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Intestinal Absorption of Stable Cyclic Dipeptides by the Oligopeptide Transporter in Rat

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Abstract

Intestinal absorption of four cyclic dipeptides was studied in the everted small intestine of the rat.

Cyclic seryltyrosine (cyclo(Ser-Tyr)) was stable enough to be transported whereas linear seryltyrosine was not. The absorption clearance of cyclo(Ser-Tyr) was concentration-dependent, and for cyclo(Ser-Tyr) at 125 μM decreased in the presence of glycylsarcosine (10 mM) or cephalexin (10 mM), which were reported to be absorbed by oligopeptide transporter. The absorption clearance was also reduced at 4°C and in the presence of 1 mM dinitrophenol. Kinetic analysis of cyclo(Ser-Tyr) absorption showed that K_m and V_{max} were 19·8 μM and 0·295 nmol min^{-1} cm^{-1}, respectively. It was also suggested that cyclic aspartylphenylalanine and cyclic histidylphenylalanine were absorbed by oligopeptide transporters, but cyclic histidylproline was not. The absorption clearance of cyclo(Ser-Tyr) in the control was much higher than the value of the correlation line representing a plot of passive transport (which was obtained from the absorption clearance of cyclic peptides in the presence of glycylsarcosine (10 mM)) against hydrophobicity (oil–water partition coefficient).

These results indicate that cyclo(Ser-Tyr) is absorbed by the oligopeptide transporter.

Peptide and protein drugs must be transported without metabolic degradation to the systemic circulation if they are to exert their pharmacological action. Although active transport of oligopeptides by the intestinal oligopeptide transporter has been reported (Ganapathy & Leiback 1985; Fei et al 1994; Tamai et al 1994), intestinal absorption of intact peptide is generally poor because of metabolic degradation by peptidase. Our previous kinetic study of the intestinal absorption of peptides such as kyotorphin (Mizuma et al 1997a) and sugar-coupled leucine enkephalin (Mizuma et al 1996) indicated that metabolic degradation in intestinal tissue during absorption was the rate-limiting factor in peptide absorption. However, the peptides were stabilized by chemical modification (Mizuma et al 1994, 1996). Cyclic glycylphenylalanine (cyclo(Gly-Phe)), which is considered to be a chemically modified form, was stable enough to be transported in any region of the small intestine, although linear glycylphenylalanine and linear phenylalanylglycine were too unstable to be transported (Mizuma et al 1997b). Because it was suggested that cyclo(Gly-Phe) was also transported by the oligopeptide transporter, the cyclic form of peptides might be candidates for prodrugs (Mizuma et al 1997b). Furthermore, cyclic peptides have also been reported to have biological potential (Walter et al 1975, 1979; Prasad et al 1977; Morley & Levin 1980; Sakurada et al 1982; Lin et al 1994). The analgesic activity of the cyclic form of kyotorphin (cyclo(kyotorphin)) is greater than that of the linear form (kyotorphin) (Sakurada et al 1982). In this report, therefore, we studied the intestinal absorption of cyclic seryltyrosine (cyclo(Ser-Tyr)), which has a structure similar to that of cyclo(Gly-Phe), but with hydroxyl groups in the side chain, and is of similar stability. In addition to cyclic seryltyrosine (cyclo(Ser-Tyr)), three cyclic peptides were studied, cyclic aspartylphenylalanine (cyclo(Asp-Phe)), which has an anionic group, cyclic histidylphenylalanine (cyclo(His-Phe)) and cyclic histidylproline (cyclo(His-Pro)) (the biologically active metabolite of thyrotropin-releasing

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hormone (Prasad et al 1977; Morley & Levin 1980)) which have cationic groups.

Materials and Methods

Materials

Cyclo(Ser-Tyr), cyclo(Asp-Phe), cyclo(His-Phe), cyclo(His-Pro) and seryltyrosine (Ser-Tyr) were purchased from Bachem Feinchemikalien AG (Switzerland). Glycylsarcosine and cephalexin were purchased from Sigma (St Louis, MO). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were of analytical grade.

