Phenylpropionic Acid-Based Cyclic Prodrugs of Opioid Peptides that Exhibit Metabolic Stability to Peptidases and Excellent Cellular Permeation

Olafur S. Gudmundsson,[1,2] Kalpana Nimkar,[1] Sanjeev Gangwar,[1,2] Teruna Siahaan,[1] and Ronald T. Borchardt[1,3]

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Purpose. To evaluate the cellular permeation characteristics and the chemical and enzymatic stability of phenylpropionic acid-based cyclic prodrugs 1 and 2 of opioid peptides [Leu^5]-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) and DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH), respectively.

Methods. The rates of conversion of cyclic prodrugs 1 and 2 to [Leu^5]-enkephalin and DADLE, respectively, in HBSS, pH 7.4 (Caco-2 cell transport buffer) and in various biological media having measurable esterase activity were determined by HPLC. The cell permeation characteristics of [Leu^5]-enkephalin, DADLE, and cyclic prodrugs 1 and 2 were measured using Caco-2 cell monolayers grown onto microporus membranes and monitored by HPLC.

Results. In HBSS, pH 7.4, cyclic prodrugs 1 and 2 degraded to [Leu^5]-enkephalin and DADLE, respectively, in stoichiometric amounts. In 90% human plasma, the rates of disappearance of cyclic prodrugs 1 and 2 were slightly faster than in HBSS, pH 7.4. These accelerated rates of disappearance in 90% human plasma could be reduced to the rates observed in HBSS, pH 7.4, by pretreatment of the plasma with paraoxon, a known inhibitor of serine-dependent esterases. In homogenates of Caco-2 cells and rat liver, accelerated rates of disappearance of cyclic prodrugs 1 and 2 were not observed. When applied to the AP side of a Caco-2 cell monolayer, cyclic prodrug 1 exhibited significantly greater stability against peptidase metabolism than did [Leu^5]-enkephalin. Cyclic prodrug 2 and DADLE exhibited stability similar to prodrug 1 when applied to the AP side of the Caco-2 cell monolayers. Prodrug 1 was 1680 fold more able to permeate the Caco-2 cell monolayers than was [Leu^5]-enkephalin, in part because of its increased enzymatic stability. Prodrug 2 was shown to be approximately 77 fold more able to permeate a Caco-2 cell monolayer than was DADLE.

Conclusions. Cyclic prodrugs 1 and 2, prepared with the phenylpropionic acid promoiety, were substantially more able to permeate Caco-2 cell monolayers than were the corresponding opioid peptides. Prodrug 1 exhibited increased stability to peptidase metabolism compared to [Leu^5]-enkephalin. In 90% human plasma but not in Caco-2 cell and rat liver homogenates, the opioid peptides were released from the cyclic prodrugs by an esterase-catalyzed reaction that is sensitive to paraoxon inhibition. However, the rate of this bioconversion appears to be extremely slow.

KEY WORDS: esterase-sensitive prodrugs; peptide delivery; opioid peptides; Caco-2 cells; membrane permeability; chemical and enzymatic stability.

INTRODUCTION

The intestinal mucosa is a physical as well as a biological barrier to the permeation of peptides (1,2). Physically, the intestinal mucosa restricts permeation of hydrophilic peptides to the paracellular pathway, whereas hydrophobic peptides can traverse this biological membrane via the transcellular route. A hydrophilic peptide [e.g., opioid peptides (3,4)], whose permeation is restricted to the paracellular route, typically exhibits an oral bioavailability of <2%, whereas hydrophobic peptides that can permeate via the transcellular pathway usually exhibit much higher bioavailability. Traditionally, the biological barrier component of the intestinal mucosa was thought to consist of proteases and peptidases (5). However, the nature of this barrier has now been expanded to include intracellular metabolism by cytochrome P450-3A4, as well as apically polarized efflux mediated by proteins like P-glycoprotein (6,7).

Recently, our laboratory introduced the concept of making cyclic prodrugs of hydrophilic peptides as a way to modify their physicochemical properties sufficiently to overcome the physical barrier of the intestinal mucosa (i.e., make them transcellular permeants rather then paracellular permeants) (8–12). More recently, we have applied this strategy to opioid peptides by synthesizing cyclic prodrugs of [Leu^5]-enkephalin and DADLE using phenylpropionic acid, coumarinic acid and acyloxyalkoxy linkers (13,14). The physicochemical properties (e.g., lipophilicity) of these compounds were measured and shown to be in a range that should permit optimal transcellular permeation (13,14). When transport studies were performed using Caco-2 cell monolayers, an *in vitro* cell culture model of the intestinal mucosa (15), significant increases were seen in the permeation characteristics of the cyclic prodrugs made with the coumarinic-acid linker compared to those of the corresponding opioid peptides (16). In contrast, very low permeation through Caco-2 cell monolayers was observed for the cyclic prodrugs made with the acyloxyalkoxy linker (17). This discrepancy between the physicochemical properties and the cell membrane permeation of the acyloxyalkoxy-based cyclic prodrugs could, however, easily be explained by the observation that these prodrugs were substrates for the apically polarized efflux systems (17). Therefore, it was of interest to measure the permeation of the phenylpropionic acid-based prodrugs and to determine if their physicochemical properties correlated with their permeation characteristics or whether they also had low cellular permeation because of substrate activity for the apically polarized efflux systems.

