Evaluation of the Permeation Characteristics of a Model Opioid Peptide, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE), and Its Cyclic Prodrugs across the Blood-Brain Barrier Using an In Situ Perfused Rat Brain Model

WEIQING CHEN, JERRY Z. YANG, RIKKE ANDERSEN, LISBETH H. NIELSEN, and RONALD T. BORCHARDT
Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas (W.C., J.Z.Y., R.T.B.); and Department of Pharmaceutics, The Royal Danish School of Pharmacy, Copenhagen, Denmark (R.A., L.H.N.)
Received April 9, 2002; accepted August 2, 2002

ABSTRACT
The permeation characteristics 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 (AOA-DADLE), coumarinic acid-based cyclic prodrug of DADLE (CA-DADLE), and oxymethyl-modified coumarinic acid-based cyclic prodrug of DADLE (OMCA-DADLE)) across the blood-brain barrier (BBB) were determined using an in situ perfused rat brain model. The rat brains were perfused with Krebs-bicarbonate buffer containing test compounds in the absence or presence of a specific P-glycoprotein inhibitor (GF-120918). Brain samples were collected after perfusion and processed by a capillary depletion method. After liquid phase extraction with acetoni-trile, samples were analyzed using high-performance liquid chromatography with tandem mass spectrometric detection. Linear uptake kinetics of DADLE and its cyclic prodrugs was observed within the range of 60 to 240 s of perfusion. The apparent permeability coefficient ($P_{app}$) of DADLE across the BBB was very low ($<10^{-7}$ cm/s), probably due to its unfavorable physicochemical properties (e.g., charge, hydrophilicity, and high hydrogen-bonding potential). All three cyclic prodrugs, however, also exhibited low membrane permeation ($P_{app} < 10^{-7}$ cm/s) in spite of their more favorable physicochemical properties (e.g., no charge, high hydrophobicity, and low hydrogen-bonding potential). Inclusion of GF-120918 (10 $\mu$M) in the perfusates fully inhibited the P-gp activity in the BBB and dramatically increased the $P_{app}$ values of AOA-DADLE, CA-DADLE, and OMCA-DADLE by approximately 50-, 460-, and 170-fold, respectively. In contrast, GF-120918 had no effect on the $P_{app}$ value of DADLE. In addition, the observed bioconversions of the prodrugs to DADLE in the rat brains after 240-s perfusion were very low (5.1% from AOA-DADLE, 0.6% from CA-DADLE, and 0.2% from OMCA-DADLE), which was consistent with the in vitro bioconversion rates determined previously in rat brain homogenates.

In an attempt to improve the blood-brain barrier (BBB) permeation of H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE), an opioid peptide (Hill and Pepper, 1978; Iyengar et al., 1987; Prokai-Tatrai et al., 1996), our laboratory has synthesized cyclic prodrugs of this peptide 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., 2002a) (Fig. 1). Unlike DADLE, which is hydrophilic and charged, AOA-DADLE (Bak et al., 1999b), CA-DADLE (Wang et al., 1999), and OMCA-DADLE (Ouyang et al., 2002a) are lipophilic and uncharged. The physicochemical properties of these cyclic prodrugs of DADLE are indicative of solutes that have good cell membrane permeation characteristics (Pauletti et al., 1997).

However, when the cell membrane permeation of these cyclic prodrugs of DADLE was evaluated using various in vitro cell culture models (e.g., Caco-2 cells and Madin-Darby canine kidney cells) (Bak et al., 1999a; Ouyang et al., 2002b; Tang and Borchardt, 2002a,b), they all exhibited low transcellular permeation characteristics. This low cell membrane permeation seems to result from their substrate activities for the apically polarized efflux transporters, including P-glycoprotein (P-gp) (Bak et al., 1999a; Ouyang et al., 2002b; Tang and Borchardt, 2002a,b). These in vitro results suggest that
the brain delivery of these cyclic prodrugs might be significantly restricted due to the high level of P-gp expressed in the BBB (Borst and Schinkel, 1998).

Initial attempts to assess the permeation characteristics of AOA-DADLE, CA-DADLE, and OMCA-DADLE across the BBB were made in vivo by measuring brain levels of these prodrugs after their i.v. administration in rats (Yang et al., 2002b). As expected from the in vitro cell permeation studies described above, the brain uptake after i.v. administration of these prodrugs in rats was shown to be very low (Yang et al., 2002b).