Intestinal absorption

The intestinal absorption of peptides was studied with the everted small intestine (Mizuma et al 1992). Briefly, male Wistar rats, 180–230 g (Japan Slc, Shizuoka, Japan), were fasted overnight, anaesthetized with ether, and the intestinal blood was removed by saline perfusion. The jejunum was removed and everted. After connection to a disposable 10 mL plastic syringe in a manner similar to that reported by Doluisio et al (1969) the everted jejunum was placed in 30 mL incubation medium (113·3 mM NaCl, 4·83 mM KCl, 1·214 mM KH_2PO_4, 1·205 mM $MgSO_4$, 16·96 mM $NaHCO_3$, 10·18 mM Na_2HPO_4, 0·645 mM $CaCl_2$, pH 7·4), containing the peptide, in a beaker through which gas (95% O_2, 5% CO_2) was bubbled at 37°C. The serosal side was filled with 5 mL incubation medium containing no peptides. The method of Doluisio et al (1969) was used to mix and sample the serosal solution. Incubation media (0·1 mL) were sampled from both the serosal and mucosal sides for up to 60 min. The samples were mixed with 0·1 mL internal standard solution (250 μM *p*-aminosalicylic acid in 10% perchloric acid) and centrifuged at 11 000 *g* for 5 min in a KM-15200 bench-top centrifuge (Kubota, Japan). The resulting supernatant was analysed by high-performance liquid chromatography (HPLC).

Oil–water partition

Peptide solution (250 μM; 2 mL) in sodium phosphate buffer (0·05 M, pH 7·4) was added to *n*-butanol (6 mL) in a glass tube. After mixing for 30 s the mixture in a glass tube was placed in an incubator at 37°C for 2 h, during which the mixture was mixed for 30 s every 10 min. The mixture was separated into butanol and aqueous phases by centrifugation for 10 min at 1500 *g*. The aqueous layer (200 μL) was mixed with internal standard solution (50 μL) and analysed by HPLC. The butanol layer (5 mL) was evaporated under reduced pressure and the resulting residue was dissolved in internal standard solution (0·2 mL) and analysed by HPLC.

HPLC assay

Peptides and their metabolites were determined by reversed-phase HPLC on a 15 cm × 6 mm i.d. 80TM ODS column (Tosoh, Japan). The chromatograph consisted of a 655A-11 pump, operated at a flow rate of 1·5 mL min^{-1}, a 655A UV detector, operated at 210 nm, and a D-2500 integrator (all from Hitachi, Tokyo, Japan). For assay of cyclo(Ser-Tyr) and Ser-Tyr the mobile phase was 5% methanol and 0·05% phosphoric acid in water. For assay of cyclo(Gly-Phe), cyclo(Asp-Phe) and cyclo(His-Pro) the mobile phase was 15% methanol and 0·05% phosphoric acid in water. For assay of cyclo(His-Pro) the mobile phase was 14% methanol, 0·05% phosphoric acid and 2·5 mM 1-heptanesulphonate sodium salt in water.

Data analysis

Absorption clearance, CL_{abs}, was calculated by use of equation 1; cyclic peptides on the mucosal side were stable and appeared on the serosal side at a constant rate from 10 to 30 min after the start of incubation.

$$CL_{abs} = \frac{\text{(rate of absorption)}}{\text{(mucosal concentration)}} \quad (1)$$

where the rate of absorption was calculated by linear least-squares fitting from the amount absorbed between 10 and 30 min. Kinetic analysis of cyclo(Ser-Tyr) absorption was performed by fitting data to equation 2 by means of the non-linear fitting program, MULTI (Yamaoka et al 1981).

$$\text{Absorption rate} = [V_{max}C/(K_m + C)] + CL_{pas}C \quad (2)$$

where V_{max} and K_m are the maximum rate of absorption and the Michaelis constant, respectively, for carrier-mediated transport. CL_{pas} and C are the absorption clearance of non-saturable (passive) transport and the mucosal concentration of cyclo(Ser-Tyr), respectively. Statistical treatment was performed by Dunnett's multiple-comparison post test after analysis of variance.

Results

Time-course of the concentrations of Ser-Tyr and cyclo(Ser-Tyr) on the mucosal and serosal sides

Ser-Tyr was eliminated from the mucosal side, but not detected on the serosal side (Figure 1). Cyclo(Ser-Tyr) was eliminated from the mucosal side more slowly than Ser-Tyr, and appeared on the serosal side.

