cell proliferation [138], and, as a result, most anticancer therapies target proliferative mechanisms within the tumor cell compartment. Evidently, the outcome of a cancer depends not solely upon the tumor's proliferative capacity but largely upon the most harmful process in tumor development, that is, the invasion of tumor cells into the surrounding tissue [140]. At this invasive front, the tumor cells undergo the so-called epithelial–mesenchymal transition (EMT) resulting in a reduced proliferative and increased migratory capacity.

EMT, as well as tumor initiation, development, and metastasis formation, does not occur only through genetic and epigenetic changes within tumor cells. It is becoming increasingly clear that signals generated by the surrounding host tissue, that is, the tumor stroma, influence tumor cells [141–145]. However, very little is known about how tumor cells respond to their microenvironment. Conversely, it is well established that tumor cells themselves influence the stroma. They can, for example, stimulate the surrounding healthy tissue to form blood vessels, which provide the tumor with oxygen and other nutrients [146]. Often, the tumor tissue also provokes an inflammatory

response that is comparable to the inflammation observed in wounding and tissue repair [139]. Most important, tumor cells can subvert the functions of normal leukocytes, fibroblasts, and endothelial cells and utilize them to their own advantage [147], which has led to the notion of tumor–stroma cross talk [148].

These observations suggest that targeting reactive stroma in addition to tumor cells may provide a potentially powerful means to control cancer growth and dissemination. Thus, finding ways to exploit tumor–stroma cross talk for new therapeutic strategies has taken center stage in cancer research [149]. One advantage of targeting stromal cells is that these cells are not genetically as unstable as cancer cells and are consequently less likely to develop drug resistance [148].

10.5.2.2 Colon Cancer

Colon cancer is one of the main causes of cancer mortality in Western societies [150]. About 15–20% of colorectal tumors are causally determined by inheritance of genetic alterations such as the hereditary nonpolyposis colorectal cancer (HNPCC) and the syndrome familial adenomatous polyposis (FAP) [151, 152]. Microsatellite instability, a characteristic of HNPCC, is caused by mutations in the genes essential for mismatch repair. The loss of mismatch repair has several consequences: most crucially, the loss of proofreading and correction of small deletions and insertions. FAP is a rare autosomal dominant syndrome caused by an inherited mutation in the *APC* gene. The disease is characterized by the development of multiple colorectal adenomas, numbering from a few polyps to several thousands.

Mutations at the *APC* locus are a common and early somatic event in polyps and cancer; that is, for some individuals, the first hit is the germline mutation, whereas for others, it is a somatic event. *APC* can also be silenced by hypermethylation. In general, this finding suggests that, although inheriting mutated copy of *APC* is associated with a highly penetrant phenotype, there are, nonetheless, both genetic and environmental influences that modify that penetrance. Other key players in colon carcinogenesis are the oncogene *KRAS*, and, besides *APC*, two other tumor suppressor genes, namely, *SMAD4* and *TP53* [150]. Potter *et al.* proposed that there are at least four separate molecular pathways involved in colon carcinogenesis that have some events in common: (1) APC–β-catenin–Tcf–MYC pathway associated with the adenoma–carcinoma sequence; (2) the HNPCC pathway characterized by the loss of DNA mismatch repair (by inherited or acquired mutation or methylation) that results in microsatellite instability in the tumors; (3) the ulcerative colitis dysplasia–carcinoma sequence that is usually not associated with *APC* mutation or polyp formation; and (4) hypermethylation silencing of the estrogen receptor gene, which may be part of a wider pattern of gene-specific hypermethylation – common in sporadic tumors [152] .

10.5.3 Transporters and Colon Cancer

Membrane transporters play key roles in cancer drug therapy. In fact, the effectiveness of cancer chemotherapy may often depend on the relative transport capacities of

tumor cells. Some transporters can affect the entry of drugs into cells and others drug exit. In particular, various ABC transporters such as ABCB1 mediate energy-dependent efflux of drugs and thereby play major roles in the development of drug resistance [153]. Most of the current chemotherapeutics such as folate antagonists, pyrimidine and purine antimetabolites, alkylating drugs, platinum agents, and DNA topoisomerase-targeting drugs act upon proliferation, while microtubule-targeting compounds additionally interfere with the migratory activity of cells. Some ABC transporters pump out a wide variety of substances of various classes (e.g., ABCB1, ABCC1, and ABCC3), while others specifically transport microtubule-targeting compounds (ABCB4 and ABCB11), thus, playing potentially a crucial role in affecting the efficacy of drugs that target cell migration [153]. Studies indicate that there might be a correlation between induction of ABCA5 and ABCB1 expression and the differentiation state of human colon tumor [154]. As far as active uptake is concerned, SLC31A1 (CTR1, copper transporter), concentrative nucleoside transporters (SLC28A family), and folate/thiamine transporters (SLC19 family) have been shown, for instance, to transport cisplatin (**XXVII**), antimetabolites, and antifolates, respectively [20, 155, 156]. While most of the time, these influx carriers are responsible for increased chemosensitivity (i.e., susceptibility of tumor cells to the cell-killing effects of chemotherapy drugs), they may also be responsible for resistance (i.e., ability of cancer cells to become resistant to the effects of the chemotherapy drugs). For instance, patients with elevated SLC28A3 levels had lower response to fludarabine therapy suggesting that resistance to fludarabine might be related to intracellular membrane localization of the SLC38A3 protein product due to increased degradation of drugs in intracellular compartments [157]. Di Pietro *et al.* showed a differential expression of transporters in colon cancer compared to healthy mucosa [158]. We have studied the influence of transporters on chemosensitivity and resistance in a genome-wide approach in a panel of 60 cell lines including colon cancer cell lines used by the National Cancer Institute [159, 160]. By correlating gene expression with the potencies of 119 anticancer drugs, we could identify new transporter–drug interactions responsible for either chemoresistance or sensitivity. Positive correlations (chemo-sensitivity) were observed between folate carriers (e.g., SLC19A1, A2, and A3) and folate analogue drugs, nucleoside transporters (e.g., SLC29A1) and nucleoside analogues, and amino acid transporters (e.g., SLC38A2) and amino acid-type drugs. Among 40 ABC transporter genes tested, 3 showed strong negative correlation (chemoresistance) with several drugs using validated array data *ABCB1*, *ABCC3*, and *ABCB5* (a novel putative drug resistance factor) ($p < 0.001$). For ABCB1, all known substrates (19 drugs) yielded significant negative correlations.

Clearly, transporters play a significant role in cancer therapy. However, in-depth knowledge about the activity of these transporters in colon cancer and more specifically in the various subpopulations of tumor cells is lacking.

Besides the role of drug carriers in cancer, some membrane transporters have been demonstrated to act as tumor suppressor genes and be silenced by DNA methylation in colon cancer, and some transporters have been shown to be involved in EMT. As a consequence, membrane transporters may be differentially expressed between

proliferating and migrating invasive tumor cells. Both aspects will be addressed in more detail later.

In summary, very little is still known about the mechanisms involved in the uptake of chemotherapeutics in solid tumors. Even less is known about the role of transporters in drug delivery with respect to the heterogeneity of a tumor. Understanding the activity of transporters within a tumor may contribute to a more efficient and specific drug therapy. Targeting the microenvironment promises new and successful strategies for drug development. As a consequence, exploiting stroma-specific transporters as drug carriers may allow targeting stromal cells and, thus, interfering with the tumor-stroma cross talk. Moreover, very little has been done to understand the role of transporters in cancer not only as simple drug carriers but also as tumor suppressor genes.

10.5.3.1 Transporters as Tumor Suppressor Genes

Some solute carriers also seem to act as tumor suppressor genes in colon cancer such *SLC5A8* and *SLC26A3* [161–163].

Searching for genes that are aberrantly methylated at high frequency in human colon cancer, Li *et al.* found the transporter SLC5A8 [162]. Transfection of *SLC5A8* suppressed colony growth in each of three SLC5A8-deficient cell lines tested, but it showed no suppressive effect on any of the early events, detectable in colon adenomas, and even on earlier microscopic colonic aberrant crypt foci. Similarly, in a follow-up study, Ueno *et al.* showed in gastric cancer that aberrant methylation and histone deacetylation were associated with silencing of SLC5A8 [164].

Similar to SLC5A8, the glutamate transporter SLC1A2 has been shown to be silenced in glial cells by DNA methylation leading to the hypothesis that malfunctioning or the loss of SLC1A2 in glial may contribute to a certain extent to the progression of malign brain tumors termed glioma [165]. As far as SLC26A3, an anion exchanger, also called DRA (downregulated in adenoma), is concerned, Schweinfest *et al.* described that this transporter is downregulated in colon adenomas and carcinomas compared to healthy mucosa [166]. Later, transfection studies in colon cancer cell lines suggested that this transporter acts as tumor suppressor. Moreover, mutation studies revealed that the loss of the anion transport is not linked to its tumor suppressor function [161].

10.5.3.2 Role of Transporters in the Tumor–Stroma Interaction

Expression of Transporters During EMT Some studies have led to the hypothesis that transporters may be differentially expressed between proliferating and migrating invasive tumor cells and play a role in EMT, such as the zinc transporter LIV-1 [167, 168]. An interesting example in this context is the role of the monocarboxylate transporter MCT4 (SLC16A3) in migration and metastasis formation. A recent study showed that increased expression of CD147, an accessory β-subunit of MCT4, is coupled to the upregulation of MCT4 and that silencing of MCT4 resulted in decreased migration of metastatic cancer cells. CD147 has been shown to induce

MMPs in fibroblasts and cancer cells [169]. There are indications that transporters may be regulated by factors originating from the microenvironment [170].

However, very little is known about the regulation of transporters. Thus, the chemosensitivity and chemoresistance for a given carrier-mediated chemotherapeutic drug may be influenced by the microenvironment and may change significantly during EMT. This aspect has never been addressed so far.

Regulation of PEPT1 and HPT1 in Cancers of the GIT The regulation of PEPT1 is influenced by many different factors such as chemically synthesized dipeptides, leptin, EGF, insulin, and high-protein meal or prolonged fasting. However, only very recently, a direct regulation via a transcription factor-binding site has been identified. Shimakura *et al*. suggested that the transcription factor CDX2 regulates PEPT1 through the functional interaction with SP1 [171]. *In vitro* experiments in Caco-2 cells showed that the mutation of the SP1-binding site diminished the effect of CDX2, and the coexpression of SP1 and CDX2 had synergistically transactivated the PEPT1 promoter. Moreover, Nduati *et al*. presented in their very recent study evidence that the interaction of CDX2 and phosphorylated CREB (CREB) transcription factors is essential for leptin-induced PEPT1 regulation ([172], cf. below).

CDX2 plays a crucial role in the differentiation of healthy intestinal absorptive cells [173, 174]. Clearly, in tumor development, the expression and activity of CDX2 are affected over time [173]. However, opinions about whether CDX2 expression is lost completely or is only in a subset of poorly differentiated tumor cells differ. The mechanisms involved are still subject of ongoing research [173]. Witek *et al*. even suggested that in 80% of the tumors they analyzed the expression of CDX2 was increased [175].

In a very recent study, CDX2 expression has been shown to be adaptable and strongly influenced by the microenvironment. As a consequence, the authors suggest that CDX2 expression might be relevant during the process of metastatic dissemination when the gene is transiently turned down. In line with these findings, Gross *et al*. reported shortly afterwards that CDX2 decreased mobility and dissemination of colon cancer cells [176]. It has already been shown that PEPT1 expression is increased in chronically inflamed colon mucosa (cf. below), a typical diseased state in which the microenvironment plays a significant role (cf. below). In addition, PEPT1 is highly expressed in Caco-2 cells, a colon cancer-derived cell line [177]. As a result, one could assume that the regulation of PEPT1 in colon cancer, which in many respects is a situation of a not-healing wound, could be differently expressed compared to the healthy colon. Nevertheless, to our knowledge, the regulation of PEPT1 expression in human colon cancer has never really been investigated. We have tested the expression in laser dissected, differentiated colon tumor cells and could not observe any expression of *PEPT1* (unpublished data). This leads to the conclusion that the regulation of PEPT1 in colon cancer may mainly be under the control of other factors than CDX2 and the ones active in chronic inflammation (cf. below). While neoplastic processes in the colon do not seem to affect PEPT1 expression, some studies suggest that

PEPT1 expression is increased in cells of the stomach undergoing metaplasia, that is, gastric mucosal cells differentiating into a more intestinal phenotype [178]. Using a mouse model, Mutoh *et al.* demonstrated that CDX2 induced not only morphological but also functional absorptive enterocytes in the intestinal metaplastic mucosa *in vivo*, suggesting that CDX2 is necessary and sufficient by itself to specify the development of intestinal absorptive enterocytes [179]. Thus, the regulation of PEPT1 could take place in metaplastic cells of the stomach under the control of CDX2.

Similar to *PEPT1*, *HPT1* (*CDH17*) is expressed in the small intestine, metaplastic cells in gastric carcinoma, and in Caco-2 cells [180]. However, in contrast to *PEPT1*, it is also expressed in colon and colon carcinoma [181–183]. Very interestingly, while overexpression of this gene in gastric cancer is associated with lymph node metastasis, its reduced expression in colon cancer is linked to the progression of colon carcinoma and lymph node metastasis [183, 184]. *CDH17* and its protein product seem to be mostly expressed in differentiated tumor cells and are downregulated in tumoral dedifferentiation [182, 183]. Similar to PEPT1, Hinoi *et al.* reported that the regulation of CDH17 seems to be under the control of CDX2 [181]. The authors actually discovered this link by applying microarray analysis. They compared the expression profiles of HT29 cells with minimal endogenous CDX2 expression and HT29 cells engineered to express exogenous CDX2. CDH17 was strongly induced in the engineered cells. Moreover, immunoprecipitation assays suggested the presence of two CDX2 responsive elements in the 5′-flanking region of CDH17. Testing the expressions of CDX2 and CDH17 in patient samples, the authors observed a close correlation. In addition, in CDX2 knockout mice, they observed a suppression of CDH17 expression in polyps arising in the proximal colon [181]. Ko *et al.* observed that in intestinal metaplasia and adenocarcinoma of the stomach CDX2 colocalizes with liver–intestine cadherin [185].

Still further investigations are necessary to elucidate the expressions of PEPT1 and CDH17 and their roles in cancers and especially colon cancer. As far as the design of new drugs using PEPT1 as a carrier is concerned, understanding the differences of regulation of this transporter between colon cancer and chronic inflammatory diseases of the large intestine will be very important. Regarding CDH17, still further studies are needed to elucidate its role as a peptide transporter and, thus, as a potential drug carrier.

In summary, we have elucidated that various factors can interfere with the expression of transporters in tumors. In addition, the regulation of transporters may be influenced by the microenvironment. Even though chronic inflammation and tumor formation have many similarities, by using PEPT1 as an example, we have seen that the expression of transporters may differ. Thus, knowing the expression pattern and regulation of transporters in these situations is very important when exploiting transporters as drug carriers. Moreover, the expression of transporters may serve as a readout for active transcription factors in these situations and, thus, serve as an indicator for the design of new drugs. Figure 10.5 presents an overview of the role of transporters in cancer.

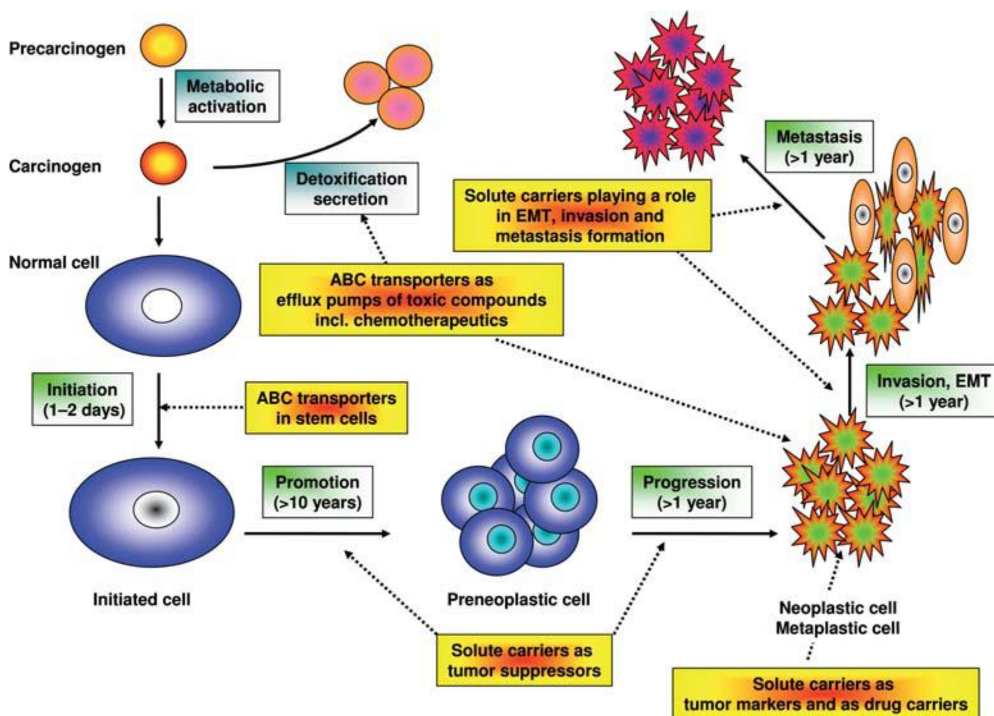


Figure 10.5 Overview of the role of transporters in cancer.

10.5.3.3 Role of Transporters in Intestinal Stem Cells

Various studies indicated that ABC transporters are expressed in stem cells, which led to the hypothesis that they are expressed in tumor-initiating cells [186, 187]. It has been shown in colon cancer that cells expressing CD133+ have such a cancer-initiating potential [188, 189]. Monzani *et al.* observed in melanoma, a coexpression of ABCG2 and CD133+ [190]. Interestingly, CD133+ is strongly expressed in Caco-2 cells and is not downregulated upon differentiation [191]. We observed that compared to other colon cancer cell lines with different stages of differentiation (i.e., T84, HT29, LS174T, SW480, and HCT116), differentiated Caco-2 and HT29 cells had the highest *CD133* expression, while the expression in SW480 cells, cells with mesenchymal characteristics, was lowest (unpublished data). Frank *et al.* showed that ABCB5 is a molecular marker for a distinct subset of chemoresistant, stem cell phenotype-expressing tumor cells among melanoma bulk populations [192]. However, the expression and the role of ABC transporters in cancer-initiating cells in solid tumors have never directly been shown.

In conclusion, various studies indicate that transporters could be exploited in solid tumors when targeting tumor-initiating cells. However, there is still a significant lack of information available regarding their expression and role in these cells. In addition, to date it is still unclear which models may be most appropriate when studying these aspects in such cells. Thus, for the design of new drugs, medical

chemistry has to take into account that most probably chemotherapeutics are subjected to secretion.

10.5.4 Role of PEPT1 in Inflammatory Bowel Disease

Intestinal epithelial and subepithelial cells are an integrated part of the immune system, being a primary barrier and detection zone for pathogens. In a study by Merlin *et al.*, it has been suggested that PEPT1 could be involved in mediating the signal between pathogen invasion and immune response via transport of bacteria-derived *n*-formyl peptides such as formyl-Met-Leu-Phe (fMLP) [118]. The PEPT1-mediated transport of fMLP induced basolateral to apical neutrophil migration in a neutrophil/Caco-2-BBE cell model [118]. In both rat jejunum *in vivo* and *in vitro* cell culture studies, the PEPT1-mediated fMLP transport induced inflammation-like responses, without inducing inflammation in normal rat colon, which does not express *Pept1* [193, 194]. PEPT1 is not normally expressed in the colon, but, under pathophysiological conditions such as chronic ulcerative colitis and Crohn's disease, colonic expression of PEPT1 is observed [193, 194]. Furthermore, *n*-formyl-peptides transported via PEPT1 induced cell surface expression of MHC class 1 in HT-29-Cl.19A cells [194]. However, a recent study in rats indicated that induced colitis does not alter the bioavailability of Pept1 substrates such as cephalexin and valaciclovir [189]. In rabbit, small intestinal inflammation decreases the transport via Pept1; however, this seems to be due to an altered affinity for the substrate rather than altered mRNA expression [196]. After 80% colonic resection in rats, upregulation of *Pept1* expression is observed, and the induction of intestinal inflammation using fMLP causes a damage of the intestinal epithelium indicating a role for PEPT1 in the generation of this disease state [72, 195, 197, 198]. Also, small bowel resection in humans, causing small bowel syndrome, leads to upregulation of PEPT1 in the colon and may be part of an adaptional process to increase dietary nitrogen absorption [195]. In another study, the long-term effects of treating rats with sublethal concentrations of the endotoxin lipopolysaccharide (LPS) was evaluated [199]. LPS is an important component of the outer membrane of Gram-negative bacteria, and, when injected in rats, it increases levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL) 1β along with decreasing levels of *Pept1* mRNA and protein in the small intestinal enterocytes [58]. Administration of dexamethasone (0.1 mg/100 g body weight), which decreased the amount of Il-1β and TNF-α in the small intestinal mucosa, could counteract the effect of LPS on Pept1 expression [199]. However, in Caco-2/BBE cells and in mice intestine, it was shown that interleukin IL-1β does not increase Gly-Sar uptake via PEPT1, although the amount of *Pept1* mRNA is increased in the colon and decreased in the small intestine [200]. On the other hand, TNF-α and interferon-γ upregulated Pept1 activity in mouse proximal and distal colon, but they had no effect on the *Pept1* mRNA in the small intestine [200]. TNF-α is able to stimulate the EGF receptor, and the long-term activation results in a decreased expression of *PEPT1* mRNA and its protein product, whereas short-term activation increases PEPT1-mediated uptake in Caco-2 cells [201, 202]. Another

hormone likely to be involved in the regulation of PEPT1 in the colon during inflammation is leptin. Leptin is an adipocyte-secreted hormone that is present at increased levels in the colon during IBD, where the cells express and release leptin on the apical membrane [203]. Recently, Nduati *et al*. have shown by promoter studies that leptin increases *PEPT1* mRNA expression and its protein product in Caco-2 BBE cells by transcriptional activation that depends on the CDX2-binding sites located −579 and −562 in the *PEPT* promoter [172]. Their results indicate that the signaling pathway leading to leptin transcriptional activation of the *PEPT* gene is via increased cAMP levels and subsequent activation of PKA that translocates into the nucleus and increases the level of phosphorylated CREB. This is then followed by binding of CDX2 and pCREB to the promoter [172].

Taken together, PEPT1 seems to play an important role in intestinal inflammation, especially in the colon, and may thus be a novel anti-inflammatory drug target for the medicinal chemist. This is stressed by the recent findings that the tripeptide Lys-Pro-Val, due to the intracellular accumulation via PEPT1, inhibits the activation of NF-κB and MAP kinase inflammation and reduces proinflammatory cytokine secretion in DSS- and TNBS-induced colitis in mice [204].

10.6 Summary and Outlook

Drug absorption has traditionally been thought to occur predominantly via passive transcellular and paracellular transport mechanisms. However, recent studies indicate that carrier-mediated drug transport may play a more important role than previously appreciated. Clearly, the bioavailability of a given drug can be increased by targeting transporters as drug carriers. We have presented a couple of examples for which the design of a prodrug – which is taken up actively – significantly contributed to an improvement of the bioavailability. Yet, the mechanisms of uptake are actually still unknown or only partially elucidated.

