Targeted Prodrug Design to Optimize Drug Delivery

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ABSTRACT Classical prodrug design often represents a nonspecific chemical approach to mask undesirable drug properties such as limited bioavailability, lack of site specificity, and chemical instability. On the other hand, targeted prodrug design represents a new strategy for directed and efficient drug delivery. Particularly, targeting the prodrugs to a specific enzyme or a specific membrane transporter, or both, has potential as a selective drug delivery system in cancer chemotherapy or as an efficient oral drug delivery system. Site-selective targeting with prodrugs can be further enhanced by the simultaneous use of gene delivery to express the requisite enzymes or transporters. This review highlights evolving strategies in targeted prodrug design, including antibody-directed enzyme prodrug therapy, gene-directed enzyme prodrug therapy, and peptide transporter-associated prodrug therapy.

INTRODUCTION

Many therapeutic drugs have undesirable properties that may become pharmacological, pharmaceutical, or pharmacokinetic barriers in clinical drug application. Among the various approaches to minimize the undesirable drug properties while retaining the desirable therapeutic activity, the chemical approach using drug derivatization offers perhaps the highest flexibility and has been demonstrated as an important means of improving drug efficacy.

The prodrug approach, a chemical approach using reversible derivatives, can be useful in the optimization of the clinical application of a drug. The prodrug approach gained attention as a technique for improving drug therapy in the early 1970s. Numerous prodrugs have been designed and developed since then to overcome pharmaceutical and pharmacokinetic barriers in clinical drug application, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity, and poor patient acceptance (bad taste, odor, pain at injection site, etc.) (1).

The term "prodrug" or "proagent" was first introduced by Albert (2) to signify pharmacologically inactive chemical derivatives that could be used to alter the physicochemical properties of drugs, in a temporary manner, to increase their usefulness and/or to decrease associated toxicity. Since Albert discussed the concept of prodrugs in the late 1950s, such compounds have also been called "latentiated drugs," "bioreversible derivatives," and "congeners," but "prodrug" is now the most commonly accepted term (3-5). Usually, the use of the term implies a covalent link between a drug and a chemical moiety, though some authors also use it to characterize some forms of salts of the active drug molecule. Although there is no strict universal definition for a prodrug itself, and the definition may vary from author to author, generally prodrugs can be defined as pharmacologically inert chemical derivatives that can be converted in vivo to the active drug molecules, enzymatically or nonenzymatically, to exert a therapeutic effect. Ideally, the prodrug should be converted to the original drug as soon as the goal is achieved, followed by the subsequent rapid elimination of the released derivatizing group (5,6).

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Targeted Prodrug Design

Prodrugs can be designed to target specific enzymes or carriers by considering enzyme-substrate specificity or carrier-substrate specificity in order to overcome various undesirable drug properties. This type of "targeted-prodrug" design requires considerable knowledge of particular enzymes or carrier systems, including their molecular and functional characteristics. Recently, advances in gene cloning and controlled gene expression techniques in mammalian cells allow the elucidation of the molecular nature of enzymes and carrier proteins and make possible more rational design of "targeted-prodrugs."

In this brief review, targeted prodrug design will be discussed in 2 categories: (1) targeting specific enzymes and (2) targeting specific membrane transporters.

Prodrug Design Targeting Enzymes

In prodrug design, enzymes can be recognized as presystemic metabolic sites or prodrug-drug in vivo reconversion sites. Usually, targeting enzymes to reduce the presystemic metabolism is more successfully achieved by irreversible chemical modification rather than by a prodrug approach. Therefore, our discussion focuses on the enzymes as in vivo reconversion targets for prodrugs.

The enzyme-targeted prodrug approach can be broadly used to improve oral drug absorption, as well as site-specific drug delivery. In the case of improving oral drug absorption, gastrointestinal enzymes may be the main targets for prodrug design, and the use of a nutrient moiety as a derivatizing group permits more specific targeting for gastro-intestinal enzymes to improve oral drug absorption (7). These prodrugs have the additional advantage of producing nontoxic nutrient byproducts when they regenerate the active drugs in vivo. There have been extensive studies on gastrointestinal enzymes, which provide necessary information (eg, enzyme distribution, activity, and specificity) for prodrug design. Because enzyme targeting to improve oral drug absorption has been reviewed elsewhere (7-10), we will discuss enzyme targeting for site-specific drug delivery in the following section.

Strategy for Site-Specific Drug Delivery

The use of prodrugs has been actively pursued to achieve very precise and direct effects at the "site of action," with minimal effect on the rest of the body. Stella and Himmelstein (11,12) suggested that at least 3 factors should be optimized for the site-specific delivery of drugs by using the prodrug approach.

1. The prodrug must be readily transported to the site of action, and uptake to the site must be rapid and essentially perfusion rate limited.

2. Once at the site, the prodrug must be selectively cleaved to the active drug relative to its conversion at other sites.

3. Once selectively generated at the site of action, the active drug must be somewhat retained by the tissue.

In the prodrug approach, site-specific drug delivery can be obtained from tissue-specific activation of a prodrug, which is the result of metabolism by an enzyme that is either unique for the tissue or present at a higher concentration (compared with other tissues); thus, it activates the prodrug more efficiently. For example, glycosidase activity of the colonic microflora offers an opportunity to design a colon-specific drug delivery system (13). Glycoside derivatives are hydrophilic and poorly absorbed from the small intestine, but once they reach the colon, they can be effectively cleaved by bacterial glycosidases to release the free drug or be absorbed by the colonic mucosa. This strategy was evaluated in rats with 2 steroid prodrugs, prednisolone 21β-D-glucoside and dexamethasone 21-β-D-glucoside (13,14). Of these glycosidic prodrugs, the dexamethasone glucoside appeared to be the better candidate, with nearly 60% of the orally administered prodrugs reaching the caecum as a free steroid, while orally administered parent steroids were absorbed almost exclusively from the small intestine. L-γ-glutamyl transpeptidase, which is highly concentrated in the kidney. Then, L-dopa was decarboxylated to dopamine by aromatic L-amino acid decarboxylase, which is also highly concentrated in the kidney. The concentration of dopamine in the kidney after administration of L-γ-glutamyl dopa was almost 5 times higher than that after an equivalent dose of L-dopa (15). This type of

site-specific drug delivery has been of particular concern in cancer chemotherapy. Appropriately designed prodrugs have been found to be effective in the treatment of animal tumors possessing high levels of an activating enzyme (17,18). However, clinical results were disappointing when it was found that human tumors containing appropriately high levels of the activating enzymes were rare and that the high levels of activating enzymes were not associated with any particular type of tumor (19). Recently, new therapies have been proposed to overcome this limitation of prodrug therapy. These new approaches are referred to as ADEPT (antibody-directed enzyme prodrug therapy) and GDEPT (gene-directed enzyme prodrug therapy), which attempt the localization of prodrug activation enzymes into specific cancer cells prior to prodrug administration.