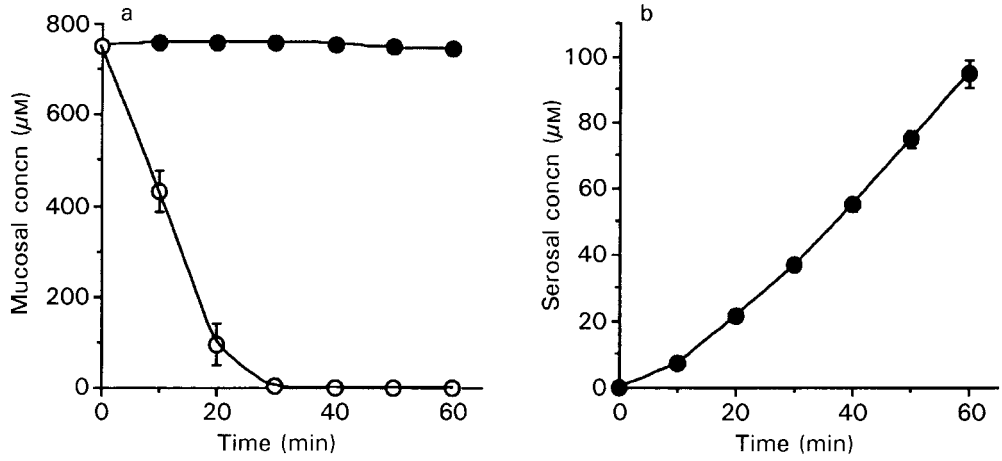

Figure 1. Time courses of the concentrations of cyclo(Ser-Tyr) (●) and SerTyr (○) on the mucosal (a) and serosal (b) sides in the absorption experiments. Data are means ± s.e. (n = 4 or 5).

Concentration-dependency of the absorption of cyclo(Ser-Tyr)

The Eadie-Hofstee plot of cyclo(Ser-Tyr) absorption is shown in Figure 2. Kinetic analysis of the data according to equation 2 showed that K_m and V_{max} were 19·8 μM and 0·295 nmol min^{-1} cm^{-1}, respectively, and that CL_{pas} was 0·516 μL min^{-1} cm^{-1}.

Effect of transport inhibitors on cyclo(Ser-Tyr) absorption

The absorption clearance of cyclo(Ser-Tyr) at 125 μM was significantly reduced in the presence of glycylsarcosine (10 mM) or cephalexin (10 mM) (Table 1). It was also reduced in the presence of dinitrophenol (DNP) (1 mM) or at 4°C.

Absorption of cyclo(Asp-Phe), cyclo(His-Phe) and cyclo(His-Pro) and the effect of transport inhibitors

Cyclo(Asp-Phe), cyclo(His-Phe) and cyclo(His-Pro) were stable on the mucosal side (data not shown), and appeared on the serosal side. Table 2 shows the absorption clearances of cyclo(Asp-Phe), cyclo(His-Phe) and cyclo(His-Pro) and the effects of transport inhibitors. The absorption clearance of cyclo(Asp-Phe) was reduced in the presence of glycylsarcosine (10 mM) or cephalexin (10 mM), though not significantly in the presence of cephalexin. The absorption clearances of cyclo(Asp-Phe) at 250 and 750 μM were lower than at 125 μM and were comparable with those at 125 μM in the presence of transport inhibitors. The absorption clearance of cyclo(His-Phe) was significantly reduced in the presence of glycylsarcosine (10 mM) and cephalexin (10 mM). The absorption clearance of cyclo(His-Phe) was also reduced as the concentration of cyclo(His-Phe) was increased. The absorption clearance of cyclo(His-Pro) at 125 μM was lower than those of any of the cyclic dipeptides, and was not reduced in the presence of glycylsarcosine (10 mM).

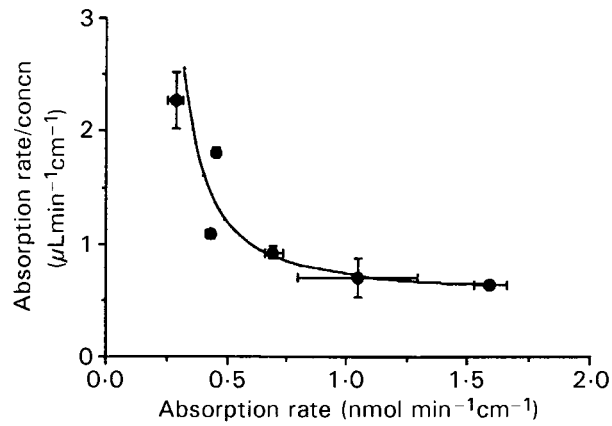

Figure 2. Eadie-Hofstee plot of cyclo(Ser-Tyr) absorption. Data are means ± s.e. (n = 3–5). The curved line represents calculated theoretical values.