In this study, we have determined the transport characteristics of the phenylpropionic acid-based prodrugs 1 and 2 of [Leu^5]-enkephalin and DADLE, respectively (Fig. 1) using Caco-2 cell monolayers and shown that, consistent with their physicochemical properties (13) and solution conformations

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66047.

[2] Present addresses: Olafur S. Gudmundsson, Bristol-Myers Squibb, Exploratory Biopharmaceutics and Drug Delivery, Pharmaceutical Reseach Institute, One Squibb Drive, P.O. Box 191, New Brunswick, New Jersey 08903-0191; Sanjeev Gangwar, Coulter Pharm., Inc., 550 California Avenue, Ste. 200, Palo Alto, California 94306.

[3] To whom correspondence should be addressed. (e-mail: borchardt@hbc.ukans.edu)

ABBREVIATION: PNPB, *p*-nitrophenyl butyrate; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle medium; AP, apical; BL, basolateral; P_{app}, apparent permeability coefficient; $t_{1/2}$, apparent half-life.

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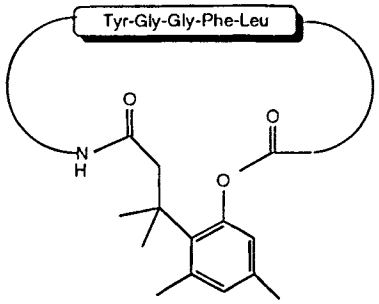

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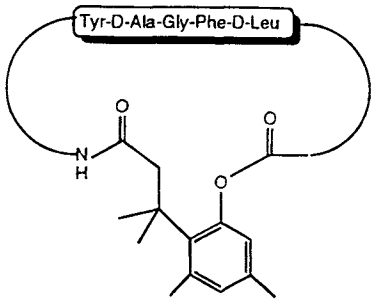

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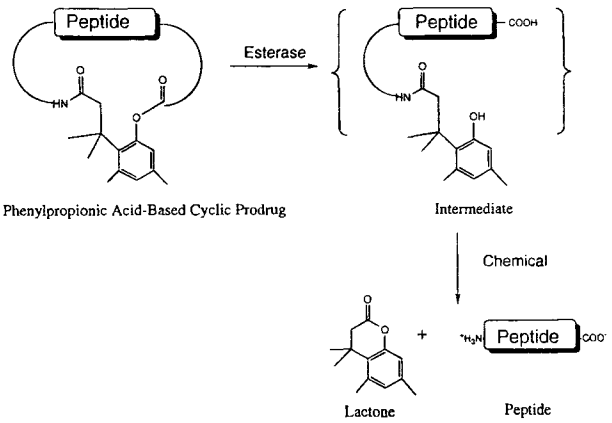
Fig. 1. Phenylpropionic acid-based cyclic prodrugs 1 and 2.

(18), they exhibit substantially higher permeation than the corresponding opioid peptides. Furthermore, we have shown that these cyclic prodrugs are not substrates for the apically polarized efflux systems. The stability of the phenylpropionic acid-based cyclic prodrugs was also assessed in biological media and compared to the chemical stability of the prodrugs in aqueous buffer at pH 7.4, to confirm their bioconversion to the opioid peptides (Scheme 1).

MATERIALS

The phenylpropionic acid-based prodrugs 1 and 2 were synthesized by procedures reported elsewhere (13). The opioid peptides, [Leu^5]-enkephalin and DADLE, diethyl *p*-nitrophenyl phosphate (paraoxon, approx. 90%), *p*-nitrophenyl butyrate (PNPB) (~98%), dimethyl sulfoxide (>99.5%), potassium cyanide, taurine (2-aminoethanesulfonic acid), bovine serum albumin (BSA, fraction V protein standard) (>98–99%), Dulbecco's phosphate buffered saline, and Hanks' balanced salts (modified) were purchased from Sigma Chemical Co. (St. Louis, MO). The naphthalene-2,3-dicarboxaldehyde (NDA) was provided by Professor John F. Stobaugh (Department of Pharmaceutical Chemistry, The University of Kansas) and used as received. Reagents and buffers used to grow Caco-2 cell monolayers and to conduct transport studies were purchased from the sources described previously by Gudmundsson *et al.* (16). All other chemicals and solvents were of high purity or analytical grade and were used as received.



Scheme 1. Proposed mechanism for the release of opioid peptides from the phenylpropionic acid-based cyclic prodrugs.

METHODS

Cell Culture

Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Rockville, MD) and were grown as described by Gudmundsson *et al.* (16). For transport experiments, cells were detached from the plastic support at approximately 80% confluence and plated on collagen-coated polycarbonate membranes (Transwells®, 24.5 mm in diameter, 3 μm pore size, Costar Corporation, Cambridge, MA) previously coated with rat tail collagen, as described by Gudmundsson *et al.* (16). All cells used in this study were between passage 44 and 49.

