A Modified Coumarinic Acid-Based Cyclic Prodrug of an Opioid Peptide: Its Enzymatic and Chemical Stability and Cell Permeation Characteristics

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Purpose. To evaluate the chemical/enzymatic stability and the cell permeation characteristics of the modified coumarinic acid-based cyclic prodrug **2** of DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH), which has an aldehyde equivalent (oxymethyl) inserted between the phenolic group of the promoiety and the carboxylic acid group of the peptide.

Methods. The rates of the chemical/enzymatic conversion of the oxymethyl-modified prodrug **2** to DADLE were measured by HPLC. The cellular permeation characteristics of DADLE and its oxymethyl-modified prodrug **2** were measured by HPLC using Caco-2 cells, wild type Madin-Darby Canine Kidney cells (MDCK-WT), MDCK cells transfected with human *MDR1* gene (MDCK-MDR1), and MDCK cells transfected with human *MRP2* gene (MDCK-MRP2) grown onto microporous membranes.

Results. The oxymethyl-modified coumarinic acid-based cyclic prodrug **2** degraded chemically to DADLE in a pH-dependent manner, i.e., rates of conversion increased with increasing pH. The prodrug **2** degraded rapidly in rat plasma ($t_{1/2}$ = 39 min) and rat liver homogenate ($t_{1/2}$ = 59.2 min), but much slower in Caco-2 cell homogenate ($t_{1/2}$ = 678.7 min) and human plasma ($t_{1/2}$ = 264.3 min). In all four cell lines used for transport studies, the flux rates of the oxymethyl prodrug **2** in the basolateral (BL)-to-apical (AP) direction ($P_{app\ BL\text{-}to\text{-}AP}$) were significantly greater than the flux rates in the AP-to-BL direction ($P_{app\ AP\text{-}to\text{-}BL}$). The $P_{app\ BL\text{-}to\text{-}AP}$ /$P_{app\ AP\text{-}to\text{-}BL}$ ratios were >116, 35.1, 21.2, and 12.6 in Caco-2, MDCK-MDR1, MDCK-MRP2, and MDCK-WT cells, respectively. The efflux of the modified prodrug could be inhibited by GF120918 (an inhibitor for P-gp) and cyclosporin A (an inhibitor for P-gp and MRP2).

Conclusions. The oxymethyl-modified coumarinic acid-based cyclic prodrug **2** of DADLE could be converted to DADLE in both chemical and enzymatic media. However, the prodrug was a good substrate for both P-gp and MRP2 suggesting that its permeation across intestinal mucosa and blood-brain barrier would be significantly restricted.

KEY WORDS: esterase-sensitive prodrugs; peptide delivery; opioid peptides; Caco-2 cells; MDCK-WT cells; MDCK-MDR1 cells; MDCK-MRP2 cells; chemical stability; enzymatic stability.

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ABBREVIATIONS: HBSS, Hank's balanced salt solution; AP, apical; BL, basolateral; P_{app}, apparent permeability coefficient; MDCK cells, Madin-Darby Canine Kidney cells; WT, wild type; P-gp, P-glycoprotein; MRP2, multi-drug resistance-related protein 2.

INTRODUCTION

The unfavorable physicochemical properties of peptides pose a great challenge to the clinical development of oral dosage forms of peptide-based drugs (1,2). Peptide-based drugs are typically charged (free C- and N- terminus) and hydrophilic (polar amino acid side chains), with high hydrogen-bonding potential. These physicochemical characteristics restrict the permeation of peptide-based drugs, which contributes to their low oral bioavailability (3). Natural peptide-based drugs are also susceptible to hydrolytic degradation in the intestinal lumen and mucosa (4), whereas peptidomimetics are suspectible to phase I metabolism in the intestinal mucosa and liver (e.g., cytochrome P450 3A4) (5). All of these unfavorable physicochemical and biochemical properties contribute to the poor oral bioavailability of peptide-based drug candidates (3). To circumvent these problems, scientists have attempted to employ formulation and prodrug strategies to improve the systemic exposure of peptides and peptidomimetics after oral administration to animals and humans (1,2,6).

Recently, our laboratory introduced the concept of cyclization of peptides and peptidomimetics as a prodrug strategy to enhance their oral bioavailability (7,8). To achieve cyclization in a bioreversible manner, three linker technologies were developed, using an acyloxyalkoxy linker (9), a phenylpropionic acid linker (10), and a coumarinic acid linker (11). When these linker technologies were applied to the derivatization of opioid peptides and Arg-Gly-Asp (RGD) peptidomimetics, the resulting prodrugs exhibited more favorable physicochemical and biochemical properties than the parent peptides and peptidomimetics for intestinal mucosal permeation (12–15).