ADEPT and GDEPT for Selective Drug Delivery

General Concept of ADEPT and GDEPT

Enzymes that activate prodrugs can be directed to human tumor xenografts by conjugating them to tumor-selective monoclonal antibodies (20-25). As illustrated in Figure 1, an antitumor antibody is conjugated to an enzyme not normally present in extracellular fluid or on cell membranes and then these conjugates are localized in the tumor via intravenous infusion. After allowing for the conjugate to clear from the blood, a prodrug is administered that is normally inert but is activated by the enzyme delivered to the tumor. This is the ADEPT procedure. Using different combinations of antibody, enzyme, and prodrug, many classes of human tumor xenograft have been shown to be very sensitive to this procedure, although in most cases they are quite resistant to conventional chemotherapy (20-25). Early clinical trials are promising and indicate that ADEPT may become an effective treatment for solid cancers for which tumor-selective antibodies are known (26,27).

Tumors have also been targeted with genes encoding prodrug-activating enzymes (28-30). This approach can use a viral vector (eg, retroviral or adenoviral) to carry a prodrug-activating enzyme gene into both tumor and normal cells (Figure 1). By linking the foreign gene downstream of tumor-specific transcription units, tumor-specific expression of the foreign enzyme gene can be achieved (28). This approach has been called virus-directed enzyme prodrug therapy (VDEPT) or more generally gene-directed enzyme prodrug therapy (GDEPT) and has shown promising results in laboratory systems (31-33). In addition to viral vectors, several methods for delivery of the genes to the target tumor, under the control of tumor-selective promoters, have been proposed using liposomes and cationic lipids (34).

Strategy of Enzyme Targeting in ADEPT and GDEPT

For the appropriate combination of an enzyme and a prodrug, the choice of enzyme is very important because the appropriate prodrugs can be designed for almost any enzyme. However, enzymes that are monomeric, have low molecular weight, and lack a requirement of glycosylation would be preferable for ease of handling and possible protein modification (35). In ADEPT and VDEPT, the preferred targets may be the enzymes of nonhuman or nonmammalian origin that could catalyze substrates not normally activated in humans. Thus, in terms of specificity, enzymes from nonhuman sources, particularly those of microbiological origin, are advantageous, and if their immunogenicity is controllable, then specificity may become the overriding consideration (20). Appropriate combinations of enzymes and prodrugs (Table 1) have been proposed for ADEPT or GDEPT (25,35). Some of these combinations are not suitable in any situation, whereas others are more suitable for ADEPT than GDEPT or vice versa. In ADEPT, prodrug activation occurs extracellularly, whereas in GDEPT it occurs intracellularly. Therefore, the choice of enzymes can be different in those 2 approaches. The best enzymes for GDEPT would appear to be monomeric enzymes of bacterial or viral origin with wide substrate specificity (25,35). One example is a bacterial nitroreductase that can convert a relatively nontoxic monofunctional alkylating agent into a 10,000 times more cytotoxic difunctional alkylating agent (36). Because nitroreductases require either NADH or NADPH as an essential reductant, activation of prodrugs can only take place within the cells. Therefore, this enzyme may be a better choice for GDEPT than for ADEPT. In contrast, charged prodrugs that are bifunctional alkylating agents are especially suitable for ADEPT (36). Because of the charge, they will not enter cells

