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Review

Transporter-Mediated Drug Delivery

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Abstract: Transmembrane transport of small organic and inorganic molecules is one of the cornerstones of cellular metabolism. Among transmembrane transporters, solute carrier (SLC) proteins form the largest, albeit very diverse, superfamily with over 400 members. It has been early on recognized that xenobiotics can directly interact with SLCs and that this interaction can fundamentally determine their efficacy, including bioavailability and intertissue distribution. Apart from the well-established prodrug strategy, the chemical ligation of transporter substrates to nanoparticles of various chemical composition has recently been used as a means to enhance their targeting or absorption. In this review, we summarize efforts in drug design exploiting interactions with specific SLC transporters to optimize their therapeutic effects. Furthermore, we describe current and future challenges as well as new directions for the advanced development of therapeutics that target SLC transporters.

Keywords: membrane transporter; SLC; solute carrier; drug design; pharmacokinetics; prodrug; nanoparticle; bile acids.

1. Introduction

In this review, we focus on the delivery of drugs via transmembrane transporters, especially the SLC solute carriers, through the cellular plasma membrane or various intracellular membranes. SLC solute carriers include all transmembrane proteins that enable the translocation of solutes such as nutrients, metabolites, ions and xenobiotics across the membrane in a facilitative or secondary active manner. Together with the ATP-dependent ABC transporters (see below), transporter proteins form an essential protein machinery for the regulation the cellular and systemic homeostasis of all solutes in our body, as well as for the maintenance of the necessary ion gradients such as the inwardly directed Na^+ gradient at the intestinal brush border membrane of enterocytes, to drive uptake of solutes in a Na^+-coupled secondary active manner, as is the case for intestinal uptake of glucose via SLGT1/SLC5A1 [1].

Transport proteins are often categorized by their mechanism of transport. ATP-binding cassette (ABC) transporters are primary active transporters that, in higher organisms, use ATP hydrolysis to drive transport of solutes across the membrane out of cells [2,3]. Co-transporters are secondary active transporters that couple solute translocation to the cotransport or countertransport of either inorganic ions or other solutes; in the latter case, they are often called exchangers. Facilitative transporters translocate solutes across the membrane according to their electrochemical gradient. Indicated earlier, secondary and facilitative transporters in both human and higher organisms are represented by the SLC solute carrier superfamily of proteins, a diverse, heterogenous group of proteins likely of polyphyletic origin [4,5].

The ABC and SLC transporter superfamilies in human consist of 48 and over 400 proteins, respectively, out of which ~11 ABC transporters [6,7] and ~26 SLC transporters [6] are thought to be directly involved in drug translocation. Many of these transporters are present in the plasma membrane of liver and kidney cells, as well as in the cells of biological barriers, and they fundamentally shape the pharmacokinetics of small-molecule

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drugs in the body [6]. Apart from exploring the interaction of existing drugs with transporters, approaches have been developed to exploit the specific cellular localizations and transport activities of transporters as gateways for the delivery of therapeutics to specific organs or across specific biological barriers. In this review, we summarize such approaches and then speculate on potential new directions and applications.

There are several factors that motivate the targeting of transporters expressed in specific tissues. For some medications, crossing through specific barriers is vital for their action, e.g., central nervous system (CNS) drugs need to cross the blood-brain barrier (BBB). For others, it provides an advantage for the therapeutic delivery, e.g., switching from intravenous to oral administration of chemotherapeutics that should be able to cross the intestinal barrier. In yet other cases, they can be beneficial for targeting drugs to specific cell types, e.g., targeting chemotherapeutics to tumor cells while sparing healthy cells. The specific and/or highly abundant expression of certain transporters in biological membranes makes them good candidates for such efforts, and in addition, transporter expression levels are typically less variable than those of receptors, making them more attractive targets [8]. Furthermore, transporters generally have a more promiscuous binding site for their ligands than receptors, making them more suitable for interaction with a broader range of small molecules [9]. In addition, the substrates of transporters are typically small molecules that are stable, readily modifiable and non-immunogenic, whereas cell surface receptors typically interact with and recognize large molecules (e.g., low-density lipoprotein, transferrin) [9]. These aspects point to the specific advantage of using transporters instead of receptors for targeted drug delivery strategies.

Knowledge of the distribution of transporter proteins in various human tissues is critical for understanding their role in drug metabolism and their usefulness as drug delivery targets. Because their importance in pharmacokinetics was recognized long ago, the presence of transporters in the intestine, liver and kidney, the major organs that determine ADMET (absorption, distribution, metabolism, excretion, toxicity) of drugs [6] as well as in the BBB, which determines the access of CNS drugs to the brain, has been studied intensively. For all oral formulations, the drugs must be able to cross the intestinal barrier after ingestion to be successfully absorbed into the body. The intestinal brush border membrane and mucus layer form the intestinal barrier [10], which is lined by numerous transporters for nutrient absorption [11,12]. After successful passage across the enterocytes, the absorbed drugs either enter the hepatic portal veins for direct delivery into the liver or, if they are lipophilic, enter the lymphatic system, thus avoiding the hepatic first pass effect. The liver has a variety of promiscuous transporters capable of taking up a wide range of xenobiotics [6,11], whereupon they are metabolized by hepatocytes into less toxic and more water-soluble small compounds [13]. The drug metabolites are then excreted back into the bloodstream or the bile via various transporters [13,14]. Polar drug metabolites are cleared from the body by the kidneys, unless a specific transport mechanism exists in the renal proximal tubule cells for their reabsorption [15].

Certain organs (e.g., brain/CNS, retina, testis) are protected by a layer of endothelial cells that form barriers with regulated permeability through tight junctions between the blood and the underlying organ tissues. Drugs that act in these organs, particularly those that act in the CNS, must cross these barriers. It is well known that some of them utilize nutrient transporters present on the endothelial cell membranes [16,17] and it is estimated that 10-15% of all proteins in the neurovascular unit are membrane transporters [18]. In certain cases, it is necessary to prevent a drug that does not act in the CNS from reaching the brain. Therefore, knowledge of the presence of transporters at such barriers is of utmost importance, both for drug targeting and for avoiding undesirable off-target effects of drugs.

2. Strategies for utilizing transporters for drug delivery

In this section, we summarize various approaches to exploit transporters present in specific tissues for targeted drug delivery or more efficient drug absorption.

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Several currently developed drugs have in their molecular design structural features that can be recognized by specific transporters abundant in certain tissues to improve their pharmacokinetic properties and minimize off-target effects [11]. A prodrug strategy is often followed in which a known substrate molecule of an uptake transporter is chemically conjugated with the active drug molecule of interest [12,19,20]. Recognition of the substrate moiety by the transporter then triggers uptake of the entire molecule into the cell. Once in the cytoplasm, the prodrug is designed to be cleaved and processed by active enzymes to release the active drug molecule (Figure 1). A review of prodrugs with targeted SLC-mediated absorption can be found in [12]. Numerous prodrug strategies have been developed targeting nutrient transporters to cross the intestinal or the blood-brain barrier. These transporters include amino acid and oligopeptide transporters (SLC7A5/LAT1, SLC15A1/PEPT1), vitamin transporters (SLC23A1/SVCT1, SLC5A6/SMVT), sugar transporters (SLC5A1/SGLT1), bile acid transporters (SLC10A2/ASBT) and carnitine transporters (SLC22A5/OCTN2). More detailed examples and recent developments of prodrugs utilizing these transporters are discussed in the next chapter.

It is often desirable that a drug is not transported into specific cells or across biological barriers to reduce off-target the side effects. For example, first generation H1 histamine receptor antagonists, such as diphenhydramine, chlorpheniramine and cyproheptadine, are cationic drugs that exhibit sedative side-effects in the CNS because they can effectively enter the brain [21]. In contrast, second generation H1 antagonists, such as fexofenadine, cetirizine or ebastine, do not exhibit sedative side-effects. Fexofenadine and cetirizine have been shown to be substrates for ABCB1/P-gp, a key drug efflux transporter at the blood-brain interface, limiting drug availability in the brain [22–24]. Ebastine is rapidly converted to carebastine, which is also pharmaceutically active [25]. However, unlike ebastine, carebastine is also a good substrate for ABCB1/P-gp, while it is a poor substrate for the uptake transporters in brain capillary endothelial cells (BCECs), thereby likely contributing to reduced CNS side-effects [25]. These examples illustrate that, in certain cases, it may be advantageous to design drugs to be good substrates for drug efflux transporters to avoid their permeation across biological barriers.

A more recently developed and highly promising strategy for drug delivery is based on the production of nanoparticles, generally smaller than one micrometer, that can encapsulate drug molecules and release them under certain conditions. The purpose of encapsulation is either to increase the solubility of the drug molecule or to protect it from oxidizing conditions in the gastrointestinal tract. Interestingly, there are physiological limitations to the size of the nanoparticles that can be used. Very small particles (<10 nm) are filtered in the renal glomerulus [26], while ~25 nm diameter is the enthalpic limit for the initiation of endocytic processes on the cellular surface according to kinetic models [27]. On the other hand, oversized particles (>200 nm) could activate the complement system, resulting in accumulation in the liver and spleen [28]. In order to enhance drug absorption or delivery, these nanoparticle scaffolds are often chemically modified. One such modification is the attachment of small molecule substrates of transporters to the surface of the nanoparticles to enhance recognition, and thus barrier passage or targeting to specific tissues (Figure 1).

A variety of chemical substances have been used as nanocarriers and substrates for further chemical modification to fine-tune pharmacokinetic properties. Among them, liposomes or liposome-based formulations such as functionalized liposomes [8,10,26,29,30], solid lipid nanoparticles/nanostructured lipid carriers [31], various polymer-based nanomicelles/nanoparticles [8,30,32–39], carbon dots [40], mesoporous silica nanoparticles [41,42] or nanoemulsions [43] have been used for functionalization and transporter-targeted drug delivery.

Cellular uptake of such nanoparticles usually occurs through binding the target transporter/receptor on the cell surface, followed by endocytosis via caveolin-dependent, clathrin-dependent or clathrin/caveolin-independent pathways such as micropinocytosis [8,10,26,29,43–45], rather than uptake by the transporter itself (Figure 1). Endocytosed

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particles can be trapped in lysosomes, which can lead to their degradation and prevent their transcytosis, thus reducing their efficiency [46,47]. After the endocytic process, transporters are restored/recycled, but the exact mechanism remains unclear [26].

In the next chapter we review transporters that have been used as targets to either the prodrug approach or to nanoparticle targeting.

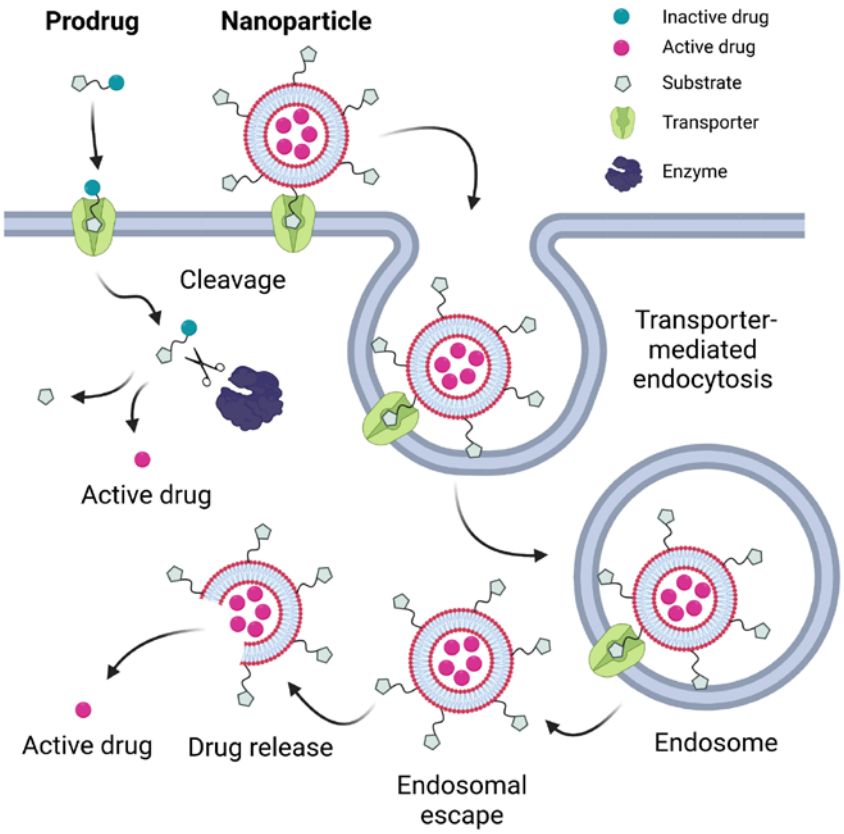

Figure 1. Illustration of the prodrug and functionalized nanoparticle strategies. In the prodrug strategy, the deliverable drug of interest is chemically linked to a known substrate of the target transporter. After uptake through the transporter, the chemical link is cleaved by intracellular enzymes, thus freeing the active drug molecule and an inert substrate. In the nanoparticle strategy, known substrates of the target transporter are chemically linked to the nanoparticle, which encapsules the active drug molecules. Due to the size of the particle, binding to the transporter typically triggers endocytosis. Endosomal escape must occur after cellular uptake to release the content of the nanoparticles, the active drug, into the cytoplasm (see text). Created with BioRender.com.

