J. Pharm. Pharmacol. 1996, 48: 270–276
Received November 25, 1994
Accepted November 15, 1995

Intestinal Absorption Studies on Peptide Mimetic α-Methyldopa Prodrugs

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Abstract

Two dipeptide mimetic prodrugs, **1** and **2**, and two tripeptide mimetic prodrugs, **3** and **4**, of L-α-methyldopa were evaluated for intestinal absorption by in-situ single-pass rat jejunal perfusion studies and by in-vitro uptake experiments in brush-border membrane vesicles (BBMVs) prepared from rat intestine. In the perfusion studies, compound **1** demonstrated a 3·5-fold increase in permeability ($P_m^* = 2{\cdot}27$) as compared with that of α-methyldopa ($P_m^* = 0{\cdot}65$), indicating that this prodrug was better absorbed in the intestine than its parent drug. Other prodrugs showed no significant improvement in intestinal permeability. The results correlated with the results of BBMV uptake studies. In the presence of an inward proton gradient, compound **1** showed Michaelis–Menton saturable kinetics of BBMV uptake with a low value of K_m ($0{\cdot}06 \pm 0{\cdot}13$ mM) and a high value of V_{max}/K_m (36·38 nmol (mg protein)$^{-1}$/30 s mM^{-1}) at a low concentration range and a linear uptake at high concentrations with $K_d = 0{\cdot}14 \pm 0{\cdot}02$ mM. Compounds **2** and **3** were mainly taken up in BBMVs via passive diffusion. Compound **4** was taken up in BBMVs basically via the carrier-mediated transport system, while the rate of uptake was much lower than that of compound **1**. The uptake of compounds **1** and **4** was significantly inhibited by dipeptides L-Gly-L-Pro and L-Gly-L-Phe, and cephradine, a β-lactam known to be transported via the dipeptide carrier system, indicating that both compounds were taken up in BBMVs via the H^+-coupled dipeptide-mediated transport system. In contrast to the complicated uptake profile of α-methyldopa, the higher rate of BBMV uptake with less variation demonstrated on compound **1** suggested that the attached nonessential amino acid moiety, D-phenylglycine, is a feasible delivery tool in carrying the parent drug through the intestine.

L-α-Methyldopa has long been used as an antihypertensive agent. However, high inter- and intra-subject variation (8–62%) in oral bioavailability was observed (Kwan et al 1976). Due to its structural similarity to amino acids, the low and variable bioavailability was thought to be due to its complicated absorption via various amino acid-mediated transport systems in the intestine (Stenbæk et al 1982). Lipophilicity has been a major concern for drug intestinal absorption; however, the absorption of hydrophilic and amphoteric compounds such as α-methyldopa, remains a challenge (Hu et al 1989; Amidon & Lee 1994).

Studies have revealed that certain dipeptide-mediated carrier transport systems are responsible for the intestinal absorption of orally absorbable amino-β-lactams (Nakashima et al 1984; Okano et al 1986a; Dantzig & Bergin 1988; Wang et al 1992). Interestingly, most of these amino-β-lactams are actually tripeptide mimetics containing D-phenylglycine or D-*p*-hydroxyphenylglycine. Since the carrier systems showed broad specificity with less structural requirement for the substrates (Bai & Amidon 1992), it is therefore rational to speculate that other di- or tripeptide mimetics containing these amino acids will also be absorbed via the transport systems. We have reported the rational design and preparation of a series of α-methyldopa prodrugs in which D-phenylglycine and D-*p*-hydroxyphenylglycine were included in the molecules as chemical delivery tools in guiding the parent drug to transport through the intestine via the dipeptide-mediated carrier systems. These di- and tripeptide prodrugs showed satisfactory stability in rat intestinal mucosa suspensions (Wang et al 1995a, b). We further report here the results of intestinal absorption studies of these prodrugs (Table 1) by virtue of in-situ single-pass perfusion on rat jejunum (Amidon & Lee 1994) and the uptake in brush-border membrane vesicles (BBMVs) prepared from isolated intestine of the rat.

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Materials and Methods

Materials

Potassium chloride, sodium chloride, sodium hydroxide, 2-(*N*-morpholino)ethanesulphonic acid (MES), [^{3}H]D-glucose, ascorbic acid, sodium taurocholate, urethane, mannitol, HEPES/Tris, trifluoroacetic acid (TFA), sodium 1-pentanesulphonate and sodium dodecyl sulphate were from Sigma, E. Merck, Aldrich and Wako Companies. All chemicals were analytical grade and were used as received. HPLC grade acetonitrile, tetrahydrofuran and methanol were purchased from Alpus Chemical Company. Male Wistar rats, 200–350 g, from The Animal Center of National Taiwan University were used in the perfusion and BBMV uptake studies.