To elucidate the mechanisms responsible for the low brain uptake of AOA-DADLE, CA-DADLE, and OMCA-DADLE, transport experiments of DADLE and its cyclic prodrugs were conducted using an in situ perfused rat brain model of the BBB (Takasato et al., 1984). By directly perfusing the rat brain with a physiological buffer containing test compound(s) in the absence and presence of inhibitors of P-gp, the mechanism responsible for the low brain uptake of these cyclic prodrugs was elucidated in these studies.

**Materials and Methods**

**Materials.** [3H]Diazepam (1 mCi/ml) and [3H]quinidine (1 mCi/ml) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]Sucrose (0.1 mCi/ml) was obtained from PerkinElmer Life Sciences (Boston, MA). GF-120908 was a gift from Dr. Kenneth Brouwer (GlaxoSmithKline, Research Triangle Park, NC). DADLE and [Leu5]-enkephalin were purchased from Sigma-Aldrich (St. Louis, MO). Prodrugs of DADLE and [Leu5]-enkephalin were synthesized in our laboratory following procedures described previously (Bak et al., 1999b; Wang et al., 1999; Ouyang et al., 2002a). All other chemicals were high grade and were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Acros Organics distributed by Fisher Scientific (Houston, TX).

**Brain Perfusion Experiments.** The in situ rat brain perfusion technique used in our laboratory was similar to that as described elsewhere (Takasato et al., 1984; Smith, 1996) with modifications. Sprague-Dawley male rats (350–400 g) used in the in situ brain perfusion experiments were purchased from Sasco (Omaha, NE). For surgical preparation, rats were given an anesthetic cocktail (56.25 mg/kg ketamine, 2.85 mg/kg xylazine, and 0.55 mg/kg acepromazine i.p.). The left common carotid artery (LCCA) was prepared for can-
nulation by ligating both the LCCA (about 1.5 cm proximal to its bifurcation) and the left external carotid artery at the bifurcation site, including the pterygopalatine artery. A polyethylene-60 catheter (20 cm; BD Biosciences, Sparks, MD) filled with heparinized saline (60 U/ml) was inserted into the LCCA toward the left internal carotid artery for perfusion. The rectal temperature of the animal was maintained at 36.5 ± 0.5°C throughout the surgery by a heat pad connected to a feedback device (model 73; YSI Inc., Yellow Springs, OH).

For perfusion experiments, the left hemisphere of the rat brain was perfused with perfusate containing DADLE or its cyclic prodrugs (20 μM) at a flow rate of 10 ml/min through the LCCA catheter that was connected to the infusion syringes via a switching valve (Hamilton, Reno, NV). The perfusion buffer (37°C, pH 7.4) consisted of bicarbonate-buffered physiological saline (NaH₂PO₄, KCl, NaHCO₃, NaCl, CaCl₂, MgSO₄, and α-glucose) and was oxygenated with a mixture air of 95% O₂ and 5% CO₂ before perfusion. Quattro LC or Quattro Micro triple quadrupole mass spectrometer using CLASS-VP version 4.2 Chromatography Data System (Shimadzu) was maintained at 36.5°C for 15 min, the pellet was discarded and the supernatant stored at 4°C for 15 min. The supernatant containing both the lipid and the aqueous phases was collected and centrifuged at 4500 g for 15 min. The supernatant of the homogenate at 4°C for 15 min, the pellet was discarded and the supernatant containing esterase and peptidases inhibitors (0.5 mM paraoxon, 10 mM phosphoramidon, amastatin, and captopril) using a glass homogenizer (Wheaton, Philadelphia, PA). A capillary depletion method (Triguero et al., 1999) was used to remove the drugs that were bound to the brain vasculature. After centrifugation of the homogenate at 4500g (model 59A Micro-Centrifuge; Fisher Scientific, Pittsburgh, PA) at 4°C for 15 min, the pellet was discarded and the supernatant containing both the lipid and the aqueous phases was collected and stored at −80°C until further extraction.