3. Targeting transporters

3.1 Facilitative glucose transporters (GLUTs)

Facilitative glucose transporters are members of the GLUT/SLC2 family, which includes 14 transporters in human [48]. Most members of this family transport six-membered ring sugars such as glucose and galactose, with different substrate specificities and tissue expression patterns, depending on their biological roles. The best characterized member, GLUT1/SLC2A1, is present to varying degrees in many different tissues and cell types. Particularly high expression levels are found in erythrocytes, endothelial cells of the blood-brain barrier (BBB) and endometrial stromal cells of the placenta, where GLUT1 fulfills vital physiological roles [48]. At the BBB, for example, GLUT1 is the major mechanism for glucose delivery to the central nervous system (CNS), and mutations in its SLC2A1 gene cause GLUT1 deficiency syndrome with seizures and other neurological symptoms [49]. Other members of the GLUT/SLC2 family have more tissue-specific expression patterns, such as GLUT5/SLC2A5 and GLUT7/SLC2A7, which are abundant in the small intestine, or GLUT4/SLC2A4, which represents the major glucose uptake

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pathway in skeletal muscle [48]. GLUT1 has attracted particular attention as its expression has been detected both at the blood-brain barrier and in malignant glioma cells that have elevated nutrient demand [50].

The prodrug strategy was used early on with GLUT1 to deliver drugs to the CNS. D-glucose or D-galactose esters of 7-chlorokynurenic acid, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, were synthesized as prodrugs to facilitate the delivery of 7-chlorokynurenic acid to the CNS and were shown to be effective against NMDA-induced seizures in mice [51,52]. Glycosyl derivatives of dopamine and L-DOPA (L-3,4-dihydroxyphenylalanine), synthesized as anti-Parkinson prodrugs, were also shown to be effective in classic dopaminergic models, indicating that the prodrugs can cross the BBB and act in the CNS after intravenous administration [53]. In later *in vitro* studies, a glucose-dopamine conjugate was shown to compete with 3-*O*-methylglucose, a non-metabolizable substrate of GLUT1, indicating direct involvement of the transporter in the uptake process [54]. Interestingly, conjugation of chlorambucil, an anticancer drug, with glucose yielded a compound that interacts with GLUT1 but is not itself transported [55].

GLUT1 has been used to deliver diagnostic and imaging markers into tumor cells, for example, [^{18}F]fluoro-2-deoxy-D-glucose is used in positron emission tomography for in vivo tumor diagnosis [56,57]. Similarly, γ-Fe_2O_3 nanoparticles were coated with dimercaptosuccinic acid and modified with 2-deoxy-D-glucose to target GLUT1-overexpressing cells for tumor imaging [58]. Liposomes loaded with the fluorescent dye coumarin 6 and decorated with glucose residues bound to cholesterol via poly(ethylene glycol) (PEG) also successfully delivered coumarin 6 to mouse brain [59]. Glucose-functionalized poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles were also developed and shown to deliver the encapsulated Cy5.5 fluorescent dye into Hep-2 cells that express GLUT1 at high levels, and enhanced antiproliferative effects were demonstrated when these nanoparticles were loaded with the chemotherapeutic agent docetaxel [36].

In addition, the GLUT1 pathway has been exploited for targeting gliomas, for which therapeutics must both cross the BBB and enhance their distribution in the tumor tissue. GLUT1 is a good candidate because it is expressed in both the BBB and various tumors. for glioma therapy, 2-deoxy-D-glucose-functionalized poly(ethylene glycol)-*co*-poly(trimethylene carbonate) (PEG-PTMC) nanoparticles were used to effectively enhance binding to GLUT1 in a dual targeting strategy involving both BBB transfer and tumor penetration [35]. Specifically, PEG-PTMC, a biodegradable aliphatic polycarbonate, was conjugated with 2-deoxy-D-glucose to target GLUT1-mediated transcytosis and deliver the encapsulated anticancer drug paclitaxel to the brain. The 2-deoxy-D-glucose nanoparticles were shown to be effectively internalized by the cells through caveolae- and clathrin-mediated endocytosis [35]. In addition, both an *in vitro* co-culture model of the BBB and *in vivo* studies with mice showed effective uptake and anti-glioma activity when the nanoparticles were loaded with paclitaxel [35]. A similar technique was used with D-glucosamine-functionalized nanoparticles, which showed enhanced tumor uptake and antiproliferative activity in cancer cells, 3D tumor spheroids and *in vivo* mouse xenografts [60]. Due to the high affinity between D-glucosamine and GLUT1, these nanoparticles could enter the tumor tissue through GLUT-mediated endocytosis with improved selectivity.

Functionalized nanoscale particles decorated with dehydroascorbic acid (DHA) have also been used to target GLUT1 for drug delivery, e.g., across the BBB or into malignant gliomas, because GLUT1 also transports DHA [61,62]. Thus, GLUT1, which is expressed on endothelial cells of the BBB, transports not only glucose but also DHA into the brain, which is subsequently reduced to ascorbic acid [63]. Because ascorbic acid is not a substrate for GLUT1, DHA transport is unidirectional, making this system ideal for drug delivery via the BBB. In one study, the authors built a "smart nanodevice" decorated with DHA using click chemistry to target malignant glioma cells [62]. The device was loaded with paclitaxel via disulfide bonds, which are reduced inside the cell due to high glutathione concentrations, triggering the release of the drug. These nanoparticles showed significantly stronger targeting of gliomas and enhanced chemotherapeutic effects than unlabeled particles or the use of paclitaxel itself due to the DHA labeling. A similar strategy

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was used to deliver itraconazole into the brain as a therapy against intracranial fungal infection. Compared to the non-conjugated micellar formulations, this strategy showed a significantly higher efficacy [61].

Multivalent glucoside ligands have also been used to functionalize liposomes for enhanced brain delivery by targeting GLUT1 [64]. The modified nanoparticles were able to deliver docetaxel into the brain in mice with significantly higher efficiency than unmodified liposomes or direct application of docetaxel alone. Modification with quin-antennary glucoside yielded the highest efficiency of delivery into the brain [64].

GLUT1 has been shown to be overexpressed not only in gliomas, but also other cancers outside the CNS as well. One example is hepatocarcinoma, against which a micellar formulation of PEG-*p*Lys-*p*Phe polymers decorated with dehydroascorbic acid has been developed, anchoring the drug via a disulfide link, the dissociation of which triggers release of the encapsulated drug due to high intracellular glutathione levels [37]. Such doxorubicin-loaded nanocarriers showed remarkable targeting abilities to hepatocarcinoma cells and enhanced anti-tumor efficacy [37]. Mesenchyme-like cancer cells were furthermore targeted by glucose-coated magnetic nanoparticles, with glucose shown to compete with nanoparticle uptake, suggesting direct involvement of sugar transporters [65].

In addition, GLUT1 has been targeted for drug delivery when treating neurodegenerative diseases, as it is abundant in the BBB, in the following ways.

Glucose-decorated nanomicelles were engineered for brain delivery of 3D6 antibody fragments (3D6-Fab) used for the clearance and reduction of Aβ plaques in Alzheimer's disease, where glucose decoration was responsible for a marked increase in cellular uptake [34]. Uptake was inhibited in a dose-dependent manner by GLUT1 inhibitor phloretin, indicating the involvement of GLUT1 in the process [34]. The highest level of brain penetration measured in mice was achieved with a 25% glucose decoration ratio, while the enhancement of Fab uptake into peripheral tissues was negligible. The delivered 3D6-Fab was also successful in preventing the aggregation of Aβ in a mouse model of Alzheimer's disease [34]. Proper orientation of the glucose molecule on the nanomicelle surface (i.e., conjugation through the C_6 position of glucose) is crucial for glucose-GLUT1 interactions and nanoparticle entry into the brain [66].

A PEG-based polymeric formulation was conjugated with galactose to enhance brain delivery of anti-BACE1 siRNA against Alzheimer's disease, based on the observation that D-glucose and D-galactose are both substrates of GLUT1 [67]. The galactose-modified nanoparticles showed cellular uptake that was inhibited by phloretin in a dose-dependent manner, indicating a dominantly GLUT1-mediated uptake pathway [68], while their brain penetration was 5.8-fold higher than that of nanoparticles not modified with galactose [68]. The effect of galactose-mediated targeting was underscored by behavioral studies in the APP/PS1 double transgenic mouse model of Alzheimer's disease, which showed that, in contrast to mice treated with non-galactose-modified nanoparticles, mice treated with the galactose-decorated anti-BACE1 siRNA-loaded nanoparticles achieved the performance of normal, healthy WT mice in the novel object recognition test [68].

It is important to emphasize that conjugation with ligand transported by a specific transporter does not automatically mean that this transporter is the primary uptake route. Glucose-coated nanoparticles have been shown to cross the primary human brain endothelium at least three times faster than non-brain endothelia, with eventual localization in astrocytes [69]. However, the GLUT1 inhibitor cytochalasin-B did had no effect on the rate of transport of these molecules. It was concluded that uptake occurs through passive diffusion, as vesicular transport could not be detected, but uptake and transfer rates were temperature dependent.

The high or specific expression of other glucose transporters such as GLUT2/SLC2A2, GLUT3/SLC2A3, GLUT12/SLC2A12 or the fructose transporter GLUT5/SLC2A5 has also been associated with various cancer types and stages [70–73]. For example, GLUT5 shows a 5-fold and 17-fold higher protein expression in MCF7 and MDA-MB-231 breast cancer cell lines, respectively, than in the 184B5 non-cancerous breast cell line [74]. However, the lack of specific binders to these transporters has hindered the development of therapeutics

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that can utilize these proteins [74]. For the case of GLUT5, fluorescently labeled glycoconjugates (2,5-anhydro-D-mannitol-coumarines) have shown high affinity and specificity of binding [75,76]. Based on these results, either mannitol directly, or mannitol-coumarin were chemically conjugated with chlorambucil, an anticancer agent [74]. These prodrugs showed selective uptake into the GLUT5-expressing MCF7 breast cancer cell line compared to the 184B5 non-cancerous mammary tissue cell line, which competed with the uptake of previously used fluorescent probes, showing a GLUT5-dependent uptake mechanism. All but one of the synthesized prodrugs also showed a cytotoxic effect [74].

Attempts were also made to target GLUT4/SLC2A4 in muscle cells. GLUT4 in the C1C12 muscle cell line was targeted with glucose-functionalized quantum dots, and uptake responded to insulin stimulation, which is known to increase the surface expression of GLUT4, and competed with 2-deoxyglucose, suggesting direct involvement of GLUT4 in the uptake process [77].

3.2 Amino acid transporters

LAT1/SLC7A5 has been in the spotlight of drug delivery efforts because it is overexpressed on both BCECs of the BBB as well as glioma and other tumor cells [78–80]. For this reason, there has been a focus on LAT1-mediated drug delivery, either with the goal of delivering therapeutics to the CNS or to specifically target cancer cells. It has been argued that LAT1 has better properties than 20 other transporters studied for delivery across the BBB in terms of high maximal capacity and appreciable binding affinity, relatively simple structural requirements for binding and relative promiscuity, and the fact that neither its use nor disruption of its activity by the possible overdose of therapeutics result in irreversible brain damage [81–83]. LAT1 is also highly and selectively expressed on both the luminal and abluminal sides of the BBB [79] and its expression is not altered by inflammatory insult [84]. In addition, it is a non-glycosylated protein [85,86], although it forms an obligate complex with an *N*-glycosylated auxiliary protein, 4F2hc/SLC3A2 [85].

