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Relationship between structure and permeability of dipeptide derivatives containing tryptophan and related compounds across human intestinal epithelial (Caco-2) cells

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Abstract—The permeability of dipeptide derivatives containing tryptophans and indole derivatives through Caco-2 cells was used as an in vitro intestinal absorption model in order to clarify structural factors which influence their intestinal epithelial permeation and metabolism. Most peptide derivatives were hydrolysed not only by the cytosolic enzymes in Caco-2 cells during permeation but also by enzymes released to the apical solution before cell permeation. The N-terminal blocked dipeptides were more resistant to hydrolases expressed in the Caco-2 cells and indole derivatives were not entirely degraded. Based on compound concentration dependency and comparison of permeability coefficients in apical-to-basolateral and basolateral-to-apical directions, the main absorption mechanism of compounds were determined. Compounds were then classified into three groups; (1) passively transported compounds, (2) actively transported compounds and (3) compounds excreted by P-glycoprotein.

1. Introduction

Clinical development of orally active peptide drugs has been restricted by their poor permeation across biological barriers such as the intestinal mucosa and by the lack of stability against enzymatic degradation. Peptidomimetics are therefore designed to enhance their oral absorption and depress enzymatic degradation.

Most drugs and peptides are transported across the intestinal epithelium by a passive diffusion process and/or carrier-mediated routes.[1] Passive absorption can occur via either the transcellular or paracellular route (Fig. 1). It is often generalized that rapidly transported hydrophobic compounds are absorbed across the membrane by a transcellular route, whereas slowly transported hydrophilic compounds are absorbed through the tight junctions via a paracellular pathway.[2] However, the small surface area of the paracellular space and the gating function of tight junctions limit the paracellular absorption of hydrophilic drugs and peptides. The radius of the tight junction was reported to be ~12 Å in Caco-2 cells.[3]

Compounds such as nutrients can utilize carrier-mediated routes, either energy-dependent or passive transporters, in the small intestine.[4–6] Recently, H^+-coupled peptide transporters, PEPT1 and PEPT2[7] have been cloned and well characterized. In the small intestine PEPT1 is expressed and localized at the brush-border membranes.[8] It has been demonstrated that di- and tripeptides are actively transported into the cells by the peptide transporters after ingestion of proteins.[8] The peptide transporters in the brush-border membranes were also shown to mediate transport of peptide-like drugs such as β-lactam antibiotics.[9]

Studies of the multidrug resistance phenomenon that accompanies cancer chemotherapy have focused on a membrane glycoprotein (termed P-glycoprotein).[10] P-Glycoprotein (P-gp) is not only highly expressed in cancer cells but also expressed in normal intestinal and colonic epithelial cells.[11] This system has been found to mediate drug transport in a secretory direction.[1] Another secretory transporters in the intestine are multidrug resistance-associated protein (MRP) family.[12] It

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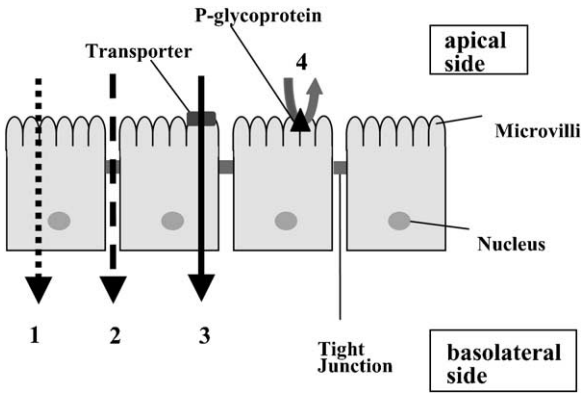

Figure 1. Pathways for drug transport across the intestinal epithelium: (1) passive transcellular, (2) passive paracellular, (3) active carrier-mediated routes, and (4) P-glycoprotein efflux system.

has been reported that P-gp transports cationic and neutral compounds as substrates while MRP family may play a role in the efflux of anionic compounds.[12]

In addition, peptides and proteins are degraded into small peptide subunits and amino acids by peptidases and proteases in the gastrointestinal tract to be absorbed across the intestinal mucosa. Di-/tripeptides are also metabolized in the epithelial cells.[13]

