Pharmaceutical Research, Vol. 13, No. 11, 1996

Research Article

Esterase-Sensitive Cyclic Prodrugs of Peptides: Evaluation of an Acyloxyalkoxy Promoiety in a Model Hexapeptide

Giovanni M. Pauletti,[1] Sanjeev Gangwar,[1] Franklin W. Okumu,[1] Teruna J. Siahaan,[1] Valentino J. Stella,[1] and Ronald T. Borchardt[1,2]

Received June 14, 1996; accepted August 23, 1996

Purpose. To evaluate a cyclic acyloxyalkoxycarbamate prodrug of a model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) as a novel approach to enhance the membrane permeation of the peptide and stabilize it to metabolism. ***Methods.*** Conversion to the linear hexapeptide was studied at 37°C in aqueous buffered solutions and in various biological milieus having measurable esterase activities. Transport and metabolism characteristics were assessed using the Caco-2 cell culture model. ***Results.*** In buffered solutions the cyclic prodrug degraded chemically to the linear hexapeptide in stoichiometric amounts. Maximum stability was observed between pH 3–4. In 90% human plasma ($t_{1/2}$ = 100 ± 4 min) and in homogenates of the rat intestinal mucosa ($t_{1/2}$ = 136 ± 4 min) and rat liver ($t_{1/2}$ = 65 ± 3 min), the cyclic prodrug disappeared faster than in buffered solution, pH 7.4 ($t_{1/2}$ = 206 ± 11 min). Pretreatment of these 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 ($t_{1/2}$ = 282 ± 25 min) was significantly more stable than the hexapeptide ($t_{1/2}$ = 14 min) and at least 76-fold more able to permeate (P_{app} = 1.30 ± 0.15 × 10^{-7} cm/s) than the parent peptide ($P_{app} \leq 0.17 \times 10^{-8}$ cm/s). ***Conclusions.*** Preparation of a cyclic peptide using an acyloxyalkoxy 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; peptide delivery; Caco-2 cells; membrane permeability; enzymatic stability; chemical stability.

INTRODUCTION

Clinical development of orally active peptide drugs has been restricted by their unfavorable physicochemical properties, which limit their intestinal mucosal permeation, and their lack of stability against enzymatic degradation (1–3). Successful oral delivery of peptides will depend, therefore, on strategies designed to alter the physicochemical characteristics of these potential drugs, without changing their biological activity, in order to circumvent the intestinal epithelial cells. In general, it is accepted that size, charge, and lipophilicity are crucial physicochemical properties in determining the ability of a peptide to permeate the intestinal mucosa (4). Solution conformation might be another important parameter affecting the transfer of peptide drugs across biological membranes (5, 6).

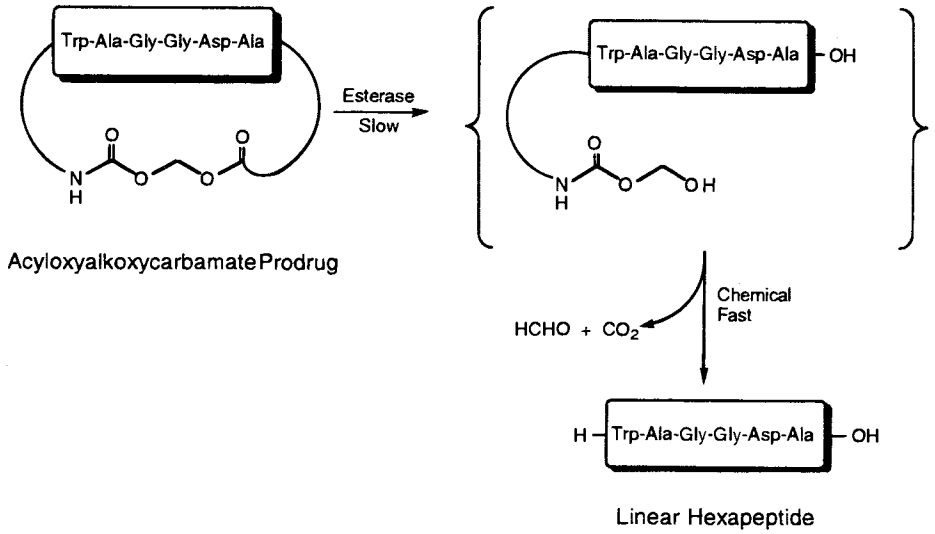
One possible approach to altering the physicochemical properties of a peptide is to employ prodrug strategies. Prodrugs are pharmacologically inactive chemical derivatives of drugs designed to overcome pharmaceutical and/or pharmacokinetic problems (for reviews see (7) and (8)). The ideal prodrug of a peptide would exhibit enhanced membrane permeation characteristics and increased stability against metabolic degradation. After crossing the membrane barrier, the prodrug should undergo spontaneous or enzymatic transformation to release the peptide, which then can exhibit its pharmacological effect. By preparing cyclic prodrugs using the functional groups of the N- and C-terminal ends of a peptide, metabolic degradation mediated by exopeptidases should be minimized (9). 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 (10).

Recently, our laboratory reported the synthesis of a cyclic prodrug of a model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) using an acyloxyalkoxy promoiety (11). The degradation of this N-terminal to C-terminal linked cyclic prodrug was designed to occur by an esterase-catalyzed reaction (Scheme 1). Esterases represent a large family of isozymes with broad substrate specificity found in a variety of tissues (e.g., blood, liver, intestinal mucosa) from different species (12, 13). In this study, we have determined the stability of the cyclic acyloxyalkoxycarbamate prodrug in buffer solutions and in various biological milieus having measurable esterase activities. Transport and metabolism of this cyclic prodrug were assessed using a cell culture model (Caco-2) of the intestinal mucosa. Caco-2

[1] Department of Pharmaceutical Chemistry, 2095 Constant Ave., The University of Kansas, Lawrence, Kansas 66047.
[2] To whom correspondence should be addressed.