Preparation of perfusion solutions

Preparation of perfusion solutions followed the procedures of Amidon et al (1986) and Lu et al (1992). To maximize the

Table 1. The prodrugs **1**–**4** of L-α-methyldopa.

Compound	Sequence[a]	Formula
1	D-Phenylglycine-L-α-methyldopa	$C_{18}H_{20}N_2O_5$
2	D-*p*-Hydroxyphenylglycine-L-α-methyldopa	$C_{18}H_{20}N_2O_6$
3	D-Phenylglycine-L-alanine-L-α-methyldopa	$C_{21}H_{26}N_3O_6$
4	D-*p*-Hydroxyphenylglycine-L-proline-L-α-methyldopa	$C_{23}H_{27}N_3O_7$

[a]The sequence represents the peptide backbone from the amino terminal to the carboxyl terminal.

absorption and to prevent the test compounds from being oxidized during perfusion, the experiments were performed at pH 6·0 with 0·02% ascorbic acid added as antioxidant and with nitrogen gas bubbled through for 10 min before each experiment. Osmolarity, measured by a Wescor 5500 vapour pressure osmometer (Wescor Company, Logan, UT, USA), was adjusted to 300 ± 10 mOsm kg^{-1} with sodium chloride. The concentration of the perfusion solutions was 0·1 mM for compounds **1**, **2** and α-methyldopa, 0·1 and 1·0 mM for compound **3**. Due to the problem of poor solubility, compound **4** (0·05 mM) was co-perfused with 10 mM sodium taurocholate.

In-situ single-pass rat jejunal perfusion

Surgery on rats followed the procedures described previously (Lu et al 1992). Male Wistar rats were fasted overnight (16–20 h) before anaesthesia. Following the induction of anaesthesia by intramuscular injection of urethane (1·5 g kg^{-1}), the rats were put on a heating pad to maintain body temperature. A midline longitudinal incision was made and a 5–10-cm jejunal segment was cannulated at the distal end to drain perfusates into collecting tubes. The entire surgical area was then covered with parafilm to minimize temperature reduction through evaporation. Tubing and syringes were covered with aluminium foil to retard the oxidation of test compounds. Perfusion solution (0·1 mM) was pumped through the jejunal segment at a flow rate of 0·2 mL min^{-1} by a syringe pump (Stoelting, KD Scientific, USA). The jejunal segment was prewashed initially with drug-free buffer for 10 min. The perfusate was collected from outlet tubing every 10 min for six collection periods after the transport of the solute and water reached steady-state and C_m and C_o, the concentrations of the test compound in the inlet and the outlet perfusate, were determined. The dimensionless effective permeability, P_{eff}*, was calculated by the following equation:

$$P_{eff}^{*} = Q(1 - C_m/C_o)/2\pi DL \quad (1)$$

where Q is the flow rate of the perfusion, D is the solute aqueous difusivity (Tucker & Nelken et al 1990), and L is the intestinal length of the perfused segment.

The aqueous permeability, P_{aq}*, of test compound was calculated from Graetz number according to the following equations:

$$P_{aq}^{*} = 1/(A^{*}Gz^{1/3}) \quad (2)$$

$$Gz = \pi DL/2Q \quad (3)$$

where A* is a predetermined constant, Q is the flow rate of the perfusion, and D is the solute aqueous diffusivity. The data of intestinal permeability are finally reported as dimensionless membrane permeability, P_m*, calculated from P_{eff}* and P_{aq}* by the following equation (Johnson & Amidon 1988):

$$P_m^{*} = P_{eff}^{*}/[1 - (P_{eff}^{*}/P_{aq}^{*})] \quad (4)$$

When inlet sample volumes are more than outlet sample volumes during the perfusion, net water absorption from the intestinal lumen is observed. Net water transport (% cm^{-1}) of the perfusate is measured experimentally as:

$$V_{inlet} - \frac{V_{outlet}}{V_{inlet}} \times \frac{100}{L} \quad (5)$$

where V represents sample volume and L is the length (cm) of the perfused jejunal segment.

