# Acyloxyalkoxy-Based Cyclic Prodrugs of Opioid Peptides: Evaluation of the Chemical and Enzymatic Stability as Well as Their Transport Properties Across Caco-2 Cell Monolayers

**Annette Bak,[1] Olafur S. Gudmundsson,[2,3] Gitte J. Friis,[1] Teruna J. Siahaan,[2] and Ronald T. Borchardt[2,4]**

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***Purpose.*** To evaluate the chemical and enzymatic stability, as well as the cellular permeation characteristics, of the acyloxyalkoxy-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 1 and 2 to [Leu$^5$]-enkephalin and DADLE, respectively, were measured by HPLC in HBSS, pH = 7.4, and in various biological media (e.g., human plasma and Caco-2 cell and rat liver homogenates) having measurable esterase activity. The cellular permeation and metabolism characteristics of [Leu$^5$]-enkephalin, DADLE and the cyclic prodrugs 1 and 2 were measured using Caco-2 cell monolayers grown onto microporous membranes and monitored by HPLC.

***Results.*** Cyclic prodrugs 1 and 2 degraded slowly but stoichiometrically to [Leu$^5$]-enkephalin and DADLE, respectively, in HBSS, pH = 7.4. In homogenates of Caco-2 cells and rat liver, as well as 90% human plasma, the rates of disappearance of the cyclic prodrugs were significantly faster than in HBSS. The stabilities of the cyclic prodrugs 1 and 2 were increased significantly in 90% human plasma and Caco-2 cell homogenates when paraoxon, a potent inhibitor of serine-dependent esterases, was included in the incubation mixtures. A similar stabilizing effect of paraoxon was not observed in 50% rat liver homogenates, but was observed in 10% homogenates of rat liver. When applied to the AP side of a Caco-2 cell monolayer, DADLE and cyclic prodrugs 1 and 2 exhibited significantly greater stability than [Leu$^5$]-enkephalin. Based on their physicochemical properties (i.e., lipophilicity), cyclic prodrugs 1 and 2 should have exhibited high permeation across Caco-2 cell monolayers. Surprisingly, the AP-to-BL apparent permeability coefficients ($P_{App}$) for cyclic prodrugs 1 and 2 across Caco-2 cell monolayers were significantly lower than the $P_{App}$ value determined for the metabolically stable opioid peptide DADLE. When the $P_{App}$ values for cyclic prodrugs 1 and 2 crossing Caco-2 cell monolayers in the BL-to-AP direction were determined, they were shown to be 36 and 52 times greater, respectively, than the AP-to-BL values.

***Conclusions.*** Cyclic prodrugs 1 and 2, prepared with an acyloxyalkoxy promoiety, were shown to degrade in biological media (e.g., 90% human plasma) via an esterase-catalyzed pathway. The degradation of cyclic prodrug 1, which contained an ester formed with an L-amino acid, degraded more rapidly in esterase-containing media than did prodrug 2, which contained an ester formed with a D-amino acid. Cyclic prodrugs 1 and 2 showed very low AP-to-BL Caco-2 cell permeability, which did not correlate with their lipophilicities. These low AP-to-BL permeabilities result because of their substrate activity for apically polarized efflux systems.

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

## INTRODUCTION

Recently, our laboratory described methodologies for the synthesis of cyclic prodrugs of the opioid peptides [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) using phenylpropionic acid or coumarinic acid linkers (1). These cyclic prodrugs were shown to have the physicochemical properties (i.e., high lipophilicity, low hydrogen bonding potential) that should permit optimal transcellular permeation (1). The predictions concerning the membrane permeation characteristics of these cyclic prodrugs were confirmed by conducting transport experiments using Caco-2 cell monolayers, a cell culture model of the intestinal mucosa (2,3). For example, the coumarinic acid-based and phenylpropionic acid-based cyclic prodrugs of DADLE were shown to be approximately 31- and 77-fold, respectively, more able to permeate Caco-2 cell monolayers than DADLE itself. In addition, these cyclic prodrugs were shown not to be substrates for efflux systems (e.g., P-glycoprotein) that could have reduced their ability to permeate the Caco-2 cell monolayers (2,3).

