In Vitro Stability and In Vivo Pharmacokinetic Studies of a Model Opioid Peptide, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE), and Its Cyclic Prodrugs

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ABSTRACT

In vitro stability and in vivo pharmacokinetic studies of a model opioid peptide, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE), and its cyclic prodrugs (acyloxyalkoxy-based cyclic prodrug of DADLE, coumarinic acid-based cyclic prodrug of DADLE, and oxymethyl-modified coumarinic acid-based cyclic prodrug of DADLE) were conducted. The enzymatic stability of DADLE and its prodrugs in various biological media was determined at 37°C in the presence and absence of paraoxon, a known esterase inhibitor. The prodrugs exhibited metabolic stability to exo- and endopeptidases, and esterase-catalyzed bioconversion of the prodrugs to DADLE was observed. For pharmacokinetic studies in rats, various biological samples (blood, bile, urine, and brain) were collected after i.v. administration of DADLE and its prodrugs. The samples were analyzed by high-performance liquid chromatography with tandem mass spectrometric detection, and the conversion from the prodrugs to intermediates to DADLE was monitored. The prodrugs exhibited similar pharmacokinetic properties and showed improved stability compared with DADLE in rat blood. This increased stability led to higher plasma concentrations of DADLE after i.v. administration of the prodrugs compared with i.v. administration of DADLE alone. In terms of elimination pathways, metabolism by endopeptidases was the major route for DADLE elimination, whereas rapid biliary excretion was the major route of elimination for the prodrugs. The rapid elimination of the prodrugs by the liver and the formation of stable intermediates after esterase hydrolysis limited the bioconversion efficiencies of the prodrugs to DADLE after i.v. administration. The substrate activity of the prodrugs for efflux transporters (e.g., P-glycoprotein) in the blood-brain barrier significantly restricted their access to the brain.

The delivery of opioid peptides to the brain has presented significant challenges to pharmaceutical scientists due to the poor biopharmaceutical properties of the peptides. These include their lability to metabolism by exo- and endopeptidases and their low permeation across the blood-brain barrier (BBB) (Fricker and Drewe, 1996; Pauletti et al., 1996a, 1997; Prokai, 1998). The issue of metabolic lability has, for all practical purposes, been solved by medicinal chemists through the design of novel peptide bond bioisosteres to replace metabolically labile peptide bonds (Sawyer, 1995). Until recently, however, medicinal chemists have had less success in manipulating the structures of opioid peptides to achieve good BBB permeation while still retaining high affinity and selectivity for opioid receptors.

Through prodrug strategies, some progress has been made recently in improving the BBB permeation characteristics of opioid peptides (Greene et al., 1996; Misicka et al., 1996; Patel et al., 1997; Prokai et al., 2000). For a prodrug strategy to be successful in delivering opioid peptides to the brain, the following criteria must be met: 1) the prodrug should have favorable physiochemical properties (e.g., hydrophobicity, low hydrogen-bonding potential, and no charge) for transcellular permeation across the BBB; 2) the prodrug should exhibit good “intrinsic” permeability across the BBB but not be a substrate for efflux transporters (e.g., P-gp); 3) the prodrug should be a good substrate for enzymes (e.g., esterases) in the brain that would catalyze its bioconversion to the opioid peptide but have less activity as a substrate for the same enzyme.

ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; DADLE, [D-Ala²,D-Leu⁵]-enkephalin; AOA-DADLE, acyloxyalkoxy-based cyclic prodrug of [D-Ala²,D-Leu⁵]-enkephalin; CA-DADLE, coumarinic acid-based cyclic prodrug of [D-Ala²,D-Leu⁵]-enkephalin; OMCA-DADLE, oxymethyl-modified coumarinic acid-based cyclic prodrug of [D-Ala²,D-Leu⁵]-enkephalin; PNPB, p-nitrophenyl butyrate; DMSO, dimethyl sulfoxide; PEG300, polyethylene glycol (ave. mol. wt. 300); HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; LC/MS/MS, high-performance liquid chromatography with tandem mass spectrometric detection; SPE, solid phase extraction; AUC, area under the curve.
enzymes in other biological media/tissues or for other enzymes that could lead to nonproductive metabolism of the prodrug (e.g., endopeptidases); and 4) the prodrug should have a reasonable plasma half-life and not be rapidly metabolized in the blood compartment or cleared by the liver or kidney or be extensively protein-bound.