Preparation of BBMVs

BBMVs were prepared using a magnesium precipitation method (Sheikh & Møller 1987). Protein content was determined (Lowry et al 1951). The purity of BBMVs was indicated by measuring the activity of the marker enzymes, alkaline phosphatase and aminopeptidase. Generally, these two enzymes were enriched 8–21-fold in the preparation. The activity of Na^+, K^+- ATPase, the marker enzyme of basolateral membranes, was very small. Normal function of BBMVs was confirmed by measuring the uptake of glucose. In the presence of Na^+ gradient ($[Na^+]_{in} < [Na^+]_{out}$), an overshoot phenomenon of glucose uptake with peak values of 9–11 times the equilibrium was routinely observed. The membrane vesicles were preloaded in the buffer solution containing 300 mM mannitol and 16 mM HEPES/Tris (pH 7·4) before perfusion. For studies on the effect of osmolarity upon drug uptake, 300, 450, 600, and 800 mM of mannitol were used.

The uptake of the drugs in BBMVs

The uptake of test compounds in BBMVs was measured by a rapid filtration technique (Okano et al 1986b; Wang et al 1992). In brief, a BBMV preparation (20 mL containing approx. 20 mg protein mL^{-1}) was added to 200 mL of a reaction buffer comprising mannitol (300 mM), HEPES/Tris buffer (25 mM adjusted to pH 7·4 by adding MES) and the test drug solutions (1–2 mM). After incubation at room temperature for the required time, an ice-cold stop solution (1·5 mL) containing NaCl (150 mM) and HEPES/Tris (16 mM, pH 7·4) was added and the solution was filtered through a filter paper (Whatman WCN, 0·45-mm pore size, 2·5-cm diam.) under vacuum. The filter paper was washed

Table 2. Summary of rat in-situ single-pass jejunal perfusion studies.

Compound[a]	No. of experiment	P_m*	Water uptake (% cm^{-1})
1	3	2·27 ± 0·19	1·26 ± 0·27
2	3	0·55 ± 0·13	0·22 ± 0·16
3	4	0·83 ± 0·12	0·74 ± 0·16
4	4	0·54 ± 0·17	0·72 ± 0·11
α-Methyldopa	4	0·65 ± 0·11	1·02 ± 0·22

[a]The concentration of the perfusate for each compound was 0·1 mM. Values given are mean ± s.e.m.

twice with 3 mL of the same stop solution. The drug remaining on the filter paper was extracted with 0·5 mL 0·01 M aqueous HCl using a vortex mixer. The solution (100 mL) was injected onto the HPLC column. Drug bound on the filter paper was determined for correction in different runs using preparations without BBMV added.

Chromatographic conditions

Assays for test compounds were carried out on a high-performance liquid-chromatography system consisting of an autosampler (Model 717, Waters, Millipore, Milford, MA, USA), a solvent delivery pump (Model 600E, Waters), a tunable absorbance detector (Model 484 or 486, Waters), and a Waters 745 data module or a PC 486 computer with chromatography manager software (Millennium 2010, Millipore). Chromatographic conditions for samples from the perfusate were as follows: a Spheri 5RP-18 column (5 μm, 250 × 4·6 mm, ABI) with a mobile phase comprising methanol:0·05 M $NH_4H_2PO_4$ buffer (pH 4·6, containing 0·02 M sodium 1-pentanesulphonate) = 1:9 (v/v) at a flow rate of 1·0 mL min^{-1} for α-methyldopa; a Nova-Pak C-18 column (4 μm, 150 × 3·9 mm, Waters) with a mobile phase comprising methanol:0·05 M $NH_4H_2PO_4$ buffer = 1:9 (v/v) at a flow rate of 1·0 mL min^{-1} for compound **1**; an Ultrasphere C-18 column (5 μm, 250 × 4·6 mm, Beckman) for compounds **2**, **3** and **4** with mobile phases methanol:0·05 M $NH_4H_2PO_4$ buffer = 15:85 (v/v) for compound **2**; 0·07% trifluoroacetic acid (v/v) in a solvent system of methanol:H_2O = 3:7 (v/v) for compound **3** and methanol:-tetrahydrofuran:0·05 M $NH_4H_2PO_4$ buffer (pH 3·7 adjusted with trifluoroacetic acid and NH_4OH) = 5:5:95 (v/v) for compound **4**.

Samples from BBMV uptake experiments were assayed on a Nova-Pak C-18 column (4 μm, 150 × 3·9 mm, Waters) except that a Lichrospher RP-18 column (5 μm, 124 × 4 mm, E. Merck) was used for assaying compound **4**. The mobile phases were as follows: Na dodecyl sulphate (0·5% w/v) in a solvent system of acetonitrile:citric acid-NH_3 buffer (pH 3) = 1:9 (v/v) at a flow rate of 0·8 mL min^{-1} for α-methyldopa, Na dodecyl sulphate (0·5% w/v) in a solvent system of acetonitrile:citric acid-NH_3 buffer (pH 3) = 3:7 (v/v) for compounds **1**, **2** and **4**, and methanol:0·1 M phosphate buffer (pH 2·5) = 1:3 (v/v) for compound **3**. Standards were injected during each assay. Linearity was validated in the usual way.