For sample extraction, brain samples were mixed with acetonitrile (1:3, v/v) and centrifuged at 21,000g for 15 min. The supernatant after centrifugation was evaporated to dryness using a Centrivap concentrator (Labconco, Kansas City, MO). The final residue was dissolved in 0.1 ml of 10% acetonitrile and centrifuged again at 10,000g and 4°C for 10 min. Thirty microliters of the supernatant was injected for high-performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) analysis.

Sample Preparation. Brain tissue samples were collected from the left frontal, parietal, and occipital cortex as well as the hippocampus and weighed. Internal standards were added to the collected brain tissues before further preparation. The internal standard used for quantification of DADLE was [Leu⁵]-enkephalin and those for cyclic prodrugs of DADLE were the cyclic [Leu⁵]-enkephalin containing the same linkers as their DADLE counterparts. Brain tissues were then homogenized (30 strokes) in ice-cold saline (1:4, v/v) containing esterase and peptidases inhibitors (0.5 mM paraoxon, 10 μM phosphoramidon, amastatin, and captopril) using a glass homogenizer (Wheaton, Philadelphia, PA). A capillary depletion method (Triguero et al., 1999) was used to remove the drugs that were bound to the brain vasculature. After centrifugation of the homogenate at 4500g (model 59A Micro-Centrifuge; Fisher Scientific, Pittsburgh, PA) at 4°C for 15 min, the pellet was discarded and the supernatant containing both the lipid and the aqueous phases was collected and stored at −80°C until further extraction.

For sample extraction, brain samples were mixed with acetonitrile (1:3, v/v) and centrifuged at 21,000g for 15 min. The supernatant after centrifugation was evaporated to dryness using a Centrivap concentrator (Labconco, Kansas City, MO). The final residue was dissolved in 0.1 ml of 10% acetonitrile and centrifuged again at 10,000g and 4°C for 10 min. Thirty microliters of the supernatant was injected for high-performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) analysis.

Sample Analysis. To determine the concentrations of DADLE and its cyclic prodrugs in the perfusates, 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. Sample separation was performed using a C18 reversed-phase column (300A, 250 × 4.6-mm i.d.; 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 detection (λ = 214 nm). The chromatographic data were acquired and analyzed using CLASS-VP version 4.2 Chromatography Data System (Shimadzu).

To determine the contents of DADLE and its prodrugs in perfused brain tissue samples, LC/MS/MS was performed using a Micromass Quattro LC or Quattro Micro triple quadrupole mass spectrometer (Micromass, Beverly, MA). The analytical method was described extensively elsewhere (Yang et al., 2002a). Briefly, the liquid chromatography was conducted using a 2690 HPLC System (Waters, Milford, MA). A reversed-phase C₁₈ column (50 × 1.0-mm i.d.; 5 μm; Vydac) was used as the analytical column and the column temperature was kept at 25°C. 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 was water with 0.1% (v/v) formic acid, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. The HPLC system was interfaced to the mass spectrometer via an electrospray interface. For sample recording, multiple reaction monitoring of several channels was used with 0.1-s dwell time and interchannel delay set at 0.03 s so that several compounds could be monitored simultaneously. Data acquisition and analysis were performed using MassLynx version 3.4 software (Micromass).

When perfusion studies were conducted using a mixture of [14C]sucrose (0.3 μCi/ml) and [3H]diazepam [1H]quinidine (1 μCi/ml) alone, the same perfusion procedures as described above were followed. Brain tissue samples collected from left cortex were weighed and digested in Solvable (Packard Biosciences, Meriden, CT) at 37°C for 24 h. Samples were then counted for radioactivity using a dual-label scintillation spectrometer (LS 6000 IC; Beckman Coulter, Inc., Fullerton, CA). To determine the original radioactivity level in the perfusate, duplicate perfusate samples (50 μl) were also collected, mixed with Solyable, and counted.

Calculations. Brain uptake of a test drug was analyzed using a simple, linear two-compartment model (the vascular blood and the brain parenchyma) in which the perfusion time was limited (<4 min) to minimize the back-flux of solutes from brain parenchyma to the “blood” (perifusate) (Takasato et al., 1984). The unidirectional transfer coefficient Kn (milliliters per second per gram) from blood to brain was estimated using the following equations:

$$Q_P/C_{pf} = K_n \times t + V_{vac}$$

or

$$K_n = (Q_P/C_{pf}) - V_{vac}/t$$

where Q_P is the amount of drug in the brain parenchyma (nanograms per gram), C_pf is the drug concentration in the perfusion fluid (nanograms per milliliter), Q_P/C_pf is defined as the apparent brain distribution volume (V_br, milliliters per gram), t is the net perfusion time (seconds), and V_vac is the brain intravascular volume. In the perfusion studies for DADLE and its cyclic prodrugs, postperfusion wash was performed after perfusion with test compound. A capillary depletion method was then used for sample preparation to minimize the residual intravascular tracer remaining in the brain parenchyma. By using the postperfusion wash and the capillary depletion step, V_vac could be excluded from eqs. 1 and 2.