Having these criteria in mind, our laboratory synthesized cyclic prodrugs of the opioid peptide H-Tyr-o-Ala-Gly-Phe-d-Leu-OH (DADLE) using an acyloxyalkoxy (AOA) linker (Bak et al., 1999b), a coumarinic acid (CA) linker (Wang et al., 1999), and an oxymethyl-modified coumarinic acid (OMCA) linker (Ouyang et al., 2002b) (Fig. 1). These prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE) were designed to undergo bioconversion to DADLE via mechanisms involving enzyme-catalyzed hydrolysis of the ester bond linking the C-terminal amino acid of the peptide to the linker. The resulting "intermediates" (Fig. 1, I–III) were then designed to degrade chemically to form DADLE. In previous studies (Bak et al., 1999a; Gudmundsson et al., 1999b; Ouyang et al., 2002a; Tang and Borchardt, 2002a,b), our laboratory has shown that these prodrugs met criterion 1 and part of criterion 3.

With respect to criterion 2, our laboratory determined the cell permeation characteristics of AOA-DADLE, CA-DADLE, and OMCA-DADLE using various cell culture models (e.g., Caco-2 cells, Madin-Darby canine kidney cells, and Madin-Darby canine kidney cells transfected with drug efflux transporters; Bak et al., 1999a; Ouyang et al., 2002a; Tang and Borchardt, 2002a,b). The results of these in vitro cell culture experiments showed that AOA-DADLE, CA-DADLE, and OMCA-DADLE exhibited poor cell permeation characteristics. Unlike the poor cell permeation characteristics of DADLE, which resulted from undesirable physiochemical properties (e.g., charge and high hydrogen-bonding potential), the poor cell permeation of the cyclic prodrugs was shown to result from their substrate activities for efflux transporters (e.g., P-gp, multidrug resistance-associated protein 2). If these efflux transporters were inhibited, the intrinsic cell permeation characteristics of AOA-DADLE, CA-DADLE, and OMCA-DADLE were significantly better than those of DADLE (Bak et al., 1999a; Ouyang et al., 2002a; Tang and Borchardt, 2002a,b), thus satisfying in part criterion 2.

These in vitro chemical/enzymatic studies and cell permeation studies have provided valuable insights into whether AOA-DADLE, CA-DADLE, and OMCA-DADLE satisfy criteria 1 and 2 mentioned above. However, to assess whether these prodrugs satisfied criteria 3 and 4, in vivo experiments must be conducted. The overall objective of the present work was to evaluate the potential of the cyclic prodrugs for delivering DADLE to the brain by investigating their in vivo stability in various biological media and by in vivo pharmacokinetic studies in rats. Therefore, experiments were designed to characterize the following biopharmaceutical properties of AOA-DADLE, CA-DADLE, and OMCA-DADLE: 1) their in vitro bioconversion rates in biological media (blood)/tissues (brain/liver), 2) their propensity to bind to plasma proteins, 3) their bioconversion rates and efficiencies in de-

![Fig. 1. Cyclic prodrug strategies.](image-url)

A, AOA-DADLE. B, CA-DADLE and OMCA-DADLE.
livering DADLE after i.v. administration, 4) their kinetics and routes of elimination after i.v. administration, and 5) their ability to permeate the BBB after i.v. administration.

Materials and Methods

Materials. The opioid peptides (DADLE and its internal standard [Leu"]-enkephalin), diethyl p-nitrophenyl phosphate (paraoxon, approx. 90%), p-nitrophenyl butyrate (PNPB, approx. 98%), guanidine hydrochloride (>99%), dimethyl sulfoxide (DMSO, >99.5%), polyethylene glycol (average mol. wt. 300) (PEG300), and Hanks’ balanced salt solution (HBSS) (modified) were purchased from Sigma-Aldrich (St. Louis, MO). Metofane (methoxyflurane; Schering-Plough, Kenilworth, NJ) was obtained from the Animal Care Unit (The University of Kansas, Lawrence, KS). Prodrugs of DADLE and prodrugs of [Leu"]-enkephalin (internal standards) were synthesized in our laboratory following procedures described elsewhere (Bak et al., 1999b; Wang et al., 1999b; Ouyang et al., 2002b). All other chemicals were of the highest purity available and used as received. All solvents were HPLC grade, including deionized, ultrafiltered water (Fisher Scientific, Fair Lawn, NJ).