Data analysis

Numerical computation on the data from solute permeability and water uptake experiments were performed on Statworks and data from BBMV uptake studies were computed with SPSS/PC$^+$ nonlinear regression and are presented as mean ± s.e.m for n experiments. Treatment differences were evaluated by Student's *t*-test.

Results and Discussion

The permeability of compounds in rat in-situ single-pass jejunal perfusion is presented as dimensionless membrane permeability (P_m*, Table 2). According to Johnson & Amidon (1988), the absorption of oral drugs can be calculated from the experimental steady-state perfusion data and reported as P_m* despite the complicated process of absorption in the gastrointestinal tract. Assuming that chemical stability, first-pass metabolism and solubility and dissolution are not the rate-controlling factors, then P_m* is the fundamental parameter for measuring human bioavailability of oral drugs (Amidon et al 1988). This correlation is independent of transport mechanism and structural class of compounds. In such correlations, a value of P_m* less than 1·0 represents incomplete absorption; whereas P_m* of 1·0 or greater correlates with 100% absorption from oral drug solutions. The P_m* value of α-methyldopa (0·65) was comparable with that reported by Amidon et al (1986). Compound **1** had a 3·5-fold increase in permeability ($P = 0·001$) as compared with that of α-methyldopa. The P_m* value of compound **3** was not significantly different from that of α-methyldopa. The P_m* values of compounds **2** and **4** were not improved over those of α-methyldopa.

In this study, the perfusion of prodrugs **1**, **3**, **4** and α-methyldopa was associated with a moderate to significant (0·72–1·26 % cm^{-1}) net water absorption under isotonic conditions (Table 2). A similar phenomenon was observed for small nutrients such as leucine, D-glucose, and α-methyldopa (Adibi et al 1972; Amidon et al 1986; Pappenheimer & Reiss 1987). Other reports have indicated that the degree of absorption of actively transported small nutrients has positive correlation with the degree of net water uptake (Matthews & Laster 1965; Amidon et al 1986; Lu et al 1992). It is generally believed that these small nutrients are transported into the luminal cells primarily by active transport, which with an as yet unknown mechanism activates a passive transport via a paracellular pathway accompanied by a significant net water uptake. Therefore, the phenomena of net water absorption associated with the perfusion may link the transport of these compounds to an active transport process.

The kinetic study of intestinal transport for compounds **1**–**4** and α-methyldopa was conducted by virtue of uptake experiments in BBMV prepared from rat isolated intestine. As shown in Fig. 1, a fast rate of BBMV uptake took place in the initial 2–3 min. Therefore, in subsequent studies, a 30-s incubation time was adopted to determine the initial rate of uptake for all the test compounds. The rate of BBMV uptake of compound **1** was much higher than that of the other three compounds (Fig. 1).

To ascertain that the uptake represents the transport of test compounds into the vesicle rather than membrane binding, the compounds were also subjected to uptake experiments for a period of 1 h in conditions of different medium osmolarity (Osm). As shown in Fig. 2, linear

