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Research Article

Esterase-Sensitive Cyclic Prodrugs of Peptides: Evaluation of a Phenylpropionic Acid Promoiety in a Model Hexapeptide

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Purpose. To evaluate a cyclic phenylpropionic acid prodrug of a model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) as a novel approach to enhance the membrane permeation of a peptide and stabilize it to metabolism.

Methods. Conversion to the linear hexapeptide was studied at 37°C in HBSS, pH 7.4, and in various biological milieus having measurable esterase activities. Transport and metabolism characteristics were assessed using the Caco-2 cell culture model.

Results. In aqueous buffered solution, pH 7.4, the cyclic prodrug degraded quantitatively ($t_{1/2}$ = 1795 ± 289 min) to the linear hexapeptide and the lactone. Substantially faster degradation of the cyclic prodrug was observed in 90% human plasma ($t_{1/2}$ = 508 ± 24 min), and in homogenates of Caco-2 cells ($t_{1/2}$ = 940 ± 13 min), the rat intestinal mucosa ($t_{1/2}$ = 1286 ± 32 min), and rat liver ($t_{1/2}$ = 840 ± 42 min). Pretreatment of these biological media with paraoxon significantly decreased the degradation rate of the prodrug. When applied to the apical side of Caco-2 cell monolayers, the cyclic prodrug was significantly more stable than the hexapeptide and at least 71-fold more able to permeate (P_{app} = 1.21 ± 0.12 × 10^{-7} cm/s) than was the parent peptide ($P_{app} \leq 0.17 \times 10^{-8}$ cm/s). In the presence of 0.1 mM palmitoyl-DL-carnitine, the transport rate of the cyclic prodrug (P_{app} = 2.19 × 10^{-6} cm/s) was 1250-fold greater than that of the linear hexapeptide.

Conclusions. Preparation of a cyclic peptide using a phenylpropionic acid promoiety reduced the lability of the peptide to peptidase metabolism and substantially increased its permeation through biological membranes. In various biological media the parent peptide was released from the prodrug by an apparent esterase-catalyzed reaction, sensitive to paraoxon inhibition.

KEY WORDS: esterase-sensitive prodrug; chemical and enzymatic stability; peptide delivery; Caco-2 cells; palmitoyl-DL-carnitine; membrane permeability.

INTRODUCTION

The major impediments to the clinical development of orally active peptides are the lack of stability against enzymatic degradation and unfavorable physicochemical properties, which limit their permeation across the intestinal mucosa (1–3). Various prodrug strategies have been employed to transiently alter the physicochemical properties of peptides in order to overcome pharmaceutical and/or pharmacokinetic problems (4,5). The ideal prodrug of a peptide would exhibit enhanced membrane permeation characteristics and increased stability against metabolic degradation. After traversing the membrane barrier, the prodrug should undergo spontaneous or enzyme-mediated transformation to release the peptide, which then can exhibit its pharmacological effect. Cyclic prodrugs prepared with a linker between the N- and C-terminal ends of a peptide are expected to exhibit greater stability against metabolic degradation mediated by exopeptidases (6). In addition, cyclization of a peptide may also restrict the conformational flexibility of the molecule, leading to a more compact structure with altered physicochemical properties (7).

Recently, we have described a promising approach in the delivery of peptides with a cyclic acyloxyalkoxycarbamate prodrug of a model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) having enhanced membrane permeation characteristics and increased metabolic stability (8). In this study, we have evaluated a unique cyclic prodrug of the same model hexapeptide that was prepared using a phenylpropionic acid [3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethyl propionic acid] promoiety. The degradation of this N- to C-terminal linked cyclic prodrug was designed to occur by an esterase-catalyzed reaction (Scheme 1). Upon the unmasking of the hydroxyl group, the intermediate undergoes spontaneous intramolecular cyclization to release the peptide attached to the carboxyl group of the

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ABBREVIATIONS: HBSS, Hanks' balanced salt solution; AP, apical; BL, basolateral.

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promoiety. The fast lactonization reaction is the result of the "trimethyl lock", which has been shown earlier to increase the rate of lactonization on the order of 10^5 to 10^7 (9–11).

The stability of the cyclic phenylpropionic acid prodrug was assessed in various biological media having measurable esterase activities and was compared to the chemical stability of the prodrug in aqueous buffered solution, pH 7.4. Transport and metabolism were determined in the presence and absence of palmitoyl-DL-carnitine using an *in vitro* cell culture model (Caco-2) of the intestinal mucosa.