In Vitro Stability and Protein Binding Studies. Human blood was obtained from the Watkins Health Center (The University of Kansas). Rat blood was obtained from male Sprague-Dawley rats (Animal Care Unit, The University of Kansas). Fresh blood was centrifuged immediately at 1800g (model 59A Micro-Centrufuge; Fisher Scientific, Pittsburgh, PA) and 4°C for 5 min and plasma was collected. For stability studies, plasma was diluted to 90% (v/v) with HBSS, pH 7.4, to maintain the pH of the solution during the experiment. Rat livers and brains were obtained from male Sprague-Dawley rats (Animal Care Unit, The University of Kansas). The tissues were blotted to dryness and cut into small pieces after weighing. The tissue pieces were homogenized immediately on ice with ice-cold HBSS (1 ml/g of tissue) using a glass homogenizer (15 strokes, pestle/wall clearance 0.25–0.76 mm; Wheaton, Philadelphia, PA). Aliquots (approx. 1.5 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. Cell debris and nuclei were removed by centrifugation at 10,000g and 4°C for 10 min using a model 59A Micro-Centrufuge (Fisher Scientific). The supernatant was collected for stability studies.

The enzymatic stability of DADLE and its cyclic prodrugs was studied in various biological media at 37°C. The disappearance of the prodrugs, possible formation of metabolites, and routes of elimination after i.v. administration, and 5) their ability to permeate the BBB after i.v. administration. Determined using a filtration method. DADLE and its prodrugs were spiked into pooled rat plasma at various concentrations (n = 3). After equilibrating at 4°C for 30 min, plasma samples were centrifuged at 5000g and 4°C for up to 3 h using Ultrafree MC-5000 NMWL filter units (Millipore Corporation, Bedford, MA) to separate the protein-bound drugs from free drugs. For control samples, drugs were spiked into filtered blank plasma as 100% recovery standards. All samples were analyzed immediately, and the percentage of protein binding for each compound was calculated based on free drug concentrations divided by total drug concentrations in control samples.

In Vivo Pharmacokinetic Studies. For pharmacokinetic studies, we used male Sprague-Dawley rats (200–250 g) with a cannula chronically implanted in the jugular vein or carotid artery (Harlan, Indianapolis, IN). The rats were housed individually and fasted overnight before use. Water was allowed ad libitum. For each compound to be studied, three to six rats were each given a 1 mg/kg i.v. dose of the drug (200–250 μl). The vehicles used for i.v. dosing were various combinations of saline, ethanol, PEG300, and DMSO, depending on the solubility of the compounds. For DADLE, saline was used as the solvent; 20% (v/v) ethanol and 80% (v/v) water were used for AOA-DADLE; 5% (v/v) DMSO, 47.5% (v/v) PEG300, and 47.5% (v/v) saline were used for CA-DADLE; and 5% (v/v) DMSO, 20% (v/v) PEG300, and 75% (v/v) saline were used for OMCA-DADLE. For i.v. injections, the rats were anesthetized with metofane, a small incision was made on the medial surface of the hind leg, and the incision was made into the femoral vein. The incision was closed with wound clips and the rats were allowed to recover from the anesthetic (5–10 min).

To determine the routes of elimination for DADLE and its prodrugs, the experiments described above were conducted using three to eight male Sprague-Dawley rats with chronically implanted bile duct cannulas (200–250 g; Harlan). The bile was collected for various time intervals (5-min intervals for 20 min, 10-min intervals up to 60 min, and 20-min intervals through 2 h) after drug administration and centrifuged immediately at 1800g and 4°C for 5 min (Micromax centrifuge; International Equipment Company, Needham Heights, MA). Plasma samples were collected and stored at −80°C until analysis.

To determine the disposition of DADLE and its prodrugs into the brain after i.v. administration, male Sprague-Dawley rats (200–250 g) were used. The rats were fasted overnight before use but were allowed access to water ad libitum. For each compound studied, three rats were given a 1 mg/kg i.v. dose of the drug. At 10 min after i.v. administration, the animals were sacrificed by decapitation and the brains removed. The samples were stored at −80°C until analysis.