Knowing the expression of transporter genes is a first step in elucidating the role of transporters in absorption. With the rise of new high-throughput technologies, significant knowledge of this aspect could be obtained. Yet, information on the protein expression and the functional activity of transporters is still needed. In addition, the influence of genotypes on drug absorption needs further investigation. However, it has become clear that the knowledge gained from genotyping and phenotyping studies can contribute to a more individualized and improved treatment.

Making an increasing amount of genomics and proteomics data publicly available will certainly deepen our understanding of how transporters are regulated and which factors are important. Consequently, drug design can be adjusted accordingly. Besides their role as drug and nutrient carriers, transporters also seem to play other important roles in diseased states of the intestine. Thus, transporters on the one hand should be considered when trying to reduce chemoresistance or improve sensitivity. On the other hand, they could also be exploited in the future as direct drug targets to treat these diseases.

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11
Hepatic Transport

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Abbreviations

4-MU	4-Methylumberiferone
ABC	ATP binding cassette
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
BSP	Bromosulfophthalein
CCK-8	Cholecystokinin octapeptide
CMV	Canalicular membrane vesicle
DHEAS	Dehydroepiandrosterone sulfate
DNP-SG	2,4-Dinitrophenyl-*S*-glucuronide
$E_2 17\beta G$	Estradiol-17β-glutathione
EHBR	Eisai hyperbilirubinemic rat
E-sul	Estrone-3-sulfate
M3G	Morphine-3-glucuronide
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug resistance
MPP^+	1-Methyl-4-phenylpyridinium
MRP	Multidrug resistance-associated protein
NTCP	Na^+-taurocholate cotransporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OST	Organic solute transporter
PFIC	Primary familial intrahepatic cholestasis
PG	Prostaglandin
P-gp	P-glycoprotein
SLC	Solute carrier
SNP	Single nucleotide polymorphism
TBuMA	Tributylmethylammonium
TEA	Tetraethylammonium

11.1 Introduction

The liver plays an important role in determining the oral bioavailability of drugs. Drug molecules absorbed into the portal vein are taken up by hepatocytes and then metabolized and/or excreted into the bile in an unchanged form. For hydrophilic drugs, several transporters located on the sinusoidal membrane are responsible for the hepatic uptake [1–3]. Biliary excretion of drugs is also mediated by the primary active transporters, referred to as ATP-binding cassette (ABC) transporters, located on the bile canalicular membrane [3, 4]. Very recently, the importance of sinusoidal efflux mediated by MRP family transporters in the hepatic transport of substrates has also emerged [3, 5, 6]. The functional changes in these uptake and efflux transporters caused by genetic polymorphisms and drug–drug interactions sometimes greatly affect the hepatic availability and clearance of drugs. Now, several *in vitro* experimental systems and methodologies for predicting the *in vivo* hepatic clearance and the contribution of each transporter to the overall hepatic clearance have been developed. In this chapter, we will review the molecular mechanisms of hepatobiliary transport of clinically used drugs and also focus on the quantitative prediction of *in vivo* drug disposition from *in vitro* data.

Please note that the nomenclature of drug transporters (ABC and SLC (solute carrier) family) has recently been established by the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/). ABC family transporters are classified into 7 groups (ABCA–ABCG) and each group consists of several isoforms, whereas SLC family transporters are classified into 47 groups (SLC1–SLC47). Exceptionally, the gene symbol of OATP (organic anion transporting polypeptide) family transporters is *SLCO* (previously named as *SLC21*). This grouping is based on the gene homology. Until then, the researchers who first succeeded in the molecular cloning of novel transporters usually put a name to the cloned transporter as they liked, so it is often observed that one transporter has several names. For example, OATP1B1 is also called as OATP-C, OATP2, LST-1, and SLC21A6. We briefly mention the aliases of each transporter frequently used previously in each section. The name of genes is italicized (e.g., *MDR1*), whereas the name of proteins is designated without italicizing (e.g., MDR1). Moreover, all letters of the transporter name in humans are capitalized (e.g., OAT1), whereas only the first letter of the transporter name in rodents is capitalized and the subsequent letters are written by lowercase (e.g., Oat1).

11.2 Hepatic Uptake

Many compounds are efficiently taken up into hepatocytes in a Na^+-dependent and -independent manner, and the effect of various inhibitors on the uptake of substrates is different depending on substrates. Now, so many transporters have been identified in the basolateral membrane of hepatocytes such as NTCP (Na^+-taurocholate cotransporting polypeptide), OATPs, and OATs (organic anion transporters)

for organic anion transport and OCTs (organic cation transporters) for organic cation transport. Major uptake transporters in basolateral membrane are listed in Table 11.1.

11.2.1 NTCP (*SLC10A1*)

NTCP is expressed exclusively in the basolateral membrane of the liver and responsible for the hepatic uptake of several kinds of bile acids in a Na^+-dependent fashion [7, 8]. NTCP generally accepts several kinds of unconjugated and conjugated bile acids including clinically used bile acids for the treatment of cholestatic disorders such as chenodeoxycholate, ursodeoxycholate, and its conjugates with glycine and taurine [9–11]. Regarding the bile acid transport, Mita *et al*. [12] have demonstrated that NTCP-mediated uptake clearances of 10 different bile acids were well correlated between human NTCP and rat Ntcp and their clearances increased in the rank order of taurine-conjugated bile acids > glycine-conjugated bile acids > unconjugated bile acids, suggesting no species differences in the properties of bile acid recognition by NTCP between rats and humans. NTCP can also transport non-bile acid compounds such as dehydroepiandrosterone sulfate (DHEAS), bromosulfophthalein (BSP), and estrone-3-sulfate (E-sul) [13]. Rat Ntcp can also accept thyroid hormones and α-amanitin (mushroom toxin) [14, 15]. Interestingly, Ho *et al*. [16] have shown that rosuvastatin is also a substrate of human NTCP, but not rat Ntcp, and that NTCP accounted for approximately 35% of the rosuvastatin uptake in human hepatocytes judging from the difference in the uptake clearance in the presence and absence of Na^+ ion. These evidences reminded us of the possible contribution of NTCP to the hepatic uptake of non-bile acid types of drugs. In general, Na^+-dependent uptake of anions is considered to be mainly mediated by NTCP; however, the presence of unidentified Na^+-dependent transporters for anionic drugs (e.g., bumetanide) has also been suggested [17].

11.2.2 OATP (*SLCO*) Family Transporters

Although several bile acids are mainly transported into hepatocytes by NTCP in a Na^+-dependent manner, many organic anions are also taken up in a Na^+-independent manner. OATP family transporters are one of the key players for the Na^+-independent hepatic uptake of anions [18–20]. The number of cloned isoforms belonging to OATP family is currently 11, 13, and 12 in humans, rats, and mice, respectively. It should be noted that OATP subtypes in humans do not always genetically correspond to those in rodents. In rats, Oatp1a1 (Oatp1) [21], Oatp1a4 (Oatp2) [22], and Oatp1b2 (Oatp4) [23] are largely responsible for the hepatic uptake, whereas in humans, OATP1B1 (OATP-C/OATP2/LST-1) [24–26] and OATP1B3 (OATP8/LST-2) [27, 28] may be the most important hepatic uptake transporters. Different from other OATP transporters, OATP1B1 and OATP1B3 are exclusively expressed in the liver and can accept a wide variety of organic anions including clinically important drugs such as HMG-CoA

Table 11.1 Major uptake transporters expressed in the basolateral membrane of human liver.

Transporter	Gene symbol	Chromosome	Reference accession (mRNA)	Amino acids	Transmembrane domains	Tissue distribution	Cellular localization	Substrates
OATP1B1	*SLCO1B1*	12p12	NM_006446	691	12	Liver	Basal	Glutathione conjugates (LTC_4), glucuronide conjugates ($E_2 17\beta G$, bilirubin glucuronide), sulfate conjugates (E-sul, DHEAS), bile acids (taurocholate, glycocholate), bilirubin, and several drugs (pravastatin, valsartan, and temocaprilat)
OATP1B3	*SLCO1B3*	12p12	NM_019844	702	12	Liver	Basal	Glutathione conjugates (LTC_4), glucuronide conjugates ($E_2 17\beta G$, bilirubin glucuronide), sulfate conjugates (DHEAS), bile acids (taurocholate, glycocholate), and several drugs (CCK-8, telmisartan, fexofenadine, and digoxin)
OATP2B1	*SLCO2B1*	11q13	NM_007256	709	12	Liver, intestine, and so on	Basal (liver), apical (intestine)	E-sul, DHEAS, pravastatin, rosuvastatin, glybenclamide, benzylpenicillin, and so on
NTCP	*SLC10A1*	14q24	NM_003049	348	7	Liver	Basal	Bile acids, DHEAS, BSP, and E-sul
OAT2	*SLC22A7*	6p21	NM_006672, NM_153320	546/548	12	Liver	Basal	α-Ketoglutarate, salicylate, methotrexate, zidovudine, 5-fluorouracil, and so on
OCT1	*SLC22A1*	6q26	NM_003057, NM_153187	554/506	12	Liver, kidney	Basal	TEA, TBuMA, MPP^+, acyclovir, ganciclovir, famotidine, metformin, and so on

reductase inhibitors (statins) and several kinds of anticancer drugs. The list of reported substrates for OATP1B1 and OATP1B3 are shown in Table 11.2. Because of their high homologies (80% in amino acids), their substrate specificities overlap each other, but some compounds are specifically recognized by specific transporter. For example, digoxin and cholecystokinin octapeptide (CCK-8) can be transported specifically by OATP1B3 but not by OATP1B1 [29, 30]. In another example, though valsartan, olmesartan, and telmisartan are in the same category of drugs, angiotensin II receptor antagonists, valsartan and olmesartan can be transported by both OATP1B1 and OATP1B3, whereas telmisartan is specifically recognized by OATP1B3 as a substrate [31–34]. Thus, the substrate specificities of OATP1B1 and OATP1B3 are very broad and similar, but sometimes strictly distinct from each other. For the endogenous compounds, OATP1B1 plays a predominant role in Na^{+}-independent hepatic uptake of several bile acids and steroid conjugates. OATP1B1 can accept bilirubin and its glucuronides as a substrate and support the hepatobiliary transport of bilirubin in combination with MRP2 [35, 36], although a contradictory result has also been published [37].

Other OATP family transporters such as OATP1A2 (OATP-A), OATP2B1 (OATP-B), OATP3A1 (OATP-D), and OATP4A1 (OATP-E) have been reported to be expressed in the liver [20]. OATP1A2 accepts a wide variety of compounds including some type II cations (bulky hydrophobic compounds) such as *N*-(4,4-azo-*n*-pentyl)-21-deoxyajmalinium, rocuronium, and *N*-methylquinine [29, 38]. However, because OATP1A2 is mainly expressed in the brain and its hepatic expression is minimal, it is still unknown whether OATP1A2 is involved in the hepatic uptake of type II cations. OATP2B1 is expressed in various tissues and its strongest expression is in the liver [29]. OATP2B1 can transport several kinds of drugs such as glibenclamide, pravastatin, atorvastatin, and pitavastatin, but its contribution to their hepatic uptake is minor or unknown because of the overlapped substrate specificities of OATP1B1 and OATP1B3 [39–42]. Other transporters are expressed ubiquitously and their substrates are almost limited to endogenous compounds (thyroid hormones, prostaglandins (PGs)), so they may not be involved in the hepatic transport of drugs [20, 43].

11.2.3 OAT (*SLC22*) Family Transporters

Though many OAT family proteins are expressed mainly in the kidney and are involved in the active renal secretion of anions, OAT2 (SLC22A7) is expressed predominantly in the basolateral membrane of the liver [44]. Human OAT2 can accept various structurally unrelated drugs (e.g., bumetanide, zidovudine, tetracycline, erythromycin, theophylline, 5-fluorouracil, methotrexate, ranitidine, paclitaxel, and allopurinol) as well as endogenous compounds (e.g., cAMP, α-ketoglutarate, L-ascorbate, prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, DHEAS, and E-sul) [45]. Though some compounds are also substrates of OATP transporters, the averaged molecular weight of substrates for OAT2 tends to be smaller than that of OATPs. The relative contribution of OAT2 to the overall *in vivo* hepatic uptake of substrates has not been

Table 11.2 Substrates of OATP1B1 and OATP1B3.

	OATP1B1	OATP1B3
Endogenous compounds		
E-sul (estrone-3-sulfate)	0.094&5.34[a]; 0.0675&7[a]; 12.5; 0.458 [35, 151, 251]	+ [29]
$E_217\beta G$	3.7–8.2 [24, 252]	5.4 [27]
PGE_2	+ [26, 252]	NT [29]
TXB_2 (thromboxane B_2)	+ [26]	
LTC_4	+ [26, 29]	+ [27, 29]
LTE_4 (leukotriene E_4)	+ [26]	
Triiodothyronine	2.7 [26, 28]	6.4 [28]
Thyroxine	3 [25, 26]	+ [29]
Taurocholate	10–33.8 [25, 26, 35]	5.8 [28]
Glycocholate	+ [29]	+ [29]
Cholate	11.4 [35]	NT [35]
DHEAS	21.5 [29, 35]	>30 [29, 35]
Bilirubin	0.0076 [35, 36]	0.0391 [36]
Bilirubin glucuronide	0.10 (mono), 0.28 (bis) [35]	0.5 (mono), NT (bis) [35]
Tauroursodeoxycholate	7.47 [9]	15.9 [9]
Glycoursodeoxycholate	5.17 [9]	24.7 [9]
Taurolithocholate sulfate	+ [177]	
Exogenous compounds		
DPDPE ([D-penicillamine (2,5)]-enkephalin)	+ [29]	+ [29]
BQ-123 (endothelin antagonist)	+ [29]	+ [29]
Pravastatin	13.7–85.7 [25, 163, 188]	
Cerivastatin	+ [188, 209]	
Fluvastatin	1.4–3.5 [181, 253]	7 [253]
Atorvastatin	12.4 [188]	
Rosuvastatin	4–8.5 [16]	9.8 [16]
Pitavastatin	3 [151]	3.25 [151]
Caspofungin	+ [254]	NT [254]
Demethylphalloin	17 [255]	7.5 [255]
Troglitazone sulfate	+ [256]	NT [256]
Rifampicin	1.5–13 [206, 257]	2.3 [206]
Arsenic	+ [258]	
Atrasentan	+ [259]	+ [259]
Bosentan	44 [219]	141 [219]
Ro 48-5033 (metabolite of bosentan)	60 [219]	166 [219]
Valsartan	1.39 [31]	18.2 [31]
Olmesartan	12.8–42.6 [33, 34]	44.2–71.8 [33, 34]
Enalapril	262 [260]	+ [260]
Methotrexate	+ [28]	24.7 [28]
Temocapril	+ [190]	
Temocaprilat	+ [190]	
DADLE ([D-Ala2, D-Leu5]-enkephalin)	+ [261]	
Microcystin-LR	7 [262]	9 [262]

Table 11.2 (*Continued*)

	OATP1B1	OATP1B3
SN-38 (active metabolite of irinotecan)	+ [263]	NT [263]
Fexofenadine	+ [92]	108 [92, 161]
Bromosulfophthalein	0.14–0.3 [29, 35]	0.4–3.3 [27, 29]
Deltorphin II	NT [29]	+ [29]
Ouabain	NT [29]	+ [29]
Digoxin	NT [29]	+ [29]
Fluo-3		+ [176]
Docetaxel	NT [264]	+ [264]
Paclitaxel	NT [264]	6.79 [264]
CCK-8 (cholecystokinin octapeptide)	NT [30]	11.1 [30]
Telmisartan	NT [32]	21.7 (1% HSA)[b] [32]
Telmisartan glucuronide	NT [179]	118 (1% HSA)[b] [179]
Benzylpenicillin	+ [252]	
Bamet-R2 (*cis*-diammine-chloro-cholylglycinate-platinum (II))	10 [265]	
Bamet-UD2 (*cis*-diammine-bisursodeoxycholate-platinum (II))	9.7 [265]	
TR-14035 (α4β1/α4 β7 integrin dual antagonist)	7.5 [266]	5.3 [266]
CDCA-NBD (7-nitrobenz-2-oxa-1,3-diazole chenodeoxycholate)	17 [267]	10 [267]
CA-NBD (7-nitrobenz-2-oxa-1,3-diazole cholate)	+ [267]	+ [267]
DCA-NBD (7-nitrobenz-2-oxa-1,3-diazole deoxycholate)	+ [267]	+ [267]
LCA-NBD (7-nitrobenz-2-oxa-1,3-diazole lithocholate)	+ [267]	+ [267]
UDCA-NBD (7-nitrobenz-2-oxa-1,3-diazole ursodeoxycholate)	+ [267]	+ [267]

The value represents the K_m values (unit: μM). +: significant uptake was observed.
NT: significant uptake was not observed.

[a]The K_m values for the high- and low-affinity site.

[b]The K_m values determined in the presence of 1% human serum albumin (HSA).

explained yet. OAT3 (SLC22A8) is detected in male rat liver [46] and transports organic anions such as ochratoxin A, E-sul, benzylpenicillin, DHEAS, and pravastatin [45]; however, its hepatic expression is minimal in mice and humans [47]. Indeed, the hepatic uptake of E-sul and *p*-aminohippurate was not different between wild-type and Oat3 knockout mice [48]. Recently, OAT5 (SLC22A10) and OAT7 (SLC22A9) have been reported to be exclusively expressed in the human liver [49, 50]. The substrate of human OAT5 has not been identified yet. OAT7 is located on the basolateral

membrane of human hepatocytes and accepts E-sul and DHEAS in a Na^+-independent manner [50]. Interestingly, OAT7-mediated transport of E-sul was transstimulated by short-chain (C3–C5) fatty acids, which implies that substrates of OAT7 are taken up in exchange for butyrate in hepatocytes [50]. However, the role of these novel OATs in the pharmacokinetics of drugs has not been demonstrated so far.

11.2.4 OCT (*SLC22*) Family Transporters

It has been shown that OCT1 (SLC22A1) and OCT3 (SLC22A3) are confirmed to express in the basolateral membrane of human hepatocytes [51, 52]. OCT1 is predominantly expressed in the liver [51], while OCT3 is expressed in several tissues such as kidney, heart, placenta, and brain [52]. OCT1 accepts type I cations, which consist of small hydrophilic compounds including tetraethylammonium (TEA), tributylmethylammonium (TBuMA), and procainamide, as well as anionic and uncharged compounds (e.g., prostaglandins) [52]. Rat Oct1 transports several endogenous compounds (choline, dopamine, serotonin, adrenaline, noradrenaline, and histamine) as well as exogenous compounds (cimetidine and 1-methyl-4-phenylpyridinium (MPP^+; neurotoxin)) [52]. Human OCT1 can transport not only endogenous and model compounds but also various marketed drugs such as antiviral drugs (acyclovir, ganciclovir), H_2-blockers (famotidine, ranitidine), and metformin [52]. The importance of OCT1 in the *in vivo* disposition of substrates has been explained by using Oct1 knockout mice [53]. By comparing the hepatic uptake of compounds between wild-type and Oct1 (−/−) mice, the uptake of TEA, metaiodobenzylguanidine (anticancer drug), and MPP^+ is mainly mediated by Oct1 [53]. However, the hepatic uptake of cimetidine and choline are not affected by knockdown of Oct1 gene [53], though they are also substrates of rat Oct1 [54]. Thus, it is possible that the uptake of cimetidine into isolated rat hepatocytes is mediated by unknown transporters other than Oct1 [55]. A series of biguanides, which are cationic and frequently used for the treatment of diabetes, is transported by Oct1 and the hepatic uptake of metformin has been drastically decreased in Oct1 (−/−) mice compared to wild-type mice (Figure 11.1) [56]. Moreover, the significant increase in serum lactic acid was observed in Oct1 (−/−) mice after administration of metformin, though its time profile of plasma concentration was similar (Figure 11.1) [57]. This suggests that metformin-induced lactic acidosis is dominated by its intrahepatic concentration and Oct1-mediated transport is a rate-limiting step in hepatic uptake of metformin. Human OCT3 also accepts several compounds such as cimetidine, dopamine, epinephrine, norepinephrine, and atropine [52], but its function in liver has not been explained yet.

11.3 Biliary Excretion

In the canalicular membrane, several ABC transporters such as MDR1 (multidrug resistance 1), MRP2 (multidrug resistance-associated protein 2), BSEP (bile salt

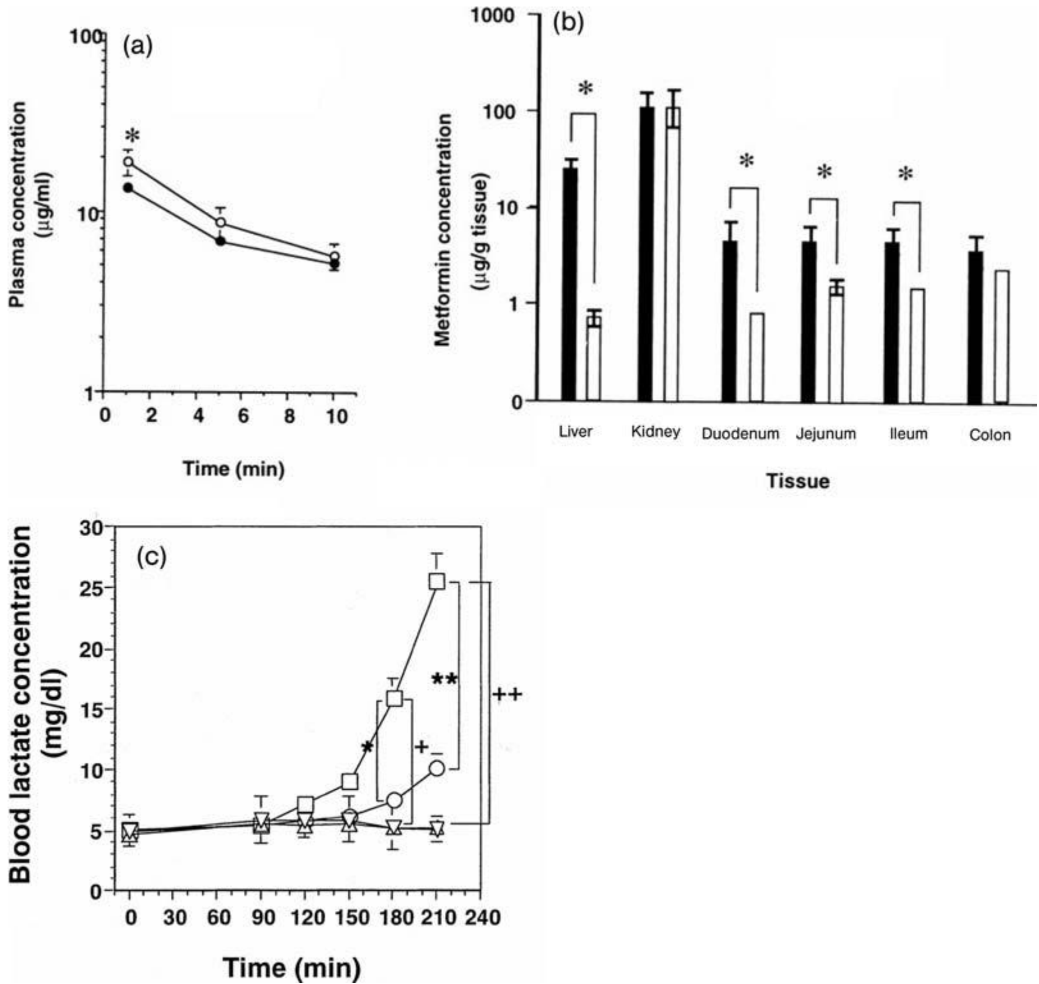

Figure 11.1 Impact of OCT1 on the pharmacokinetics and subsequent toxicological effect of metformin [56, 57]. (a) Plasma concentration profile and (b) tissue distribution of metformin in Oct1 (−/−) (open symbols) and wild-type mice (closed symbols) at 10 min after the i.v. administration of 5.0 mg/kg metformin. (c) Time profile of lactate concentration in wild-type and Oct1 (−/−) mice during intravenous infusion of 150 mg/h/kg metformin. The whole blood lactate concentration in wild-type (□) and Oct1 (−/−) mice (○) was compared with that in saline-treated wild-type (▽) and Oct1 (−/−) mice (△).

export pump), and BCRP (breast cancer resistance protein) are expressed and are involved in the biliary excretion of endogenous and exogenous compounds. Recently, novel SLC transporter, MATE (multidrug and toxic compound extrusion) may also play roles in biliary excretion of cationic compounds. Major efflux transporters in canalicular membrane are listed in Table 11.3.