MATERIALS

The cyclic phenylpropionic acid prodrug was prepared as described elsewhere (12). The linear model hexapeptide, H-Trp-Ala-Gly-Gly-Asp-Ala-OH, was obtained by solid phase synthesis using standard Fmoc-chemistry, purified by preparative HPLC (>98%), and characterized by FAB^+-MS and ^{1}H-NMR (7). Diethyl *p*-nitrophenyl phosphate (=paraoxon, approx. 90%), *p*-nitrophenyl butyrate (~98%), palmitoyl-DL-carnitine chloride, bestatin hydrochloride, captopril, Dulbecco's phosphate buffered saline, and Hanks' balanced salts (modified) were purchased from Sigma Chemical Co. (St. Louis, MO). L-Glutamine 200 mM (100x), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and non-essential amino acids 10 mM (100x) in 0.85% saline were obtained from Gibco BRL, Life Technologies (Grand Island, NY). Dulbecco's modified Eagle medium and trypsin/EDTA solution (0.25% and 0.02%, respectively, in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution) were purchased from JRH Biosciences (Lenexa, KS). Rat tail collagen (type I) was obtained from Collaborative Biomedical Products (Bedford, MA), and fetal bovine serum from Atlanta Biologicals (Norcross, GA). Diprotin A was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). All other chemicals and solvents were of high purity or analytical grade and used as received.

METHODS

Cell Culture

Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Rockville, MD) and cultured as described previously (8,13). Briefly, cells were grown in a controlled atmosphere of 5% CO_2 and 90% relative humidity at 37°C in a culture medium consisting of Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, and 1% L-glutamine. For transport studies, cells were detached from the plastic support when approximately 80% confluent by partial digestion using trypsin/EDTA solution and were plated on collagen-coated polycarbonate membranes (Transwell®, 3 μm pore size, 24.5 mm diameter) at a density of 8.0×10^4 cells/cm^2. Caco-2 cells were fed with culture medium every other day for 7 days and then daily (AP volume 1.5 ml, BL 2.6 ml). Cells were used in this study between passage 50 and 64.

Lipophilicity

The lipophilicities of the cyclic prodrug and the linear hexapeptide were estimated by determining their partition coefficients between 0.02 M phosphate buffer, pH 7.4/acetonitrile (80:20, v/v), and an immobilized artificial membrane (IAM.PC.DD column, 10 cm × 4.6 mm I.D., Regis Technologies, Inc., Morton Grove, IL) as described by Liu and colleagues (14). Aliquots (5 μl) of the peptide solutions (~20 μg/ml, in running buffer) were injected on the column (flow-rate 1.0 ml/min) and solutes were detected with a UV detector (λ = 220 nm) or with a fluorescence detector as described below.

Molecular Size

Diffusion coefficients of the peptides were experimentally measured by NMR spectroscopy using an inverse Z-gradient probe (coil constant determined with water at 25°C = 5.2 G · cm^{-1} · amp^{-1}) interfaced to a PC-driven gradient generator specifically designed for diffusion studies (Digital Specialties, Chapel Hill, NC). During the experiment, the spin echo delay was held constant at 200 ms and the gradient current at 1 amp. The duration of the gradient pulses was sequentially (i.e., 0, 1, 4, 5, 6, 7, and 8 ms) increased as described elsewhere (15,16). All the NMR spectra were processed on an IRIS Indigo using FELIX, a program from Biosym Tech. Inc. (San Diego, CA). By linear regression analysis ($r^2 \geq 0.99$), the diffusion coefficient at 25°C was obtained from the slope in a semilogarithmic plot of the intensity vs. pulse gradient. The molecular radius was then calculated according to the Stokes-Einstein equation using η = 1.0951 cP as the viscosity of D_2O at 25°C.

Structural Analysis

Structural analysis of the cyclic prodrug was performed by circular dichroism (CD) using an AVIV CD-60DS. The data for samples prepared in methanol (final concentration ~0.5 mg/ml) were collected from 280 to 190 nm in steps of 1 nm and smoothed. For secondary structure analysis, ellipticity values were taken every nanometer. Evaluation of the CD data was performed by the convex constraint analysis method (CCA) according to Perczel and co-workers (17). Spectra were appended to the reference data set in the CCA program and deconvoluted assuming 3, 4 and 5 components in order to find the weight of different secondary structural elements of the peptide. The four component deconvolution resulted in the best fit with the experimental CD spectrum, and conformational weights were obtained from this analysis. The component curves were assigned to different secondary structural elements such as α-helix, β-sheet or β-turn by comparison with CD curves in the literature (18).

Chemical Stability

The chemical stability of the cyclic phenylpropionic acid prodrug was determined in Hanks' balanced salt solution (HBSS), pH 7.4. Solutions of the prodrug (~18 μg/ml) were incubated in sealed vials at 37.0 ± 0.5°C in a temperature-controlled shaking water bath (60 rpm). Periodically, 20 μl aliquots were removed and immediately analyzed by HPLC. Rate constants were calculated by linear regression ($r^2 \geq 0.96$) from pseudo first-order plots of prodrug concentration vs. time measured up to 1440 min.