ABBREVIATIONS: HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle medium; AP, apical; BL, basolateral.

0724-8741/96/1100-1615$09.50/0



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

cells, which spontaneously undergo differentiation into confluent monolayers (14), have been shown to exhibit both the physical and metabolic barrier properties of intestinal mucosal cells to peptides (15–17). Our results are discussed in light of the physicochemical properties and the conformational structure of the cyclic prodrug.

MATERIALS

The cyclic acyloxyalkoxycarbamate prodrug was prepared as described elsewhere (11). 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 ^1H-NMR (10). Diethyl *p*-nitrophenyl phosphate (=paraoxon, approx. 90%), *p*-nitrophenyl butyrate (~98%), dimethyl sulfoxide (>99.5%), 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 (100×), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and non-essential amino acid 10 mM (100×) 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 1) was obtained from Collaborative Biomedical Products (Bedford, MA), and fetal bovine serum from Atlanta Biologicals (Norcross, GA). D-1[^{14}C]Mannitol (spec. act. = 2.07 GBq/mmol) was purchased from Moravek Biochemicals (Brea, CA), and 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 were obtained from American Type Culture Collection (Rockville, MD) at passage 18. As described previously (14), cells were grown in a controlled atmosphere of 5% CO_2 and 90% relative humidity at 37°C in 150 cm^2 culture flasks using a culture medium consisting of Dulbecco's modified Eagle medium (DMEM) 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. When approximately 80% confluent (i.e., 3–5 days), cells were detached from the plastic support by partial digestion using trypsin/EDTA solution and either subcultured in new flasks or 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 until transport experiments were performed (apical volume 1.5 ml, basolateral 2.6 ml). Cells were used in this study between passages 40 and 43.

Tissue Homogenate and Human Plasma

Rat Intestinal Homogenate

Male Sprague-Dawley rats (Animal Care Unit, The University of Kansas, Lawrence, KS) weighing 250–350 g were anesthetized with Metofane® and an abdominal incision was performed. The small intestine, from ~5 cm distal to the pylorus to ~5 cm proximal to the caecum, was immediately removed and rinsed with ice-cold oxygenated (95% O_2 and 5% CO_2) Hanks' balanced salt solution (HBSS), pH 7.4. After flushing out the intestinal contents with ice-cold oxygenated HBSS, segments of approximately 3 cm were isolated and cut open. The intestinal mucosa was scraped off with a glass slide by placing the everted intestine on a glass plate that was kept on a layer of ice. The mucosa was homogenized on ice with 10 ml HBSS using a 15 ml Wheaton glass homogenizer (15 strokes, pestle/wall clearance 0.25–0.76 mm). Aliquots of 1.5 ml were frozen and kept at −80°C until used. Before an experiment, the homogenate was quickly thawed and rehomogenized on ice with an equal volume of HBSS (10 strokes, pestle/wall clearance 0.64–0.75 mm). Cell debris and nuclei were removed at 4°C

by centrifugation for 10 min at 10,000 rpm (9000 × g) using a Marathon 21K/BR centrifuge (Hermle AG, Gosheim, FRG).

Rat Liver Homogenate

Rat livers were obtained from the same animals used to prepare the intestinal homogenate. The tissue was blotted to dryness and after weighing was sliced in small pieces with a scalpel. Homogenates were prepared in HBSS (1 ml per g tissue) as described for the preparation of rat intestinal homogenate.

Caco-2 Cell Homogenate

Confluent Caco-2 cell monolayers (21–28 days) were washed 3× with ice-cold HBSS and carefully scraped from the filter support with a rubber spatula. Cells of 6 monolayers were collected in 2 ml of ice-cold HBSS and homogenized on ice using a 15 ml Wheaton glass homogenizer (15 strokes, pestle/wall clearance 0.64–0.76 mm). Cell debris and nuclei were removed as described above.

Human Plasma

Human blood, stabilized with CPDA-1 solution USP, was obtained from the Topeka Blood Bank (Topeka, KS). Plasma was separated from the erythrocytes at 4°C by centrifugation for 10 min at 4700 rpm (2000 × g). For stability studies, human plasma was diluted to 90% (v/v) with HBSS, pH 7.4, in order to maintain the pH of the solution during the experiment.

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, and an immobilized artificial membrane (IAM.PC.DD column, 10 cm × 4.6 mm I.D., Regis Technologies, Inc., Morton Grove, IL) according to Liu *et al.* (18). Aliquots (20 μl) of the peptide solutions (~15 μg/ml, in running buffer) were injected on the column (flow rate 1.0 ml/min) and solutes detected with a UV detector (λ = 220 nm) or with a fluorescence detector as described below.

Molecular Size

The average hydrodynamic volumes of the peptides were estimated by high-resolution size exclusion chromatography performed in 0.02 M phosphate buffer, pH 7.4, with 0.25 M NaCl using a Superdex Peptide 10/30 HR column (10 × 300 mm, Pharmacia Biotech, Uppsala, Sweden). The peptide solutions (50 μl, ~10 μg/ml, in running buffer) were injected on the column (flow rate 1.0 ml/min), and solutes were detected with a UV detector at λ = 220 nm as described below.