Stability Studies

Chemical Stability

The chemical stability of cyclic prodrugs 1 and 2 was determined at 37°C in HBSS, pH 7.4, or HBSS buffer, pH 7.4, containing 1.35 mg/ml bovine serum albumin (BSA) using a slight modification of the procedure described earlier by Gudmundsson *et al.* (16). Solutions of the prodrugs (~20 μM) containing 5% DMSO were incubated in sealed vials at 37.0 ± 0.5°C in a temperature-controlled shaking water bath (60 rpm). Periodically, aliquots (20 μl) were removed and diluted with 150 μl of a 1:1 (v/v) mixture of methanol and freshly prepared 6 N guanidinium hydrochloride solution in acidified HBSS [HBSS containing 0.01% (v/v) phosphoric acid], and immediately analyzed by HPLC (see HPLC Analysis Section). Plots were made of log prodrug concentration remaining vs. time and fitted by linear regression ($r^2 = 0.97$) assuming pseudo first-order reaction. Amounts remaining (% remaining) after 10 hr were then calculated using the best fit equation.

Enzymatic Stability

The stability of cyclic prodrugs 1 and 2 in Caco-2 cell and rat liver homogenates and in 90% human plasma was determined at 37°C in the presence and absence of paraoxon, a potent esterase inhibitor. The preparation of 90% human plasma and Caco-2 cell and rat liver homogenates, as well as the determination of total esterase activity, using PNPB, and the determination of total protein concentrations in these biological media have been extensively described elsewhere (10). The

stabilities of cyclic prodrugs 1 and 2 were determined in these biological media using slight modifications of the procedures described previously by Gudmundsson *et al.* (16). Cyclic prodrugs 1 and 2 (~20 μM, final concentrations) were incubated with the biological matrix containing 5% DMSO, and samples were maintained for 10 hr in a temperature-controlled shaking water bath (60 rpm, 37.0 ± 0.5°C). To test the effect of an esterase inhibitor on the rate of degradation of the cyclic prodrugs, the biological medium was preincubated with paraoxon (final concentration 1 mM) for 15 min at 37°C before the prodrugs were added. At various times, aliquots (20 μl) were removed and the esterase activity immediately quenched by adding 150 μl of a 1:1 (v/v) mixture of methanol and freshly prepared 6 N guanidinium hydrochloride solution in acidified 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 7,500 rpm (5,000 × g) for 90 min (4°C). Aliquots (75 μl) of the filtrate were diluted with a 1:1 methanol/6 N guanidinium hydrochloride solution (v/v) and analyzed by HPLC (See HPLC Analysis Section). Recoveries for the cyclic prodrugs were ~94%. Plots were made of log prodrug concentration remaining vs. time, and fitted by linear regression (r^2 = 0.91) assuming pseudo first-order reaction. Amounts remaining (% remaining) after 10 hr were then calculated using the best fit equation.

Transport Experiments

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 leakage of [^{14}C]-mannitol in representative cell monolayers (n = 3) by the methods described by Gudmundsson *et al.* (16). The transport of the cyclic prodrugs and peptides across Caco-2 cell monolayers was determined as described earlier (16) except the concentration of the cyclic prodrugs was ~1μM. Samples removed from the donor and the receiver sides were stabilized by the addition of aliquots of acetonitrile and diluted phosphoric acid [final concentration 10% (v/v) and 0.01% (v/v), respectively]. This acidic mixture (pH ~3) was immediately frozen in a dry-ice/acetone bath and kept at −80°C until HPLC analyses (see HPLC Analysis section). Immediately prior to HPLC analysis, the cyclic prodrug samples were rapidly thawed and aliquots (40 μl) of 1 N NaOH were added to the samples to hydrolyze the cyclic prodrugs to the opioid peptides and the promoiety. After 10 min, the solution was quenched with aliquots (40 μl) of 1 N HCL prior to being derivatized by NDA (see HPLC Analysis section). The extent of degradation of the cyclic prodrugs during transport experiments was estimated by direct analysis of samples taken from both AP and BL sides.

Transport experiments in the AP-to-BL direction as well as in the BL-to-AP direction were performed in triplicate at 37°C in a shaking water bath (60 rpm).

HPLC Analysis

Apparatus

Chromatographic analyses were carried out on a Shimadzu LC-10A gradient system (Shimadzu, Inc., Tokyo, Japan) consisting of LC-10AD pumps, a SCL-10A controller, a SPD-10A UV detector, a SIL-10A autoinjector equipped with a sample cooler, two FCV-2AH high pressure switching valves and a RF-535 fluorescence detector. Data were analyzed by the Class VP-4 chromatography software system (Shimadzu). All systems and the column switching events were controlled by a Shimadzu SCL-10A or a SCL-6A system controllers.

Analyses of Stability Samples

Aliquots from a refrigerated sample tray (4°C) were injected onto a C-18 reverse-phase column (Vydac 218TP, 300 Å, 250 × 4.6 mm I.D.) equipped with a guard column. The eluents were detected by fluorescence (emission λ = 310 nm; excitation λ = 283 nm). Gradient elution was performed at a flow rate of 1 ml/min from 26–90% (v/v) acetonitrile in water using trifluoroacetic acid (0.1%, v/v) as the ion-pairing agent.