Caco-2 cells, a cell line derived from human colon carcinoma, have been previously used in various transport experiments.[14–16] Good correlation between oral drug absorption in humans and measured permeability coefficients in Caco-2 cells was reported.[17] It is known PEPT1 is expressed in the human intestinal cell line Caco-2.[8] Using a series of peptide analogues, the effect of peptide structure on transport across Caco-2 cells have been studied.[8,18–21] The structural features which appear to be required by the intestinal peptide transport system have also been clarified.[22] However, the mechanism of absorption and metabolism of structurally diverse peptide derivatives across epithelial cells have not been fully characterized. To further clarify structural factors of peptides and their derivatives which influence their epithelial permeation and metabolism should provide information for the design of peptidomimetics.

In this study, we measured permeability of dipeptide derivatives containing tryptophans (Trp) and related structurally diverse compounds such as protected peptides, cyclic peptides and indole compounds in an in vitro transport system. Trp is an essential amino acid for humans and plays an important role in the nervous system. The possible transport mechanism of the dipeptide derivatives and related compounds is discussed.

2. Results

Most peptide derivatives are hydrolysed not only by cytosolic enzymes of Caco-2 cells during permeation but also by enzymes released into the apical (AP) solution prior to cell permeation. Thus, original peptide derivatives and their hydrolysed amino acid derivatives are detected after cell permeation. Table 1 shows RA_{AP}, the relative residual amount (%) to the initially applied amount of peptide derivatives in the AP solution after 2 h and RA_{BL}, the relative amount (%) of each detected compound to the total amount of compounds in the basolateral (BL) solution after 2 h. To simplify calculation of the permeability coefficient, P_{app} of peptide derivatives, the coefficient was determined combining the amounts of peptide derivatives and their metabolites detected in the BL solution. Indole compounds were not degraded at all before and during permeation. The values of P_{app} of dipeptide derivatives and indole compounds are listed in Table 1.

2.1. Permeability coefficient of Trp-X

Permeability coefficients of 0.5 and 2 mM Trp-X (X: Gly, Ala, Leu, Phe, Tyr, Trp, Glu) were measured in the AP-to-BL direction (Table 1). These compounds were hydrolyzed in Caco-2 cells, and Trp was mainly detected after permeation (more than 95%. See RA_{BL} of Table 1). Trp-X was also degraded in the AP solution (the 13–50% of Trp-X was degraded after 2 h. See RA_{AP} of Table 1). The permeability coefficient of Trp-X was determined combining the amounts of Trp and Trp-X detected in the BL solution. In general, the 'combined' permeability coefficient at 0.5 mM of each peptide (5.7–23.3 $\times$ 10^{-6} cm/s) was approximately equal to or higher than that at 2 mM (2.8–14.7 $\times$ 10^{-6} cm/s). The permeability coefficient of Trp-Gly in the BL-to-AP direction was also measured. It was 50 times less than that in the AP-to-BL direction.

2.2. Permeability coefficient of X-Trp

Permeability coefficients of 0.5 and 2 mM X-Trp (X: Gly, Ala, Leu, Phe, Tyr) were measured in the AP-to-BL direction. These compounds were also hydrolysed by the brush-border membrane and cytosolic enzymes of Caco-2 cells and Trp (and N-terminal amino acids) was mainly detected after permeation [more than 98% except for Gly-Trp (87%)]. The X-Trp derivatives were more easily degraded in the AP solution (50–100% degradation after 2 h) than the Trp-X derivatives. The permeability coefficient of X-Trp was determined in a similar manner to that of Trp-X. The permeability coefficients ranged from 2.9 to 13.5 $\times$ 10^{-6} cm/s at 0.5 mM and from 3.5 to 10.9 $\times$ 10^{-6} cm/s at 2 mM. Degradation of Gly-D-Trp having D-amino acid was suppressed as expected (RA_{AP} = 83%, RA_{BL} = 56%) and its permeability coefficient was almost same as that of Gly-Trp.