Sample Analysis. For in vitro stability studies, high-performance liquid chromatography (HPLC) with UV detection was performed using an LC-10A gradient system (Shimadzu, Tokyo, Japan) consisting of two LC-10AS pumps, an SCL-10A system controller, and an SIL-10A autoinjector with a sample cooler. The chromatographic data were acquired and analyzed using CLASS-VP version 4.2 Chromatography Data System (Shimadzu). The HPLC analysis was conducted using a C18 reversed-phase column (250 × 4.6 mm i.d., 300 Å; Vydac, Hesperia, CA) equipped with a C18 guard column (Vydac). Gradient elution was performed at a flow rate of 1 ml/min from 26 to 58% (v/v) acetonitrile in water with 0.1% (v/v) trifluoroacetic acid. The eluents were detected by UV (λ = 214 nm). For sample preparation, aliquots (−150 μl) of the sample mixtures were transferred to Ultrafree MC-5000 NMWL filter units (Millipore Corporation) and centrifuged at 5000g and 4°C for up to 3 h (Micromax centrifuge). The filtrate was collected and kept at 4°C for HPLC analysis. The disappearance of the prodrugs, possible formation of any stable intermediates, and appearance of DADLE were monitored.
For in vivo pharmacokinetic studies, HPLC with tandem mass spectrometric detection (LC/MS/MS) methods were developed. These methods have been described extensively elsewhere (Yang et al., 2002). Briefly, a Quattro LC or Quattro Micro triple quadrupole mass spectrometer (Micromass, Beverly, MA) was used. The liquid chromatography was conducted using a 2690 HPLC System (Waters, Milford, MA). Data acquisition and analysis were performed using MassLynx version 3.5 software (Micromass). A C18 reversed-phase column (50 × 1.0 mm i.d., 300 Å; Vydac) was used as the analytical column, with the column temperature maintained at 25°C to ensure reproducible separation. Two mobile phases were used to generate a linear gradient to allow simultaneous analysis of DADLE and its prodrugs in a single run. Mobile phase A consisted of water and 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. For sample recording, multiple reaction monitoring was used so that several compounds could be monitored simultaneously. Even though accurate concentrations of the intermediates (Fig. 1, I–III) could not be determined because the intermediates converted to DADLE during the mass spectrometric analysis, it was possible to estimate their amounts based on the levels of DADLE detected by LC/MS/MS. For intermediate and metabolite identification during in vitro stability studies, mass spectrometric analysis was conducted using the same LC/MS/MS system under similar conditions. A full scan covering a broad mass range instead of multiple reaction monitoring was conducted to identify possible metabolites and confirm the formation of any stable intermediates.

For in vivo studies, different sample preparation methods were used, depending on the biological matrix. For plasma samples, protein precipitation with acetonitrile was used. To a 100-μl plasma sample, 200 μl of acetonitrile containing internal standards was added to precipitate the plasma proteins. After vortex mixing, the precipitated proteins were removed by centrifugation at 3000g and 4°C for 5 min. The supernatant was evaporated to dryness using a Centrivap concentrator (Labconco, Kansas City, MO), and the residue was reconstituted in 100 μl of 10% (v/v) acetonitrile. It was centrifuged again at 10,000g and 4°C for 5 min, and a 50 μl supernatant was injected for LC/MS/MS analysis. For brain samples, solid phase extraction (SPE) had to be used due to the complicated biological matrix. First, the whole brain was quickly homogenized on ice with 5 ml of ice-cold HBSS using a glass homogenizer (30 strokes; Wheaton) after weighing. Aliquots (approx. 1.5 ml) were immediately frozen and kept at −80°C until used. Before sample preparation, the homogenate was quickly thawed and 3 ml of ice-cold acetonitrile along with 10 μl of the internal standard solution mixture was added to 1 ml of homogenate to precipitate the proteins. After vortex mixing, the precipitated proteins were removed by centrifugation at 5000g and 4°C for 5 min. Because the supernatant had high organic content, it was evaporated and reconstituted with water for compatibility with SPE. A Centrivap concentrator was used, and the organic solvent was evaporated under vacuum. After approx. 2 h, the concentrated supernatant was diluted with 1 ml of deionized water, and the solution was loaded onto the cartridges for SPE (Yang et al., 2002). After DADLE and its prodrugs along with their internal standards were extracted from the brain samples, the final solution was evaporated to dryness and the residue was reconstituted in 100 μl of 10% (v/v) acetonitrile. It was centrifuged again at 10,000g and 4°C for 1 min, and 50 μl of the supernatant was injected for LC/MS/MS analysis. Bile and urine samples were diluted and injected directly for LC/MS/MS analysis because they had relatively high drug concentrations.

Data Analysis. For in vitro stability studies, mass balance was monitored to study the bioconversion mechanisms of the prodrugs. The apparent half-lives (t1/2) of the prodrugs were calculated using SigmaPlot (SPSS Science, Chicago, IL) from the pseudo first order rate constants obtained by linear regression of plots of log drug concentration remaining versus time. All results are presented as mean ± S.D.

For in vivo pharmacokinetic studies, the plasma data were fitted to a two-compartment model, and various pharmacokinetic parameters were calculated using WinNonlin (Pharsight, Mountain View, CA). To quantify the bioconversion efficiency of the cyclic prodrugs, the relative bioavailability of DADLE after i.v. administration of the prodrugs was calculated. The values were expressed as the ratio of the AUC of DADLE converted from the prodrug versus AUC of DADLE administered alone adjusted by dose. All results are presented as mean ± S.E.

