

Carrier-Mediated Mechanisms for Cellular Drug Transport

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1. Introduction to Transport Proteins

Transporters are a group of polytopic membrane proteins that are involved in almost all biological functions in the cell. In brief, they mediate the entry of nutrients; facilitate uptake and release of hormones, neurotransmitters, and various signaling molecules; participate in transfer of nucleic acids across the cell membrane; and participate in secretion of proteins, lipids, and carbohydrates into and beyond the cytoplasmic membrane. These membrane-transport proteins also represent key targets for therapeutic applications. Implementation of membrane transporters as therapeutic targets for drug delivery requires an understanding of their normal physiological role and the means by which they recognize ligands at the molecular level.

According to the Transporter Commission (TC) nomenclature, membrane transporters can be classified primarily into four main types, i.e., channels, porters, primary active transporters, and group translocators. The latter three types can be grouped together as carriers (1). Channels are integral membrane proteins that form a hydrophilic pore that allows specific inorganic ions and molecules to pass through by the formation of a concentration gradient between the extracellular and intracellular contents. Ion channels are said to be “gated” if they can be opened or closed in response to external stimuli or environmental changes. The rate of transport through a channel is many magnitudes higher (10^8 ions/s) than that of a carrier (10^3 ions/s). Based on the source of energy, carriers can be further subdivided into three groups: (1) uniporters, which are also called facilitated diffusion carriers. They transport a single substrate that would not normally be able to pass through the lipid bilayer membrane (e.g., charged or very hydrophilic molecules), in a passive process driven by the substrate’s own electrochemical gradient. (2) Primary active transporters, which transport their substrates across the membrane against their concentration gradient using energy derived from adenosine 5'-triphosphate (ATP) hydrolysis, e.g., ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp). (3) Secondary active transporters, which also transport molecules against their concentration gradient, but are coupled to the transport of a second substrate moving down its electrochemical gradient, usually ions such as H^+ , Na^+ , or Cl^- , which forms the driving force. All active transporters can be

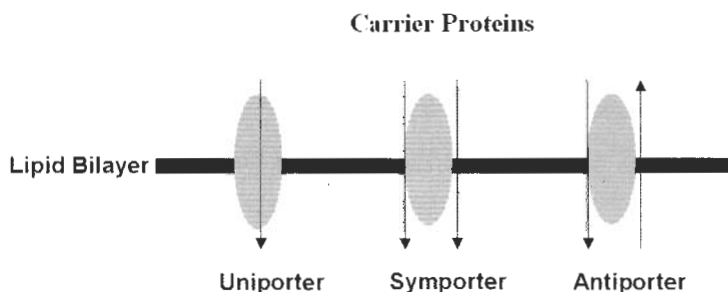


Fig. 1. Active carriers. All active transporters can be symporters, antiporters, or uniporters. Uniporters transport a single molecular species opposite its concentration gradient. A symporter cotransports a solute with an ion moving down its electrochemical gradient, whereas an antiporter countertransports a solute in the opposite direction of an ion that flows down its concentration gradient.

symporters, antiporters, or uniporters (Fig. 1). This chapter will focus briefly on the distinct transporter superfamilies, some of the transporters and the prodrug approaches that have been used to make these transporters pharmaceutically relevant.

1.1. Transporter SuperFamilies

The genome-sequencing data from the human-gene nomenclature committee and a number of biochemical and molecular genetic investigations have led to the classification of the transporters into distinct transporter superfamilies. Currently there exist about 250 entries, each of which may correspond to a single family. Superfamilies form when these individual families are very large. Some examples of superfamilies are the voltage-gated ion channel family (VIC), major facilitator superfamily (MFS), and the ATP-binding cassette superfamily (ABC). The latter two families will be discussed below.

1.1.1. The Major Facilitator Superfamily

This group of transporters primarily comprises single-polypeptide secondary carriers that are capable of transporting small solutes in response to a chemiosmotic ion gradient. The MFS transporters have been classified into 17 families (Table 1). They were originally believed to function as sugar transporters, but later studies revealed that they could transport a variety of small solutes in response to a chemiosmotic gradient. The members of each family share 25–30% sequence similarity (1). The MFS members have been classified by: (1) recognition of the significance of the family to cell physiology; (2) the application of biochemical, biophysical, and molecular genetic information from a few members to all members of the family; (3) use of a rational system for MFS protein classification; (4) evolutionary pathways responsible for the development of structural and functional diversity; and (5) unification of various mechanistic models in order to extend it to the maximum number of transporters possible (1). Almost all MFS transporters transport a wide array of small molecules. These include sugars, drugs, amino acids, metabolites, vitamins, nucleosides, and both organic and inorganic cations and anions. The MFS proteins possess either 12 or 14 putative transmembrane spanning regions and are one of the oldest protein families from an evolutionary standpoint.

1.1.2. The ABC Transporter Superfamily

The ABC superfamily consists of the largest number of transmembrane proteins. To date, 48 known ABC transporters are present and have been grouped into seven subfamilies (2). These proteins bind ATP and use the resultant energy as a driving force to transport various molecules across the cell membrane. The ABC proteins transport a variety of molecules, including large protein molecules and phospholipids. All ABC transporters contain an ATP binding domain, also called the nucleotide binding domain (NBF) and two sets of transmembrane domains. Each set of the transmembrane domain consists of approximately six transmembrane spanning α -helices. The ATP-binding domain has three conserved regions: Walker A and B motifs and a signature sequence LSGGQ, which is a feature distinct to the ABC transporters and distinguishes them from other ATP-binding proteins (3). The signature sequence consists of a loop that forms the point of interaction with the transmembrane domains. The Walker A motif is present just before the first three residues of helix 1 and has an α structure and interacts with the phosphate moieties of ATP, whereas the Walker B motif is located in strand 9 and has a β -sheet conformation (Fig. 2).

The various ABC genes are grouped into the different subfamilies based on gene-structure similarity, sequence homology with regard to the ATP-binding domain, and transmembrane regions. Table 2 lists in all known human ABC gene subfamilies.

Because these transporters are known to perform important physiological functions, mutations in their genes result in a variety of genetic disorders. Most of these disorders are allele-recessive and result in severe reduction or a complete loss of protein function. One such disorder, cystic fibrosis (CF), the most fatal childhood disease among Caucasian children, is the result of a defective CFTR (cystic fibrosis TM conductance regulator) protein. This ABC protein is a cAMP-regulated chloride ion channel. Patients with CF mutations fail to secrete chloride ions in response to a number of stimulations (4). The majority of CF chromosomes (70%) have a single codon deletion that translates to a missing phenylalanine residue at position 508 (δ F508) of the protein (5).