Enzymatic Stability

The stability of the phenylpropionic acid prodrug in 90% human plasma and various tissue homogenates was determined

at 37°C in the presence and absence of paraoxon, a potent esterase inhibitor. The preparation of 90% human plasma and homogenates of Caco-2 cells, rat intestinal mucosa and rat liver, as well as the determination of total esterase activity in these biological media using *p*-nitrophenyl butyrate have been extensively described elsewhere (8). The phenylpropionic acid prodrug was incubated at ~20 μM with the biological matrix, and samples were maintained for 6 hr in a temperature-controlled (37.0 ± 0.5°C) shaking water bath (60 rpm). To test the effect of an esterase inhibitor on the rate of degradation of the cyclic prodrug, the biological medium was preincubated with paraoxon (final concentration 1 mM) for 15 min at 37°C before adding the prodrug. At various time points, aliquots (20 μl) were removed and the esterase activity immediately quenched by adding 150 μl of a freshly prepared 6 N guanidinium hydrochloride solution in acidified HBSS (HBSS containing 0.01% (v/v) phosphoric acid). Aliquots (150 μl) of the acidic mixture (pH ~ 3) were then transferred to an Ultrafree®-MC 5000 NMWL filter unit (Millipore, Bedford, MA) and centrifuged at 7500 rpm (5000 × g) for 60 min (4°C). Aliquots (50 μl) of the filtrates were diluted with mobile phase and injected on the HPLC column. Recoveries for the peptides were ≥96.4%. Apparent half-lives ($t_{1/2}$) for the disappearance of the prodrug were calculated from the rate constants obtained by linear regression ($r^2 \geq 0.96$) from pseudo first-order plots of prodrug concentration vs. time.

Transport Studies

Caco-2 cell monolayers grown on collagen-coated polycarbonate filters (Transwells®) for 21 to 28 days were used for transport experiments. The integrity of each batch of cells was first tested by measuring the flux of [^{14}C] mannitol in representative cell monolayers ($n = 3$). AP-to-BL flux for this paracellular marker never exceeded values of 0.4%/hr ($P_{app} \leq 1.27 \times 10^{-7}$ cm/s). The transport of the peptides across Caco-2 cell monolayers was determined as described earlier (8). Briefly, the peptide solution (90–100 μM in HBSS) was applied to the donor compartment (AP, 1.5 ml or BL, 2.6 ml) of the cell monolayer and HBSS added to the receiver compartment. For transport studies under perturbed conditions, HBSS, pH 7.4, was supplemented with 0.1 mM PC. Samples (120 μl, receiver side; 20 μl donor side) were removed at various times up to 180 min from both sides. The volume removed from the receiver side was always replaced with fresh, prewarmed HBSS. Samples were stabilized with an aliquot of acetonitrile and diluted phosphoric acid (final concentration 10% (v/v) and 0.01% (v/v), respectively) and kept frozen (−80°C) until HPLC analysis.

Transport experiments, from the AP-BL side as well as from the BL-AP side, were performed in triplicate at 37°C in a shaking water bath (60 rpm). Permeation of the linear model hexapeptide through Caco-2 cell monolayers was assessed in the presence and absence of a "cocktail" of three peptidase inhibitors (i.e., 0.29 mM bestatin, 1 mM captopril, and 1 mM diprotin A).

HPLC Analysis

Chromatographic analyses were carried out on a Shimadzu LC-10A gradient system (Shimadzu, Inc., Tokyo, Japan) consisting of LC-10AD pumps, a SCP-6 controller, a SPD-10A UV detector and a RF-535 fluorescence detector connected to LCI-100 integrators (Perkin-Elmer, Norwalk, CT). Samples from a refrigerated sample tray (4°C) were injected by a Perkin-Elmer ISS-100 autoinjector on a Dynamax C_{18} reverse-phase column (5 μm, 300 Å, 25 cm × 4.6 mm I.D., Rainin Instruments, Woburn, MA) equipped with a guard column. The fluorescence of the eluent was monitored at emission λ = 345 nm (excitation λ = 285 nm). Gradient elution of the peptides was performed at a flow-rate of 1 ml/min from 11.6–90.0% (v/v) acetonitrile in water using trifluoroacetic acid (0.1%, v/v) as the ion-pairing agent. Under these conditions, the retention times of the linear model hexapeptide, the cyclic prodrug and the lactone were 6.9 min, 20.2 min and 24.3 min, respectively.