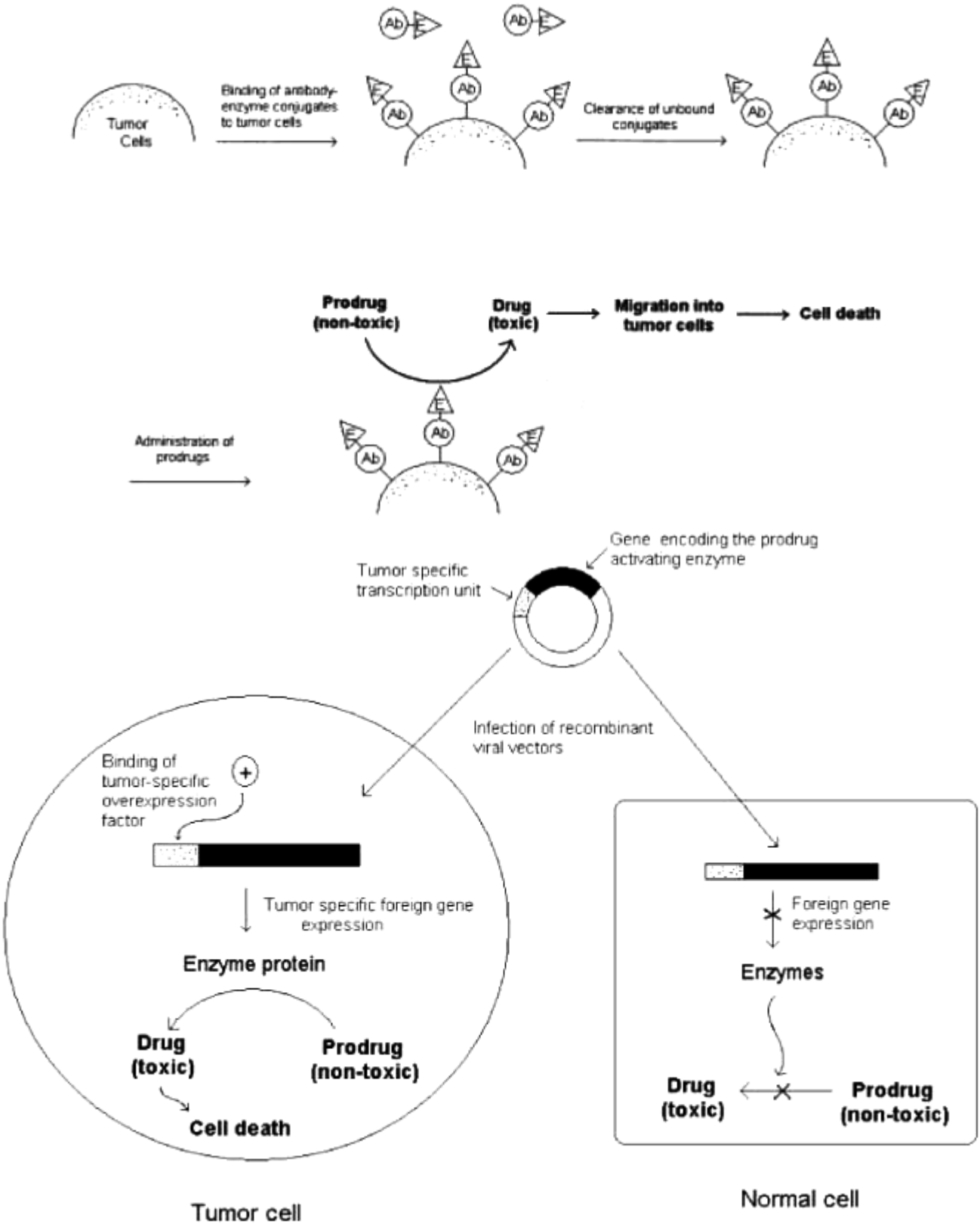

Figure 1. Schematic representation of ADEPT and VDEPT.

TABLE I

Enzymes and prodrugs that have been proposed for cancer therapy (Adapted from ref. 35 with permission)

Enzyme	Prodrug	Drug
DT diaphorase	5-(Aziridine-1-yl)-2,4-nitrobenzamide (CB 1954)*	5-(Aziridin-1-yl)-4-hydroxyl-amino-2-nitrobenzamide
Plasmin	Peptidyl-p-phenylenediamine-mustard*	Phenylenediamine-mustard
CarboxypeptidaseG2	Benzoic acid mustard glutamates*	Benzoic acid mustards(various)
Thymidine kinase(viral)	Ganciclovir*	Ganciclovir triphosphate
	6-Methoxypurine arabinonucleoside (araM)	Adenine arabinonucleoside Triphosphate(araATP)
Cytosine deaminase	5-Fluorocytosine*	5-Fluorouracil
Glucose oxidase	Glucose	Hydrogen peroxide
Xanthine oxidase	Hypoxanthine	Superoxide, hydrogen peroxide
Carboxypeptidase A	Methotrexate-alanine	Methotrexate
α-Galactosidase	N-[4-(-D-galactopyranosyl) Benzyloxycarbonyl]-daunorubicine	Daunorubicin
β-Glucosidase	Amygdalin	Cyanide
Azoreductase	Azobenzene mustards*	Phenylenediamine mustards(various)
γ-Glutamyl transferase	γ-Glutamyl-p-phenylenediamine mustard	Phenylenediamine mustard
β-Glucuronidase	Phenolmustard-glucuronide*	Phenolmustard
	Epirubicin-glucuronide	Epirubicin
β-Lactamase	Vinca-cephalosporin*	4-Desacetylvinblastine-3-carboxyhydrazide
	Phenylenediamine mustard-cephalosporin*	Phenylenediamine mustard
	Nitrogen-mustard-cephalosporin	Nitrogen mustards (various)
Alkaline phosphatase	Phenolmustard phosphate*	Phenolmustard
	Doxorubicin phosphate*	Doxorubicin
	Mitomycin phosphate*	Mitomycin alcohol
	Etoposide phosphate*	Etoposide
Penicillin amidase	Palytoxin-4-hydroxyphenyl-acetamide	Palytoxin
	Doxorubicin-phenoxyacetamide	Doxorubicin
	Melphalan-phenoxyacetamide	Melphalan
Cytochrome P-450	Cyclophosphamide	Phosphoamide mustard
	Ifosfamide	(+acrolein)
Nitroreductase	CB 1954	5-(Aziridin-1-yl)-4-hydroxyl amino-2-nitrobenzamide
	4-Nitrobenzyloxycarbonyl derivatives	Eg actinomycin D, mitomycin C

* Data obtained in vivo

and are nontoxic because the essential target for alkylating agents is intracellular DNA. After extracellular activation of prodrugs in tumor cells, the active drug will be freely diffusible and attain a high intracellular concentration. Thus, alkylating prodrugs activated by β-glucuronidase or carboxypeptidase G2 as glucuronic or glutamic acid derivatives should be excellent for ADEPT (37,38).

Prodrug Design Targeting the Membrane Transporters

Although the classical approach to improve membrane permeability of polar drugs uses lipophilic derivatives to increase passive membrane penetration, the targeted prodrug approach uses transporters designed for facilitating membrane transport of polar nutrients such as amino acids and peptides. There is direct and indirect evidence for the participation of carrier-mediated membrane transport mechanisms, where several hydrophilic compounds seem to be absorbed efficiently via specific transporters (39). Therefore, targeting specific membrane transporters is particularly important when prodrugs are polar or charged. From this point of view, use of intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs seems to be an attractive strategy for improving the bioavailability of poorly absorbed drug molecules. Prodrugs can be designed to resemble the intestinal nutrients structurally and to be absorbed by specific carrier proteins. In this case, prodrugs may have the additional advantage of producing nontoxic nutrient byproducts in which prodrugs are converted to the parent drug molecules. There have been many attempts to improve drug absorption targeting specific membrane transporters, including amino acid, peptide, and glucose transporters. Mizuma et al examined the intestinal absorption of the beta- and alpha-anomers of the glucoside and galactoside of p-nitrophenol to find a more suitable prodrug for poorly absorbed drugs (40,41). Using the everted sac technique, p-nitrophenyl-β-D-glucopyranoside was found to be actively absorbed by glucose transporters, and its permeation was comparable with that of D-glucose (40). In addition, glucose conjugates of p-nitrophenol were more permeable across rat everted jejunum than galactose conjugates of p-nitrophenol, which is consistent with the finding that D-glucose has a higher affinity to glucose transporters than D-galactose (41). The brain uptake of the potent glycine-NMDA receptor antagonists, such as 7-chlorokynurenic acid and 5,7-dichlorokynurenic acid, was significantly improved by their respective prodrugs, L-4-chlorokynurenine and L-4,6-dichlorokynurenine, which are amino acids (42). L-4-chlorokynurenine was shown to be rapidly delivered into the brain by the large neutral amino acid transporter of the blood-brain barrier and to be converted intracellularly to its parent drug, 7-chlorokynurenic acid (42). Furthermore, there have been some reports on prodrug design targeting peptide transporters, including peptidyl derivatives of a-methyldopa and alafosfalin and tripeptidyl prodrugs of foscarnet (43-45).