Table 1. Inhibitory effect of transport inhibitors on the absorption clearance of cyclo(Ser-Tyr) (125 μM).

Conditions	Absorption clearance (μL min^{-1} cm^{-1})
Control	2·272 ± 0·250
+ 10 mM Glycylsarcosine	0·660 ± 0·039*
+ 10 mM Cephalexin	0·765 ± 0·169*
+ 1 mM DNP	1·373 ± 0·144*
At 4°C	0·367 ± 0·060*

Data are means ± s.e. (n = 3 or 4). *$P < 0·05$, significantly different from the control result.

Correlation of absorption clearances of cyclo(Ser-Tyr) and other cyclic dipeptides with their hydrophobicity

The absorption clearances of 125 μM cyclic peptides in the control and in the presence of 10 mM

Table 2. Inhibitory effect of transport inhibitors on the absorption clearance (μL min^{-1} cm^{-1}) of cyclo(aspartylphenylalanine), cyclo(histidylphenylalanine) and cyclo(histidylproline) (125 μM).

Conditions	Cyclo(aspartylphenylalanine)	Cyclo(histidylphenylalanine)	Cyclo(histidylproline)
Control (125 μM)	1·036 ± 0·031	0·928 ± 0·037	0·678 ± 0·067
+ 10 mM Glycylsarcosine	0·588 ± 0·065*	0·506 ± 0·031*	0·715 ± 0·057
+ 10 mM Cephalexin	0·920 ± 0·036	0·610 ± 0·048*	NA
Control (250 μM)	0·669 ± 0·008*	0·777 ± 0·062	0·553 ± 0·040
Control (750 μM)	0·665 ± 0·061*	0·640 ± 0·066*	NA

Data are means ± s.e. (n = 3 or 4). NA, not assayed. *$P < 0·05$, significantly different from the control result.

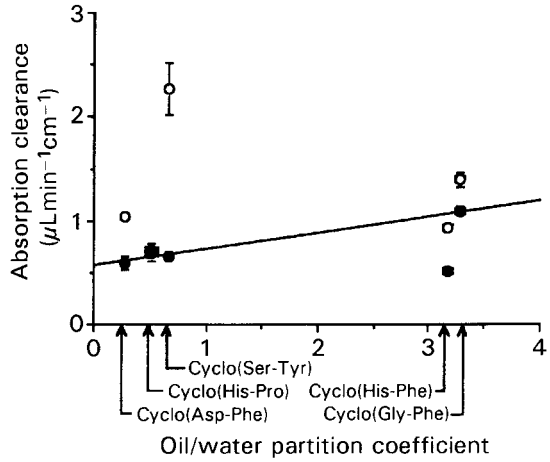

Figure 3. Correlation between absorption clearance and oil–water partition coefficient. Absorption clearance of cyclic dipeptides (125 μM) is shown in the absence (○) and the presence (●) of 10 mM glycylsarcosine. Partition coefficient data are means ± s.e. (n = 3 or 4). The line represents the correlation plot $y = 0·577 + 0·157x$ ($r^2 = 0·968$). The data for cyclo(Gly-Phe) are unpublished results.

glycylsarcosine were plotted against their oil-water coefficients. Absorption clearances of cyclic peptides (except cyclo(His-Phe)) in the presence of 10 mM glycylsarcosine, which were considered to be derived from passive transport, were linearly correlated with their hydrophobicity ($y = 0·577 + 0·157x$; $r^2 = 0·968$; Figure 3). Absorption clearances of cyclo(Ser-Tyr), cyclo(Asp-Phe) and cyclo(Gly-Phe) in the control were higher than for the correlation line, whereas absorption clearance of cyclo(His-Pro) was on the correlation line. Absorption clearances of cyclo(His-Phe) in the absence and presence of 10 mM glycylsarcosine were lower than for the correlation line.