Chemical Stability

The chemical stability of the cyclic acyloxyalkoxycarbamate prodrug was assessed at 37°C over the pH range 3.0–9.6. Solutions of the prodrug (~15 μg/ml) were prepared in buffers at an ionic strength of 0.5 M adjusted with NaCl. The buffers (0.1 M) used for each pH were as follows: potassium bitartrate at pH 3.0; sodium acetate at pH 4.0; sodium phosphate at pH 5.0–8.0; and sodium borate at pH 9.0 and 9.6. Samples were maintained at 37.0 ± 0.5°C in a temperature-controlled shaking water bath (60 rpm) in sealed vials. Periodically, 20 μl aliquots were removed and immediately analyzed by HPLC. Rate constants were calculated by linear regression from pseudo first-order plots of prodrug concentration vs. time measured for at least two half-lives.

Enzymatic Stability

The stability of the acyloxyalkoxycarbamate prodrug in various tissue homogenates and 90% human plasma was determined at 37°C in the presence and absence of paraoxon, a potent esterase inhibitor. Total esterase activity in the biological media was assessed using *p*-nitrophenyl butyrate (PNPB) as substrate. *p*-Nitrophenol, the final product of this enzymatic reaction, was quantitated spectrophotometrically at λ = 420 nm using a HP 8452 diode array spectrophotometer equipped with a temperature-controlled cuvet holder (25°C). Initial velocities were calculated by linear regression ($r^2 \geq 0.99$) and corrected for non-enzymatic hydrolysis. Esterase activities were expressed as units per milligram protein (U/mg protein). One unit represents the amount of enzyme that catalyzes the formation of 1 μmol *p*-nitrophenol per minute in HBSS, pH 7.4 (25°C). Conditions of linearity for the enzymatic hydrolysis of PNPB in HBSS, pH 7.4, (25°C) were maintained for 600 s between 0.02–2 U/ml. Total protein concentration in the biological media was determined using the Bio-Rad® Protein Assay (Bio-Rad Laboratories; Richmond, CA) with bovine serum albumin as standard.

The acyloxyalkoxycarbamate prodrug (final concentration ~24 μM) was incubated for 6 hours with 1 ml of homogenates of rat intestinal mucosa, rat liver, Caco-2 cells or 90% human plasma in a temperature-controlled (37.0 ± 0.5°C) shaking water bath (60 rpm). 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 that 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 ≥97%.

The effect of an esterase inhibitor on the rate of degradation of the cyclic prodrug in the various biological media was determined using paraoxon. Paraoxon (final concentration 1 mM) was first preincubated with the respective biological matrix for 15 min at 37°C before adding the prodrug or PNPB. Apparent half-lives ($t_{1/2}$) for the disappearance of the prodrug were calculated from the rate constants obtained by linear regression ($r^2 \geq 0.97$) from pseudo first-order plots of prodrug concentration vs. time.

Transport Studies

Caco-2 cell monolayers grown on collagen-coated polycarbonate filters (Transwells®) were used for transport experiments between days 21 and 28. The integrity of each batch of cells was first tested by measuring the leakage of [^{14}C]-mannitol in representative cell monolayers (n = 3). Apical (AP)-to-basolateral (BL) flux for this paracellular marker never

exceeded values of 0.4%/hour. Routinely, cell monolayers were washed 3× with prewarmed HBSS, pH 7.4, and the peptide solution (~100 μM in HBSS) was applied to the donor compartment (AP, 1.5 ml or BL, 2.6 ml). HBSS was added to the receiver compartment. 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. To stabilize the samples, an aliquot of acetonitrile and diluted phosphoric acid (final concentrations 10% (v/v) and 0.01% (v/v), respectively) was added. This acidic mixture (pH ~3) was immediately frozen in a dry-ice/acetone bath and kept at −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 10.8–74.0% (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 and the cyclic prodrug were 7.7 and 11.8 min, respectively.

Data Analysis

Permeability coefficients (P_{app}) of the linear model hexapeptide were calculated according to equation 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 $t = 0$.

Data analysis for the acyloxyalkoxycarbamate prodrug was based on the assumption that the change in the amount of the prodrug in the donor compartment is due only to passive diffusion from the donor to the receiver compartment and chemical hydrolysis in the donor compartment. Similarly in the receiver compartment, the change in mass of the prodrug is assumed to be related only to the passive diffusion of the prodrug from the donor to the receiver compartment and chemical hydrolysis in the receiver compartment. Under these conditions, the following equations were obtained using Laplace transformation:

$$\frac{M_D}{M_D(0)} = e^{-(A \cdot P_{app}/V_D + k)t} \quad (2)$$

$$\frac{M_R}{M_D(0)} = e^{-kt} - e^{-(A \cdot P_{app}/V_D + k)t} \quad (3)$$

V_D = volume of the donor compartment and k = rate constant characterizing the chemical hydrolysis of the cyclic prodrug during transport experiments. M_D and M_R represent the amount of prodrug in the donor and receiver compartments, respectively, and $M_D(0)$ = the initial amount of prodrug applied to the donor compartment at $t = 0$. P_{app} for the acyloxyalkoxycarbamate prodrug was obtained by a simultaneous curve-fitting procedure using equation 2 for the receiver and equation 3 for the donor compartment.

Statistical Analysis

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 $p < 0.05$.