Developing prodrugs targeting specific membrane carriers requires considerable knowledge of the carrier proteins, including their distribution and substrate specificity. Recently, advances in molecular biology have allowed the cloning and controlled expression of carrier proteins and have further elucidated the functional and structural characteristics and regulation of carrier proteins. However, limited information is available on the specific molecular nature of the protein component(s) involved in the transport process at this time. The individual membrane transporters have been reviewed elsewhere (39,46).

Among various membrane transporters, peptide transporters are attractive targets in prodrug design to improve oral drug absorption because they have several advantages. First, peptide transporters have broad substrate specificity and high capacity (47). This characteristic is essential for their normal physiologic function. Theoretically, hundreds of dipeptides and thousands of different tripeptides can be generated from 20 amino acids that are chemically and structurally diverse (47). Therefore, the physiologic advantage of the broad substrate specificity of peptide transporters is obvious. Second, peptide transporters have been more extensively studied than other transporters, and considerable information is available about them. Therefore, structural modifications targeting peptide transporters can be optimized to a much greater degree compared to other transporters. Finally, recent

cloning and controlled expression in mammalian cell systems allow us to attempt rational drug design to target the peptide transporters.

PEPT1 and PEPT2 Transporters

Among the transport proteins responsible for translocation of organic solutes across cell membranes in animals, microbes, and plants, proton-coupled peptide transporters represent a distinct protein family with significant sequence homologies in the primary structure of various members of the family (48). So far, 2 distinct peptide transporters, PEPT1 and PEPT2, have been cloned from animals and humans (49-52).

A general characterization of the structural requirements for peptide transporters has been based on uptake, transport, and competitive inhibition experiments. Recently, direct evidence has been provided through the cloning and functional expression of PEPT 1 and PEPT 2 in mammalian cells (49-52). In general, peptide transporters have broad substrate requirements and tolerate diverse chemical modification. In addition to the endogenous peptides, various therapeutic drugs, including nonpeptidyl drugs, can be recognized as substrates by peptide transporters. As shown in Figure 2, β-lactam antibiotics, ACE inhibitors, renin inhibitors, and bestatin are well-known substrates for peptide transporters (39,53,54) and possess peptide-like chemical structures with a peptide bond, an N-terminal α-amino group, and a C-terminal carboxyl group. Substitution of an N-terminal α-amino group or a C-terminal carboxyl group of the peptidyl substrates may significantly reduce the affinity for the peptide transport system (55-57), but these groups are still recognized as substrates of peptide transporters. For example, without having an N-terminal α-amino group, peptidyl prodrugs (eg, α-methyldopa-L-phenylalanine), β-lactam antibiotics (eg, cefixime or cefdinir), and ACE inhibitors (eg, captopril, enalapril, quinapril, or benazepril) have been shown to be transported via the intestinal peptide transport system (43,55,58-61). Also, thyrotropin-releasing hormone and some renin inhibitors lacking a free C-terminal carboxyl group are reported as the substrates of peptide transporters (62,63). Therefore, an N-terminal α-amino group and a C-terminal carboxyl group do not appear to be

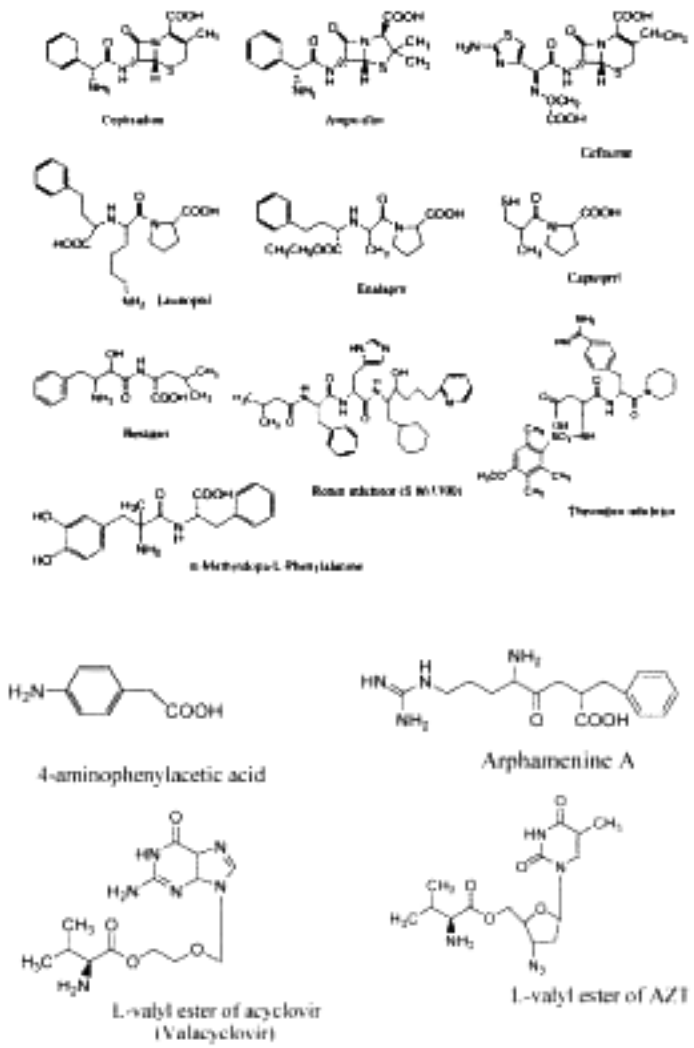

Figure 2. Substrates of peptide transporters.

critical requirements for the peptide transporters, although modification of these groups generally diminishes the substrate affinity to the transporters.