Data Analysis

Permeability coefficients (P_{app}) of the linear model hexapeptide and the cyclic prodrug were calculated according to Eq. 1:

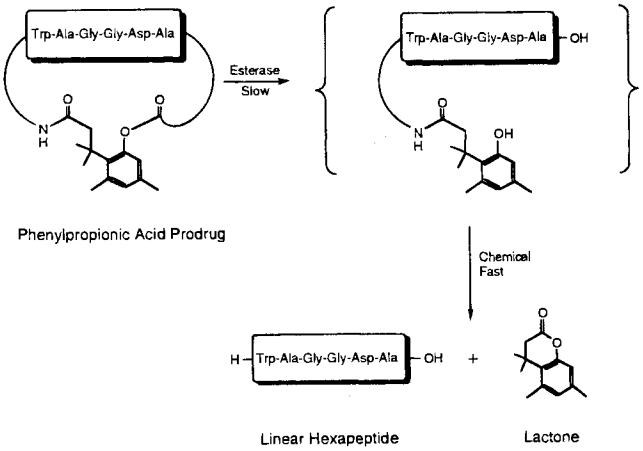
$$P_{app} = \frac{\Delta Q/\Delta t}{A \cdot c(0)} \quad (1)$$

where $\Delta Q/\Delta t$ = linear appearance rate of mass in the receiver solution, A = cross-sectional area (i.e., 4.71 cm^2), and c(0) = initial peptide concentration in the donor compartment at t = 0. The results of experiments performed in triplicate are presented as mean ± SD. Statistical significance was tested by one-way analysis of variance (ANOVA) using Tukey's family error at $p < 0.01$.

RESULTS

Chemical and Enzymatic Stability

The cyclic phenylpropionic acid prodrug was designed to undergo enzyme-mediated hydrolysis of the ester bond followed by a fast chemical step resulting in release of the parent peptide and formation of a lactone (see Scheme 1). The time courses of the disappearance of the cyclic prodrug at 37°C in HBSS, pH 7.4, together with the appearance of the linear hexapeptide



Scheme 1. Proposed mechanism for the release of the linear hexapeptide from the cyclic phenylpropionic acid prodrug.

and the lactone are shown in Fig. 1. After an incubation period of 840 min, mass balance was achieved ≥94.8% for the linear hexapeptide and ≥85.5% for the lactone, respectively. Table I shows that the rates of disappearance of the cyclic prodrug, when incubated in 90% human plasma and in homogenates of Caco-2 cells and rat liver, are significantly faster than the rate observed in HBSS, pH 7.4. Although considerable esterase activity was determined in rat intestinal homogenate, the apparent half-life of the prodrug in this biological matrix was statistically ($p \leq 0.01$) not different from the respective half-life calculated in HBSS, pH 7.4.

Incorporation of paraoxon, a potent esterase inhibitor, in these biological media resulted generally in a substantial decrease in the specific esterase activity. Based on the spectrophotometric assay using *p*-nitrophenyl butyrate as substrate, residual esterase activity after a 15 min preincubation period with paraoxon at 37°C was ≤1.6%, except in 90% human plasma, where the activity was only reduced to 73.7% of the initial activity. Nevertheless, in the presence of paraoxon, the rates of disappearance of the cyclic prodrug were statistically not different from the respective degradation rate obtained for chemical degradation in HBSS, pH 7.4. It should be mentioned that in contrast to the stoichiometric conversion of the cyclic prodrug to the linear hexapeptide in HBSS, 7.4, mass balance in the various biological media was not achieved. The linear hexapeptide never exceeded values of 0.02% of the prodrug equivalent during the entire incubation period of 360 min. This can be explained by the fact that the linear hexapeptide rapidly degrades (e.g., $t_{1/2}$ in 90% human plasma = 3.7 min; data not shown) due to the presence of peptidases in these biological media.

Transport Across Caco-2 Cell Monolayers

Cell permeability characteristics of the cyclic phenylpropionic acid prodrug and the linear hexapeptide were assessed across Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa. When the linear hexapeptide was applied to the AP side of the cell monolayer, no measurable amounts of the peptide were detected in the receiver compartment (BL side of the monolayer) (Fig. 2A). Based on the limits of detection of our analytical method for the linear hexapeptide, a maximum apparent permeability coefficient (P_{app}) of 0.17×10^{-8} cm/s was calculated. Only in the presence of a mixture of three peptidase inhibitors (i.e., bestatin, captopril, diprotin A) could AP-to-BL transport of the linear hexapeptide be observed (Fig. 2A). In contrast, the AP-to-BL transport of the cyclic prodrug was readily detectable (Fig. 2A). The P_{app} value for the cyclic prodrug in the BL-to-AP direction ($12.63 \pm 1.49 \times 10^{-8}$ cm/s) was statistically not different from the P_{app} value ($12.09 \pm 1.24 \times 10^{-8}$ cm/s) observed for the flux of this prodrug in the AP-to-BL direction. Based on the estimated P_{app} value for the linear hexapeptide (in the absence of the peptidase inhibitors) and the determined P_{app} value for the cyclic prodrug (Table II), the cyclic prodrug is at least 71-fold more able to permeate the cell monolayer. Even when the linear hexapeptide is stabilized to metabolism by inclusion of peptidase inhibitors, the cyclic prodrug is still 3 times more able to permeate the cell monolayer.