RESULTS

Chemical Stability

The degradation kinetics of the cyclic prodrug at 37°C was followed in aqueous buffered solutions, pH 3.0–9.6, for at least two half-lives. The only degradation product that appeared in the reaction mixture was the linear hexapeptide, which was stable at all pH values studied. Mass balance (≥97.2%) was achieved in all experiments. A representative time course of the disappearance of the cyclic prodrug and the appearance of the linear hexapeptide is presented in Fig. 2A. From the disappearance of the cyclic prodrug, degradation rate constants, k_{obs}, were calculated by linear regression of pseudo first-order plots ($r^2 \geq 0.98$). Fig. 1 shows a plot of the log k_{obs} vs. pH, which appears to consist of two distinct portions. Maximum stability of the cyclic prodrug was found at pH values ≤4

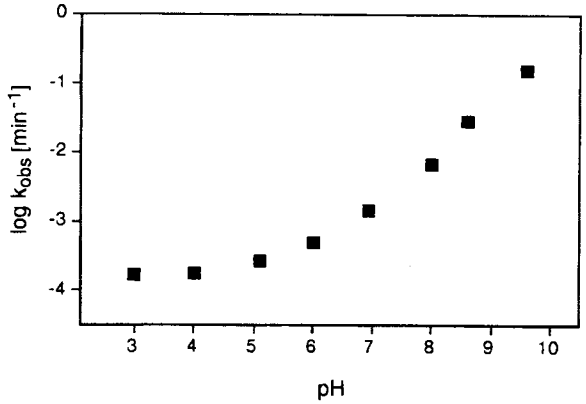


Fig. 1. The pH/rate profile for the chemical degradation of the cyclic prodrug determined in 0.1 M buffer solutions pH 3.0–9.6 (μ = 0.5 M) at 37°C. Apparent first-order rate constants, k_{obs}, were calculated from the disappearance of the prodrug as described in Materials and Methods.

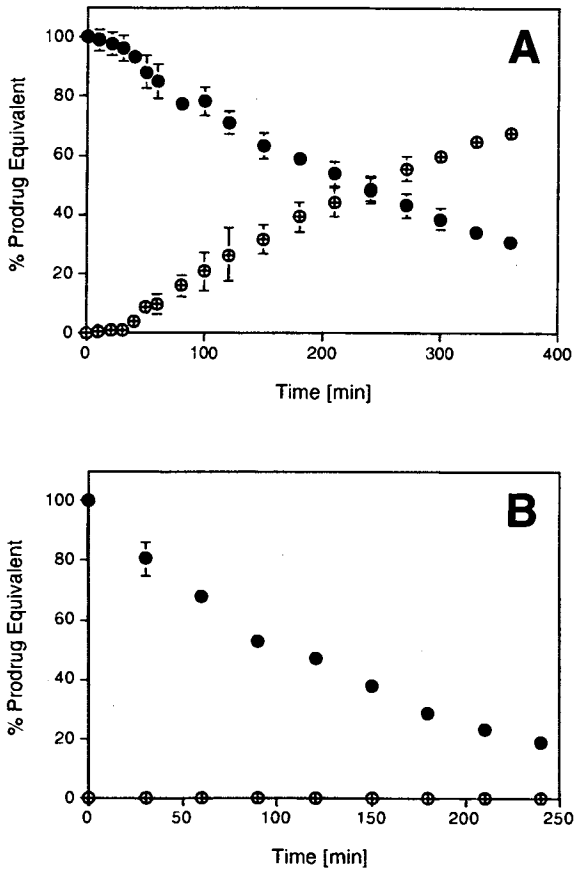


Fig. 2. Stability of the cyclic prodrug at 37°C in HBSS, pH 7.4, and 90% human plasma. Panel A shows the time course of the disappearance of the cyclic prodrug (●) and the appearance of the linear hexapeptide (⊕) in HBSS, pH 7.4; Panel B shows the result of a similar experiment performed in 90% human plasma. Experiments were performed in triplicate (average ± SD).

(k_{obs} = 1.61 × 10^{-4} min^{-1}). With increasing pH, the cyclic prodrug degrades progressively faster to the linear model hexapeptide. At pH values ≥8, the slope in the pH/rate-profile is approximately 1.

Enzymatic Stability

The acyloxyalkoxycarbamate prodrug was designed to undergo enzyme-catalyzed hydrolysis of the ester bond followed by two fast chemical steps to release the parent peptide (11) (see Scheme 1). Therefore, the stability of the cyclic prodrug was determined in various biological media (i.e., rat intestinal homogenate, rat liver homogenate, 90% human plasma and Caco-2 cell homogenate) having measurable esterase activities, and these rates were compared to the chemical degradation in HBSS, pH 7.4.

Comparison of the relative esterase activities present in these biological media as determined by PNPB hydrolysis reveals that rat intestinal homogenate contains the highest esterase activity per mg protein, followed by rat liver homogenate, Caco-2 cell homogenate and 90% human plasma. After a 15 min preincubation at 37°C with the esterase inhibitor paraoxon (1 mM), enzyme activities in all matrices were significantly reduced (Table I). However, the extent of inhibition was not related to the total enzyme activity measured in the absence of paraoxon, but seems to be species-dependent (rat >> human) and, within the same species (i.e., rat), tissue-specific (intestine > liver). In 90% human plasma, residual esterase activity after incubation with paraoxon was still 30% of the initial activity, whereas in rat liver homogenate only a minimum residual activity of 1.3% could be determined. In rat intestinal homogenate, no esterase activity at all was detectable after pretreatment with paraoxon.