Table 11.3 Major efflux transporters expressed in the bile canalicular membrane of human liver.

Transporter	Gene symbol	Chromosome	Reference accession (mRNA)	Amino acids	Transmembrane domains	Tissue distribution	Cellular localization	Substrates
MDR1	*ABCB1*	7q21	NM_000927	1280	12	Liver, brain, kidney, intestine, and so on	Apical	Neutral and cationic hydrophilic drugs such as anticancer drugs (CPT-11, doxorubicin, and vinblastine, etc.), digoxin, dexamethasone, loperamide, phenytoin, and so on, and fexofenadine (zwitterion)
MRP2	*ABCC2*	10q24	NM_000392	1545	17	Liver, kidney, and intestine	Apical	Glutathione conjugates (DNP-SG, LTC_4), glucuronide conjugates (bilirubin glucuronide, and $E_217\beta G$), and several anionic drugs (pravastatin, temocaprilat, SN-38, and methotrexate)
BCRP	*ABCG2*	4q22	NM_004827	655	6	Various organs	Apical	Mitoxantrone, topotecan, E-sul, DHEAS, imatinib, pitavastatin, and so on
BSEP	*ABCB11*	2q24	NM_003742	1321	12	Liver	Apical	Bile acids, doxorubicin, fexofenadine, and pravastatin
MATE1	*SLC47A1*	17p11	NM_018242	570	12	Liver, kidney, and skeletal muscle	Apical	Cationic compounds (TEA, MPP^+, cimetidine, metformin, creatinine, guanidine, etc.)

11.3.1
MDR1 (P-glycoprotein; *ABCB1*)

MDR1, also known as P-glycoprotein (P-gp), is expressed in various tissues such as liver, kidney, intestine, placenta, and blood–brain barrier and is responsible for the limited intestinal absorption and brain distribution of drugs and enhanced clearance in the liver and kidney. Humans have only one *MDR1* gene, while in rodents, there are two types of *Mdr1* genes, *Mdr1a* and *Mdr1b*, which are expressed in a tissue-specific manner [58–60]. For example, only *Mdr1a* is expressed in the brain and intestine in mice, whereas both *Mdr1a* and *Mdr1b* are expressed in the liver and kidney [58]. MDR1 can accept a wide variety of structurally diverse compounds, most of which are basic or uncharged, and relatively hydrophobic [61]. But organic anions such as fexofenadine and estradiol-17β-glucuronide ($E_2$17βG) are also reported to be recognized by MDR1 as a substrate [62–64]. The information about substrates of MDR1, Mdr1a, and Mdr1b has been published [61].

The direct evidence of the important role of Mdr1 in the hepatic transport of drugs has come from several studies demonstrating the comparison of the biliary excretion between normal and *Mdr1* gene knockout mice. Since Mdr1b is induced in Mdr1a (−/−) mice [65], we had better use Mdr1a/1b double knockout mice rather than Mdr1a (−/−) mice for the analyses [66, 67]. For example, typical type I cations, such as TBuMA and azidoprocainamide methoiodide, and type II cations, such as vecuronium, are excreted into bile via MDR1 [68]. In particular, the role of MDR1 may be emphasized for vecuronium as more than 40% of the administered dose is excreted into bile, largely mediated by Mdr1 [68]. Digoxin is proven to be excreted into bile mainly via Mdr1 [69]. In mice, 45% of the dose is excreted into bile in an unchanged form. The biliary clearance of digoxin in wild-type mice is about 2.7-fold more than that in Mdr1a (−/−) mice. Mdr1 is also responsible for the biliary excretion of doxorubicin and vinblastine [70]. Though the fraction of dose excreted into bile in an unchanged form of doxorubicin and vinblastine is only 13 and 5%, respectively, due to the extensive metabolism, the excreted amount of unchanged doxorubicin and vinblastine into bile in wild-type mice is three- to fivefold more than that in Mdr1a (−/−) mice.

MDR3, a homologue of MDR1, is responsible for the biliary excretion of phospholipids, and a hereditary defect in this gene results in the acquisition of progressive familial intrahepatic cholestatis type 3 (PFIC3) [71].

11.3.2
MRP2 (*ABCC2*)

While MDR1 accepts many kinds of neutral and cationic compounds, MRP2 is thought to be the transporter responsible for the biliary excretion of many organic anions including conjugated metabolites. Originally, the importance of MRP2 in the biliary excretion of several drugs has been explained by comparing the *in vivo* biliary clearance and *in vitro* uptake into the bile canalicular membrane vesicles (CMVs) between wild-type and Mrp2-hereditary deficient rats such as Eisai hyperbilirubinemic rats (EHBRs) and GY/TR^- rats [72, 73]. MRP2 can recognize a

wide variety of endogenous and exogenous compounds including clinically important drugs such as HMG-CoA reductase inhibitors (statins), angiotensin II receptor antagonist (valsartan and olmesartan), methotrexate, temocaprilat, BQ-123 (cyclic peptide for the endothelin antagonist), and cefodizime [74]. Interestingly, the substrate specificities of MRP2 are very similar to those of OATPs, which do not share homology with MRP2; therefore, the efficient hepatobiliary transport of organic anions is thought to be realized with the cooperation of hepatic uptake transport mediated by OATP transporters and biliary efflux via MRP2. Using this transport system, pravastatin can undergo efficient enterohepatic circulation, which leads to its retention in the liver, a pharmacological target of statins, and avoidance of excessive systemic exposure [75]. It is also reported that the impaired biliary excretion of S3025 (1-[2-(4-chlorophenyl)-cyclopropylmethoxy]-3,4-dihydroxy-5-(3-imidazo-[4,5]b pyridine-1-3-yl-3-[4-carboxy]-phenyl-acryloyloxy)-cyclohexancarboxylic acid), a chlorogenic acid derivative, in Mrp2-deficient rats resulted in the prolonged pharmacological action (the inhibition of hepatic glucose production) compared to wild-type rats [76]. MRP2 can also transport many kinds of conjugates with glutathione, glucuronate, and sulfate (e.g., 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), leukotriene C_4 (LTC_4), acetaminophen glucuronide, and lithocholate-3-sulfate) [74]. Several endogenous compounds are also reported to be transported via MRP2 [74]. For example, reduced glutathione is excreted into the bile mainly via MRP2, which is the driving force of the bile salt-independent bile flow [77, 78]. Bilirubin glucuronide is physiologically pumped out by MRP2, and a hereditary defect in MRP2 expression results in the acquisition of Dubin–Johnson syndrome in humans, which exhibits hyperbilirubinemia due to a lack in its biliary excretion [79]. It was suggested that OATP1B1 and MRP2 play important roles in the detoxification of bilirubin. The species difference in the transport function of MRP2 has been investigated. Ishizuka *et al.* [80] have demonstrated that transport activity of temocaprilat (an Mrp2 substrate) into canalicular membrane vesicles prepared from several species was largely different. Niinuma *et al.* [81] have reported that the transport activity per milligram membrane vesicle protein is virtually identical in humans and rats for glucuronide conjugates, but the transport activity of non-conjugated organic anions and glutathione conjugates in humans was 10–20% of that in rats. Ninomiya *et al.* [82] have compared the substrate specificities and their transport activity of MRP2 among four different species, rats, mice, monkey, and dogs, and concluded that their substrate specificities are similar; however, their intrinsic transport activity differs from one species to another due to not only the difference in the K_m and V_{max} values but also the qualitatively different mode of substrate and modulator recognition exhibited by different species. Zimmermann *et al.* [83] have also indicated the species difference in the modulation of transport by other compounds between human and mouse MRP2.

Recently, Mrp2 knockout mice have been established and we can directly evaluate the role of Mdr1, Mrp2, and Bcrp in pharmacokinetics of drugs in mice without considering the species difference in rat and mouse Mrp2 when using EHBRs or GY/TR$^-$ rats [84]. Previous reports have demonstrated the biliary excretion of

glucuronides and sulfates of 4-methylumbelliferone (4-MU), hermol, and acetoaminophen in Mrp2 knockout mice [85]. As a result, the biliary excretion of only 4-MU glucuronide was reduced. This is distinct from the results from rats, showing that biliary excretion of sulfates is shared by Mrp2 and Bcrp, whereas excretion of glucuronides is mainly determined by Mrp2 in rats [86–89]. In other examples, the biliary excretion of fexofenadine in EHBRs was not changed compared to wild-type mice [90], whereas the biliary excretion in Mrp2 knockout mice was partly decreased, though the part of clearance mediated by unidentified transporters is still remained [91, 92]. However, the disposition of irinotecan and its active metabolite, SN-38, was not changed by the knockout of Mrp2 [93], while biliary excretion of irinotecan and SN-38 was reduced in Mrp2-deficient rats [94]. We cannot say which one is a better model for predicting the role of MRP2 in the disposition of drugs in humans, Mrp2-deficient rats or Mrp2-knockout mice.

11.3.3 BCRP (*ABCG2*)

BCRP is sometimes called "half transporter" since it has 6 putative transmembrane domains, whereas other ABC transporters have 12 or 17 transmembrane domains, and it functions as homodimer [95, 96]. The domain organization of BCRP is also unique because ABC region is located in the N-terminus, whereas ABC region of other ABC transporters is located in the C-terminus. Since BCRP was cloned from drug-resistant cancer cells, various kinds of anticancer drugs (mitoxantrone, anthracyclines (doxorubicin and daunorubicin), etoposide, camptothecins, indolocarbazoles, methotrexate, and imatinib) are originally recognized as substrates of BCRP mostly by observing the drug resistance in BCRP-overexpressing cells [95, 96]. Currently, the transport studies have revealed that BCRP can transport many compounds with different physicochemical properties such as sulfate and glucuronide conjugates (E-sul, DHEAS, 4-MU sulfate, and 4-MU glucuronide), antibiotics (ciprofloxacin, erythromycin, rifampicin, and nitrofurantoin), flavonoids (genistein, quercetin), carcinogens (2-amino-1-methyl-6-phenolimidazo(4,5-b)pyridine (PhIP)), sulfasalazine, and cimetidine [95, 96]. The substrate specificity of BCRP overlaps that of P-gp and MRP2. Recently, the role of BCRP in the biliary excretion of compounds has been clarified in the use of Bcrp (−/−) mice. Regarding the biliary excretion, in the case of pitavastatin, interestingly its biliary excretion was not changed in EHBRs compared to wild-type rats [97], unlike pravastatin, which is excreted into the bile predominantly by Mrp2 [98]. However, biliary excretion of pitavastatin was drastically decreased in Bcrp (−/−) mice, suggesting that pitavastatin may be excreted into the bile mainly via Bcrp [97]. However, the biliary excretion of rosuvastatin partially decreased in both Bcrp (−/−) mice and Mrp2-deficient EHBRs (in-house unpublished data). Therefore, the important transporters for the biliary excretion might be different among three unmetabolized statins. The biliary clearance of fluoroquinolones (grepafloxacin, ulifloxacin, ciprofloxacin, and ofloxacin) was also decreased in Bcrp (−/−) mice [99].

11.3.4
BSEP (*ABCB11*)

BSEP mediates the biliary excretion of unconjugated bile salts such as taurocholate, glycocholate, and cholate as well as conjugated bile salts with glycine and taurine [10, 100, 101]. Mita *et al.* [12] have demonstrated that BSEP-mediated uptake clearances of 10 different bile acids were well correlated between human and rat BSEP, and their clearances of taurine-conjugated bile acids were larger than glycine-conjugated and unconjugated bile acids. Thus, BSEP is responsible for the formation of the bile salt-dependent bile flow [10, 100, 101]. Its hereditary defect results in the acquisition of PFIC2, a potentially lethal disease that requires liver transplantation [102]. Hayashi *et al.* [103] have demonstrated that two mutations of BSEP frequently observed in PFIC2 patients, E297G and D482G, resulted in impaired membrane trafficking, whereas the transport functions of these mutants remained largely unchanged. They also demonstrated that 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated BSEP [104], which suggests that 4-phenylbutyrate has a potential to improve cholestatic diseases by the enhanced redistribution of internalized BSEP to the cell surface. The inhibition of BSEP function by some drugs sometimes results in the drug-induced cholestasis [105]. Recent reports have indicated that BSEP also transports non-bile acid substrates such as doxorubicin, pravastatin, and fexofenadine [92, 106, 107], though its impact on their pharmacokinetics remains to be cleared.

11.3.5
MATE1 (*SLC47A1*)

Very recently, MATE1 has been cloned as a mammalian homologue of bacterial multidrug resistance conferring MATE family [108, 109]. It is supposed to be a cation–proton antiporter that operates in both directions. In humans, two isoforms, MATE1 (highly expressed in the liver, kidney, and skeletal muscle) [109] and MATE2-K (mainly expressed in the kidney) [110], have been identified, while in rodents, Mate1 (mainly expressed in the kidney and placenta) [111, 112] and Mate2 (testis-specific) [113] have been cloned so far. MATE1 can transport several organic cations such as TEA, MPP^+, cimetidine, metformin, creatinine, guanidine, procainamide, cisplatin, oxaliplatin, paraquat, and topotecan [109, 114–116]. Interestingly, acyclovir and ganciclovir (uncharged) and E-sul (anion) are also recognized by MATE1 as a substrate [115]. However, so far the role of MATE1 in the biliary excretion of drugs has not been explained yet.

11.4
Sinusoidal Efflux

Recently, some MRP family transporters such as MRP1, 3, 4, and 6 are located on the basolateral membrane and involved in the sinusoidal efflux of certain compounds

and their conjugates from hepatocytes to blood circulation. Clarifying the role of these transporters in the pharmacokinetics of drugs has just started with the aid of knockout mice. Major efflux transporters in basolateral membrane are listed in Table 11.4.

11.4.1 MRP3 (*ABCC3*)

MRP3 is expressed in a wide range of tissues including liver, intestine, and kidney and it is confirmed to be localized on the basolateral membrane of human liver [5, 6]. Rat Mrp3 is expressed at low level in normal liver and its expression markedly increases in EHBR (Mrp2-deficient rat) [117]. Later, human MRP3 is also induced by several cholestatic disorders [118–120], suggesting that the physiological role of MRP3 has been believed to provide protection to hepatocytes from intrahepatic toxins such as bile acids and bilirubin only under pathological conditions. However, MRP3 is highly expressed in the liver under normal condition in mice [121] and is not largely upregulated in Mrp2 (−/−) mice and under cholestatis conditions [84, 93, 121] compared to humans and rats, maybe due to the little room for upregulation. MRP3 expression is also detected in human liver under physiological condition [118, 122, 123], so at least in humans and mice, MRP3 can partly modulate the hepatic transport of several substrates even under physiological condition. The substrate specificities of MRP3 is narrower compared to MRP2, but several organic anions such as DHEAS, bilirubin glucuronide, methotrexate, LTC_4, and $E_217\beta G$ can be substrates of MRP3 [6]. Recently, Mrp3 knockout mice have been established [121, 124]. The serum levels of bilirubin glucuronide in Mrp3 (−/−) mice was lower than those of the wild-type mice under cholestatic condition induced by bile duct ligation, but bile acid homeostasis was not modified by knockout of Mrp3 in mice [121, 124]. As far as the transport of xenobiotics is concerned, Mrp3 (−/−) mice cannot excrete morphine-3-glucuronide (M3G) from the liver to the blood, which is the major hepatic elimination route for morphine, leading to the increased concentration of M3G in the liver and bile and 50-fold reduction in its plasma level (Figure 11.2) [125]. Also, the hepatic basolateral efflux clearance of glucuronide conjugates of 4-MU, acetaminophen, and harmol was drastically decreased and that of sulfate conjugates of these three compounds was partly decreased in perfused liver of Mrp3 (−/−) mice [126]. Recently, the biliary excretion rate and the hepatic clearance of non-metabolized drug, fexofenadine were increased in Mrp3 (−/−) mice compared to the wild-type mice [92, 127], suggesting the significant role of Mrp3 in the pharmacokinetics of certain kinds of substrate drugs.

11.4.2 MRP4 (*ABCC4*)

Similar to MRP3, MRP4 is a multispecific efflux transporter expressed in a wide variety of tissues such as the liver, kidney, and brain [5, 6]. Interestingly, MRP4 is localized in the basolateral membrane of human liver [128], whereas it is localized in

Table 11.4 Major efflux transporters expressed in the basolateral membrane of human liver.

Transporter	Gene symbol	Chromosome	Reference accession (mRNA)	Amino acids	Transmembrane domains	Tissue distribution	Cellular localization	Substrates
MRP3	*ABCC3*	17q22	NM_003786	1527	17	Liver, intestine, and kidney	Basal	Bile acids (taurocholate, glycocholate), glucuronide conjugates ($E_2 17\beta G$, E3040-glu), sulfate conjugates (taurolithocholate-3-sulfate), methotrexate, and fexofenadine
MRP4	*ABCC4*	13q32	NM_005845	1325	12	Liver, kidney, and brain	Basal (liver, BCSFB), apical (kidney, BBB)	$E_2 17\beta G$, cAMP, cGMP, 6-mercaptopurine, folate, prostaglandins, and so on
OSTα/β		3q29/15q22	NM_152672/ NM_178859	340/128	7/1	Liver, intestine	Basal	Bile acids
MRP1	*ABCC1*	16p13	NM_004996, NM_019862, NM_019898, NM_019899, NM_019900	1531/1472/ 1475/1416/1466	17	Various organs	Basal	Glutathione conjugates (LTC_4, ethacrynic acid-SG), GSH, GSSG, glucuronide conjugates (bilirubin glucuronide, $E_2 17\beta G$), vincristine, daunorubicin, methotrexate, and so on
MRP5	*ABCC5*	3q27	NM_005688	1437	12	Various organs	Basal	cGMP, cAMP, folate, and methotrexate
MRP6	*ABCC6*	16p13	NM_001171	1503	17	Liver, kidney	Basal	BQ-123, LTC_4, ethylmaleimide-SG, and DNP-SG

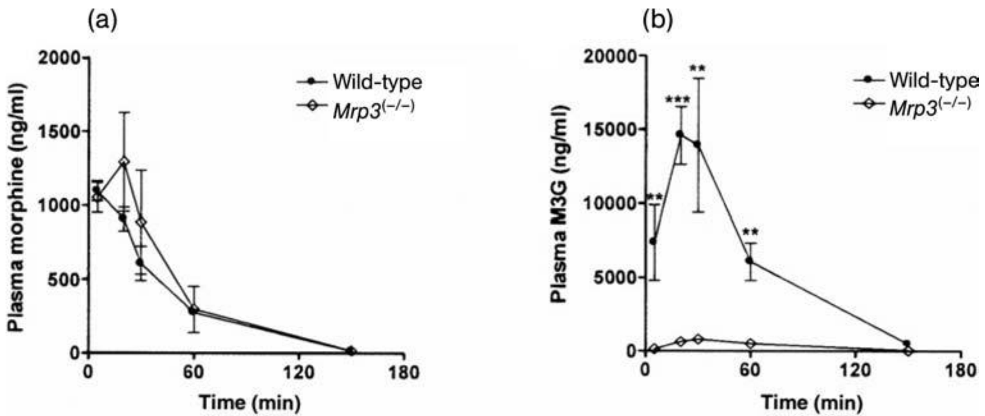

Figure 11.2 Impact of Mrp3 on the pharmacokinetics of morphine [125]. Wild-type mice (closed circle) and Mrp3 (−/−) mice (open circle) received a dose of 15 mg of morphine per kg i.p., and plasma concentrations of morphine (a) and morphine-3-glucuronide (b) were determined at the indicated time points.

the apical membrane of kidney and brain capillary endothelial cells [129–133]. The substrate specificity of MRP4 is broad and it can accept several endogenous compounds including prostaglandins, bile acids, sulfated steroids, and uric acid [5, 6]. Especially, glutathione stimulated the MRP4-mediated bile acid transport and it is cotransported with bile acids [128, 134]. It is worth noting that MRP4 also accepts relatively small compounds such as nucleoside analogues (cGMP, cAMP, adefovir, tenofovir, azidothymidine (AZT), and zidovudine) [5, 6]. Similar to MRP3, MRP4 is also upregulated under cholestatic condition [135, 136], and after the bile duct ligation, serum bile acid concentration was fourfold lower in Mrp4 (−/−) mice compared to wild-type mice, while serum bilirubin level was the same as the control mice [135], suggesting that MRP4 modulates the bile acids but not bilirubin. Also, the hepatic basolateral efflux clearance of sulfate conjugates of 4-MU, acetaminophen, and harmol was partly decreased, but that of glucuronide conjugates was not changed in perfused liver of Mrp4 (−/−) mice [126]. The importance of MRP4 in the pharmacokinetics of drugs is still unknown.

11.4.3 Other Transporters

In the basolateral membrane of liver, MRP1 (ABCC1), MRP5 (ABCC5), and MRP6 (ABCC6) are also localized [137]. The detailed information about these transporters is found in other review article [137]. The expression of MRP1 and MRP5 is very low under normal condition, but severe liver injury induces MRP1 and MRP5 [138, 139], implying that these transporters might be involved in the protection of liver. MRP6 transports LTC_4, DNP-SG, and BQ-123 [140, 141], and the hereditary deficiency of MRP6 gene causes the pseudoxanthoma elasticum, though its molecular mechanism

has not been clarified yet [142–144]. Ostα/Ostβ (organic solute transporter) heterodimer expressed in the kidney, small intestine, and liver is thought to be involved in the efflux transport of some compounds including taurocholate, E-sul, digoxin, PGE_2, and DHEAS [100]. This transporter is also upregulated under the cholestatic conditions through the transactivation of FXR by bile acids [145–147]. Previous reports suggested that OATP family transporters can transport bidirectionally, implying that OATPs can also work as an efflux transporter of anions [148, 149], but its *in vivo* role has not yet been elucidated.