Unfortunately, the lack of sufficient crystal structure information limits our understanding of such transporters from their functional and organizational point of view. Such information may provide crucial insight into the treatment of such diseases. To this end, Locher and colleagues (6) recently obtained the structure of the vitamin B₁₂ transporter (an *Escherichia coli* ABC transporter) at 3.2 Å, wherein they have described the structure and potential transport mechanism of the transporter (Fig. 2). Improved insight of the actual transport process and the residues involved should result from such studies.

1.1.3. The Solute Carrier (SLC) Superfamily

The ABC and MFS superfamilies account for nearly half of the solute transporters (1). Originally, MFS and ABC were two distinct superfamilies each containing SLCs, but the increasing number of solute carriers has led HGNC (HUGO Gene Nomenclature Committee) to classify these proteins into a separate superfamily, SLC. The SLC proteins comprise integral membrane proteins involved in the transport of various solutes like amino acids, sugars, vitamins, and bile acids across the cell membrane. They are mainly secondary active carriers (co-transporters) that catalyze, (1) transport of single molecular species (uniport), (2) cotransport of a solute with a cation (symport, e.g., ASBT), (3) countertransport of a solute against a cation (antiport), or (4) bring

Table 1
The Major Facilitator Superfamily

#	Family name	Abbrev	Members (#)	Source(s) ^a	Size range	TM#	Mechanism(s)	Polarity	Substrates	Representative example(s)
1	Sugar porter	SP	133	Bac, Ar, Pr, Y, An, Pl	404–818	12	Sugar uniport Sugar:H ⁺ symport Sugar:sugar antiport	None In Both	Monosaccharides (hexoses, pentoses), disaccharides, quinate, organocations, inositols	XylE of <i>E. coli</i>
2	Drug:H1 antiporter (14 TMS)	DHA14	30	Bac, Y		14	Drug:H1 antiport	Out	Multiple or single drugs	QacA of <i>S. aureus</i>
3	Drug:H1 antiporter (12 TMS)	DHA12	46	Bac, Ar, Y, An		12	Drug:H1 antiport	Out	Multiple or single drugs	Bmr of <i>B. subtilis</i>
4	Organophosphate: antiport	OPA	12	Bac, An	439–495	12	Organo phosphate:Pi antiport	Both	Sugar-phosphates, glycerol phosphate, phospho-glycerates, phosphoenolpyruvate	UhpT of <i>E. coli</i>
5	Oligosaccharide:H1 symporter	OHS	6	Bac	415–425	12	Sugar:H ⁺ symport Sugar:sugar antiport	In Both	Di- and trisaccharides	LacY of <i>E. coli</i>
6	Metabolite:H1 symporter	MHS	16	Bac	425–500	12	Solute:H ⁺ t symport	In	Citrate, α-ketoglutarate, proline, betaine, methylphthalate, dicarboxylates	KgtP of <i>E. coli</i>
7	Fucose-galactose-glucose:	FGHS	4	Bac	404–438	12	Hexose uniport Hexose:H ⁺ symport	None In	L-Fucose, glucose, galactose	FucP of <i>E. coli</i>
8	H1 symporter	NNP	13	Bac, Y, Pl	395–547	12	Nitrite uniport? Nitrate:H ⁺ symport?	Out In	Nitrite, nitrate	NarK of <i>E. coli</i>
9	Nitrate/nitrite proter	PHS	11	Y, Pl	518–587	12	Pi:H ₁ symport	In	Inorganic phosphate	Pho-5 of <i>N. crassa</i>

(continued on next page)

10	Phosphate:H1 symporter	NHS	2	G2 Bac	418	12	Nucleoside:H ⁺ symport	In	Nucleosides	NupG of <i>E. coli</i>
11	Nucleoside:H1 symporter	OFA	5	Bac, Ar, An	373–470	12	Anion:anion antiport	Both	Oxalate, formate	OxlT of <i>O. formigenes</i>
12	Oxalate:formate antiporter	SHS	3	G2 Bac	407–496	14	Substrate:H ⁺ symport	In	Sialate	NanT of <i>E. coli</i>
13	Sialate:H1 symporter Monocarboxylate porter	MCP	13	Y, An	450–808	12	Substrate:H ⁺ symporter	In	Pyruvate, lactate, mevalonate	Mct of <i>H. sapiens</i>
14	Anion:cation symporter	ACS	40	Bac, Y, An	411–596	12	Substrate:H ⁺ or NaI symporter	In	Glucarate, hexuronate, tartrate, 4-hydroxyphenyl acetate inorganic phosphate, allantoate	ExtU of <i>E. coli</i>
15	Aromatic acid:H1 symporter	AAHS	7	Bac	418–460	12	Substrate:H ⁺ symport	In	Muconate, benzoate; 4-OH-benzoate; 2,4-dichlorophenoxy-acetate, protocatechurate, 3-hydroxypropionate	PcaK of <i>P. putida</i>
16	Unknown major facilitator	UMF	6	Y	600–637	14	Unknown	N/A	Unknown	Yh1040c of <i>S. cereisiae</i>
17	Cyanate permease	CP	3	Bac	393–402	12	Substrate:H ⁺ symport?	In	NCO	CynX of <i>E. coli</i>
18	Proton-dependent	POT	24	Bac, Y, An, PI	463–783	12	Substrate:H ⁺ symport	In	Peptides, amino acids, nitrate, chlorate, nitrite	DtpT of <i>L. lactis</i>

After ref. 1.

