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

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Purpose. To evaluate the cellular permeation characteristics and the chemical and enzymatic stability of coumarinic acid-based cyclic prodrugs 1 and 2 of the 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 the 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 chemically to intermediates that further degraded to [Leu^5]-enkephalin and DADLE, respectively, in stoichiometric amounts. In 90% human plasma and rat liver homogenate, the disappearance of cyclic prodrugs 1 and 2 was significantly faster than in HBSS, pH 7.4. The half-lives in 90% human plasma and in rat liver homogenate were substantially longer after pretreatment with paraoxon, a known inhibitor of serine-dependent esterases. 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 similar stability when applied to the AP side of the Caco-2 cell monolayer. Prodrug 1 was 665-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 31 fold more able to permeate a Caco-2 cell monolayer than was DADLE.

Conclusions. Cyclic prodrugs 1 and 2, prepared with the coumarinic 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 various biological media, the opioid peptides were released from the prodrugs by an esterase-catalyzed reaction, which is sensitive to paraoxon inhibition.

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

INTRODUCTION

In recent years, significant advances have been made in opioid research, particularly with respect to the synthesis of peptides with high affinity and selectivity for the different types (μ, δ, κ) of opioid receptors (1,2). These opioid receptor-specific peptides have unique pharmacological properties that warrant their development as therapeutic agents. However, in many cases, the clinical development of these opioid peptides has been prevented because of their poor biopharmaceutical properties, which include metabolic lability and poor cell membrane permeation characteristics. The problem of metabolic lability has for all practical purposes been resolved by medicinal chemists through structural manipulation of the peptides (i.e., introducing peptidase-resistant bonds). Until recently, medicinal chemists have had less success in manipulating the structures of opioid peptides so as to achieve good cell membrane permeation while still retaining high affinity and high selectivity for opioid receptors. Through drug design strategies (3–5), prodrug strategies (6–9), and targeting strategies (10) some progress has been made in recent years in improving the cell membrane permeation characteristics of opioid peptides, particularly their blood-brain barrier (BBB) permeation. However, little progress has been made in developing strategies to improve the permeation of opioid peptides through the intestinal mucosa; thus, improving their oral bioavailability.

Permeation of peptides, including opioid peptides, across the intestinal mucosa can occur via the paracellular pathway or the transcellular pathway. In general, hydrophilic peptides are restricted to the paracellular pathway, which consists of aqueous pores (average size in the small intestine approx. 7–9 Å) created by the cellular tight junctions (11). These aqueous pores restrict peptide permeation based on the size and charge of the molecule (11,12). A hydrophilic peptide, whose permeation is restricted to the paracellular pathway, typically exhibits an oral bioavailability of <1–2% (11). Unfortunately, opioid peptides can be included in the category of paracellular permeants; therefore, they exhibit poor oral bioavailabilities (1,2,13).

In contrast to hydrophilic peptides, hydrophobic peptides that lack charge and exhibit a low hydrogen-bonding potential, can traverse the intestinal mucosa by passive diffusion via the transcellular pathway (14,15). Unfortunately, many of the structural features of opioid peptides [e.g., free N-terminal amino and C-terminal carboxyl groups and side chain carboxyl (e.g., Asp, Glu), amino (e.g., Lys, Arg) and hydroxyl (e.g., Ser, Thr, Tyr) groups] that bestow upon the molecules affinity and specificity for the different opioid receptors also endow the molecules with undesirable physicochemical properties (e.g.,

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ABBREVIATIONS: BBB, blood-brain barrier; PNPB, *p*-nitrophenyl butyrate; PC, palmitoyl-DL-carnitine; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle medium; BSA, bovine serum albumin; AP, apical; BL, basolateral; P_{app}, apparent permeability coefficient; $t_{1/2}$, apparent half-lives.

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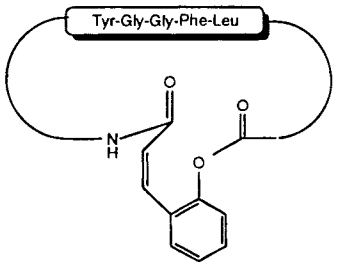
charge, hydrophilicity, high hydrogen bonding potential) that limit their permeation via the transcellular mute.

To succeed in developing orally bioavailable opioid peptides, it will be necessary to incorporate structural features into the molecules that will optimize the pharmacological (i.e., high receptor affinity) as well as the biopharmaceutical (e.g., membrane permeability) properties. In recent years, this approach to drug design has been successfully applied to some opioid peptides (3–5,16). As an alternative approach, unfavorable biopharmaceutical properties of opioid peptides can be transiently modified using prodrug strategies. In general, prodrug strategies applied to peptides (11,17,18), including opioid peptides (6–9), have focused on modification of a single functional group (e.g., N-terminal end). More recently, our laboratories (17,19–24) have introduced the concept of making cyclic prodrugs of peptides as a way to modify the physicochemical properties of the molecules sufficiently to overcome these biopharmaceutical barriers (e.g., intestinal mucosa, BBB).