However, crucial to the ultimate success of any prodrug strategy is the optimization of the bioconversion rate of the prodrug to the parent drug *in vivo*. Preliminary *in vitro* experiments (16,17) suggested that the cyclic prodrugs of the opioid peptide DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH) underwent bioconversion in rat plasma when using the acyloxyalkoxy or coumarinic acid linkers. However, when these prodrugs were administered i.v. to rats, the elimination rates of the prodrugs by the liver were much faster than their bioconversion rates to DADLE; thus, lower than expected blood levels of this opioid peptide were observed from these prodrugs. (J.Z. Yang, W. Chen and R.T. Borchardt, unpublished data).

Therefore, we attempted to modify the structure of the ester bond between the carboxylic acid group at the C-terminal end of DADLE and the phenolic group of the coumarinic acid linker. This ester bond is the "biological trigger" that, when hydrolyzed by plasma esterases, leads to the bioconversion of the coumarinic acid-based prodrug **1** to DADLE (12,15,16) (Fig. 1). Since this ester bond is sterically hindered and the rates of hydrolysis of ester bonds by esterases are sensitive to steric hindrance (18), we synthesized a modified coumarinic acid-based cyclic prodrug **2** of DADLE (19). Prodrug **2** of DADLE has an aldehyde equivalent (oxymethyl) inserted between the phenolic group of the coumarinic acid promoiety and the carboxylic acid group of the C-terminal of the opioid peptide (Fig. 1). By inserting the oxymethyl moiety, the ester bond of prodrug **2** should be less

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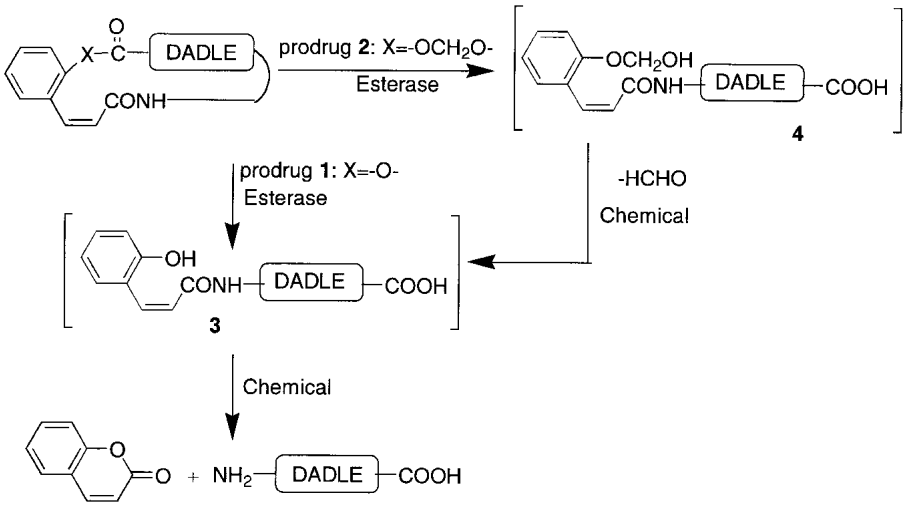

Fig. 1. Pathway for bioconversion of the coumarinic acid-based (**1**) and oxymethyl-modified coumarinic acid-based (**2**) prodrug of DADLE.

sterically hindered than the ester bond of prodrug **1**, which is formed by direct linkage of the phenolic group of coumarinic acid to the carboxylic acid moiety of DADLE. In this paper, we describe the chemical/enzymatic stability and cell membrane permeation characteristics of prodrug **2** and compare these to the characteristics of prodrug **1**.

MATERIALS

The oxymethyl-modified coumarinic acid-based cyclic prodrug of DADLE (**2**) was synthesized following procedures reported elsewhere (19). DADLE, diethyl *p*-nitrophenyl phosphate (paraxon 90%), *p*-nitrophenyl butyrate (PNPB) (98%), dimethyl sulfoxide (>99.5%), Dulbecco's phosphate buffered saline, and Hank's balanced salts (HBSS) (modified) were purchased from Sigma Chemical Co. (St Louis, Missouri). 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, New York). Dulbecco's modified Eagle medium (DMEM) and trypsin/EDTA solution [0.25% and 0.02%, respectively, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution] were purchased from JRH Bioscience (Lenexa, Kansas). Rat-tail collagen (type I) was obtained from Collaborative Biomedical Products (Bedford, Massachusetts), and fetal bovine serum (FBS) from Atlanta Biologicals (Norcross, Georgia). D-1-[^{14}C]-mannitol (specific activity 2.07 GBq/mmol) was purchased from Moravek Biochemicals (Brea, California). All other chemicals and solvents were used as received.