For statistical analysis, analysis of variance and Student’s t test were used where appropriate. A probability of less than 0.05 (P < 0.05) was considered statistically significant.

Results

In Vitro Stability and Protein Binding Studies. The enzymatic stability of DADLE and its cyclic prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE) was studied in various biological media, including human plasma, rat plasma, rat liver homogenate, and rat brain homogenate. The bioconversion profiles for DADLE and its prodrugs in rat plasma are presented in Fig. 2. DADLE disappeared slowly in rat plasma. The metabolites of DADLE that were identified by LC/MS/MS seem to result from hydrolysis of the pentapeptide catalyzed by endopeptidases (data not shown). The half-lives of all three prodrugs in rat plasma were much shorter than that of DADLE due to rapid ester bond cleavage catalyzed by esterases. Conversion to DADLE was observed for all these prodrugs, but complete mass balance was not achieved. Some interesting differences were observed between the three prodrugs in terms of the formation of intermediates. For example, whereas intermediate I from AOA-DADLE was not detected, significant accumulation of intermediate II from CA-DADLE and intermediate II (and probably III) from OMCA-DADLE was observed (Fig. 2).

Table 1 summarizes the half-lives of DADLE and its prodrugs in various biological media. DADLE was relatively stable in human and rat plasma, but its half-lives were much shorter in rat liver and brain homogenates. For the cyclic prodrugs, conversion to DADLE was observed in all biological media but with different half-lives. It is interesting to note that AOA-DADLE and OMCA-DADLE, which have the same local structure around the ester bond, had similar half-lives in all of the biological media/tissues tested. In contrast, CA-DADLE, which has a different structure around the ester bond, exhibited quite different stability in these biological media and tissues. Using PNPB as a substrate, the specific esterase activities for the four biological media were determined to be 0.52 U/mg protein (rat liver homogenate) >0.20 U/mg protein (rat brain homogenate) >0.05 U/mg protein (rat plasma) >0.02 U/mg protein (human plasma). Of the three prodrugs, CA-DADLE had half-lives that decreased as the esterase activities of the biological media increased; no such correlation was observed for the other two prodrugs. The half-lives of AOA-DADLE and OMCA-DADLE were generally longer than that of CA-DADLE in the biological media. With the addition of paraoxon, the esterase activities for all biological media were reduced to less than 0.02 U/mg protein. Although paraoxon had no significant effects on the stability of DADLE, the half-lives of all prodrugs were much longer in the presence of paraoxon, and different inhibition effects were observed for the three different prodrugs.

In protein binding studies, the prodrugs generally had much higher bound fractions than did the parent drug.
The protein binding percentages in rat plasma were 47\% for AOA-DADLE, 88\% for CA-DADLE, 60\% for OMCA-DADLE, and only 21\% for DADLE.

In Vivo Pharmacokinetic Studies. The pharmacokinetic properties and bioconversion efficiencies of the prodrugs were studied in rats after i.v. administration of DADLE and its prodrugs. Pharmacokinetic parameters (AUC, clearance, $V_{ss}$, $k_e$, and $t_{1/2}$) were calculated and are provided in Table 2. After i.v. administration, DADLE disappeared rapidly from the plasma with a short elimination half-life (Fig. 3A). In comparison, the prodrugs were more stable and had longer elimination half-lives (Fig. 3, B–D). Continuous generation of DADLE from all three prodrugs was observed during the time course of the experiment. How-

**TABLE 1**

Stability of DADLE and its cyclic prodrugs in various biological media and tissues

The stability of DADLE and its cyclic prodrugs in human and rat plasma and various rat tissue homogenates was determined ($n = 3$) at 37°C in the presence and absence of paraoxon, a known esterase inhibitor. The disappearance of the prodrugs and their conversion to DADLE was monitored by HPLC with UV detection. See Materials and Methods for experimental details.

<table>
<thead>
<tr>
<th>Medium</th>
<th>DADLE</th>
<th>AOA-DADLE</th>
<th>CA-DADLE</th>
<th>OMCA-DADLE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$t_{1/2}$ (min, mean ± S.D.)$^a$</td>
<td>$t_{1/2}$ (min, mean ± S.D.)$^a$</td>
<td>$t_{1/2}$ (min, mean ± S.D.)$^a$</td>
<td>$t_{1/2}$ (min, mean ± S.D.)$^a$</td>
</tr>
<tr>
<td>Human plasma$^b$</td>
<td>&gt;15 days</td>
<td>&gt;10 days</td>
<td>124 ± 7</td>
<td>141 ± 8</td>
</tr>
<tr>
<td>Rat plasma$^b$</td>
<td>509 ± 28</td>
<td>451 ± 9</td>
<td>38 ± 4</td>
<td>124 ± 5</td>
</tr>
<tr>
<td>Rat liver$^c$</td>
<td>25 ± 1</td>
<td>30 ± 1</td>
<td>217 ± 11</td>
<td>473 ± 144</td>
</tr>
<tr>
<td>Rat brain$^c$</td>
<td>22 ± 1</td>
<td>48 ± 2</td>
<td>676 ± 7</td>
<td>1070 ± 17</td>
</tr>
</tbody>
</table>

$^a$ Calculated from first-order rate constants.

