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Preparation and Intestinal Absorption of L-Dopa-D-phenylglycine

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ABSTRACT

L-Dopa-D-phenylglycine was synthesized in this laboratory as L-dopa derivative for improving its intestinal absorption. As designed for transport through the intestine via oligopeptide transporter (PepT1), the competition of this dipeptide with known substrates for PepT1 in brush-border membrane vesicle (BBMV) was investigated. At the presence of L-Glycinyl-L-proline (L-Gly-L-Pro), L-Glycinyl-L-phenylalanine (L-Gly-L-Phe) or cephradine, the uptake of L-dopa-D-phenylglycine in BBMV was reduced to 54.1 ± 4.5%, 57.6 ± 5.2% or 62.9 ± 10.2%, respectively. The inhibition by these dipeptides and the tripeptide mimetic amino-β-lactam was significantly higher than by amino acids L-Phenylalanine (L-Phe) or L-dopa. The results suggested that the intestinal H^+-coupled PepT1 was involved in the uptake of L-dopa-D-phenylglycine. The steady state plasma concentrations of L-dopa-D-phenylglycine and L-dopa in rats after a single pass *in-situ* jejunal perfusion with 0.1 mM perfusate were 104.0 ± 12.90 μg/mL and 1.24 μg/mL respectively. L-Dopa-D-phenylglycine demonstrated a 50.1 fold higher plasma concentration, in terms of molar ratio, than that of L-dopa. D-Phenylglycine was proved to be a satisfactory moiety for the improvement of L-dopa absorption in the intestine.

Key words: L-Dopa-D-phenylglycine, dipeptide mimetics, PepT1, intestinal absorption

INTRODUCTION

L-Dopa has long been used as a prodrug of dopamine in the treatment of Parkinson's disease[1,2]. However, clinically this drug illustrated wide range of inter- and intra-patient variations in the rate and extent of absorption[3,4]. The variation of oral bioavailability due to its interaction with food is in part attributed to its complicated absorption through amino acid transport systems with amino acids in the diet[5,6]. A rational approach in improving the oral bioavailability of L-dopa is thus to prevent this drug from being absorbed via these routes.

Studies revealed the existence of H^+-coupled oligopeptide transporter (PepT1) in brush-border membrane of duodenum, jejunum and ileum of the small intestine[7,8]. This transporter was responsible for the absorption of dipeptide mimetic angiotensin converting enzyme (ACE) inhibitors[9,10], and orally absorbable tripeptide mimetic amino-β-lactams[11-19]. Based on the thought that D-phenylglycine is the common moiety in the molecules of orally absorbable amino-β-lactams, we proved that D-phenylglycine significantly increased the uptake of α-methyldopa in BBMV and might be a feasible delivery tool for transporting drugs of poor bioavailability through the intestine via PepT1[20].We further used D-phenylglycine to prepare a series of tripeptide mimetic L-dopa derivatives as dopamine prodrugs[21]. In this report we describe the synthesis of a dipeptide L-dopa-D-phenylglycine and its transport via PepT1. The intestinal absorption of this dipeptide in rats with that of L-dopa is also compared.

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MATERIALS AND METHODS

I. *Materials and Instruments*

Chemicals, reagent grade for synthesis and analytical grade for biological studies, were from Sigma (Saint Louis, Missouri, U. S. A), E. Merck (Darmstadt, Germany), Aldrich (Milwaukee, Wisconsin, U. S. A) and Wako (Osaka, Japan). Melting points were determined in Buchi 510 capillary melting point apparatus and were uncorrected. IR spectra were determined on a Perkin-Elmer 1760 FT-IR instrument (Shelton, Connectitut, U. S. A). Proton NMR spectra were determined on a Bruker 80 MHz or Bruker 400 MHz spectrometer (Madison, Wisconsin, U. S. A) with chemical shifts recorded in parts per million relative to tetramethylsilane. Mass and high-resolution mass (HRMS) were measured on Finnigan MAT 4510 and JEOL JNS-D300 spectrometer respectively. Branson Sonifier 450 sonicator, Kubota 2010 and Eppendorf 5415C centrifuge, Model 905 incubator (Cherng Huei Instrument Co.) and Ystral Laboratory series x10/20 Homogenizer were used in the preparation of intestinal mucosal suspension. Osmolarity of test solutions was determined with Wescor 5500 vapor pressure osmometer (Wescor Company, Logan, UT, U.S.A.). High performance liquid chromatography system used in the assay of biological samples consisted of an autosampler (AS950, Jasco, Tokyo,