11.5 Prediction of Hepatobiliary Transport of Substrates from *In Vitro* Data

11.5.1 Prediction of Hepatic Uptake Process from *In Vitro* Data

Hepatic transport properties can be investigated by several *in vitro* methods. To evaluate the hepatic uptake of compounds, isolated hepatocytes are very useful. Now, we can purchase several batches of human cryopreserved hepatocytes from several commercial sources. Shitara *et al.* [150] have indicated that we clearly observed the time-dependent saturable uptake of $E_2 17\beta G$ (OATP substrate) and taurocholate (NTCP substrate) in human cryopreserved hepatocytes, though the change in uptake clearance before and after cryopreservation exhibited a large interbatch variability among five preparations of human hepatocytes probably due to both the interindividual difference of intrinsic transport activity and the artifact caused by the different condition of isolation and cryopreservation of hepatocytes. Thus, in our usual case, before investigating the transport properties of several compounds using human cryopreserved hepatocytes, we prescreened the uptake clearance of $E_2 17\beta G$ and taurocholate in many batches and selected at least three batches of hepatocytes with large transport activity in advance [151]. Cultured hepatocytes can also be used due to the easy handling; however, we must keep in mind that several reports have indicated that long-term (>1 day) culture on collagen-coated dish results in the drastic reduction of the mRNA and protein levels of several transporters and uptake activity of organic anions such as pravastatin [152–154].

Based on the pharmacokinetic theory, the hepatic uptake intrinsic clearance can be estimated simply by scaling up the uptake clearance in hepatocytes *in vivo*. By multiplying the uptake clearance per unit cell number by cell number per gram of liver (e.g., 1.25×10^8 cells/g liver (rat)), it was possible to extrapolate the *in vitro* uptake data to the *in vivo* intrinsic uptake clearance. Miyauchi *et al.* [155] have demonstrated that the uptake clearance of 15 drugs in isolated hepatocytes correlated well with that estimated by *in situ* multiple indicator dilution (MID) method, though *in situ* clearance appeared to reach an upper limit possibly because the diffusion of compounds in unstirred water layer became the rate-determining process. Kato *et al.* [156] have also showed that the uptake clearance of four types of endothelin antagonists obtained from integration plot analysis after i.v. administration of compounds in rats is almost

comparable to that calculated from the uptake clearance in isolated rat hepatocytes assuming the well-stirred model. These evidences suggested that isolated hepatocytes are a good model for predicting the hepatic uptake clearance.

11.5.2
Prediction of the Contribution of Each Transporter to the Overall Hepatic Uptake

The transport property of each transporter can be evaluated by using several kinds of gene expression systems (e.g., mammalian cells, *Xenopus* oocytes). However, there are many transporters expressed on the same membrane and their substrate specificities often overlap one another. In this case, all of the transporters that can transport one compound are not always important for the overall hepatic uptake if their relative contribution is very minor compared to that of other major transporters. Therefore, it is essential to know the quantitative contribution of each transporter to the hepatic uptake to show the importance of each transporter in *in vivo* condition. When the function and/or expression level of one transporter caused by genetic polymorphisms, pathophysiological conditions, and transporter-mediated drug–drug interactions is changed, the information about the contribution is necessary to predict the change in the *in vivo* pharmacokinetics from *in vitro* data.

Kouzuki *et al.* [157, 158] have proposed a method using reference compounds to determine the contribution of rat Oatp1a1 and Ntcp to the hepatic uptake of bile acids and organic anions. This concept is originally established in the field of metabolic enzymes by Crespi *et al.* [159] and they named it "relative activity factor (RAF)" method. In this method, they checked the transport activity of both test compounds and the reference compounds, which should be specific substrates for single transporters, in short-term cultured rat hepatocytes and transporter-expressing COS-7 cells. Then, they estimated the contribution from the following equations:

$$\text{Contribution}\,(\%) = \frac{R_{\text{COS}}}{R_{\text{hep}}} \times 100, \tag{11.1}$$

$$R_{\text{cos}} = \frac{\text{CL}_{\text{uptake,COS(test)}}}{\text{CL}_{\text{uptake,COS(reference)}}} \tag{11.2}$$

$$R_{\text{hep}} = \frac{\text{CL}_{\text{uptake,hep(test)}}}{\text{CL}_{\text{uptake,hep(reference)}}} \tag{11.3}$$

where $\text{CL}_{\text{uptake,COS(test)}}$ and $\text{CL}_{\text{uptake,COS(reference)}}$ represent the uptake clearances of test compounds and reference compounds in transporter-transfected COS-7 cells, respectively, and $\text{CL}_{\text{uptake,hep(test)}}$ and $\text{CL}_{\text{uptake,hep(reference)}}$ represent the uptake clearances of test compounds and reference compounds in isolated rat hepatocytes, respectively. To estimate the contribution of rat Oatp1a1 and Ntcp, they used taucholate for Ntcp and $E_2 17\beta G$ for Oatp1a1 as reference compounds. As a result, rat Ntcp was responsible for the hepatic uptake of bile acids. However, some organic anions were partially taken up via Oatp1a1, but the hepatic uptake of other anions such as pravastatin and DNP-SG could not be explained by Oatp1a1-mediated

transport, suggesting that uptake transporters other than Oatp1a1 are involved in their uptake. Now, other hepatic uptake transporters such as Oatp1a4, Oatp1b2, and Oat2 have also been characterized and they can accept various kinds of anions [22, 23, 160]. Therefore, $E_2 17\beta G$ can no longer be used as a reference compound for Oatp1a1, but their concept can be applied to estimate the relative contribution.

Hirano *et al.* [151] have applied this concept to human hepatocytes to estimate the relative contribution of OATP1B1 and OATP1B3 to the hepatic uptake of $E_2 17\beta G$ and pitavastatin, a novel HMG-CoA reductase inhibitor, in cryopreserved human hepatocytes. They used E-sul for OATP1B1 and CCK-8 for OATP1B3 as reference compounds (Figure 11.3a). As with the previous method, they calculated the ratio of the uptake clearance of reference compounds in human hepatocytes to that in expression systems and defined as "R_{act}" for OATP1B1 and 1B3, and by multiplying the R_{act} value by the uptake clearance of test compounds (CL_{test}), we could estimate the uptake clearance of test compounds mediated by specific transporter in human liver. Assuming that the hepatic uptake clearance (CL_{hep}) could be explained by OATP1B1- and OATP1B3-mediated transport, the following equation should be correct:

$$CL_{hep} = R_{act,OATP1B1} \times CL_{test,OATP1B1} + R_{act,OATP1B3} \times CL_{test,OATP1B3}. \quad (11.4)$$

They have demonstrated that both pitavastatin and $E_2 17\beta G$ were taken up mainly by OATP1B1 in three independent batches of human hepatocytes and that the observed uptake clearance in human hepatocytes was almost comparable to the sum of the estimated clearance mediated by OATP1B1 and 1B3.

They also confirmed their results by two different approaches [42, 151]. One is to directly estimate the ratio of the expression level of OATP1B1, 1B3, and 2B1 in human hepatocytes to that in expression systems by comparing the band density of Western blot analysis and estimated their contributions using that ratio instead of R_{act} value shown above [42, 151] (Figure 11.3b). The other approach is to estimate the inhibitable portion of the uptake of test compounds in human hepatocytes in the presence of specific inhibitor for each transporter [42] (Figure 11.3c). We used E-sul as a specific inhibitor for OATP1B1. The uptake of pitavastatin was completely inhibited by 100 microM E-sul, indicating the major role of OATP1B1 in their hepatic uptake (Figure 11.4a), whereas that of telmisartan was not inhibited by E-sul (Figure 11.4b). Each approach has both advantages and disadvantages, and so we recommend that users compare the results obtained from different methods and validate their results. Though some anionic drugs shared the same pharmacokinetic properties in which they are efficiently accumulated in the liver, the relative contribution of each transporter depends on individual substrates. According to our estimation, valsartan and olmesartan are taken up via both OATP1B1 and OATP1B3, while fexofenadine and telmisartan are transported predominantly by OATP1B3 [31–33, 161].

Gene silencing techniques such as antisense, ribozyme, and RNA interference (RNAi) are also powerful tools to determine the transport activity of a specific protein. Hagenbuch *et al.* [162] have investigated the effect of coinjection of transporter (Ntcp or Oatp1a1)-specific antisense oligonucleotide on the uptake of BSP and taurocholate

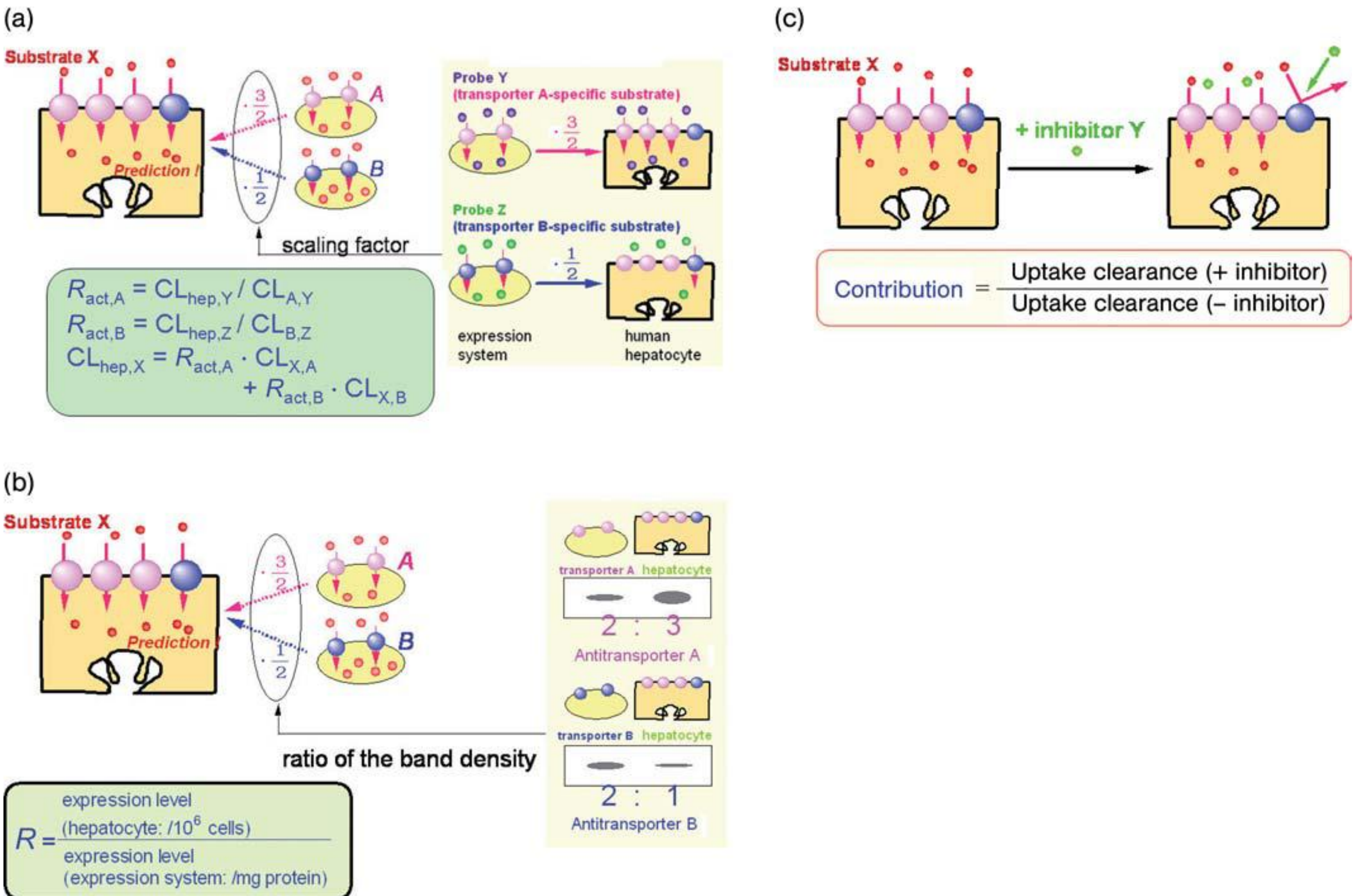

Figure 11.3 Schematic diagram of the methods for estimating the contribution of each transporter to the overall hepatic uptake [250] (a) using reference compounds; (b) using the relative expression levels estimated from Western blot analysis; and (c) using transporter-specific inhibitors. The details are described in the Section 11.5.2.

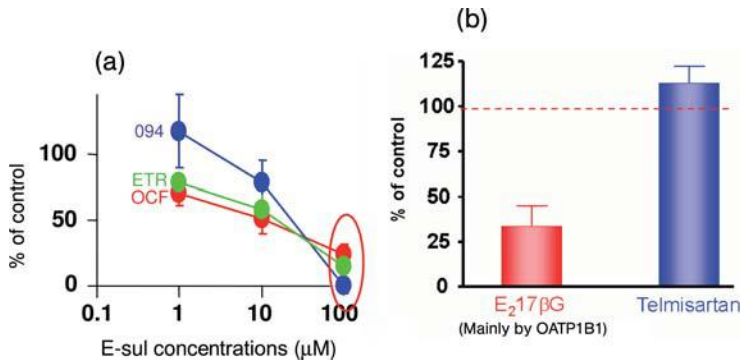

Figure 11.4 The inhibitory effect of E-sul on the hepatic uptake of pitavastatin (a) and $E_2 17\beta G$ and telmisartan (b) [32, 42]. (a) The transport of pitavastatin (0.1 microM) into human hepatocytes was determined in the presence or absence of E-sul at the designated concentrations. Three different independent batches of human hepatocytes were used in this study. The values are expressed as a percentage of the uptake of pitavastatin in the absence of E-sul. (b) Saturable uptake of telmisartan into human hepatocytes was determined after the subtraction of nonsaturable uptake (evaluated as the uptake clearance of 40 microM telmisartan) from the uptake of 0.1 microM telmisartan in the presence or absence of E-sul (30 microM). The incubation buffer contains 0.3% human serum albumin to avoid the nonspecific adsorption of telmisartan.

in the *Xenopus* oocytes injected with total rat liver mRNA. They succeeded in the significant reduction of the expression level of target transporter specifically and concluded that Na^+-dependent and independent uptakes of taurocholate were almost accounted for by Ntcp and Oatp1a1, respectively, while only half of the BSP uptake could be explained by Oatp1a1. Nakai *et al.* [163] took the same approach to show the importance of OATP1B1 to the hepatic uptake of pravastatin and $E_2 17\beta G$ in humans by adding antisense oligonucleotide to human liver poly(A) mRNA-injected oocytes. Recently, some groups have succeeded in constructing small interference RNAs (siRNAs) that can efficiently decrease the expression level of specific transporters such as MDR1 and MRP2 [164, 165]. However, it is fairly difficult to apply these gene-silencing techniques to the primary cultured hepatocytes because long-term culture dramatically decreases the expression level of several transporters [152–154], though generally it takes a few days to knockdown the protein by the depletion of mRNA expression, and the optimization of the culture condition will be required for this analysis.

11.5.3
Prediction of Hepatic Efflux Process from *In Vitro* Data

One of the popular experimental systems to investigate the hepatic efflux process is canalicular membrane vesicle (CMV). It is difficult to evaluate the transport activity of efflux transporters in cell systems because substrates cannot easily access the intracellular compartment, so CMV system is often used to rapidly determine the ATP-dependent efflux transport of substrates across bile canalicular membrane.

Aoki *et al.* [166] have compared the *in vitro* transport clearance of nine substrates in rat CMVs, defined as the initial velocity for the ATP-dependent uptake divided by the substrate concentration of the incubation medium with *in vivo* biliary excretion clearance defined as the biliary excretion rate normalized by protein unbound concentration in rat liver at steady state, and found a significant correlation between *in vitro* and *in vivo* clearance, suggesting that *in vivo* biliary excretion clearance can be predicted from the *in vitro* transport study using CMVs. Some transporter-specific inhibitors for efflux transporters may be useful to understand the contribution of each efflux transporter to the overall biliary excretion. Ko143 preferentially inhibits the BCRP-mediated transport [167], while PSC833 and LY335979 inhibit the MDR1-mediated transport more potently than the transport via other efflux transporters [168, 169]. By evaluating the effect of transporter-specific inhibitors on the ATP-dependent transport of test compounds in human CMVs, the relative contribution of each transporter to the biliary excretion might be clarified. Recently, LeCluyse *et al.* [170] have demonstrated that a collagen-sandwich culture enables the hepatocytes to form bile canalicular pocket between the adjacent cells and depletion with Ca^{2+} from the incubation medium rapidly disrupts the bile canaliculi [171]. The advantage of this culture configuration is that the polarity and the expression level of uptake and efflux transporters are well retained for several days unlike the normal culture on the rigid collagen and that biliary excretion of compounds can be evaluated in intact cell systems by differential cumulative uptake in the monolayers preincubated with Ca^{2+}-containing buffer and Ca^{2+}-free buffer [171, 172]. Liu *et al.* [173] have found that the *in vitro* biliary clearance of five compounds (inulin, salicylate, methotrexate, [D-pen^{2,5}] enkephalin, and taurocholate) in rat hepatocytes calculated by the amount excreted into bile canalicular pocket divided by the area under the incubation medium concentration–time profile was well correlated with their *in vivo* intrinsic biliary clearance, suggesting that this system is useful for the prediction of *in vivo* biliary excretion of compounds. Recently, Bi *et al.* [174] have evaluated the biliary excretion of several substrates for various efflux transporters in sandwich-cultured cryopreserved human hepatocytes. By scaling up the *in vitro* biliary excretion clearance by multiplying the clearance per cell to the cell number per gram liver, it is possible that *in vivo* clearance may be estimated in humans from *in vitro* data. Ghibellini *et al.* [175] have recently succeeded in the extrapolation of human *in vivo* biliary clearance of three compounds estimated by gamma scintigraphy and direct aspiration of duodenal secretions from *in vitro* biliary clearance in sandwich-cultured human hepatocytes.

11.5.4 Utilization of Double (Multiple) Transfected Cells for the Characterization of Hepatobiliary Transport

A brand-new approach to evaluate the uptake and efflux processes simultaneously is to use double-transfected cells that express both uptake and efflux transporters (Figure 11.5). Originally, Cui *et al.* and Sasaki *et al.* established OATP1B3/MRP2 and OATP1B1/MRP2 double transfectants, respectively [176, 177]. If a compound is a bisubstrate of uptake and efflux transfectants, basal-to-apical transcellular transport

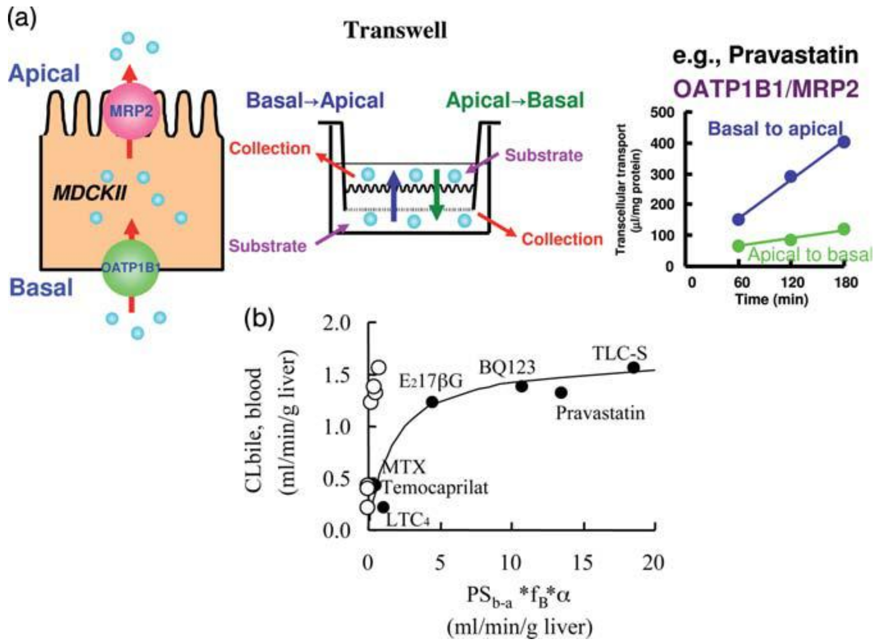

Figure 11.5 Vectorial transcellular transport of drugs in double-transfected cells. (a) The double-transfected cells expressing OATP1B1 (uptake transporter) on the basal side and MRP2 (efflux transporter) on the apical side have been established. If a compound is a bisubstrate of uptake and efflux transporters such as pravastatin, its basal-to-apical transcellular transport is significantly higher compared to the apical-to-basal transport [177]. (b) Prediction of *in vivo* biliary clearance of several bisubstrates of rat Oatp1b2 and Mrp2 from their *in vitro* transcellular transport clearance in rat Oatp1b2/Mrp2 double transfectant [178].

was significantly greater than that in the opposite direction. Therefore, this system is suitable for the high-throughput screening of bisubstrates. To extrapolate the *in vivo* biliary excretion clearance from *in vitro* experiments, Sasaki *et al.* [178] have measured the basal-to-apical transcellular transport clearances of seven bisubstrates in rat Oatp1b2/Mrp2 double transfectants and their *in vivo* biliary clearances ($CL_{bile,blood}$) calculated from the biliary excretion rate normalized by blood concentration at steady state. They proposed that the *in vivo* and *in vitro* clearance can be well described as the following equation:

$$CL_{bile,blood} = \frac{Q_H \cdot f_B \cdot \alpha \cdot PS_{b\rightarrow a}}{Q_H + f_B \cdot \alpha \cdot PS_{b\rightarrow a}}, \quad (11.5)$$

where Q_H, f_B, and $PS_{b\rightarrow a}$ represent the hepatic blood flow rate, protein unbound fraction of the compounds in blood, and transcellular transport clearance in double transfectants corrected by the fact that 1 g of liver contains 160 mg protein, respectively. And, α means the scaling factor to predict *in vivo* clearance from *in vitro* results quantitatively. When α was 17.9, all data were well fitted to the theoretical curve

described as Equation 11.5 (Figure 11.5). Now, we constructed several kinds of double-transfected cells such as human and rat NTCP/BSEP and (OATP1B1 or OATP1B3)/(MRP2, MDR1, or BCRP) double-transfected cells and the methodology for extrapolating the *in vivo* clearance from *in vitro* data will be constructed and validated [12, 64, 179, 180]. Kopplow *et al.* [181] have established the quadruple-transfected cells expressing OATP1B1, OATP1B3, OATP2B1, and MRP2 to screen the transcellular transport of organic anions in human hepatocytes. Very recently, Nies *et al.* [182] have constructed OCT1/MDR1 double transfectant and observed the transcellular transport of cationic plant alkaloid, berberine. To construct a set of double transfectants to mimic the transcellular transport in each organ is important for the understanding of the involvement of transporters in the pharmacokinetics of drugs.

11.6 Genetic Polymorphism of Transporters and Its Clinical Relevance

Genetic polymorphism of transporters is one of the important factors for determining the interindividual difference in the pharmacokinetics and subsequent pharmacological action of substrate drugs. Recently, many mutations including single nucleotide polymorphisms (SNPs) have been identified in several transporters and their impact on the drug transport has been rapidly analyzed by *in vitro* and clinical studies. Excellent reviews about genetic polymorphisms of transporters have been published and we only mention some of the examples here.