Several drugs already utilize LAT1 for crossing the BBB (e.g., melphalan [87], levodopa [78], gabapentin [88], pregabalin [89], methyldopa, baclofen [26]). The approach to generate LAT1-transported prodrugs mostly utilizes the conjugation of drugs with large and/or hydrophobic amino acids, such as aliphatic amino acids, L-Phe or L-Tyr, and has been applied to drugs such as ketoprofen [90], ferulic acid [82], dopamine [91], valproic acid [92], nipecotic acid [93], phosphonoformate [94], flurbiprofen, salicylic acid, ibuprofen, naproxen [86], and probenecid [95] (for a recent review, see [96]). In addition, gemcitabine has also been conjugated with threonine to target LAT1 [97]. There is also a prodrug strategy to enable the brain penetration of 7-chlorokynurenic acid, a NMDA receptor antagonist, by converting it into the prodrug of 4-chlorokynurenine [98]. This compound is an amino acid that is readily taken up by the L system (LAT1/SLC7A5) through the BBB into the CNS [98]. In terms of substrate recognition by LAT1, analysis of competent substrates has suggested that a free amino and a free carboxyl group are required for recognition by LAT1 [99–101]. A pharmacophore study has later found that the free amino group can also interact with LAT1 through a H-bond interaction instead of purely through the positive charge [102]. The model has also pointed out the preference of aromatic vs. lipophilic moieties, as well as an optional H-bond acceptor region that can enhance affinity [102]. Later, a quantitative structure-activity relationship (QSAR) model has been developed for designing potent binders of LAT1, which suggests that phenylalanine attached at the meta position of the aromatic ring with an amide bond has the highest ability to utilize LAT1 [103]. It should be noted that prodrugs designed to be substrates for LAT1 can also be substrates for other uptake transporters, such as monocarboxylate transporter 8 and 10 (MCT8/SLC16A2 and MCT10/SLC16A10, respectively), or organic anion transporter proteins (OATPs, SLCO/SLC21 family) [104,105]. Eadie-Hofstee plots can be applied to find uptake systems that transport a particular compound, and such second transport systems have been found for a number of prodrugs at higher concentrations [86].

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Nanoparticles have also been functionalized using LAT1 substrates to focus their targeting. In particular, LAT1/SLC7A5 has been used to deliver anticancer medication through the BBB for the treatment of glioma, either for imaging/detection/staging purposes, or for developing anti-cancer therapeutics.

Methionine was used as a bait conjugated with gold nanoclusters that were functionalized with a fluorescent dye as well as doxorubicin to carry this cargo into tumor cells [106]. The authors suggested that LAT1/SLC7A5 and LAT2/SLC7A8 are involved in the uptake process [106].

The functionalized nanoparticle strategy was used to target LAT1 by L-DOPA-decorated amphiphiles [107]. These liposomes, when loaded with NIR-dye, showed preferential accumulation in brain tissue, and while carrying WP1066, a STAT3 inhibitor [108], enhanced overall survival in a glioblastoma mouse model [107].

An interesting approach is presented by Mintz *et al.*, who synthesized carbon nanodots from tryptophan and 1,2-ethylenediamine, which were able to cross the BBB in zebrafish [40]. The authors hypothesized that residual tryptophan bound to the surface of the carbon dots facilitated uptake through the BBB via the LAT1 transporter [40].

Phenylalanine-coupled solid lipid nanoparticles have been prepared that can deliver doxorubicin into glioma with higher efficiency than without phenylalanine coupling [109]. However, these efforts have been criticized because the conjugation was performed on the α-carboxyl group of phenylalanine, which was previously reported to be essential for recognition by LAT1 [102,110]. Moreover, phenylalanine tends to be entrapped in the core of solid lipid nanoparticles due to its hydrophobicity [101]. Later, another strategy used linkage through the γ-carboxyl group of glutamate to the surface of liposomes and PLGA nanoparticles for the same purpose, also reporting effective uptake into glioma cells and crossing of the BBB [101]. A similar approach was also applied to PLGA nanoparticles to target breast cancer cells through LAT1-mediated delivery [111].

Liposomes composed of egg phosphatidylcholine (EPC) and dioleoyl phosphatidylethanolamine (DOPE) modified with an L-tyrosine conjugated polymer showed enhanced uptake into HeLa cells, which strongly express LAT1, compared to liposomes with unconjugated polymer [112]. Interestingly, the polymer used, poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide), is a thermoresponsive polymer that exhibits a phase transition at 32°C leading to changes in hydrophobicity, associated with its hydration and dehydration. At temperatures above the phase transition temperature, the uptake of the formulated nanoparticles into HeLa cells was observed because the polymer surface of the liposomes becomes hydrophobic [112]. Compared to this, L-tyrosine modification has further enhanced the cellular uptake of these nanoparticles [112].

In addition to LAT1, another commonly targeted amino acid transporter is SLC6A14/ATB$^{0,+}$. SLC6A14 is a relatively promiscuous transporter that transports a wide range of neutral and cationic amino acids [113] and is highly concentrative [9]. It is highly expressed in the lungs [113] but is also found in the small intestine [114,115], making it a candidate for mediating oral drug delivery. In addition, SLC6A14 has been shown to be upregulated in various tumor types [116–119].

SLC6A14 has been targeted by prodrugs because, as it can transport a wide range of substrates, conjugating to an amino acid by esterification or acylation transforms many drugs into substrates of SLC6A14 [120]. Examples include the L-valyl esters valacyclovir and valganciclovir, the L-glutamic acid γ-ester of acyclovir [120], and Val-SN-38, the valyl ester prodrug of SN-38, a derivative of the topoisomerase inhibitor irinotecan [121]. Interestingly, the Val-SN-38 prodrug was also taken up by SLC38A1, SLC38A2 and ASCT2/SLC1A5, highlighting that several transporters with overlapping substrate specificity can contribute to prodrug uptake [121]. The quaternary ammonium substrate of SLC6A14, L-carnitine, has also been used as a vehicle for the delivery of small molecules in a prodrug approach. Butyrate is a bacterial metabolite that has been attributed with preventive effects against inflammation in the large intestines, as well as tumor suppressing and immunosuppressive effects relevant to the treatment of inflammatory bowel disease (IBD) [122]. While there is no information on whether the normal uptake routes of

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butyrate are altered in IBD, it has been shown that SLC6A14 is upregulated under IBD conditions [122]. Since L-carnitine is also a substrate of SLC6A14, an ester prodrug of butyrate conjugated with L-carnitine has been developed to target colon epithelial cells [122]. Butyrate-L-carnitine has been shown to interact with both SLC6A14 and the intestinal carnitine transporter OCTN2/SLC22A5 [122] (see also the section about organic cation transporters).

In a study using liposomes conjugated with small molecules, different amino acids (Gly, Asp, Lys) were evaluated for their targeting efficacy, with lysine showing the highest efficacy [29,123]. Lysine-conjugated liposomes have been shown to be taken up by carcinoma cell lines [29]. The authors suggest that binding of the lysine-conjugated particle leads to a sustained occluded state of the transporter, which induces endocytosis [29]. The preparation further showed selective accumulation of lysine-conjugated liposomes in tumor compared to non-conjugated liposomes. Aspartate conjugates also showed efficacy when the conjugation occurred via the β-carboxyl group of the aspartate side chain.

In addition, L-valine conjugated PLGA nanoparticles have been used to improve oral delivery of insulin, presumable through uptake by amino acid transporters in the small intestine [124]. However, in this case, the authors mention that L-valine was conjugated with the PLGA particles through its amino group, which could preclude their binding to amino acid transporters.

Attempts were also made to target both LAT1 and SLC6A14 with a single nanoparticle [125]. Liposomes loaded with irinotecan were functionalized with polyethylene glycol monostearate conjugated with different amino acids. Interestingly, while liposomes functionalized with glutamate and lysine were able to target LAT1 and SLC6A14, respectively, tyrosine-functionalized liposomes were able to target both transporters simultaneously. These dual-targeting liposomes showed highest uptake efficiency in BxPC-3 and MCF-7 cancer cells, which highly express both LAT1 and SLC6A14. The tumor inhibition rate of the tyrosine-functionalized liposomes was also higher than that of unfunctionalized liposome formulations. The mechanism of uptake was confirmed as LAT1- and SLC6A14-mediated endocytosis [125].

Another amino acid transporter, ASCT2/SLC1A5, has been shown to be overexpressed in various carcinomas, making it an ideal target for cancer-specific drug delivery [126–130]. ASCT2 is an amino acid transporter of the SLC1 family that transports L-alanine and L-glutamine as well as other small neutral amino acids [131–134]. Glutamine transport activity is particularly important for cancer cells, in which glutamine addiction can develop due to the Warburg effect [135]. For this reason, radiolabeled [^{18}F](2*S*,4*R*)4-fluoroglutamine has been used as a positron emission tomography (PET) probe for tumor imaging [136,137]. Similarly, non-glutamate based amino acid radiotracers have been developed for use with PET/CT-based cancer diagnosis, such as anti-1-amino-3-[^{18}F]fluorocyclobutane-1-carboxylic acid (FACBC, fluciclovine) [138,139], which is also partly transported by ASCT2 [140]. The prodrug strategy has additionally been used to generate a series of glutamine-linked Pt(IV) prodrugs that have shown anticancer activity, albeit to a lesser extent than their parent compound, cisplatin. However, for one of the compounds, the authors expect less off-target accumulation, as it is mainly taken up by ASCT2-overexpressing tumor cells [141].

Functionalized nanoparticles targeting ASCT2 via glutamine have also been generated. In one study, glutamine-β-cyclodextrin was synthesized and loaded with doxorubicin and was shown to accumulate specifically in highly ASCT2-expressing triple negative breast cancer cell lines (MDA-MB-231 and BT549). Uptake of the compound was attenuated by L-γ-glutamyl-*p*-nitroanilid, a specific inhibitor of ASCT2, demonstrating the involvement of the transporter in the uptake process [142]. In another study, a polyglutamine-based co-polymer gene delivery system was developed for cancer therapy to deliver interfering siRNA agents against multidrug resistance protein 1 (MDR1/P-gp/ABCB1) and Survivin. The nanoparticles were shown to be taken up by clathrin-mediated endocytosis, which was partially ASCT2-dependent, as inhibition of ASCT2 attenuated uptake, while glutamine deprivation enhanced it [143]. Interestingly, nanoparticle binding resulted in a

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significantly decrease in intracellular glutamine levels due to competition for glutamine, which in turn resulted in a remarkable upregulation of ASCT2. *In vivo*, polyglutamine-based nanoparticles were shown to be specifically taken up into the lung parenchyma after intravenous injection, likely due to the high expression of ASCT2 in those tissues [143].

LAT1 and other SLC7 amino acid transporters form obligate complexes with type II single-transmembrane domain glycoproteins of the SLC3 family [129,144–146]. Thus, SLC3A2 (also known as CD98), the obligate interaction partner of LAT1, although not a transporter itself, is a potential target as well due to its elevated expression in various carcinomas and neoplasms, and as a consequence of intestinal inflammation [147,148].

Targeting and silencing of SLC3A2 in colorectal cancer was the basis for the development of a new oral nanoparticle strategy that improves the efficacy of anticancer drugs. While SLC3A2 is only weakly expressed on the basolateral membranes in healthy colon epithelial cells, it is distinctly overexpressed on both apical and basolateral membranes in colon cancer, where it plays a special role in the development of colon cancer [149]. This suggests that SLC3A2 can serve as a receptor for targeted drug delivery in colon cancer cells and that its downregulation in combination with anticancer drug treatment increases the therapeutic efficacy of the anticancer drug. For this purpose, SLC3A2 siRNA and the anti-cancer alkaloid camptothecin were co-loaded into SLC3A2 Fab-functionalized nanoparticles [149]. These nanoparticles showed enhanced drug delivery, anticancer and antimigration effects in *in vitro* and *in vivo* experiments compared to drug-only loaded nanoparticles or non-functionalized nanoparticles, demonstrating the potential of this targeted nanoparticle combination therapy [149]. A similar antibody-mediated targeting strategy was used with PLGA nanoparticles to deliver SLC3A2 siRNA into intestinal cells of mice with colitis [150], thereby targeting SLC3A2 on the surface of colon epithelial cells and macrophages, where it is overexpressed due to inflammatory processes [148,150].