Recently, our laboratories (24) have described methodology for the synthesis of the coumarinic acid-based cyclic prodrugs 1 and 2 (Fig. 1) of [Leu^5]-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) and its metabolically stable analog DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH), respectively. These coumarinic acid-based cyclic prodrugs were designed to undergo bioconversion *in vivo* to the opioid peptides via a two-step process involving an initial esterase-catalyzed reaction (Scheme 1). In this study, we have demonstrated that cyclic prodrugs 1 and 2 can indeed undergo the bioconversion process shown in Scheme 1 when incubated *in vitro* with biological media having measurable esterase activities. In addition, using Caco-2 cell monolayers, an *in vitro* cell culture model of the intestinal mucosa (25), we have shown that, consistent with their physicochemical properties, cyclic prodrugs 1 and 2 exhibit substantially higher cell permeation than the corresponding opioid peptides and that these prodrugs permeate via the transcellular rather than the paracellular pathway.



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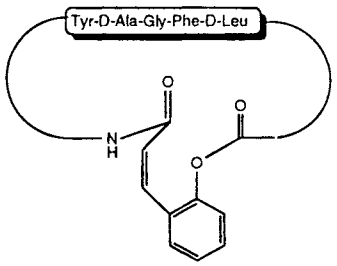

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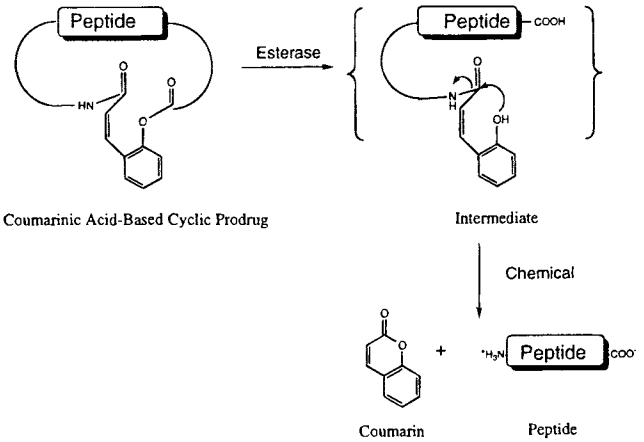
Fig. 1. Coumarinic acid-based cyclic prodrugs 1 and 2.



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

MATERIALS

The coumarinic acid-based prodrugs 1 and 2 where synthesized by Dr Binghe Wang and colleagues at North Carolina State University, following procedures reported elsewhere (24). The opioid peptides, [Leu^5]-enkephalin and DADLE, diethyl *p*-nitrophenyl phosphate (paraoxon, approx. 90%), *p*-nitrophenyl butyrate (PNPB) (~98%), dimethyl sulfoxide (> 99.5%), palmitoyl-DL-carnitine (PC) chloride, 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 acids [10 mM (100×) in 85% saline] were obtained from Gibco BRL, Life Technologies (Grand Island, NY). Dulbecco's modified Eagle medium (DMEM) and trypsin/EDTA solution [0.25% and 0.02%, respectively, in Ca^{+2}- and Mg^{+2}-free Hanks' balanced salt solution (HBSS)] 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). D-1[^{14}C] Mannitol (spec. act. = 2.07 Gbq/mmol) was purchased from Moravek Biochemicals (Brea, CA). 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 (21). Cells were grown in a controlled atmosphere at 5% CO_2 and 90% relative humidity at 37°C in a culture medium consisting of DMEM supplemented with 10% heat-inactivated fetal calf serum, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin and 1% glutamine. For transport experiments, cells were detached from the plastic support when approximately 80% confluent by digestion using trypsin/EDTA solution and were plated on collagen-coated

polycarbonate membranes (Transwells®, 24.5 mm in diameter, 3 μm pore size, purchased from Costar Corporation, Cambridge, MA) that had previously been coated with rat tail collagen at a density of 8.0×10^4 cells/cm^2. The culture medium was replaced every other day for the first 7 days and daily thereafter [apical (AP) volume 1.5 ml, basolateral (BL) 2.6 ml]. All cells used in this study were between passage 52 and 68.