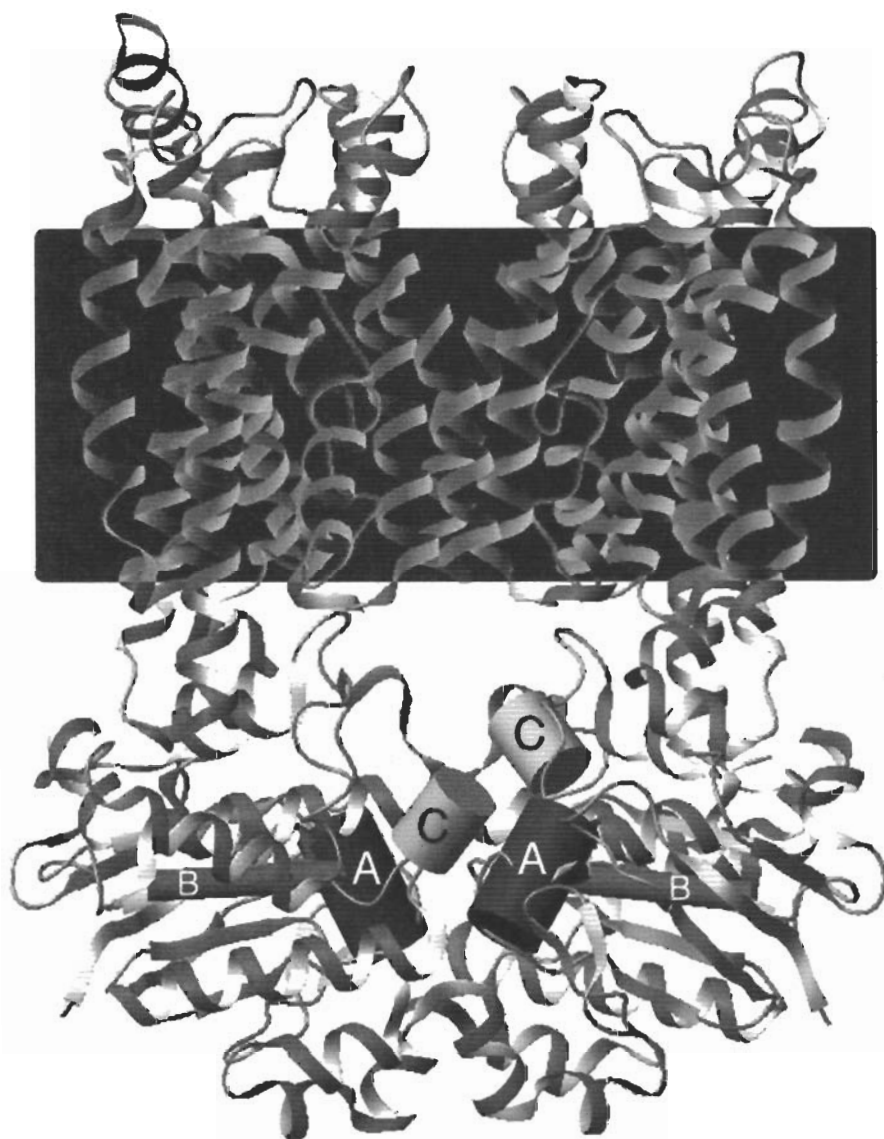


Fig. 2. Crystal structure of the Vitamin B12 transporter. Visualization of protein databank accession number 1L7V (www.rcsb.org) using the molecular-graphics program MolMol. The protein backbone is shown as a ribbon plot; cylinders visualize the following protein motifs: (A) Walker A motif; (B) Walker B motif; (C) ABC signature sequence. The dark background box demarcates the putative boundaries of the cell membrane.

about the exchange of one solute for another (Fig. 1). Currently the SLC superfamily contains 37 families and 237 members. By the end of the Human Genome project, the Human Genome Organization committee anticipates approx 2300 members to make up this class of transporters (7). Because high-resolution structural information is lacking, most SLC proteins have their structures limited to their primary sequence. Nonetheless, the crystal structure of bacteriorhodopsin at 1.55Å resolution was recently obtained and can be used as a starting point for the structural determination of proteins

Table 2
Members of the ATP-Binding Cassette (ABC) Transporter Family

ABC gene subfamily	Description
ABCA (ABC1)	Twelve full transporters. Divided into two subgroups based on intron structure-ABCA1, -2, -3, -4, -7, -12, -13 in one subgroup and ABCA5, -6, -8, -9, -10 in the other. ABCA1 and -4 proteins are involved in cholesterol transport disorder and visual cycle, respectively.
ABCB (MDR/TAP)	Four full and seven half transporters. ABCB1 confers multidrug resistance phenotype to cancer cells. ABCB4 and -11 secrete bile acids and are located in the liver. ABCB2 and -3 are half transporters.
ABCC (CFTR/MRP)	Twelve full transporters involved in ion transport, signal transduction, and toxin secretion. CFTR is a c-AMP regulated chloride ion channel and mutations in this gene cause cystic fibrosis. The MRP proteins transport glutathione and many drug conjugates.
ABCD (ALD)	Four half transporters and are involved in regulation of long-chain fatty-acid transport.
ABCE (OABP)	Not involved in membrane transport functions because they lack the transmembrane domains. Only have ATP-binding domains derived from ABC transporters.
ABCF (GCN20)	Same as ABCE.
ABCG (white)	Six half "reverse" half transporters with ATP-binding domain at the N-terminal and the transmembrane domain at the C-terminal. Involved in cholesterol transport regulation.

Adapted from refs. 2,108.

with similar membrane topology (8). Recently, Zuniga and colleagues used the topology of bacterial lac permease to prepare a homology model of Glut1 that was used successfully to explain certain amino acid point mutations (9). However, alternate techniques like membrane-insertion scanning, Ala-scanning mutagenesis, and *N*-glycosylation scanning have been used to elucidate the topology of these transporters (10).

SLC proteins have significant therapeutic relevance because they play a significant role in cellular processes. They are able to transport a variety of pharmacologically active compounds, enhancing their oral absorption and bioavailability and influencing their PK/PD. Mutations in these transporters have resulted in disruption of transporter

function. For example, a single P290S mutation in the Apical Sodium-dependent bile acid transporter results in a complete loss of the bile acid-uptake function (11).