Derivatization Procedure

Samples from the transport experiments were converted to the corresponding opioid peptides as described above and then derivatized precolumn with the fluorogenic agent NDA in the presence of cyanide ion to form the corresponding N-substituted 1-cyanobenz[f]isoindole (CBI) derivatives (19,20). Initially, the pH of the transport samples was adjusted to pH 6.8 by addition of an aliquot of phosphate buffer (50 μl, 200 mM, pH 6.8). The derivatization reaction was then initiated by adding aliquots of a potassium cyanide stock solution (20 μL, 50 mM) and a NDA stock solution (20 μL, 50 mM). The reaction was allowed to proceed for 15 min on ice (0–4°C) and was then quenched by the addition of taurine (20 μL, 450 mM). The reaction mixture were stored for an additional 10 min. During the derivatization procedure, samples were protected from light. The samples were then loaded onto a refrigerated sample tray (4°C) that was protected from light and were stored for up to 10 hours.

Analyses of Transport Samples

Samples from the transport studies were derivatized with NDA as descibed above and aliquots were immediately injected from a refrigerated sample tray (4°C) protected from light onto a multidimensional chromatographic system where the CBI derivatives were transferred as a single fraction from one column to a second to obtain maximum separation. This technique has been extensively described elsewhere (20), and was used with adjustments appropriate for the sample matrix of interest. Briefly, aliquots were injected onto a diphenyl column (Column 1: Vydac 219TP54, 300 Å, 250 × 4.6 mm I.D.) equipped with a guard column. Typically, 1.5-ml elution fractions, containing the analyte of interest, were transferred from column 1 to column 2 by use of high pressure switching valves. A C-18 reverse-phase column (Vydac 218TP, 300 Å, 250 × 4.6 mm I.D.) was used as column 2. Elution of both columns was performed isocratically at a flow rate of 1 ml/min. A mobile phase of acetonitrile:water (37:63 v/v) using trifluoroacetic acid (0.1%, v/v) as the ion-pairing agent was used to elute column 1, whereas a mobile phase of acetonitrile:water:tetrahydrofuran (45:51:4 v/v/v) using trifluoroacetic acid (0.1%, v/v) as the ion-pairing agent was used to elute column 2. The CBI derivatives were

detected by fluorescence (emission λ = 490 nm; excitation λ = 420 nm).

Data Analysis

Permeability coefficients (P_{app}) of the compounds 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.

RESULTS

Chemical and Enzymatic Stability

The phenylpropionic acid-based cyclic prodrugs 1 and 2 were designed to undergo enzyme-catalyzed hydrolysis of the ester bond linking the *C*-terminal carboxyl acid of the peptide to the phenolic group of the promoiety (Scheme 1). The resulting intermediate should then undergo rapid chemical hydrolysis of the amide bond linking the *N*-terminal amino group of the peptide to the carboxylic acid of the promoiety via an intramolecular reaction to yield the opioid peptide and a lactone (Scheme 1). Initial experiments were conducted in HBSS, pH 7.4, to determine the chemical stability of cyclic prodrugs 1 and 2. In Fig. 2 is shown the time course of disappearance of the cyclic prodrug 2 at 37°C in HBSS at pH 7.4. A similar time course for the degradation of cyclic prodrug 1 of [Leu^5]-enkephalin was observed (data not shown). With both cyclic prodrugs, mass balance was achieved (~98.7 %) relative to the formation of the opioid peptides and the lactone for incubations up to 10 hr. The estimated amount of cyclic prodrugs 1 and 2 remaining in HBSS, pH 7.4, after 10 hr were 91% and 90%, respectively. It should be noted that no intermediates could be detected in these hydrolytic processes. Stability studies were also conducted in HBSS, pH 7.4, in the presence of 1.35 mg/ml of bovine serum albumin (BSA), to determine the possible effect of protein binding on the stability of the cyclic prodrugs.

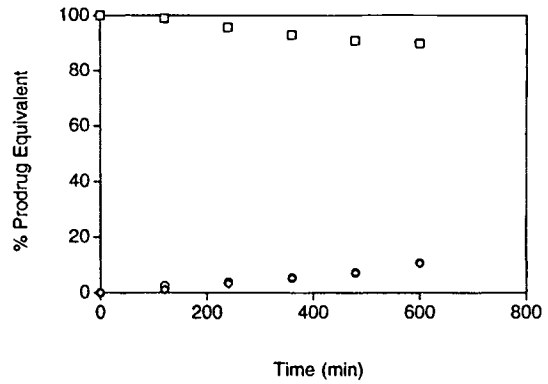


Fig. 2. Stability of cyclic prodrug 2 at 37°C in HBSS buffer, pH 7.4, showing the time course of disappearance of the cyclic prodrug (□) and the appearance of DADLE (○) and the lactone (◇).

Amounts remaining (% remaining) of cyclic prodrugs 1 and 2 after 10 hr were determined to be 89% and 90%, respectively (Table I). These values are similar to the values observed when cyclic prodrugs 1 and 2 were incubated in HBSS, pH 7.4, buffer in the absence of BSA.