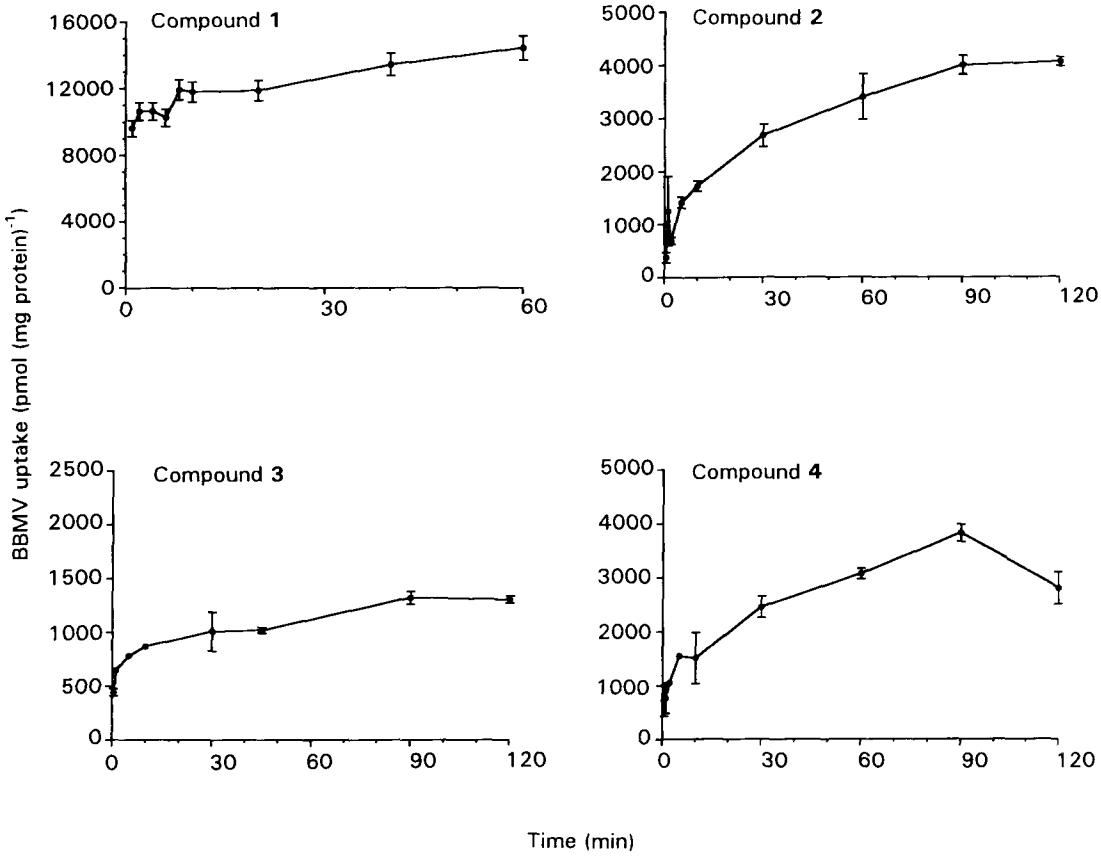

FIG. 1. The uptake of compounds **1**–**4** in BBMVs. Each point represents the mean ± s.e.m. of three to six experiments.

relationship between the uptake and 1/Osm was observed for compounds **1**–**4** ($r^2 = 0.989$, 0·913, 0·995 and 0·968, respectively). The intercept of uptake vs 1/Osm for compounds **1** and **2** was close to zero, indicating that surface binding in BBMV was minimal (Fig. 2). The intercept for compound **3** was below zero, probably due to an experimental error. On the contrary, extensive surface binding was observed for compound **4**, as indicated from the intercept of the uptake vs 1/Osm (1·14 nmol (mg protein)$^{-1}$).

The uptake of compounds **1**, **2**, **3**, **4** and α-methyldopa in BBMV for the first 30 s was measured, respectively, over a concentration range of 0·50–20 mM. Since both passive diffusion and carrier-mediated transport process might be involved in the uptake, the parameter K_d, designated to indicate passive diffusion and the Michaelis-Menten kinetic parameters, V_{max} and K_m, were estimated by SPSS/PC$^+$ nonlinear regression according to the following equation (Okano et al 1986b):

$$v = V_{max}[S]/(K_m + [S]) + K_d[S] \quad (1)$$

with $r^2 = 0.88$, 0·98, 0·98 and 0·79 for compounds **1**, **2**, **3** and **4**, respectively (Table 3). The curves of concentration-dependent uptake were thus generated (Fig. 3). Both passive diffusion and carrier-mediated transport processes were involved in the uptake of compound **1**. The rate of uptake was very fast at low concentrations. After the transport system was saturated (> 2 mM), passive diffusion processes dominated and the uptake profile became linear. Assay of compound **1** at low concentrations reached the detection limit and therefore caused a high value of standard error of mean of K_m (0·06 ± 0·13 mM). Tsuji et al (1990) reported the BBMV uptake of L-α-methyldopa-L-phenylalanine. Although this compound and compound **1** are both dipeptides containing α-methyldopa, the peptide backbones have opposite amino acid sequences. compared with that of L-α-methyldopa-L-phenylalanine, compound **1**, with a free NH_2 group on the D-phenylglycine moiety, is structurally closer to the orally absorbable amino-β-lactams. The maximum rate of uptake (V_{max} 2·18 ± 0·28 nmol (mg protein)$^{-1}$/30 s) for compound **1** in our study is about 19 times that reported for L-α-methyldopa-L-phenylalanine. The high value of V_{max}/K_m (36·38 nmol (mg protein)$^{-1}$/30 s mM^{-1}) in comparison with the value of K_d (0·14 ± 0·02 nmol (mg protein)$^{-1}$/30 s mM^{-1}) suggested that a carrier transport process dominates the absorption of compound **1** at low concentrations.