Several studies have indicated that the intestinal and renal peptide transporters are stereoselective (64-66). Peptidomimetic drugs as well as peptides containing L-amino acids interact with peptide transporters with greater affinity than do those containing D-amino acids. In addition, a D-amino acid at the N-terminal end of a peptide may have a greater effect on transport than one at the carboxyl terminal end (67).

Until recently, the presence of a peptide bond in the substrate has been considered as a prerequisite for recognition by peptide transporters. However, recent findings on the nonpeptidyl substrates of peptide

transporters such as arpharmenine A (68,69), 4-aminophenylacetic acid (70), and amino acid ester prodrugs of acyclovir and zidovudine (AZT) (71,72) strongly challenge the obligatory need for a peptide bond (Figure 2). Arphamenine A, an Arg-Phe analogue without a peptide bond, appeared to be the substrate of peptide transporters in Caco-2 cells, as well as renal brush border membrane vesicles (68,69). In addition, using renal brush border membrane vesicles and Xenopus oocytes expressing PEPT1, Temple et al demonstrated that 4-aminophenylacetic acid, a small nonpeptidyl drug, was translocated by peptide transporters (70). These studies on the nonpeptidyl substrates of peptide transporters suggest that a peptide bond is not a prerequisite for recognition by peptide transporters. Recently, Han et al successfully demonstrated the prodrug approach targeting the peptide transporters with nonpeptidyl prodrugs to improve oral drug absorption of polar nucleosides (71,72). These results encourage the prodrug design targeting peptide transporters with great flexibility in chemical modification.

Peptide Transporter Associated Prodrug Therapy

As previously described, peptide transporters have broad substrate specificity and are a good target for prodrug development to improve oral drug absorption. As shown in Figure 3, Amidon et al have developed the prodrug strategy targeting peptide transporters that is referred to as Peptide Transporter Associated Prodrug Therapy (PTAPT). A polar drug with low membrane permeability through passive diffusion is converted into a prodrug that is absorbed via the peptide transporter into the mucosal cell. This prodrug may still be very polar to assure sufficient solubility in the gastrointestinal lumen but may be well absorbed across the intestinal epithelium via the peptide transporters. Following membrane transport, the prodrug may be hydrolyzed by enzymes in the mucosal cell, blood, or liver. This prodrug strategy has been demonstrated to be effective for improving the membrane permeability and systemic availability of the polar a-methyldopa through peptidyl derivatives (43,73). The low membrane permeability of L-α-methyldopa, which is poorly absorbed via Na+-coupled neutral amino acid transporter (74), has been significantly improved by the peptidyl prodrugs such as Phe-α-methyldopa, α-methyldopa-Phe, and α-methyldopa-Pro in rats, with absorption via peptide transporters (43,73). To minimize the extensive metabolism of L-dopa in the gut wall, tri-peptide prodrugs of L-dopa, p-glu-L-dopa-pro were designed to be absorbed via peptide transporters and converted to L-dopa by peptidases (75).

Recently, Amidon et al broadened the application of PTAPT to nonpeptidyl type prodrugs, such as amino acid ester prodrugs. They synthesized several amino

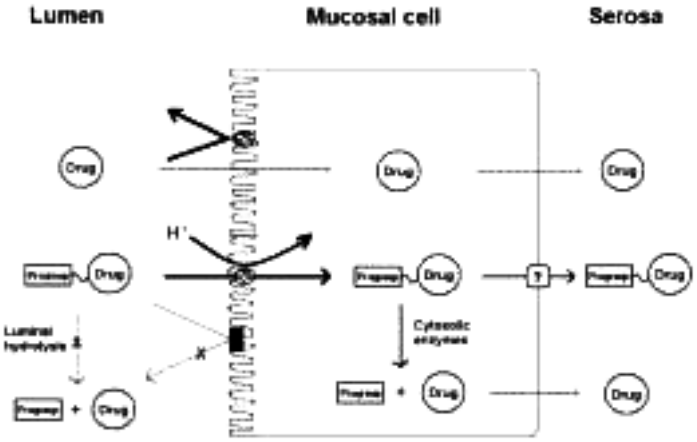

Figure 3. Schematic representation of PTAPT to increase intestinal drug absorption of polar drugs.

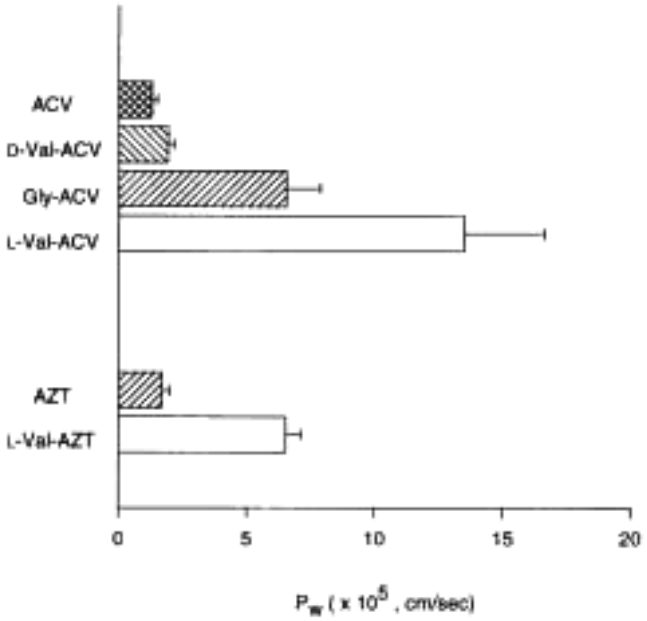

Figure 4. Intestinal membrane permeabilities of amino acid ester prodrugs and their parent drugs in rats (0.01 mM, Mean + SE). ACV and AZT: n = 6; the others: n = 4 (adapted from Han et al. [ref. 71], with permission).