The stabilities of the cyclic prodrugs (expressed as % remaining after 10 hr) in various biological media (i.e., 90% human plasma and homogenates of Caco-2 cells and rat livers) having measurable esterase activities were also determined and are presented in Table I. These results show that the cyclic prodrugs in the presence of 90% human plasma were slightly less stable than in HBSS, pH 7.4 (Table I). In Caco-2 cell and rat liver homogenates, the stabilities of the cyclic prodrugs 1 and 2 were similar to those observed in HBSS and HBSS with BSA in spite of the fact that these biological media contained measurable esterase activities.

To determine if the slightly increased instability of cyclic prodrugs 1 and 2 observed in 90% human plasma was catalyzed by esterases, the effect of paraoxon, a potent esterase inhibitor, on their stability in plasma was determined. In separate experiments (16), paraoxon was shown to significantly reduce the esterase activity present in homogenates of Caco-2 cells and rat livers as well as in 90% human plasma (Table I). When the stabilities of cyclic prodrugs 1 and 2 were determined in human plasma pretreated with 1 mM paraoxon for 15 min at 37°C, the amounts of the cyclic prodrugs remaining after a 10 hr incubation were increased and the stability was similar to values seen for the chemical stability of the cyclic prodrugs in HBSS buffer (Table I). These results suggest that the cyclic prodrugs most likely degrade in 90% human plasma by hydrolysis of the ester bond mediated by esterases. However, it should be mentioned that, in this biological medium, mass balance was not achieved. This was particularly true for cyclic prodrug 1, since [Leu^5]-enkephalin is rapidly metabolized in this biological medium (16). Paraoxon pretreatment of Caco-2 cell and rat liver homogenates had no significant effect on the stability of cyclic prodrugs 1 and 2 (Table I). Similarly, inclusion of paraoxon in HBSS, pH 7.4, or HBSS with BSA had no significant effect on the extent of hydrolysis of cyclic prodrugs 1 and 2 (Table I).

Transport Across Caco-2 Cell Monolayers

Cell permeability characteristics of [Leu^5]-enkephalin, DADLE and cyclic prodrugs 1 and 2 were assessed using Caco-2 cell monolayers, an *in vitro* model of the intestine (15). As described by Gudmundsson *et al.* (16), [Leu^5]-enkephalin is extremely unstable when applied to the apical side of Caco-2 cell monolayers ($t_{1/2}$ = 15 min). Based on the limits of detection of our analytical method, its maximum apparent permeability value would be 0.31×10^{-8} cm/s. In contrast, DADLE, when applied to the AP side of Caco-2 cell monolayers, is relatively stable (<10% degradation in 180 min) and has a P_{app} value of 7.8×10^{-8} cm/s (16).

Cyclic prodrug 1 was significantly more stable than [Leu^5]-enkephalin when applied to the AP side of Caco-2 cell monolayers (Fig. 3A). The stability of cyclic prodrug 2 when applied to the AP side of Caco-2 cell monolayers was also higher than that observed with DADLE, and was more similar to what was seen for cyclic prodrug 1 (Fig. 3A). Consistent with the results described above with a Caco-2 cell homogenate, no detectable

Table I. Percent of Cyclic Prodrugs 1 and 2 Remaining After 10 Hours in HBSS and Various Biological Media

Incubation mixture	Specific activity[a] [U/mg protein]	Enzyme concentration [U/ml][a]		% remaining after 10 hr incubation[b]			
				1		2	
		−paraoxon	+paraoxon	−paraoxon	+paraoxon	−paraoxon	+paraoxon
HBSS, pH 7,4	0	0	0	91 ± 1	92 ± 2	90 ± 2	91 ± 1.5
BSA in HBSS[c]	0	0	0	89 ± 0.5	90 ± 1	90 ± 1	91 ± 0.5
Human Plasma[d]	0.006	0.517	0.15	79 ± 1	90 ± 1	79 ± 1.5	91 ± 1.5
Homogenates							
Caco-2 cells	0.18	0.31	0.01	92 ± 2	92 ± 1	93 ± 1	92 ± 0.5
Rat liver	1.05	31.5	0.85	86 ± 1	92 ± 2	85 ± 1.5	91 ± 2

[a] Determined for matrixes containing 5% DMSO at 25°C in HBSS, pH 7.4 using PNBP.
[b] Estimated from pseudo-first order rate constants as described in the Materials and Methods Section.
[c] Bovine serum albumin 1.35mg/ml in HBSS, pH 7.4.
[d] Human plasma diluted to 90% with HBSS, pH 7.4.

bioconversion of cyclic prodrugs 1 and 2 to [Leu^5]-enkephalin and DADLE, respectively, was observed in these transport experiments. Furthermore, approximately 90% of the total amount of cyclic prodrugs 1 and 2 could be accounted for in the donor and receiver solutions after 180 min, suggesting minimal adsorption of the cyclic prodrugs to the cell monolayer or the Transwell® system itself at the concentration employed for these experiments.

Based on the estimated Papp value for [Leu^5]-enkephalin and the determined P_{app} value for cyclic prodrug 1, the cyclic prodrug 1 is approximately 1677-fold more able to permeate through the Caco-2 cell monolayers than the opioid peptide (Table II). In comparison, cyclic prodrug 2 is approximately 77-fold more able to permeate through Caco-2 cell monolayers than is DADLE. It should be noted that the Papp values for DADLE and cyclic prodrugs 1 and 2 determined in the BL-to-AP direction were nearly identical to the P_{app} values determined in the AP-to-BL direction (Table II).