METHODS

Cell Cultures

Caco-2 cells were obtained from American Type Culture Collection (Rockville, Maryland) at passage 18. As described previously (20), cells were grown in a controlled atmosphere of 5% CO_2 and 90% relative humidity at 37°C in 150 cm^2 culture flask using a culture medium consisting of 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% confluency (i.e., 3 to 5 days) was reached, 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 (AP) volume 1.5 ml; basolateral (BL) volume 2.6 ml]. Cells were used in this study between passages 35 and 45.

Wild type Madin-Darby Canine Kidney cells (MDCK-WT), MDCK cells transfected with human *MDR1* gene (MDCK-MDR1), and MDCK cells transfected with human *MRP2* gene (MDCK-MRP2) were obtained as gifts from Professor Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The various MDCK cells were cultured for 6 days under the conditions used to culture Caco-2 cells.

Tissue Homogenates and Plasmas

Caco-2 Cell Homogenate

Confluent Caco-2 cell monolayers (21–28 days) were washed 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 0.25–0.76mm). Cell debris and nuclei were removed as described later in the "Rat Liver Homogenates" Section.

Rat Liver Homogenates

Male Sprague-Dawley rats (Animal Care Unit, The University of Kansas, Lawrence, KS) weighing 250–350 g were sacrificed and an abdominal incision was made to remove the liver. The liver was blotted to dryness, weighed, and sliced into small pieces with a scalpel and homogenized on ice with HBSS (1 ml HBSS/g of liver) using a 15 ml Wheaton glass homogenizer (15 strokes, pestle/wall clearance 0.25-0.76 mm). Aliquots (1.5 ml) of the liver homogenates were frozen and kept at –80°C until used for stability studies. Before conduct-

ing stability experiments, 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 9000 × g using a Marathon 21 K/BR centrifuge (Hermle AG, Gosheim, Germany).

Rat Plasma

Rat blood was freshly drawn from male Sprague-Dawley rats (Animal Care Unit, The University of Kansas, Lawrence, Kansas) weighing 250–350 g anesthetized with Metofane®. Plasma was separated from the erythrocytes at 4°C by centrifuging for 10 min at 2000 × g. For stability experiments, rat plasma was diluted to 90% (v/v) with HBSS, pH 7.4, to maintain the pH of the solution during the experiment.

Human Plasma

Human blood was freshly drawn from a healthy volunteer by the nurse from Student Health Center of the University of Kansas. The blood was processed prior to conducting stability experiments in the same manner as the rat blood.

Chemical Stability

The chemical stability of the cyclic prodrugs was assessed at 37°C in different buffer solutions (sodium acetate, pH 4.0; sodium phosphate, pH 7.0; sodium borate, pH 10.0). The 0.05 M buffer solutions were adjusted to an ionic strength of 0.15 M with NaCl. The prodrug was then dissolved in the buffer to a final concentration of 12.5 μM, and the samples were maintained at 37.0 ± 0.5°C in a temperature-controlled shaking water bath (60 rpm) in sealed vials. Aliquots (20 μl) were removed at various time intervals and immediately stabilized in a solution of 20% acetonitrile in HBSS (v/v) with 0.01% phosphoric acid. Samples were analyzed by HPLC and rate constants were obtained by regression from pseudo-first-order plots of the prodrug concentration vs. time measured for at least two half-lives.

Enzymatic Stability

The stability of the prodrug in various tissue homogenates and rat and human plasma was determined at 37°C in the presence and absence of paraoxon, a potent esterase inhibitor. Total esterase activity in the biologic media was assessed using *p*-nitrophenyl butyrate (PNPB) as a substrate. *p*-Nitrophenol, the final product of this enzymatic reaction, was quantitated spectrophotometrically at $\lambda = 420$ nm using an HP 8452 diode array spectrometer equipped with a temperature-controlled cuvette holder (25°C). Initial velocities were calculated by linear regression and corrected for nonenzymatic hydrolysis. Esterase activities were expressed as units per milligram of protein. One unit represents the amount of enzyme that catalyzes the formation of 1μmol *p*-nitrophenol per minute in HBSS, pH = 7.4 at 25°C. Conditions of linearity for the enzymatic hydrolysis of PNPB were maintained for 300 s between 0.02–2 U/ml. Total protein concentration in the biologic media was determined using Bio-Rad® Protein Assay (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin as a standard.