3.3 Bile acid transporters

Bile acid conjugation was used as early as 1948 as a strategy for targeting hepatocytes to treat germ and viral infections attacking the liver [10,151,152]. Bile acids are polyhydroxylated steroidal acids derived from cholesterol that are secreted by the liver into the bile canaliculus via the ABCB11/BSEP transporter and stored in the gallbladder [153]. The majority chemical species in bile are the primary bile acids cholic acid and chenodeoxycholic acid, which are conjugated with either glycine or taurine [153]. After emptying into the small intestine following the ingestion of a meal, they help solubilize and break down large dietary lipid droplets by converting them into small ones, thus improving accessibility to pancreatic lipases. After enzymatic digestion of the components of the micelles, the lipids are converted into common hydrolysis products such as fatty acids, monoacylglycerols, phospholipids and free cholesterol [10,154]. These products remain associated with the bile acids in mixed micelles, which facilitates their passage through the intestinal mucus layer, one of the important barriers to overcome when developing drug delivery strategies [10,155]. The ingredients of the micelles are then taken up by the enterocytes in the upper part of the small intestine, either via passive diffusion or by specific transporter proteins [10,156–158]. After uptake across the brush border membrane, the resulting intracellular lipid droplets bind to fatty acid binding protein (FABP), and the lipid digestion products migrate to the endoplasmic reticulum, where they are re-esterified to generate triglycerides, phospholipids, cholesterol esters, etc. After transport from the ER to the Golgi apparatus, lipids are packaged together with apolipoproteins to form chylomicrons, with apolipoproteins playing an important role in chylomicron synthesis [159,160]. The chylomicrons are then extruded from the Golgi apparatus in exocytic vesicles and released across the basolateral membrane into the lacteals in the villi of the small intestine, and thence into the lymphatic vessels and thoracic duct to enter systemic circulation [10,156,157]. Similar transport pathways may exist for certain fat-soluble vitamins, carotenoids and other lipophilic compounds that rely on the formation of bile acid micelles.

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As for the absorption of therapeutic drugs via the lipid absorption pathway, most of them enter the portal vein after transcytosis of enterocytes. However, it is also known that the bioavailability of several highly lipophilic drugs depends significantly on lymphatic transport [157,161,162]. In general, it is the high lipophilicity and the excessive particle size that favor the lymphatic system over the portal vein [10]. Drug uptake via the lymph has several advantages, such as the ability to bypass first-pass metabolism in the liver and the avoidance of rapid distribution of drugs in organs and tissues, resulting in reduced toxicity [10,157,161]. The lymphatic delivery pathway can also be exploited via the microfold M cells located in the intestinal epithelium [162]. These are specialized immune cells distributed among the epithelial cells covering mucosa-associated lymphoid tissues such as Peyer's patches [10]. Their normal function is to rapidly take up antigens from the intestinal lumen in order to initiate an immune response [10,163,164]. They lack microvilli and a mucus layer and are coated by a thinner glycocalyx than enterocytes, which allows them easier access to the contents of the intestinal lumen, making them ideal candidates for developing advanced oral bioavailability strategies for therapeutics [10,163].

While the pathway of lipid absorption facilitated by bile acid micelles occurs in the upper part of the small intestine, the conjugated bile acids are taken up via the luminal sodium-coupled bile acid transporter SLC10A2/ASBT located in the distal ileum. There, 95% of conjugated bile acids are absorbed as part of the recycling of bile salt called enterohepatic circulation, which is important because the liver is unable to synthesize enough bile salts to meet the daily requirements [10,165]. As part of the recycling process, bile acids taken up through the apical membrane of epithelial cells via SLC10A2/ASBT bind to the ileal bile acid binding protein IBABP, which then shuttles them to the basolateral membrane. From there they exit via the heteromeric organic solute transporter OSTα/β (SLC51A/B) into the portal vein [166]. From there, conjugated bile acids travel back to the liver where they are taken up by the hepatic sodium-dependent taurocholic transporter SLC10A1/NTCP [166]. Unconjugated bile acids can be taken up by OATP transporters (SLCO/SLC21 family).

The intestinal barrier poses a major challenge in the development of new strategies to improve oral drug availability. While most small molecule drugs administered orally are believed to be substrates of one or more uptake transporters expressed in the intestines [167,168], various approaches have been used to attempt to move non-transport substrates or poor substrates more efficiently across the brush border membrane. Bile acids and their derivatives were among the first molecules used to aid drug absorption in the intestines. Specifically, it was found that the conjugation of chemically modified cholic acid with peptides of different lengths resulted in the uptake of some of these peptides into bile [169,170], while competitively inhibiting the uptake of taurocholate. This was one of the first studies in this field when the molecular identity of the bile acid transporter in the ileum was still unknown. It was identified as ASBT/SLC10A2 in 2003 [171,172].

Subsequent studies have actively used the prodrug-based strategy to enhance either intestinal absorption or targeting to the liver. In the case of the antiviral agent ribavirin used to hepatitis C infections, the aim was to lessen off-target effects in erythrocytes that cause hemolytic anemia. To this end, ribavirin was conjugated with bile acids to target the liver bile acid transporter NTCP/SLC10A1 [172]. This strategy reduced the ribavirin concentration in erythrocytes 16.7-fold and exposure of ribavirin in erythrocytes, plasma and kidneys 1.8-fold, while exposure in the liver was similar to ribavirin itself [173].

Another antiviral agent, valacyclovir, was conjugated with chenodeoxycholate to improve oral bioavailability. The conjugate resulted in a more than 10-fold increase in intestinal uptake compared to the parent acyclovir in a cell line model [174]. In addition, the acyclovir molecule was recovered from the urine of rats after the administration of the conjugate, indicating that acyclovir was successfully cleaved and activated in the organism.

Yet another example illustrating the prodrug strategy to improve targeting into the liver is floxuridine, an antimetabolite used to treat metastatic liver disease. In order to enhance its hepatic uptake, it was conjugated with chenodeoxycholic acid using glutamic

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acid as a linker between the drug and the bile acid [175]. Two isomers were synthesized, and both were found to be substrates of NCTP/SLC10A1 [175]. The compounds show stability in rat plasma but rapid release of the drug in rat liver. This suggest that glutamic acid is a promising linker for conjugation of bile acids with liver-targeted drugs because the ester bond remains stable in plasma but is readily metabolized in the liver [175].

Conjugation of cytarabine, an anti-cancer agent that has poor oral bioavailability, with various bile acids has been explored as a way to improve both intestinal absorption and liver targeting to optimize liver cancer treatment [176]. The ursodeoxycholic acid derivative of cytarabine showed prolonged half-life *in vivo* and increased oral bioavailability compared to cytarabine itself [176]. This reaffirms the benefit of the bile acid transporter-based prodrug strategy to enhance oral absorption.

Bile acid conjugation was also used to improve the oral bioavailability of heparin by conjugation deoxycholic acid with low molecular weight heparin (LMWH) [177]. The formulation was effective *in vivo* [178,179], and a competition study with free bile acid indicated that the uptake process was mediated by ASBT/SLC10A2 [179]. A similar strategy was later used for insulin by linking it to succinimido deoxycholate and succinimido bisdeoxycholyl-L-lysine [180]. The resulting conjugates retained high binding affinity to the insulin receptor and showed prolonged biological activity than normal insulin when administered intravenously to rats [180].

An interesting approach was taken by Lu and coworkers by linking paclitaxel to a PEG linker via a disulfide bond, which in turn was linked to cholic acid via an amide bond [181]. This targeted prodrug approach is based on the knowledge that elevated glutathione levels in tumor cells reduce the disulfide bond and activate the drug [181–184]. The resulting formulation was resistant to acidic *in vitro* conditions mimicking those in the stomach, and the prodrug was able to enter MDA-MB-231 breast cancer cells, with uptake reduced by addition of sodium taurocholate, indicating the involvement of ASBT/SLC10A2 [181]. *In vivo* studies in rats showed a higher plasma concentration of the prodrug than paclitaxel administered alone [181]. This prodrug approach gives chemotherapeutic agents with limited solubility and permeability an optimized oral delivery and tumor-specific release.

Early on, comparative molecular field analysis (CoMFA) has suggested that substitution at positions 3, 7, 12 and 24 of bile acids lead to reasonable binding to the bile acid transporters [185]. Subsequent structure-activity studies confirmed that the C_2-C_3 positions of bile acids can be successfully conjugated without affecting the interaction with bile acid transporters [186]. In fact, C_3 does not appear to specifically interact with ASBT/SLC10A2 and thus offers a preferred conjugation site [10,187], even though the C_3 hydroxyl group seems to be essential for binding to the IBABP [10]. In contrast, position 24 has been frequently used for conjugation, probably due to its easy chemical accessibility [187–189]. It is generally believed that the negative charge at the C_{24} position is not essential for transport, but significantly increases the affinity to bile acid transporters [187,189]. However, its modification might lead to a lower uptake rate [187].

Even though 3D-QSAR models successfully predict the interaction of small molecules with ASBT/SLC10A2 [190], the atomic-resolution structure of a bacterial homolog of ASBT from *Neisseria meningitidis* suggests that the cavity of ASBT is relatively small, and therefore it is questionable whether bile acid conjugates are actually transported [191]. On the other hand, it has also been claimed that the substrate-binding site of ASBT is much bigger than the size of bile acids, and that larger substrates can also be accommodated. For example, even a tetrameric form of deoxycholic acid shows high affinity for ASBT and improves the oral bioavailability of heparin upon conjugation [192]. Nevertheless, whether these large substrates actually enter the cell via the bile acid transporter or whether uptake occurs via endocytosis has not yet been determined.

In addition to the classical prodrug strategy involving direct chemical linking of small molecule drugs to bile acids, the nanocarrier strategy involving nanoparticles functionalized with bile acid molecules has also been developed. In particular, the decoration

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of various types of nanoparticles with bile acids has been widely used to enable oral bioavailability of heparin and insulin.

Deoxycholic acid-conjugated chitosan particles were loaded with insulin for successful delivery into the portal vein [193–195]. Chitosan is a natural polysaccharide derived from marine crustaceans and chitosan-based nanomaterials have proven effective for advanced delivery approaches such as protein/peptide delivery, as they offer several advantages, including high encapsulation efficiency and favorable biocompatibility. Insulin-loaded deoxycholic acid-conjugated chitosan particles were shown to undergo ASBT/SLC10A2-mediated endocytosis, followed by sequestration to the basolateral membrane via IBABP and release at the basolateral membrane [193]. Another, different formulation based on the same idea has also been developed [196,197]. Polymer coating increases the stability of liposomes while enabling their conjugation with various small molecule ligands. Chitosan-coated and deoxycholic acid-modified liposomes have been successfully used to deliver insulin to rats *in vivo* [198], suggesting that delivery of proteins/peptides via the bile acid uptake route is possible.

In another study, an attempt was made to deliver insulin by developing PEGylated polyhydroxybutyrate copolymeric nanoparticles conjugated with deoxycholic acid [199]. To avoid the release of insulin from the nanoparticles due to the harsh acidic and enzymatic milieu in the stomach, the nanoparticles were coated with a hydrophobic polymer, Eudragit S-100. While the encapsulation has prolonged *in vivo* insulin release beyond 24 hours, deoxycholic acid ligation caused significantly higher intestinal uptake of the nanoparticles [199].

Heparin conjugated to nanomaterials has been explored in view of its expected versatility in surface functionalization and embedding of biomolecules [200]. Nanocarriers were developed using heparin-taurocholic acid nanoparticles loaded with docetaxel [201,202]. The self-assembling formulation enabled effective oral absorption and anti-cancer activity in tumor-bearing mice and absorption could be blocked by the administration of taurocholic acid, confirming the involvement of the bile acid pathway.