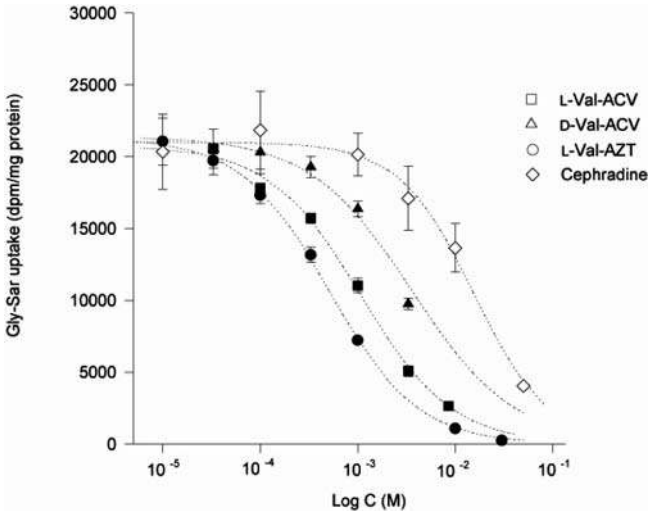

Figure 5. Inhibition of Gly-Sar uptake in CHO/hPEPT1 cells. Log C represents the inhibitor concentration (adapted from Han et al. [ref. 71], with permission).

acid ester prodrugs of the nucleoside antiviral drugs acyclovir and AZT and examined their intestinal absorption characteristics in 3 different experimental systems such as an in situ rat perfusion model, stably transfected CHO/hPEPT1 cells, and adenovirus mediated Caco-2/hPEPT1 cells (71,72). Amino acid ester prodrugs significantly (3-10 fold) increased the intestinal absorption of their parent drugs via a peptide transporter mediated mechanism, even though they do not have a peptide bond in their structures (Figures 4 and 5). Following the membrane transport, these prodrugs were rapidly converted to the active parent drugs by intracellular hydrolysis (72). These studies also demonstrated that the hPEPT1 transporter can recognize various amino acid progroups with stereoselectivity and that the nucleoside component can be varied. These results provide a new rationale for nonpeptidyl prodrug design targeting peptide transporters with great flexibility in structural modification. Recently, other research groups (76-79) have independently reported the peptide transporter-mediated membrane transport of the L-valylester prodrug of acyclovir (valacylovir) and confirmed the high potential of PTAPT in nonpeptidyl as well as peptidyl drug design. Consequently, PTAPT is a very useful strategy to improve the intestinal absorption of polar drugs.

CONCLUSIONS

The prodrug approach has been used to overcome various undesirable drug properties and to optimize the clinical drug application. Recent advances in molecular biology provide direct availability of enzymes and carrier proteins, including their molecular and functional characteristics. Thus prodrug design is becoming more elaborate in the development of efficient and selective drug delivery systems. Hence, prodrug design can no longer be considered as just a chemical modification to solve problems associated with drugs. The targeted prodrug approach, which can be combined with gene delivery and controlled expression of enzymes and carrier proteins, is a promising strategy for precise and efficient drug delivery and the enhancement of therapeutic efficacy.

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REFERENCES

1. Stella V. Pro-drugs: an overview and definition. In: Higuchi T, Stella V, eds. *Prodrugs As Novel Drug Delivery Systems.* ACS Symposium Series. Washington, DC: American Chemical Society; 1975:1-115.

2. Albert A. Chemical aspects of selective toxicity. Nature. 1958;182:421-423.

Harper NJ. Drug latentiation. Prog Drug Res. 1962;4:221-294.

3. Roche EB. *Design of Biopharmaceutical Properties through Prodrugs and Analogs.* Washington, DC: American Pharmaceutical Association; 1977.

4. Sinkula AA, Yalkowsky SH. Rationale for design of biologically reversible drug derivatives: prodrugs. J Pharm Sci. 1975;64:181-210.

5. Stella VJ, Charman WN, Naringrekar VH. Prodrugs. Do they have advantages in clinical practice? Drugs. 1985;29:455-473.

6. Banerjee PK, Amidon GL. Design of prodrugs based on enzymes-substrate specificity. In: Bundgaard H, ed. *Design of Prodrugs.* New York: Elsevier; 1985:93-133.

7. Amidon GL, Leesman GD, Elliott RL. Improving intestinal absorption of water-insoluble compounds: a membrane metabolism strategy. J Pharm Sci. 1980;69:1363-1368.

8. Fleisher D, Stewart BH, Amidon GL. Design of prodrugs for improved gastrointestinal absorption by intestinal enzyme targeting. Methods Enzymol. 1985;112:360-381.

9. Bai JP, Amidon GL. Structural specificity of mucosal-cell transport and metabolism of peptide drugs: implication for oral peptide drug delivery. Pharm Res. 1992;9:969-978.

10. Stella VJ, Himmelstein KJ. Prodrugs and site-specific drug delivery. J Med Chem. 1980;23:1275-1282.

11. Stella VJ, Himmelstein KJ. Critique of prodrugs and site specific delivery. In: Bundgaard H, ed. *Optimization of Drug Delivery.* Alfred Benzon Symposium 17. Copenhagen, Munksgaard; 1982:134-155.

12. Friend DR, Chang GW. A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. J Med Chem. 1984;27:261-266.

13. Bohme H, Ahrens KH, Hotzel HH. Properties and reactions of N-(alpha-hydroxyalkyl)-thionamides. Arch Pharm. (Weinheim) 1974;307:748-755.

14. Wilk S, Mizoguchi H, Orlowski M. Gamma-glutamyl dopa: a kidney-specific dopamine precursor. J Pharmacol Exp Ther. 1978;206:227-232.

15. Mizoguchi H, Orlowski M, Wilk S, Green JP. Gamma-glutamyl DOPA and gamma-glutamyl dopamine: effect on plasma glucose levels. Eur J Pharmacol. 1979;57:239-245.

16. Connors TA, Whisson ME. Cure of mice bearing advanced plasma cell tumours with aniline mustard: the relationship between glucuronidase activity and tumour sensitivity. Nature. 1966;210:866-867.

17. Cobb LM, Connors TA, Elson LA, Khan AH, Mitchley BC, Ross WC, et al. 2,4-Dinitro-5-ethyleneiminobenzamide (CB1954): a potent and selective inhibitor of the growth of the Walker carcinoma 256. Biochem Pharmacol. 1969;18:1519-1527.