The cyclic prodrug at a final concentration of 12.5 μM was incubated with the biological matrix containing 1% DMSO; the samples were maintained for at least two half-lives in a temperature-controlled shaking water bath (60 rpm, 37.0 ± 0.5°C). When the esterase inhibitor was included in the experiment, the biologic medium was preincubated with paraoxon (final concentration 1 mM) for 15 min at 37°C before the prodrug was added. At various time intervals, aliquots (20 μl) were removed and the esterase activity immediately quenched by adding 180 μl of freshly prepared 6 N guanidinium hydrochloride solution in acidified HBSS containing 0.01% (v/v) phosphoric acid. The acid mixtures were transferred to an Ultrafree®MC 5000 NMWL filter unit (Millipore, Bedford, Massachusetts) and centrifuged at 7500 × g for 90 min at 4°C. Aliquots of the filtrate were analyzed by HPLC (see HPLC analysis section). Apparent half-lives for the disappearance of the prodrugs were calculated from the pseudo first-order rate constants obtained by a non-linear regression model.

Transport Experiments

Cell monolayers were grown on collagen-coated polycarbonate filters (Transwell®). All studies were done in triplicate in a shaking water bath at 37°C. The integrity of each batch of cells was tested by measuring [^{14}C]-mannitol flux in representative monolayers and the activity of P-glycoprotein (P-gp) was monitored by measuring the bi-directional flux of [^{3}H]-digoxin, i.e., AP-to-BL and BL-to-AP. Cell monolayers were washed three times with prewarmed HBSS, pH 7.4. The prodrug (50 μM) dissolved in HBSS with 1% DMSO was applied to the donor compartment (AP: 1.5 ml; BL: 2.6 ml). HBSS with 1% DMSO was added to the receiver compartment and samples were taken at intervals up to 90 min (donor: 10 μl; receiver: 100 μl). The samples were stabilized in acetonitrile (10% v/v) and diluted phosphoric acid (0.01% v/v) in HBSS. This mixture was immediately frozen and kept at −80°C until analysis was done by HPLC. Permeability coefficients (P_{app}) of the compounds were calculated by: $P_{app} = (\Delta Q/\Delta t)/(A \times C_0)$, where $\Delta Q/\Delta t$ is the linear appearance rate of mass in the receiver solution, A is the cross-section area (4.71 cm^2), and C_0 is the initial concentration of the donor side at $t = 0$.

[^{3}H]-Digoxin, a substrate for P-gp (22–23), and [^{3}H]-vinblastine, a substrate for both P-gp and MRP2 (22–23) were used to check the functional efflux activity of P-gp and MRP2 in these cell models (22–23). The ratios of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ of [^{3}H]-digoxin across Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers were 14, 8.4, and 33, respectively. These ratios were decreased to 1.1 in the presence of P-gp specific inhibitor GF102918 (2 μM) (24). The ratios of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ of [^{3}H]-vinblastine across Caco-2, MDCK-WT, and MDCK-MRP2 cell monolayers were 18, 15, and 36, respectively. These ratios were decreased to 1.9, 1.7, and 16, respectively, when the P-gp inhibitor GF 120918 (2 μM) was included in the incubation media.

HPLC Analysis

A Shimadzu 10A gradient system (Shimadzu. Inc, Tokyo, Japan) consisting of LC-10AS pumps and an SCL-10A controller connected to an SPD-10A UV detector was employed for HPLC analysis. A Shimadzu SIL 10A autoinjector was used to inject samples into a reversed-phase C_{18} column (Vydac 218TP54, 5 μM, 250 × 4.6 mm). The gradient was set

at flow rate of 1 ml/min from 26 to 90% (v/v) acetonitrile in water using trifluoroacetic acid (0.1% v/v) as the ion-pairing agent. The low limit of quantification (LLQ) of the peptide prodrug was 10 nM. The recovery of the peptide prodrug in the transport experiments was consistently > 95%.

RESULTS

Chemical and Enzymatic Stability

The chemical stabilities of the cyclic peptide prodrugs **1** and **2** were determined in aqueous buffer solutions at pH = 4.0, 7.0, and 10.0, at 37°C. The disappearance of prodrugs **1** and **2** followed pseudo-first order kinetics at all three pH values. However, both prodrugs, during their degradation, exhibited the buildup of an intermediate as detected by HPLC (see Fig. 3). In addition, the appearance rates of DADLE lagged behind the disappearance rates of the prodrugs (e.g., in HBSS, pH 7.4, Fig. 2). Although the conversion of the prodrug **2** to DADLE may involve an additional intermediate **4** and an additional chemical step (i.e., **4** to **3**, release of formaldehyde) (Fig. 1), qualitatively, prodrugs **1** and **2** exhibited identical degradation product profiles as determined by HPLC at all three pH values studied (e.g., in phosphate buffer, pH = 7.0, Fig. 3). Prodrug **2** is slightly less stable than prodrug **1** at pH = 4.0 and 7.0, whereas, both prodrugs became very unstable when the pH was increased to 10.0 (Table I).