Japan), a Model 600E (Waters, Milford, Massachussetts, U.S.A.) solvent delivery pump, a Model LC-4C electrochemical detector with a glassy-carbon electrode (Bioanalytical Systems, Inc., West Lafayette, Indiana, U.S.A.), and an integrator (Macintegrator I, Curperpino, San Francisco, U. S. A.).

II. *Animals*

Male Wistar rats weighing 200-350 g were used in preparing intestinal mucosal suspension and in intestinal perfusion studies. Animals were housed in a 12 hr light/dark with 21 ± 2°C constant temperature environment prior to study. The rats were fasted for 18 hr before the experiment. Water was supplied *ad libitum*.

III. *Synthesis*

L-N(Boc)-dopa-D-phenylglycine benzyl ester: A solution of L-*N*(Boc)-dopa prepared according to Bloom *et al.*[22], D-phenylglycine benzyl ester hydrochloride[23], dicyclohexylcarbodiimide (DCC), hydroxybenztriazole (HOBt) and sodium carbonate in molar ratio 1.0:1.0:1.0:1.1:1.1 in dioxane was stirred at room temperature for 16 hr until the reaction was judged complete by TLC. The solid precipitate was filtered off, the filtrate was concentrated in vacuo and the residue was partitioned between ethyl acetate and aqueous potassium hydrogen sulfate ($KHSO_4$) solution (5%). The combined ethyl acetate solution was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo. The solid residue was chromatographed to afford L-*N*(Boc)-dopa-D-phenylglycine benzyl ester (yield 65%) as colorless solid; mp 69-70°C; IR (KBr) n_{max} 3500-3300 (br), 1736, 1719, 1702, 1686 cm^{-1}; ^{1}HNMR (400 MHz, $CDCl_3$) δ1.36 (s, 9H, *t*-butyl), 2.79-2.90 (m, 2H, catechol-C$\underline{H}_2$-), 4.37 (br, 1H, CONH), 5.03-5.20 (m, 3H, Ph-C$\underline{H}$+Ph-C$\underline{H}_2$-), 5.52 (m, 1H, C$\underline{H}$), 6.42-6.70 (m, 3H, catechol Hs), 7.21-7.26 (m, 10H, Ph-Hs) ppm.

L-Dopa-D-phenylglycine: A solution of L-*N*(Boc)-dopa-D-phenylglycine benzyl ester in trifluoroacetic acid and dichloromethane (1:1, v/v) was stirred at room temperature for 3 hr. The volatile components were removed in vacuo and *n*-hexane was added to facilitate precipitation. A methanolic solution of the L-dopa-D-phenylglycine benzyl ester intermediate was subjected to hydrogenolysis in the presence of palladium hydroxide in charcoal ($Pd(OH)_2/C$ containing 20% of Pd) under hydrogen (14.7 psi). When the reaction was judged complete by TLC, the catalyst was removed by filtration and the solvent was evaporated to dryness. The crude product was purified by reversed-phase MPLC (Lobar RP-18, 40-63 mm) using a solvent system containing aqueous trifluoroacetic acid (0.1%) and MeOH as the mobile phase to give the desired L-dopa-D-phenylglycine (yield 59%) as colorless solid; mp 216°C (dec.); IR (KBr) n_{max} 3500-3300 (br), 1661, cm^{-1}; ^{1}H NMR: (400 MHz, D_2O+TFA): δ 2.72-2.90 (m, 2H, -C$\underline{H}_2$-), 5.30 (s, 1H, Ph-C$\underline{H}$-), 6.34-6.62(m, 3H, catechol H), 7.21 (s, 5H, Ph H) ppm; HRMS (FAB^-) *m/z* 329.1134 $(M-H)^-$ (calcd for $C_{17}H_{17}N_2O_5$ *m/z* 329.1137).