2.3. Permeability coefficient of Ac-Trp-X

Permeability coefficients of 0.5 and 2 mM Ac-Trp-X (X: Ala, Leu, Phe, Trp) were measured in the AP-to-BL direction. Since about 60% of Ac-Trp-X was detected in the BL solution after permeation, Ac-dipeptides were relatively stable against cytosolic hydrolases of Caco-2 cells compared to unprotected peptides. Ac-peptides were not degraded in the AP solution. However, their permeability was 10–100 times lower than that of unprotected dipeptides including their metabolites.

Table 1. Permeability coefficients of peptide derivatives containing Trp and related compounds, and relative amounts of their metabolites

	Compd	P_{app} ($\times 10^{-6}$)[a] (cm/s)			RA_{AP} (%)[b]	RA_{BL} (%)[c]			
		AP-to-BL		BL-to-AP		Compd	Trp	Ac-Trp	Trp-NH_2
		0.5 mM	2 mM	2 mM					
Trp-X	Trp-Gly	16.6 (1.4)	10.8 (0.5)	0.2 (0.0_4)	68	4	96	—	—
	Trp-Ala	14.4 (1.4)	14.7 (1.0)	—	64	4	96	—	—
	Trp-Leu	23.3 (2.4)	6.4 (0.5)	—	53	5	95	—	—
	Trp-Phe	10.9 (1.2)	10.3 (1.2)	—	50	3	97	—	—
	Trp-Tyr	10.6 (1.7)	2.8 (0.2)	—	53	3	97	—	—
	Trp-Trp	10.9 (0.5)	3.4 (0.3)	—	67	2	98	—	—
	Trp-Glu	5.7 (0.4)	8.0 (0.8)	—	87	5	95	—	—
X-Trp	Gly-Trp	2.9 (0.0_3)	3.5 (0.2)	—	4	13	87	—	—
	Ala-Trp	3.0 (0.0_8)	4.1 (0.5)	—	0	0.5	99.5	—	—
	Leu-Trp	8.1 (0.0_2)	10.9 (0.8)	—	16	0.3	99.7	—	—
	Phe-Trp	13.5 (0.0_6)	8.7 (0.4)	—	50	1.7	98.3	—	—
	Tyr-Trp	9.8 (0.0_6)	4.7 (0.4)	—	4	0.1	99.9	—	—
	Gly-D-Trp	2.7 (1.5)	1.0 (0.1)	—	83	56	44	—	—
Ac-Trp-X	Ac-Trp-Ala	0.1 (0.0_1)	0.1 (0.0_2)	—	100	60.5	39.3	0.2	—
	Ac-Trp-Leu	0.1 (0.0_1)	0.1 (0.0_3)	—	100	59	40	1	—
	Ac-Trp-Phe	0.1 (0.0_2)	0.1 (0.0_3)	—	100	64.5	35.0	0.5	—
	Ac-Trp-Trp	0.1 (0.0_1)	0.1 (0.0_4)	—	100	59.5	38.7	1.8	—
X-Trp-NH_2	Gly-Trp-NH_2	6.0 (0.7)	6.7 (0.6)	—	17	2	23	—	75
	Phe-Trp-NH_2	15.9 (0.7)	10.4 (1.0)	2.0 (0.1)	6	Trace	23	—	77
Ac-dipeptide-NH_2	Ac-Trp-Val-NH_2	0.2 (0.0_4)	0.2 (0.0_4)	1.4 (0.0_5)	100	80	20	Trace	—
	Ac-D-Trp-Val-NH_2	0.2 (0.0_7)	0.1 (0.0_7)	1.6 (0.0_6)[d]	100	80	20	0	—
Cyclo(Trp-X)	Cyclo(Trp-Gly)	—	0.8 (0.0_9)	0.7 (0.0_2)	100	85	15	—	—
	Cyclo(Trp-Trp)	1.2 (0.2)[e]	—	20.6 (0.2)[e]	100	54	46	—	—
Indole derivatives	Indole	—	57.3 (8.7)	52.8 (5.6)	100	100	—	—	—
	3-Methylindole	89.0 (2.8)	—	56.3 (1.1)[d]	100	100	—	—	—
	Indole-3-acetic acid	—	14.2 (0.6)	8.2 (0.6)	100	100	—	—	—
	Indole-3-propionic acid	—	21.3 (5.2)	7.7 (0.2)	100	100	—	—	—
	Indole-3-butyric acid	—	52.2 (1.3)	39.7 (2.9)	100	100	—	—	—
	Indole-2-carboxylic acid	—	23.1 (1.0)	20.2 (0.4)	100	100	—	—	—
	Indole-3-carboxylic acid	—	27.5 (0.7)	14.5 (0.0_3)	100	100	—	—	—
	Indole-3-carboxyaldehyde	—	102.8 (3.6)	67.2 (3.9)	100	100	—	—	—
	Indole-3-acetamide	—	59.0 (2.6)	43.3 (2.6)	100	100	—	—	—
	Tryptophol	—	57.5 (9.2)	69.4 (2.5)	100	100	—	—	—