Earlier, our laboratory described the use of an acyloxyalkoxy linker to make a cyclic prodrug of a model hexapeptide (4). This acyloxyalkoxy-based cyclic prodrug was shown to undergo bioconversion to the parent peptide via an esterase-sensitive pathway (Fig. 1) and they were also shown to have improved metabolic stability to peptidases and better permeation characteristics than the model hexapeptide (5). In addition, the enhanced membrane permeation characteristics of this cyclic prodrug could be attributed in part to its more favorable physicochemical properties (i.e., lipophilicity) (5) and in part to the formation of unique solution structures that reduced its hydrogen bonding potential compared to that of the parent hexapeptide (6).

Recently, our laboratory (7) has applied this acyloxyalkoxy linker technology to the preparation of cyclic prodrugs 1 and 2 of [Leu$^5$]-enkephalin and DADLE, respectively (Fig. 2). In this manuscript, we describe the chemical and enzymatic stability, as well as the cellular permeation characteristics, of cyclic prodrugs 1 and 2. Surprisingly, in spite of their optimal physicochemical properties (i.e., high lipophilicity, low hydrogen bonding potential) for transcellular permeation, these cyclic prodrugs were shown to have very low permeation across Caco-2 cell monolayers, which could be attributed to their substrate activities for apically polarized efflux systems (e.g., P-glycoprotein).

[1] Department of Analytical and Pharmaceutical Chemistry, The Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark.

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

[3] Present address: Exploratory Biopharmaceutics and Drug Delivery, Bristol-Myers Squibb Pharmaceutical Research Institute, One Squibb Drive, P.O. Box 191, New Brunswick, New Jersey 08903-0191.

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

**ABBREVIATIONS:** HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle medium; AP, apical; BL, basolateral; CsA, cyclosporin; $P_{App}$, apparent permeability coefficient; $t_{1/2}$, apparent half-lives.

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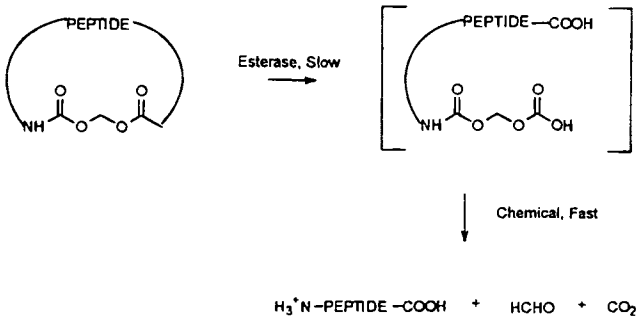



**Fig. 1.** Proposed pathway of bioconversion of acyloxyalkoxy-based cyclic prodrugs of peptides in esterase-containing medium.

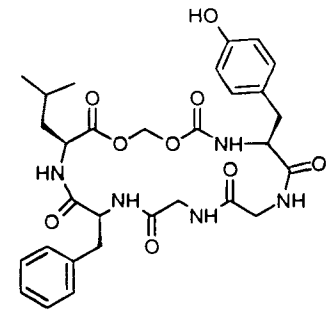
## MATERIALS AND METHODS

### Materials

The acyloxyalkoxy-based cyclic prodrugs 1 and 2 were prepared as described elsewhere (7). The opioid peptides, [Leu$^5$]-enkephalin and DADLE, as well as diethyl p-nitrophenyl phosphate (paraoxon, approx. 90%), p-nitrophenyl butyrate (approx. 98%), cyclosporin (CsA), and Hanks' balanced salts (modified) (HBSS) were purchased from Sigma Chemical Co. (St. Louis, MO). Commercial sources of the materials used to culture Caco-2 cells are described in detail elsewhere (2).