DISCUSSION

Chemical and Enzymatic Stability

A fundamental requirement of a successful peptide prodrug is that it can be converted to the parent peptide by chemical and/or enzymatic reaction (8). The phenylpropionic acid-based cyclic prodrugs described in this study were designed to release the opioid peptides by enzymatic hydrolysis of the ester bond (Scheme 1) (12). The resulting intermediate would then undergo a fast chemical cleavage of the amide bond to yield the peptide and lactone (12). Since the ester bond can be cleaved enzymatically as well as chemically, the stabilities of cyclic prodrugs 1 and 2 were determined in BHSS buffer, pH 7.4, as well as in biological media known to contain esterase activity.

The chemical stability of cyclic prodrugs 1 and 2 in HBSS, pH 7.4, revealed stoichiometric conversion of both cyclic prodrugs to the corresponding linear opioid peptides. Apparent rate constants calculated for the disappearance of the prodrugs and the appearance of the opioid peptides were statistically not different (data not shown). These values are consistent with values observed for a cyclic prodrug formed with the phenylpropionic acid promoiety and a linear hexapeptide studied earlier by our laboratory (11). The time course of disappearance of cyclic prodrug 2 at 37°C in HBSS, pH 7.4, as well as the appearance of DADLE and the lactone, is shown in Fig. 2. Since an intermediate (Scheme 1) was not detected by HPLC during the chemical stability experiments, and the rates of formation of the opioid peptides were kinetically equivalent to the rates of disappearance of the cyclic prodrugs, we concluded that the rate-determining step in the cascade of reactions leading to the release of the opioid peptides from the cyclic prodrugs is indeed the hydrolysis of the ester promoiety. This conclusion is supported by an earlier kinetic analysis done by our laboratory, using a phenylpropionic acid-based prodrug of a model hexapeptide, in which the formation of an intermediate was not detected (11).

It is interesting to compare the chemical stabilities of the phenylpropionic acid-based cyclic prodrugs 1 and 2 with those of their coumarinic acid-based counterparts (16). Of particular significance is the fact that with the coumarinic acid-based cyclic prodrugs, an intermediate was detected during their chemical conversion to the opioid peptides suggesting that the intramolecular lactonization reaction is slower with the coumarinic acid-based system than with the phenylpropionic acid-based system. It is also of interest to note that the phenylpropio-

Table II. Permeability Coefficients (P_{app}) of [Leu^5]-Enkephalin and DADLE and Their Phenylpropionic Acid-Based Cyclic Prodrugs 1 and 2 Across Caco-2 Cell Monolayers in the AP-to-BL Direction and in the BL-to-AP Direction

Peptide	P_{app} values	
	AP-to-BL $P_{app} \times 10^6$ [cm/s]	BL-to-AP $P_{app} \times 10^6$ [cm/s]
[Leu^5]-enkephalin	<0.0031[a]	<0.0031[a]
Cyclic prodrug 1	5.20 ± 0.08	13.3 ± 0.426
DADLE	0.078 ± 0.007[a]	0.086 ± 0.005[a]
Cyclic prodrug 2	5.98 ± 0.15	8.27 ± 0.51

[a] Taken from Gudmundsson *et al.* (16).

nic acid-based cyclic prodrugs are substantially more stable (approx. 7-fold) than the coumarinic acid-based cyclic prodrugs in HBSS, pH 7.4.

The protein binding of cyclic prodrug 2 (20 μM) was estimated in the presence of 1.35 mg/ml bovine serum albumin in HBSS buffer by allowing the solution to equilibrate for 15 min and then either directly injecting a solution into an HPLC system or first filtering the samples through a Ultrafree®MC 5000 NMWL filter unit (Millipore, Bedford, MA) and then analyzing them by HPLC. These studies suggest that cyclic prodrug 2 was approximately 90% protein bound. This is in comparison with a 65% protein binding for the coumarinic acid-based cyclic prodrug of DADLE (16). To investigate the effect of protein binding on the chemical stability of cyclic prodrugs 1 and 2, experiments were conducted in HBSS buffer, pH 7.4, containing 1.35 mg/ml BSA (Table I). As expected, the BSA does not appear to affect the chemical stability of the phenylpropionic acid-based prodrugs.

In a biological milieu, cyclic prodrugs 1 and 2 would be expected to degrade faster to the opioid peptides because of the presence of esterases. To assess the susceptibility of these prodrugs to esterase-catalyzed hydrolyses, the rates of prodrug conversion to the corresponding opioid peptides were determined in Caco-2 cell and rat liver homogenates and in 90% human plasma. Based on a spectrophotometric assay using PNPB as a substrate, all biological media used in these studies exhibited measurable levels of esterase activity (Table I).