Both cyclic prodrugs **1** and **2** were designed to undergo bioconversion to DADLE by an initial enzyme-catalyzed step which leads to the hydrolysis of the ester bond linking the peptide to the coumarinic acid linker, resulting in intermediates **3** and **4**, respectively (Fig. 1). In an attempt to estimate the enzymatic stability of the ester bonds in these two prodrugs, the rates of bioconversion of prodrug **2** to DADLE were determined in various biologic media (i.e., Caco-2 cell and rat liver homogenates, and rat and human plasma) and compared to the bioconversion rates reported for prodrug **1**. The activity of esterases in the various biologic media were indirectly determined using PNPB as a substrate (21). The esterase activity (determined using PNPB as a substrate) detected in the various biologic media in this study (Table II) is consistent with data reported previously by our laboratory (16) (i.e., rat liver homogenate> rat plasma>> Caco-2 cell homogenate = human plasma). When the stability of prodrug **2** was determined in the various biologic media, the order of instability was shown to be: rat plasma> rat liver homogenate> human plasma> Caco-2 cell homogenate (Table II). It is interesting to note that prodrug **2** was more stable in human plasma and Caco-2 cell homogenates than in HBSS, pH 7.4, in spite of the presence of significant esterase activity in these biologic media. When paraoxon, a potent esterase inhibitor, was added to all of the biologic media, the stability of prodrug **2** increased significantly, particularly in rat liver homogenate and rat plasma.

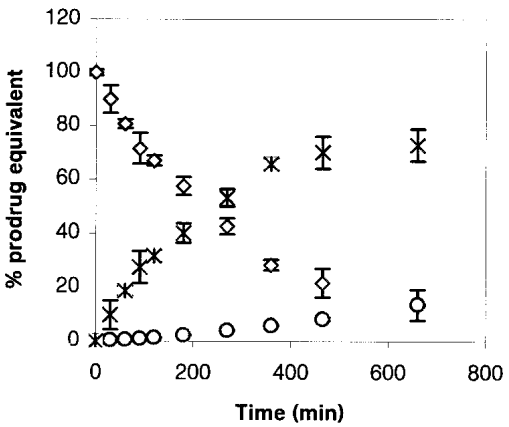

Fig. 2. Chemical stability of prodrug **2** at 37°C in HBSS at pH 7.4. Experiments were performed in triplicate (average ± S.D.). ◇—◇, prodrug 2; ○—○, DADLE; ✶—✶, intermediate.

Transport Study

To determine the permeation characteristics of the oxymethyl-modified coumarinic acid-based prodrug **2**, four cell lines (Caco-2, MDCK-WT, MDCK-MDR1, and MDCK-MRP2) were employed. Initial experiments were conducted using Caco-2 cells since this cell line has been used by our laboratory to determine the permeation characteristics of the coumarinic acid-based prodrug **1** (22). MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cells were used to characterize the substrate specificity of prodrug **2** for P-glycoprotein (P-gp) vs. multidrug resistance-related protein (MRP2) in a manner similar to that reported recently by our laboratory for the coumarinic acid-based prodrug **1** (22) and the acyloxyalkoxy-based prodrug of DADLE (23).

When prodrug **2** was applied to either the AP or BL side of the Caco-2 cell monolayers for up to 2 h, the formation of DADLE was not detected (data not shown). When prodrug **2** was applied to the AP side of Caco-2 cell monolayer, no detectable prodrug or DADLE appeared on the BL side of the monolayer. Based on the limits of detection of the HPLC assay, a maximal $P_{app\ AP\text{-}to\text{-}\ BL}$ of 1×10^{-7} cm/sec was calculated.

In contrast, when prodrug **2** was applied to the BL side of Caco-2 cell monolayers, significant amounts of the prodrug appeared with time on the AP side. From these data, a $P_{app\ BL\text{-}to\text{-}AP}$ value of $1.16 \pm 0.13 \times 10^{-5}$ cm/sec was calculated. The highly polarized transport (i.e. BL-to-AP >> AP-to-BL flux) of prodrug **2**, which is reflected in the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of > 116, suggests that this molecule is a substrate for efflux transporter(s) in Caco-2 cells. Further evidence in support of this conclusion was obtained by determining the transport of prodrug **2** in the presence of GF120918, a known inhibitor of P-gp (24), and cyclosporin A, a known inhibitor of both P-gp and MRP2 (25,26). In the presence of GF120918 and cyclosporin A, permeation of prodrug **2** in the AP-to-BL direction was detected and the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was reduced to approximately 10 (Table III).