### Stability Studies

The chemical and enzymatic stability of cyclic prodrugs 1 and 2 were determined in duplicate at 37°C in HBSS, pH 7.4, Caco-2 cell homogenates, rat liver homogenates (diluted to 10% or 50% with HBSS) and human plasma (diluted to 90% with HBSS). The preparation of 90% human plasma and homogenates of Caco-2 cells and rat liver, as well as the determination of the total esterase activity in the biological media (assessed using p-nitrophenyl butyrate as a substrate) and the protein concentration [assessed using the Bio-Rad® protein assay (Bio-Rad Laboratories, Richmond, CA)] have been described elsewhere (2). For esterase inhibition studies, all the media were preincubated for 15 min at 37°C with paraoxon (final conc.: 1 mM), an inhibitor of type B esterases. Cyclic prodrugs 1 and 2 were incubated at a concentration of 200 μM in prewarmed HBSS or the respective biological matrix containing 1% DMSO in a temperature-controlled shaking water bath (60 rpm) at 37°C. Samples (20 μl) withdrawn at various time points were quenched immediately with 150 μl ice-cold acidified 6 N guanidine hydrochloride in HBSS containing 0.01% (v/v) phosphoric acid. Samples withdrawn from incubation mixtures containing rat liver homogenate and human plasma were filtered through an Ultrafree® MC 5000 NMWL filter by centrifugation at 7500 rpm and diluted with ice-cold acetonitrile as previously described (2). All the samples were immediately frozen in a dry-ice/acetone bath and kept at −80°C until HPLC analysis. Aliquots (100 μl) of each sample were analyzed by HPLC using uv detection as described below and apparent half-lives for the disappearance of the prodrugs were calculated from the rate constants obtained by linear regression from pseudo-first-order plots of prodrug concentration vs. time. Reactions were followed for at least two half-lives.



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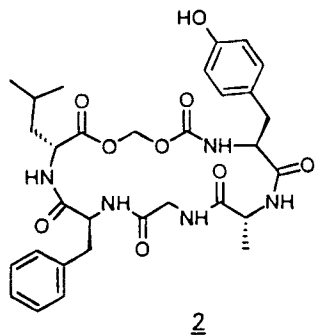

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**Fig. 2.** Structures of the acyloxyalkoxy-based cyclic prodrugs 1 (derived from [Leu$^5$]-enkephalin) and 2 (derived from DADLE).

### Transport Experiments

Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Rockville, MD) and cultured on collagen-coated polycarbonate filters (Transwells®, Costar Corporation, Cambridge, MA) as described previously (2,8). Monolayers, 21–28 days old, were used for transport experiments. All studies were done in triplicate in a shaking water bath (60 rpm) at 37°C. The integrity of each batch of cells was first tested by measuring the flux of [$^{14}$C]-mannitol in three representative monolayers as described by Gudmundsson *et al.* (2). The transport characteristics of the peptides across Caco-2 cell monolayers were determined as described earlier (2) with minor modifications. Briefly, cell monolayers were washed three times with prewarmed HBSS, pH 7.4. Solutions of [Leu$^5$]-enkephalin, DADLE and cyclic prodrugs 1 and 2 (final concentration ~ 75 μM) were prepared in HBSS containing 0.25% DMSO. Aliquots (AP, 1.5 ml or BL 2.6 ml) of these solutions were applied to the donor compartment. HBSS was added to the receiver compartment (AP, 1.5 ml or BL 2.6 ml). For transport studies assessing the role of P-glycoprotein, the HBSS solutions (AP and BL) were supplemented with CsA (final concentration = 25 μM). Samples were withdrawn at various times up to 180 minutes from both sides (receiver, 120 μl and donor, 20 μl). Samples withdrawn from the receiver side were replaced with equal amounts of prewarmed HBSS or HBSS containing 25 μM CsA. To stabilize the samples taken from the donor and receiver compartments, 120 μl and 10 μl of an ice-cold solution of acetonitrile in diluted phosphoric acid [final concentration 10% (v/v) and 0.01 (v/v), respectively] were added. The mixtures were immediately frozen in a dry-ice/acetone bath and kept at −80°C until HPLC analysis. Aliquots (100 μl) of

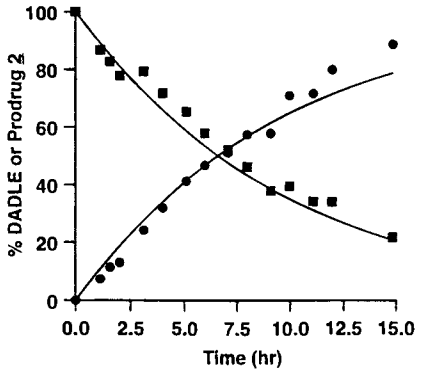


**Fig. 3.** Stoichiometry of conversion of prodrug 2 to DADLE in HBSS, pH 7.4, 37°C. Solid lines: best fit of the data (see Materials and Methods). Disappearance of cyclic prodrug 2, ■ and appearance of DADLE, ●.

each sample were analyzed by HPLC using fluorescence detection.