Discussion

Cyclo(Ser-Tyr) was stable on the mucosal side, and appeared on the serosal side (Figure 1). However, the linear peptide Ser-Tyr was degraded on the mucosal side, and did not appear on the serosal side. Because all the cyclic peptides studied in this report, and cyclo(Gly-Phe), were stable in intestine, it was inferred that the cyclic form is stable against peptidases. The absorption clearance was reduced in the presence of DNP and at 4°C, indicating energy-dependent transport (Table 1). The reduction in absorption clearance of cyclo(Ser-Tyr) in the presence of glycylsarcosine or cephalexin indicates saturable transport by the oligopeptide transporter, because glycylsarcosine and cephalexin were reported to be transported by the oligopeptide transporter (Matthews & Burston 1984; Nakashima et al 1984; Ganapathy & Leiback 1985; Fei et al 1994). Kinetic analysis of the absorption of cyclo(Ser-Tyr) revealed that K_m was 19·8 μM, indicating that the affinity of cyclo(Ser-Tyr) for the oligopeptide transporter was higher than that of the linear dipeptides ($K_m = 0·081–5·1$ mM; Fei et al 1994).

The inhibitive effects of glycylsarcosine or cephalexin, and concentration-dependency, were also observed in the absorption clearance of cyclo(Asp-Phe) and cyclo(His-Phe) (Table 2), suggesting that these peptides also were transported by the oligopeptide transporter. However, absorption clearance of cyclo(His-Pro) was lower than that of cyclic peptides, and was not reduced in the presence of 10 mM glycylsarcosine, suggesting that the oligopeptide transporter was probably not contributing to absorption.

The absorption clearance of cyclic peptides in the control and in the presence of oligopeptide transport inhibitors (10 mM glycylsarcosine) in relation to their hydrophobicity were studied to clarify the relative contributions of passive transport and carrier-mediated transport to their absorption. Figure 3 shows that absorption clearance by passive transport, obtained in the presence of the transport inhibitor, correlated well with hydrophobicity ($R^2 = 0·968$), except for cyclo(His-Phe). This is in accordance with the well known positive correla-

tion between passive transport and hydrophobicity (lipophilicity) (Martin 1981). The value of the y intercept (0·577 μL min^{-1} cm^{-1}) was considered to be absorption clearance attributable to paracellular transport (Madara & Pappenheimer 1987).

The absorption clearance of cyclo(Ser-Tyr) in the control was much higher than the value on the correlation line. This also implies oligopeptide transporter-mediated absorption as described above (Figure 2 and Table 1). The absorption clearances of cyclo(Asp-Phe) and cyclo(Gly-Phe) were also higher than the respective values on the correlation line, but the differences were smaller than for cyclo(Ser-Tyr), implying that the contribution of the oligopeptide transporter to the absorption of cyclo(Asp-Phe) and cyclo(Gly-Phe) was small. Thus cyclo(Ser-Tyr) is considered to be a typical cyclic peptide transported by the oligopeptide transporter.

However, the absorption clearance of cyclo(His-Pro) in the control and in the presence of glycylsarcosine was on the correlation line, suggesting hardly any contribution of the oligopeptide transporter to absorption.

On the other hand, the absorption clearance of cyclo(His-Phe) in the absence or presence of 10 mM glycylsarcosine was lower than the value on the correlation line. This was considered to be a result of reverse-transport of cyclo(His-Phe) from the epithelial cell to the mucosal side by an efflux system such as P-glycoprotein. P-glycoprotein for vincristine (Meyers et al 1991) and etoposide (Leu & Huang 1995) and Gp170 for hydrophobic cations (Hsing et al 1992) have been reported as examples of intestinal efflux systems.

Although their transport characteristics varied, all the cyclic peptides in this study were stable in the intestine. Because cyclic peptides have been reported to have biological activity (Walter et al 1975, 1979; Prasad et al 1977; Morley & Levin 1980; Sakurada et al 1982; Lin et al 1994), this study suggests the potential of orally active cyclic peptides. Furthermore, the results presented in this report also utimulate the study of intestinal transportability of cyclic peptides for designing peptidomimetic drugs sufficiently stable for transport by the oligopeptide transporter.

In conclusion, cyclo(Ser-Tyr), cyclo(Asp-Phe), cyclo(His-Phe) and cyclo(His-Pro) were stable enough to be absorbed. The absorption of cyclo(Ser-Tyr) was mediated in part by the oligopeptide transporter. It is also suggested that the absorption of cyclo(Asp-Phe) and cyclo(His-Phe) was partly mediated by the oligopeptide transporter, but that of cyclo(His-Pro) was not.