Equation 1 was used for multiple time point analysis, in which the perfusions were conducted for various periods of time and measured V_br values were plotted versus perfusion time. The K_n was determined as the slope from the linear regression of the measured V_br values at multiple time points. Equation 2 was used for single time point analysis, in which the perfusions were conducted for a single time period. The K_n was determined by dividing the V_br value by perfusion time.

The K_n values were converted to PA values using the Crone-Renkin model of capillary transfer (Crone and Levitt, 1984):

$$PA = -F \ln (1 - K_n/F)$$

where F is the regional cerebral perfusion fluid flow, which was determined using [3H]diazepam in separate experiments (0.025 ml/s/g); P is the apparent permeability coefficient of the BBB for a test compound; and A is the rat brain capillary surface area (130 cm²/g), which was reported previously (Metzer et al., 1980). P was calculated by dividing PA by A.
**Statistical Analysis.** All data are presented as mean ± S.D. for three rats. A Student’s t test was used to compare individual means and a P value ≤0.05 is considered to be statistically significant.

**Results**

**Physical Integrity of the BBB.** The perfusion conditions used herein for studying BBB permeation by DADLE and its cyclic prodrugs were first validated by monitoring brain uptake of various radiolabeled markers, including 1) \(^{3}H\)diazepam, which was used to determine regional cerebral fluid flow; and 2) \(^{14}C\)sucrose, which was used as a nonpermeable marker to monitor the physical integrity of the BBB. As shown in Fig. 2, \(^{3}H\)diazepam exhibited linear uptake kinetics and high brain permeation. In contrast, \(^{14}C\)sucrose showed very low BBB permeation (Papp = 2.21 ± 0.56 × 10⁻⁷ cm/s). The permeation of \(^{14}C\)sucrose across the BBB remained low even when DADLE or its cyclic prodrugs (20 μM) were included in the perfusate.

![Graph A](image1.png)  ![Graph B](image2.png)

**Fig. 2.** \(V_{br}\) values of diazepam (A) and sucrose (B) in rat brains versus perfusion time. Rat brains were perfused with buffers containing \(^{3}H\)diazepam (1 μCi/ml) and \(^{14}C\)sucrose (0.3 μCi/ml) for various times (15, 30, 60, and 240 s). The rats were decapitated and the brains removed and dissected on ice. Brain tissue samples collected from left cortex were weighed and digested in Sowable at 37°C for 24 h. Samples were counted for radioactivity using a dual-label scintillation spectrometer. The \(V_{br}\) values were determined using multiple time point analysis, as described under Materials and Methods. The solid line in A is the linear regression of the mean \(V_{br}\) values (n = 3) at each time point.
(data not shown). However, the BBB permeation of $[^{14}]$C)sucrose was increased slightly (approx. 2.3-fold) when GF-120918 (10 μM), a P-gp inhibitor, was included in the perfusate (Table 1).

**Efflux Activity of P-gp in the BBB.** On the basis of data published previously by our laboratory (Bak et al., 1999a; Ouyang et al., 2002a,b; Tang and Borchardt, 2002a,b), AOA-DADLE, CA-DADLE, and OMCA-DADLE all exhibited substrate activities for efflux transporters in cell culture models. Therefore, studies were designed to determine whether the substrate activity of these cyclic prodrugs limited their permeation of the BBB using this perfused brain model. According to the literature, the major efflux transporter present in the BBB is P-gp (Regina et al., 1998; Zhang et al., 2000). Therefore, the efflux activity of the BBB was evaluated by determining the permeation of $[^{3}H]$quinidine, a known substrate of P-gp (Kusuhara et al., 1997). To determine the effect of GF-120918 on the BBB permeation of $[^{3}H]$quinidine, rat brain perfusion studies of this solute were determined in the absence and presence of different concentrations of this P-gp inhibitor. The $P_{app}$ values for $[^{3}H]$quinidine increased significantly as the concentrations of GF-120918 in the perfusate increased from 0 to 1 μM. The $P_{app}$ values eventually plateaued at GF-120918 concentrations above 4 μM (Fig. 3). When the $P_{app}$ values of $[^{3}H]$quinidine in the absence and presence of GF-120918 were fitted to a modified Michaelis-Menten equation, an IC$_{50}$ value of 0.37 ± 0.31 μM and a $P_{app}$max of 2.1 × 10$^{-5}$ cm/s were calculated. At the highest concentration of GF-120918 used in these studies, the $P_{app}$ value of quinidine increased approximately 10-fold (Table 1).