The reported mutations in *MDR1* gene observed in humans are more than 100 sites [183]. One of the most famous SNPs is C3435T (Ile1145Ile) in exon 26, which is a synonymous mutation. Its frequency in Japanese and Caucasians is 42.0 and 54.2%, respectively. Hoffmeyer *et al.* [184] have reported that the protein expression level of MDR1 in duodenum in subjects with C3435T mutation was significantly smaller and the plasma AUC after oral administration of digoxin was increased compared to subjects without C3435T mutation. This mutation is closely linked to G2677T (Ala893Ser) and C1236T (Gly412Gly), and extensive clinical studies to observe the effects of these SNPs or haplotypes on the pharmacokinetics of several kinds of MDR1 substrates have been performed. Overall, C3435T tended to increase the plasma AUC of MDR1 substrates, though contradictory results have also come out [185]. There is no evidence showing SNPs in *MDR1* affected the biliary excretion of drugs.

SLCO1B1 (gene product: OATP1B1) has more than 40 naturally occurring mutations and A388G (Asn130Asp) and T521C (Val174Ala) are the most famous and frequent SNPs among them [186]. The allele frequency in each ethnicity (A388G: Asian = 64%, African-American = 74%, and Caucasian = 40%; T521C: Asian 16%, Caucasian = 14%, and African-American = 1%) may lead to racial difference in the pharmacokinetics of OATP1B1 substrate drugs. Nishizato *et al.* [187] have first demonstrated that T521C is highly linked to A388G, and they found a haplotype named *SLCO1B1**15 in Japanese, and that plasma AUC of orally administered

Table 11.5 The effect of *SLCO1B1* polymorphisms on the clinical pharmacokinetics of drugs.

Drugs	Mutations in *SLCO1B1*	Reference
Pravastatin	*1b/*1b < *1b/*15	[187]
	*1a/*1b or *1b/*1b < *1a/*1a < *1a/*5	[268]
	−11187 G/G < G/A	[269]
	521 T/T < T/C	
	*15B noncarriers < carriers	
	*17 noncarriers < carriers	
	*1b/*1b < *1a/*1a	[190]
	*1b/*15 < *1a/*15	
	*17 noncarriers < carriers	[270]
	*1a/*1a < *1a/*15 < *15/*15	[271]
	*15 or *17 noncarriers < carriers	[272]
Pitavastatin	*1b/*1b < *1a/*1a or *1a/*1b < *1a/*15 or *1b/*15	[273]
	*1b/*1b < *1b/*15 < *15/*15	[274]
Rosuvastatin	521 T/T < T/C < C/C (whites)	[275]
	*15 noncarriers < *15 heterozygotes < *15 homozygotes	[276]
	521 T/T < C/C	[277]
Simvastatin	521 T/T or T/C < C/C (acid form)	[278]
Atorvastatin	521 T/T or T/C < C/C	[277]
Fluvastatin	521 T = C	[279]
Repaglinide	521 T/T < T/C < C/C	[192]
Nateglinide	521 T/T < T/C, T/T < C/C	[280]
Fexofenadine	521 T/T < T/C < C/C	[281]
Valsartan	*1b/*1b < *1a/*1a (trend)	[190]
	*1b/*15 < *1a/*15 (trend)	
Temocapril	*1b/*1b < *1a/*1a (trend)	[190]
	*1b/*15 < *1a/*15 (trend)	
Pioglitazone	521 T = C	[282]
Rosiglitazone	521 T = C	[282]
Atrasentan	521 T/T < T/C < C/C	[259]
Mycophenolic acid	No relationship (*1a, *1b, *15)	[283]
Irinotecan	*1a or *1b < *15 (SN-38)	[193]
	*1a/*1a < *1b/*15+*15/*15 (irinotecan)	[284]
	*1a/*1a. < *1b/*15+*15/*15 (SN-38)	
	*1a/*1a > *1b/*15+*15/*15 (SN-38glu)	
Ezetimibe	*1a < *15	[285]
Talinolol	*1b < *1a (trend)	[286]
Torsemide	521 T/T < T/C < C/C	[287]

pravastatin is significantly higher in subjects with *15 alleles compared to *1b alleles (A388G). After that, several clinical studies supported this finding. The results of clinical studies investigating the relationship between SNPs in *SLCO1B1* and pharmacokinetics of drugs are summarized in Table 11.5. In most cases, T521C mutation is thought to decrease the transport function of OATP1B1, which results in the reduction of hepatic clearance [186]. These outcomes are supported by *in vitro* studies demonstrating that cells expressing *15 mutant showed the decrease in the

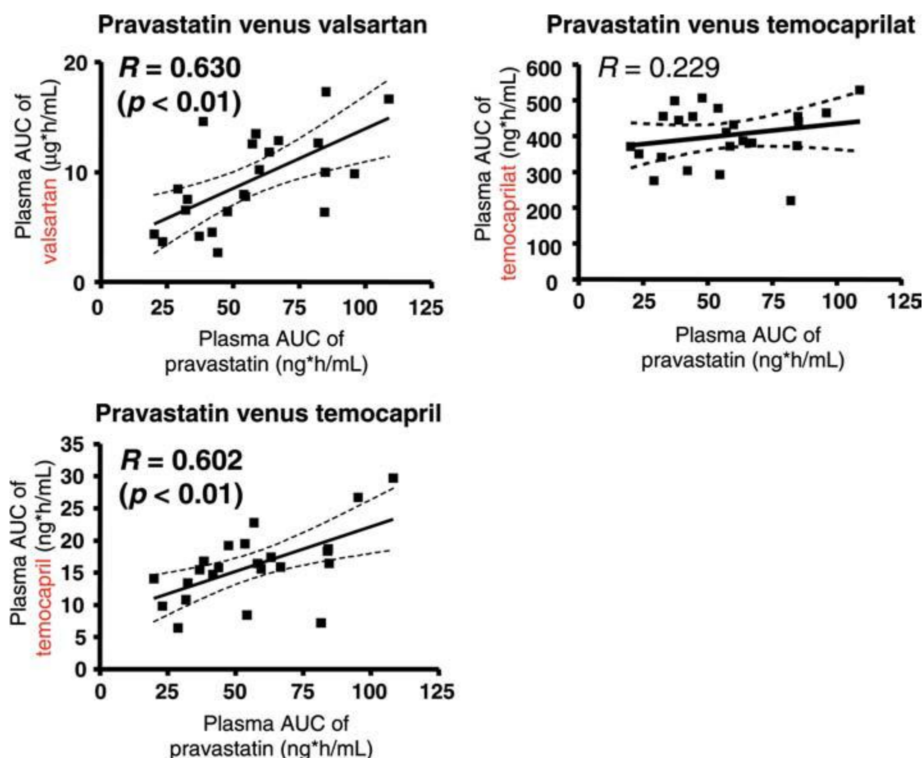

Figure 11.6 The correlation between the plasma AUC of pravastatin in each subject and that of valsartan, temocapril, and temocaprilat [190]. Each point represents AUC values of three drugs for each subject after oral administration of pravastatin (10 mg), valsartan (40 mg), or temocapril (2 mg). Solid lines represent fitted lines calculated by linear regression analysis and dotted lines represent 95% confidence intervals of correlations.

transport activity compared to wild-type OATP1B1 [16, 188, 189]. On the other hand, the subjects with *SLCO1B1**1b alleles showed the lower plasma AUC of pravastatin, valsartan, and temocapril than those with *SLCO1B1**1a alleles, and the plasma AUC of pravastatin in each subject was correlated well with that of valsartan and temocapril (Figure 11.6), which suggested that the clearance mechanism of pravastatin may be shared with valsartan and temocapril and *SLCO1B1**1b may increase the transport function in human liver, though *in vitro* analyses do not simply explain this clinical outcome [190]. Recently, some reports have mentioned that T521C mutation may relate to the pharmacological and toxicological actions of substrate drugs such as decrease in cholesterol-lowering effect of pravastain [191], increase in the reduction of blood glucose level by repaglinide [192], and increased frequency of neutropenia expression induced by irinotecan [193] (Figure 11.7). Other major hepatic OATP transporter, *SLCO1B3* (gene product: OATP1B3) has two frequent SNPs, T334G (Ser112Ala) and G699A (Met233Ile), but currently we have no reports showing these SNPs affected the transport function of substrates [194, 195].

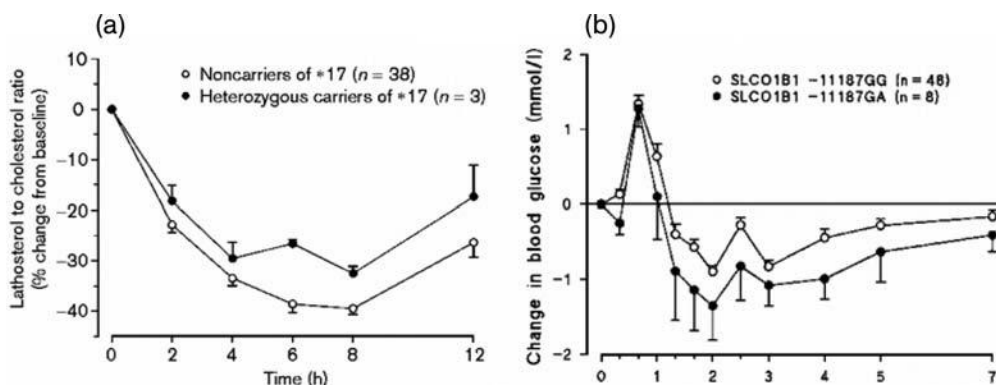

Figure 11.7 Impact of *SLCO1B1* mutations on the pharmacological effects of pravastatin and repaglinide. (a) Plasma lathosterol/cholesterol ratio after single oral administration of 40 mg pravastatin in healthy subjects with or without *SLCO1B1**17 (G-11187A + Asn130Asp + Val174Ala) allele [191]. (b) Change in blood glucose level after single oral administration of 0.25 mg repaglinide in healthy subjects with or without G-11187A mutation in *SLCO1B1* [192].

BCRP has also one frequent SNP, C421A (Gln141Lys). This mutation is more frequently observed in Asians (∼35%) than Caucasians (10%). Clinical studies indicated that C421A mutation decreased the total clearance of diflomotecan, rosuvastatin, and sulfasalazine [196–198]. BCRP is expressed in the small intestine as well as the liver and it is difficult to identify whether these clinical outcomes come from a decrease in the transport function in the small intestine or in the liver. However, the pharmacokinetics of diflomotecan was changed after both i.v. and p.o. administration, which indicates that this may be caused partly by the decrease of its biliary excretion mediated by BCRP [198]. *In vitro* analyses revealed that the transport function normalized by the protein expression level of BCRP C421A mutant is apparently normal [199], but another report showed that C421A mutation significantly decreased the protein expression level of BCRP in human placenta [200]. Thus, the decreased function by C421A mutation is thought to be caused by its decreased expression.

In the case of MRP2, though some SNPs are found in coding region, recent studies have indicated the impact of SNPs in the upstream regions on the toxicological aspects of drugs. For example, two kinds of haplotypes (-1774delG, G-1549A and C-24T) of *MRP2* increased the frequency of drug-induced hepatitis [201]. Other report has demonstrated that C-24T mutation increased the frequency of diclofenac-induced hepatotoxicity [202]. These may be caused by the decrease in hepatic clearance of toxicants, which results in the high exposure of toxicants in liver. A-1019G mutation in *MRP2* has also reported to decrease the frequency of severe diarrhea related to irinotecan [203], which suggested that A-1019G may decrease the biliary excretion of SN-38, an active metabolite of irinotecan, and decreased the exposure of SN-38 in the intestine. Very recently, mutations in *OCT1* have been reported to result in the increase in plasma concentration of metformin and subsequent attenuation of its glucose-lowering effect [204, 205].

11.7 Transporter-Mediated Drug–Drug Interactions

11.7.1 Effect of Drugs on the Activity of Uptake Transporters Located on the Sinusoidal Membrane

Recently, several reports have suggested that the inhibition of hepatic uptake transporters by coadministered drugs results in the decrease in the hepatic clearance and increase in the plasma concentration of victim drugs. For example, antituberculosis agents (rifamycin SV and rifampicin) reduced the clearance of BSP and also induced hyperbilirubinemia due to the inhibition of OATP1B1-mediated bilirubin uptake [206]. These results may be accounted for by considering the fact that the Ki values of rifamycin SV are 2 and 3 microM for OATP1B1 and OATP1B3, respectively, whereas those of rifampicin are 17 and 5 microM for these transporters, and that the peak unbound plasma concentrations of rifamycin SV and rifampicin may be 5–10 microM [206]. The fact that rifamycin SV induces a more potent increase in plasma unconjugated bilirubin concentrations than rifampicin may also be accounted for by these kinetic considerations. The interaction between cerivastatin and gemfibrozil has also been highlighted because of the appearance of severe side effects including rhabdomyolysis and death (Figure 11.8) [207]. This led to the withdrawal of cerivastatin from the market. On the other hand, after coadministration of cyclosporin A, the plasma concentration of cerivastatin was increased 3.8-fold (Figure 11.8) [208]. *In vitro* experiments revealed that cyclosporin A potently inhibited the uptake of cerivastatin in human hepatocytes and OATP1B1-expressing MDCKII cells with Ki values of 0.280–0.685 microM and 0.238 microM, respectively [209]. In contrast, cerivastatin metabolism mainly mediated by CYP2C8 and 3A4 was not affected by cyclosporin A at concentrations up to 30 microM [209]. These results suggest that the inhibition of cerivastatin uptake into hepatocytes results in an increased blood concentration of cerivastatin, which may lead to the severe side effects (Figure 11.8) [209]. According to the recent clinical studies, coadministration of cyclosporin A also increased the plasma concentration of several OATP1B1 substrate drugs such as other non-metabolized statins, pravastatin (5–7.9-fold) [210], rosuvastatin (7.1-fold) [211] and pitavastatin (4.5-fold) [212], repaglinide (4-fold) [213], and bosentan (30-fold) [214], which indicates that we pay attention to the drug interaction between cyclosporin A and OATP1B1 substrates in clinical situations. However, gemfibrozil and its glucuronide also have the potency to inhibit OATP1B1-mediated uptake with a Ki value of 4–72 and 24 microM, respectively [215]. Though Shitara *et al.* [215] have concluded that the main mechanism of this drug interaction is the inhibition of CYP2C8-mediated cerivastatin metabolism by gemfibrozil glucuronide (Figure 11.8), gemfibrozil is thought to slightly inhibit the OATP1B1-mediated uptake because it increased the plasma AUC of non-metabolized statins such as pravastatin (2.02-fold) [216], pitavastatin (1.45-fold) [217], and rosuvastatin (1.88-fold) [218], which is lower than that of cerivastatin (4.36-fold) [207]. Several compounds are recognized as bisubstrates of both metabolic enzymes and transporters

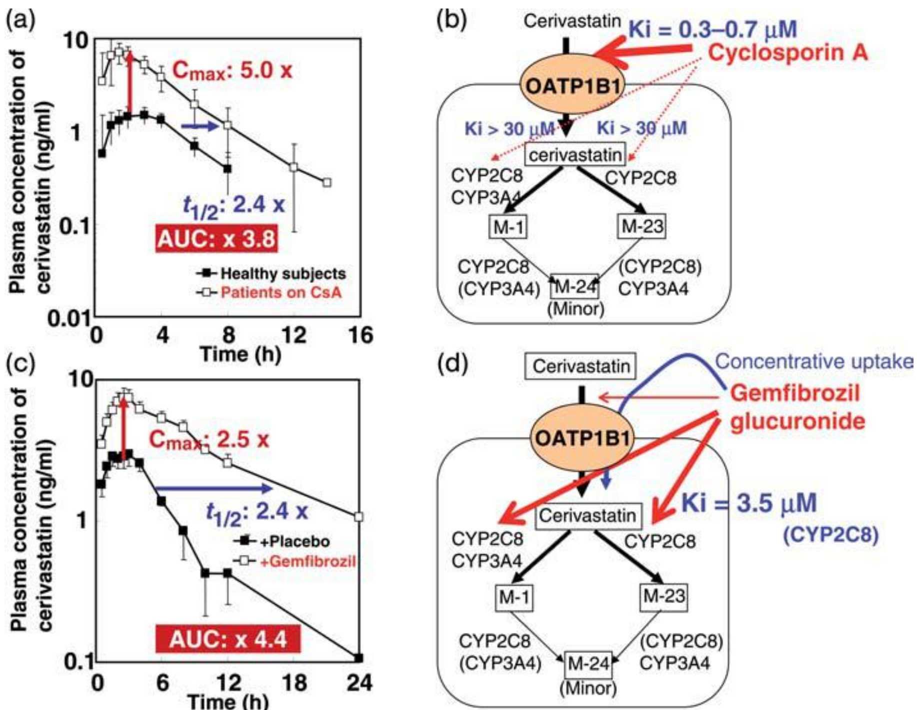

Figure 11.8 Drug–drug interaction between cerivastatin and cyclosporin A and between cerivastatin and gemfibrozil. (a, b) Cyclosporin A increased the plasma concentration of cerivastatin, which is mainly caused by the inhibition of OATP1B1-mediated uptake of cerivastatin by cyclosporin A [208, 209]. (c, d) Gemfibrozil increased the plasma concentration of cerivastatin, mainly caused by the inhibition of CYP2C8-mediated metabolism of cerivastatin by gemfibrozil glucuronide, which may be concentrated in hepatocytes, and partly caused by its inhibition of OATP1B1-mediated uptake [207, 215].

such as atorvastatin, bosentan, and repaglinide [188, 192, 219, 220]. In that case, we should consider the rate-limiting step of overall clearance to predict the drug–drug interaction. Figure 11.9 shows the effects of the coadministration of itraconazole (CYP3A4 inhibitor) and cyclosporin A (CYP3A4 and OATP1B1 inhibitor) on the pharmacokinetics of three different types of statins [210, 221–224]. Pravastatin is a substrate of OATP1B1 [25, 163, 188], but not metabolized, whereas simvastatin uptake is thought to occur without any aid of transporters due to the high lipophilicity of simvastatin lactone. However, atorvastatin is taken up into the liver by OATP1B1 [188, 220] and subsequently metabolized by CYP3A4. Cyclosporin A inhibited the hepatic clearance of these statins, but the inhibition of CYP3A4 by itraconazole greatly affected the plasma AUC of simvastatin, and modestly changed that of pravastatin and atorvastatin, though both simvastatin and atorvastatin are substrates of CYP3A4. This apparent discrepancy can be explained by the rate-limiting step of overall clearance of two statins. For hydrophilic atorvastatin, OATP1B1 is

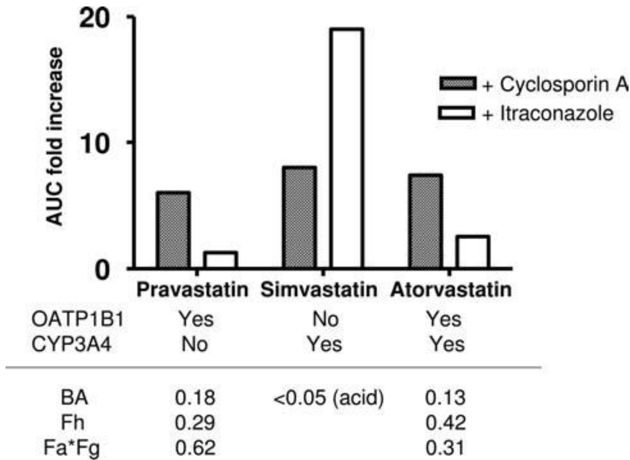

Figure 11.9 Different effects of itraconazole and cyclosporin A on the plasma AUC of pravastatin, simvastatin, and atorvastatin [210, 221–224]. The fold-increase in the plasma AUC of statins was expressed after coadministration of cyclosporin A or itraconazole. The data were obtained from the previous literature. The details are described in the Section 11.7.1. BA: bioavailability, $F_a{}^*F_g$: fraction of dose absorbed from the small intestine to the portal vein, F_h: hepatic availability.

involved in its hepatic uptake, and overall intrinsic clearance is solely determined by the uptake clearance. Thus, the decrease in the hepatic clearance of atorvastatin was almost the same as that of pravastatin. However, hydrophobic simvastatin can permeate the membrane passively, so the overall intrinsic clearance approximates the metabolic intrinsic clearance, thus the inhibition of CYP3A4 greatly decreased the hepatic clearance of simvastatin. Recently, Ki values of several drugs for OATP1B1 and OATP1B3 have been published [42, 92]. Comparing the Ki values with the maximum unbound plasma concentration at the inlet to the liver in humans, OATP1B1 may be inhibited by several drugs such as cyclosporin A, indinavir, ritonavir, rifamycin SV, rifampicin, and clarithromycin [42], whereas OATP1B3 may also be inhibited by cyclosporin A and rifampicin [92]. Bilirubin is also a substrate of OATP1B1 [35, 36], so the inhibition of OATP1B1 causes the hyperbilirubinemia. Campbell *et al.* [225] have shown that the potency of several compounds that inhibited OATP1B1-mediated transport was correlated well with the incidence of drug-induced hyperbilirubinemia. In another example, clearance of theophylline was decreased after coadministration of erythromycin, which is thought to be partly caused by inhibition of OAT2-mediated uptake of theophylline by erythromycin [226, 227].

The drug-induced change in the expression levels of transporters also modifies the pharmacokinetics of substrate drugs. Though detailed mechanisms have not been explained yet, after repetitive dosing of rifampicin, efavirenz, and ritonavir + saquinavir, the plasma concentration of pravastatin was decreased [228–230]. Because all compounds are PXR ligands, OATP1B1 may be induced by PXR-mediated mechanism and uptake clearance of substrate drugs may be increased.

11.7.2
Effect of Drugs on the Activity of Efflux Transporters Located on the Bile Canalicular Membrane

A variety of inhibitory effects of drugs on the function of efflux transporters in the bile canalicular membrane have also been reported. Though much of the severe drug-induced cholestasis results from immune reactions, part may also be caused by the inhibition of BSEP [11, 231]. Cyclosporin A, rifamycin SV rifampicin, and glibenclamide were reported to *cis*-inhibit the BSEP function in BSEP-expressing membrane vesicles [105]. Bosentan (endothelin antagonist) also often causes cholestasis in humans. The bosentan-induced cholestasis was reported to be reproduced in rats, and bosentan and its metabolite (Ro-47-8634) could inhibit the BSEP-mediated taurocholate transport with Ki values of 12 and 8.5 microM, respectively [105]. Though the predicted plasma concentration of bosentan does not reach its Ki value, its inhibition of the BSEP-mediated efflux of bile acids may contribute to the expression of cholestasis. Troglitazone was withdrawn from the market due to the expression of lethal hepatotoxicity. Though detailed mechanisms have not been clarified, troglitazone and its sulfate potently inhibited the BSEP function [232]. Male rats were shown to have higher troglitazone sulfate levels than those in female rats (due to a higher sulfotransferase activity in male rats), thus more profound cholestasis seen in males after troglitazone administration might be caused by an inhibition of BSEP by troglitazone sulfate [233].