[a] The measurement was repeated at least four times. Values in parentheses are the standard deviation.
[b] The relative residual amount (%) to the initially applied amount of peptide derivatives in the apical solution after 2 h.
[c] The relative amount (%) of each detected compound to the total amount of compounds in the basolateral solution after 2 h.
[d] Measured at 0.5 mM.
[e] Measured at 0.1 mM.

2.4. Permeability coefficient of X-Trp-NH_2

Permeability coefficients of 0.5 and 2 mM X-Trp-NH_2 (X: Gly, Phe) were measured in the AP-to-BL direction. In contrast with N-terminal protected peptides, C-terminal amide derivatives had comparable permeability with unprotected peptides but were degraded before and during permeation. After permeation, Trp-NH_2 was mainly detected.

2.5. Permeability coefficient of Ac-Trp-Val-NH_2 and Ac-D-Trp-Val-NH_2

Permeability coefficients of 0.5 and 2 mM Ac-Trp-Val-NH_2 and Ac-D-Trp-Val-NH_2 were measured in the AP-to-BL direction. These compounds were rather stable against cytosolic hydrolases. Both compounds were not degraded in the AP solution before permeation. Eighty percent of the original compound, 20% of Trp and a trace of Ac-Trp were detected after permeation. Their permeability coefficient was the same order of magnitude as that of Ac-Trp-X, one to two orders lower than that of unprotected peptides. The permeability of Ac-Trp-Val-NH_2 was 7 times higher in the BL-to-AP direction than that in the AP-to-BL direction. There was no effect of modification of L-Trp to D-Trp on degradation and permeability of these peptide derivatives.

2.6. Permeability coefficient of cyclo(Trp-X)

Permeability coefficients of 2 mM cyclo(Trp-Gly) and 0.1 mM cyclo(Trp-Trp) which were not sufficiently soluble in aqueous solutions were measured in the both AP-to-BL and BL-to-AP directions. These cyclic peptides were not only stable in the AP solution but also 50–80% of them remained after permeation. Both compounds had similar P_{app} values in the AP-to-BL direction. The permeability of cyclo(Trp-Trp) was 20 times higher in the BL-to-AP direction than that in the AP-to-BL direction whereas the permeability of cyclo(Trp-Gly) in both directions was nearly the same.

2.7. Permeability coefficient of indole derivatives

Permeability coefficients of 2 mM indole derivatives: indole, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, indole-2-carboxylic acid, indole-3-carboxylic acid, indole-3-carboxyaldehyde, indole-3-acetamide and tryptophol, were measured in the both AP-to-BL and BL-to-AP directions. Permeability coefficients of 3-methylindole were measured at 0.5 mM because of its low solubility in aqueous solution. Because these compounds were not entirely degraded, their P_{app} values were determined as usual. These compounds showed high cell permeability in both directions (7.7–102.8 $\times$ 10^{-6} cm/s). The permeability of 3-methylindole, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, indole-3-carboxylic acid, indole-3-carboxyaldehyde and indole-3-acetamide was higher in the AP-to-BL direction than in the BL-to-AP direction. For indole, indole-2-carboxylic acid and tryptophol, the AP-to-BL permeability was approximately the same as or slightly lower than the BL-to-AP permeability.