IV. *BBMV Uptake Studies*

Brush-border membrane vesicles (BBMV) were prepared using a magnesium precipitation method and protein content was determined[24]. The purity of BBMV 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 BBMV was confirmed by measuring the uptake of D-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 (16 mM, pH 7.4) before the experiment. For studies on the effect of osmolarity upon drug uptake, 300, 450, 600, and 800 mM of mannitol were used. The uptake of test compounds in BBMV was measured by a rapid filtration technique. A BBMV preparation (20 mL containing approximately 20 mg protein / mL) was added into 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 acquired 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 diameter) under a vacuum. The filter paper was washed twice with 3 mL of the same stop solution. The drugs remaining on the filter paper were extracted with 0.5 mL of 0.01 M aqueous HCl solution by virtue of a vortex motion. The solution (100 mL) was injected onto the HPLC column. The drug bound on the filter paper was determined for correction in different runs using preparations without BBMV added.

V. *Degradation of L-Dopa-D-phenylglycine in Intestinal Mucosa Suspension*

Mucosa suspension was prepared from the intestine of male Wistar rats according to the method of Hu et al and was stored in an ice bath before use[25]. Degradation studies were conducted immediately after the mucosa suspension was prepared. A methanolic solution (100 μL) of the test compound (1 mg/mL) was diluted with an isotonic mannitol buffer solution (pH 6.5, 2.4 mL) as the stock solution. This stock solution (1000 μL) was mixed with the mucosal suspension (1000 μL). The mixture was incubated in a water bath at 37°C and subjected to sampling at intervals between zero to 60 minutes. Each sampled solution (200 μL) was denatured with 0.8 mL of MeOH and centrifuged at 6600 *g* for 5 minutes. Each of the supernatant (20 -100 μL) was subjected to HPLC assay.

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VI. *In Situ Rat Perfusion*

Preparation of jejunal segments and the perfusion solutions followed the procedure of Lu *et al*[26]. In order to simulate the intestinal microclimate, the experiments were performed at pH 6.0 phosphate buffer solution. To maximize the absorption and to prevent the test compounds from being oxidized during perfusion, 0.02% ascorbic acid was added as an antioxidant and nitrogen gas was bubbled through for 10 min before each experiment. Concentration of the test compounds in the perfusion solutions was 0.1 mM. The entire surgical area of the jejunal segment was covered with parafilm to minimize temperature reduction through evaporation. Tubing and syringes were covered with aluminum foil to retard the oxidation of testing compounds. Perfusion solution was pumped through the jejunal segment at a flow rate of 0.2 mL/min by a syringe pump (Stoelting, KD Scientific, Pennsylvania, U.S.A.). The jejunal segment was prewashed with drug-free buffer for 10 min before the drug solution was pumped in. Outlet tubing samples were collected every 10 min for 6 collection periods after water and solute transport reached steady-state. After 90 min of perfusion, blood samples were centrifuged at 1415 *g* for 10 min. The plasma sample separated was stored at –20˚C before analysis. The samples were filtered through 0.2 μm membrane filters before injecting into HPLC system.