Several experimental systems to check the inhibition potency of bile acid transport have been characterized. Using sandwich-cultured human hepatocytes, bosentan, cyclosporin A, CI-1034 (endothelin-A receptor antagonist), glyburide, erythromycin estolate, and troleandomycin could inhibit the taurocholate efflux to the bile pocket [234]. Moreover, Mita *et al.* [235] constructed NTCP/BSEP double-transfected cells and some cholestasis-induced compounds inhibited both the NTCP-mediated uptake and the BSEP-mediated efflux of taurocholate. Then, they have found fluorescent bile acids whose transcellular transport was clearly observed, which may be used for the rapid identification of inhibitors of NTCP and BSEP in drug screening process [235].

Previous reports have suggested that BSEP may be transinhibited by Mrp2 substrates such as $E_2 17\beta G$ by comparing the inhibitory effect of Mrp2 substrates on BSEP function in isolated bile canalicular membrane vesicles in normal and Mrp2-deficient EHBRs and in membrane vesicles expressing only BSEP and those expressing both BSEP and MRP2 [236, 237]. The finding that MRP2 substrates with a cholestatic nature do not cause cholestasis in EHBRs might be consistent with this hypothesis [238].

Regarding the drug–drug interaction, SN-38 is formed in hepatocytes following the hydrolysis of irinotecan and then excreted into the bile. The severe diarrhea is one of the dose-limiting toxicity for irinotecan. One of the reasons for that toxicity is the high exposure of SN-38 in the intestine. Because SN-38 is excreted into bile mainly via MRP2 [94, 239, 240], Horikawa *et al.* [241] have proposed that inhibition of the biliary excretion of SN-38 by pronebecid, an inhibitor for MRP2, causes a reduction in the incidence of diarrhea but does not affect plasma concentration of SN-38. Thus, it is possible to control adverse reactions of irinotecan by utilizing the drug–drug interaction mediated by efflux transporter, MRP2.

Drug interaction between digoxin and quinidine has been reported previously [242]. Quinidine reduced both renal and biliary excretion of digoxin. The Ki value of quinidine for MDR1-mediated efflux was assumed to be 5 microM [243]. However, the maximum unbound plasma concentration of quinidine at the inlet to the liver is estimated to be 4 microM, which suggests that quinidine may inhibit the MDR1-mediated efflux of digoxin due to the similar concentration to Ki value and possible accumulation of quinidine into hepatocytes [244].

11.7.3
Prediction of Drug–Drug Interaction from *In Vitro* Data

If the unbound drug concentrations in plasma (In) are higher than the Ki values for transporters, transporter function may be significantly decreased [244]. To predict the potency of drug–drug interaction quantitatively, the estimation of the inhibitor concentration at the interacting site (e.g., vicinity of transporters) should be needed. To avoid the false-negative prediction, Ito *et al.* [245] have proposed the equation for the calculation of the maximum unbound concentration of inhibitor at the inlet to the liver. For the accurate prediction, physiologically based pharmacokinetic models for both substrates and inhibitors should be constructed, and the plasma concentration–time profiles of substrates and inhibitors can be simulated [75]. The possible target sites of drug interaction in the liver are uptake and efflux processes. However, it is fairly difficult to discriminate which processes are responsible for its interaction.

Ueda *et al.* [246] have established the quantitative prediction method for alteration in pharmacokinetics of drugs caused by the inhibition of uptake as well as efflux transport. They tried to predict the drug–drug interaction between methotrexate and probenecid (Figure 11.10). In this strategy, the inhibitory effect of probenecid for the hepatic uptake of methotrexate was evaluated by using the isolated rat hepatocytes and that for its biliary excretion was examined by bile CMVs. The degree of inhibition of the uptake and efflux processes *in vivo* was comparable to that predicted from *in vitro* experiments. Then, R values $(= 1 + \frac{\mathrm{In}}{\mathrm{Ki}})$ for uptake (R_{uptake}) and efflux process ($R_{\mathrm{excretion}}$) could be calculated. The net degree of inhibition (R_{net}) can be described by the following equation:

$$R_{\mathrm{net}} \leq R_{\mathrm{uptake}} \times R_{\mathrm{excretion}}. \tag{11.6}$$

They showed that the degree of the reduction in the hepatic clearance was overestimated by a simple calculation of the product of the reduction in the hepatic uptake and biliary excretion (Equation 11.6) and this method is useful to avoid the false-negative predictions [246].

11.8
Concluding Remarks

The information summarized in this chapter describes how transporters play an important role in the hepatobiliary excretion of drugs, which is also one of the

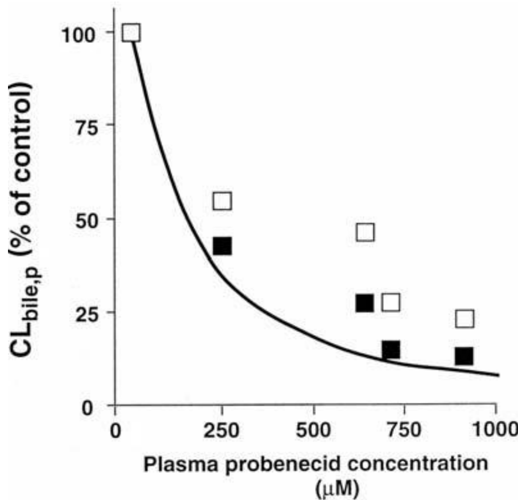

Figure 11.10 Extrapolation of the drug interaction involving both hepatic uptake and biliary excretion processes from *in vitro* data [246]. *Y*-axis represents the biliary excretion clearance of methotrexate in the presence of several concentrations of probenecid with respect to circulating plasma. Open square shows the $CL_{bile,p}$ values of methotrexate observed *in vivo*. Closed square and solid line represent the predicted $CL_{bile,p}$ values derived from intrinsic biliary excretion clearance estimated by using the unbound concentration of the inhibitor in the liver and in the plasma, respectively.

determinants for the bioavailability of drugs. The several *in vitro* systems may be useful for identifying the substrates and inhibitors for each transporter with desired transport properties during the screening of drug candidates. By utilizing these *in vitro* data, we can construct the physiologically-based pharmacokinetic (PBPK) model to predict the time-dependent whole-body pharmacokinetics *in vivo* by integrating the kinetic parameters for each transporter and metabolic enzyme and physiological parameters such as blood flow rate and protein unbound fraction of drugs in plasma into the model. Because especially in humans, it is impossible to measure the concentration in each tissue and the site of actions, it is useful to predict the pharmacological and toxicological effects of drugs by PBPK modeling [75]. Also, with the aid of positron emission tomography (PET) or single-photon emission computed tomography (SPECT), we can obtain the detailed data on tissue distribution directly in humans. Some reports have succeeded in quantifying the drug concentration in human brain [247, 248]. In the field of metabolic enzymes, a set of "probe drug," which is a specific substrate of each enzyme, has been established and metabolic activities of specific P450s can be directly estimated in human *in vivo* condition by measuring the metabolites in the blood and urine after administration of several kinds of probe drugs ("cocktail approach") [249]. To establish a set of clinically applicable probe drug and evaluation method for each transporter is important for the phenotyping of transporter function in humans. Utilizing these tools, more accurate prediction of the transport function and pharmacokinetics of drugs will be realized in future.

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12
The Importance of Gut Wall Metabolism in Determining Drug Bioavailability

Christopher Kohl

Abbreviations

ACAT	Advanced compartmental absorption and transit
AUC	Area under the curve
CES	Carboxylesterase
CYP	Cytochrome P450
FDA	Food and Drug Administration
NAT	*N*-Acetyltransferase
NCE	New chemical entity
PAMPA	Parallel artificial membrane permeability assay
PBPK	Physiologically-based pharmacokinetics
QSAR	Quantitative structure–activity relationship
SULT	Sulfotransferase
TMF	Transport, metabolism, and blood flow
UGT	UDP glucuronyltransferase

Symbols

CL	Clearance
CL/F	Oral clearance
CL_h	Hepatic clearance
CL_{int}	Intrinsic clearance
$CL_{int,g}$	Intestinal intrinsic clearance
CL_{perm}	Permeability clearance
D	Dose
f_{abs}	Fraction absorbed
F	Oral bioavailability
F_g	Fraction escaping gut wall extraction
F_h	Fraction escaping liver extraction

F_l	Fraction escaping lung extraction
$f_{u,b}$	Fraction unbound in blood
h	Hour
k_a, k_{01}	Absorption rate constant
ima	Drug administration into the superior mesenteric artery
ip	Drug administration into the portal vein
IV	Drug administration via a peripheral vein
min	Minutes
ml	Milliliters
po	Drug administration via the oral route
Q_g	Gut mucosal blood flow
Q_h	Liver blood flow
Q_{villi}	Blood flow supplying the villi of the intestinal mucosa

12.1 Introduction

Over the past 15 years, the intestinal tract and its drug-metabolizing capacity have attracted increasing attention from the biomedical community. A number of clinical reports have surmised claims of a substantial contribution of gut wall first-pass metabolism to the limited oral bioavailability of well-established drugs such as cyclosporine, midazolam, nifedipine, and verapamil [1]. These clinical observations have been supplemented by elegant *in vitro* studies using molecular biology and biochemical approaches to identify the abundance and substrate selectivity of drug-metabolizing enzymes located in the mucosa of the gastrointestinal tract. Furthermore, physiologically based mathematical models have been refined in an attempt to predict the gut wall first-pass effect of drugs and NCE from *in vitro* data [2–5]. In this chapter, we will explain the physiological context that applies to such models, describe the enzymes thought to be most important for gut wall metabolism, and review the utility and appropriateness of the approaches most commonly used. We will also critically appraise the evidence hitherto available that highlights the relative relevance of gut wall metabolism in comparison to hepatic metabolism (Figure 12.1).

12.2 Physiology of the Intestinal Mucosa

To assess the impact of drug metabolism in the gut wall on oral bioavailability and develop adequate models for gut wall metabolism, it is necessary to understand the physiology of the intestinal mucosa.

The greatest activity of drug-metabolizing enzymes in the gut wall is located in the epithelium of the mucosa, its superficial lining that faces the lumen of the bowel. The mucosal epithelium consists of a single layer of enterocytes lining both the crypts and the villi. The apical membrane of the enterocytes extends in numerous projections (microvilli) forming the brush border membrane and increasing its surface area

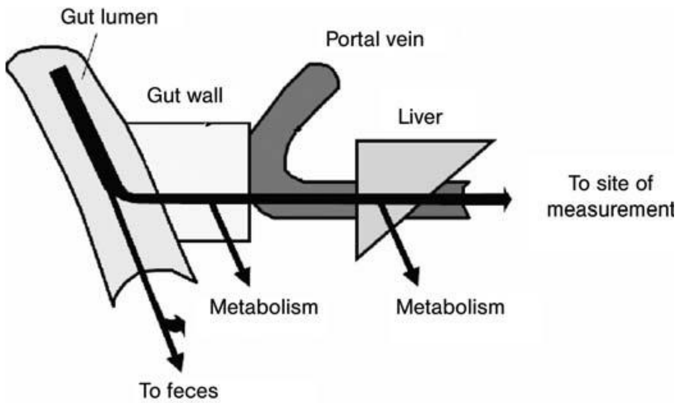

Figure 12.1 Schematic representation of gut wall and liver first-pass effects.

20-fold. These enterocytes have a programmed life span and are continuously generated in the depth of the crypts. During their maturation, they migrate to the tip of the villi, and it is here the highest activity of drug-metabolizing enzymes is found. The time the crypt cells take to mature and ascend to the tip of the villi is about 2–6 days, and one to two enterocytes are renewed per 100 cells and hour. The blood supply of the villus is provided by arterioles that pass to the tip of the villus (see Figure 12.2). Here, they spread into many small capillaries, which then drain into a villus venule. In the villus, afferent arterioles and efferent venules are in very close vicinity to each other (within 20 μm) so that passive diffusion of oxygen, nutrients, and drugs can occur while effectively bypassing the capillary network at the villus tip where the majority of drug-metabolizing enzymatic activity is located. This phenomenon is called countercurrent exchange and could have implications for the impact of metabolism in the gut wall after IV dosing [6].

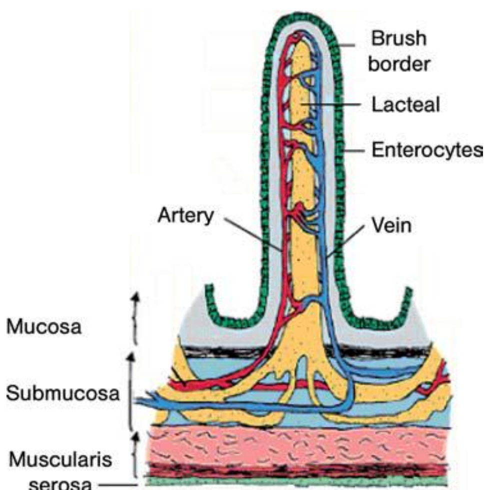

Figure 12.2 Cross section of a villus in the human mucosa of the small intestine.

The small intestine and the proximal half of the colon are supplied with blood by the superior mesenteric artery. The blood flow through the superior mesenteric artery is about 12% of cardiac output (500–700 ml/min in humans). Within the walls of the small intestine, the mesenteric circulation is organized in in-series and in-parallel relationships. The circulation of the mucosa is in series with the submucosa from which the mucosal vessels originate, but it is in parallel with the muscularis layer of the gut wall. Hence, it is important to consider the mucosal blood flow only and not the total intestinal blood flow when estimating intestinal clearance or intestinal extraction (see below). The structure of the mesenteric circulation makes an internal redistribution of intestinal blood flow possible without necessitating changes in the overall blood supply of the organ. The blood flow to each layer of the intestinal wall is constantly adapted to the metabolic demands and activity status prevailing. During absorption, blood flow increases by 30–130%, but the hyperemia is limited to the exposed segment of the intestine. During exercise, sympathetic vasoconstriction can shut off the intestinal blood supply for limited time spans in favor of a redistribution of blood to skeletal muscle and heart. Conversely, after meal intake, the blood flow to the small intestine is increased, and, as the portal vein is in series with the superior mesenteric artery, hepatic blood flow is also increased. As we will see later, increased blood flow may well influence the extent of oral bioavailability. The blood flow supplying the intestinal mucosa can be estimated at 248 ml/min [7].

12.3 Drug-Metabolizing Enzymes in the Human Mucosa

As outlined above, the main drug-metabolizing activity in the gut wall with respect to phase I and phase II enzymes is located in the enterocytes at the tip of the villi. Enzymatic activities of colonocytes, the cells lining the colon, are generally lower; however, the expression of apical efflux transport proteins such as P-glycoprotein in colonocytes is significant and exceeds than that in enterocytes (see Chapter 10).

12.3.1 Cytochrome P450

The predominant CYP in human enterocytes has been found to be CYP3A4 taking up about 80% of all intestinal CYP. The CYP3A4 expression appears to slightly decrease along the length of the small intestine, reaching very low levels in the colonocytes of the large intestine (only about 1/40 of those in the small intestine). The abundance of CYP2C9 equating to about 15% of all intestinal CYP follows a similar pattern. Prototypical substrates of CYP2D6 and CYP2C19, such as metoprolol [8] and omeprazol [9], are also metabolized in the intestinal mucosa. Considerable activity of CYP3A5 has been detected in the gut wall of some individuals within the human population [10] with CYP3A5 expression being higher in the colon than that of CYP3A4 [1]. CYP3A5 appears to metabolize the same substrates as CYP3A4 [11], but it is less prone to inhibition [12, 13] and it may express regioselectivity differently from CYP3A4 [14]. For instance, CYP3A5

metabolizes alprazolam to similar amounts at the 4- and 1′-positions, whereas CYP3A4 favors the 4-position by about 10-fold [14]. The large between-subject variability in the expression levels of CYP3A5 is thought to contribute to the largely varying oral clearance (CL/*F*) values across the patient population seen with many CYP3A4 substrates [15]. This is seen in the case of the immunosuppressant tacrolimus where oral clearance is faster depending on the number of CYP3A51 alleles carried [11].

The percentage contributions to the overall intestinal CYP protein are as follows: CYP3A4 80%, CYP2C9 15%, CYP2C19 2.9%, CYP2J2 1.4%, and CYP2D6 1% [10]. Table 12.1 gives a survey of CYPs identified in the human intestinal tract and examples of some drugs metabolized by them. The oral bioavailability is quoted in this table to help make assumptions regarding the propensity of these drugs toward first-pass metabolism in general and gut wall first-pass metabolism in particular.

12.3.2
Glucuronyltransferase

The major isozymes of the UGT family expressed in the gut wall are UGT1A1, UGT1A3, UGT1A4, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 [58]. Of these, UGT1A8 and UGT1A10 are selectively expressed with the intestinal wall. Table 12.2 gives an exemplary survey of drugs predominantly cleared via glucuronidation (contribution to total $CL > 50\%$) and the UGT isozymes involved.

UGT expression data assessing the abundance along the intestine appear to be less robust compared to CYP data, but several UGT isozymes are expressed along the whole length of the gastrointestinal tract (Figure 12.3, [80]). There is no general trend in terms of the regional activity. Some of the intestinal UGT activities are in the range of those in the liver, for example, for ezetimibe [68] and mycophenolic acid [81], when CL_{int} values are compared on the basis of milligram microsomal protein. If these values are scaled up to the whole organs, however, gastrointestinal glucuronidation rarely exceeds 5% of that of the liver [58].

12.3.3
Sulfotransferase

Studies investigating the expression and activities of SULT in human gut are scarce. One recent study found that the sulfotransferases SULT1A1, 1A3, 1E1, and 2A1 are abundant in the cytosol of the gut wall mucosa [82]. SULT1A3 appears to be not expressed in the liver. In the ileum, higher expression levels (on the basis of ng SULT/mg of cytosolic protein) compared to the liver were found for SULT1A1, SULT1A3, and SULT1B1 [82]. Table 12.3 gives drug examples metabolized by gut wall SULT.

12.3.4
Other Enzymes

About a decade ago, microsomal esterases were classified into four families (CES1–4) based on their sequence homology [95]. Of these, only CES2 is expressed in the

Table 12.1 Drug examples found to be metabolized in the gut wall by CYP.

Drug	CYP isozyme	Metabolic routes	Oral *F* (%)
Alfentanil	3A4	N-Dealkylation (noralfentanil, *N*-phenylpropionamide) [16]	42 ± 15 [17]
Alprazolam	3A4	Hydroxylation (1′-hydroxy, 4-hydroxy) [18]	80–100 [19]
Bufuralol	2D6	Hydroxylation (1′-hydroxy) [18, 20]	46 [20]
Buspirone	3A4	N-Dealkylation (1-pyrimidylpiperazine), hydroxylation (6-hydroxy) [21, 22]	3.9 [23]
Cyclosporin	3A4	AM1-, AM9-, AM4-N-oxidation [24]	22 [25]
Cisapride	3A4	N-Dealkylation (norcisapride), hydroxylation (2-hydroxy, 4-hydroxy) [26]	42 ± 11 [27]
Diclofenac	2C9	Hydroxylation (4′-hydroxy) [28]	54 ± 2 [29]
Erythromycin	3A4	N-Dealkylation [30]	35 ± 2 [29]
Ethinylestradiol	3A4	Hydroxylation (2-hydroxy) [31]	51 ± 9 [29]
Felodipine	3A4	Ring oxidation [32]	15 ± 8 [29]
Flunitrazepam	3A4, 2C19	N-Dealkylation (Norflunitrazepam), hydroxylation (3-hydroxy) [18, 33]	≥80 [34]
Flurazepam	3A4	N-Dealkylation [35]	30–60 [36]
Indinavir	3A4	N-Dealkylation, hydroxylation (*trans*-indan-OH) [37]	60–65 [38]
Lovastatin	3A4	Hydroxylation (6′-exo-methylene, 6′-hydroxy, 3″-hydroxy) [39]	<5 [29]
Metoprolol	2D6	O-Demethylation, hydroxylation [40, 41]	38 ± 14 [29]
Midazolam	3A4	Hydroxylation (1′-hydroxy, 4-hydroxy) [42]	44 ± 17 [29]
Nicardipine	3A4	Ring oxidation [32]	18 ± 11 [29]
Nifedipine	3A4	Ring oxidation [43]	50 ± 13 [29]
Omeprazole	3A4, 2C19	Hydroxylation (CYP2C19), S-oxidation (CYP3A4) [44]	53 ± 29 [29]
Propafenone	3A4, 2D6	N-Dealkylation (CYP3A4), hydroxylation (CYP2D6) [45]	5–50 [29]
Quinidine	3A4	Hydroxylation (3*S*-hydroxy), N-oxidation [46]	71–81 [47]
Rifabutin	3A4	O-Dealkylation (27-*O*-demethyl), hydroxylation (20-hydroxy, 31-hydroxy, 32-hydroxy) [48]	20 [49]
Saquinavir	3A4	Hydroxylations [50]	4 [29]
Tacrolimus	3A4	O-Demethylation [51]	16 ± 7 [29]
Terfenadine	3A4	Hydroxylation, N-dealkylation [52]	<5 [29]
Testosterone	3A4	Hydroxylation (6β-hydroxy) [53]	2–4 [54]
Tolbutamide	2C9	Hydroxylation (4-methyl-hydroxy) [55]	93 ± 10 [36]
Triazolam	3A4	Hydroxylation (1′-hydroxy, 4-hydroxy) [42]	44 [29]
Verapamil	3A4, 2C8	N-, O-dealkylation [56, 57]	22 ± 8 [29]

human small intestine, in contrast to the liver that contains enzymes of both CES1 and CES2 families [96]. Interestingly, the CES activity in the human intestine exhibits no significant proximal-to-distal gradient, in contrast to CYP [96]. Both CES1 and CES2 also cleave amides [95], as evident by their considerable butanilicaine and isocarboxazid hydrolase activity (Figure 12.4).

Table 12.2 Drug examples found to be metabolized in the gut wall by UGT.

Drug	UGT isozyme	Metabolic routes	Oral *F* (%)
Codeine	2B4, 2B7	6-*O*-Glucuronide [59]	50 ± 7 [60]
Morphine	2B4, 2B7	3-*O*-Glucuronide, 6-*O*-glucuronide [59]	24 ± 12 [61]
	1A3	3-*O*-glucuronide [62]	
	1A8	3-*O*-glucuronide, 6-*O*-glucuronide [63]	
Naloxone	2B7	3-*O*-Glucuronide [64]	2 [58]
	1A8	3-*O*-Glucuronide [63]	
Zidovudin	2B7	5′-*O*-Glucuronide [59]	75 ± 15 [65]
Paracetamol	1A1, 1A6, 1A9, 1A10	Phenolic *O*-glucuronide [66]	89 ± 4 [67]
Ezetimibe	1A1, 1A3, 2B15	Phenolic 4′-*O*-glucuronide [68]	35–60 [69]
Rac-ketoprofen	1A3, A9, 1A10, 2B7	Acyl glucuronide [70]	85 ± 21 [71]
Mycophenolic Acid (sodium salt)	1A8, 1A9	Phenolic 7-*O*-glucuronide [72], acyl glucuronide (minor) [73]	72 [74]
	2B7	Acyl glucuronide (minor) [73]	
Raloxifene	1A1, 1A8	4′-*O*-Glucuronide (major), 6-*O*-glucuronide (minor) [75]	2 [76]
	1A10	4′-*O*-glucuronide [75]	
Resveratrol	1A6, 1A8, 1A10	4′-*O*-Glucuronide, 3-*O*-glucuronide [77]	<5 [78]
	1A1	3-*O*-Glucuronide [79]	
	1A9	4′-*O*-Glucuronide [79]	

The wide distribution of esterase activity in the mucosa of stomach, small intestine, and colon has been exploited in designing numerous ester prodrugs [97–101]. Examples of successful ester prodrugs hydrolyzed, at least in part, in the gastrointestinal mucosa are ramipril [102], simvastatin [103], and bacampicillin [104].