In a further attempt to explore bile acid transporter-mediated delivery routes to improve oral administration of poorly water-soluble drugs, self-assembled hybrid nanoparticles of sodium-taurocholate and polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol (Soluplus®, BASF Pharma, Germany) were prepared, and the calcium channel blocker felodipine was selected as a model drug [203]. The permeability of felodipine depended on the presence of taurocholate on the particles and was inhibited by excess sodium taurocholate or inhibition of ASBT/SLC10A2. A fluorescence approach was used to verify that the Soluplus nanoparticles were taken up intact by the ileum. These results confirm the potential use of this approach to enhance the oral bioavailability of poorly soluble drugs [203].

Taurocholic acid-modified nanostructured lipid carriers based on polyethylene glycol 100-monostearate have been developed for improving the oral delivery of the cancer preventing agent curcumin [31]. Taurocholine modification has also been used on nanoparticles to deliver siRNA of Akt2 for the treatment of colorectal cancer metastases in the liver [204].

Conjugation with glycocholic acid has also been shown to increase drug bioavailability. This has been shown for the 39-amino acid peptide exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist and incretin mimetic used to treat type 2 diabetes. However, its therapeutic benefit is limited due to the frequent injections required. To address this issue, liposomes coated with glycocholic acid-conjugated chondroitin sulfate and loaded with exendin-4 were used to facilitate oral administration [205]. The efficiency of the nanoparticle formulation was similar to that of subcutaneous injection of exendin-4 in a rat model of type 2 diabetes. Interestingly, the site of absorption of the modified liposomes relocated from duodenum to ileum, most likely as a result of coating with bile acids [205].

To avoid problems with bioavailability of the chemotherapeutic agent etoposide, a topoisomerase II inhibitor, the medication was embedded in a nanoemulsion based on

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low molecular weight methylcellulose, which also contains the ion pair of the anionic 1,2-didecanoyl-*sn*-glycero-3-phosphate, a lipid, and the cationic N^{α}-deoxycholyl-L-lysyl-methylester, a derivative of deoxycholic acid [43]. This formulation showed improved cellular permeability in Caco-2/HT29-MTX-E12 cells and also higher oral bioavailability in *in vivo* studies in rats [43]. Inhibition of ASBT with actinomycin D and the heteromeric organic solute transporter OST α/SLC51A and OST β/SLC51B with clofazimine reduced permeability, indicating the involvement of bile acid transporters in this process [43].

It should be noted that the hydrophobic nature of certain bile acids, such as deoxycholic acid, causes the molecule to be preferentially buried in the liposomes or micelles, which could limit its efficacy by obstructing binding to bile acid transporters [9]. Similarly, while carriers such as bilosomes, which are liposome-like systems with bile acids present directly in the bilayer membrane, do show advantages for oral delivery, whether they directly interact with bile acid transporters is still unclear [10].

3.4 Choline transporters

While choline is an important precursor for phospholipid production in all cell types, it plays a special role in the brain for the synthesis of the neurotransmitter acetylcholine [206,207]. To fulfill the high demands of the brain for choline, and due to the cationic charge of the choline molecule, it has long been recognized that a dedicated transport system should be available at the BBB for choline. However, the identity of the transporter has long remained elusive. While the high-affinity Na^+-dependent choline transporter CHT1/SLC5A7 was shown to be highly expressed in cholinergic nerve endings [208], it is not expressed in brain capillary endothelial cells that form the BBB [209,210]. Instead, the choline transporter-like proteins CTL1/SLC44A1 and CTL2/SLC44A2 were shown to be expressed on the plasma membrane of human brain microvascular endothelial cells (hBMEC) as well as human brain cortical sections [210]. Upon the knockdown of CTL1/SLC44A1 by RNA interference in cultured rat astrocytes, the Na^+-independent choline uptake activity vanished, indicating that CTL1 likely transports choline in a Na^+-independent fashion [211]. Due to the presence of choline transporters in the BBB, they stand at the focus of drug-transporter interactions and serve as gateways for the delivery of therapeutic agents across the BBB. In addition to normal brain function, glioma cells have an increased demand for choline to synthesize phospholipids, which are essential for cell proliferation [9]. Therefore, targeting choline transporters could be beneficial both for delivering drugs into the CNS and for targeting glioma cells in the brain.

Even before the identification of the choline transporter at the BBB, pharmacophore models were proposed to study the chemical modifiability of choline while retaining affinity to its transporter [212,213]. While earlier studies suggested that both the quaternary ammonium and the free hydroxyl groups are necessary for the recognition by the transporter [213], it was later found that bis-quaternary ammonium compounds can also inhibit transport [214]. Based on this, various linker lengths and types have been explored to develop high-affinity binders to the choline transporter at the BBB [215,216]. One of these compounds was shown to efficiently accumulate in the brain when linked with the BODIPY dye, while a nanodelivery system based on dendrigraft poly-L-lysines (DLGs) decorated with the compound was able to successfully deliver plasmid DNA into the brain [216]. Interestingly, even though the uptake of conjugated nanoparticles was inhibited by excess choline, inhibition by filipine suggested a non-specific adsorptive endocytosis mechanism of uptake [216].

Similar nanoparticles were later used to deliver a gadolinium chelate contrast enhancer for the localization of glioma by magnetic resonance imaging (MRI) [217], and also for the simultaneous delivery of doxorubicin and a vector carrying a gene encoding the hTRAIL (human tumor necrosis factor-related apoptosis-inducing ligand) protein [218]. Both applications showed superior brain penetration and activity of the formulations compared to non-conjugated controls. Similarly, a micellar preparation based on linking the above-mentioned choline derivative to a PEG segment conjugated with 8 doxorubicin

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molecules was prepared and showed higher glioma accumulation compared to the same formulation without the choline derivative compound [219].

3.5 Vitamin transporters

Vitamins are essential compounds that play a role as cofactors or precursors in a variety of fundamental physiological processes. Since vitamins are essential nutrients, there are several vitamin transporters in the intestines for their absorption. Some of these vitamin transporters have been exploited as routes to enhance the oral absorption of drugs.

Ascorbic acid (vitamin C) is an important cofactor in various enzymatic processes and typically acts as an electron donor. It also scavenges and neutralizes free radicals such as reactive oxygen species [220]. This antioxidant activity of ascorbic acid is especially important during the inflammatory reaction to protect immune cells [220]. The byproduct of the antioxidant activity is the oxidized form of ascorbic acid called dehydroascorbic acid (DHA) [220]. Different transport systems exist for the two forms. While L-ascorbic acid is taken up by the Na^+-coupled vitamin C transporter SVCT1/SLC23A1 and SVCT2/SLC23A2, DHA can cross the membrane through facilitated diffusion with the help of GLUT/SLC2 transporters (see the section above about facilitative glucose transporters).

While SVCT1/SLC23A1 is expressed in epithelial tissues such as the small intestine or kidney and is responsible for regulating whole-body ascorbic acid levels, SVCT2/SLC23A2 is broadly expressed. Importantly, SVCT2 is also highly expressed in epithelial cells of the choroid plexus [221,222], suggesting that it enables the transport of ascorbic acid into the brain [220,223]. This function is especially important because the blood levels of the oxidized form of vitamin C, DHA, which could serve as an alternative source of vitamin C supply to the brain, are negligible under normal physiological conditions [224]. Vitamin C taken up by SVCT2 in the epithelial choroid plexus cells was recently shown to exit the cells into the cerebrospinal fluid (CSF) via GLUT12/SLC2A12, a facilitative transporter that is also highly expressed in the choroid plexus [225]. In further support of the concept that SVCT2 and GLUT12 provide vitamin C to the brain via the choroid plexus and the CSF, earlier autoradiographic studies confirmed that ^{14}C-labeled ascorbic acid slowly accumulates in the central nervous system after intravenous injection and that radioactivity leaving the choroid plexus reaches the highest levels in the central nervous system 6 days after intravenous injection in mice [226]. How exactly ascorbic acid enters the brain from the CSF has not yet been clarified.

Since ascorbic acid can cross both the intestinal barrier and get delivered into the CSF via SVCT/GLUT ascorbic acid transporters, the conjugation of ascorbic acid to various compounds has been explored as a strategy for brain delivery.

Earlier studies to generate ascorbic acid transporter-specific ligands have shown that the C_5 and C_6 positions are modifiable without significantly affecting the interaction with the transporter [227–229]. Additionally, the oxidized forms of these derived compounds showed no interaction with GLUT transporters GLUT1 or GLUT3, which transport DHA, confirming their specificity for SVCT transporters [227,228]. Some of these derivatives have been developed for medical imaging but have proven to be of limited use [230,231]. Prodrugs of nipecotic acid (an SLC6 GABA transporter uptake inhibitor), kynurenic acid (a neuroactive intermediate of L-tryptophan metabolism) and diclofenac acid (a non-steroidal anti-inflammatory drug) conjugated with ascorbic acid have also been developed in order to improve their brain penetration [232–234]. The nipecotic acid derivative was also tested in a mouse model of epilepsy induced by pentylenetetrazole (a GABAA receptor antagonist) and was found to prolong the latency for the onset of tonic seizures, whereas the application of nipecotic acid itself had no such effect [233,234]. The γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenylacetyl)-(*S*)-alanyl]-(*S*)-phenylglycine tert-butyl ester (DAPT), a potential therapeutic against Alzheimer's disease, has also been linked chemically to ascorbic acid in order to improve its bioavailability in the CNS and to reduce potential off-target effects [235]. One of the developed compounds showed accumulation in the brain while retaining the inhibitory activity on γ-secretase [235]. A prodrug of the

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anti-inflammatory drug ibuprofen was also developed by conjugating ibuprofen with ascorbic acid to enhance its delivery to the brain via SVCT2 allowing it to be used for the treatment of CNS disorders such as Alzheimer's disease [236]. The prodrug accumulated in the brain to a greater extent than ibuprofen and became activated in the brain, releasing ibuprofen [236]. The uptake competed with the transport of free ascorbic acid, consistent with the involvement of SVCT2 in the uptake process [236].

To further exploit the potential of SVCT2 for improved drug delivery into the brain, nanocarriers, that is liposomes and lipid-core polymeric micelles were developed to target SVCT2. For this, the nanocarriers were decorated with ascorbate by modifying 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-amino-PEG with ascorbic acid [223]. The nanocarriers showed enhanced targeting to SVCT2-expressing glioma cells based on the delivery of rhodamine into these cells, which could be significantly inhibited by the presence of free ascorbic acid in the medium, indicating SVCT2-mediated uptake [223]. In another study, poly(D,L-lactic-*co*-glycolic acid)-*block*-poly(ethylene glycol) (PLGA-*b*-PEG)-based nanoparticles were functionalized with ascorbic acid and loaded with galantamine, an acetylcholinesterase inhibitor used to treat Alzheimer's disease [237]. Ascorbic acid conjugation enhanced uptake of the nanoparticles into SVCT2-expressing cells in *in vitro* studies. The functionalization also reduced the accumulation of galantamine in the liver, spleen, lungs and kidneys and improved the outcome in scopolamine-induced amnesic rats [237].

SMVT/SLC5A6 is a Na^+-coupled vitamin transporter expressed in absorptive tissues such as the intestine, kidney and placenta [238,239]. It plays a role mainly in the uptake of the vitamins pantothenate and biotin, as well as lipoate from the diet [239]. Even before the identification of this transporter, it was observed that conjugation of biotin with various molecules enhanced their cellular uptake [240–242]. In these studies, the cellular uptake of Tat protein (trans-activator of transcription) of human immunodeficiency virus 1 (HIV-1) or its fragments were enhanced by biotin conjugation. The fragment proteins were developed to display Tat antagonistic activity, and in a later study, a retro-inverso derivative of this peptide was developed that exhibited high resistance to proteolysis in serum [243]. All of these Tat-derived biotinylated peptides were shown to use SMVT as an uptake route [243,244].

Similarly, a camptothecin topoisomerase inhibitor-PEG-biotin conjugate was developed, which showed enhanced cytotoxic activity compared to camptothecin alone [245]. Since the PEG and PEG-biotin fragments alone did not induce cell death, it was concluded that the improved efficiency of the conjugate was likely due to enhanced solubility, stability and SMVT-mediated uptake of camptothecin [245].