### HPLC Analysis

A Shimadzu 10A gradient system (Shimadzu, Inc., Tokyo, Japan) consisting of LC-10AS pumps, an SCL-10A controller connected to an SPD-10A uv detector or an RF-535 fluorescence detector was employed for HPLC analysis. Samples from a refrigerated sample tray (4°C) were injected by a Shimadzu SIL 10A auto injector on a reversed phase $C_{18}$ column (Vydac 218TP54, 5 μm, 250 × 4.6 mm) equipped with a guard column. Gradient elution was performed at a flow rate of 1 ml/min from 26–90% v/v acetonitrile in water using 0.1% v/v trifluoroacetic acid as ion-pairing agent. The uv absorbance was monitored at 254 nm and the fluorescence emission at 305 nm (excitation 283 nm). Under these conditions the retention time of 1, 2, [Leu$^5$]-enkephalin and DADLE were 12.31 min, 12.55 min, 6.96 min, and 8.96 min, respectively.

### Data Analysis

Apparent permeability coefficients ($P_{app}$) were calculated using Eq. 1:

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

where $\Delta Q/\Delta t$ is the linear appearance rate of mass in the receiver solution, A is the cross-sectional area and c(0) is the initial peptide concentration in the donor compartment at t = 0.

## RESULTS

### Chemical and Enzymatic Stability

The acyloxyalkoxy-based cyclic prodrugs 1 and 2 were shown to degrade stoichiometrically to [Leu$^5$]-enkephalin and DADLE, respectively, in HBSS, pH 7.4. The time course for the conversion of prodrug 2 to DADLE is shown in Fig. 3. The apparent half-lives ($t_{1/2}$) for cyclic prodrugs 1 and 2 in HBSS are provided in Table I. [Leu$^5$]-enkephalin and DADLE were stable in HBSS, pH 7.4 (data not shown).

As shown in Table I, prodrug 1 was more unstable in Caco-2 cell homogenate, 50% rat liver homogenate and 90% human plasma than in HBSS. The $t_{1/2}$'s of 1 decreased as the esterase activity of the medium increased (Table I). As expected, stoichiometric conversion of prodrug 1 to [Leu$^5$]-enkephalin was not observed because of the instability of this opioid peptide in these biological media (e.g., $t_{1/2} < 1$–2 min in human plasma) (9–10). Paraoxon, a type B esterase inhibitor, was shown to significantly slow down the degradation of 1 in Caco-2 cell homogenate and 90% human plasma, but not in 50% rat liver homogenate (Table I). However, when 10% rat liver homogenate was used, paraoxon was shown to decrease the degradation rate of cyclic prodrug 1 (data not shown).

Prodrug 2 also degraded more rapidly in Caco-2 cell homogenate, 90% plasma and 50% rat liver homogenate than in HBSS. However, the $t_{1/2}$'s were longer than the corresponding half-lives for prodrug 1 (Table I). Furthermore, paraoxon had no significant effect on the $t_{1/2}$'s of cyclic prodrug 2 in Caco-2 cell homogenate and 90% human plasma (Table I).

### Transport Across Caco-2 Cell Monolayers

Cell permeability characteristics of the acyloxyalkoxy-based cyclic prodrugs 1 and 2 were determined across Caco-2 monolayers, an in vitro model of the intestinal mucosa and compared to the permeability characteristics of [Leu$^5$]-enkephalin and DADLE. As previously described, when [Leu$^5$]-enkephalin is applied to the AP side of Caco-2 monolayers, no measurable amounts of this opioid peptide are detectable on the BL side (2). This can be explained by the fact that [Leu$^5$]-enkephalin degrades rapidly ($T_{1/2}$ = 15 min) when applied to

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

| Medium | Specific activity[a] U/mg protein | Enzyme concentration[a] U/ml | | $t_{1/2}$ (min)[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1 | | 2 | |
| | | − paraoxon | + paraoxon | − paraoxon | + paraoxon | − paraoxon | + paraoxon |
| HBSS | 0 | 0 | 0 | 444 ± 26 | 330 ± 17 | 442 ± 11 | 371 ± 7.6 |
| Human plasma[c] | 0.04 | 1.84 | 0 | 77.8 ± 0.5 | 116 ± 6 | 215 ± 23 | 216 ± 13 |
| Rat liver homogenate[d] | 1.49 | 39.60 | 2.24 | 6.1 ± 2.6 | 5.5 ± 1.2 | 100 ± 10 | 130 ± 1 |
| Caco-2 cell homogenate | 0.24 | 0.32 | 0 | 52.7 ± 1.6 | 264 ± 3 | 359 ± 31 | 353 ± 18 |

[a] Specific activity and enzyme concentration, determined at 25°C in HBSS, pH 7.4.
[b] Calculated from first-order rate constants.
[c] Human plasma was diluted to 90% with HBSS, pH 7.4.
[d] Rat liver homogenate diluted to 50% with HBSS, pH 7.4.