2.8. Effect of transporter substrates on permeability coefficients

To investigate interaction of compounds with transporter systems, the AP-to-BL permeability coefficient of Trp-Ala was determined in the presence of Phe or Gly-Pro, which are known as substrates of neutral amino acid transporters or PEPT1, respectively, in the AP solutions as shown in Figure 2. Significant inhibition of the AP-to-BL permeation of Trp-Ala by Gly-Pro but not by Phe was observed. This suggests that Trp-Ala is actively transported by a peptide transporter such as PEPT1.

2.9. Effect of P-gp inhibitor, verapamil on permeability coefficients

To investigate involvement of P-gp in permeation of cyclo(Trp-Trp), verapamil, a P-gp inhibitor, was added to the both AP and BL solutions. As shown in Figure 3, the AP-to-BL permeability was increased whereas the BL-to-AP permeability was decreased in the presence of verapamil, suggesting involvement of P-gp.

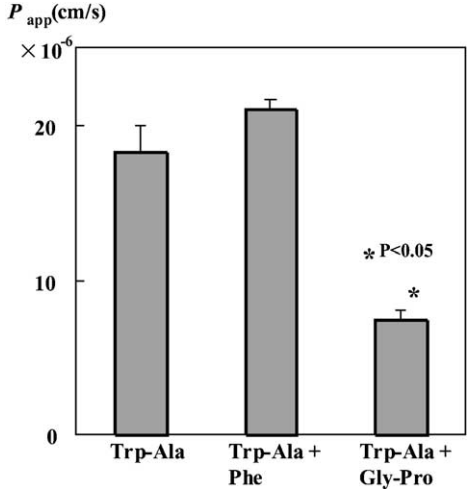

Figure 2. The AP-to-BL permeability coefficient of Trp-Ala in the presence of Phe or Gly-Pro.

2.10. The pH dependence of permeability coefficients

The AP-to-BL permeability coefficient of the 2 mM Trp-Gly, cyclo(Trp-Gly) was measured at pH 6.3 and compared with that at pH 7.3. The permeability coefficient of Trp-Gly at pH 6.3 (22.2 $\times$ 10^{-6} cm/s) was higher than that at pH 7.3 (10.8 $\times$ 10^{-6} cm/s). This result suggests involvement of an H^+-dependent transporter such as PEPT1 in the transport of Trp-Gly. The permeability coefficient of cyclo(Trp-Gly) did not depend on pH as expected for neutral compounds at neutral pHs without carrier-mediated transport (0.9 $\times$ 10^{-6} cm/s at pH 6.3 and 0.8 $\times$ 10^{-6} cm/s at pH 7.3).

3. Discussion

As previously described, Caco-2 cells express various transporters including the P-gp and MRP efflux systems. Therefore, compounds are passively transported, actively transported and/or excreted by the efflux systems in Caco-2 cells. Determining bi-directional permeability coefficients, Stenberg et al. suggested that efflux mechanism was involved in transport of the compounds if they had several fold higher P_{app} values in the BL-to-AP than in the AP-to-BL direction.[23] In contrast, if the AP-to-BL permeability of a compound is higher than the BL-to-AP permeability, the compound is thought to be transported by an active transporter mechanism.[23] Furthermore, involvement of saturable transporters is implicated for compounds having the higher P_{app} value at lower concentrations because the P_{app} value declines with increasing concentration before saturation of the transporter system.

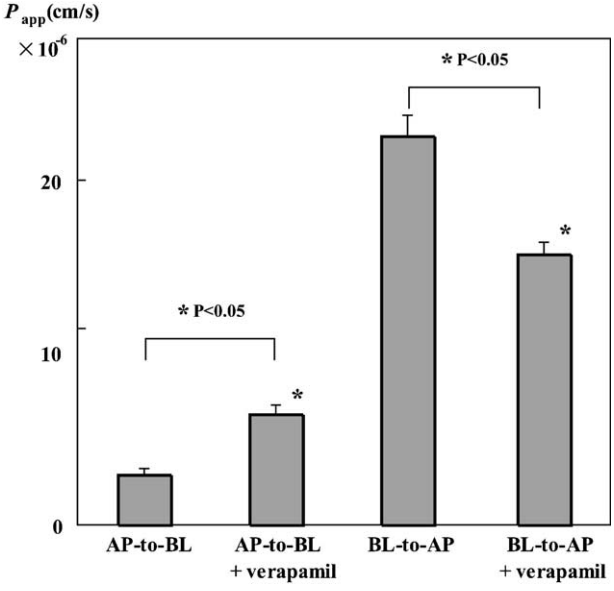

Figure 3. The AP-to-BL and BL-to-AP permeability coefficient of cyclo(Trp-Trp) in the presence of verapamil, a P-glycoprotein inhibitor.