To determine the transport characteristics of the cyclic prodrug and the linear hexapeptide under conditions where the cell monolayers exhibit larger intercellular pore radii and, therefore, represent more accurately the physiological situation in the small intestine, transport experiments across Caco-2 cell monolayers were performed in the presence of 0.1 mM palmitoyl-DL-carnitine (PC), a tight junction modulator. Fig. 2B shows the time courses for the appearance of the peptides in

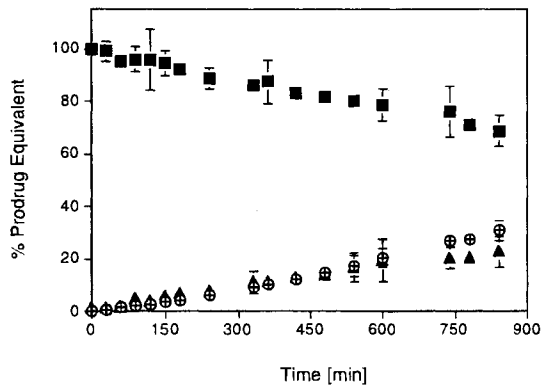


Fig. 1. Stability of the cyclic phenylpropionic acid prodrug at 37°C in HBSS, pH 7.4. Time courses are shown for the disappearance of the cyclic prodrug (■) and the appearance of the linear hexapeptide (⊕) as well as the lactone (▲). Experiments were performed in triplicate (average ± SD).

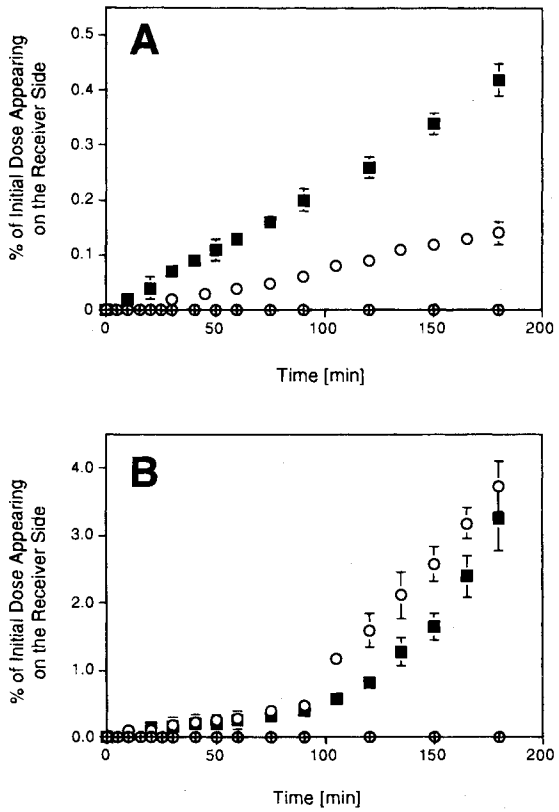


Fig. 2. Transport of the cyclic phenylpropionic acid prodrug (■) and the linear hexapeptide (in the presence (○) and absence (⊕) of peptidase inhibitors) across Caco-2 cell monolayers. Panel A shows the appearance of the cyclic prodrug and the linear hexapeptide (data taken from (8)) in the receiver compartment in the absence of palmitoyl-DL-carnitine. In Panel B the time profiles of the peptides under perturbed conditions (i.e., in the presence of 0.1 mM palmitoyl-DL-carnitine) are presented. Experiments were performed in triplicate (average ± SD).

Table I. Apparent Half-Lives of the Cyclic Prodrug in HBSS, pH 7.4, Rat Intestinal Homogenate, Rat Liver Homogenate, 90% Human Plasma and Caco-2 Cell Homogenate in the Presence and Absence of Paraoxon (1 mM) at 37°C

Incubation Mixture	Specific Activity[a] [U/mg protein]	Enzyme Concentration [U/ml]		Apparent Half-Life[b] [min]	
		−paraoxon	+paraoxon	−paraoxon	+paraoxon
HBSS, pH 7.4	0	0	0	1795 ± 289	1870 ± 149
Caco-2 cell homogenate	0.29	0.48	0	940 ± 13*	1820 ± 194
90% human plasma[c]	0.004	0.19	0.14	508 ± 24*	1729 ± 245
rat intestinal homogenate	7.19	16.54	0	1286 ± 32	1788 ± 442
rat liver homogenate	1.75	20.59	0.33	810 ± 42*	2089 ± 107