Prodrug derivatives of acyclovir conjugated with both various lipids and biotin were also developed, and the addition of both the hydrophobic moiety and biotin appeared to have an additive effect in increasing cellular uptake of the compounds [246,247]. The effect of biotin conjugation could be significantly reduced by competition with biotin, indicating the involvement of SMVT in this process [246,247]. Computational docking of the biotinylated prodrug was also performed using a structural model of human SMVT, suggesting a possible mode of interaction of the generated compounds with the transporter [247].

Biotin has also been used to functionalize various nanocarriers in order to improve the oral bioavailability of biomolecules and drugs. Insulin encapsulated in biotinylated liposomes showed about twice the bioavailability of those in conventional liposomes [248]. The formulation exhibited a mild hypoglycemic effect that lasted longer than subcutaneous insulin injection [248]. Nanostructured lipid carriers functionalized with biotin were also developed for intestinal absorption of oridonin, a natural compound with anti-inflammatory and anti-cancer effects, which otherwise exhibits low solubility and bioavailability [249]. However, the involvement of SMVT in the uptake of these formulations has not been tested.

Several types of cancers exhibit increased uptake of vitamins, indicating that vitamin transporters or receptors could be used for selective cancer targeting [250–252]. In one study, a hydrophobized polysaccharide, pullulan acetate, was used to generate self-

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assembling nanoparticles functionalized with biotin to enhance cancer cell targeting [253]. The biotin-conjugated nanoparticles showed increased uptake in cells of the HepG2 carcinoma cells line compared to unconjugated particles [253]. Biotin-coated nanodiamonds for drug delivery were developed and tested against streptavidin binding, but cell-based *in vitro* studies were not performed [254]. Another type of nanoparticles formed from poly(amido)amine (PAMAM) dendrimers conjugated to biotin and labeled with fluorescein isothiocyanate (FITC) were shown to be taken up by HeLa cells much more effectively than unmodified PAMAM [255,256]. However, the uptake of these nanoparticles was only partially mediated by SMVT as it proceeded predominantly through nonspecific absorption that could not be inhibited by biotin [256,257]. While biotin conjugation did confer an advantage for FITC dye delivery, no increase in delivery was observed between biotinylated and non-biotinylated particles when the nanoparticles were loaded with cisplatin [257]. Biotin-conjugated polymeric micelles were also developed as delivery agents for doxorubicin. The corresponding study showed that biotin labeling enhanced both cellular uptake and drug efficacy when tested in the MCF-7 breast cancer cell line [258]. Similar results were reported for biotinylated cubosomes carrying paclitaxel into HeLa adenocarcinoma cells [259] and for biotinylated polyurethane-urea nanoparticles loaded with a reporter gene-encoding vector and either sunitinib or phenoxodiol as anticancer agents [260]. However, none of these studies tested the involvement of SMVT in the uptake process.

In an interesting study that shows that biotin targeting is likely to be receptor-mediated, rat erythrocytes were used as nanocarriers functionalized with *N*-hydroxysuccinimide ester of biotin [261]. Upon injection into rats, the modified erythrocytes accumulated predominantly in the liver and spleen, which was attributed to a clearance process of the biotinylated erythrocytes that depends on C3b receptors of the complement system present on liver and spleen macrophages, which then leads to opsonization and excretion by the liver and spleen [261]. Subsequently, methotrexate was encapsulated into the erythrocytes using the pre-swell dilution procedure and was shown to accumulate in the liver at higher levels one hour after application of the biotin-labeled erythrocytes compared to using unlabeled nanoparticles [261].

Vitamin B6 has been used as a conjugate to enhance the uptake of nanoparticles into cancer cells [262], as increased vitamin B6 metabolism is associated with cancer risk, especially in lung cancer, and elevated expression of the vitamin B6-dependent enzyme serine hydroxymethyltransferase (SMHT) is associated with increased requirement for DNA synthesis as part of the metabolic adaptation of cancer cells to support growth and proliferation [263,264]. Nanoparticles consisting of a poly(ester amine)-based gene delivery system were decorated with the active form of vitamin B6, pyridoxal 5'-phosphate. The decorated system exhibited higher transfection rates in lung cancer cells than normal lung cells, resulting in enhanced gene delivery within the rapidly proliferating cancer cells. The nanoparticles utilized an uptake mechanism with relatively high affinity, followed by an endocytic internalization mechanism. Moreover, the uptake of the nanoparticles could be inhibited by the vitamin B6 antagonist, 4'-deoxypyridoxine [262]. Whether uptake involves one of the known H^+-coupled thiamine transporters SLC19A2 and SLC19A3, which mediate transmembrane translocation of the positively charged pyridoxine [265], or whether another, yet unidentified transporter is involved is still unclear.

Conjugation of folic acid has been used both to improve the oral bioavailability of therapeutics, as a means to target cancer cells and to deliver drugs into the brain via the blood-cerebrospinal fluid barrier. The first folate transporter identified was the reduced folate carrier (RFC/SLC19A1), which shows high affinity for reduced folates most abundant in systemic circulation, such as 5-methyltetrahydrofolate. It is widely expressed, and it can mediate folate uptake from the bloodstream [266]. Absorption of dietary folic acid occurs in the duodenum and upper jejunum predominantly as a carrier-mediated process with a low-pH optimum [267]. This transporter has been identified as the H^+-coupled folate transporter PCFT/SLC46A1 [268]. It enables folates to be absorbed across the brush-border membrane. PCFT is also expressed in the choroid plexus and is required for the

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transport of folates into the CSF. Loss of function of this transporter causes autosomal recessive hereditary folate malabsorption, a syndrome characterized by severe systemic and cerebral folate deficiency [268]. The folate receptor alpha (FRα) is expressed in the choroid plexus as well, and its loss of function results in an autosomal recessive disorder that solely leads to cerebral folate deficiency [268]. One theory to account for the requirement of both PCFT and FRα in the transepithelial flow of folate from blood to CSF is that folate binds from the side of the blood to the receptor at the basolateral membrane where PCFT is also expressed. This would be followed by internalization and the forming of a vesicle containing both receptor and PCFT, which would traffic to the apical membrane and be released into the CSF as an exosome from which folates are exported via PCFT [269]. An alternative pathway would comprise the PCFT-mediated export of folates from acidified endosomes within the intracellular compartment, followed by export into the CSF via the RFC/SLC19A1 reduced folate/organic phosphate antiporter [270].

Folate-functionalized PLGA nanoparticles have been successfully used to deliver an Hsp90 heat shock protein inhibitor to mouse Colon-26 epithelial-like and Raw 264.7 macrophage-like cells [271]. The formulation was also shown to be taken up by inflamed colon cells in a mouse model of ulcerative colitis and attenuate both inflammation as well as colitis-associated cancer [271]. In contrast, similar nanoparticles without folic acid conjugation did not show therapeutic efficacy. Due to the expression of folate receptors on the inflamed colon cells, the cellular uptake was suggested to be a receptor-mediated process [271].

Interestingly, PCFT/SLC46A1 was shown to be upregulated in proximal intestinal epithelial cells of diabetic rats [272]. Based on this observation, folate-grafted chitosan nanoparticles have been generated and loaded with insulin. The resulting nanoparticles could be taken up by Caco-2 cells highly expressing PCFT, transported through the Golgi pathway, and the uptake was attenuated by free folic acid. In contrast, in Caco-2 cells expressing lower amounts of PCFT, the nanoparticles were endocytosed but mainly degraded in lysosomes [272]. *In vivo* studies with diabetic rats also showed that the nanoparticles can successfully deliver insulin into the bloodstream reaching an oral bioavailability of 14.4% [272].

In human, the receptor-mediated uptake pathway of folate can be mediated by three different folate receptors paralogs, α, β and γ [273]. While all three folate receptors are reported to be expressed in the small intestines only at negligible levels [267], folate receptor α is expressed in epithelial cells of the proximal tubules of the kidney and the choroid plexus, as well as in various cancers [273,274]. Due to this, folate-linked therapeutics have been developed for targeting cancer cells, which predominantly use the receptor mediated pathway.

Folate-conjugated *N*-trimethyl-chitosan chloride (TMC) nanoparticles have been engineered for targeting tumor cells, which could be loaded with anti-cancer proteins [274]. In this study, the nanoparticles were loaded with FITC-BSA (bovine serum albumin), and folate functionalization showed 3.7-fold increase in uptake compared to non-functionalized nanoparticles [274]. The dependency of the uptake on folate receptor expression was confirmed by competition with free folate in the buffer and by using the A549 cell line, which is a folate receptor-deficient cell line [274].

Multi-walled carbon nanotubes have been coated with chitosan that has previously been functionalized with folic acid to generate a nanodelivery agent [275]. The nanoparticle was able to deliver a plasmid encoding green fluorescent protein (GFP) into HeLa and MCF-7 cancer cells, and the chitosan-folic acid coating improved the transfection efficiency 1.5-fold [275]. The uptake was suggested to be mediated by a folate receptor, but this has not been examined in detail.

Recently, a new route for delivery of nanomedicine to the CNS has been described using the folic acid transport pathway of the choroid plexus [276]. This was based on the observation that folate uptake in neuroepithelial cells in mouse embryos is dependent on the presence of the low-density lipoprotein (LDL) receptor-related protein 2 (LRP2) in the cellular membrane [277]. It was hypothesized that the direct interaction of the folic acid-

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bound soluble FRα with LRP2 triggers the endocytosis of the receptor complex and thus enables folic acid uptake [277]. To exploit this pathway, nanoparticles made of poly-(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) were surface-modified with the folic acid receptor α/folic acid complex (FRα-FA). The uptake of FRα-FA conjugated nanoparticles by human choroid plexus epithelial cells (HCPEpiCs) was determined *in vitro* by inverted optical fluorescence and confocal microscopy. FRα modified nanoparticles were internalized by HCPEpiCs to a greater extent and the apparent permeability coefficient was significantly higher than that of their unmodified counterparts [276]. The biodistribution of unmodified and FRα-FA-modified nanoparticles following intravenous administration was compared in ICR albino mice and showed that conjugation of the FRα-FA complex to the nanoparticle surface promoted higher accumulation in the brain, highlighting the potential of FRα-FA-modified nanoparticles as a strategy for delivering molecules from the blood to the CNS. However, the mechanism cellular uptake and transport of nanoparticle across the choroid plexus and whether folic acid transporters play a role in this process remain unclear and require further investigation.

3.6 Oligopeptide transporters (PEPT1/PEPT2)

The conjugation of amino acids or dipeptides to existing drugs has been a long-standing strategy to improve their oral bioavailability [278–281]. One of the first and most extensively studied prodrug of this type is valacyclovir, the L-valine conjugate of acyclovir, a potent antiviral agent [282]. Conjugation of acyclovir with amino acids such as L-valine significantly improved oral bioavailability as tested in rats [282] and also in human volunteers [283].

The intestinal transport system responsible for the increased permeability was identified as the oligopeptide transporter PEPT1/SLC15A1 [284,285]. It is highly expressed in the small intestines and is responsible for the absorption of dietary di- and tripeptides as well as a variety of peptide-like drugs such as aminocephalosporins, angiotensin-converting enzyme inhibitors, antiviral prodrugs in a proton-coupled manner [284,286]. Its closest paralog with 50% sequence identity [287], PEPT2/SLC15A2, is expressed in the proximal tubules of the kidney, where it reabsorbs oligopeptides, as well as peptide-like drugs, including prodrugs [286]. PEPT2 is also expressed in adult rat brain by astrocytes, ependymal cells, subependymal cells and by the epithelial cells of the choroid plexus [288]. Also, retinal Müller cells and peripheral satellite cells express this transporter [288]. Two further paralogs in human, SLC15A3/PHT2 and SLC15A4/PHT1, are less well-characterized and are highly expressed in various immune cells, where they are thought to operate as histidine and peptide transporters [286,289]. SLC15A3 and SLC15A4 are preferentially expressed by cells within the lymphoid system, including dendritic cells, and are upregulated in response to toll-like receptor (TLR) stimulation [290,291]. Recent studies have shown that SLC15A4 contributes to the trafficking of TLRs and their ligands to endolysosomes, wherein recognition and signaling are initiated [292].