Compounds **2** and **3** showed a much lower rate of carrier-mediated uptake. The uptake curve is close to linear throughout the whole concentration range, suggesting that passive diffusion might be a major process for these two compounds (Fig. 3). Compound **4** had carrier-mediated transport kinetics at low concentrations and reached a plateau when the concentration increased. The passive

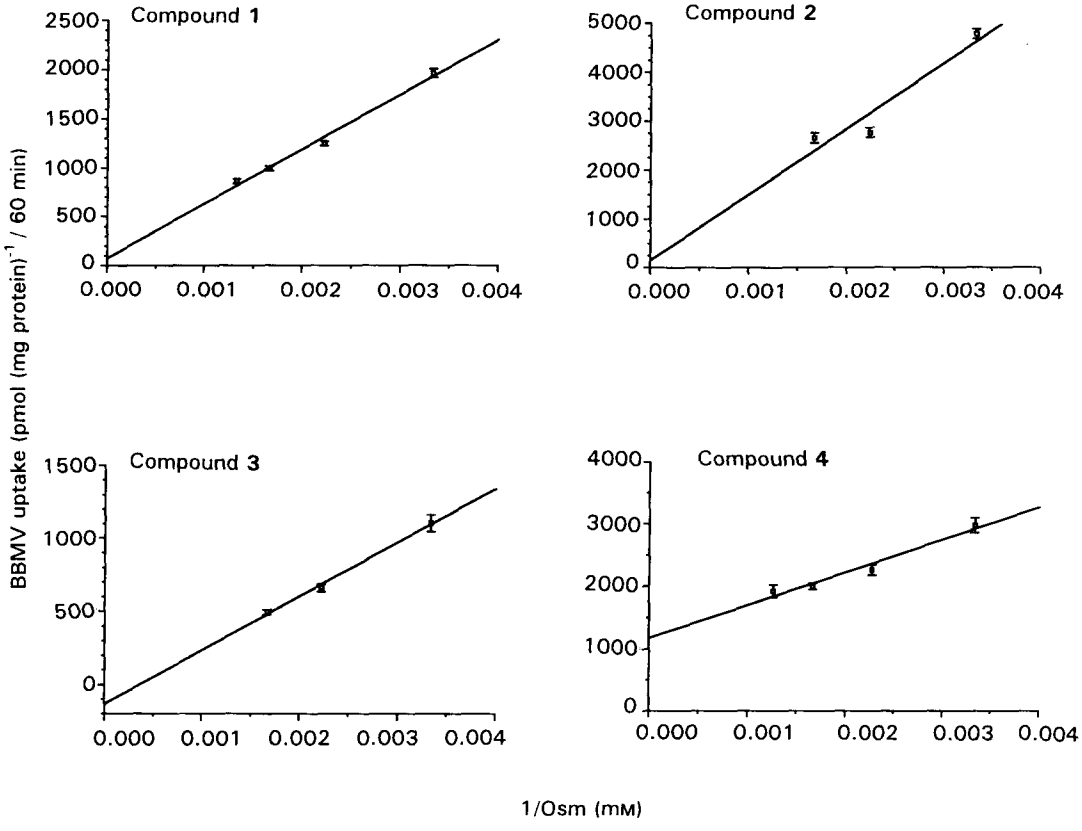

FIG. 2. Plot of the uptake of compounds **1**–**4** against the reciprocal of medium osmolarity.

diffusion process seems not important in the uptake of this compound. The uptake of α-methyldopa was more complex (Fig. 3). A large variation was observed at the same concentration in separate experiments. Neither linear nor non-linear regression fits the uptake profile, suggesting that the transport of α-methyldopa might be through a complex or mixed type of transport process.

In the presence of a proton gradient, significant inhibition of BBMV uptake for compounds **1** and **4** by dipeptides L-Gly-L-Pro (20 mM) and L-Gly-L-Phe (20 mM), and cephradine (20 mM), the β-lactam known to be transported via the H^+-coupled dipetide transport system, was observed (Fig. 4, $P < 0.05$). α-Methyldopa and amino acid L-phenylalanine had no such inhibitory effect. The results suggested that the H^+-coupled dipeptide-mediated transport system is involved in the uptake of compounds **1** and **4** in BBMVs.

Table 3. Kinetic parameters[a] of BBMV uptake of α-methyldopa prodrugs.