**Table II.** Apparent Permeability Coefficients ($P_{App}$) of [Leu$^5$]-Enkephalin, DADLE, 1 and 2 Across Caco-2 Cell Monolayers Determined in the Presence and Absence of CsA

| Compound | Membrane interaction potential[a] (log $k'_{IAM}$) | $P_{App}$(cm/s) × $10^{8}$[b] AP-to-BL | $P_{App}$(cm/s) × $10^{8}$[b] BL-to-AP | $P_{App}$ (BL-to-AP)/ $P_{App}$ (AP-to-BL) |
|---|---|---|---|---|
| [Leu$^5$]-enkephalin | 0.17 | <0.31[c] | <0.31[c] | 1 |
| DADLE | 0.43 | 7.80 ± 0.71 | 7.80 ± 0.71 | 1 |
| 1 | 1.36 | 1.80 ± 0.89 | 65.1 ± 11.4 | 36 |
| 2 | 1.63 | 1.86 ± 1.19 | 96.9 ± 4.90 | 52 |
| 1 + CsA[d] | n.d. | 2.39 ± 0.08 | 10.4 ± 0.80 | 4.4 |
| 2 + CsA[d] | n.d. | 4.42 ± 0.43 | 66.3 ± 6.10 | 15 |

[a] Data taken from Bak *et al.* (7).
[b] See Materials and Methods section for a description of $P_{App}$.
[c] Based on the limits of detection of the analytical method.
[d] Transport experiments were done in the presence of 25 μM CsA added to donor and receiver side.

the AP side of Caco-2 cell monolayers, due to the presence of peptidases in the brush border of the monolayer (2). The maximum apparent permeability coefficient ($P_{APP}$) (Table II) for [Leu$^5$]-enkephalin was estimated based on the limits of detection of our analytical method. In contrast, DADLE (2) and prodrugs 1 and 2 were shown to be fairly stable (< 30% degradation in 3 hr) when applied to the AP side of Caco-2 cell monolayers, and detectable levels of these peptides were measured on the BL side (Table II). Surprisingly, when the $P_{App\ BL\text{-}to\text{-}AP}$ values for 1 and 2 were measured, they were 36 and 52 times greater, respectively, than the $P_{App\ AP\text{-}to\text{-}BL}$ values (Table II). This polarity in flux is illustrated in Fig. 4 for cyclic prodrug 2. A similar polarity in flux was not observed for DADLE (2). The much higher BL-to-AP permeation of 1 and 2 compared to that in the AP-to-BL direction (Table II) suggested that these prodrugs are substrates for apically polarized efflux systems.

To confirm the involvement of apically polarized efflux systems in limiting the AP-to-BL permeation of the cyclic prodrugs 1 and 2 across Caco-2 cell monolayers, CsA, a known inhibitor of P-glycoprotein was evaluated for its ability to increase the AP-to-BL flux and decrease the BL-to-AP flux of these prodrugs. Inclusion of CsA (25 μM) in the incubation media on the AP and BL sides of Caco-2 cell monolayers reduced the $P_{App\ BL\text{-}to\text{-}AP}$ / $P_{App\ AP\text{-}to\text{-}BL}$ ratio, suggesting that this

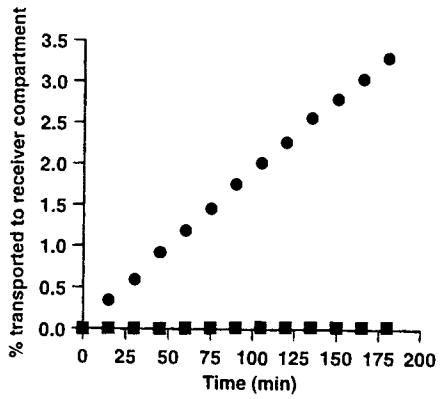


**Fig. 4.** Transport of prodrug 2 in the AP-TO-BL (■) and BL-to-AP (●) directions across Caco-2 cell monolayers (see Materials and Methods).