Since the identification of the PEPT1/SLC15A1 transporter as a promiscuous intestinal peptide uptake mechanism, a wealth of scientific literature has focused on the production of amino acid, dipeptide and tripeptide prodrugs to enhance intestinal absorption of drugs that are not readily absorbed by the oral route. Drug classes for which this strategy has been used include antiviral agents (acyclovir [282,283,285,293–296], gancyclovir [297], levovirin [298], oseltamivir [299,300], zanamivir [301], peramivir [302], lopinavir [303], cidofovir [304], zidovudine [305]), chemotherapy medications (cytarabine [306], paclitaxel [307], floxuridine [308–313], gemcitabine [314], melphalan [315]), anti-inflammatory agents (5-aminosalicylic acid [316], nabumetone [317], ibuprofen [318]), natural products (oleanolic acid [319–321], glucosamine [322]), L-DOPA and L-methyldopa [20,323–326], as well as tricin [327], pterostilbene [328], alendronate [329,330], and various other drugs [331]. An interesting strategy was the development of a dipeptide-like thiopeptide "carrier", which is a small molecule binder of PEPT1 that was intended to be used as a general drug carrier [317]. This carrier was chemically conjugated to several different drugs and

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many of the conjugates showed high-affinity binding to PEPT1 and the ability to permeate into cells and through a Caco-2 cellular monolayer [318,331].

Additionally, many studies have focused on exploring the selectivity of binding to PEPT1 to optimize interactions between prodrugs and the transporter. In a study using a dipeptide-conjugated azidothymidine library to screen the ability of dipeptides to compete with the known ligand cephalexin, certain dipeptides, such as Phe-Gly or Val-Ser were found to be highly effective, in line with previous studies [332]. Peptides whose first amino acid is Ile or Ala have also shown binding to PEPT1, as has been previously found for certain prodrugs [332]. In addition, several other dipeptides (Arg-Ile, Ile-Ala, Leu-Ile, Phe-Ala, Phe-Lys, Pro-Ile, Ser-Pro, Ser-Glu, Thr-Ala, Val-Arg) have shown strong binding to PEPT1 [332], which could also be used in drug modifications to improve intestinal permeability. Other studies have also shown that L-valine or L-isoleucine conjugation often gives the most efficient cellular permeability and oral bioavailability [282,293,298,299,301,302,306,307,310,312,314,319,320,328]. In turn, D-amino acid-containing dipeptides appear to be less well absorbed by the oligopeptide transport system, and their affinity is also lower [325].

Interestingly, as the structural basis of valacyclovir [333] and valganciclovir [334] binding to homologs of PEPT have been experimentally elucidated, it was found that the two drugs bind in different orientations in these structures despite their very close chemical similarity. It was suggested that the binding orientation of these prodrugs may depend on structural differences between the prokaryotic DtpA protein and mammalian PEPT proteins used in the above studies [335]. With the recent resolution of an outward-open state of rat PepT2, molecular docking suggested a binding mode for valacyclovir consistent with that of tripeptides [335]. Using the same protein structure, a very similar binding mode and set of interacting residues were proposed for valganciclovir, and these might correspond to an initial binding conformation [335]. Nevertheless, this mode of binding and the set of interacting residues do not completely overlap with the experimentally observed binding mode of valacyclovir to the inward-open structure of a bacterial homolog of hPEPT1, $Pept_{Sh}$, which might represent a substrate conformation at a later point in the transport cycle [333]. Variation in binding modes has also been observed previously for di- and tripeptide substrates [336,337]. As for dipeptides with positively charged lysine and arginine sidechains, it was proposed that they could bind in a conformation similar to that of tripeptides [338]. The conserved position of the amino group in the above-mentioned binding modes of valacyclovir and valganciclovir [335] as well as di- and tripeptides [336,337] suggests that the N-terminus of substrate peptides is the primary binding site of the substrate, confirming previous findings and the importance of a free amino group in high-affinity substrates [339].

The chemical requirements for a PEPT1 substrate have been the subject of a number of studies. Early on, it was shown that PEPT1 substrates must have at least the two oppositely charged head groups separated by at least four methylene groups [340]. Based on structural information of rat PepT2, it was proposed that Glu622 and Arg57 are responsible for binding the N- and C-terminus, respectively, of the bound peptide substrate [335], corresponding to Glu595 and Arg27, respectively, in hPEPT1. The same cryo-EM structure has also confirmed that the ideal distance between these two binding points is about 6 Å, in line with previous suggestions [335,340]. In addition, it has been proposed that interaction with the first carbonyl group of the substrate through Asn192 (Asn171 in hPEPT1) and a series of tyrosine and tryptophan residues in the protein contributes to the promiscuity of the binding pocket by forming hydrophobic and polar subpockets as required [335,341]. The importance of a carbonyl moiety near the primary amino group of the substrate has also been shown to be important in previous studies of the structure-activity relationship [342].

It should be noted that in some instances, amino acids were conjugated with drugs via their amino groups while bearing a free carboxyl group, resulting in compounds that did not comply with the binding mechanism described above [302,327,328,343]. In one such case, although the uptake of such a prodrug competed with the PepT1 model

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substrate glycylsarcosine (Gly-Sar), uptake was also inhibited by estrogen-3-sulfate, which is a typical substrate of the organic anion-transporter OATP2B1/SLCO2B1 that is also expressed in the Caco-2 cells used in the assay [328]. Consequently, anionic amino acid prodrugs intended to be substrates for PEPT1 may also utilize other cellular uptake pathways.

PEPT1 has also been targeted by nanoparticle formulations to enhance intestinal absorption of drugs. One such example is the targeting of PEPT1 for the delivery of docetaxel by developing dipeptide-linked (L-valyl-valine, L-valyl-phenylalanine) PLGA nanoparticles [344]. The formulation was tested in HeLa cells stably transfected with hPEPT1, as well as in Caco-2 cells, and showed improved uptake compared to unmodified nanoparticles. In addition, *in situ* small intestinal perfusion as well as *in vivo* oral absorption experiments showed that the modified nanoparticles can elicit higher uptake of the drug and higher plasma half-life. Interestingly, the application of the formulation was observed to decrease the expression of PEPT1 at both the mRNA and protein levels [344].

PEPT1 was targeted using poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) nanoparticles linked with valine, Gly-Sar, valyl-glycine and tyrosyl-valine to improve the oral delivery of acyclovir [345]. *In vitro* uptake of the functionalized nanoparticles competed with the PEPT1 model peptide Gly-Sar, indicating the involvement of the transporter in the uptake of the nanoparticles [345]. Encapsulation of acyclovir did not alter the rate of absorption, based on the assessment of the C_{max}/AUC ratio, but increased half-life and mean residence time (MRT) following oral administration in *in vivo* mouse studies [345].

3.7 Organic cation transporters (OCTNs)

OCTN2/SLC22A5 is a Na^+-coupled L-carnitine transporter expressed in the small intestine and is responsible for the uptake of dietary L-carnitine [346–348] together with $ATB^{0,+}$/SLC6A14 [349,350]. The carnitine uptake pathway has been used to improve oral absorption of various drugs. The prodrug strategy was used to synthesize compounds linking L-carnitine to gemcitabine through the carboxyl group. This formulation was able to increase the oral bioavailability of gemcitabine by up to 4.9-fold [351]. A potential prodrug of butyrate, butyrate-L-carnitine, has also been synthesized for the treatment of IBD because of its anti-inflammatory effect [122]. However, under pathophysiological conditions, OCTN2 was shown to be downregulated while SLC6A14 is expressed at higher levels, so the uptake of the prodrug likely proceeded via SLC6A14 [122] (see also the section on amino acid transporters). L-carnitine conjugation has also been used to increase the hydrophilicity and to prolong the pulmonary residence time of prednisolone, a drug often administered by inhalation for the treatment of bronchial asthma (BA) [352]. The non-tumorigenic human airway epithelial cell line BEAS-2B has been shown to express both OCTN2/SLC22A5 and OCTN1/SLC22A4 carnitine transporters [352,353]. Of two prodrugs, the one retaining both the free choline and carboxyl groups of L-carnitine showed higher uptake in BEAS-2B cells, indicating that both charged groups are probably important for substrate recognition by carnitine transporters [352].

L-carnitine conjugation has also been used to guide the targeting of various nanoparticle formulations. PLGA nanoparticles conjugated with L-carnitine were engineered to be taken up by Caco-2 cells [354]. It was found that the optimal surface density of L-carnitine is about 10% to achieve the highest uptake efficacy [354]. It was also found that the uptake of nanoparticles competes with free L-carnitine in the buffer and that the uptake process is dependent on the presence of Na^+, which is in line with the Na^+-cotransporter mechanism of OCTN2 [354]. The uptake of the labeled nanoparticles was also inhibited by various endocytosis inhibitors such as indomethacin and chlorpromazine, indicating that endocytosis is involved as well [354]. Nanoparticles with 10% L-carnitine labeling also showed the most favorable pharmacokinetic parameters, including maximum plasma concentration (C_{max}) and oral bioavailability compared to unlabeled PLGA nanoparticles [354].

OCTN2 is also highly expressed in brain capillary endothelial cells that make up the BBB [355–358]. L-carnitine was used as a carrier to deliver nipecotic acid, an

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anticonvulsant, through the BBB [359]. The conjugate prodrug was shown to be taken up and activated *in vivo* in the mouse brain and to prolong the latency duration of convulsions triggered by pentylenetetrazole [359].

Cells from the cell fibroblast-like cell line glioblastoma T98G multiforme have also shown robust expression of OCTN2/SLC22A5 [44]. This cell line has been used as a model for targeting glioma and the BBB with PLGA nanoparticles conjugated with L-carnitine. [44]. Various linker lengths were explored, with PEG-1000 showing highest cellular uptake [44]. Uptake of L-carnitine conjugated nanoparticles followed the endocytic pathway and was dependent on Na^+ and competed with free L-carnitine, in contrast to unlabeled particles [44]. L-carnitine conjugated PLGA nanoparticles loaded with paclitaxel were found to be 11-fold more enriched in mouse brain compared to non-conjugated nanoparticles. In addition, the cytotoxicity increased with the application of L-carnitine conjugated nanoparticles compared to non-conjugated nanoparticles and to Taxol, presumably due to enhanced cellular uptake, as shown by cytotoxicity assays in T98G cells. Finally, *in vitro* anti-glioma efficacy was evaluated in T98G spheroids, with paclitaxel-loaded L-carnitine conjugated nanoparticles showing enhanced toxicity [44].

Since SLC6A14 can also transports L-carnitine, albeit with a lower affinity than OCTN2/SLC22A5, similar nanoparticles have also been shown to bind to and utilize both transporters in various cancer cell lines [360]. Similar dual targeting has also been used to target LAT1 and SLC6A14 with a single nanoparticle formulation [125] (see in the section on amino acid transporters).

3.8 Organic anion transporters (OATPs)

Transporters of the SLCO/SLC21 family (also called organic anion transporter family) typically exhibit a broad substrate range with a preference for negatively charged substrates. Because of these properties, they readily interact with a great variety of endogenous compounds and xenobiotics [361,362]. OATP1B1/SLCO1B1 and OATP1B3/SLCO1B3 are two members of the SLCO family that are considered to have liver-specific expression [363]. In certain cases, these transporters have been targeted to enhance liver-specific drug delivery in order to reduce off-target effects in other tissues.

The enzyme glucokinase is present in liver, pancreas and brain, converts glucose to glucose-6-phosphate for further metabolism, and plays a central role in glucose homeostasis [11]. Its activators are among the potential next generation therapies for type 2 diabetes [364,365]. However, these compounds can cause hypoglycemia due to overactivation of glucokinase in the pancreas leading to the overproduction of insulin [11]. One of the strategies to reduce off-target effects was the development of hepatoselective glucokinase activators by targeting OATPs highly expressed in the liver [364,366]. With this in mind, a structure-activity relationship (SAR) study of *N*-heteroarylacetamides revealed a hepatoselective glucokinase activator that, through the incorporation of a carboxyl group, enables hepatoselective uptake via OATPs while minimizing passive cellular uptake of the compound [364]. One of the resulting compounds proved to be a substrate for OATP1B1 and OATP1B3 and showed enhanced activity in isolated hepatocytes compared to systemic activators. In addition, was able to normalize fasting plasma glucose levels in a diabetic rat model without causing hypoglycemia as did systemic activators [364]. The hepatoselective compound displayed a tissue distribution with a liver-to-pancreas ratio of 75-fold for rat and 58-fold for dog [364].