Tissue Homogenate and Human Plasma

Rat Liver Homogenate

Rat livers were obtained from male Sprague-Dawley rats (Animal Care Unit, The University of Kansas, Lawrence, KS) weighing 250–350 g. The tissue was blotted to dryness and after weighing was sliced in small pieces with a scalpel. The liver pieces were then quickly homogenized on ice with ice-cold HBSS (1 ml per 1 g tissue) using a Wheaton glass homogenizer (15 strokes, pestle/wall clearance 0.25–0.76 mm). Aliquots (1 ml) were frozen and kept at −80°C until used. Before each experiment, the homogenate was quickly thawed and rehomogenized on ice with an equal volume of ice-cold HBSS (10–15 strokes, pestle/wall clearance 0.64–0.76 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).

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 from 6 monolayers were collected in 1 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). Before each experiment, the homogenate was quickly thawed and rehomogenized on ice and the cell debris and nuclei were removed by centrifugation as described above.

Human Plasma

Human plasma, stabilized by CPDA-1 solution USP, was obtained from the Topeka Blood Bank (Topeka, KS). Plasma was separated from erythrocytes at 4°C by centrifugation for 10 min at 4,700 rpm (2,000 × g) immediately prior to its use. 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.

Stability Studies

Chemical Stability

The chemical stability of cyclic prodrugs 1 and 2 was determined at 37°C in HBSS, pH 7.4. Solutions of the prodrugs (~100 μM) containing 1% 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 immediately analyzed by HPLC (See HPLC Analyses Section). Apparent half-lives ($t_{1/2}$) for the disappearance of the prodrugs were calculated from the pseudo first-order rate constants obtained by linear regression ($r^2 = 0.97$) of plots of log prodrug concentration remaining vs. time.

Enzymatic Stability

The stability of cyclic prodrugs 1 and 2 in various tissue homogenates and 90% human plasma was determined at 37°C in the presence and absence of paraoxon, an esterase inhibitor. The methodology for determination of total esterase activity using PNPB, as well as the determination of total protein concentrations in these biological media, has been extensively described elsewhere (21). Cyclic prodrugs 1 and 2 (~100 μM, final concentrations) were incubated with the biological matrix containing 1% DMSO, and samples were maintained for 6 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 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 7,500 rpm (5,000 × g) for 90 min (4°C). Aliquots (75 μl) of the filtrate were diluted with methanol and analyzed by HPLC (see HPLC Analysis Section). Apparent half-lives ($t_{1/2}$) for the disappearance of the prodrugs were calculated from the pseudo first-order rate constants obtained by linear regression ($r^2 = 0.93$) of plots of log prodrug concentration remaining vs. time.

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). AP—to—BL flux for this paracellular marker never exceeded values of 0.4%/hr (Papp = 1.27×10^{-7} cm/s). Routinely, cell monolayers were washed 3× with prewarmed HBSS, pH 7.4. Peptide and prodrug solutions prepared in HBSS (~100 μM for opioid peptides and ~20 μM for prodrugs) containing 0.25% DMSO were applied to the donor compartment (AP, 1.5 ml or BL, 2.6 ml). HBSS was added to the receiver compartment (BL or AP) and, subsequently, samples were removed at various intervals up to 180 min from both sides (receiver, 120 μL; donor, 20 μL). The sample volume removed from the receiver side was always replaced with fresh, prewarmed HBSS. To stabilize the samples, aliquots of acetonitrile and diluted phosphoric acid [final concentration 10% (v/v) and 0.01% (v/v), respectively] were added. This acidic mixture (pH ~3) was immediately frozen in a dry-ice/acetone bath and kept at −80°C until HPLC analysis (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 analyzed by HPLC. The extent of

degradation of the cyclic prodrugs during transport experiments was estimated by direct HPLC analysis of samples taken from both the AP and BL sides.

Transport experiments from the AP—to—BL side as well as from the BL—to—AP side were performed in triplicate at 37°C in a shaking water bath (60 rpm). The permeation of the compounds was also assessed in the presence of 100 μM palmitoyl-DL-carnitine (PC).

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, a SIL-10A autoinjector equipped with a sample cooler, and a RF-535 fluorescence detector connected to a CR-4 integrator (Shimadzu, Inc., Tokyo, Japan). 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 uv (λ = 254 nm) or 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.

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 triplicates are presented as means ± SD.