Surprisingly, cyclic prodrugs 1 and 2 were only slightly more unstable in 90% human plasma then in HBSS, pH 7.4 (Table I). This, however, suggests the possibility that some esterase-catalyzed bioconversion is occurring in 90% human plasma. Additional experimental evidence to support this hypothesis was the observation that inclusion of paraoxon, a potent esterase inhibitor in the plasma samples did increase the stability of cyclic prodrugs 1 and 2 to values comparable to their chemical stability in HBSS buffer, pH 7.4. In Caco-2 cell and in rat liver homogenates, in the presence or absence of paraoxon, the rates of degradation of cyclic prodrugs 1 and 2 were similar to rates observed in HBSS, pH 7.4. It is interesting to compare these stability data in biological media for the phenylpropionic acid-based cyclic prodrugs with the stability data for their coumarinic acid counterparts (16). Of particular significance is the observation that the coumarinic acid-based cyclic prodrugs of [Leu^5]-enkephalin and DADLE were significantly more unstable in 90% human plasma as well as in rat liver homogenates (16) than were the corresponding phenylpropionic acid-based cyclic prodrugs. This could result from the more extensive protein binding observed with the phenylpropionic acid-based prodrugs and/or that they are just poorer substrates than the coumarinic acid-based prodrugs for esterases. The poor substrate activity of the phenylpropionic acid-based cyclic prodrugs may result from the steric hindrance contributed by the gem dimethyl group adjacent to the phenolic ester that undergoes hydrolysis. Finally, it is interesting to note that both the phenylpropionic acid-based and the coumarinic acid-based cyclic prodrugs are stable in Caco-2 cell homogenates.

Transport Across Caco-2 Cell Monolayers

The intestinal mucosa significantly restricts oral absorption of opioid peptides into the systemic circulation (16). Tight intracellular junctions restrict the flux of opioid peptides to the paracellular route (physical barrier), and brush border peptidases (biological barrier) rapidly metabolize opioid peptides (e.g., [Leu^5]-enkephalin) to their corresponding amino acids (16). The phenylpropionic acid cyclic prodrug strategy evaluated in this study could have a positive impact on cell membrane permeability since these compounds have more favorable physicochemical properties (i.e., increased lipophilicity, reduced hydrogen bonding potential) (13) created in part by the formation of a unique solution structure (18). Furthermore, these cyclic prodrugs should have significantly increased stability to exo- and endopeptidases since their N-terminal and C-terminals are derivatized. Therefore, it was of interest to investigate the transport and metabolism of the phenylpropionic acid-based cyclic prodrugs in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa that has been shown to be a physical barrier to peptide permeation (2), and the fact that permeability values determined using Caco-2 cell monolayers correlate well with human intestinal permeability values (21). In addition to being a physical barrier, Caco-2 cell monolayers have also been shown to be a biological barrier consisting of metabolic enzymes and apically polarized efflux systems (e.g., P-glycoprotein) (7,22).

When the opioid peptides, themselves, were applied to the AP side of Caco-2 cell monolayers, [Leu^5]-enkephalin was shown to degrade rapidly ($t_{1/2}$ = 15 min), while DADLE was substantially more stable (<10% degradation in 180 min) (16). In contrast, cyclic prodrugs 1 and 2 were shown in this study to be very stable when applied to the AP side (Fig. 3A), indicating that cyclization prevented degradation by brush border exopeptidases (e.g., amino- and carboxypeptidases) and endopeptidases (e.g., enkephalinase). Furthermore, no appearance of the opioid peptides was observed in the receiver side, indicating that the transcellularly routed cyclic prodrugs are also stable to cytosolic enzymes present in Caco-2 cell monolayers. This, however, does not exclude the possibility that the cyclic prodrugs may be degraded by non-peptidase-mediated cytosolic enzymes like cytochrome P450 3A4 (CYP3A4) that are not expressed in significant amounts in Caco-2 cells (6).

Characterization of the physicochemical properties of cyclic prodrugs 1 and 2 (Table III) showed them to be significantly more lipophilic than [Leu^5]-enkephalin and DADLE (13). Furthermore, these phenylpropionic acid-based cyclic prodrugs are also more lipophilic than other cyclic prodrugs of the same opioid peptides made with coumarinic acid (13) and acyloxyalkoxy (14) linkers. These physicochemical characteristics of the phenylpropionic acid-based cyclic prodrugs are consistent with their significant cell membrane permeating abilities. To better understand their lipophilic character and their ability to permeate cell membranes, we conducted conformational studies on these compounds using one- and two-dimensional NMR and circular dichroism (CD) (18). These studies revealed that the phenylpropionic acid-based cyclic prodrugs exhibited more well-defined, rigid and compact secondary structures composed of β-turns that are partially stabilized by formation of intramolecular hydrogen bonds than did the opioid peptides. The formation of the intramolecular hydrogen bonds, as well as the incorporation of the lipophilic phenylpropionic acid promoiety into the relatively hydrophobic peptide sequence, may account