VII. *Chromatography*

Samples from BBMV uptake experiments, stability studies and the perfusion experiments were assayed in HPLC coupled with an electrochemical detector (0.75 V; filter 0.1 Hz). L-Dopa-D-phenylglycine was eluted in a Lichrospher RP-18 column (5 mm, 250 mm × 4 mm, E. Merck, Germany) with a solvent system comprising acetonitrile and ammonium phosphate buffer (0.1M, pH 3.3) at a ratio of 2:8 and sodium dodecyl sulfate (0.05%, w/v) at a flow rate of 1.2 mL/min. L-Dopa was eluted in a Spheris S5 SCX column (5μm, 250 x 4.6 mm, Spherisob, USA) with a solvent system comprising 1.84 mM tetrabutylammonium hydrogen sulfate, 1.04 mM sodium dodecylsulfate in 7.5 % (v/v) phosphate buffer (pH 2.0, 0.1 M) at a flow rate of 1.5 mL/min. Isoproterenol and dihydroxyl-benzylamine were used as internal standards for L-dopa-D-phenylglycine and L-dopa, respectively. The retention time of L-dopa-D-phenylglycine and L-dopa were 8.420 and 3.585 min, respectively. The precision and accuracy of assay methods were validated by intra-day and inter-day analysis of serum standards over a period of 6 days.

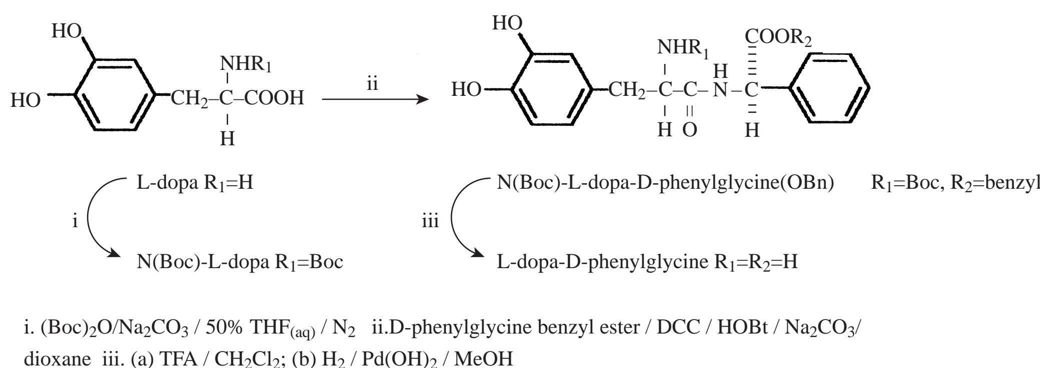
VIII. *Data Analysis*

Data analysis were performed on Visual dBase and SPSS/PC$^+$ and were represented as mean ± S.D. for n experiments. Treatment differences were evaluated by paired Student's t-test.

RESULTS AND DISCUSSION

Preparation of L-*N*(Boc)-dopa-D-phenylglycine benzyl ester from L-*N*(Boc)-dopa and D-phenylglycine benzyl ester followed general procedures of peptide synthesis using dicyclohexylcarbodiimide (DCC) as coupling reagent and 1-hydroxybenzotriazole (HOBt) for the prevention of epimerization. Removal of Boc and benzyl protecting groups by treatment with trifluoroacetic acid followed by catalytic hydrogenolysis in the presence of $Pd(OH)_2$ lead to L-dopa-D-phenylglycine (Scheme 1).

Typical HPLC chromatograms of L-dopa-D-phenylglycine and L-dopa from plasma samples are shown in Figure 1. Recovery of drugs was estimated by comparison of peak areas in drug-free plasma extracts spiked with standard solutions. The plasma recovery rates were 74.9% ± 3.0 (n = 3) and 82.0% ± 5.6 (n = 3), respectively, for L-dopa-D-phenylglycine and L-dopa. Replicate standards (*n* = 6) were analyzed to assess the intra-day and inter-day variability of the assay and the precision expressed as coefficient of variation (CV), as well as accuracy expressed as relative errors (RE) were calculated. The standard curve of L-dopa-D-phenylglycine was linear over the plasma concentration range of 0.1 to 30 μg/mL (r^2 = 0.998) with the limit of quantitation (LOQ) at 0.1 μg/mL. The overall intra-day and inter-day accuracy (RE) were between –7.44 and +4.74% and the CV were less than 20%. The standard curve of L-dopa was linear over the plasma concentration range of 0.05 μg/mL to



i. $(Boc)_2O/Na_2CO_3$ / 50% $THF_{(aq)}$ / N_2 ii.D-phenylglycine benzyl ester / DCC / HOBt / Na_2CO_3/ dioxane iii. (a) TFA / CH_2Cl_2; (b) H_2 / $Pd(OH)_2$ / MeOH