RESULTS

Chemical and Enzymatic Stability

The coumarinic acid-based cyclic prodrugs 1 and 2 were designed to undergo esterase catalyzed hydrolysis of the ester bond linking the C-terminal carboxyl acid of the peptide to the phenolic group of the promoiety. The resulting intermediate should then undergo rapid chemical hydrolyses 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 peptide and coumarin (Scheme 1). Initial experiments were conducted in HBSS, pH 7.4, to determine the chemical stability of cyclic prodrugs 1 and 2 (Fig. 1). The time course of disappearance of cyclic prodrug 2 at 37°C in HBSS, pH 7.4, is shown in Fig. 2. A buildup and disappearance of the intermediate (shown in Scheme 1) was detected followed by the appearance of DADLE and coumarin. A similar time course for the degradation of cyclic prodrug 1 was observed (data not shown). With both cyclic prodrugs, complete hydrolysis to the corresponding opioid peptide and coumarin was observed and mass balance was maintained. The apparent half-lives ($t_{1/2}$s)

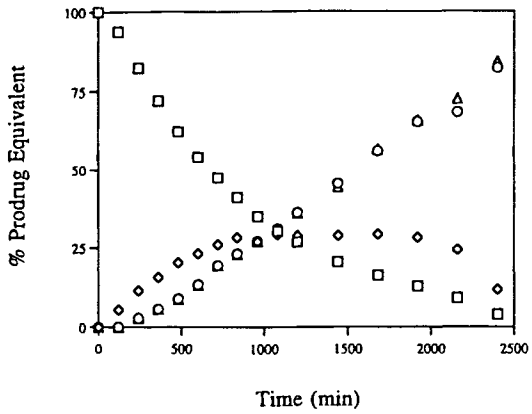


Fig. 2. Stability of cyclic prodrug 2 at 37°C in HBSS buffer, pH 7.4, showing the time course of disappearance of the cyclic prodrug (□) and the appearance of the intermediate (◇), DADLE (○) and coumarin (Δ).

for bioconversion of cyclic prodrugs 1 and 2 to [Leu^5]-enkephalin and DADLE, respectively, are given in Table I.

The stability of cyclic prodrugs 1 and 2 in various biological media (i.e., homogenates of Caco-2 cells and rat liver, and 90% human plasma) having measurable esterase activities were also determined and the $t_{1/2}$ values are presented in Table I. These results show that the $t_{1/2}$s of the cyclic prodrugs in the presence of 90% human plasma and rat liver homogenates were significantly shorter than the $t_{1/2}$s observed in HBSS at pH 7.4. In homogenate of Caco-2 cells, the $t_{1/2}$s of disappearance of cyclic prodrugs 1 and 2 were similar to the values observed in HBSS in spite of the fact that this biological media contained measurable esterase activities.

The effect of paraoxon, a potent esterase inhibitor, on the $t_{1/2}$s of cyclic prodrugs 1 and 2 was determined to prove that the accelerated rates of disappearance observed in human plasma and rat liver homogenates were indeed due to esterase activity. In separate experiments, paraoxon was shown to inhibit the esterase activity present in homogenates of Caco-2 cells, rat liver and in 90% human plasma (Table I). When the stability of cyclic prodrugs 1 and 2 was determined in rat liver homogenates and human plasma pretreated with 1 mM paraoxon for 15 min at 37°C, the $t_{1/2}$s of disappearance of the cyclic prodrugs were significantly longer than those in rat liver homogenates and human plasma not treated with this esterase inhibitor (Table I). Paraoxon pretreatment of the Caco-2 cell homogenate had no significant effect on the $t_{1/2}$s of disappearance of cyclic prodrugs 1 and 2 (Table I). Similarly, inclusion of paraoxon in HBSS, pH 7.4, had no significant effect on the chemical hydrolysis of cyclic prodrugs 1 and 2 (Table I). It should be mentioned that, in contrast to the stoichiometric conversion of cyclic prodrugs 1 and 2 to the opioid peptides observed in HBSS, pH 7.4 (Fig. 2), complete mass balance was not observed in the various biological media employed in these studies (data not shown). This was particularly true of cyclic prodrug 1, since [Leu^5]-enkephalin is rapidly metabolized in these biological media, presumably by amino and carboxyl peptidases (i.e., $t_{1/2} \approx$ 1–2 min in 90% human plasma) (26–27). With cyclic prodrug 2, the formation of DADLE could be detected in the various biological media employed in the study. However, it slowly underwent metabolism, presumably by endopeptidases; thus, complete mass balance was not observed.

Table I. Apparent Half-Lives ($t_{1/2}$) of the Cyclic Prodrugs 1 and 2 in HBSS and Various Esterase Containing Biological Media

Incubation mixture	Specific activity[a] [U/mg protein]	Enzyme concentration [U/ml]		$t_{1/2}$[b] (min)			
				1		2	
		−paraoxon	+paraoxon	−paraoxon	+paraoxon	−paraoxon	+paraoxon
HBSS, pH 7,4	0	0	0	299 ± 11	314 ± 18	375 ± 2	379 ± 22
Human plasma[c]	0.004	0.28	0.11	108 ± 1	260 ± 7	113 ± 8	247 ± 30
Homogenates:							
Caco-2 cells	0.24	0.32	0	295 ± 10	325 ± 11	306 ± 6	354 ± 14
Rat liver[d]	1.15	34.88	0.90	5 ± 1	244 ± 18	7 ± 2	231 ± 4

[a] Determined at 25°C in HBSS, pH 7.4 using PNPB.
[b] Calculated from first-order rate constants.
[c] Human plasma diluted to 90% with HBSS, pH 7.4.
[d] Rat liver homogenates diluted to 50% with HBSS, pH 7.4.