Apparent half-lives for the disappearance of the cyclic prodrug in the various biological media are presented in Table I. With the exception of Caco-2 cell homogenate, the rate of disappearance of the cyclic prodrug in these biological media was substantially faster than in HBSS, pH 7.4. In Caco-2 cell homogenate, the cyclic prodrug disappeared at approximately the same rate as that observed in HBSS, although esterase activity was measured in this biological matrix.

In the presence of paraoxon, the disappearance of the cyclic prodrug in all biological media, except for Caco-2 cell homogenate, was significantly slower than in the absence of the esterase inhibitor. This indicates that the cyclic prodrug most likely degrades by hydrolysis of the ester bond mediated by esterases. However, it should be mentioned that in all biological media, mass balance for the conversion of the cyclic prodrug to the linear hexapeptide was not achieved. The amount of the linear hexapeptide never exceeded values of 0.05% of the prodrug equivalent as shown in Fig. 2B for the degradation of the cyclic prodrug in 90% human plasma. This can be explained by the fact that, in contrast to the experiments performed in aqueous buffered solutions, 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 in Caco-2 Cell Monolayers

To determine the cell permeability characteristics of the linear hexapeptide and the cyclic prodrug, permeation of these peptides was assessed in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa. Fig. 3A shows that the linear hexapeptide rapidly disappears from the AP side of Caco-2 cell monolayers ($t_{1/2}$ = 14 min), suggesting high susceptibility to enzymatic degradation by peptidases. To test this hypothesis, transport experiments were conducted in the presence of a mixture of three potent peptidase inhibitors (i.e., bestatin, captopril, and diprotin A) (19). Under these conditions, the disappearance of the linear hexapeptide was indeed slower, so that more than 70% of the initially applied peptide concentration was still present on the AP side of the monolayer after a 180 min incubation. The cyclic prodrug, in contrast, was significantly more stable than the linear hexapeptide when applied to the AP side of the Caco-2 cell monolayers (Fig. 3A). In the absence of peptidase inhibitors, 66.9 ± 1.0% of the prodrug initially added to the donor compartment was present after a 3-hr incubation period. A comparison of the disappearance rate of the cyclic prodrug from the AP side of Caco-2 cell monolayers ($t_{1/2}$ = 282 ± 25 min) with the respective rate determined for the chemical hydrolysis of the cyclic prodrug in HBSS ($t_{1/2}$ = 206 ± 11 min, see Table I) revealed that the cyclic prodrug is

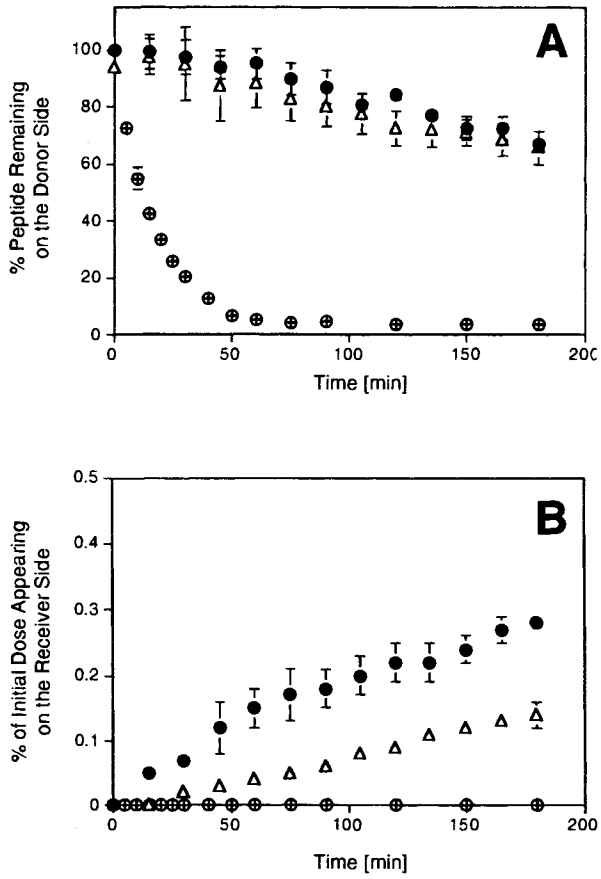


Fig. 3. Time course of disappearance of the cyclic prodrug (●) and appearance of the linear hexapeptide (in the presence (△) and absence (⊕) of peptidase inhibitors) when applied to the apical side of Caco-2 cell monolayers and incubated up to 3 hours at 37°C. Panel A shows time profiles of the peptides remaining in the donor compartment (i.e., apical side), and Panel B represents the peptides transported to the receiver compartment (i.e., basolateral side). Experiments were performed in triplicate (average ± SD).

metabolically stable when applied to the AP side of Caco-2 cell monolayers.

In the absence of peptidase inhibitors, the linear hexapeptide did not permeate the Caco-2 cell monolayer in measurable amounts (Fig. 3B). As a consequence, the apparent permeability coefficient (P_{app}) was estimated based on the analytical detection limit for the peptide (Table II). The intrinsic permeability of the linear hexapeptide was obtained from the linear flux (0.08%/hr) that was observed from the AP to the BL compartment and vice versa in the presence of the peptidase inhibitors. The cyclic prodrug, in contrast, was able to cross the cell barrier in the absence of peptidase inhibitors at an initial rate of 0.15%/hr (calculated within the first 45 min). At later time points, the increase in the amount of prodrug measured in the BL compartment deviates from linearity because the prodrug is undergoing chemical hydrolysis as described earlier in this paper. When compared to the P_{app} of the parent peptide, the cyclic prodrug is at least 76-fold more able to permeate than is the linear hexapeptide (Table II). Transport in the AP to BL direction was statistically not different from the transport in the opposite direction (i.e., BL-AP). The cyclic acyloxyalkoxycarbamate prodrug was also approximately 3 times more able to permeate than the linear model hexapeptide in the presence of potent peptidase inhibitors.