Scheme 1. Synthesis of L-dopa-D-phenylglycine.

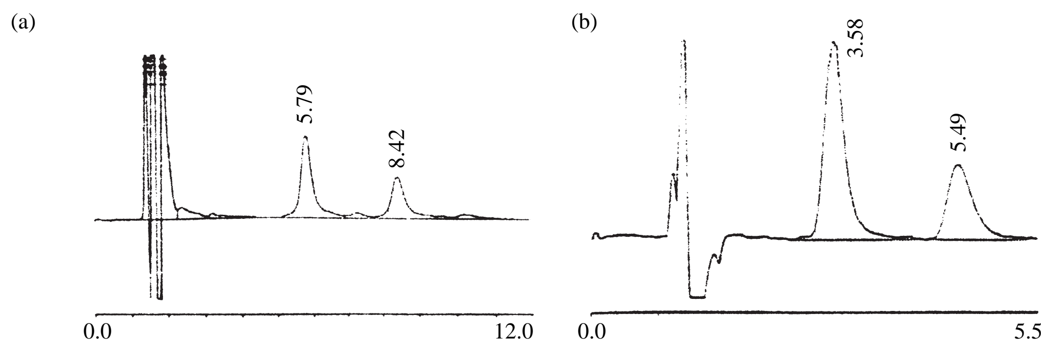


Figure 1. HPLC chromatograms of plasma samples containing (a) L-dopa-D-phenylglycine (retention time 8.42 min) with isoproterenol (retention time 5.79 min) as internal standard or (b) L-dopa (retention time 5.49 min) with dihydroxyl-benzylamine (retention time 3.58 min) as internal standard.

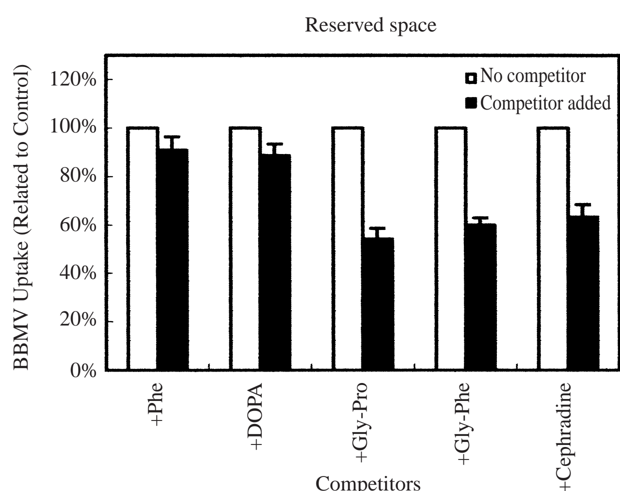


Figure 2. The uptake of L-dopa-D-phenylglycine in BBMV was significantly reduced to 54.1 ± 4.5%, 57.6 ± 5.2% and 62.9 ± 10.2%, respectively, in the presence of L-Gly-L-Pro, L-Gly-L-Phe or cephradine ($p<0.05$), while no significant reduction of the uptake of L-dopa-D-phenylglycine was observed while L-Phe or L-dopa was present.