**Permeation of DADLE and Its Cyclic Prodrugs across the BBB.** Brain uptake of DADLE and its cyclic prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE) in the absence of GF-120918 was evaluated by determining the $V_{br}$ values at 60, 120, and 240 s of perfusion (multiple time point analysis). Figure 4 shows the linear uptake kinetics of DADLE and its cyclic prodrugs. Linear regression analysis was done on the mean $V_{br}$ values at each perfusion time ($n = 3$) for each compound, giving the $K_{m}$ values that were then used to calculate the apparent $P_{app}$ values. In the absence of GF-120918, the $P_{app}$ values for the BBB permeation of the cyclic prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE) were approximately equal to the $P_{app}$ value determined for DADLE (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$P_{app} \times 10^{5}$ (cm/s)$^{a}$</th>
<th>$P_{app}$</th>
<th>cLogP$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DADLE</td>
<td>0.5 ± 1.4</td>
<td>0.6 ± 0.1</td>
<td>-0.32</td>
</tr>
<tr>
<td>AOA-DADLE</td>
<td>1.2 ± 1.0</td>
<td>60.5 ± 25.6</td>
<td>3.32</td>
</tr>
<tr>
<td>CA-DADLE</td>
<td>0.4 ± 0.7</td>
<td>185 ± 65.3</td>
<td>4.96</td>
</tr>
<tr>
<td>OMCA-DADLE</td>
<td>0.7 ± 0.6</td>
<td>119 ± 12.8</td>
<td>4.95</td>
</tr>
<tr>
<td>Quinidine</td>
<td>16.2 ± 1.1</td>
<td>169 ± 65.3</td>
<td>3.36</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.2 ± 0.6</td>
<td>5.2 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ In the case of AOA-DADLE, CA-DADLE, and OMCA-DADLE, $P_{app}$ values are based on the sum of prodrug, intermediate, and DADLE present in brain tissue.

$^{b}$ A 10 μM concentration of GF-120918 was used in these studies because at this concentration the efflux of quinidine was totally inhibited.

The cLogP values for AOA-DADLE, CA-DADLE, and OMCA-DADLE were calculated using a Sybyl 6.5 program, and the cLogP value for quinidine was reported by Voigt et al. (1988).

**Discussion**

The in situ perfused rat brain model, which was used in these studies, has been used by other investigators to elucidate the mechanisms (e.g., passive diffusion, passive diffusion modulated by efflux transporters, and active transport) of transport of a variety of drugs across the BBB (Smith, 1996). However, before initiation of our studies designed to elucidate the mechanisms of BBB transport of DADLE and its three prodrugs, validation of this model in our laboratory
needed to be done. Linear characteristics of brain uptake of a solute in this two-compartment model (blood and brain) were confirmed by kinetic studies of \( ^{3}H \) diazepam (Fig. 2), which is known to undergo passive diffusion via the transcellular route across the BBB (Rapoport et al., 1979; Takasato et al., 1984). Because the permeation of \( ^{14}C \) sucrose, which is known to permeate the BBB by passive diffusion via the paracellular route, was extremely low (Fig. 2), the integrity of the tight junctions of the BBB was demonstrated. Furthermore, the permeation of \( ^{14}C \) sucrose remained low in the presence of DADLE or its three prodrugs (data not shown), demonstrating that the test compounds did not alter the integrity of the tight junctions.