Physicochemical Properties

Physicochemical properties (lipophilicities and average hydrodynamic volumes) of the linear hexapeptide and the cyclic prodrug, which might be crucial in determining their permeation characteristics, were determined by chromatographic techniques and are shown in Table II. The negative log k'_{IAM} value determined for the linear peptide reflects moderate interaction between the hydrophobic portion of the immobilized phosphatidylcholine analogs and the peptide. Incorporating the peptide into the cyclic prodrug resulted in stronger interactions to the lipophilic stationary phase, as indicated by a larger log k'_{IAM} value. This suggests higher lipophilicity for the cyclic prodrug as compared to the linear hexapeptide.

Based on the capacity factors, K_d, that were determined for the peptides by high-resolution size exclusion chromatography (Table II), the cyclic prodrug appears to have a smaller molecular hydrodynamic volume than the linear hexapeptide, although its molecular weight is larger.

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	206 ± 11	201 ± 4
Caco-2 cell homogenate	0.29	0.48	n.d.	209 ± 8	n.d.
90% human plasma[c]	0.01	0.37	0.11	100 ± 4*	171 ± 13*
rat intestinal homogenate	7.37	10.32	0	136 ± 4*	215 ± 4
rat liver homogenate	1.53	13.77	0.18	65 ± 3*	193 ± 3

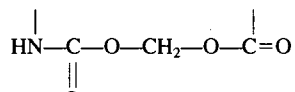
Note: n.d. = not determined. *Significantly different from the control in HBSS, pH 7.4 ($p < 0.05$).

[a] Determined at 25°C in HBSS, pH 7.4 using *p*-nitrophenyl butyrate as substrate.

[b] Calculated from apparent first-order rate constants.

[c] Human plasma diluted to 90% (v/v) with HBSS, pH 7.4.

Table II. Physicochemical Properties and Transport Characteristics Determined Through Caco-2 Cell Monolayers of the Cyclic Prodrug and the Linear Hexapeptide

Compound	MW	Size[a] [K_d]	Lipophilicity[b] [log k'_{IAM}]	Permeability coefficients, P_{app} [$\times 10^8$ cm/s]	
				AP-BL	BL-AP
H-Trp-Ala-Gly-Gly-Asp-Ala-OH	574	0.59	−1.10	<0.17	<0.17
H-Trp-Ala-Gly-Gly-Asp-Ala-OH + peptidase inhibitor "cocktail"[c]				4.05 ± 0.24	4.66 ± 0.60
	631	0.83	−0.32	12.97 ± 1.48[d]	11.59 ± 0.91[d]

[a] Determined by high-resolution size exclusion chromatography.
[b] Partition coefficient between 0.02 M phosphate buffer, pH 7.4, and an immobilized artificial membrane of phosphatidylcholine analogs (IAM.PC.DD).
[c] Bestatin 0.29 mM, captopril 1 mM, diprotin A 1 mM.
[d] Calculated from the cumulative flux in the receiver compartment that was corrected for chemical degradation of the prodrug in HBSS, pH 7.4 (see Materials and Methods).

DISCUSSION

A fundamental requirement of a prodrug is that it can be converted to the parent drug by either a chemical or enzymatic reaction (7). The acyloxyalkoxycarbamate prodrug described in this study was designed to release the model hexapeptide by enzymatic hydrolysis of the ester moiety (see Scheme 1). Since ester bonds are not only enzymatically but also chemically labile, we determined the stability of the cyclic prodrug both in buffered solutions, pH 3.0-9.6, and in biological media known to contain esterase activity.

With respect to chemical instability, this cyclic prodrug was more stable under moderate acidic conditions than in basic solutions (Fig. 1). The plateau region between pH 3 and 4 suggests spontaneous or water-catalyzed hydrolysis, whereas the progressively faster disappearance at pH values ≥5 implies a specific base-catalyzed hydrolysis of the ester moiety as the major degradation mechanism. It is important to note that the linear hexapeptide can be formed from the cyclic prodrug by chemical hydrolysis, i.e., in the absence of any esterase activity. Since the rate constants for the formation of the linear model hexapeptide (e.g., HBSS, pH 7.4, $t_{1/2}$ = 213 ± 13 min) were nearly identical with those obtained for the disappearance of the cyclic prodrug (e.g., HBSS, pH 7.4, $t_{1/2}$ = 206 ± 11 min), we concluded that the rate-determining step in the cascade of reactions leading to the release of the parent peptide is indeed the ester hydrolysis, followed by rapid elimination of formaldehyde and carbon dioxide (Scheme 1).

In a biological milieu, the cyclic prodrug would be expected to degrade to the linear hexapeptide by both chemical and enzyme-catalyzed reactions (Scheme 1). To assess the susceptibility of this prodrug to esterase-catalyzed hydrolysis, several biological media were selected and the rates of prodrug conversion determined. Based on the spectrophotometric assay using PNPB as a substrate, all biological media that were used in this study exhibited measurable levels of esterase activity (Table I). The specific esterase activity, however, was remarkably species-dependent and, within the same species, found to differ significantly between various tissues (Table I). Studies performed earlier with different substrates indicate that rat tissue, in general, exhibits much higher esterase activities than human tissues (12, 20, 21). Within the same species, liver, plasma and intestinal mucosa are considered the most important sites of metabolism for esterase-sensitive drugs, although the ranking order in activity is unquestionably affected by substrate specificity (22).