Systemic stearoyl-CoA desaturase-1 (SCD1) is an enzyme that catalyzes the introduction of a cis-double bond between the C_9 and C_{10} positions of various long chain saturated fatty acid-CoA esters, which has made it a promising target for the treatment of type 2 diabetes, dyslipidemia, obesity and metabolic diseases [367,368]. However, systemic inhibition of SCD1 causes side effects such as dry skin and hair loss, in addition to its targeted effect of reducing *de novo* production of oleic acid in liver [367]. Since SCD1 expression is highest in the liver, hepatoselective inhibitors were developed by exploiting transport through liver-specific OATPs [367,369]. The OATP-targeting homing moiety used was either a tetrazole acetic acid [367] or a nicotinic acid [369], both of which bear a free carboxyl

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group. Each of these compounds has been shown to be substrates of OATP1B1 and OATP1B3 [367,369]. The tetrazole acetic acid derivative displayed a liver-to-plasma distribution ratio of >10-fold and a liver-to-skin ratio of >30-fold in various preclinical animals [367]. In turn, both compounds showed improved blood glucose clearance in an obese mouse model [367,369].

3.9 Monocarboxylate transporters (MCTs)

MCT1/SLC16A1 is a monocarboxylate transporter that has been shown to be highly expressed on both the apical and basolateral membranes of enterocytes along the intestinal tract, particularly in the colon and rectum [370–372]. MCT1 is responsible for the uptake of short-chain fatty acids such as acetate, propionate and butyrate, which are important metabolites for maintaining a healthy colon with anti-inflammatory effects. They are produced by bacterial fermentation of undigested fibers from complex carbohydrates by the intestinal microflora [373–375]. Due to its localization, MCT1 has gained focus as a possible entry pathway for therapeutics across the intestinal barrier.

XP13512 is a prodrug of gabapentin designed to be transported by intestinal nutrient transporters to enhance oral bioavailability of gabapentin at therapeutic doses for treating neuropathic pain [376]. The acyloxy-alkyl carbamate modification changes the zwitterionic state of gabapentin due to conjugation at the amino group, while the free carboxyl group remains unmodified. The prodrug was subsequently found to act as a substrate for both MCT1 and SMVT transporters and to compete with their natural substrates [376]. *In vitro* transport studies in Caco-2 and MDCK cellular monolayers indicate that the prodrug is able to effectively cross both cellular layers [376]. This prodrug was later marketed as gabapentin enacarbil, an extended release formulation, and approved for use for the treatment of moderate to severe primary restless legs syndrome (RLS) [377–379].

In another study, 5-fluorouracil was conjugated with dicarboxylic acids to target MCT1 in order to enhance oral bioavailability [380]. The octanedioic acid ester derivative of 5-fluorouracil showed superior uptake properties in Caco-2 cells as well as in monolayers compared to the unmodified drug [380]. The uptake could be inhibited with known inhibitors and substrates of MCT1 such as quercetin and butyrate, respectively, indicating the involvement of the transporter in the uptake process [380]. The prodrug also showed improved uptake rates according to *in situ* perfusion measurements as well as a 4.1-fold increase in oral bioavailability in rats [380].

A similar strategy was used for gemcitabine, an anti-cancer agent, to develop prodrugs with various linker lengths to improve oral bioavailability by targeting MCT1 in the intestine [381]. Out of five prodrugs, all showed uptake competing with butyric acid, indicating the involvement of MCT1, with prodrug **2** showing highest affinity to MCT1 [381]. Interestingly, all prodrugs showed superior permeation properties in Caco-2 monolayers compared to previous gemcitabine prodrugs that utilize PEPT1 [314] or OCTN2 [351] transporters for uptake. Prodrug **2** with a 6-carbon linker also showed the highest oral bioavailability in rats [381].

In addition, MCT1/SLC16A1 was also found to be highly expressed in tumors [382]. For delivery into cancer cells, *O*-carboxymethyl chitosan nanoparticles were modified with acetic acid to help target them to MCT1-abundant membranes [39]. The resulting nanoparticles showed significantly higher uptake rates of carried docetaxel in Caco-2 cells than unmodified liposomes [39]. Conjugation of acetic acid significantly improved oral bioavailability, while drug uptake was competitively attenuated by free acetic acid, suggesting the involvement of MCT1 in the process [39]. The conjugated nanoparticles also showed significantly higher anti-tumor efficacy than the unmodified liposome formulation of docetaxel [39]. The best uptake results were achieved with 45.14% conjugation with acetic acid, likely due to steric crowding effects that limit transporter binding, similarly to other reports [354,383,384].

Butyrate itself was also used to decorate PEG-based nanoparticles to enhance intestinal delivery [385]. The functionalized nanoparticles showed up to 2.84-fold increase in uptake in both E12 and Caco-2 cells compared to non-functionalized ones [385]. Free

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butyrate, as well as lactate and pravastatin were found to attenuate the endocytosis of the functionalized nanoparticles, indicating the involvement of MCT1 in the uptake process [385]. The decorated nanoparticles also showed 2-fold increased uptake in an *ex vivo* ligated intestinal loop assay compared to normal nanoparticles [385]. The nanoparticle formulations were used to deliver insulin into rats, and it was found that butyrate conjugation enhances oral bioavailability of insulin to 3-fold higher levels compared to unconjugated nanoparticles, while the nanoparticle encapsulation prolonged its release [385].

In the BBB, MCT1/SLC16A1 is expressed at both the luminal and abluminal membranes of the brain capillary endothelial cells and it plays an important role at the luminal membrane for the influx of lactate from the blood stream into the brain [386,387]. Therefore, it has also been a target for the delivery of formulations to the brain. To this end, β-hydroxybutyrate conjugated solid lipid nanoparticles loaded with docetaxel were tested [388]. They showed significantly increased uptake in brain epithelial cells, which was inhibited by β-hydroxybutyrate, indicating the involvement of MCT1. The particles also showed an effective increase in docetaxel distribution in the brain [388].

Pluronic-85, a tri-block copolymer that self-assembles into micelles, has been used as a nanocarrier to delivery drugs through the intestinal and blood-brain barriers, as well as into tumors [389]. While this material has been at the focus of drug nanocarrier development due to its low toxicity and inhibition of several ABC transporters related to multidrug resistance [390], it has also been shown to interact with OCTN2 and MCT1 in bovine brain microvascular endothelial cells (BBMEC), which often serve as a model system for the BBB [374,391]. However, whether MCT1 is involved in the uptake of the various formulations based on Pluronic-85 has not yet been systematically studied [374].

4. Conclusions and outlook

In general, the rational design of targeted delivery systems has mainly focused on specific organs with well-characterized physiology (e.g., liver) and key biological barriers (e.g., blood-brain barrier, intestinal barrier). However, based on similar principles, transporter-based targeting of other organs (e.g., kidney, lung, pancreas, prostate, ovary, bone) or barriers (e.g., the blood-cerebrospinal fluid barrier, the blood-testis barrier, the blood-retina barrier) should also be possible, even though only limited information on applications is available and this is often hindered by the lack of a detailed characterization and quantitation of the transporters available on these organs or barriers [11].

There are certain routes of drug administration that offer viable alternatives to oral delivery. For example, nasal delivery of therapeutics through the nasal cavity is attractive because olfactory neurons exposed in the nasal cavity provide direct access to the CNS, thus avoiding initial metabolism in the liver [392]. To date, many ABC and SLC drug transporters have been reported to be present in the nasal cavity [393,394]. While there is evidence that some of these transporters, such as the equilibrative nucleoside transporter ENT1/SLC29A1, can readily mediate the uptake of substrates such as [^{18}F]fluorothymidine into the brain through the nasal route [395], these mechanisms appear to be underutilized in the development of new formulations. While certain nanoparticle formulations designed for nasal-to-brain delivery use functionalization through receptor ligands, there do not appear to be any reports of targeting transporters in the nasal cavity for drug delivery [396].

Similarly, the pulmonary route of administration through inhalation could be an interesting alternative because of the thin epithelial barrier, large surface area [397] and lower expression of metabolic enzymes compared to liver [397,398]. While drug transporters are expressed in mammalian airway epithelia, their exploitation through rational design to enhance delivery has remained scarce [399–401]. In addition, OCTN2 expressed in the trachea has been used for lung-specific drug targeting by developing an L-carnityl ester conjugate of prednisolone for pulmonary administration against bronchial asthma [352]. This novel prodrug also showed improved efficacy in an *in vivo* model of asthma

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[402]. However, other transporters in different cell types of the lung have not, to our knowledge, been used for organ-specific drug delivery.

Along the same lines, many other transporters besides those mentioned in this review could also be potential targets for tissue- or barrier-specific targeting of medications. Specifically in the intestines, several known uptake systems are available, including the cholesterol transporter SLC65A2/NPC1L1 (Niemann–Pick C1-Like 1) [403], the fatty acid transporter CD36 [404] and the long-chain fatty acid transporter proteins SLC27/FATP [405]. In terms of brain and CNS targeting, one vitamin that must enter the brain from the blood via the choroid plexus and CSF is riboflavin (vitamin B2). Riboflavin is an essential component of the brain and is not synthesized in mammalian tissues. Based on *in vitro* studies it was shown that there is a potent active transport system for riboflavin in the isolated rabbit choroid plexus [406] but its molecular identity appears to be unknown. The known riboflavin transporters belong to the SLC52 family, but these do not appear to provide active transport [407].

Recently, many SLC transporters have been shown to have very specific expression patterns, in contrast to non-transporter protein families [408]. Nevertheless, many SLC transporters remain relatively understudied, and more information about their localization and tissue expression patterns would be beneficial. With respect to transporter expression levels, it is important to note that species-specific differences in expression may limit the usefulness of preclinical animal models and bridging the gap between them and clinical trials is an important challenge to be addressed [32].

In terms of the exploitation of individual transporters, the lack of specific binders to transporters with similar substrate specificity can be a bottleneck [74], and their development would enable the generation of more advanced drug delivery systems. Once specific binders are available, dual targeting can also be an interesting approach, especially when targeting heterogenous cell populations such as in tumors [26,125].

Transporters can also module the efficacy of nanoparticle uptake. In this context, it is important to note that systematic database analyses suggest that many more transporter-like proteins may be encoded in mammalian genomes than previously thought [5]. For example, MFSD2A/SLC59A1, a key transporter for docosahexaenoic acid uptake at the BBB, regulates caveolae-mediated transcytosis by modifying the lipid composition of the plasma membrane [409]. Transcytosis in BBB endothelial cells is exceedingly suppressed compared to peripheral endothelial cells [409]. Priming the BBB with MFSD2A inhibitors such as tunicamycin, followed by the application of transcytosis-employing nanoparticles carrying doxorubicin showed that doxorubicin was taken up 4.3-fold more effectively in this manner [409].

In addition to their advantages, many of the current transporter-targeted delivery formulations still have to overcome certain challenges. One such obvious barrier in targeting nutrient transporters is the occurrence of off-target effects due to the abundant expression of nutrient transporters in healthy cells in a variety of tissues [8]. On the other hand, pathological conditions can also lead to changes in the expression pattern of certain transporters [32], for example, GLUT1 expression in the BBB is decreased in patients in an early stage of Alzheimer's disease [30,410]. It should also be taken into account that the application of transporter-targeted formulations may itself also affect the expression level of the corresponding transporter, including depletion from the cell surface [8,26,29,101,111,123,344,360]. Transporter-targeting formulations are typically also expected to compete with the endogenous substrate of the transporter and thus block its uptake, which can lead to efficacy or safety concerns [32]. Overall, most transporter-targeted formulations are limited by their specificity, potential toxicity and absorption efficiency [10].

While the efficacy of absorption and a phenotypic readout are often used to evaluate the success of transporter-targeted delivery, the underlying mechanism of uptake is often not known in detail, especially in relation to the recycling of the targeted transporters [8,26]. In addition, transporter-targeted nanocarrier formulations often get trapped in lysosomes, which can cause their degradation and prevent their successful transcytosis to

the basolateral membrane [10]. However, the specific mechanism of lysosomal escape is not known. Therefore, more extensive investigation of the intracellular fate, including the mechanism of absorption and processing of nanocarrier formulations, is warranted.

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