In addition to its physical barrier properties (e.g., tight junctions and lipid bilayers), the BBB also expresses high levels of efflux transporters (e.g., P-gp) (Jette and Beliveau, 1993; Anderson, 1996; Borst and Schinkel, 1998) that can modulate the transcellular passive diffusion of lipophilic permeants (e.g., cyclic prodrugs of DADLE). To assess P-gp-mediated efflux activity in this in situ rat brain perfusion model, the transport of \( ^{3}H \) quinidine, a known substrate of P-gp (Kusuhara et al., 1997), was determined in the absence and presence of GF-120918, a known P-gp-specific inhibitor (Hyafil et al., 1993). Compared with the \( P_{\text{app}} \) value of \( ^{3}H \) quinidine in the absence of P-gp inhibitor, the \( P_{\text{app}} \) value of \( ^{3}H \) quinidine in the presence of GF-120918 (10 \( \mu \)M) increased by approximately 10-fold (Table 1). In addition, the effect of GF-120918 on brain permeation of quinidine was concentration-dependent with the \( P_{\text{app}} \) values of quinidine plateauing at GF-120918 concentrations above 4 \( \mu \)M (Fig. 3). These results with \( ^{3}H \) quinidine demonstrated the presence of a functional P-gp in this in situ perfused rat brain model and that the efflux activity of this P-gp can be inhibited by GF-120918. It should be noted that inclusion of GF-120918 (10 \( \mu \)M) in the perfusion buffer increased brain permeation of \( ^{14}C \) sucrose by about 2.3-fold (Table 1). However, the contribution of this "loosening" of the tight junctions to the overall permeation of \( ^{3}H \) quinidine would be considered minor because this solute is highly lipophilic (cLogP = 3.36; Voigt et al., 1988) and thus considered to be a transcellular permeant.

After optimization of the in situ rat brain perfusion conditions, the permeation characteristics of DADLE and its cyclic prodrugs across the BBB were determined. Similar to \( ^{3}H \) diazepam, linear uptake kinetics of DADLE and its cyclic prodrugs was observed (Fig. 4). As expected, the permeation of DADLE across the BBB was very low due to its unfavorable physiochemical properties (e.g., hydrophilicity, charge and high hydrogen bonding potential) (Table 1). On the basis of our previous observations using cell culture models (Bak et al., 1999a; Ouyang et al., 2002b; Tang and Borchardt,
2002a,b), we were not surprised to observe the low $P_{\text{app}}$ values of the three cyclic prodrugs across the BBB, which were comparable with the $P_{\text{app}}$ values of DADLE and sucrose (a nonpermeable marker) (Table 1). The poor BBB permeation of these prodrugs did not correlate with their physicochemical properties (e.g., no charge, high hydrophobicity, and low hydrogen-bonding potential) (Bak et al., 1999b; Wang et al., 1999; Ouyang et al., 2002a), but they were very consistent with their poor cell permeation characteristics observed in cell culture models (Bak et al., 1999a; Ouyang et al., 2002b; Tang and Borchardt, 2002a,b).

The previous cell permeation studies showed that all three prodrugs were substrates for the efflux transporters, particularly P-gp (Ouyang et al., 2002b; Tang and Borchardt, 2002a,b). Because P-gp is known to be expressed at high levels in the BBB (Cordon-Cardo et al., 1989; Regina et al., 1998), this efflux transporter was considered most likely responsible for the low BBB permeation of these cyclic prodrugs. To study the possible role of P-gp on prodrug permeation into the brain, GF-120918 was added to the perfusion buffer containing DADLE or its prodrugs during the brain perfusion experiments. In the presence of 10 $\mu$M GF-120918, the permeation of AOA-DADLE, CA-DADLE, and OMCA-DADLE into the brain was enhanced tremendously (Table 1). These results confirmed that brain uptake of these cyclic prodrugs of DADLE was being significantly limited by their substrate activities for P-gp in the BBB. It should be noted that GF-120918 had no effect on brain permeation of DADLE, which was consistent with the observation that this peptide is a paracellular permeant and does not exhibit favorable interaction with the lipid bilayers of cell membrane where P-gp resides.