Apparent half-lives of the acyloxyalkoxycarbamate prodrug in human plasma and rat tissue homogenates were significantly less than in HBSS, pH 7.4. This suggests that the disappearance of the cyclic prodrug may be catalyzed by esterases. Due to rapid metabolism of the linear hexapeptide in biological media (*e.g.*, $t_{1/2}$ in 90% human plasma = 3.7 min; data not shown), it was not clear whether the disappearance of the cyclic prodrug was based on the mechanism proposed in Scheme 1 or mediated by endopeptidases. The latter pathway could be ruled out by the observation that a N-terminal-to-C-terminal-linked analog of the hexapeptide showed ≤0.3% degradation after a 6-hr incubation in rat liver homogenate (data not shown). Hence, we have reasonable confidence that the cyclic prodrug is converted to the linear model hexapeptide by an esterase-mediated reaction.

Additional experimental evidence for the proposed mechanism shown in Scheme 1 was obtained from studies using paraoxon, a potent esterase inhibitor. Apparent half-lives of the cyclic prodrug in 90% human plasma and rat tissue homogenates were significantly longer after preincubation with the potent esterase inhibitor. In Caco-2 cell homogenate, however, the rate of disappearance of the cyclic prodrug in the absence of paraoxon was not different than the chemical hydrolysis rate, in spite of the fact that this biological medium had high esterase activity (Table I). This suggests that the cyclic prodrug can be cleaved only by a certain family of isozymes that are present in 90% human plasma and rat tissue homogenates but not in

Caco-2 cells. Esterases that are sensitive to paraoxon inhibition are classified as B-esterases (23). Based on the almost complete inhibition of the enzymatic hydrolysis of PNPB in rat tissue homogenates in the presence of paraoxon, we conclude that these biological media contain predominantly B-esterases. Human plasma, in contrast, showed still more than 30% residual esterase activity after pretreatment with paraoxon, suggesting the presence of a considerable fraction of A-and/or C-esterases in this medium. Since the rate of disappearance of the cyclic prodrug in 90% human plasma in the presence of paraoxon was significantly different from chemical hydrolysis, the acyloxyalkoxycarbamate prodrug seems to have affinity for B-esterases as well as for "non"-B-esterases (i.e., A- and/or C-esterases). However, carboxypeptidases are also known to cleave ester bonds (24) and, therefore, it is not known whether the release of the parent peptide from the cyclic prodrug is mediated only by esterases.

The intestinal mucosa represents a significant barrier to oral delivery of peptides into the systematic circulation (1, 4). 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 (1–3). Therefore, it was of interest to investigate the transport and metabolism of the acyloxyalkoxycarbamate prodrug in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa that has been shown to exhibit physical (14, 17) and metabolic (16) barrier properties similar to the *in vivo* situation.

As expected, the linear hexapeptide disappeared rapidly from the AP side of Caco-2 cell monolayers, indicating high susceptibility to peptidase metabolism (Fig. 3A). HPLC analysis revealed Trp and Trp-Ala as a major metabolites (data not shown). This suggests metabolism by aminopeptidases and dipeptidyl peptidase IV, respectively. Since the linear hexapeptide was not detected in the BL compartment, we investigated whether the metabolic or the physical barrier function of the intestinal mucosa is the major detriment to the permeation of this peptide. In the presence of a peptidase inhibitor "cocktail" that has been successfully used to stabilize the metabolism of delta sleep-inducing peptide (DSIP) on Caco-2 cells (19), approximately 70% of the initially applied amount of peptide remained after a 3-hr incubation. Under these conditions, a linear increase in the amount of peptide transported to the BL compartment was observed, indicating that the metabolic rather than the physical barrier properties restrict the permeation of the linear hexapeptide across Caco-2 cell monolayers.

The cyclic prodrug, in contrast, was significantly more stable than the linear hexapeptide when placed on the AP side of Caco-2 cell monolayers (Fig. 3A). This indicates that the cyclic prodrug approach using the acyloxyalkoxy promoiety can markedly reduce the lability of the peptide toward metabolic degradation mediated by intestinal peptidases. In addition, the cyclic prodrug appears to exhibit more favorable physicochemical properties to permeate the physical barrier of the intestinal mucosa. This conclusion was drawn from the observation that the permeation of the acyloxyalkoxycarbamate prodrug is approximately 3 times greater than the intrinsic permeability determined for the linear model hexapeptide in the presence of peptidase inhibitors. The cyclic prodrug was also ~3 times more permeable than the metabolically stable N-terminus acetylated and C-terminus amidated analog of the linear hexapeptide (10).

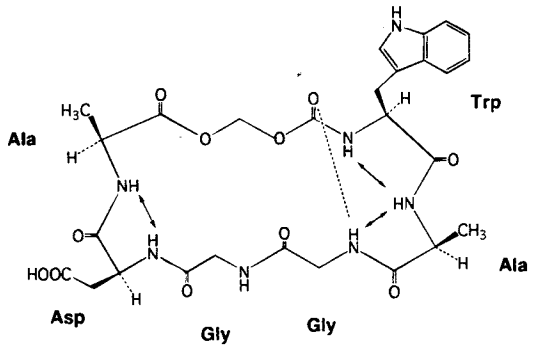


Fig. 4. Secondary structure of the cyclic prodrug in solution (with permission (25)). Dashed line indicates the intramolecular hydrogen bond of a β-turn and arrows symbolize the close proximity (≤ 4Å) of amide protons in the peptide backbone.