[a] Determined at 25°C in HBSS, pH 7.4 using *p*-nitrophenyl butyrate as substrate.
[b] Calculated from apparent first-order rate constants (see Materials and Methods).
[c] Human plasma diluted to 90% (v/v) with HBSS, pH 7.4.
* Significantly different from the control in HBSS, pH 7.4 ($p < 0.01$).

the receiver compartment when the monolayer was perturbed by PC. In the absence of peptidase inhibitors, transport of the linear hexapeptide was still very slow and below the detection limits of our analytical system. However, in the presence of peptidase inhibitors, the linear hexapeptide showed a steep linear increase in its flux to the receiver compartment after a lag-phase of about 90 min (Fig. 2B). A similar time profile was observed for the cyclic prodrug in the absence of peptidase inhibitors (lag-phase ≤ 105 min). When compared to the unperturbed monolayer (i.e., in the absence of PC), the intrinsic permeability for the cyclic prodrug increased by 18-fold and for the linear hexapeptide by 46-fold, due to the controlled opening of the tight junctions by PC (Table II).

Physicochemical Properties and Structural Characteristics

The lipophilicity of the peptides was determined by IAM chromatography, and the results are shown in Table II. The calculated log k'_{IAM} values suggest that the cyclic prodrug is significantly more lipophilic than the linear hexapeptide. The molecular size, however, appears to be approximately the same for the two peptides as revealed by NMR analysis (Table II). The experimental circular dichroism spectrum of the cyclic prodrug is shown in Fig. 3. The CD spectrum was processed and deconvoluted to give four components, and conformational weights were obtained for each of the components. The component curves were assigned to different secondary structural elements such as α-helix, β-sheet or β-turn by comparison with curves in the literature as described in Materials and Methods. The conformational weights for the unordered and ordered structures were approximately 65% and 30%, respectively. The CD spectrum of the cyclic acyloxyalkoxycarbamate prodrug, in contrast, shows a characteristic minimum at λ = ~220 nm, indicating a significant contribution of a β-turn structure (~60% (19)).

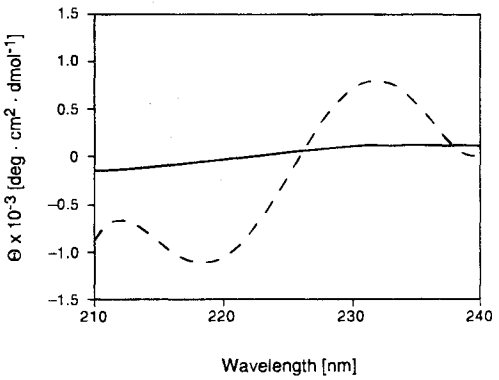


Fig. 3. Structural analysis of the cyclic phenylpropionic acid prodrug performed by circular dichroism (CD). Solid line indicates the CD spectrum of the cyclic phenylpropionic acid prodrug in methanol, dotted line represents the CD spectrum obtained for the cyclic acyloxyalkoxycarbamate prodrug in the same solvent (with permission (19)).

DISCUSSION

A fundamental requirement for the prodrug approach to be successful is reliable conversion to the parent drug by either enzyme-catalyzed or non-enzymatic reactions (5). The cyclic phenylpropionic acid prodrug described in this study was designed to release the linear hexapeptide by enzyme-mediated hydrolysis of the ester bond (see Scheme 1). The chemical stability assessed in HBSS, pH 7.4, revealed stoichiometric conversion of the cyclic prodrug to the linear hexapeptide. Apparent rate constants calculated for the disappearance of the prodrug ($k_{obs} = 3.86 \pm 0.54 \times 10^{-4}$ min^{-1}) and the appearance of the parent peptide ($k_{obs} = 3.76 \pm 0.52 \times 10^{-4}$ min^{-1}) were statistically not different ($p < 0.01$). Since the hydroxy intermediate (Scheme 1) was not detected by HPLC during the entire stability study, and the formation of the lactone was kinetically equivalent to the disappearance of the cyclic prodrug ($k_{obs} = 3.18 \pm 0.65 \times 10^{-4}$ min^{-1}), we conclude that the rate-limiting step in the cascade of reactions leading to the release of the linear hexapeptide is indeed the hydrolysis of the ester moiety. This conclusion is supported by earlier kinetic analyses of the spontaneous intramolecular cyclization of phenylpropionic acid derivatives containing the "trimethyl lock" (9–11). Amsberry and Borchardt (20) have reported an apparent half-life of 65 s for the lactonization of 4-methoxyaniline 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid amide.