inhibitor of P-glycoprotein was partially inhibiting the efflux system responsible for altering the permeation of cyclic prodrugs 1 and 2. The limited solubility of CsA prohibited addition of higher concentrations of this P-glycoprotein inhibitor; thus, we were unable to determine if complete inhibition of the efflux systems modulating the flux of these cyclic prodrugs could be achieved. Since only partial inhibition of efflux could be achieved with 25 μM CsA, we cannot rule out the possibility that the cyclic prodrugs 1 and 2 may be substrates for other polarized efflux systems (in addition to P-glycoprotein) present in Caco-2 cells.

## DISCUSSION

### Chemical and Enzymatic Stability

Crucial to the success of a peptide prodrug strategy is the prodrug's ability to be converted quantitatively to the parent peptide *in vivo* by an enzyme-catalyzed and/or chemical reaction (11–13). The acyloxyalkoxy-based cyclic prodrugs 1 and 2 described in the present study were shown to convert quantitatively to [Leu$^5$]-enkephalin and DADLE, respectively, in HBSS by chemical hydrolysis (illustrated for prodrug 2 in Fig. 3). As seen from the data presented in Table I, the $t_{1/2}$ values for 1 and 2 in HBSS in the presence of paraoxon are slightly shorter than the half-lives in the absence of paraoxon. A possible explanation for this observation could be a transesterification between paraoxon, a phosphate ester, and the ester bond in the promoiety. This type of transesterification has previously been observed during the synthesis of the acyloxyalkoxy prodrugs, when intermediates containing the promoiety were stored in ethyl acetate (Bak *et al.*, unpublished data).

It is interesting to compare the chemical stabilities of the acyloxyalkoxy-based cyclic prodrugs 1 and 2 with their coumarinic acid-based (2) and phenylpropionic acid-based (3) counterparts. Like the phenylpropionic acid-based cyclic prodrugs (3), an intermediate was not detectable with the acyloxyalkoxy-based cyclic prodrugs 1 and 2 during their chemical conversion to [Leu$^5$]-enkephalin and DADLE, respectively. This is in contrast to the coumarinic acid-based cyclic prodrugs of these opioid peptides where intermediates were detectable (2). It is also of interest to note that the acyloxyalkoxy-based cyclic prodrugs 1 and 2 had stabilities in HBSS, pH = 7.4, similar

to the stabilities of the coumarinic acid-based cyclic prodrugs of the same opioid peptides (2) ($t_{1/2} < 400$ min). The cyclic prodrugs having the acyloxyalkoxy and coumarinic acid linkers are substantially less stable chemically than the phenylpropionic acid-based cyclic prodrugs (3).

In biological media containing esterase activity, the acyloxyalkoxy-based cyclic prodrugs 1 and 2 were designed to degrade to the parent peptides by esterase-catalyzed reactions (Fig. 1). However, no peak in the HPLC chromatograms corresponding to [Leu$^5$]-enkephalin was observed when prodrug 1 was incubated in human plasma or homogenates of Caco-2 cells and rat liver (data not shown). This is probably due to the rapid degradation of [Leu$^5$]-enkephalin by peptidases present in these biological media (e.g., [Leu$^5$]-enkephalin has a $t_{1/2} \approx <1-2$ min in 90% human plasma). In contrast, the generation of DADLE from prodrug 2 could be detected in esterase-containing media (e.g., human plasma, data not shown). The half-lives of prodrug 1 decrease as the esterase activity of the medium increases (Table II), which is to be expected for a prodrug designed to be esterase-sensitive (Fig. 1). Paraoxon, an inhibitor of type B esterases, was able to significantly slow down the degradation of prodrug 1 in Caco-2 cell homogenate (Table I), 90% human plasma (Table I) and 10% rat liver homogenate (data not shown), but not in 50% rat liver homogenate (Table I). In the 50% rat liver homogenate experiment, paraoxon was not detectable by HPLC (data not shown), suggesting that it had been degraded by esterases in the rat liver homogenate. Esterases which can hydrolyze organophosphates such as paraoxon without being inhibited themselves are termed type A esterases. High levels of A esterases are found in the liver and plasma of a range of mammalian species (14). Therefore, it is highly probable that type A esterases are also contributing to the residual esterase activity present in human plasma and rat liver homogenate after treatment with paraoxon and to the hydrolysis of paraoxon seen in the 50% rat liver experiment.