Transport Across Caco-2 Cell Monolayers

The permeability characteristics of [Leu^5]-enkephalin, DADLE and the cyclic prodrugs 1 and 2 were determined using Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa (25). When [Leu^5]-enkephalin was applied to the AP side of Caco-2 cell monolayers, it was rapidly metabolized by peptidases, and no measurable amount of the opioid peptide could be detected in the receiver compartment (BL side) (Fig. 3A and 3B). A maximum apparent permeability (P_{app}) value of 0.31×10^{-8} cm/s was calculated based on the limits of detection of our analytical method for [Leu^5]-enkephalin. In contrast, DADLE, when applied to the AP side of Caco-2 cell monolayers, was relatively stable and could be detected on the receiver side (BL side) of the Caco-2 cell monolayers (Fig. 4A and 4B). Based on these data, a P_{app} value of $7.8 \pm 0.7 \times 10^{-8}$ cm/s was calculated for DADLE.

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

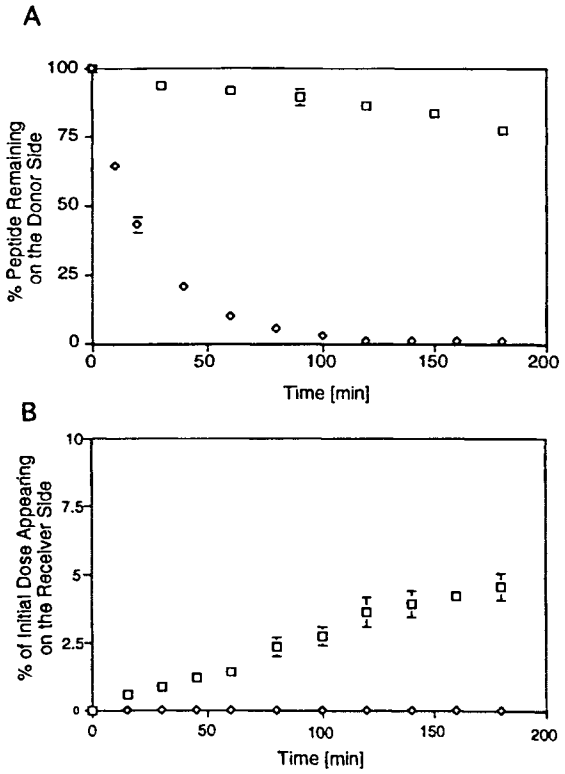


Fig. 3. Time course for the disappearance of cyclic prodrug 1 (□) and [Leu^5]-enkephalin (◇) and their appearance on the BL side when applied to the AP side of a Caco-2 cell monolayers and incubated up to 3 hours at 37°C. Panel A shows time profiles of the amount remaining in the donor compartment (i.e., AP side); Panel B represents the amount transported to the receiver side (i.e., BL side). Experiments were performed in triplicate (average ± SD).

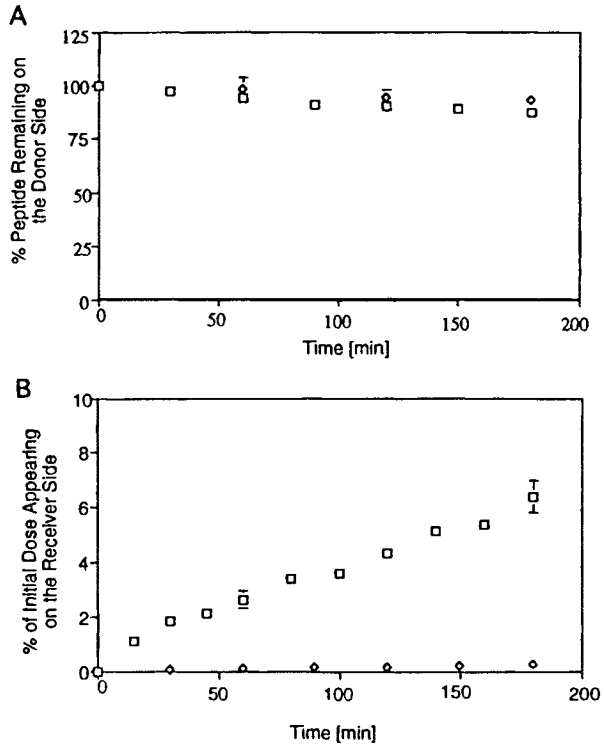


Fig. 4. Time course for the disappearance of cyclic prodrug 2 (□) and DADLE (◇) and their appearance on the BL side when applied to the AP side of a Caco-2 cell monolayers and incubated up to 3 hours at 37°C. Panel A shows time profiles of the amount remaining in the donor compartment (i.e., AP side); Panel B represents the amount transported to the receiver side (i.e., BL side). Experiments were performed in triplicate (average ± SD).