2. Carrier-Mediated Transport and Prodrugs

To exert their pharmacological activity, drugs must reach their sites of action with certain minimal effective concentration. Most drugs act on specific cellular targets, and in order to reach these targets they must first reach the systemic circulation by penetrating the epithelial barriers covering the absorptive surfaces of the body, such as the skin, intestine, and lung. Transepithelial transport mechanisms for drug molecules include the paracellular route and the transcellular route. In paracellular transport, molecules cross the epithelium through the cells, whereas molecules move across the epithelium through the intercellular junctions between adjacent cells when they use the transcellular pathway. Transcellular transport can be further characterized into passive diffusion and either receptor mediated or active carrier-mediated translocation, the focus of this discussion. In active carrier-mediated transport, a membrane-embedded transport protein transports molecules against a concentration gradient using an energy supply provided by either ATP hydrolysis, or cotransport of ions moving down their concentration gradient, often Na^+ or H^+ . In most biological epithelia, drug molecules confront two obstacles in reaching the systemic circulation: (1) a biochemical barrier resulting from enzymatic degradation, and (2) a physical barrier originating from the lipid bilayer. The first obstacle can be overcome by changing the route of administration or drug formulation, for instance, by encapsulating the drugs in vehicles impenetrable to metabolic enzymes. However, for hydrophilic drugs and drugs with high molecular weight, especially macromolecules, epithelial membranes still impose a formidable barrier. Several methods have been employed to increase the transport of a solute across an epithelial membrane: (1) opening the tight junctions or changing the epithelial lipid bilayer membrane (penetration enhancement); (2) oral lipid-based formulations; and (3) design of prodrugs with increased membrane permeability by lipophilization or targeting to a carrier-mediated transport system. Penetration enhancers have had limited success mainly owing to the limited clinical application because of the nonspecific behavior of many tight junctional perturbants. Lipid-based oral drug delivery has been a more promising means of drug delivery with several formulations currently available on the market, including Sandimmune®Neoral (cyclosporine microemulsion), Norvir® (ritonavir), and Fortovase® (saquinavir). Finally, in recent years significant attention has been placed on the construction of pro-drugs.

Historically, the term prodrug was first introduced by Albert (12), who used the word prodrug or proagent to describe compounds that undergo biotransformation before exhibiting their pharmacological effects. Nowadays, the term prodrug is used to describe a compound that is converted to the pharmacologically active substance after administration (13). Today, rational prodrug design is widely used to overcome problems of absorption, distribution, and biotransformation associated with certain drug molecules. The prodrug concept has been most successfully applied in order to accomplish the following:

- Facilitate absorption and distribution of drugs with poor lipid solubility
- Stabilize against metabolism during oral absorption
- Increase the duration of action of drugs that are rapidly eliminated
- Overcome problems of poor product acceptance by patients

- Eliminate stability and other formulation problems
- Promote site-specific delivery of a drug
- Increase the aqueous solubility
- Lower the toxicity of a drug

In the rational design and synthesis of the ideal prodrug derivative, several factors should be considered: (1) prodrugs should be easily synthesized and purified, (2) prodrugs must be stable in bulk form and dosage form, and (3) neither the prodrug nor its metabolic derivative should be toxic. The vast majority of prodrugs are designed to increase the intestinal absorption of polar drugs—that is, to increase lipophilicity. Prodrug design by increasing lipophilicity has been proven successful. Various ester prodrugs of 6-azauridine (6-AZA), a compound used to treat psoriasis, such as 2',3',5'-triacyl-6-AZA and 2',3',5'-tribenzoyl-6-AZA have been developed. Orally administered 2',3',5'-triacyl-6-AZA caused the same clinical effects as an equivalent intravenously administered dose of 6-AZA (14).

Charged hydrophilic compounds and pharmaceutical macromolecules encounter difficulties in permeating the cell membrane. However, the systemic absorption of many water-soluble nutrients (e.g., sugars, vitamins), endogenous proteins (e.g., insulin, growth factors), and toxins (e.g., ricin, cholera toxin) appears to be highly efficient. The effective transcellular movement of these molecules is facilitated by specialized transport processes in the epithelia, that is, carrier-mediated transport and receptor mediated endocytosis/transcytosis. Both processes are operated by specific membrane-associated proteins, which are concentration-, energy-, and temperature-dependent.

Apart from naturally occurring substrates, it is now well-recognized that many drugs can be selectively taken up by active transport processes. For pharmaceutical scientists, these membrane transporters provide alternative routes for the delivery of drugs that would normally be impermeable to the biological barriers. Using a method similar to the conventional prodrug approach, absorption is enhanced by formation of conjugates between drugs and the endogenous ligands of the membrane transporters. Consequently, through specific interactions between the moiety and its transporter, drug candidates can be shuttled across or into the cells and eventually be released from the ligands.

Furthermore, taking advantage of recent advances in molecular biology and computer modeling, scientists are now starting to design prodrugs based on structural requirements of the transporter systems. In general, prodrug strategies involving carrier-mediated pathways have the advantage of high uptake capacity. However, the size of drug conjugates is relatively limited (~1000 Da), probably because larger conjugates fail to be shuttled through the restricted space within the carrier protein. For peptide and protein delivery, carrier-mediated pathways could facilitate peptides only up to four amino acids, although further studies are under way to explore the full potential of this promising pathway. In the following sections, various transporters and prodrug approaches targeting individual transporters will be discussed.

2.1. Sodium-Dependent Multivitamin Transporter (SMVT)

Water-soluble vitamins are not synthesized by the body and therefore must be provided in the diet in order to allow for normal growth and development. Often many of these coenzyme precursors are absorbed into the body through passive diffusion in the intestine. However, uptake of biotin, pantothenate, and the cofactor lipoate occurs by means of an active, carrier-mediated process. Furthermore, it has been reported that

this process is dependent on a Na^+ -gradient and is not stimulated by an inside-negative membrane potential. Although the name appears to be a misnomer, because the protein transports many other compounds beside vitamins, it has been designated the SMVT (15).

SMVT is a protein of 635 amino acids with 12 transmembrane domains and is responsible for the transport of the water-soluble vitamins biotin, pantothenate, and lipoic acid (16,17). It is expressed in the placenta, intestine, brain, liver, lung, kidneys, and heart in rats, rabbits, and humans (17). Substrate specificity studies indicate that the transporter is specific for the aforementioned compounds as well as biotin analogs with a Na^+ :substrate stoichiometry of 2:1. It appears that the carboxylate group of the long side-chain of the substrate molecule serves as the point of interaction for the transporter (16).