Compound	K_m (mM)	V_{max}[b]	V_{max}/K_m[c]	K_d[c]
1	0·06 ± 0·13	2·18 ± 0·28	36·38	0·14 ± 0·02
2	3·52 ± 0·60	3·12 ± 0·19	0·89	0·19 ± 0·01
3	2·24 ± 0·31	0·41 ± 0·08	0·18	0·58 ± 0·02
4	2·97 ± 0·65	3·84 ± 0·28	1·29	0

[a]Data are represented as mean ± s.e.m. of 3 to 6 experiments. [b]The unit for V_{max} is nmol (mg protein)$^{-1}$/30 s. [c]The units for V_{max}/K_m and K_d are nmol (mg protein)$^{-1}$/30 s mM^{-1}.

Conclusion

The intestinal absorption of two dipeptide mimetic prodrugs (**1** and **2**), two tripeptide mimetic prodrugs (**3** and **4**) and the parent drug, L-α-methyldopa, were compared by virtue of in-situ single-pass rat jejunal perfusion studies and by determining the kinetics on BBMV uptake. Prodrug **1** showed higher permeability than that of L-α-methyldopa in the perfusion studies, while the permeability of prodrugs **2**, **3** and **4**, was not improved compared with the parent drug. This result correlates with the results of BBMV uptake studies where compound **1** showed a much higher rate of uptake than that of compounds **2**, **3** and **4**. The low value of K_m and the high value of V_{max}/K_m, in comparison with K_d, suggested that this compound had high affinity for the carrier transport system and the system dominates the transport of this compound. In the presence of an inward H^+ gradient, inhibition of the uptake of compound **1** by dipeptides L-Gly-L-Pro, L-Gly-L-Phe, and cephradine suggested that this compound was taken up in BBMV by the H^+- coupled dipeptide-mediated transport system. Similar results for compound **4** also point to its absorption via an H^+- coupled carrier transport system. As for compounds **2** and **3**, the BBMV uptake studies suggested that passive diffusion is the major route for their intestinal absorption. Compound **1** had a higher rate of BBMV uptake with less variation than that of α-methyldopa in the concentration range 0·50–10 mM. The attached nonessential amino acid moiety, D-phenylglycine, seemed to be a feasible delivery tool in improving the intestinal BBMV uptake for