A peptide transporter, PEPT1, which is localized at the brush-border membranes is expressed in the Caco-2 cells.[8] Zwitterionized dipeptides, Trp-X and X-Trp showed relatively high permeability although they are very hydrophilic. These peptides seem to be transported by peptide transporters such as PEPT1. The result that their P_{app} value at 0.5 mM was higher than or nearly equal to that at 2 mM also suggests involvement of saturable peptide transporters. However, a portion of the peptides were hydrolyzed in AP solutions and transported as free amino acids by amino acid transporters. As a result, the value of the permeability coefficient of Trp-X and X-Trp in Table 1 was summation of fractions of each permeability of dipeptides and their metabolite, Trp degraded in AP solutions. Thus, it is not easy to discuss the relationship between the structure of constituent amino acids and the permeability coefficient. Since Gly-D-Trp which is relatively stable in the AP solution showed higher permeability coefficient at 0.5 than at 2 mM, this dipeptide having D-Trp is also considered to be a substrate of peptide transporters.

Two X-Trp-NH_2 derivatives were degraded before and during permeation but showed high permeability coefficient values. Their P_{app} value at 0.5 mM was higher than or nearly equal to that at 2 mM. These peptide-amides may also be transported via a carrier-mediated route.

On the other hand, the N-terminal blocked dipeptides, Ac-Trp-X and Ac-dipeptide-NH_2 were rather resistant to hydrolases expressed in the Caco-2 cells, whereas their permeability coefficient was 10-100 times lower than that of zwitterionized peptides. The N-terminal blocked dipeptides having the similar P_{app} values at 0.5 and 2 mM appear to be passively transported via either the transcellular or paracellular route.

Cyclic peptides, cyclo(Trp-X) were stable to hydrolases. In addition, their permeability was 10 times higher than that of acetylated peptides probably because of their higher hydrophobicity. Based on the higher BL-to-AP permeability and the effect of verapamil, cyclo(Trp-Trp) may be excreted by P-gp. Ac-Trp-Val-NH_2 and Ac-D-Trp-Val-NH_2 were also transported faster in the BL-to-AP than in the AP-to-BL direction. It has been reported that P-gp binds to and transports neutral or positively charged hydrophobic compounds.[12,24] Cyclo(Trp-Trp), Ac-Trp-Val-NH_2, and Ac-D-Trp-Val-NH_2 are neutral at pH 7.3 and likely sufficiently hydrophobic to bind to P-gp.

All indole compounds tested in this report showed high permeability. Since our measurements showed that 3-methylindole, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, indole-3-carboxylic acid, indole-3-carboxyaldehyde and indole-3-acetamide had higher AP-to-BL permeability than BL-to-AP permeability, they were likely actively transported, at least partially, whereas indole, indole-2-carboxylic acid and tryptophol were likely passively transported.

Bai and Amidon reviewed intestinal mucosal-cell exopeptidases and endopeptidases.[13] Most exopeptidases are aminopeptidases. An L-configuration and a free N-terminal α-amino group are required for activity. An endopeptidase in the brush border membrane has been characterized which hydrolyzes peptide bonds on the N-terminal side of hydrophobic amino acids such as tryptophan, phenylalanine, tyrosine, valine, and leucine. N-Terminal protected peptides, which also have N-terminal Trp, were thus resistant against brush-border membrane and cytosolic peptidases. Since X-Trp derivatives are possibly substrates of aminopeptidases and endopeptidases, the compounds may be more easily degraded than Trp-X derivatives. Cyclo(Trp-Trp) also seems to be hydrolyzed by endopeptidases even if it is blocked at the N-terminus.