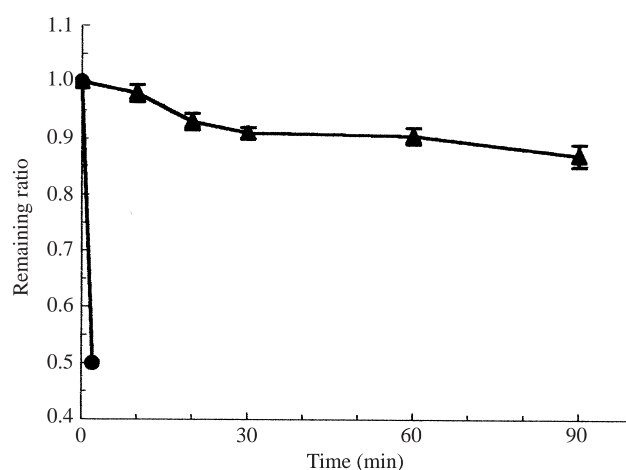


Figure 3. The degradation of L-dopa-D-phenyglycine (-▲-) in rat intestinal mucosa suspension in comparison with L-Gly-L-Phe (-●-). The recovery of each test compound was presented as remaining ratio (mean ± S.D.) of 3 experiments.

2.5 μg/mL (r^2 = 0.995) with the limit of quantitation (LOQ) at 0.05 μg/mL. The overall intra-day and inter-day RE were between –9.23 and +8.31% with CV less than 14%.

The H^+-coupled transport system PepT1 exists in brush-border membrane of duodenum, jejunum and ileum of the small intestine(8). Studies of the transport of L-dopa-D-phenylglycine and L-dopa via PepT1 was thus conducted by measuring their uptake in the brush-border membrane vesicle (BBMV) prepared from isolated jejunum segment of rat intestine according to literature method. As L-dopa-D-phenylglycine was designed to be absorbed in the intestine via PepT1, its transport via this transporter was investigated by the competition of BBMV uptake with L-Gly-L-Pro, L-Gly-L-Phe and cephradine, known to be transported via this transport system(14,17). This compound was incubated in BBMV with the presence of ten times the molar ratio of L-Phenylalanine (20 mM), L-dopa (20 mM), L-Gly-L-Pro (20 mM), L-Gly-L-Phe (20 mM) or cephradine (20 mM), respectively. No significant inhibition on the BBMV uptake of L-dopa-D-phenylglycine was observed when it was incubated with amino acids L-Phe or L-dopa. On the other hand, its uptake was reduced to 54.1 ± 4.5%, 57.6 ± 5.2% and 62.9 ± 10.2%, respectively, in the presence of L-Gly-L-Pro, L-Gly-L-Phe or cephradine (Figure 2). The inhibition was significantly higher than by L-Phe. The results suggested that the intestinal H^+-coupled PepT1 was involved in the uptake of the dipeptide mimetic L-dopa-D-phenylglycine.

Stability of L-dopa-D-phenylglycine in rat intestine was determined prior to the absorption study by virtue of measuring its degradation in a mucosal preparation isolated from rat intestine(3). In order to simulate the intestinal microclimate pH, test compounds, L-dopa-D-phenylglycine or L-Gly-L-Phe for comparison, was incubated with the mucosal suspension in a pH 6.0 isotonic buffer solution. Samples were withdrawn for analysis in HPLC at time intervals between zero and 90 min of incubation. L-Gly-L-Phe, used as a reference to ensure the enzymatic activity of the mucosal preparation, degraded rapidly with only 19% of recovery after 2 min of incubation. L-Dopa-D-phenylglycine on the other hand was fairly stable with more than 85% of recovery after 90 min of incubation, indicating its stability in the intestine (Figure 3).

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Table 1. Dimensionless membrane permeabilities (P_m*) and steady state plasma concentrations of L-dopa-D-phenylglycine and L-dopa in rats measured by *in situ* single-pass jejunal perfusion

Compound[a]	No. of Experiments	P_m*	plasma conc.[b] (μg/mL)	molar ratio[c] relative to L-dopa
L-dopa-D-phenylglycine	4	1.32 ± 0.33	104.0 ± 12.9	50.1
L-dopa	3	0.94 ± 0.10	1.24[d]	1.0

[a] Concentration of the perfusate of each compound was 0.1 mM. [b] Data presented are mean ± S.D. of n experiments.
[c] Plasma concentration in molar ratio relative to L-dopa.
[d] L-Dopa was detected in one of three test rats. It was below detection limit in the other two rats.