to the AP side of Caco-2 cell monolayers was similar to that observed with DADLE (Fig. 4A). Consistent with the results described above with Caco-2 cell homogenates, little or no bioconversion of cyclic prodrugs 1 and 2 to [Leu^5]-enkephalin and DADLE, respectively, were observed in these transport experiments.

Based on the estimated P_{app} values for [Leu^5]-enkephalin and the determined P_{app} value for cyclic prodrug 1, the cyclic prodrug is approximately 665-fold more able to permeate the Caco-2 cell monolayers than is the opioid peptide (Table II). In comparison, the cyclic prodrug 2 is approximately 31-fold more able to permeate Caco-2 cell monolayers than is the opioid peptide. It should be noted that the P_{app} values for DADLE and cyclic prodrugs 1 and 2 determined in the BL—to—AP direction were identical to the P_{app} values determined in the AP—to—BL direction (Table II).

To determine the route of permeation of the opioid peptides and the cyclic prodrugs across Caco-2 cell monolayers, transport experiments were performed in the absence and presence of PC. In earlier studies (28), our laboratory showed that PC is a modulator of tight junctions in Caco-2 cells and that pretreatment of these cell monolayers with 0.1 mM PC increased the aqueous pore radius from 4.7 Å to 11.3 Å. As shown in Table II, preincubation of a Caco-2 cell monolayers with PC had no effect on the AP—to—BL flux of [Leu^5]-enkephalin, probably because of its extensive metabolism. However, when Caco-2 cell monolayers were incubated with PC, the AP—to—BL permeation of the metabolically stable analog DADLE increased by 60-fold as compared to untreated monolayers, suggesting that this peptide permeates via a paracellular pathway (Table II). In contrast, the permeation of the cyclic prodrugs 1 and 2 increased by only 3.0— and 2.3—fold, respectively, in the presence of PC, suggesting that these molecules traverse the monolayers by the transcellular route (Table II).

DISCUSSION

Chemical and Enzymatic Stability

A fundamental requirement for a successful peptide prodrug is the complete conversion of the prodrug to the peptide by either chemical and/or enzymatic reactions (18). The coumarinic acid—based cyclic prodrugs 1 and 2 were designed to release [Leu^5]-enkephalin and DADLE, respectively, by enzymatic hydrolysis of the ester promoiety to form an intermediate that then undergoes lactonization to release the parent peptide (Scheme 1) (24). The chemical stability assessment done in HBSS, pH 7.4, revealed stoichiometric conversion of the cyclic prodrugs to the opioid peptides (Fig. 2). Since the intermediate shown in Scheme 1 was detected by HPLC during the stability study, we concluded that the rate-determining step in the cascade of reactions leading to the release of the opioid peptide is the lactonization reaction. The observation of the intermediate in HBSS buffer was unexpected, as it is well known that the rate-limiting step for the lactonization of coumarinic acid derivatives, such as of the intermediate in this reaction (Scheme 1), is the collapse of the tetrahedral intermediate, which is general-acid catalyzed (29). Changing the buffer composition could certainly change the ability of the buffer to afford such general-buffer catalyses and, thus, the $t_{1/2}$ of the intermediate. However, detailed examination of the mechanism of this observation is beyond the scope of this study.

The protein binding of cyclic prodrug 2 (20 μM) was estimated in the presence of 1.35 mg/ml bovine serum albumin (BSA) in HBSS buffer, by allowing the cyclic prodrug to equilibrate in this solution for 15 min. The solution, containing the cyclic prodrug 2 and BSA, was then either injected directly onto a HPLC or first filtered through a Ultrafree®MC 5000 NMWL filter unit (Millipore, Bedford, MA) to remove the BSA and then analyzed by HPLC. These studies suggested that cyclic prodrug 2 was approximately 65% protein bound.

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 investigate the susceptibility of these prodrugs to esterases, several biological media were selected, and the stability of the prodrugs was determined. Within the same species, liver, plasma and intestinal mucosa are considered the most important sites of metabolism for esterase—sensitive drugs (30). Therefore, these biological milieus were selected for the stability studies. Based on the spectrophotometric assay using PNPB as a substrate, all of the biological media that were used in this study exhibited measurable levels of esterase activity (Table I).