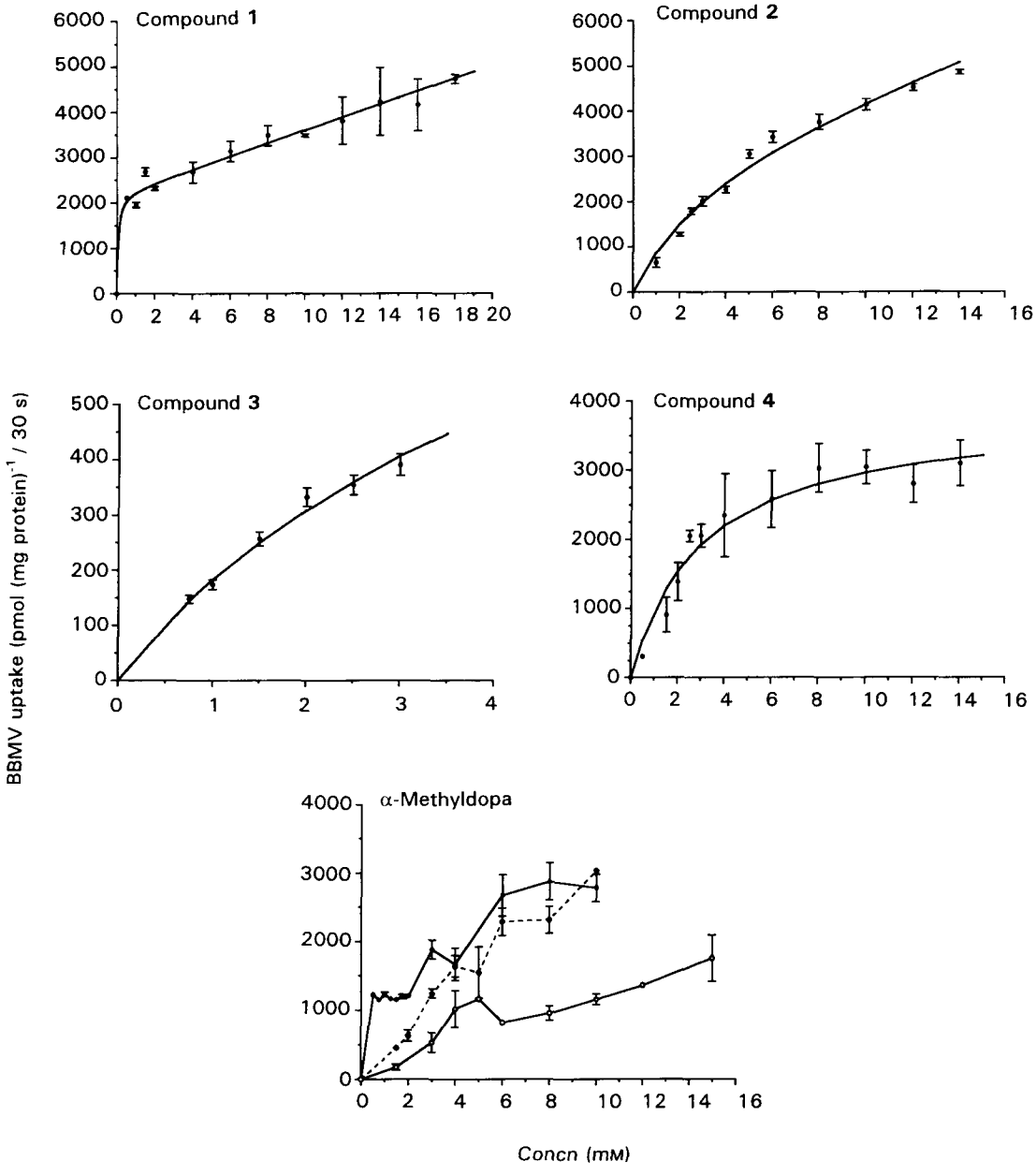

FIG. 3. Concentration dependence of the BBMV uptake of compounds **1**, **2**, **3**, **4** and α-methyldopa. Each point represents the mean ± s.e.m. of three to six experiments. The solid and dashed lines in the uptake of α-methyldopa represent three separate experiments.

α-methyldopa; however, the structure-absorption relationship analysis from the limited number of prodrugs tested could not lead to a conclusion on whether the dipeptide or the tripeptide mimetics are better oral prodrugs of α-methyldopa for improving the intestinal absorbtion.

Acknowledgement

This study was supported by grant NSC 82-0412-B002-112 from the National Science Council, the Republic of China.

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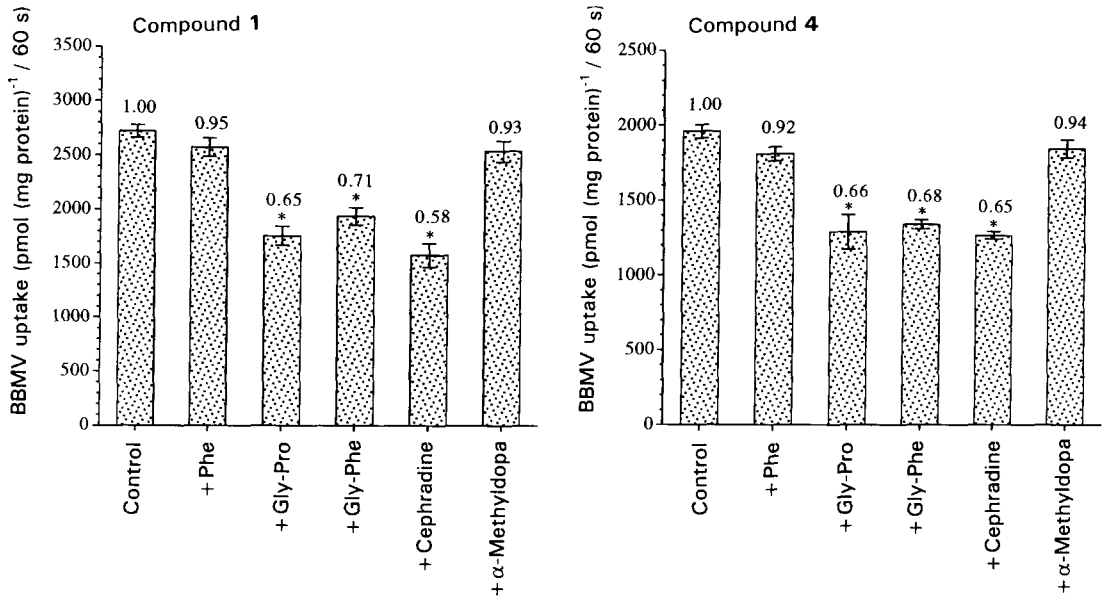

FIG. 4. The effect of amino acid L-Phe, dipeptides L-Gly-L-Pro and LGly-L-Phe, β-lactam cephradine, and α-methyldopa (20 mM each) on the BBMV uptake of compound **1** (2 mM) and compound **4** (2 mM). The uptake for the initial 60 s was determined in each experiment. Data presented are mean ± s.e.m. of three experiments. The asterisk marked on the bars represents a significant difference of BBMV uptake from that of the control experiments ($P < 0.05$).

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