$^b$ Human and rat plasma samples were diluted to 90% (v/v) with HBSS, pH 7.4.

$^c$ Rat liver and brain homogenates were diluted to 50% (v/v) with HBSS, pH 7.4.

DADLE. The protein binding percentages in rat plasma were 47 ± 11\% for AOA-DADLE, 88 ± 6\% for CA-DADLE, 60 ± 6\% for OMCA-DADLE, and only 21 ± 4\% for DADLE.

**Fig. 2.** Stability of DADLE (A), AOA-DADLE (B), CA-DADLE (C), and OMCA-DADLE (D) in rat plasma in vitro ($n = 3$). The prodrugs (●–●), DADLE (○–○), and intermediates (▼–▼) were analyzed by HPLC. See Materials and Methods for experimental details.
ever, the DADLE concentrations arising from the prodrugs were always significantly lower than the observed prodrug concentrations (Fig. 3). Assuming the bioavailability of DADLE after i.v. administration was 100%, the bioconversion efficiencies to DADLE were 35/100% for AOA-DADLE, 39/100% for CA-DADLE, and 5/100% for OMCA-DADLE. Even with incomplete conversions, the prodrugs still provided sustained release of DADLE in the plasma, which led to higher DADLE concentrations over longer duration compared with administration of DADLE alone. Interestingly, the intermediates (Fig. 1, II and probably III) arising from esterase-catalyzed hydrolysis of CA-DADLE and OMCA-DADLE were also detected in plasma (Fig. 3, C and D). As predicted from the in vitro results, intermediate I arising from AOA-DADLE was not detected (Fig. 3B).

Pharmacokinetic experiments were also conducted using bile duct-cannulated rats to determine the routes of elimination for DADLE and its prodrugs. Table 3 summarizes the results of biliary clearance for DADLE and its prodrugs. Although the bile recovery amount for DADLE was low, extensive biliary excretion was observed for the prodrugs, with more AOA-DADLE being recovered in the bile than the

<table>
<thead>
<tr>
<th>PK Parameters (mean ± S.E.)</th>
<th>DADLE</th>
<th>AOA-DADLE</th>
<th>CA-DADLE</th>
<th>OMCA-DADLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (ng · min/ml)</td>
<td>11,754 ± 1,073</td>
<td>12,942 ± 2,091</td>
<td>17,136 ± 2,488</td>
<td>12,758 ± 2,958</td>
</tr>
<tr>
<td>Clearance (ml/min)</td>
<td>20.4 ± 1.7</td>
<td>20.9 ± 4.0</td>
<td>14.5 ± 2.2</td>
<td>20.4 ± 5.3</td>
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<tr>
<td>$V_{ss}$ (ml)</td>
<td>93.4 ± 17.6</td>
<td>221.6 ± 29.1</td>
<td>269.1 ± 63.0</td>
<td>238.4 ± 103.9</td>
</tr>
<tr>
<td>$k_{el}$ (1/min)</td>
<td>0.35 ± 0.08</td>
<td>0.20 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.18 ± 0.04</td>
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<tr>
<td>$t_{1/2}$ (min)</td>
<td>4.65 ± 0.58</td>
<td>14.4 ± 1.4</td>
<td>24.2 ± 3.6</td>
<td>17.6 ± 4.0</td>
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![Fig. 3](https://placebotox.aspetjournals.org/doi/10.1124/jpet.117.235184/f3)

**Fig. 3.** Time course for disappearance of DADLE (A), AOA-DADLE (B), CA-DADLE (C), and OMCA-DADLE (D) after i.v. administration of the respective drugs ($n = 3–6$). The prodrugs ($\bullet$), DADLE ($\circ$), and intermediates ($\nabla$) were analyzed by LC/MS/MS. See Materials and Methods for experimental details.
other two prodrugs (Fig. 4). The intermediates of CA-DADLE and OMCA-DADLE also contributed to the overall clearance, but less than 10% of intermediates II and III were recovered in bile. In renal clearance studies, less than 5% of DADLE and its prodrugs were recovered in urine (data not shown).