In an attempt to determine which efflux transporter (P-gp or MRP2) is involved in limiting the permeation of prodrug **2** in Caco-2 cells, we conducted experiments with MDCK-MDR1 and MDCK-MRP2 cells. We also employed MDCK-WT as a control. As seen in Table III, MDCK-WT cells also showed polarized efflux of prodrug **2** (i.e., $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ = 12.6). When transport studies were conducted in MDCK-MDR1 and MDCK-MRP2 cell monolayers, the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios increased to 35.1 and 21.2, respectively (Table III). The $P_{app\ BL\text{-}to\text{-}AP}$/

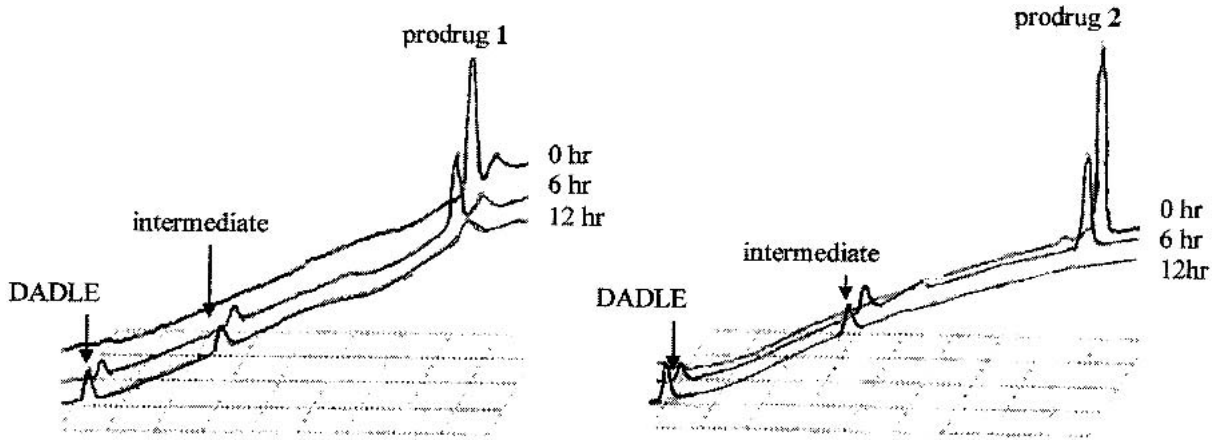

Fig. 3. Typical HPLC chromatograms of prodrug **1** (part A) and prodrug **2** (part B) in 0.05 M phosphate buffer solution at pH 7.0 (μ = 0.15) at 37°C. Samples were taken at various time intervals, and were analyzed using a gradient set at flow rate of 1ml/min from 26 to 90% (v/v) acetonitrile in water with trifluoroacetic acid (0.1% v/v) as the ion-pairing agent.

$P_{app\ AP\text{-}to\text{-}BL}$ ratios of prodrug **2** in MDCK-WT, MDCK-MDR1, and MDCK-MRP2 could be reduced to 1.9, 6.9, and 7.6, respectively, in the presence of GF120918 and cyclosporin A.

DISCUSSION

Chemical and Enzymatic Stability

A crucial requirement for the success of a peptide prodrug is its ability to be converted quantitatively to the parent peptide by chemical and/or enzymatic reactions (27). Therefore, we conducted chemical and enzymatic stability studies of prodrugs **1** (16) and **2** that were designed to assess their potential to undergo bioconversion *in vivo*.

From the chemical stability studies, we observed that both prodrugs **1** and **2** degraded to a common intermediate and to DADLE at all three pH values studied (Fig. 2, Fig.3). Earlier, our laboratory showed that prodrug **1** degraded chemically and enzymatically to DADLE through the intermediate **3** (15,16). Because the intermediate generated from prodrug **2** has a retention time identical to that of intermediate **3** generated from prodrug **1**, we believe that prodrugs **1** and **2** degrade via this common intermediate. However, we cannot totally rule out the possibility that the intermediate generated from prodrug **2** is intermediate **4** or a mixture of intermediates **3** and **4** that happened to co-elute on this HPLC system. If the intermediate generated from the chemical degradation of prodrug **2** is intermediate **3**, then the rate-determining step in its conversion to DADLE is the lactonization of **3**, not the conversion of **4** to **3**. Based on data published by Cox and Yates (28), who studied the chemical degradation of $PhOCH_2OAc$, it is reasonable to hypothesize that the chemical breakdown of $PhOCH_2OH$ to formaldehyde and phenol is a fast reaction.