The knowledge and characterization of this transporter has led to SMVT targeting as an approach to intestinal drug absorption by chemical modification of drugs to prodrugs and analogs. Recently, Sinko and colleagues characterized biotin conjugates for the treatment of cancer and HIV (18–20). Tat9-C is a 10-amino acid inhibitor of HIV-1 Tat protein that binds to the TAR domain of viral RNA preventing Tat-dependent gene expression in cultured cells. Tat9-C(biotin) and Tat9-K(biotin), biotinylated forms of Tat9-C were taken up 30-fold more efficiently by Jurkat cells than Tat9-C (21). Subsequently, Sinko and colleagues used a retro-inverso derivative of Tat9-K(biotin) denoted R.I.-K(biotin)-Tat9, which was more resistant to proteolysis and had similar TAR RNA binding activity to Tat9-K(biotin) in a mechanistic study of transport to determine its suitability for oral administration (18). They demonstrated both the inhibition of R.I.-K(biotin)-Tat9 transport across Caco-2 cells and the inhibition of R.I.-K(biotin)-Tat9 uptake in CHO/hSMVT cells in the presence of biotin, implicating that the compound as a substrate for SMVT. Similar studies confirmed SMVT in the transport of the 29 kDa peptide-loaded bioconjugate (PEG:[R.I.-Cys-K{biotin}-Tat9]₈).

In addition to the biotinylation of HIV inhibitors, the conjugation of biotin to camptothecin (CPT) has been explored (20). CPT is an anticancer agent whose mechanism of action is the conversion of DNA topoisomerase I into a DNA-damaging agent. Biotinylation of CPT-PEG resulted in a significant increase of cytotoxicity in human ovarian carcinoma cells as compared to CPT-PEG and CPT alone, suggesting the involvement of SMVT in uptake. Thus the targeting of SMVT transporters provides an alternate approach to increasing the bioavailability of compounds especially after oral delivery.

2.2. Sugar Transport

Intestinal sugar transporters are responsible for transporting the monosaccharides glucose, galactose, and fructose from the intestinal lumen to the blood. The sodium ion-dependent glucose transporter (SGLT1) was the first transporter to be cloned and is responsible for the active transport of glucose across the intestinal brush border (22). SGLT1 is a hydrophobic integral membrane protein with approx 12 putative membrane-spanning domains. In addition to SGLT1, there exist other intestinal monosaccharide transporters, including GLUT5 and GLUT2, which are sodium ion-independent facilitative transporters. GLUT5 is responsible for the transport of fructose from the lumen to the cytosol, whereas GLUT2 transports glucose, fructose, and galactose from the cytosol to the blood. Also, a homolog of SGLT1, designated SGLT2 and sharing 59% amino acid homology, has been identified in the human kidney and is responsible for the reabsorption of hexoses (23,24).

The ability to conjugate sugars transported by SGLT1 or GLUT5 to drug molecules provides a means of getting otherwise poorly absorbed drugs into the blood stream. Various research has been performed to test this hypothesis. Using *p*-nitrophenol and 2-naphthol linked to various sugars, it was verified that these compounds moved across everted rat jejunum in a similar manner to that of *D*-glucose (25,26). Consistent with sodium dependent transport, replacement of sodium ions with potassium ions in the incubation media or the presence of phlorizin significantly inhibited transport. Furthermore, the importance of the type of sugar and the nature of the linkage were investigated, and demonstrated that glucose conjugates were most efficiently transported and β -anomers were more effective than α -anomers.

Conjugation of sugars to peptides was also investigated as a means of increasing absorption. The everted sac technique was used to examine intestinal transport of the tripeptide, tyrosyl-glycyl-glycine (YGG), a component of enkephalin, coupled to glucose and several disaccharides (27). Uncoupled YGG was hydrolyzed by aminopeptidase and no transport was observed. However, sodium ion-dependent transport from the mucosal to the serosal side was observed using the sugar derivatives and was greatest with glucose coupled YGG. Nomoto and colleagues used the tetrapeptide Gly-Gly-Tyr-Arg (GGYR) glycosylated with *p*-(succinylamido)phenyl α or β -*D*-glucopyranoside (α , β -SAPG) to examine uptake into brush border membrane vesicles (BBMV) and transport through the intestinal membrane using the everted sac method (28). As with YGG, these studies implicated SGLT1 in the uptake and transport of these compounds.

A more clinically relevant approach was used by conjugating the peptide insulin (INS) with succinylamidophenyl (SA)- α -*D*-glucopyranoside (SAPG), SA- α -*D*-mannopyranoside, or SA- α -*L*-arabinopyranoside, and determining the hypoglycemic effect in rats (29). The greatest effect of the modification of insulin occurred with SAPG-INS, resulting in a 56% decrease in blood glucose levels when administered intra-intestinally. Furthermore, the digestion of monosaccharide derivatives by pepsin and chymotrypsin indicated that the resistance of insulin to enzymatic degradation was increased by its modification with monosaccharide. Thus sugar conjugates may be a means to enhance the hypoglycemic effect of insulin by increased stability and targeting the sugar transporter.

In addition to using the prodrug approach for intestinal transport work has been done investigating the transport of peptides across the blood-brain barrier (BBB) with sugar-coupled peptides (30,31). GLUT1 is a facilitated glucose transporter similar in characteristics to GLUT2 and GLUT5 and has been used as target for sugar-coupled enkephalins, endogenous peptides that control brain function. For instance, the δ -selective glycosylated Leu-enkephalin amide 2, H_2N -Tyr-D-Thr-Gly-Phe-Leu-Ser(β -*D*-Glc)-CONH₂, produced analgesic effects similar to morphine. Also, Battaglia and colleagues report the use of *D*-glucose conjugates of 7-chlorokynurenic acid in rats to protect against seizures (32). The development and use of further such compounds promises enhanced delivery of membrane-impermeable drugs through the BBB for the treatment of migraines as well as neurological disorders.

2.3. Amino Acid Transport

As expected, there are numerous amino acids transporters, especially in the intestine. These include sodium-dependent transporters such as systems B, IMINO, α , A, and ASC, and sodium-independent systems y^+ , b, asc, and L. The focus of this section is merely an overview of the targeting of amino acid transporters for drug delivery. For a more detailed characterization of the various amino acid transporters, see refs. (33,34).

System L, including LAT1, which is responsible for the transport of large neutral amino acids in a sodium-independent manner, has been widely studied because of its broad substrate selectivity and its ability to transport amino acids through the intestine as well as the BBB (35). It has been demonstrated that substrates for LAT1 must have a free carboxyl and an amino group. The carbonyl oxygen closer to the amino group needs a computed charge of -0.55 to approx -0.56 and must not participate in hydrogen bonding. Furthermore, the hydrophobic interaction between the substrate side chain and the substrate binding site of LAT1 is crucial for the substrate binding (36).