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References

Doluisio, J. T., Billups, N. F., Dittert, L. W., Sugita, E. T., Swintosky, J. V. (1969) Drug absorption I: an in situ rat gut technique yielding realistic absorption rates. J. Pharm. Sci. 58: 1196–1200

Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leiback, F. H., Romero, M. F., Singh, S. K., Boron, W. F., Hediger, M. A. (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368: 563–566

Ganapathy, V., Leiback, F. H. (1985) Is intestinal peptide transport energized by a proton gradient? Am. J. Physiol. 249: G153–G160

Hsing, S., Gatmaitan, Z., Arias, I. M. (1992) The function of Gp170, the multidrug-resistance gene product, in the brush border of rat intestinal mucosa. Gastroenterology 102: 879–885

Leu, B.-L., Huang, J. (1995) Inhibition of intestinal P-glycoprotein and effects on etoposide absorption. Cancer Chemother. Pharmacol. 35: 432–436

Lin, L., Okada, S., York, D. A., Bray, G. A. (1994) Structural requirements for the biological activity of enterostatin. Peptides 15: 849–854

Madara, J. L., Pappenheimer, J. R. (1987) Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. J. Membrane Biol. 100: 149–164

Martin, Y. C. (1981) A practitioner's perspective of the role of quantitative structure-activity analysis in medicinal chemistry. J. Med. Chem. 24: 229–237

Matthews, D. M., Burston, D. (1984) Uptake of a series of neutral dipeptides including L-alanyl-L-alanine, glycylglycine and glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 67: 541–549

Meyers, M. B., Scotto, K. W., Sirotnak, M. F. (1991) P-Glycoprotein content and mediation of vincristine efflux: correlation with the level of differentiation in luminal epithelium of mouse small intestine. Cancer Commun. 3: 159–165

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

Mizuma, T., Sakai, N., Awazu, S. (1994) Na^+-Dependent transport of aminopeptidase-resistant sugar-coupled tripeptides in rat intestine. Biochem. Biophys. Res. Commun. 203: 1412–1416

Mizuma, T., Ohta, K., Koyanagi, A., Awazu, S. (1996) Improvement of intestinal absorption of leucine enkephalin by sugar coupling and peptidase inhibitors. J. Pharm. Sci. 85: 854–857

Mizuma, T., Koyanagi, A., Awazu, S. (1997a) Intestinal transport and metabolism of analgesic dipeptide, kyotorphin: rate-limiting factor in intestinal absorption of peptide as drug. Biochim. Biophys. Acta 1335: 111–119

Mizuma, T., Masubuchi, S., Awazu, S. (1997b) Intestinal absorption of stable cyclic glycylphenylalanine: comparison with the linear form. J. Pharm. Pharmacol. 49: 1067–1071

Morley, J. E., Levin, A. S. (1980) Thyrotropin-releasing hormone (TRH) suppresses stress-induced eating. Life Sci. 27: 269–274

Nakashima, E., Tsuji, A., Mizuo, H., Yamana, T. (1984) Kinetics and mechanism of in vitro uptake of amino-β-lactam antibiotics by rat small intestine and relation to the intact-peptide transport system. Biochem. Pharmacol. 33: 3345–3352

Prasad, C., Matsui, T., Peterkofsky, A. (1977) Antagonism of ethanol narcosis by histidyl-proline diketopiperazine. Nature 268: 142–144

Sakurada, S., Sakurada, T., Jin, H., Sato, T., Kisara, K., Sakai, Y., Suzuki, K. (1982) Antinociceptive activities of synthetic dipeptides in mice. J. Pharm. Pharmacol. 34: 750–751

Tamai, I., Tomizawa, N., Kadowaki, A., Terasaki, T., Nakayama, K., Higasida, H., Tsuji, A. (1994) Functional expression of intestinal dipeptide/β-lactam antibiotic transporter in *Xenopus laevis* oocytes. Biochem. Pharmacol. 48: 881–888

Walter, R., Hoffman, P. L., Flexner, J. B., Flexner, L. B. (1975) Neurohypophyseal hormones, analogs, and fragments: their effect on puromycin-induced amnesia. Proc. Natl Acad. Sci. USA 72: 4180–4184

Walter, R., Ritzmann, R. F., Bhargava, H. N., Flexner, L. B. (1979) Prolyl-leucyl-glycinamide, cyclo(leucylglycine), and derivatives block development of physical dependence on morphine in mice. Proc. Natl Acad. Sci. USA 76: 518–520

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T. (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharmacobiodyn. 4: 879–885