Table I. The pH/Rate Profile for the Chemical Degradation of Cyclic Peptide Prodrug 1 and 2 Determined in 0.1 M Buffer Solution at pH = 4.0, 7.0, and 10.0 (μ = 0.15) at 37°C

	$t_{1/2}$ (min)[a]		
	pH 4.0	7.0	10.0
prodrug 1	477.8 (46.8)	364.3 (5.0)	9.4 (0.4)
prodrug 2	387.7 (21.8)	291.8 (1.4)	10.8 (0.9)

Note: Apparent half-lives were calculated from the disappearance of the prodrugs as described in Methods.

[a] Results were presented as $t_{1/2}$ (S.D) (min) of triplicate determinations.

To estimate the bioconversion rate of prodrug **2** *in vivo*, its enzymatic stability was determined *in vitro* in several biologic media (Table III). Prodrug **2** was shown to undergo bioconversion to DADLE in all biologic media tested. In the presence of paraoxon, an esterase inhibitor, the bioconversion rates were significantly decreased, suggesting that the esterases in these media were responsible for the transformation of prodrug **2** to DADLE. It is interesting to note that the $t_{1/2}$ values of prodrug **2** in human plasma (264.3 min) and in Caco-2 cell homogenate (678.7 min) were longer than the $t_{1/2}$ in HBSS pH = 7.4 (218.0 min). Since significant esterase activity exists in human plasma and Caco-2 cell homogenates, a possible explanation for these observations is that prodrug **2** is highly protein bound in these biologic media, limiting the prodrug's "free fraction", which ultimately is available for bioconversion by esterase. In rat plasma and rat liver homogenate, the $t_{1/2}$ values of prodrug **2** (39 min in rat plasma and 59.2 min for rat liver homogenate) were shorter than the $t_{1/2}$ in HBSS pH 7.4, which is consistent with the esterase activity present in these biologic media.

Transport Study

The intestinal epithelium constitutes a major barrier for oral delivery of peptides and peptidomimetics (3). In considering the possible pathways of penetration of a peptide or peptidomimetic across the intestinal epithelium, one needs to consider not only passive diffusion (29) and passive diffusion facilitated by a transporter (e.g., peptide transporters) (30,31) but also passive diffusion restricted by efflux transporter(s) (e.g., P-gp and MRP2) (32).

In humans, P-gp is a 170-kDa protein encoded by *MDR1* gene (33,34). It is located on the AP side of the some cell membranes (e.g., intestinal epithelial cells) and acts as a secretary detoxifying system to protect cells from the damage of environmental toxic agents (34). P-gp is not only overex-

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

	Specific activity (U/mg protein)[a]	Enzyme concentration (U/ml)		$t^b_{1/2}$(min)	
		w/o paraoxon	w/ paraxon	w/o paraoxon	w/ paraxon
HBSS (pH = 7.4)	0	0	0	218.0(39.2)	247.2(26.0)
Caco-2 homogenates	0.04	0.02	0.005	678.7(68.8)	1039.4(122.7)
Rat liver homogenates[c]	0.5	17.5	0.5	59.2(0.9)	573.0(122.7)
Rat plasma[d]	0.2	6.1	0.1	39(2.0)	164(6)
Human plasma[d]	0.02	0.8	0.4	264.3(66.6)	396.4(109.7)

[a] Determined at 25°C in HBSS, pH 7.4, using PNPB as a substrate as described in the Experimental Section.
[b] Calculated from first-order rate constants.
[c] Diluted to 50% with HBSS, pH 7.4.
[d] Diluted to 90% with HBSS, pH 7.4.

pressed in tumor cells but is also distributed throughout normal tissues, including liver, kidney and intestinal mucosa (35). Therefore, P-gp in the intestinal mucosa acts as a biologic barrier to the absorption of some drugs and acts as a clearance pathway in the liver and kidney (32,36). MRP2 is also localized on the AP side of canalicular membranes of hepatocytes and on the AP side of intestinal mucosa and kidney cells (37). It has been reported to function to produce drug resistance in cancer cells and restrict the absorption of orally administered drugs through the intestinal mucosa (37,38).

Caco-2 cells, a cell culture model of the intestinal mucosa, have been used not only to determine the passive diffusion characteristics of drug molecules (39) but also to determine the substrate activity of a drug for efflux transporters (40). Since Caco-2 cells express multiple efflux systems (e.g., P-gp and MRP2) (41,42), it is difficult to determine the "efflux transporter substrate/inhibition profile" of a drug using this cell culture model. Therefore, MDCK cells have been transfected with human genes for efflux transporters (e.g., *MDR1* and *MRP2*), leading to overexpression of that transporter and thus a potential model for determining the "efflux transporter substrate/inhibition profile" of a drug (43,44).