18. Connors TA. Prodrugs in cancer chemotherapy. Xenobiotica. 1986;16:975-988.

19. Bagshawe KD. Antibody-directed enzyme prodrug therapy (ADEPT). Adv Pharmacol. 1993;24:99-121.

20. Bagshawe KD. Antibody directed enzymes revive anti-cancer prodrugs concept. Br J Cancer. 1987;56:531-532.

21. Bagshawe KD, Springer CJ, Searle F, Antoniw P, Sharma SK, Melton RG, Sherwood RF. A cytotoxic agent can be generated selectively at cancer sites. Br J Cancer. 1988;58:700-703.

22. Bagshawe KD. The first Bagshawe lecture. Towards generating cytotoxic agents at cancer sites. Br J Cancer. 1989;60:275-281.

23. Senter PD, Saulnier MG, Schreiber GJ, Hirschberg DL, Brown JP, Hellstrom I, et al. Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. Proc Natl Acad Sci. U S A. 1988;85:4842-4846.

24. Connor TA, Knox RJ. Prodrugs in cancer chemotherapy. Stem cells. 1995;13:501-511.

25. Sharma SK, Bagshawe KD, Melton RG, Sherwood RF. Human immune response to monoclonal antibody-enzyme conjugates in ADEPT pilot clinical trial. Cell Biophys. 1992;2:109-120.

26. Springer CJ, Poon GK, Sharma SK, Bagshawe KD. Identification of prodrug, active drug, and metabolites in an ADEPT clinical study. Cell Biophys. 1993;22:9-26.

27. Harris JD, Gutierrez AA, Hurst HC, Sikora K, Lemoine NR. Gene therapy for cancer using tumour-specific prodrug activation. Gene Ther. 1994;1:170-175.

28. Huber BE, Richards CA, Austin EA. Virus-directed enzyme/prodrug therapy (VDEPT): selectively engineering drug sensitivity into tumors. Ann NY Acad Sci. 1994;716:104-114.

29. Culver KW, Van Gilder J, Link CJ, Carlstrom T, Buroker T, Yuh W, et al. Gene therapy for the treatment of malignant brain tumors with in vivo tumor transduction with the herpes simplex thymidine kinase gene/ganciclovir system. Hum Gene Ther. 1994;5:343-379.

30. Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science. 1992;256:1550-1552.

31. Mullen CA, Kilstrup M, Blaese RM. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc Natl Acad Sci U S A. 1992;89:33-37.

32. Barba D, Hardin J, Ray J, Gage FH. Thymidine kinase-mediated killing of rat brain tumors. J Neurosurg. 1993;79:729-735.

33. Deonarain MP, Spooner RA, Epenetos AA. Genetic delivery of enzymes for cancer therapy. Gene Ther. 1995;2:235-244.

34. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. Gene Ther. 1995;2:702-709.

35. Anlezark GM, Melton RG, Sherwood RF, Coles B, Friedlos F, Knox RJ. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)—I. Purification and properties of a nitroreductase enzyme from Escherichia coli—a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochem Pharmacol. 1992;44:2289-2295.

36. Haisma HJ, Boven E, van Muijen M, de Jong J, van der Vijgh WJ, Pinedo HM. A monoclonal antibody-ß-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. Br J Cancer. 1992;66:474-478.

37. Eccles SA, Court WJ, Box GA, Dean CJ, Melton RG, Springer CJ. Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2p185. Cancer Res. 1994;54:5171-5177.

38. Tsuji A, Tamai I. Carrier-mediated intestinal transport of drugs. Pharm Res. 1996;13:963-77.

39. Mizuma T, Ohta K, Hayashi M, Awazu S. Intestinal active absorption of sugar-conjugated compounds by glucose transport system: implication of improvement of poorly absorbable drugs. Biochem Pharmacol. 1992;43:2037-2039.

40. Mizuma T, Ohta K, Hayashi M, Awazu S. Comparative study of active absorption by the intestine and disposition of anomers of sugar-conjugated compounds. Biochem Pharmacol. 1993;45:1520-1523.

41. Hokari M, Wu HQ, Schwarcz R, Smith QR. Facilitated brain uptake of 4-chlorokynurenine and conversion to 7-chlorokynurenic acid. Neuroreport. 1996;8:15-18.

42. Hu M, Subramanian P, Mosberg HI, Amidon GL. Use of the peptide carrier system to improve the intestinal absorption of L-alpha-methyldopa: carrier kinetics, intestinal permeabilities, and in vitro hydrolysis of dipeptidyl derivatives of L-alpha-methyldopa. Pharm Res. 1989;6:66-70.

43. Swaan PW, Tukker JJ. Carrier-mediated transport mechanism of foscarnet (trisodium phosphonoformate hexahydrate) in rat intestinal tissue. J Pharmacol Exp Ther. 1995;272:242-247.

44. Grappel SF, Giovenella AJ, Nisbet LJ. Activity of a peptidyl prodrug, alafosfalin, against anaerobic bacteria. Antimicrob Agents Chemother. 1985;27:961-963.

45. Oh D-M, Han H-k, Amidon GL. Drug transport and targeting. Intestinal transport. In Amidon GL, Sadee W, eds. *Membrane Transporters as Drug Targets.* New York: Plenum Press, 1999;59-88.

46. Ganapathy V, Brandsch M, Leibach FH. Intestinal transport of amino acids and peptides. In Johnson LR, ed. *Physiology of the Gastrointestinal Tract.* New York, Raven Press; 1994:1773-1794.

47. Ganapathy V, Leibach FH. Peptide transporters. Curr Opin Nephrol Hypertens. 1996;5:395-400.

48. Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng L, et al. Human intestinal H+/peptide cotransporter: cloning, functional expression and chromosomal localization. J Biol Chem. 1995;270:6456-6463.

49. Saito H, Okuda M, Terada T, Sasaki S, Inui K. Cloning and characterization of a rat H+/peptide cotransporter mediating absorption of beta-lactam antibiotics in the intestine and kidney. J Pharmacol Exp Ther. 1995;275:1631-1637.

50. Liu W, Ramamoorthy S, Fei YJ, Ganapathy NE, Hediger MA, Ganapathy V, et al. Molecular cloning of PEPT2, a new member of the H+/peptide cotransporter family from human kidney. Biochim Biophys Acta. 1995;1235:461-466.