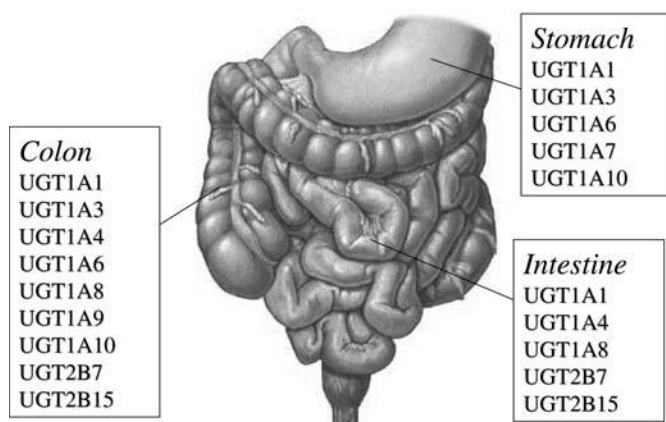

Figure 12.3 Distribution of UGT isozymes in the human gastrointestinal mucosa.

Table 12.3 Drug examples found to be metabolized in the gut wall by SULT.

Drug	SULT isozyme	Metabolic routes	Oral *F* (%)
Paracetamol	1A1 [83]	4-*O*-Sulfate	89 ± 4 [67]
Minoxidil	1A1 [83]	1-*N*-Sulfate	95 [36]
Dobutamine	1A3 [84]	Phenolic sulfate	No data
Apomorphine	1A3 [84]	Phenolic sulfate	<4 [85]
	1A1 [86]	Phenolic sulfate	
Ethinylestradiol	1E1 [82]	3-*O*-Sulfate	51 ± 9 [29]
Tibolone	2A1 [87]	17-*O*-Sulfate [88]	No data
Terbutaline	1A1, 1A3 [89, 90]	Catechol-*O*-sulfate	10–15 [91]
Isoproterenol	1A3 [84, 92]	Catechol-*O*-sulfate	No data
Salbutamol	1A1 [83], 1A3 [93]	4-*O*-Sulfate	49 [94]

In addition, the activation of the anticancer drug irinotecan to its activeprincipal SN-38 occurs by CES2 [105–107], and its gastrointestinal toxicity has been associated with local SN-38 liberation in the gut mucosa [106].

The human Caco-2 cell line has been shown to be of limited value as an *in vitro* model for the absorption and enzymatic cleavage of ester prodrugs in the gut wall mucosa because the expression pattern of CES1 and CES2 resembles more closely to the liver rather than the intestine [107].

For CES, there appear to be no regional differences in the small intestine for the *N*-acetyltransferase (NAT) activity [108]. The presence of both NAT isozymes has been demonstrated in the human gut mucosa by using the prototypical substrates *p*-aminobenzoic acid (NAT1) and sulfamethazine (NAT2) [109, 110]. The active metabolites 5-aminosalicylic acid and sulfapyridine of the prodrug sulfasalazine undergo extensive presystemic acetylation in the small intestine [111].

Alcohol dehydrogenases (ADHs), epoxide hydrolase (hydratase), *S*-methyltransferase, thiopurine methyltransferase, and glutathione *S*-transferases (GSTs) are also expressed in the gastrointestinal mucosa [29], but they are of relatively minor importance for the metabolism of drugs and will not be considered here.

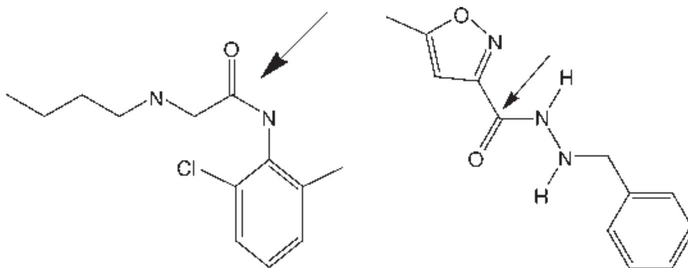

Figure 12.4 Chemical structures of butanilicaine (left) and isocarboxazid (right). The arrow indicates the site of hydrolysis.

12.4 Oral Bioavailability

The FDA definition given in CFR 21.320.1 refers to bioavailability as "the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of action." This phrasing has led to a considerable confusion and sometimes misinterpretation of published data [19], and, as a consequence, the terms oral bioavailability and oral absorption are often used interchangeably in the scientific literature [112]. However, in the context of a more mechanistic interpretation of pharmacokinetic data, the separation of the two expressions is desired.

We will refer to oral absorption as the "movement of drug across the outer mucosal membranes of the GI tract" [112], whereas oral bioavailability is "that fraction of the dose that reaches the general circulation unchanged" [113]. The general circulation is defined experimentally in terms of a blood vessel in the peripheral circulation.

As in humans ethical constraints limit sampling sites to peripheral blood vessels, "absorption" cannot directly be estimated. Hence, the "absorption rate" (k_a, k_{01}) as calculated from peripheral plasma concentration data by compartmental or noncompartmental models reflects rather an "oral bioavailability rate," unless any gut wall and liver first-pass elimination is ruled out [112].

The oral bioavailability F consists of the fractions that survive the various barriers posed by successive organs [114]:

$$F = f_{abs} \cdot F_g \cdot F_h \cdot F_l, \tag{12.1}$$

where f_{abs} is the fraction absorbed from the intestinal lumen and F_g, F_h, and F_l are the fractions escaping extraction in the intestinal mucosa, liver, and lung, respectively. Unfortunately, standard PK sampling regimes do not allow the estimation of f_{abs}. However, the estimation of the product of $f_{abs} \cdot F_g$ is possible in the preclinical setting after administration of an oral and intraportal dose according to

$$f_{abs} \cdot F_g = \frac{D^{ip}}{D^{po}} \cdot \frac{AUC^{po}}{AUC^{ip}}, \tag{12.2}$$

where D^{ip} and D^{po} are the intraportal and oral dose, respectively, and AUC^{po} and AUC^{ip} denote the AUC after oral and intraportal administration [113] (see Figure 12.5).

f_{abs} can be estimated according to Equation 12.3 by administering a dose into the superior (cranial) mesenteric artery (D^{ima}) and relating the AUC measured from peripheral vein sampling (AUC^{ima}) to the AUC measured after an oral dose (AUC^{po}):

$$f_{abs} = \frac{D^{ima}}{D^{po}} \cdot \frac{AUC^{po}}{AUC^{ima}}. \tag{12.3}$$

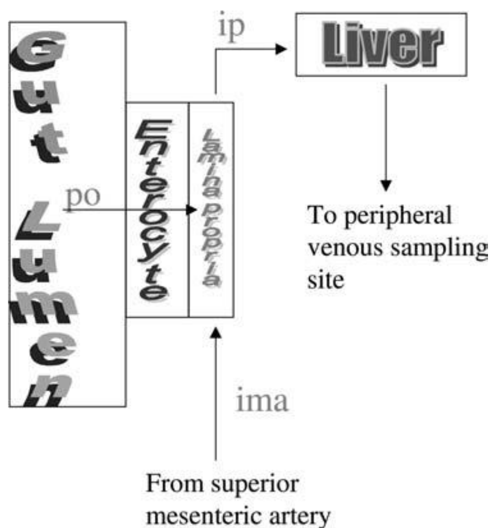

Figure 12.5 Schematic diagram of relative location of administration and sampling sites (for abbreviations see text).

Finally, F_g is derived experimentally from peripheral venous plasma concentrations after dosing to the hepatic portal vein and the superior mesenteric artery, respectively, according to Equation 12.4:

$$F_g = \frac{D^{ip}}{D^{ima}} \cdot \frac{AUC^{ima}}{AUC^{ip}}. \quad (12.4)$$

As these experimental approaches are clearly not applicable to clinical testing, indirect approaches have been used to delineate $f_{abs} \cdot F_g$ [115–118], often making assumptions not necessarily valid (e.g., $f_{abs} = 1$) or using PBPK models not entirely adequate [2].

12.4.1
In Vivo Approaches to Differentiate Between Intestinal and Hepatic First-Pass Metabolism

Owing to ethical constraints, the direct assessment of intestinal metabolism occurring in humans *in vivo* can only be undertaken at rare occasions, as it requires access to hepatic portal blood. For instance, transumbilical portal vein catherization can be justified only in patients requiring portal venous system radiography or regional cancer chemotherapy [118]. If the fraction absorbed is known, the extent of intestinal metabolism can then be estimated from drug and metabolite measurements in hepatic portal blood. This way, Lund *et al.* have demonstrated the almost complete hydrolysis of pivampicillin to ampicillin by the gut wall [119]. Mahon *et al.* found higher concentrations of mono- and didesethyl metabolites of flurazepam in the portal venous blood compared to hepatic venous or peripheral arterial blood following duodenal flurazepam administration to two intensive care patients [120].

The evaluation of the extent of drug metabolism in the intestinal wall can also be carried out in patients with a portocaval anastomosis where portal blood (i.e., all the venous return from the stomach, small intestine, and colon) bypasses the liver. In these "anhepatic" patients, no hepatic first-pass metabolism should occur. Comparing AUC after duodenal administration of a drug dose during the "anhepatic phase" and another dose given after the liver is in place will make it possible to calculate $f_{abs} \times F_g$ (Equation 12.1). Unless a further IV dose can be administered, F_g can be estimated only under the assumption that absorption is complete ($f_{abs} = 1$). Other assumptions to be made are linear pharmacokinetics, negligible enterocytic metabolism following drug supply via the mesenteric artery (i.e., after IV administration), and no differential influence of the general anesthesia on intestinal and hepatic clearance [4]. The few drugs that have been investigated in anhepatic patients include propranolol [121], midazolam [122], and cyclosporin [123]. For propranolol, no significant gut wall metabolism was detected suggesting that the low oral bioavailability is rather a result of extensive hepatic first-pass metabolism [121]. In the case of midazolam, however, Paine *et al.* were able to show very significant gut mucosal metabolism (mean intestinal first-pass extraction fraction of 43%) and estimated F_g as 0.57 ± 0.27 [122]. Similarly, Watkins and coworkers found unambiguously the primary cyclosporine metabolites in the hepatic portal vein and systemic blood of anhepatic patients during liver transplantation and concluded that these metabolites were of intestinal mucosal origin [123], assuming that other organs, such as the lungs, did not contribute to extrahepatic metabolism.

Another way to estimate presystemic metabolism in the gut wall is more amenable to routine clinical practice and involves drug administration via intravenous and oral routes. In addition to the assumptions outlined for anhepatic patients, this method assumes an average liver blood that does not change during treatment and uses a simple PBPK model to estimate F_g according to Equation 12.5:

$$F_g = \frac{F}{f_{abs} \cdot F_h}, \tag{12.5}$$

where F is determined from the experimental data after IV and po administration, f_{abs} is assumed to be 1, and F_h is estimated from the IV data according to (12.6), correcting total CL for renal CL and assuming that all nonrenal CL is hepatic.

$$F_h = 1 - \frac{CL_h}{Q_h}. \tag{12.6}$$

With this method, Tateishi *et al.* estimated F_g of midazolam as 0.53 ± 0.22, which is in good agreement with the anhepatic data [124].

The indirect approach by po/IV comparison can be refined by interaction studies attempting to selectively inhibit or induce gut wall metabolism. Grapefruit juice is assumed to selectively cause a mechanism-based inhibition of gut wall CYP3A enzymes, and Kupferschmidt *et al.* have used this method to derive F_g of 0.69 ± 0.31 for midazolam [125]. Similarly, Benet and coworkers have extended the original work on cyclosporin by Watkins' group [123] through interaction studies with the CYP3A4 inducer rifampin [126, 127] and the CYP3A4 inhibitor ketoconazole [128]. Other

CYP3A4 substrates whose poor oral bioavailability has been ascribed to extensive intestinal (next to hepatic) first-pass extraction are verapamil and nifedipine. In both cases, the IV/po approach combined with administration of the inducer rifampin was used to argue for extensive gut wall extraction [129, 130]. The results of the former trial have been confirmed using St John's wort as an inducer and controling absorption by jejunal single-pass perfusion [131]. Alfentanil, metabolized almost exclusively by CYP3A4, exhibited substantial gut wall extraction ($F_g = 0.56 \pm 0.18$) as adjudged from IV/po studies and coadministration of rifampin (CYP3A4 induction), troleandomycin (CYP3A4 inhibition), or grapefruit juice [17].

In summary, recent clinical studies have indicated that the small intestine contributes substantially to the overall first-pass metabolism of CYP3A4 substrates such as cyclosporin, nifedipine, midazolam, verapamil, and alfentanil. Much of the evidence has been derived indirectly from comparisons of areas under the plasma concentration curves (AUCs) after IV and po administrations and additional studies investigating the effect of coadministered CYP3A4 inhibitors and inducers. The absence of well-established clinical inhibitors/inducers for non-CYP3A enzymes may help rationalize the relative lack of clinical studies indicating significant involvement of other drug-metabolizing enzymes in gut wall first-pass extraction. Clearly, some of the assumptions made in those indirect studies have not yet been tested. For instance, it is often assumed that the systemic clearance of a drug after an IV dose reflects only hepatic elimination and that metabolism of the systematically available drug by intestinal enzymes is negligible. Only then can hepatic extraction be calculated directly from the observed total CL after IV administration and the reported values of hepatic blood flow. This allows then the indirect estimation of F_g according to Equation 12.5. Owing to the potential countercurrent exchange and plasma protein binding, the fraction of the systemically available drug metabolized by the intestine may indeed not be as great as that which occurs during absorption. However, it appears unrealistic to neglect completely the intestinal metabolism of drugs when delivered from the systemic circulation. For instance, Paine *et al.* [122] estimated F_g of midazolam in anhepatic patients after IV administration as 0.92 (0.75–1). The neglect of the contribution of the intestinal mucosa to total clearance after IV administration may hence lead to an overestimation of the gut wall first-pass effect. Further uncertainties include the variability in hepatic blood flow and f_{abs} [115]. In this context, it should also be borne in mind that the clinical methods available allow only a realistic (i.e., without assumption regarding f_{abs}) estimation of the product $f_{abs} \times f_g$.

The obvious clinical limitations in directly assessing gut wall metabolism in humans underline the importance of modeling approaches. These will be briefly reviewed in Section 12.4.3.

12.4.2
In Vitro Approaches to Estimate Intestinal Metabolism

Owing to the limitations of the methods available to study intestinal metabolism in a clinical setting (see Section 12.4.1), *in vitro* approaches with human systems give important, often the only information accessible on the intestinal metabolism of a drug or an NCE. Human *in vitro* systems suitable for the determination of gut

mucosal CL_{int} values serving as input parameters for computational models (see Section 12.4.3) comprise whole-tissue models (Ussing chamber, precision-cut intestinal slices), cellular models (shed enterocytes), and subcellular fractions (gut mucosal microsomes).

Complete human intestinal mucosal sheets can be investigated in the Ussing diffusion chamber. This experimental setup makes it possible not only to investigate mucosal metabolism in different gut segments along the length of the intestine but also to conduct vectorial transport of drug and metabolites formed *in situ* [132]. If carried out competently, experiments with Ussing preparations can be characterized by high metabolic activity, good activity of transport proteins, and high tissue viability including membrane integrity. Issues are the supply of suitable, fresh human tissue, and the need for rapid, but careful, tissue preparation including the removal of muscle layers and serosa.

Precision-cut human mucosal slices are prepared with a Krumdieck tissue slicer [132]. A study comparing precision-cut slices and intestinal sheets in the Ussing chamber found comparable utility of both approaches to assess intestinal metabolism [132]. Formation rates of midazolam 1′-hydroxylation (CYP3A4/5), diclofenac 4′-hydroxylation (CYP2C9), bufuralol hydroxylation (CYP2D6), 7-hydroxycoumarin sulfation, and glucuronidation were found to be constant for up to 4 h in both precision-cut slices and Ussing chamber setup [132].

Shed enterocytes constitute a very elegant cellular *in vitro* model of human gut metabolism [133]. About 15–30 million enterocytes are shed per minute into the lumen of the gastrointestinal tract, and these cells can be collected by segmental jejunal perfusion from healthy volunteers. The majority of shed enterocytes collected this way were nonapoptotic and metabolically fully competent. Obstacles to more widespread use of this tool are the still very limited availability and very high price.

Gut mucosal microsomes are the most widespread *in vitro* tool because of their ease to use, commercial availability, and their amenability to long-term storage. Over the past decade, considerable improvements in the preparation methods of intestinal microsomes have been made resulting in a better preservation of CYP activity. Intestinal microsomes prepared by enterocyte elution [134] appear to have generally higher activities compared to those prepared from mucosal scrapings [135].

12.4.3 Computational Approaches to Estimate and to Predict Human Intestinal Metabolism

The lack of generally applicable direct methods to measure intestinal metabolism in humans (see Section 12.4.1) emphasizes the need for reliable computational models to differentiate intestinal metabolism from hepatic first-pass metabolism. Historically, the well-stirred (venous equilibrium) model has been adapted to the gut [1, 29] and used for these predictions according to Equation 12.7:

$$F_g = \frac{Q_g}{(f_{u,b} \cdot CL_{int,g}) + Q_g}, \tag{12.7}$$

where Q_g represents the mucosal blood flow, $f_{u,b}$ the unbound fraction in blood, and $CL_{int,g}$ the intestinal intrinsic clearance. It has been questioned whether the

well-stirred model is adequate for intestinal first-pass metabolism, because the drug is not delivered by the blood to the mucosa, and the influence of plasma protein binding on the vectorial movement of drug from the intestinal lumen to the vasculature is unknown [2, 4, 5]. This approach has been defended by pointing out that mucosal blood flow and plasma protein binding do influence the residence time in enterocytes by drawing drug away from the metabolizing enzymes [29]. However, the main argument against the "intestinal well-stirred model" is its poor predictivity [2, 4, 5]. Tummel *et al.* [29] predicted F_g of 12 CYP3A4 substrates and found up to 15-fold prediction errors with a trend for overpredicting F_g [5]. The poor predictivity has been confirmed by Yang *et al.*, particularly when $f_{u,b}$ was included in the model [4]. Some of the apparent inadequacy of the well-stirred gut model can possibly be explained by the limitations of the "measured" data (see Section 12.4.1) and the use of suboptimal intestinal microsomal data to estimate $CL_{int,g}$ (see Section 12.4.2). It has also been put forward that an intestinal parallel-tube model should better reflect mucosal physiology, as less stirring of the blood is expected compared to that of the liver [5].

To overcome the shortcomings of the intestinal well-stirred model, Yang *et al.* proposed the "Q_{gut} model" [4]. This model maintains the basic equation of the well-stirred model, but expands the flow term into a hybrid of both permeability through the enterocyte membrane defined by CL_{perm} and villous blood flow (Q_{villi}) according to Equation 12.8:

$$Q_{gut} = \frac{Q_{villi} \cdot CL_{perm}}{Q_{villi} + CL_{perm}}. \tag{12.8}$$

CL_{perm} can be derived from *in vitro* permeability experiments (Caco-2, MDCK cells, and PAMPA) or *in silico* from polar surface area and the number of hydrogen-bond donors [4]. Yang *et al.* found a clear superiority of the Q_{gut} model over the well-stirred model in terms of accuracy and precision, particularly when plasma protein binding was ignored [4]. This simplified the model to (12.9):

$$F_g = \frac{Q_{villi}}{Q_{villi} + CL_{int,g} \cdot (1 + (Q_{villi}/CL_{prem}))}. \tag{12.9}$$

The Q_{gut} model can be regarded as the minimal version of the TMF (transport, metabolism, and blood flow) model proposed by Mizuma [2, 136]. An intestinal permeability term was also incorporated by Fagerholm into his approach to predict gut wall first-pass extraction [5]. Instead of relying on scaling factors to convert *in vitro* CL_{int} into *in vivo* CL_{int} and on assumptions regarding blood flow values, Fagerholm used verapamil as a reference compound with known mucosal extraction and known *in vitro* CL_{int}. Using his method, Fagerholm predicted the first-pass *in vivo* gut wall extraction of midazolam, bromocriptine, nifedipine, and diltiazem with little error [5].

These simple models based on the assumption of a single intestinal compartment have been refined to the advanced compartmental absorption and transport model that allows transit and differential expression of enzymes and transporters down the length of the gastrointestinal tract including pH, fluid, and blood flow differences [3]. The ACAT model is based on a series of integrated differential equations and has been implemented in the commercial software Gastroplus (see Chapter 17).

12.5 Clinical Relevance of Gut Wall First-Pass Metabolism

Gut wall metabolism as a major determinant for poor oral bioavailability is still a matter of debate within the scientific community [1, 115, 117]. In general, the metabolic capacity of the intestinal tract appears inferior to that of the liver, but for a number of drug molecules such as midazolam, nifedipine, verapamil, tacrolimus, and saquinavir, gastrointestinal first-pass elimination is substantial. This appears to be particularly true for CYP3A substrates, though the total intestinal CYP3A amount is only about 1% of that in the liver [10]. However, it has been argued that drug exposure to the enterocytic metabolizing enzymes is probably greater than that in the liver where it is limited by fast blood flow and the impact of binding to blood components [4]. In contrast, all drug absorbed into the enterocyte could be freely accessible to drug-metabolizing enzymes, limited only by the flux through the cell and assuming negligible intracellular binding. The higher substrate accessibility might result in more likely enzyme saturation in the mucosa, but this could be offset by recycling and dilution via efflux transport proteins. On the other hand, saturation of intestinal efflux transporters following oral dosing and high luminal concentrations is also a realistic possibility. Owing to experimental limitations, clinically relevant gut wall first-pass metabolism is often discovered only after studies utilizing the complete inhibition of intestinal CYP3A following the ingestion of grapefruit juice are done. In this setting, significant increases in oral exposure have been observed for midazolam [125], buspirone [137], felodipine [138], lovastatin [139], simvastatin [140], cyclosporin [25], nifedipine [141], and cisapride [142]. It is tempting to speculate that detection of clinically relevant intestinal metabolism of non-CYP3A substrates has been hindered by missing gut-selective inhibitors of those enzymes.

Still, from a pharmaceutical industry perspective, drug candidate attrition rates owing to an unexpectedly high gastrointestinal first-pass effect are low, because the majority of intestinal metabolic liabilities will have been readily detected in systems geared toward hepatic metabolism.

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13
Modified Cell Lines

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Abbreviations

ASBT	Apical sodium-dependent bile acid transporter (also designated as SLC10A2)
BCRP	Breast cancer resistance protein (also designated as ABCG2)
Caco-2	Human colon adenocarcinoma cell line used as absorption model
cDNA	Complementary deoxyribonucleic acid
CYP	Cytochrome P450
CYP-OR	Cytochrome P450:NADPH oxidoreductase
LLC-PK_1	Porcine kidney cell line used as absorption model
MCT	Monocarboxylate transporter (also designated as SLC16)
MDCK	Madin–Darby canine kidney cell line used as absorption model
MDR1	Multidrug resistance protein 1 (also designated P-gp and ABCB1)
MRP1	Multidrug resistance associated protein 1 (also designated ABCC1)
MRP2	Multidrug resistance associated protein 2 (also designated ABCC2)
OATP	Organic anion transporting polypeptide (also designated as SLCO or SLC21)
PEPT	Peptide transporter (also designated as SLC15)
TEER	Transepithelial electrical resistance

13.1
Introduction

The extent of oral absorption of a drug depends on physical properties of the drug (e.g., solubility and membrane permeability) and its interaction with various enzymatic processes of an organism. The two most prominent enzymatic systems are drug metabolic enzymes and drug transporters. Both metabolic enzymes and transporters exist as many related forms that generally have distinct, yet potentially overlapping, substrate specificities.