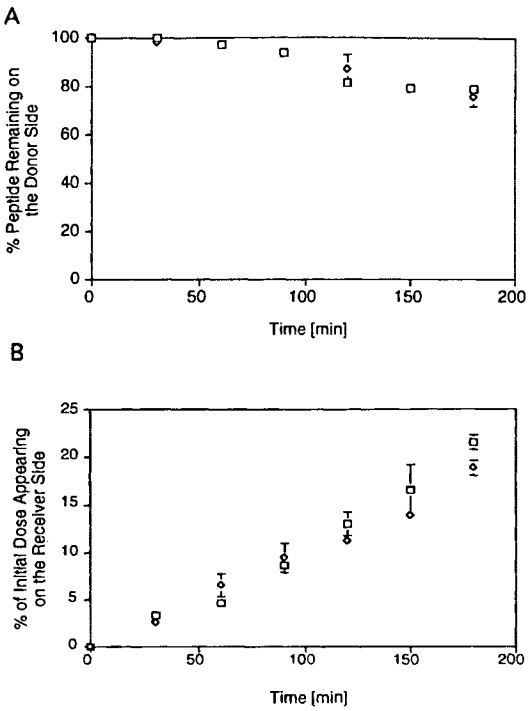


Fig. 3. Time course for the disappearance of cyclic prodrug 1 (◇) and cyclic prodrug 2 (□) and their appearance on the BL side when applied to the AP side of a Caco-2 cell monolayer and incubated up to 3 hours at 37°C. Panel A shows time profiles of the amount remaining in the donor compartment (i.e., AP side); Panel B represents the amount transported to the receiver side (i.e., BL side). Experiments were performed in triplicate (average ± SD).

for the significant membrane interaction potential of these cyclic prodrugs as determined by IAM chromatography (Table III) (13). Furthermore, the existence of these well-defined secondary structures in cyclic prodrugs 1 and 2, particularly the existence of an intramolecular hydrogen bond, also correlates well with their lipophilicity and their ability to permeate the Caco-2 cell monolayers.

It is well known that peptides having favorable physicochemical properties (no charge, small size, hydrophobicity, low hydrogen bonding potential) may permeate cell monolayers mainly by the transcellular pathway (2). The passive flux of peptides permeating the cell monolayers via the transcellular pathway may, however, be limited by their substrate properties for apically polarized efflux systems (e.g., P-glycoprotein) or intracellular enzymes (e.g., cytochrome P450 isozymes) (6,23). The literature suggests that lipophilicity (7), cationic character (7), and the presence of certain peptide bioisosteres (24) tend to predispose a molecule to be a substrate for efflux systems. In addition, it has been reported that some water solubility is required for the recognition (23). As shown in this study, cyclic prodrugs 1 and 2 are not substrates for the polarized efflux systems in Caco-2 cell monolayers in spite of the fact that they are transcellularly transported and very lipophilic. In fact, the phenylpropinic acid-based cyclic prodrugs are significantly more lipophilic than the coumarinic acid-based (16) and acyloxyalkoxy-based cyclic prodrugs (17). It is extremely interesting to note that the least lipophilic cyclic prodrugs in this series (i.e. the acyloxyalkoxy-based prodrugs) are the only compounds that are substrates for the efflux systems in Caco-2 cells (17). It should also be pointed out that acyloxyalkoxy prodrugs are also the most water soluble and, furthermore, they have very different solution conformations than the coumarinic acid and phenylpropionic acid linked prodrugs (25).

Furthermore, it is important to note that many proteins and peptides are rapidly removed from the systemic circulation by the liver (26,27). Hepatic clearance of peptides is known to involve uptake by receptor-mediated endocytosis or carrier-mediated transport by a basolateral multispecific transporter. One of the carrier-mediated systems with potential relevance to transport of hydrophobic peptides is the multidrug resistance (MDR) gene product (27), also known as P-glycoprotein, which is the same protein limiting intestinal mucosa permeation. At this point, *in vivo* studies have not been performed on these compounds. However, we believe that since these cyclic prodrugs are not substrates for P-glycoprotein in Caco-2 cells, they will not be extensively cleared by this mechanism in the liver. *In vivo* studies to confirm this are currently in progress in our laboratory. This does not exclude the possibility that these cyclic prodrugs may be recognized by other carrier-mediated or receptor-mediated processes in the liver and, thus, may be extensively cleared into the bile.

In conclusion, the experimental results presented in this paper illustrate that the use of the phenylpropionic acid promoiety may be a promising approach to increase membrane permeation of opioid peptides by shifting their pathway of permeation to the transcellular route, and simultaneously increasing their metabolic stability. Compared to the coumarinic acid promoiety the disadvantage of using the phenylpropionic acid promoiety may be its high protein binding as well as the slow rate of bioconversion in biological media.

Table III. Physicochemical Properties of [Leu^5]-Enkephalin, DADLE and Cyclic Prodrugs 1 and 2

Compound	MW	Size[a] Å	Membrane interaction potential[b] [log k'_{IAM}]
[Leu^5]-enkephalin	556	5.01	0.17
Cyclic prodrug 1	742	5.01	3.32
DADLE	571	4.77	0.43
Cyclic prodrug 2	756	5.16	3.32

[a] Diffusion coefficients were measured by NMR in DMSO-d_6 and molecular size were calculated by the Stoke-Einstein equation (13).
[b] Capacity factors (k'_{IAM}) were determined by measuring the partitioning of the compounds between 0.01 M phosphate buffer, pH 7.4 and an immobilized artificial membrane column (IAM.PC.DD, 10 cm × 4.6 mm I.D. Regis Technologies, Inc. Morton Grove, IL) (13).

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