Based on the results in this report tested compounds were placed into three classes according to their main transport mechanism; (1) passively transported compounds, (2) actively transported compounds and (3) compounds excreted by P-gp. The N-terminal blocked dipeptides; Ac-Trp-X, Ac-dipeptide-NH_2, cyclo(Trp-Gly), and the indole compounds; indole, indole-2-carboxylic acid, and tryptophol are included in the group (1). The zwitterionized dipeptides; Trp-X and X-Trp, the peptide amides; X-Trp-NH_2, and the indole compounds; 3-methylindole, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, indole-3-carboxylic acid, indole-3-carboxyaldehyde and indole-3-acetamide are categorized in the group (2). Cyclo(Trp-Trp), Ac-Trp-Val-NH_2, and Ac-D-Trp-Val-NH_2 are likely to be included in the group (3).

A number of predictive models of Caco-2 cell permeability using physicochemical properties of molecules such as hydrophobicity, hydrogen bonding ability, and polar surface areas have been reported.[1,23,25–27] However, in these previous studies only passive transport through membranes were focused on and discussed. Since the compounds that are transported by various absorption mechanisms are included in this report, contribution by each transport mechanism to the total permeability of compounds should be separately considered. For instance, indole-3-carboxyaldehyde, which is actively transported, showed the highest permeability in the AP-to-BL direction. It is difficult to determine which structural features of the compounds will be responsible for interaction with the transporters because multiple transporters are expressed in Caco-2 cells. Indole-3-carboxyaldehyde is a neutral molecule and the passive transcellular route may also contribute to permeation of this compound. Although Caco-2 cells are a useful model system to evaluate metabolism and absorption of compounds, understanding the role of passive transport phenomena are important. Future studies using artificial lipid membranes may provide insight into permeability via a passive transcellular route.

4. Conclusion

The possible metabolism and transport mechanism of dipeptide derivatives and Trp related compounds were

indicated based on their absorption behavior through Caco-2 cells. For the compounds hydrolyzed by enzymes inside cells, the relative amount of each metabolite was determined. Tested compounds were classified into three classes according to their main absorption mechanism; (1) passively transported compounds, (2) actively transported compounds and (3) compounds excreted by P-gp.

5. Experimental

5.1. Materials

Peptides and their derivatives were purchased from Nacalai Tesque (Kyoto, Japan), Kokusan Chemical Co Ltd. (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), Bachem AG (Bubendorf, Switzerland), Kanto Chemical (Tokyo, Japan), or Sigma-Aldrich Japan (Tokyo, Japan). Acetyl-dipeptides and acetyl-dipeptide-amides were synthesized by either standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase method or solution-phase techniques.[28] The structure of each synthesized peptide was confirmed by high-resolution electron bombardment ionization mass spectrometry. Table 2 presents the high-resolution mass spectral data for the peptides. 'Nissui 2' autoclavable Dulbecco's modified Eagle medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). The potassium salt of penicillin G and streptomycin sulfate for tissue culture medium were purchased from JCN Biomedicals Inc. (OH, USA) and Wako Pure Chemical Industries, respectively. Transwell polycarbonate filters (12 mm in diameter, 0.4 μm-diameter pore size) were obtained from Corning (MA, USA). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries or Nacalai Tesque.

5.2. Cells

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in flasks as described previously.[15] After trypsinization, the cells were seeded on Transwell polycarbonate filters for transport experiments at 4.5×10^5 cells/cm^2. The cells were cultivated for 20–22 days in DMEM with 10% fetal calf serum. The medium was changed three times a week. We used filters with electrical resistance of more than 300 Ω cm^2. No mycoplasma was detectable on a DNA testing by Toray Research Center (Tokyo, Japan) after DNA extraction from the cells with a DNA Isolation kit (Wako Pure Chemical Industries).