In a biological milieu, the cyclic prodrug would be expected to degrade faster to the linear hexapeptide due to the esterase-catalyzed hydrolysis of the ester bond. Based on the spectrophotometric assay using *p*-nitrophenyl butyrate as a substrate, all biological media that were used in this study exhibited measurable levels of esterase activity (Table I). As observed

earlier (8), the specific esterase activity was remarkably species-dependent (rat >> human) and, within the same species, was found to differ significantly between various tissues (e.g., rat intestinal mucosa > rat liver). Preincubation with paraoxon reduced the esterase activity in all biological media by a least 98%, except in the diluted human plasma. Here, more than 70% residual esterase activity was found after pretreatment with paraoxon, suggesting the presence of a considerable fraction of A- and/or C-esterases (21). In the absence of paraoxon, the rates of disappearance of the cyclic prodrug in these biological media were substantially faster than expected from the chemical stability of the prodrug. Inclusion of the esterase inhibitor, however, resulted in significantly greater half-lives, similar to the half-life of the prodrug observed in HBSS, pH 7.4 (Table I). This implies that in biological media the cyclic prodrug degrades faster due to an esterase-catalyzed reaction. Based on the sensitivity of this reaction to paraoxon inhibition, it seems that predominantly B-esterases are involved in this enzyme-mediated hydrolysis (21). Nevertheless, differences in substrate specificity and the presence of various isozymes in these biological media might be responsible for faster disappearance of the cyclic prodrug in human plasma than in rat intestinal homogenate although total esterase activity in the latter medium was significantly greater based on the *p*-nitrophenyl butyrate assay (Table I). Similar results were observed earlier with another cyclic prodrug of the same model hexapeptide (8).

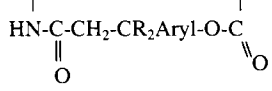
The phenylpropionic acid promoiety was used earlier in our laboratory to prepare linear, esterase-sensitive amide prodrugs (20,22). The hydrolysis of linear amide prodrugs in human plasma was significantly faster than that observed for the cyclic prodrug in this study. Furthermore, the amide prodrug described by Amsberry and co-workers was sensitive to hydrolysis mediated by porcine liver esterase, EC 3.1.1.1 ($t_{1/2}$ = 12 min; enzyme concentration = 0.1 U/ml). The cyclic prodrug prepared with the linear hexapeptide, in contrast, was stable when incubated with porcine liver esterase up to 56 U/ml (data not shown). These differences imply that the cyclic structure has a significant impact on the rate of the enzyme-catalyzed hydrolysis of the ester moiety. It seems that the larger cyclic phenylpropionic acid prodrug does not fit as easily into the active side of the enzyme and, consequently, the hydrolysis is limited to a smaller family of isozymes.

The major barrier to oral delivery of peptides into the systemic circulation is the intestinal mucosa (1,23). Tight intercellular junctions limit paracellular flux of a peptide (physical barrier), and peptidases associated with the brush-border membrane and the cytoplasm (metabolic barrier) rapidly metabolize peptides to their constituent amino acids (1,2,24). Therefore, it was of interest to investigate the transport and metabolism of the cyclic phenylpropionic acid prodrug in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa that has been shown to exhibit physical (25,26) and metabolic (27) barrier properties similar to the *in vivo* situation.

When applied to the AP side of Caco-2 cell monolayers, the linear hexapeptide degrades rapidly with a $t_{1/2}$ = 14 min (8). The phenylpropionic acid prodrug, in contrast, is significantly more stable than the linear hexapeptide. After a 3-hr incubation period, 103.4 ± 6.3% of the prodrug initially applied to the AP compartment was still present (data not shown). This indicates that the cyclic prodrug approach using the phenylpropionic acid promoiety can markedly reduce the lability of the peptide toward metabolic degradation mediated by intestinal peptidases. In the absence of peptidase inhibitors, the flux of the cyclic prodrug was at least 71-fold greater than the flux estimated for the linear hexapeptide (Table II). When compared to the transport of the linear hexapeptide in the presence of a "cocktail" of potent peptidase inhibitors, the prodrug was still approximately 3 times more able to permeate Caco-2 cell monolayers than was the parent hexapeptide (Table II). The flux of the cyclic prodrug was also ~3 times greater than determined for the metabolically stable, N-terminus acetylated and C-terminus amidated analog of the linear hexapeptide (7). Since the linear hexapeptide and the cyclic prodrug are approximately of the same molecular size (Table II) and the cyclic prodrug is more lipophilic, the greater flux of the prodrug implies different pathways of permeation for the two peptides.