The disposition of DADLE and its cyclic prodrugs into the brain was determined after i.v. administration of drugs. After sample preparation and analysis, the amounts of DADLE and its prodrugs in the brain were determined (Table 4). Brain uptake of DADLE was very low, and the prodrugs did not show better permeation characteristics than DADLE itself. However, bioconversion of the cyclic prodrugs to DADLE was observed in the brain (Table 4).

Discussion

In Vitro Stability and Protein Binding Studies. In all of the various biological media and tissues used in these studies, conversion of the prodrugs to DADLE was observed, but the rates of those bioconversions differed. These dissimilarities could arise because the prodrugs have different substrate activities for the same esterases or they are substrates for different esterases. Differences were also observed between prodrug half-lives and esterase activities (based on the rates of PNPB hydrolysis) in the biological media/tissues, indicating that several types of esterases might be involved in the hydrolysis of the cyclic prodrugs. It is well known that there are three predominant types of esterases (type A, B, and C), and PNPB is a substrate for type B esterase (Walker and Mackness, 1987; Takahashi et al., 1995; Huang et al., 1996). Because the half-lives of CA-DADLE correlated with the type B esterase activities of the biological media, this prodrug is probably a substrate mainly for this form. For AOA-DADLE and OMCA-DADLE, it is more likely that both prodrugs were hydrolyzed by type A and/or type C esterases.

For the overall bioconversion process, the prodrugs seem to have different rate-limiting steps to the formation of DADLE. As shown in Fig. 2, degradation of AOA-DADLE led to the formation of DADLE only. The intermediate I was not detectable, suggesting that it very rapidly degrades to DADLE, CO₂, and formaldehyde. Therefore, the ester bond cleavage of AOA-DADLE seems to be the rate-limiting step in the bioconversion to DADLE. In contrast, CA-DADLE degraded to DADLE with the formation of intermediate II and OMCA-DADLE converted to DADLE with the formation of intermediates II and III (Fig. 1B). However, in both in vitro (Fig. 2) and in vivo (Fig. 3) experiments, only intermediate II was detectable because the lactonization reaction seems to be slow. Therefore, the conversion of II to DADLE is the rate-limiting step in the overall bioconversion process for both CA-DADLE and OMCA-DADLE.

A successful prodrug strategy for the delivery of an opioid peptide to the brain has to include the preferential conversion of the prodrug to the parent drug via either chemical or enzymatic reactions in the target tissue (e.g., brain). In these studies, we have shown that the cyclic prodrugs AOA-DADLE, CA-DADLE, and OMCA-DADLE are substrates for esterases in all of the biological media/tissues tested (Table 1). The prodrugs also exhibited increased metabolic stability to exo- and endopeptidases because DADLE and not fragments of DADLE were detected during the in vitro bioconversion experiments (Fig. 2). However, two problems still exist with regard to the bioconversion of these prodrugs. The first is that AOA-DADLE and OMCA-DADLE tend to undergo more rapid bioconversion in rat plasma and liver than in brain (Table 1). CA-DADLE seems to have more favorable bioconversion characteristics because the half-lives of this prodrug in rat plasma and brain homogenate are approximately equal (Table 1). However, the bioconversion in liver homogenate is extremely fast (Table 1). In addition, the CA linker and OMCA linker have the added complication of generating intermediates (Fig. 1, II and III) that seem to limit their “bioconversion efficiencies” in vivo. To overcome these problems, our laboratory is currently synthesizing analogs of the three linkers in an attempt to alter the rates of
esterase-catalyzed bioconversion (i.e., increase bioconversion in brain and reduce bioconversion in blood). Because the long half-lives of the intermediates (Fig. 1, II and III) arising from CA-based and OMCA-based cyclic prodrugs seem to also be a significant problem with these linkers, modified linkers having more rapid rates of conversion of the intermediate(s) to DADLE are also being developed in our laboratory.

Plasma protein binding can exert a significant influence on the pharmacokinetic properties and biological activities of a drug, especially for brain-targeted compounds (Raub et al., 1993). For DADLE and its prodrugs, more lipophilic compounds had higher fractions bound in the order CA-based and OMCA-based cyclic prodrugs, especially for brain-targeted compounds (Raub et al., 1993). For DADLE and its prodrugs, more lipophilic compounds had higher fractions bound in the order CA-DADLE > OMCA-DADLE > AOA-DADLE > DADLE, but none of the prodrugs had protein-binding values high enough to substantially affect the distribution of the compounds.