It is interesting to note that prodrug 2 was found to be less prone to degradation by esterases than was prodrug 1 (Table I). This probably results because the ester bond in prodrug 2 is formed with a D-amino acid (D-Leu), whereas in prodrug 1 the ester bond is formed with a L-amino acid (L-Leu). Carboxylesterases, which are type B esterases, are known to be sensitive to the substituent in the β position of the acyl group in aliphatic esters (15). Furthermore, it has been shown that the carboxylesterases present in rat plasma and rat liver homogenates hydrolyze ester prodrugs of propranolol in a stereospecific manner (16). Thus, the degradation of prodrug 2 in these biological media may be catalyzed by other esterases, i.e., type A or type C esterases, and/or the affinity of type B esterases for prodrug 2 may be lower than for prodrug 1. Since paraoxon, an inhibitor of type B esterases, was unable to inhibit the degradation of prodrug 2 significantly (Table I), it is more likely that prodrug 2 is hydrolyzed by type A and/or type C esterases.

A comparison of the stability of the acyloxyalkoxy-based cyclic prodrug 1 with its coumarinic acid-based counterpart (2) in esterase-containing biological media showed many similarities. For example, both the acyloxyalkoxy-based and coumarinic acid-based cyclic prodrugs of [Leu$^5$]-enkephalin were rapidly degraded in rat liver homogenate and less rapidly degraded in human plasma. Interestingly, the acyloxyalkoxy-based cyclic prodrug 1 showed increased instability in Caco-2 cell homogenates compared to HBSS, whereas the coumarinic acid-based cyclic prodrug showed no enhanced rate of degradation in this biological media. An interesting difference between the prodrug systems was the observation that the coumarinic acid-based cyclic prodrugs did not show a difference between the rates of degradation of the L-amino acid-([Leu$^5$]-enkephalin) and D-amino acid-(DADLE) containing peptides whereas a difference was observed in the acyloxyalkoxy-based prodrugs of the same opioid peptides.

### Caco-2 Cell Transport Characteristics

Based on the physicochemical properties (e.g., high lipophilicity, low hydrogen bonding potential) of the acyloxyalkoxy-based cyclic prodrugs 1 and 2 (7), it was expected that these cyclic prodrugs of [Leu$^5$]-enkephalin and DADLE would have favorable permeation characteristics across Caco-2 cell monolayers. This hypothesis was based on our earlier observations with the phenylpropionic acid- and coumarinic acid-based cyclic prodrugs of the same opioid peptides. These cyclic prodrugs exhibited favorable physicochemical properties (1,2), which correlated well with their excellent Caco-2 permeation characteristics (3,4). However, it should be noted that, while these phenylpropionic acid- and coumarinic acid-based cyclic prodrugs of [Leu$^5$]-enkephalin and DADLE were highly lipophilic, they were shown not to be substrates for apically polarized efflux systems in Caco-2 cells (2,3), which could have restricted their transcellular permeation (17).