The action of drug metabolizing enzymes may decrease oral bioavailability by metabolizing a portion of the drug that has been absorbed before it enters circulation. Human drug metabolizing enzymes have been extensively studied for decades and are now well characterized. Metabolic enzyme-specific substrate, inhibitor, and antibody *in vitro* probe reagents as well as authentic standards are available for most of the major enzymes. These allow the establishment of the relative and absolute amounts of metabolism by individual enzymes and prediction of drug–drug interactions. In addition, a number of *in vivo* probes are available. *In vivo* studies are essential to validate predictive models based upon *in vitro* measurements.

There are a number of drug transporters that are expressed in various tissues including the intestine. Intestinal drug transporters can either increase or decrease oral bioavailability depending on whether the transporter facilitates or impedes drug uptake into the enterocyte (which is generally regarded as rate limiting). The impact of transporters on drug bioavailability and drug disposition has been investigated extensively in the last few years. However, the research is still hampered by the lack of comprehensive set of reagents (specific substrates and inhibitors) and standards. As with the metabolic enzymes, the goal for drug transporters is to develop predictive *in vitro* models.

To conduct basic research and address the need for reagents and standards, cell lines have been modified so that the function of metabolizing enzymes and transporters can be examined individually. These cell lines can serve as a source of active protein to validate a chemical as a substrate or inhibitor or as a source of protein to validate the specificity of an antibody. For this approach to be robust for establishing specificity (and to minimize false-negative findings), all of the key proteins need to be available and active in the system. However, as specific probes are being identified and developed, useful mechanistic studies can be performed on transporters and substrates/inhibitors, which are currently available.

There is also a need for cell systems that express a multiplicity of metabolic enzymes and transporters to assess drug disposition *in vivo*. Cell line modification through cDNA expression can be used to add missing functions to cells that are known to express only a subset of needed enzymes or transporters known to be present *in vivo*. Cell lines can also be modified by deleting a natively expressed transporter so that the cell lines are more selective for other transporters. In both ways, the *in vitro* testing models can be improved.

This chapter describes some of the modified mammalian cell-based systems that have been developed to express intestinal cytochrome P450 enzymes and intestinal transporters. The reader should be aware that other experimental systems, such as transporter expression and drug uptake studies in *Xenopus laevis* oocytes and baculovirus expression system, have shown considerable promise [1–3].

13.2
Cell/Vector Systems

Modified mammalian cell systems for the study of the role of transporters and/or metabolism in oral absorption consists of two main components, the cell line and the

vector bearing a cDNA encoding the protein of interest. The cell line serves two roles: first, to support an adequate expression of the cDNA and second, to provide a barrier function that is generally critical in transporter function assays.

The key function of the vector is to introduce the cDNA under the control of a strong promoter and, if a stable cell line is to be developed, also to introduce resistance to a compound that is otherwise toxic to the host cell. This facilitates selection (only vector-bearing cells grown in special media) of a minority of cells that have incorporated the vector. There are many adequate expression vectors available from a number of commercial suppliers. Several classes of vector systems appear appropriate for transporter expression as integrating, episomal, retroviral, Vaccinia virus-, and Adenovirus-based systems have all been used successfully in studies referenced in this chapter.

Analysis of cytochrome P450 function is most commonly performed by analyzing metabolite formation in culture. This presents an analytical challenge, as during the drug discovery/lead optimization phases, radiolabeled compounds or authentic metabolite standards are generally not available. Although the extent of metabolism could theoretically be measured by the loss of parent compound, the extent of the loss is generally less than 20%, which is difficult to detect reliably and provides a very narrow dynamic range to rank compounds.

Bidirectional transport assay is most commonly performed to determine transporter function by analyzing rates of drug transport through cell monolayers grown on membrane support systems (e.g., microporous polycarbonate or polyethylene terephthalate membrane-containing cell culture inserts). Typically, efflux transporters such as MDR1 are preferentially expressed on the apical face of the cell and the apparent permeability of an MDR1 substrate is substantially higher in the basolateral-to-apical direction compared to that in the apical-to-basolateral direction. In the transport assay, rates of drug permeation are measured in two directions. Figure 13.1 provides a diagram of the assay. A polarization ratio is calculated as the apparent permeability in the basolateral-to-apical direction divided by the apparent permeability in the apical-to-basolateral direction. Presumably, polarization ratios greater than unity imply an active efflux transport. However, in practice, polarization ratios greater than 2 are used as an indictor for solid active efflux transport due to experimental errors. The parent compound is the analyte in these assays, and automation-compatible membrane insert systems (24-well) have been available for a number of years. More recently, 96-well insert systems have been introduced.

Alternatively, the transporter function can be analyzed by accumulation or efflux assay. Accumulation assay is generally used to determine substrate accumulation rate into the cells mediated by uptake transporters, whereas efflux assay is generally used to determine substrate efflux rate of the cells mediated by efflux transporters. For efflux assay, cells are preincubated with the substrate.

For efflux transporters, however, the functional interaction between the drug and the transporter occurs in the intracellular space. Some substrates, particularly the negatively charged MRP2 substrates, have very low permeability and in the absence of an uptake transporter may not give detectable transport. In these cases, double-transfected cell lines have been developed so that compounds with low permeability

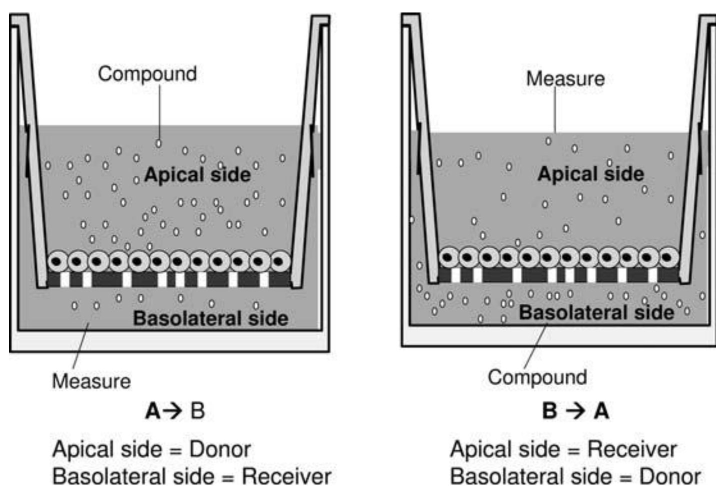

Figure 13.1 Schematic diagram of drug transport assay in cell monolayers cultured on a culture insert containing permeable membrane.

can get in cells via uptake transporters [4]. Another system that has been analyzed uses membrane vesicles prepared from transfected (and control) cells [5]. Generally, studies with membrane vesicles require the use of radiolabeled substrate material, which is generally not available for all compounds in a drug discovery/lead optimization program.

Two properties determine the usefulness of a cell line as a particular host for cDNA expression. These are

(1) The extent to which the cell line supports an appropriate functional expression of the cDNA. The level of expression achieved is determined by interactions of the vector/expressed protein with the cell. These interactions include the strength of the promoter (weaker promoters can be compensated for by using a vector that is present at high copy number), the adequacy of the selective agent (not all agents are toxic to all cells), the stability of the expressed protein (some proteins may be rapidly degraded in some cells), and whether the expressed protein exerts any deleterious effect on the viability of host cells (some efflux transporters could deplete the cell of essential components, such as GSH). If cytochromes P450 are to be expressed, the necessary redox partners (oxidoreductase (OR) and cytochrome b_5) also need to be expressed natively in the host cell (coexpression is also an option). Finally, for bidirectional transport assay, transporters must be expressed in a polarized manner in the host cell (i.e., preferentially either on the basolateral side or apical side of the cell).

(2) The extent to which the cell line is appropriate for drug metabolism and transport studies. If drug bidirectional transport studies are to be performed, the cell line needs to form monolayers with tight cell–cell junctions. If cell–cell junctions are loose, background (or nontransporter-dependent) paracellular drug transport

will be high. In addition, if a panel of cells expressing individual transporters is being developed, expression of any native drug metabolism enzymes or transporters should be low as these native processes introduce background to the system.

Three cell lines, Caco-2, MDCK, and LLC-PK_1, have been most commonly used for cDNA expression. All three of these cell lines have very low levels of oxidative drug metabolism, although variants of Caco-2 and LLC-PK_1, cultured under appropriate conditions, have been reported to express significant levels of CYP3A subfamily enzymes [6–8]. Beside MDR1, Caco-2 cells have been reported to express a significant number of uptake and efflux transporters, such as MRP2-6, BCRP, OATP1A2, OATP2B1, OCT1, MCT1, and PEPT1 [9–13]. It should be noted that mRNA expression levels may change in different Caco-2 clones [12, 13], so transporter levels should be carefully characterized in a particular Caco-2 cell clone.

Owing to the overlapped substrate selectivity of transporters expressed in Caco-2 cells, a particular transporter can be knocked out by using RNAi technology to improve assay selectivity [14].

Using putative MDR1 substrates, MDCK cells express substantially higher levels of native efflux transporters than those of LLC-PK_1 cells. Everything else being equal, the LLC-PK_1 model is preferred because it forms high-quality monolayers and has low levels of native transporters.

13.3 Expression of Individual Metabolic Enzymes

Most interest has focused on oxidative enzymes of the cytochrome P450 (CYP) class, which are expressed not only in the liver to the highest degree but also in the intestine to a significant degree. Conjugating enzymes are also expressed in the intestine [15, 16]. Human intestine appears to be relatively rich in CYP3A4 (which is also abundant in the liver), and intestinal CYP3A4 can significantly contribute to the first-pass metabolism of drugs such as midazolam, cyclosporin A, and verapamil [17]. Human intestine has also been reported to contain lower levels of CYP2C9, CYP2C19, CYP2D6, and CYP1A1 [18, 19]. However, because of low abundance, it appears unlikely that the presence of these enzymes in the intestine significantly reduces oral bioavailability (relative to the impact on first-pass metabolism in the liver).

Catalytic activity of CYP enzymes requires functional coupling with its redox partners, cytochrome P450:NADPH oxidoreductase and cytochrome b_5. Measurable levels of these two proteins are natively expressed in most mammalian cell lines. Therefore, introduction of only the CYP cDNA is generally needed for detectable catalytic activity. However, the levels of expression of the redox partner proteins may be low and may not support maximal CYP catalytic activity; therefore, enhancement of OR levels may be desirable. This approach has been used successfully with an Adenovirus expression system in LLC-PK_1 cells [20].

Because of its importance in first-pass metabolism, there has been a considerable interest in introducing CYP3A4 into cell systems. This protein is normally not expressed in cell lines and was introduced into Caco-2 cells with the goal of improving this common screening model. The first reported expression of a drug-metabolizing enzyme in a drug permeability model was of CYP3A4 in Caco-2 cells [21]. CYP3A4 catalytic activities were increased about 100-fold relative to control cells but still well below than that found in the intestine. However, it was noted that expression of CYP3A4 markedly reduced the proliferative capacity of the Caco-2 cells (relative to CYP2A6-expressing and control cells). Nonetheless, expression of CYP3A4 did not alter transepithelial electrical resistance (TEER) values or the permeability of model compounds. Coexpressing OR did not substantially elevate CYP3A4 catalytic activity (on a milligram cellular protein basis) [22]. However, 4-fold and 16-fold higher catalytic activities were obtained using the same expression system with MDCK and LLC-PK_1 cells as hosts [23]. Similarly, high levels of expression were obtained in the LLC-PK_1 model using an Adenovirus vector [20].

Levels of CYP3A4 activity in LLC-PK_1 models appear to be comparable to those found in human intestine [23], and a significant first-pass effect has been observed as CYP3A4 substrates pass through monolayers. For example, in the LLC-PK_1 model, up to 19% of nifedipine was metabolized as it passed through the monolayer. The LLC-PK_1/CYP3A4 systems appear to be a reasonable model for assessing the extent of any first-pass effect during permeation through the intestine. However, given the modest amount of metabolite formed for nifedipine (an excellent CYP3A4 substrate), there appears to be little reason to adopt these cells as an "improved" screening model. Alternative approaches, such as measuring the rates of parent compound loss in CYP3A4 microsomal incubations, should be as informative and easier to implement.

Over the last few years, the impact of interplay between intestinal metabolic enzymes and transporters on drug first-pass bioavailability has been reported. CYP3A4-transfected Caco-2 was originally developed to evaluate the first-pass metabolism [21]. Since it coexpresses both CYP3A4 and MDR1, the cell line has provided a pivotal experiment model to investigate the influence of interplay between CYP3A4 and MDR1 on drug metabolic extent and extraction ratio of K77 and sirolimus, substrates of both CYP3A4 and MDR1 [24, 25]. The results indicate that inhibition of intestinal apical efflux transporters MDR1 and metabolic enzyme CYP3A4 was synergistic, both reducing drug metabolic extent and extraction ratio. The results also suggested that, by dosing substrates at the basolateral membrane side, CYP3A4-transfected Caco-2 cells could be used to assess the interplay between CYP3A4 and MDR1 in hepatocytes [24–26]. It is expected that the extent of metabolism in intestine could also be affected by the interplay between other apical efflux transporters and enzymes [26]. However, the research is limited by the availability of appropriate experiment models.

It has been reported that the CYP3A4 activity in CYP3A4-transfected Caco-2 cells can be stabilized and improved by adding 5-azacytidine in the culture medium. Cell monolayer normal growth was not affected by the treatment [22].

13.4 Expression of Transporters

13.4.1 Efflux Transporters

The identities and roles of many drug transporters are discussed in other chapters in this volume and are not extensively reintroduced here. The goal is to develop a comprehensive panel of cells expressing individual, functional transporters as research reagents. To simplify data interpretation, the set of transporters should be expressed in the same host cell line and the abundance of functional proteins in the cell line should be known relative to the corresponding *in vivo* values. However, useful mechanistic data can be obtained from less comprehensive systems.

As stated earlier, there are many drug transporters expressed in the intestine [27, 28]. Although the expression levels of some transporters have been examined, the protein levels remain to be further defined and the methods need to be standardized. The export proteins MDR1, MRP1, MRP2, and BCRP have been of particular interest because they are expressed at relatively high levels in the intestine [27, 28] and are known to function to efflux drugs.

The most extensively studied protein is MDR1, natively expressed in Caco-2 cells. MDCK and LLC-PK_1 cells expressing high levels of cDNA-derived human (and rodent) MDR1 have been developed in several laboratories [29, 30]. The development of these cell lines has been facilitated by the fact that MDR1 expression confers resistance to cytotoxic drugs such as vinblastine. This allows direct growth selection of cells expressing high levels of MDR1. MRP2 and BCRP have been successfully expressed in MDCK cells [31, 32]. Like MDR1, MRPs and BCRP expression can confer resistance to cytotoxic drugs that can facilitate isolation of cells expressing high levels of functional protein. Many MRP2 and BCRP substrates have very poor permeability and when added to the extracellular space cannot reach the active site of the transporter. The issue of substrate access to the MRP2 and BCRP active site has been addressed by coexpressing one or multiple uptake transporters, such as OATP1B1 or OATP1B3, which allows an efficient access to the substrate within the intracellular space [4, 33, 34]. These cell lines were developed primarily to address drug clearance in hepatocytes. Although the expression level ratio of the transporters may be different from that under physiological conditions, the substrate transport rank order would not be affected (absolute transport values are affected) since drug uptake is generally the rate-determining step. This has been demonstrated by *in vivo* results showing that the uptake across the sinusoidal membrane determined the biliary clearance of BQ123 [35]. Results from MDCK cell line coexpressing rat Oatp4 and Mrp2 also suggested that Oatp2-mediated uptake across basolateral membrane was the rate-determining process for the transcellular transport of $E_217\beta G$ and pravastatin [36]. That protein expression level ratio is not critical in determining transport rank order was further confirmed by the good correlation between the transport activity in human and rat double-transfected cell lines, and close prediction of *in vivo* biliary clearance from *in vitro* results obtained from double-transfected cell line [36].

An alternative method to overcome this issue is to perform uptake studies by using membrane vesicles prepared from cDNA-expressing and control cells [5]. Vesicle preparation and vesicle assays are, however, labor intensive. BCRP, MRPs, and BSEP inside-out vesicles prepared from insect cells by using baculovirus system are currently available from BD Biosciences, Solvo, and GenoMembrane.

MDR1-expressing cells have been used extensively to study the extent and rates of drug transport. Generally, a larger polarization ratio (rate of drug permeation basolateral to apical divided by the rate of drug permeation apical to basolateral) in MDR1-expressing cells relative to control cells (or MDR1 cells incubated in the presence of an MDR1 inhibitor) is considered an evidence for active transport by MDR1. Polarization ratio values are determined by both the drug transporting activity of MDR1 in the system for transporting the drug of interest and the intrinsic permeability of the molecule. Compounds with higher intrinsic permeability give lower polarization ratios. In addition, because transport processes are saturable, polarization ratios tend to decrease with increasing drug concentration. Therefore, care must be taken in interpreting the significance of polarization ratio values.

There is a need to develop a framework to better understand the conditions under which efflux transporters will significantly modulate oral bioavailability. Although it is clear that transport by MDR1 can reduce oral bioavailability, many successful, orally bioavailable drugs are *in vitro* substrates for efflux transporters such as MDR1 (e.g., digoxin, fexofenadine, cyclosporin A, and erythromycin). One *in vivo* indication of role of MDR1 in oral bioavailability has been pharmacokinetic drug–drug interactions between MDR1 inhibitors and nonmetabolized MDR1 substrates such as digoxin and fexofenadine. Another *in vivo* manifestation may be nonproportional pharmacokinetics. For example, the high-affinity MDR1 substrate UK-343 664 (K_m 7.3 μM) shows no systemic exposure at doses below 10 mg. Between 30 and 800 mg, the systemic exposure increased about 250-fold [37]. It seems reasonable that there is a region defined by drug dose, drug permeability, efflux enzyme affinity, and efflux enzyme activity where efflux transport will reduce oral bioavailability. Given that transporters are saturable, drugs administered at low doses and/or drugs with low intrinsic permeability are most likely to have lower oral bioavailability reduced by the action of efflux transporters. Similarly, drugs with low concentration, low permeability, and high affinity to efflux transporters generally have difficulties to cross the blood–brain barrier. Published data for more drug compounds are needed to define the combination of conditions that allow MDR1 to significantly reduce oral bioavailability.

Owing to the potentially profound impact of intestinal efflux transporters on drug bioavailability [26], a clear priority remains to build up a panel of intestinal efflux transporters that are expressed individually in modified cell lines. These research tools will be instrumental in identifying and validating selective probe transporter substrates and inhibitors. The availability of such probes will allow a better understanding of the influence of transporters on *in vivo* pharmacokinetics. A similar set of probes has been instrumental in increasing our understanding of the role cytochrome P450 plays in human pharmacokinetics and in avoiding issues associated with these enzymes.

In addition, the availability of specific probes for transporters will help generate data for creating transporter structure–activity models for the transporters [38] and thus provide the ability to rationally design around any transporter-related issues associated with a drug candidate series. In addition, specific probe substrates (and inhibitors) provide a means to relate the levels of transporter function *in vitro* to the levels of transporter function *in vivo*. A quantitative understanding of this relationship is key to developing accurate *in vitro* to *in vivo* extrapolation models. At present, a number of relatively selective probes have been reported but there are major gaps in terms of overall coverage and the degree of specificity. There is also a need to define the conditions (dose, intrinsic permeability, efflux transporter activity, and affinity) that efflux transport requires to significantly limit oral bioavailability. This will permit the analysis of transport and pharmacokinetic parameters for more compounds such as UK-343 664.

13.4.2
Uptake Transporters

Uptake transporters expressed in intestine can function to increase drug bioavailability. Drugs with low intrinsic permeability achieve acceptable oral bioavailability because they are substrates for uptake transporters that normally function in nutrient uptake. Compared to efflux transporters, less is known about intestinal drug uptake transporters. So far, the most prominent example is the peptide transporter, PEPT1, which is active toward peptidomimetic antibiotics such as cephalexin, the antiviral agent valacyclovir [39], and other drugs. Caco-2 cell is a suitable model for intestinal peptide transport studies since it expresses the intestinal peptide transporter PEPT1, but not the renal peptide transporter PEPT2 [40]. H^+/peptide transport activity was also observed in MDCK, but not in LLC-PK_1 [41]. Further studies indicated that, in addition to PEPT1 expressing at the apical membranes, a different peptide transporter expresses natively at the basolateral membranes of both Caco-2 and MDCK monolayers [42, 43]. Recombinant human PEPT1 has also been expressed in Chinese hamster ovary cells and MDCK cells [44, 45].

The reabsorption of conjugated bile acids is mediated by ASBT, which is localized on the apical membrane of ileal enterocytes in mammals. ASBT is a drug target not only to lower plasma cholesterol level but also to improve intestinal permeability [46]. Although available monolayer cell lines do not express ASBT, it has been expressed in MDCK cells [47]. Human intestine also expresses multiple MCT isoforms [48]. These MCTs are responsible for the absorption of short-chain fatty acid. Expression of MCT in Caco-2 allows it to be an appropriate model to study short-chain fatty acid transport [9, 49, 50].

In addition to the above uptake transporters that predominately express in the intestine, uptake transporters that function in other tissues also express in the intestine. For example, brain transporter OATP1A2, hepatic transporter OATP1B1, and OATP1B3 have been detected in human intestine [51]. In the intestine, OATP1A2 is localized on the apical membrane, and is the key uptake transporter for fexofenadine [51] and levofloxacin [12]. OATP1A2 was reported to be natively expressed in

Caco-2 cells and was thought to be the transporter for levofloxacin uptake in Caco-2 cells [12].

13.5 Summary and Future Perspectives

Intestinal transporters and metabolic enzymes influence drug oral bioavailability. The function of these transporters and enzymes has been investigated by using cell lines that either natively express or are modified to express transporters and drug metabolic enzymes. Cell lines expressing major intestinal metabolic enzymes and transporters, such as CYP3A4, MDR1, MRP2, BCRP, PEPT1, OATP1B1, and OATP1B3, have been developed. Although the expression levels of some transporters have been examined, the protein levels remain to be further defined and experimental standards need to be set up to better interpret results with respect to *in vivo* impact. Interplay between intestinal enzymes and transporters also affects drug oral bioavailability. However, due to the lack of model systems, research into this effect is currently limited to the interplay between MDR1 and CYP3A4. Development of cell lines that coexpress other intestinal metabolic enzymes and transporters will facilitate these studies. RNAi technology has been used to knock out a particular transporter to improve assay selectivity of cell lines. Owing to the limited number of specific substrates and inhibitors for transporters, this technology can be used as an alternative strategy to identify and define substrates and inhibitors for transporters.

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