In human plasma and rat liver homogenate, the apparent $t_{1/2}$s were significantly less for both cyclic prodrugs 1 and 2 than in HBSS at pH 7.4 (Table I). This indicates that the disappearance of the prodrugs may indeed be catalyzed by esterases. Further experimental evidence for the proposed esterase-catalyzed hydrolyses was obtained from using paraoxon, a potent inhibitor of type B esterases (31). Preincubation with paraoxon reduces esterase activity in all biological media by at least 93%, except in human plasma where more then 40% residual activity was found after pretreatment with paraoxon. The $t_{1/2}$ values of the cyclic prodrugs in human plasma and rat liver homogenate were significantly greater after incubation with the esterase inhibitor and more similar to the values observed for the chemical stability of the prodrugs in HBSS buffer at pH 7.4. In Caco-2 cell homogenate, however, the $t_{1/2}$ of disappearance of the cyclic prodrugs in the absence of paraoxon was not different from that in HBSS at pH 7.4, in spite of the fact that this biological medium had high esterase activity. This suggests that the cyclic prodrugs can be cleaved only by a certain family of isozymes that are present in human

Table II. Apparent Permeability Coefficients (P_{app}) of [Leu^5]-Enkephalin and DADLE and Their Coumarinic Acid-Based Cyclic Prodrugs 1 and 2 Across Caco-2 Cell Monolayers in the AP-to-BL Direction, Determined in the Presence and Absence of 0.1 mM Palmitoyl-DL-Carnitine (PC), and in the BL-to-AP Direction

	P_{app} values		
	AP-to-BL $P_{app} \times 10^6$ [cm/s]		BL-to-AP
Peptide	−0.1 mM PC	+0.1 mM PC	$P_{app} \times 10^6$ [cm/s]
[Leu^5]-enkephalin	<0.0031	<0.0031	<0.0031
Cyclic prodrug 1	2.06 ± 0.02	6.14 ± 0.29	2.66 ± 0.12
DADLE	0.078 ± 0.007	4.73 ± 0.39	0.086 ± 0.005
Cyclic prodrug 2	2.42 ± 0.18	5.46 ± 0.27	3.16 ± 0.26

plasma and rat liver homogenate but not in Caco-2 cell homogenate.

Preincubation with paraoxon almost completely inhibited the enzymatic hydrolysis of PNPB in the rat tissue homogenate, indicating that this biological medium contains mainly B-type esterases (31). In contrast, human plasma still showed about 40% residual esterase activity after pretreatment with paraoxon, suggesting the presence of a considerable fraction of A-and/or C-type esterases in this medium (31). Since the rate of disappearance of the cyclic prodrugs in human plasma after preincubation with paraoxon is not the same as the rate of disappearance in HBSS at pH 7.4, the cyclic prodrugs seem to serve as a substrate for B-type esterases as well as for A- and/or C-type esterases. These observations are in agreement with earlier work performed in our laboratory with other cyclic prodrugs (21–22). However, carboxylpeptidases are also known to cleave ester bonds (32); thus, it is not known whether the release of the opioid peptides from the cyclic prodrugs is mediated only by esterases.

Transport Across Caco-2 Cell Monolayer

The major barrier to oral delivery of opioid peptides into the systemic circulation is the intestinal mucosa (11). As previously discussed, tight intercellular junctions limit the flux of opioid peptides to the paracellular route (physical barrier), and brush border peptidases (biological barrier) rapidly metabolize opioid peptides to their corresponding amino acids (11). The coumarinic acid-based cyclic prodrug strategy evaluated in this study could have a positive impact on cell membrane (i.e., intestinal mucosa) permeation by increasing the stability of the opioid peptides to exo- and endopeptidases. Furthermore, cyclic prodrugs 1 and 2 have more favorable physicochemical properties (i.e., size, lipophilicity, hydrogen bonding potential) (see Table III) and solution structures for membrane permeation than do the opioid peptides.

It was, therefore, of interest to investigate the transport and metabolism of the coumarinic acid-based cyclic prodrugs in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa that has been shown to exhibit physical restriction to permeation (25). In addition to being a physical barrier, it is also a biological barrier consisting of metabolic enzymes and apically polarized efflux systems (e.g., P-glycoprotein) (33–34). The permeation of drugs through this culture model has, furthermore, been shown to correlate well with human intestinal permeability (35).

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	683	4.35	2.70
DADLE	571	4.77	0.43
Cyclic prodrug 2	697	4.77	2.91

[a] Diffusion coefficients were measured by NMR in DMSO-d_6 and molecular size were calculated by the Stoke-Einstein equation (data taken from reference 37).