51. Saito H, Terada T, Okuda M, Sasaki S, Inui K. Molecular cloning and tissue distribution of rat peptide transporter PEPT2. Biochim Biophys Acta. 1996;1280:173-177.

52. Bai JPF, Stewart BH, Amidon GL. Gastrointestinal transport of peptide and protein drugs and prodrugs. In: Welling PG, Balant LP, eds. *Handbook of Experimental Pharmacology.* Heidelberg: Springer-Verlag; 1994:110:189-206.

53. Leibach FH, Ganapathy V. Peptide transporters in the intestine and the kidney. Annu Rev Nutr. 1996;16:99-119.

54. Bai P-F, Subramanian P, Mosberg HI, Amidon GL. Structural requirements for the intestinal mucosal-cell peptide transporter: the need for N-terminal alpha-amino group. Pharm Res. 1991;8:593-599.

55. Samanen J, Wilson G, Smith PL, Lee CP, Bondinell W, Ku T, et al. Chemical approaches to improve the oral bioavailability of peptidergic molecules. J Pharm Pharmacol. 1996;48:119-135.

56. Hidalgo IJ, Bhatnagar P, Lee CP, Miller J, Cucullino G, Smith PL. Structural requirements for interaction with the oligopeptide transporter in Caco-2 cells. Pharm Res. 1995;12:317-319.

57. Tsuji A, Tamai I, Nakanishi M, Terasaki T, Hamano S. Intestinal brush-border transport of the oral cephalosporin antibiotic, cefdinir, mediated by dipeptide and monocarboxylic acid transport systems in rabbits. J Pharm Pharmacol. 1993;45:996-998.

58. Tsuji A, Terasaki T, Tamai I, Hirooka H. H + gradient-dependent and carrier-mediated transport of cefixime, a new cephalosporin antibiotic, across brush-border membrane vesicles from rat small intestine. J Pharmacol Exp Ther. 1987;241:594-601.

59. Friedman DI, Amidon GL. Passive and carrier-mediated intestinal absorption components of two angiotensin converting enzyme (ACE) inhibitor prodrugs in rats: enalapril and fosinopril. Pharm Res. 1989;6:1043-1047.

60. Yee S, Amidon GL. Intestinal absorption mechanism of three angiotensin-converting enzyme inhibitors: quinapril, benazepril and CGS 16617. Pharm Sci. 1990;7:S-155.

61. Kramer W, Girbig F, Gutjaha U, Kleemann H-W, Leipe I, Urbach H, et al. Interaction of renin inhibitors with the intestinal uptake system for oligopeptides and beta-lactam antibiotics. Biochim Biophys Acta. 1990;1027:25-30.

62. Humphrey MJ, Ringrose PS. Peptides and related drugs: a review of their absorption, metabolism, and excretion. Drug Metab Rev. 1986;17:283-310.

63. Tamai I, Ling HY, Timbul SM, Nishikido J, Tsuji A. Stereospecific absorption and degradation of cephalexin. J Pharm Pharmacol. 1988;40:320-324.

64. Lister N, Sykes AP, Bailey PD, Boyd CA, Bronk JR. Dipeptide transport and hydrolysis in isolated loops of rat small intestine: effects of stereospecificity. J Physiol. (Lond). 1995;484:173-182.

65. Ganapathy V, Leibach FH. Peptide transport in rabbit kidney. Studies with L-carnosine. Biochim Biophys Acta. 1982;691:362-366.

66. Boyd CA, Ward MR. A micro-electrode study of oligopeptide absorption by the small intestinal epithelium of Necturus maculosus. J Physiol. 1982;324:411-428.

67. Daniel H, Adibi SA. Functional separation of dipeptide transport and hydrolysis in kidney brush border membrane vesicles. FASEB. 1994;8:753-759.

68. Enjoh M, Hashimoto K, Arai S, Shimizu M. Inhibitory effect of arphamenine A on intestinal dipeptide transport. Biosci Biotech Biochem. 1996;60:1893-1895.

69. Temple CS, Stewart AK, Meredith D, Lister NA, Morgan KM, Collier ID, et al. Peptide mimics as substrates for the intestinal peptide transporter. J Biol Chem. 1998;273:20-22.

70. Han H-k, de Vrueh RLA, Rhie JK, Covitz K-MY, Smith PL, Lee C-P, et al. 5'-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm Res. 1998;15:1154-1159.*

71. Han H-k, Oh D-M, Amidon GL. Cellular uptake mechanism of amino acid ester prodrugs in Caco-2/hPEPT1 cells overexpressing a human peptide transporter. *Pharm Res. 1998;15:1382-1386.*

72. Bai JP, Hu M, Subramanian P, Mosberg HI, Amidon GL. Utilization of peptide carrier system to improve intestinal absorption: targeting prolidase as a prodrug-converting enzyme. J Pharm Sci. 1992;81:113-116.

73. Hu M, Borchardt RT. Mechanism of L-a-methyldopa transport through a monolayer of polarized human intestinal epithelial cells (Caco-2). Pharm Res. 1990;7:1313-1319.

74. Bai JPF. PGlu-L-dopa-pro: a tripeptide prodrug targeting the intestinal peptide transporter for absorption and tissue enzymes for conversion. Pharm Res. 1995;12:1101-1104.

75. Ganapathy ME, Huang W, Wang H, Ganapathy V, Leibach FH. Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. Biochem Biophys Res Commun. 1998;246:470-475.

76. Balimane PV, Tamai I, Guo A, Nakanishi T, Kitada H, Leibach FH, et al. Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. Biochem Biophy Res Commun. 1998;250:246-251.

77. Sinko PJ, Balimane PV. Carrier-mediated intestinal absorption of valacyclovir, the L-valyl ester prodrug of acyclovir: 1. Interactions with peptides, organic anions and organic cations in rats. Biopharm Drug Dispos. 1998;19:209-217.

78. de Vrueh RL, Smith PL, Lee CP. Transport of L-valine-acyclovir via the oligopeptide transporter in the human intestinal cell line, Caco-2. J Pharmacol Exp Ther. 1998;286:1166-1170.