Therefore, it was very surprising to observe that the acyloxyalkoxy-based cyclic prodrugs 1 and 2 of [Leu$^5$]-enkephalin and DADLE, respectively, exhibited $P_{App\ AP\text{-}to\text{-}BL}$ values across Caco-2 cell monolayers that were approx. 200–300 times lower than the values observed previously with the phenylpropionic acid- and coumarinic acid-based cyclic prodrugs of the same opioid peptides (2,3). In fact, the acyloxyalkoxy-based cyclic prodrug 2 of DADLE had an $P_{App\ AP\text{-}to\text{-}BL}$ value approx. 4 times lower than that of DADLE itself (Table II) in spite of having a membrane interaction potential (log $k'_{IAM}$) approx. 4 times higher (7). However, an explanation for the lower permeation of cyclic prodrugs 1 and 2 across Caco-2 cells became obvious when the $P_{App\ BL\text{-}to\text{-}AP}$ values for these prodrugs were determined. The much higher $P_{App\ BL\text{-}to\text{-}AP}$ values compared to the $P_{App\ AP\text{-}to\text{-}BL}$ values (Table II, Fig. 4) for cyclic prodrugs 1 and 2 suggest that these acyloxyalkoxy-based prodrugs are substrates for apically polarized efflux systems (17). Further evidence in support of the hypothesis that cyclic prodrugs 1 and 2 are substrates for an apically polarized efflux system was obtained by conducting competition experiments using CsA, a known inhibitor of P-glycoprotein. Inclusion of CsA in the donor and receiver compartments of a Caco-2 cell transport experiment resulted in a slight increase in the $P_{App\ AP\text{-}to\text{-}BL}$ values and a significant decrease in the $P_{App\ BL\text{-}to\text{-}AP}$ values for cyclic prodrugs 1 and 2. These results suggest that P-glycoprotein may be involved in modifying the permeation of these cyclic prodrugs across Caco-2 cell monolayers. However, since only partial inhibition of the efflux of these cyclic prodrugs in Caco-2 cells was achieved with CsA, this could suggest that other efflux systems may also be involved. Alternately, these results with CsA could suggest that cyclic prodrugs 1 and 2 are much better substrates for P-glycoprotein than CsA and that concentrations of CsA sufficient to totally inhibit P-glycoprotein in Caco-2 cells were not employed in these experiments.

The substrate activity of the acyloxyalkoxy-based cyclic prodrugs of [Leu$^5$]-enkephalin and DADLE for efflux systems

in Caco-2 cells versus the nonsubstrate activity of the phenylpropionic acid- and coumarinic acid-based cyclic prodrugs of the same opioid peptides is very intriguing. Based on their lipophilicities as measured by their abilities to partition into an artificial membrane column (log $k'_{IAM}$ values), the acyloxyalkoxy-based prodrugs were the least lipophilic and the phenylpropionic acid-based prodrugs were the most lipophilic (1,7). The coumarinic acid-based prodrugs had intermediate lipophilicity (7). These results would have suggested, based on what is known about the substrate activity of P-glycoprotein (17), that the phenylpropionic acid- and coumarinic acid-based prodrugs would be more likely to be substrates for P-glycoprotein than the acyloxyalkoxy-based prodrugs. Obviously, these predictions were wrong because neither the phenylpropionic acid- (4) nor the coumarinic acid-based (3) prodrugs were substrates for efflux systems (e.g., P-glycoprotein), whereas the acyloxyalkoxy-based prodrugs were excellent substrates. These results suggest that for this series of compounds, lipophilicity alone was not a good predictor of substrate activity for P-glycoprotein. One possible difference between the acyloxyalkoxy-based prodrugs and the phenylpropionic acid- and coumarinic acid-based prodrugs is their solution conformations. While the phenylpropionic acid- and coumarinic acid-based prodrugs exist in well-defined solution conformations with significant secondary structure (e.g., β-turns) (18), the acyloxyalkoxy-based prodrugs have two major conformers that exist in equilibrium as determined by NMR (19). These conformers arise from the cis-trans isomerization of the carbamate junction between the promoiety and the peptide (19). Therefore, it may be possible that the acyloxyalkoxy-based prodrugs in the lipid environment of the cell membrane adopt structures more conducive to interactions with P-glycoprotein. Alternately, the acyloxyalkoxy linker itself cannot be ruled out at this time as the structural feature recognized by P-glycoprotein (19).

## CONCLUSIONS

In conclusion, we have evaluated the chemical and enzymatic stability as well as the transport properties across Caco-2 monolayers for the acyloxyalkoxy-based prodrugs 1 and 2. The ester linkage in prodrug 1 is very sensitive to the esterases present in Caco-2 cell homogenate, human plasma and rat liver homogenate. The ester linkage in prodrug 2 is less sensitive to the same esterases because the ester is formed through a D-amino acid. Prodrug 1 is probably degraded by type B as well as other types of esterases. Prodrug 2 is mainly degraded by esterases other than type B. Prodrugs 1 and 2 are substrates for one or more apically polarized efflux systems present in Caco-2 cells. One of these efflux systems is apically polarized P-glycoprotein. At this time, it is unclear why the acyloxyalkoxy-based prodrugs are substrates for P-glycoprotein and/or other efflux systems, but we believe it may be related to their solution conformations.

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