[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) (data taken from reference 37).

When the opioid peptides were applied to the AP side of Caco-2 cell monolayers, [Leu^5]-enkephalin degraded rapidly ($t_{1/2}$ = 15 min) (Fig. 3A), while DADLE was substantially more stable (<10% degradation in 180 min) (Fig. 4A). Compared to [Leu^5]-enkephalin, cyclic prodrugs 1 and 2 were quite stable (<10% degraded in 180 min) when applied to the AP side (Figs. 3A and 4A), indicating that cyclization prevented degradation by brush border exopeptidases (e.g., aminopeptidases and carboxypeptidases) and endopeptidases (e.g., enkephalinase). It should also be noted that, for the cyclic prodrugs that traverse the monolayers via the transcellular pathway, no appearance of the opioid peptides was observed in the receiver side, indicating that the cyclic prodrugs were also stable to cytosolic enzymes. This does not, however, exclude the possibility that the cyclic prodrugs may be degraded by non-peptidase-mediated cytosolic pathways like cytochrome P450 3A4 (CYP3A4) *in vivo*. Unfortunately, CYP3A4 is not expressed in appreciable quantities in Caco-2 cells (36). It should also be noted that there is considerable overlap in substrate specificity for CYP3A4 and P-glycoprotein; i.e., compounds that are substrates for CYP3A4 are usually also substrates for P-glycoprotein (36).

Characterization of the physicochemical properties of cyclic prodrugs 1 and 2 (Table III) showed them to be more lipophilic than [Leu^5]-enkephalin and DADLE (37). These results are consistent with their enhanced cell membrane permeation characteristics (Table II). In an attempt to understand how the solution structure of cyclic prodrugs 1 and 2 might influence their passive diffusion and shift in pathway of permeation, complete conformational studies using one- and two-dimensional NMR, circular dichroism (CD), and molecular dynamics (MD) were performed (38). These studies indicated that the cyclic prodrugs had a more compact and rigid secondary structure composed of a β-turn that is partially stabilized by formation of intramolecular hydrogen bonds, than did the opioid peptides. The existence of this well-defined secondary structure in cyclic prodrugs 1 and 2, particularly the existence of an intramolecular hydrogen bond, also correlates well with the enhanced ability of these prodrugs to permeate the Caco-2 cell monolayers.

For peptides that have favorable physicochemical properties (no charge, small size, hydrophobic, low hydrogen bonding potential), the transcellular route of passive diffusion can predominate (11). However, this passive flux of peptides across the intestinal mucosa by the transcellular route may be limited by their substrate properties for a polarized efflux system (e.g., P-glycoprotein) or intracellular metabolic enzymes (e.g., cytochrome P450 isozymes) (36,39). P-glycoprotein is thought to serve as a cellular efflux pump, actively removing substances from the cytosolic compartment, thus lowering the intracellular concentration of cytotoxins and thereby protecting the cell. The exact functional role of the efflux system and its integration with drug metabolism by CYP3A4 are currently the subject of extensive investigation (36,39). The literature suggests that lipophilicity (34), cationic character (34), and the presence of certain peptide bioisosteres (40) 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 (39). Cyclic prodrugs 1 and 2 are not substrates for the polarized efflux systems even though they are transcellularly transported and very lipophilic. This, however, does not exclude the possibility that cyclic prodrugs of the same opioid peptides formed by different promoieties may still be substrates due to different physicochemical properties and/or solution structures. In fact, we have recently observed that acyloxyalkoxy-based cyclic prodrugs of [Leu^5]-enkephalin and DADLE are substrates for apically polarized efflux systems in Caco-2 cells (41).

Another factor that can significantly influence the overall oral bioavailability of peptides is their rate of clearance by the liver into the bile (42). In recent years, significant progress has been made in elucidating the molecular basis of these clearance mechanisms (42). It is important to note that one of the major pathways by which hydrophobic peptides are cleared by the liver into the bile is mediated by P-glycoprotein, which is the same protein limiting intestinal mucosa and BBB permeation. At this point *in vivo* studies have not been performed on these cyclic prodrugs. However, we hypothesize 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 hypothesis are currently in progress in our laboratory.

In conclusion, the experimental results presented in this paper illustrate that the use of the coumarinic acid promoiety to prepare esterase sensitive cyclic prodrugs may be a promising approach to increasing membrane permeation of peptides by shifting their pathway of permeation to the transcellular route and simultaneously increasing their metabolic stability. It should also be noted that the end product from the promoiety, coumarin, is known to be relatively non-toxic (43). This fact should help to alleviate concern about potential toxic effects introduced by the prodrug promoiety.

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