The transport data generated in this study (Table III) indicate that prodrug **2** is a substrate for one or more efflux transporters. In Caco-2 cells, the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio, which is used as an indicator of the efflux activity, of prodrug **2** (>116) indicated that it was a good substrate for P-gp and/or MRP2 present in this cell line (45,46). In addition, the result that the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of prodrug **2** is higher than the ratio of prodrug **1** ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ = 71) (22) suggested that prodrug **2** is a better substrate for the efflux transporters presented in this cell line. In the presence of inhibitors of efflux transporters (i.e., GF120918, a P-gp specific inhibitor (24), and cyclosporin A, an inhibitor for both P-gp and MRP2(26,47)), polarized efflux of prodrug **2** in Caco-2 cells was substantially reduced (Table II). The results with GF120918 and cyclosporin A suggested that the transporters in Caco-2 cells mediating the efflux of prodrug **2** are probably P-gp and MRP2. To elucidate the "efflux transporter substrate profile" for prodrug **2**, experiments similar to those described with prodrug **1** (22) were conducted using MDCK-MDR1 and MDCK-MRP2 cells.

MDCK-MDR1 cells overexpress human P-gp and exhibit greater polarized efflux of P-gp substrates than do MDCK-WT cells (46). In MDCK-MDR1 cells, prodrug **2** showed a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 35.1, which was higher than that in MDCK-WT cells (12.6), suggesting that prodrug **2** was a substrate for P-gp. Similarly, MDCK-MRP2 cells over express MRP2 and exhibit greater polarized efflux of MRP2 substrates than do MDCK-WT cells (45). The $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio in MDCK-MRP2 of 21.2 is higher than the ratio in the MDCK-WT (12.6), suggesting that prodrug **2** was also a substrate for MRP2. GF120918 and cyclosporin A inhibited the polarized efflux of prodrug **2** in all three MDCK cell lines (Table II), producing further support for our hypothesis that prodrug **2** is a substrate for both P-gp and MRP2.

MDR1 and MRP2 are expressed in the cell membrane of intestinal epithelial cells and brain capillary endothelial cells, and in the canalicular membrane of hepatocytes (35,37). These efflux transporters act as detoxifying clearance systems in these tissues (35,37). Previous studies from our laboratory showed that prodrug **1** was a substrate for both P-gp and MRP2 in Caco-2 cells and MDCK cells (22). This substrate activity of prodrug **1** for these efflux transporters leads to its low permeation across the intestinal mucosa as confirmed using an *in situ* perfused rat ileum model and its low permeation across the blood-brain barrier as confirmed using an *in*

Table III. Apparent Permeability Coefficients (P_{app}) of Prodrug 2 across Different Cell Monolayers Determined in the Presence and Absence of MDR1 and MRP2 Inhibitors

	P_{app}, cm/s ($\times 10^7$) (w/o inhibitor)[a]			P_{app}, cm/s ($\times 10^7$) (w/ GF 120918 + Cyclosporin A)[a]		
	AP-to-BL	BL-to-AP	Ratio	AP-to-BL	BL-to-AP	Ratio
Caco-2	<0.10	116.56(13.95)	>116.6	4.41(0.73)	44.45(2.81)	10.1
MDCK-WT	1.30(0.71)	16.38(1.80)	12.6	2.65(0.32)	4.93(0.12)	1.9
MDCK-MDR1	0.43(0.12)	15.25(0.52)	35.1	1.11(0.25)	7.65(0.57)	6.9
MDCK-MRP2	0.53(0.21)	11.30(0.98)	21.2	1.66(0.19)	12.59(0.54)	7.6

[a] Results were presented as average $P_{app} \times 10^{-7}$ cm/s (S.D.) of triplicate determinations.

situ perfused rat brain model (Weiqing Chen, unpublished data). Moreover, the substrate activity of the prodrug for efflux transporters also leads to its high biliary clearance rate (approx. 30%) as observed in pharmacokinetic studies (Jerry Yang, Weiqing Chen, and R. T. Borchardt, unpublished data). Because prodrug **2** exhibits an "efflux transporter substrate profile" similar to that of prodrug **1** (i.e., P-gp and MRP2), we expect that prodrug **2** will also have restricted permeation across the intestinal mucosa and blood-brain barrier and high biliary clearance in pharmacokinetic studies (these studies are currently in progress in our laboratory).

In conclusion, the oxymethyl-modified coumarinic acid-based cyclic prodrug **2** of DADLE appeared to be sensitive to esterases. However, like prodrug **1**, the oxymethyl-modified coumarinic acid-based cyclic prodrug **2** exhibited substrate activity for efflux transporters (i.e., P-gp and MRP2), which *in vivo* would be predicted to limit its permeation across the intestinal mucosa and blood-brain barrier and facilitate its clearance by the liver.

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