System L has been used to transport amino acid-analogs such as L-dopa, a therapeutic drug for the treatment of Parkinson's disease (37); the opioid baclofen (38); as well as the anticancer agents melphalan and acivicin (39,40). Yang and colleagues synthesized amino acid ester prodrugs of acyclovir and investigated the nasal uptake of these compounds. They demonstrate improvement of nasal absorption of the L-aspartate β -ester prodrug of acyclovir by the amino acid transporter, demonstrating the potential of designed prodrugs for efficient transmucosal delivery (41). More recently, Balakrishnan provided evidence of the presence of a large, neutral amino acid transport system on the corneal epithelium. The rabbit corneal cell line (SIRC) as well as freshly excised rabbit cornea were utilized as a model, and uptake studies were performed with ^3H -L-tyrosine to demonstrate involvement of the amino acid transporter (42).

2.4. Nucleoside Transport

Nucleosides are the precursors of nucleotides and form the building blocks of nucleic acids. Natural nucleosides are hydrophilic molecules (43), and cannot easily cross the cell membrane by nonfacilitated diffusion, and therefore nucleoside transporters are necessary for the permeation of nucleosides across biological membranes. Thus nucleosides permeate most animal cells via multiple transporters present in the plasma membrane of most cell types (44,45). These transporters can be grouped into two broad categories: (1) Na^+ -dependent concentrative transporters that mediate only the influx of nucleosides and (2) the Na^+ -independent equilibrative transporters that mediate both influx and efflux of nucleosides. The various subtypes of the two major nucleoside transporters are listed below (see Table 3).

The equilibrative nucleoside transporters (ENTs) are broadly selective accepting both purines and pyrimidines, have low affinity, and exhibit high rates of transport properties. This group of transporters is further subdivided based on their sensitivities to nitrobenzylmercaptapurineriboside (NBMPR) (46) into *es* (equilibrative-sensitive) and *ei* (equilibrative-insensitive) transporters. Equilibrative transporters are widely distributed among various cell types and only very recently have the human *es* and the *ei* subtypes been identified by cloning (47), through the use of placental and HeLa cell cDNA libraries (48). They have been designated as human equilibrative transporters 1 and 2 (hENT1 and hENT2), respectively. Both these transporters contain 456 amino acid residues, share 50% sequence identity, and are thought to contain 11 transmembrane spanning regions with a cytoplasmic N-terminus and an extracellular C-terminus (49). The regions important for inhibitor binding have been identified by creating chimeras from hENT1, rENT1, and rENT2 cDNAs (50), and performing transport-inhibition studies in *Xenopus laevis* oocytes. These studies indicated that transmembrane domains 3–6 comprising amino acids 100–231 in the hENT1 are important for interaction with dilazep and dipyrindamole (51).

Table 3
Characteristics of Nucleoside Transport System

Transporter	Property	Substrate selectivity ^a	Tissue Distribution ^b	NBMPR ^c	Na ⁺ -dependent?
SPNT	Concentrative	Pu, U	Rat liver, jejunum	No	Yes
rCNT1	Concentrative	Py, A	Intestine, kidney	No	Yes
hCNT1	Concentrative	Pu, U	Kidney, intestine	No	Yes
hCNT2	Concentrative	Py, A	Kidney, brain, heart, liver	No	Yes
hCNT3	Concentrative	Pu, Py	Bone marrow, intestine	No	Yes
N4	Concentrative	Py, G	ND	Yes	Yes
N5	Concentrative	G, FB	ND	Yes	Yes
hENT1	Equilibrative	Pu, Py	ubiquitous	Yes	No
hENT2	Equilibrative	Pu, Py	Skeletal Muscle, heart, pancreas	No	No

Adapted from ref. 44.

^aPu, purine; U, uridine; Py, pyrimidines; A, adenosine; G, guanosine; FB, formycin B.

^bND, not determined.

^cDenotes whether the selected transporter is sensitive to NBMPR.

Na⁺-dependent concentrative nucleoside transporters (CNT) are widespread and have been expressed in the intestine, kidney, liver, spleenocytes, and many cultured cell lines (52). The first member of the CNT was cloned from rat jejunum and designated as the rat concentrative nucleoside transporter1 (rCNT1) (53). Since then, the family has expanded rapidly to include rCNT2 from the rat intestine and the human counterparts to both the rat transporters, hCNT1, and hCNT2, respectively (54). This group of transport processes has been further divided into five classes (N1-N5) on the basis of their substrate specificity (*see* Table 3). The SPNT/hSPNT1 (previously named N1 [cif]) system is widely distributed and is purine specific with high selectivity towards uridine, guanosine, and adenosine. hCNT1/rCNT1 (previously N2 [cit]) is more selective towards pyrimidine, especially thymidine, whereas N3 (cib) exhibits broad specificity and transport both purines and pyrimidines. N4 is found in the human kidney and is similar to N2, but additionally transports guanosine. Finally, N5 has been found in leukemic cells, with specificity toward guanosine (43), but is more sensitive to inhibition by NBMPR compared with other subtypes (55). The mammalian CNT family proteins are about 650 amino acids in length and exhibit about 64% identity (56). Recent studies have shown that the mammalian CNTs possess 13 transmembrane segments and a very long glycosylated extracellular C-terminus (57). Residues 319 and 320 in hCNT1 were found to be important for the pyrimidine nucleoside specificity (58).

Structurally modified nucleosides are pharmacologically active molecules that have been used in the treatment of cancer and as antiviral therapies (59). They have the ability to interfere with normal nucleotide metabolism and DNA replication, promoting antiproliferative effects and preventing replication of the viral DNA in infected cells. The transport of nucleoside analogs across the gastrointestinal tract is mediated by the transporters described earlier (i.e., CNT1 and SPNT). Most of these nucleoside analogs inhibit natural nucleoside transport by competitive inhibition (60,61). All nucleoside analogs must be phosphorylated to their triphosphate form in order to be biologically active. This is accomplished by various enzymes including the salvage

enzyme deoxycytidine kinase (62). Incorporation of the triphosphate analogs interferes with DNA elongation during replication and repair of DNA damage (63). In addition to their DNA chain terminating effect, it is believed that both expression and activity of nucleoside transporters may be affected by treatment with the analogs (59).