Structural analysis performed by CD as well as by one- and two-dimensional NMR spectroscopy revealed that the cyclic prodrug contains well defined elements of secondary structure (25), whereas the linear hexapeptide exists in a dynamic equilibrium between random coil and β-turns (10). Fig. 4 shows the secondary structure determined for the cyclic prodrug in solution (25). The cyclic nature of the prodrug seems to favor the formation of intramolecular hydrogen bonds. This might account for an overall higher lipophilicity index (i.e., log k'_{IAM}) of the cyclic prodrug as determined by IAM chromatography (Table II). In addition, cyclization of a linear molecule can also lead to a reduction in size. Based on the K_d values obtained by size exclusion chromatography (Table II), it appears that the cyclic prodrug is significantly smaller than compounds of equivalent molecular weight. As suggested by these results, the smaller and less hydrophilic cyclic prodrug comprises more favorable physicochemical properties to traverse biological membranes than does the linear model hexapeptide. With respect to the mechanism of permeation of the cyclic prodrug through Caco-2 cell monolayers, reduction in molecular size could have increased the paracellular flux of the peptide through aqueous pores, and/or the higher lipophilicity of the prodrug could have shifted its pathway of permeation to a larger contribution toward the transcellular route.

In conclusion, the experimental results presented in this paper illustrate that the use of the acyloxyalkoxy promoiety to prepare esterase-sensitive cyclic prodrugs may represent a promising approach in drug delivery to enhance the membrane permeation of peptides and, simultaneously, increase their metabolic stability.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Norman F. H. Ho (Pharmacia & Upjohn Company, Kalamazoo, MI) for his valuable help in the data analysis and Manoj M. Nerurkar (Department of Pharmaceutical Chemistry, The University of Kansas) for his assistance in animal surgery. This work was supported by research grants from Glaxo, Inc., The United States Public Health Service (DA09315), Costar Corporation, and a fellowship to G. M. P. from the Swiss National Science Foundation.

REFERENCES

1. V. H. L. Lee and A. Yamamoto. *Adv. Drug Delivery Rev.* **4**:171–207 (1990).
2. V. Bocci. *Adv. Drug Delivery Rev.* **4**:149–169 (1990).
3. X. H. Zhou. *J. Controlled Release* **29**:239–252 (1994).
4. G. M. Pauletti, S. Gangwar, G. T. Knipp, M. M. Nerurkar, F. W. Okumu, K. Tamura, T. J. Siahaan, and R. T. Borchardt. *J. Controlled Release* **41**:3–17 (1996).
5. R. A. Gray, D. G. Vander Velde, C. J. Burke, M. C. Manning, C. R. Middaugh, and R. T. Borchadt. *Biochemistry* **33**:1323–1331 (1994).
6. W. A. Banks, A. J. Kastin, D. H. Coy, and E. Angulo. *Brain. Res. Bull.* **17**:155–158 (1986).
7. H. Bundgaard. *Adv. Drug Delivery Rev.* **8**:1–38 (1992).
8. R. Oliyai and V. J. Stella. *Ann. Rev. Pharmacol. Toxicol.* **32**:521–544 (1993).
9. J. K. McDonald and A. J. Barrett. *Mammalian Proteases: A Glossary and Bibliography*, Vol. 2, Exopeptidases, Academic Press, New York, 1986.
10. F. W. Okumu, G. M. Pauletti, D. G. Vander Velde, T. J. Siahaan, and R. T. Borchardt. *Pharm. Res.* **12**:S–302 (1995).
11. S. Gangwar, G. M. Pauletti, T. J. Siahaan, V. J. Stella, and R. T. Borchardt. *J. Org. Chem.* (submitted).
12. M. Inoue, M. Morikawa, M. Tsuboi, and M. Sugiura. *Jpn. J. Pharmacol.* **29**:9–16 (1979).
13. F. M. Williams. *Clin. Pharmacokinet.* **10**:392–403 (1985).
14. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. *Gastroenterology* **96**:736–749 (1989).
15. P. Artursson. *J. Pharm. Sci.* **79**:476–482 (1990).
16. M. Pinto, S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Tradou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. *Biol. Cell* **47**:323–330 (1983).
17. G. Wilson, I. F. Hassan, C. J. Dix, I. Williamson, R. Shah, and M. Mackay. *J. Controlled Release* **11**:25–40 (1990).
18. H. Liu, S. Ong, L. Glunz, and C. Pidgeon. *Anal. Chem.* **67**:3550–3557 (1995).
19. P. F. Augustijns and R. T. Borchardt. *Drug Metab. Dispos.* **23**:1372–1378 (1995).
20. C. S. Cook, P. J. Karabatsos, G. L. Schoenhard, and A. Karim. *Pharm. Res.* **12**:1158–1164 (1995).
21. W. N. Aldridge. *Biochem. J.* **53**:117–124 (1953).
22. K. Takahashi, S. Tamagawa, H. Sakano, T. Katagi, and N. Mizuno. *Biol. Pharm. Bull.* **18**:1401–1404 (1995).
23. W. N. Aldridge. *Biochem. J.* **53**:110–117 (1953).
24. D. S. Auld and B. Holmquist. *Biochemistry* **13**:4355–4361 (1974).
25. S. Gangwar, S. D. S. Jois, T. J. Siahaan, D. G. Vander Velde, V. J. Stella, and R. T. Borchardt. *Pharm. Res.* **13**: 1657–1662 (1996).