Assuming that P-gp activity was totally inhibited in the presence of 10 $\mu$M GF-120918, as indicated from the quinidine studies (Fig. 3), the $P_{\text{app}}$ values determined in the presence of GF-120918 then represent the intrinsic BBB permeability coefficients of cyclic prodrugs. As expected, these intrinsic BBB permeability coefficients of AOA-DADLE, CA-DADLE, and OMCA-DADLE are significantly greater than the intrinsic permeability coefficient of DADLE. It is interesting to note that these intrinsic $P_{\text{app}}$ values correlated well with the lipophilic characteristics of DADLE and its cyclic prodrugs as indicated by their cLogP values (Table 1). These results suggest that the physicochemical properties designed
into these cyclic prodrugs of DADLE do provide the molecules with good intrinsic permeation across the BBB, and it is their substrate activity for P-gp that ultimately limits their access to the brain. However, the current results do not exclude the contribution of other transporters in the BBB to their active efflux.

For a prodrug strategy ultimately to be successful in delivering drugs to the brain, other factors in addition to good cell membrane permeation need to be considered. Although it is generally beneficial to make prodrugs more lipophilic to improve their intrinsic cell membrane permeation, one also needs to monitor their potential substrate activities for the efflux transporters as demonstrated in these studies. Recent work by Seelig et al. (2000) and Seelig and Landwojtowicz (2000) suggested that polar surface area characteristics of a molecule may be important in determining substrate activity for these efflux transporters. Therefore, studies continue in our laboratory in an effort to identify chemical linkers that do not bestow efflux transporter substrate properties on the cyclic prodrugs.

Another desirable characteristic of a successful prodrug for brain delivery is preferential bioconversion of the prodrug in the brain. The current rat brain perfusion studies provided some insight into this bioconversion. Very low levels of DADLE derived from its prodrugs were observed in the brain after 240-s perfusion. These results are consistent with the in vitro stability studies reported previously by our laboratory (Yang et al., 2002b). According to a simulation model described by Anderson (1996) for prodrug-to-drug bioconversion in plasma and the brain, brain selectivity of prodrug bioconversion is a prerequisite for enhancement of brain delivery of a parent compound. Using a prodrug with a brain bioconversion rate 10-fold greater than its plasma bioconversion rate and a half-life of approximately 1 to 2 min, optimal brain delivery of parent drug was achieved (Anderson, 1996). In a previous study using rat plasma and brain homogenates (Yang et al., 2002b), we showed that these prodrugs were bioconverted in plasma much more rapidly than in brain (or liver). In addition, we observed from in vivo pharmacokinetic studies that these cyclic prodrugs were rapidly cleared by the liver, probably due to their substrate activity for efflux transporters (Yang et al., 2002b). Therefore, for the cyclic prodrug strategy to be successful in delivering a pharmacologically significant amount of DADLE into the brain, selectivity for brain bioconversion and an optimal bioconversion rate need to be incorporated into the cyclic prodrug design. Our laboratory is currently modifying the three chemical linkers in an attempt to alter the rates of esterase-catalyzed bioconversion (i.e., increase bioconversion in brain and reduce bioconversion in blood).

In summary, the cyclic prodrugs of DADLE for targeted brain delivery have satisfied some of the criteria for successful prodrugs, including 1) more favorable physiochemical properties, 2) higher intrinsic BBB permeation, 3) improved stability in vivo, and 4) bioconversion to the parent drug. However, some undesirable characteristics associated with

Fig. 5. Effect of GF-120918 on brain uptake of DADLE and the cyclic prodrugs AOA-DADLE, CA-DADLE, and OMCA-DADLE. Brain samples were collected after perfusion and processed by a capillary depletion method. After liquid phase extraction with acetonitrile, samples were analyzed using LC/MS/MS. The $V_{br}$ values for DADLE and its cyclic prodrugs were determined using single time point analysis, as described under Materials and Methods and are presented as the mean ± S.D. (n = 3). The asterisks (*) indicate that the difference between the $V_{br}$ values in the absence and presence of GF-120918 was statistically significant ($P < 0.05$) according to paired Student's t test.
the prodrugs significantly limited their ability to deliver DADLE to the brain, i.e., their substrate activities for P-gp in the BBB and low bioconversion efficiency in the brain. Therefore, for this cyclic prodrug strategy to be successful, these undesirable characteristics will need to be designed out of molecules by altering the chemical linkers.

Acknowledgments

We thank Dr. Kenneth Brouwer (GlaxoSmithKline) for providing a sample of GF-120918.

References