In order to improve the bioavailability of polar drugs such as nucleoside antivirals, membrane transporters located in the intestinal epithelium are usually targeted. However, because the nucleoside transporters themselves are of high-affinity/low-capacity, alternate approaches have to be sought. The intestinal PcpT1, which will be discussed in more detail later, is one such transporter. It has been targeted to improve the bioavailability of nucleoside amino acid ester prodrugs of acyclovir and AZT (3'-azido-3'-deoxythymidine), because this transporter is characterized by high capacity and low affinity (64). AZT was the first drug approved for the treatment of AIDS and has an oral bioavailability of up to 70% (65). AZT uptake has been found to be Na⁺-dependent in rat jejunum (54), but the primary mode of AZT absorption was found to be by passive diffusion (66). Many chemotherapeutic drugs used in the treatment of cancer are also nucleoside derivatives (59), such as the pharmacologically active fluoropyrimidines, which inhibit thymidylate synthase, causing a reduction of phosphorylated forms of deoxythymidylate, and resulting in apoptosis (67). Gemcitabine (2',2'-difluorodeoxycytidine dFdC), a very recently developed agent in the treatment of solid tumors, has been found to penetrate the cell via *es* nucleoside transporters, and Ara-C is yet another significant anticancer drug that is a substrate for nucleoside transporters (68).

Various mammalian expression systems like the COS-1 cells and oocytes have been used to study the interactions of the nucleoside analogs with the nucleoside transporters (44,69). More recently computer simulations have been utilized to study such actions. Our laboratory recently reported three-dimensional quantitative structure-activity relationship (3D-QSAR) models of each of the nucleoside transporters, based on their available binding profiles. We used comparative molecular field analysis (CoMFA) and GOLPE to generate models that relate the differences among the substrates to their bioactivity (69a). Such information could provide future insight into the design and use of agents targeting the nucleoside transporters. However, further studies need to be carried out to understand completely the interactions between the transporters and the nucleoside derivatives.

2.5. The Intestinal Peptide Transporter

The intestinal oligopeptide transporter (PepT1) is located in the brush-border membrane of the intestine. It provides the main pathway for absorption of dietary nitrogen by mediating the cellular uptake of dipeptides and tripeptides (70). It also transports a number of peptidomimetics such as β -lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors and, possibly, renin inhibitors (71). As a result of this broad substrate specificity and its ability to transport a variety of peptidomimetic drugs, PepT1 has captured much attention as a target for mucosal drug delivery (72).

PepT1 belongs to a larger family of oligopeptide transporters, the proton/oligopeptide transporter (POT) family, in which only two human members hPepT1 and hPepT2 have been identified (73). It is an integral membrane protein, 707 amino acids long with a molecular weight of 79 kDa and 12 putative membrane spanning regions with a cytoplasmic N- and C-terminal (74). First cloned in rabbit by expression cloning (75), it was later expressed in rat (76) and human (77). The protein has several N-glycosylation sites, and the rat and human PepT1 have one and two protein kinase C

(PKC) phosphorylation sites, respectively. Transport studies indicated that uptake was electrogenically coupled to an inward flow of protons with a 1:1 stoichiometry (78). The transporter has a low affinity, with a K_m value in the millimolar range (79), and a very broad substrate specificity (64,80). The high degree of tolerance of the transporter for structurally diverse substrates necessitates the need to investigate its substrate-binding domains. The lack of a crystal structure has led to the development and use of several experimental and computational approaches in order to obtain information about the structural specificity toward its substrates (72). In brief, a systematic investigation of the structural influence on uptake using a number of di- and tripeptides, β -lactam antibiotics in isolated brush-border membrane vesicles (81), molecular modeling to identify the pharmacophore for the substrate binding (82), site-directed mutagenesis (83), and engineered chimeras between PepT1 and PepT2 (84) are a few of the methods that have been employed.

The human H⁺-coupled oligopeptide transporter has been suggested as an excellent target for the delivery of pharmacologically active compounds because of its ability to accommodate a wide variety of molecules with varied size, hydrophobicity, and charge at its substrate-binding site (85). Most substrates for the peptide transporter, as implied by its nomenclature, are peptide in nature. For example, cephalixin resembles Phe-Cys-Val and enalapril resembles Phc-Ala-Pro, whereas β -lactams are dipeptide analogs. The intestinal peptide transporter has been employed to improve the intestinal absorption of certain drugs by converting them to di- or tripeptide type prodrugs (86). For example the low bioavailability of α -methyldopa, an antihypertensive agent (interestingly, in itself a substrate for the structurally restrictive sodium-coupled amino acid transporter) has been improved by converting it into a dipeptide prodrug, α -methyldopa-L-phenylalanine, which is then efficiently taken up by PepT1 (87). However, in 1998, Balimane and colleagues reported that the drug valacyclovir, which lacks a peptide bond, acts as a substrate for PepT1 (88). Valacyclovir is the water-soluble 5'-L-valyl ester prodrug of acyclovir. Acyclovir, a nucleoside analog, was the first antitherapeutic agent capable of selectively inhibiting the replication of herpes simplex virus (HSV), producing little or no adverse side effects (43,88). However the low oral bioavailability of acyclovir hampered the efficient use of this drug. The prodrug valacyclovir has a mean oral bioavailability that is three to four times greater than that of acyclovir, with a dose-dependent rate of absorption (89). The mechanism of intestinal absorption was studied in three different experimental systems: *in situ* rat perfusion model, CHO/hPEPT1 cells, and Caco-2 cells (64). The results indicated a high affinity of the prodrug to the peptide transporter. Furthermore, uptake was significantly inhibited by the most common substrate (Gly-Sar) of PepT1, implicating it in the transport of the amino acid esters.

Based on this peptidomimetic approach, it may be possible to create other polar antiviral and anticancer therapeutic agents with increased oral bioavailability. In patients suffering from cytomegalovirus (CMV) infection, clinical trials have shown valganciclovir, the valyl ester of ganciclovir increases the bioavailability of ganciclovir when taken orally. Cloned hPEPT1 was expressed in *X. laevis* oocytes and exposed to varying concentrations of ganciclovir and valganciclovir, to detect currents. Exposure to ganciclovir did not produce any detectable currents, however inward currents were associated with valganciclovir exposure. Their experiments demonstrated that valganciclovir has a high affinity for hPEPT1 and is readily converted to its pharmacologically active parent compound ganciclovir in the intestine, once transported by hPEPT1 through the brush border membrane into the mucosal cells.