The intestinal absorption of L-dopa-D-phenyglycine and L-dopa were compared by measuring the steady-state plasma concentration in rats by *in situ* single-pass jejunal perfusion. The steady-state plasma concentration of the perfused drugs was monitored. Amidon's dimensionless membrane permeability (Pm*) was also measured as an indication for drug disappearance from the intestine(26). According to the theoretical analysis of Johnson and Amidon, Pm* calculated from experimental steady-state perfusion data, is the fundamental parameter for measuring oral bioavailability of drugs, assuming that chemical stability, first-pass metabolism and solubility/dissolution are not rate controlling factors(27,28). With equal molar concentration of compounds perfused, L-dopa-D-phenyglycine demonstrated higher Pm* than that of L-dopa ($p<0.025$, Table 1). The plasma concentration of L-dopa-D-phenyglycine (104.0 ± 12.90 μg/mL) was 50.1 fold, in terms of molar ratio, higher than that of L-dopa (1.24 μg/mL).

CONCLUSION

D-Phenylglycine was designed as a delivery tool for transporting L-dopa through the intestine via intestinal PepT1 transporter. In an inward proton gradient, dipeptides L-Gly-L-Pro, L-Gly-L-Phe, and tripeptide mimetic amino-β-lactam cephradine inhibited the BBMV uptake of this dipeptide, suggesting that this compound was taken up in the intestine by PepT1. This dipeptide was better absorbed than L-dopa in rats as the steady-state plasma concentration was measured by *in situ* single-pass jejunal perfusion. The approach to use D-phenylglycine as a drug delivery tool for improving the intestinal absorption of L-dopa was proved successful. Biotransformation and pharmacokinetic profiles from this dipeptide prodrug to dopamine and the anti-Parkinsonism activity after oral administration of this prodrug need to be further investigated.

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L-Dopa-D-phenylglycine之合成及小腸吸收

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摘 要

本研究合成雙胜肽L-dopa-D-phenylglycine，作為dopamine之前驅藥。設計原理為以D-phenylglycine作為藥引子，導引L-dopa透過小腸小胜肽運輸體（oligopeptide transporter, PepT1）吸收。我們由大鼠小腸黏膜製作刷狀緣膜包囊（brush border membrane vesicle, BBMV），藉由L-dopa-D-phenylglycine與L-Gly-L-Pro, L-Gly-L-Phe及cephradine對PepT1的競爭，測量其BBMV之吸收量，探討L-dopa-D-phenylglycine是否透過小腸小胜肽運輸體吸收。在雙胜肽L-Gly-L-Pro, L-Gly-L-Phe及擬三胜肽cephradine的存在下，L-dopa-D-phenylglycine在BBMV之吸收分別降為54.1 ± 4.5%, 57.6 ± 5.2%及62.9 ± 10.2%，胺基酸L-Phe則不影響其吸收，顯示L-dopa-D-phenylglycine可透過小腸小胜肽運輸體吸收。在活體Wistar大鼠進行空腸灌流試驗達到血中濃度恆定時，L-dopa-D-phenylglycine的血中濃度（104.0 ± 12.9 μg/mL）以當量數計為L-dopa血中濃度（1.24 μg/mL）的50.1倍，顯示L-dopa-D-Phenylglycine在大鼠小腸具有優於L-dopa的吸收。這個雙胜肽前驅藥在大鼠的吸收動力學研究證明D-Phenylglycine具有藥引子功能，可促進L-dopa藉由小腸PepT1吸收。

關鍵詞：L-dopa-D-phenylglycine，雙胜肽前驅藥，小腸小胜肽運輸體