Recently, Adson and colleagues (28) proposed a biophysical model for the delineation of the paracellular and transcellular contributions to the flux of a solute across a cell monolayer. Following their protocols, we first characterized the average

Table II. Physicochemical Properties and AP-to-BL Transport Characteristics Determined for the Cyclic Prodrug and the Linear Hexapeptide in the Presence and Absence of 0.1 mM Palmitoyl-DL-carnitine (PC) across Caco-2 Cell Monolayers

Compound	MW	Size[a] [Å]	Lipophilicity[b] [log k'_{IAM}]	Permeability Coefficient, $P_{app} \times 10^8$ [cm/s]	
				−0.1 mM PC	+0.1 mM PC
H_2N-Trp-Ala-Gly-Gly-Asp-Ala-OH	574	3.6	−1.10	<0.17[d]	<0.17
H_2N-Trp-Ala-Gly-Gly-Asp-Ala-OH + peptidase inhibitor "cocktail"[c]				4.05 ± 0.24[d]	186.1 ± 17.9
Trp-Ala-Gly-Gly-Asp-Ala 	761	3.6	−0.32	12.09 ± 1.24	212.9 ± 30.7

[a] Stokes-Einstein radius calculated from the diffusion coefficient in D_2O (see Materials and Methods).
[b] Capacity factor determined from the partitioning of the solute between 0.02 M phosphate buffer, pH 7.4/acetonitrile (80:20, v/v) and an immobilized artificial membrane of phosphatidylcholine analogs (IAM.PC.DD).
[c] Bestatin 0.29 mM, captopril 1 mM, diprotin A 1 mM.
[d] From Pauletti et al., 1996 (8).

pore radius of the junctional complex in our Caco-2 cell monolayers (i.e., 4.4 ± 1.1 Å; data not shown). The maximal paracellular contribution to the flux of the peptides was then estimated using the Renkin molecular sieving function for cylindrical pores (28). Within the limitations of this model, the linear hexapeptide was found to traverse the cell monolayer mainly via the paracellular (>50%) pathway, whereas the cyclic peptide appears to permeate mainly via the transcellular route (≥82%). This result is consistent with the increased lipophilicity determined for the cyclic prodrug by IAM chromatography.

The increased lipophilicity of a cyclic acyloxyalkoxycarbamate prodrug prepared using the same model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) was attributed to intramolecular hydrogen bonding, which was observed in the cyclic prodrug but not in the linear hexapeptide (19). In contrast, the cyclic phenylpropionic acid prodrug was found to exist predominantly in a unordered structure as revealed by circular dichroism (Fig. 3) and NMR spectroscopy (data not shown). Therefore, we conclude that the phenylpropionic acid promoiety per se, when attached to this linear hexapeptide, is responsible for the change in lipophilicity.

Under normal physiological conditions, the pore radius of the aqueous pathway for passive diffusion in the human jejunum varies between 8 and 13 Å (29). The average pore radius of the Caco-2 cell monolayers used in this study was found to be significantly smaller (i.e., 4.4 ± 1.1 Å). As a consequence, the flux of solutes traversing this cell monolayer predominantly via the paracellular route is expected to be substantially lower than the flux across the human jejunum. Recently, it has been demonstrated by our laboratory (30) that palmitoyl-DL-carnitine (PC) is a useful tight junction modulator that allows one to control the paracellular pore radius in Caco-2 cell monolayers without completely destroying the physical barrier properties of the cell monolayer. Therefore, we performed transport experiments in the presence of 0.1 mM PC, which increased the average pore radius of our Caco-2 cell monolayers to 12.0 ± 3.1 Å (data not shown). Under these perturbed conditions, the cyclic prodrug and the linear hexapeptide, which was metabolically stabilized by the presence of peptidase inhibitors, permeate the cell monolayer approximately to the same extent via the paracellular route (62% for the cyclic prodrug and 71% for the linear hexapeptide, respectively). In the absence of peptidase inhibitors, the linear hexapeptide was not detected in measurable amounts in the receiver compartment. Since the apparent half-life of the linear hexapeptide in the donor compartment under these conditions ($t_{1/2}$ = 16 min) was not substantially different from the respective half-life determined under unperturbed conditions ($t_{1/2}$ = 14 min, (8)), we conclude that PC affects the physical but not the metabolic barrier of Caco-2 cells. It is important to note that in the presence of 0.1 mM PC, which seems to reflect more closely the physiological situation in the human jejunum with respect to the aqueous pore radius of the intestinal mucosa, the cyclic prodrug is approximately 1250-fold more able to permeate the cellular barrier than is the linear hexapeptide.

In conclusion, the phenylpropionic acid promoiety used to prepare esterase-sensitive cyclic prodrugs appears to be a promising approach in the delivery of peptide drugs to enhance their membrane permeation and, simultaneously, increase their metabolic stability.

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