2.6. The Apical Sodium-Dependent Bile-Acid Transporter (ASBT)

The metabolism of cholesterol to bile acids is an important route for the elimination of cholesterol from the body. Bile acids are secreted from the gallbladder into the proximal jejunum, where they are passively absorbed by jejunal and ileal enterocytes. At the distal ileum, a sodium-dependent bile-acid transporter (ASBT) exists on the apical surface of the enterocyte. This transporter accepts bile acids, which then enter into the portal system where they complete enterohepatic circulation. ASBT is officially referred to as SLC10A2, an approximately 4 Kb mRNA message that codes for a 348 amino acids protein. ASBT was first cloned from hamster by expression cloning (90). Later human (11), rat, rabbit, and mouse (91) were also cloned. The interspecies protein homology is approx 85–90%, however, the K_m values of hamster and human ASBT are 33 and 17 μM , respectively (90). ASBT is an electrogenic active transporter with a 2:1 Na^+ : bile-acid coupling stoichiometry (92). The apical sorting signal for ASBT is present at the C-terminal of ASBT (93). The vectorial transport of conjugated bile acids is efficiently regulated by two bile-acid transporters, NTCP (sodium taurocholate transporting protein) and ASBT in the liver and intestine, respectively. These two transporters share a 35% sequence identity and an overall similarity of 63%. Studies have shown that NTCP and ASBT topologies are similar and almost overlap on hydropathy plot analysis (94). Based on hydropathy analysis and an exoplasmic N-terminus and a cytoplasmic C-terminus (95), ASBT was proposed to have a seven-transmembrane model. However, Hallén and colleagues suggested the possibility of a 9TM model based on membrane-insertion scanning analysis (96). This group predicted TM3 and TM 4 region to be made up only 9–10 amino acids each, which suggests that these regions must adopt a β -sheet conformation in order to span the membrane effectively. Also they predicted that TM2 and 3 and TM8-9 are linked by two and four extramembraneous amino acids, respectively, suggesting a very sharp turn in helical structure. To address the topology controversy, our laboratory recently carried out *N*-glycosylation scanning analysis that strongly supports a 7TM topology model for hASBT (97).

In order to elucidate the transport mechanism of ASBT, it is essential to first identify the residues/regions involved in the transport mechanism. It is believed that the charged residues on the extracellular side of the transporter may be involved in the initial substrate recognition and binding. Several studies probed its functional regions by chemical modifications using amino specific reagents (98). Studies using a 3D QSAR pharmacophore model also predicted several important interactions between bile acids and ASBT (99). Their studies on rabbit ASBT led to the development of a 3D pharmacophore model. The model predicts that ring D, the methyl group at position 18 and the α -hydroxyl groups at positions 7 and 12 are essential for ASBT affinity and binding. The hydroxyl group at position 3 allows for certain flexibility and did not interfere with active transport.

ASBT is a transporter with significant pharmaceutical relevance. It has been targeted for drug delivery and has recently been studied as a pharmacological target for cholesterol-lowering therapy (100,101). Kramer and group have shown that bile-acid transporters can efficiently transport bile acid-drug conjugates. Swaan and colleagues conjugated small peptides at the C-24 position, which could also be transported by the bile ASBT (102). Based on these studies, they developed a 3D QSAR model for ASBT using comparative molecular-field analysis (103). The carrier-mediated bile acid transport for targeted drug-delivery purposes can be applied to the liver and gall bladder for

directed delivery. It can also be used to enhance oral absorption of various prodrugs and lower serum cholesterol levels (73). Bile acid-transport systems have been used successfully to target various inhibitors such as HMG-Co A reductase inhibitors to the liver. These inhibitors or drug molecules, when conjugated to bile acids, seem to retain their affinity with the bile-acid carrier. ASBT has also been targeted for lowering of serum cholesterol levels. Previously HMG-CoA reductase inhibitors and various bile-acid sequestrants, such as cholestyramine, were used to lower cholesterol levels (104). Bezothiazepine derivative was able to inhibit active ileal bile-acid absorption in mice rats, monkeys, and humans (105). It has also been shown that inhibition of ASBT by SC-435 significantly reduced plasma low-density lipoprotein (LDL) cholesterol levels through enhanced LDL receptor-mediated LDL apoB clearance (106). Inhibition of the intestinal bile-acid transporter is thought to result in an increased fecal bile-acid excretion and lower plasma LDL levels after oral administration of the various drugs.

3. Conclusion

A substantial role has been recognized for transport proteins in oral absorption and drug bioavailability; drug resistance; excretion of drugs and their metabolites, mediated by transporters in the kidney and liver; drug toxicity; and drug pharmacokinetics and pharmacodynamics. Furthermore, the pathophysiology of several hereditary diseases has been attributed to mutations in transport proteins. Overall, solute transporters play an invaluable role in fundamental cellular processes in health and disease, and function as important mediators governing all aspects of drug therapy.

Although the importance of transporters in shuttling drugs across intestinal and other tissues was recognized in the late 1970s, research in the transporter field did not start in earnest until after the initial expression cloning of the sodium-dependent glucose transporter, SGLT1, in 1986 (22). The resulting "transporter revolution" led to the cloning of numerous pharmacologically important transporters, as evidenced by an exponential increase in the number of publications on transporters over the last decade.

Despite the apparent clinical importance of transporter proteins, the knowledge of their structure and mechanism of action has lagged far behind the knowledge of these properties of proteins in general. As a result, we are unable to predict the interaction of drugs with this important class of membrane proteins *a priori*, and detection of drug-transporter interactions remains unacceptably serendipitous. It has been clearly recognized that transporters and their natural substrates may provide an excellent target for increasing the epithelial transfer and systemic availability of therapeutic moieties. The prodrug approach has been applied successfully to target several transporters, but the widespread application of this technique by means of "rational design" is hampered by a lack of structural data. Therefore, it is imperative that scientists direct their attention to investigating structure-function and structure-activity relationships of membrane transporters and, eventually, solve their molecular structure using high-resolution techniques. Clearly, *in silico* transporter models are a first step in this direction, and the increased availability of data sets from single transporter expression systems should facilitate the construction of more reliable and predictive 3D-QSAR models (107).

Finally, it should be noted that certain transporters can serve as "anti-targets," i.e., systems that negatively impact drug therapy, such as the drug resistance-associated transporters P-gp, MRP, and BCRP. By screening compounds for potential interaction with these systems *in silico*, one can avoid low response rates and, ultimately, optimize drug therapy.

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