Table 2. High-resolution mass spectral data for the synthesized peptides

Peptide	Formula	MH^+ (m/z)	
		Calcd	Found[a]
Ac-Trp-Ala	$C_{16}H_{19}N_3O_4$	317.1376	317.1373 (0.0015)
Ac-Trp-Leu	$C_{19}H_{25}N_3O_4$	359.1845	359.1826 (0.0006)
Ac-Trp-Phe	$C_{22}H_{23}N_3O_4$	393.1689	393.1678 (0.0021)
Ac-Trp-Trp	$C_{24}H_{24}N_4O_4$	432.1798	432.1785 (0.0022)
Ac-Trp-Val-NH_2	$C_{18}H_{24}N_4O_3$	344.1848	344.1852 (0.0016)
Ac-D-Trp-Val-NH_2	$C_{18}H_{24}N_4O_3$	344.1848	344.1842 (0.0014)

[a] The measurement was repeated at least five times. Values in parentheses mean the standard deviation.

5.3. Transport experiments

The transport experiments were done with Transwell filters (multiwell plates) as previously described.[16] Hank's balanced salt solution (HBSS) including 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), pH 7.3, was used as the AP and BL solutions. Compounds of 0.1–2 mM were dissolved in the either AP or BL solution. Compounds which were not sufficiently soluble in HBSS were dissolved in 0.5–2% dimethyl sulfoxide (DMSO) in HBSS.

In AP-to-BL absorption experiments, after aspiration of the medium from the AP side, the filters were put in 1.2 mL of HBSS. Five hundred microliters of the AP solution was added to the filters, followed by incubation for 10–15 min at 37 °C. The AP solutions were aspirated off after pre-incubation, and the filters were then put in 1.2 mL of the fresh BL solution. The transport experiments were started when 0.5 mL of the compound solution was added to the AP side. One hundred microliters of the AP solution was sampled soon after addition of the solution. The concentration of the solution was taken as the initial concentration. At 40-min intervals, 200 μL of the BL solution was sampled, and the same volume of fresh HBSS was added. In BL-to-AP absorption experiments, after pre-incubation, the filters were put in 1.3 mL of the compound dissolved in the BL solution. Four hundred microliters of the AP solution was added to the AP side and then 100 μL of the apical solution was sampled at 40-min intervals, and the same volume of fresh HBSS was added. In a similar way to AP-to-BL absorption, the initial concentration was that of the BL solution which was sampled (0.1 mL) soon after addition of the AP solution.

The amount of peptides and their derivatives as well as their metabolites in the sample was measured by their fluorescence after HPLC (Shimadzu LC-10AS, Japan) according to the following conditions.

HPLC conditions: C_{18} column (COSMOSIL ODS-A 4.6 × 150 mm, Cadenza CD-C-18 4.6 × 75 mm, or YMC-Pack ODS-A 4.6 × 150 mm); detector: fluorescence (Ex. 285 nm, Em. 345 nm); solvent: 25–80% MeOH or 20–30% CH_3CN in H_2O containing 0.05% TFA; flow rate: 0.8 mL/min; retention time: 2.9–10.2 min.

The apparent permeability coefficient, P_{app}, of compounds was calculated using the following equation:

$$P_{app} = 1/(AC_0) \cdot dQ/dt \quad (1)$$

where A is the area of the monolayer, which was 1.13 cm^2 (12 mm in diameter), C_0 is the initial concen-

tration of compounds in the donor side, Q is the amount of compounds transported to the receiver solution, and t is the time. The measurement was repeated at least four times. The standard error was shown in Table 1.

5.4. Inhibition of permeation by substrates of transporters

The AP-to-BL permeability coefficient of 0.2 mM Trp-Ala was determined in the presence of 2 mM Phe and Gly-Pro in the AP solutions.

5.5. Inhibition of permeation by verapamil

The AP-to-BL and BL-to-AP permeability coefficient of 0.02 mM cyclo(Trp-Trp) was determined in the presence of 0.2 mM verapamil, a P-glycoprotein inhibitor in the both AP and BL solutions.

5.6. Effect of pH on transport

The AP-to-BL permeability coefficient of the 2 mM Trp-Gly and cyclo(Trp-Gly) was measured at pH 6.3 as well as pH 7.3. HBSS including 25 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.3, was used as the AP solutions. The BL solutions were held at pH 7.3.

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