In Vivo Pharmacokinetic Studies. DADLE and its prodrugs disappeared rapidly after i.v. administration, but the prodrugs had improved stability in rat blood compared with DADLE (Fig. 3). At the same time, continuous but incomplete conversion of the prodrugs to DADLE was observed. As predicted from the in vitro results, intermediate I arising from AOA-DADLE was not detected, but intermediates II and III from CA-DADLE and OMCA-DADLE were present in significant amounts in blood after i.v. administration of these cyclic prodrugs. The slow conversions of III to II in OMCA-DADLE and II to DADLE in CA-DADLE impact negatively on their abilities to deliver this opioid peptide in rat blood as well as to target tissue (i.e., brain).

On the basis of drug clearance results, only a small fraction of DADLE was recovered in the bile and urine; therefore, metabolism by endopeptidases seems to be the major route of elimination for DADLE. The fast elimination of the prodrugs could be attributed mainly to extensive bile excretion with minor kidney clearance, whereas metabolism or deep tissue distribution was not a significant factor (Table 3). The prodrug concentrations in the bile were generally 50 to 100 times higher than their plasma concentrations, which indicates an active bile clearance mechanism, possibly mediated by efflux systems (e.g., P-gp). In contrast, DADLE and the intermediates derived from the prodrugs showed significantly lower bile excretion rates and seemed not to be substrates for P-gp.

As discussed in the Introduction, for successful opioid peptide delivery to the brain, a prodrug strategy should meet four criteria. On the basis of results from previous and present studies, we now have enough information to evaluate how AOA-DADLE, CA-DADLE, and OMCA-DADLE meet these criteria. The prodrugs meet criterion 1 and were shown to have transcellular permeation due to favorable physicochemical properties (e.g., increased lipophilicity and no charge) and unique solution structures (e.g., β-turns) that reduce their hydrogen-bonding potential (Bak et al., 1999b; Gudmundsson et al., 1999a,b,c; Ouyang et al., 2002b). With respect to criterion 2, all three prodrugs exhibited significantly better intrinsic cell permeation characteristics than DADLE itself (Bak et al., 1999a; Ouyang et al., 2002b; Tang and Borchardt, 2002a,b). However, in vitro cell culture experiments suggest that the BBB permeation of the cyclic prodrugs may be significantly restricted by their substrate activities for efflux transporters (Bak et al., 1999a; Ouyang et al., 2002a; Tang and Borchardt, 2002a,b). On the basis of the in vitro stability studies, the prodrugs partially satisfy criterion 3 with good substrate activities for esterases and stability to exo- and endopeptidases; however, the sites of esterase-catalyzed bioconversion and the long half-lives of the intermediates (Fig. 1, II and III) arising from CA-based and OMCA-based cyclic prodrugs may limit their bioconversion efficiencies in the brain. Finally, with respect to criterion 4, although the prodrugs have improved plasma half-lives compared with DADLE, their biliary excretion rates are still too rapid, and the problems associated with intermediates II and III generated from CA-DADLE and OMCA-DADLE also decreased their bioconversion efficiencies in vivo.

The brain uptake studies of the cyclic prodrugs described herein were conducted to evaluate their ability to deliver DADLE to the brain after i.v. administration. As expected based on their substrate activities for P-gp (Bak et al., 1999a; Ouyang et al., 2002a; Tang and Borchardt, 2002a,b), the amount of DADLE found in the brain after prodrug administration was very small. To investigate the BBB permeation of the prodrugs and their substrate activities for P-gp, studies using an in situ-perfused rat brain model were conducted, and the results are reported in Chen et al. (2002).

In summary, esterase-catalyzed bioconversion of the prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE) to DADLE was observed both in vitro and in vivo. The cyclic prodrugs showed similarities in their pharmacokinetic parameters and displayed improved stability compared with the parent drug DADLE in vivo. However, the prodrugs were unable to deliver significant amounts of DADLE to the brain because of their rapid biliary excretion, poor BBB permeation, and slow conversion in the brain. Therefore, to improve their potential to diffuse across the BBB for better DADLE delivery into the brain, modifications will need to be made in the linkers used to make these prodrugs to 1) stabilize them to esterases in the blood compartment while increasing their bioconversion rates in the brain, 2) decrease the chemical stability of the intermediates to improve their bioconversion efficiencies to DADLE, 3) reduce their substrate activities for efflux transporters to achieve better “apparent” permeation properties, and 4) reduce their substrate activities for the transporter(s) in the liver to limit their biliary clearance. Studies are currently ongoing in our laboratory to improve the biopharmaceutical properties of these cyclic prodrugs.

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