Pharmacodynamic and Pharmacokinetic Characterization of Poly(Ethylene glycol) Conjugation to Met-Enkephalin Analog [\(\text{D-Pen}^2,\text{D-Pen}^5\)]-enkephalin (DPDPE)

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ABSTRACT

Poly(ethylene glycol), or PEG, conjugation to proteins and peptides is a growing technology used to enhance efficacy of therapeutics. This investigation assesses pharmacodynamic and pharmacokinetic characteristics of PEG-conjugated [\(\text{D-Pen}^2,\text{D-Pen}^5\)]-enkephalin (DPDPE), a met-enkephalin analog, in rodent (in vivo, in situ) and bovine (in vitro) systems. PEG-DPDPE showed increased analgesia (i.v.) compared with nonconjugated form (\(p < 0.01\)), despite a 172-fold lower binding affinity for the \(\delta\)-opioid receptor. \([^{125}\text{I}]\)PEG-DPDPE had a 36-fold greater hydrophilicity (\(p < 0.01\)) and 12% increase in the unbound plasma protein fraction (\(p < 0.01\)), compared with \([^{125}\text{I}]\)DPDPE. \([^{125}\text{I}]\)PEG-DPDPE had a 2.5-fold increase in elimination half-life (\(p < 0.01\)), 2.7-fold decrease in volume of distribution (\(p < 0.01\)), and a 7-fold decrease in plasma clearance rate (\(p < 0.01\)) to \([^{125}\text{I}]\)DPDPE. Time course distribution showed significant concentration differences (\(p < 0.01\)) in plasma, whole blood, liver, gallbladder, gastrointestinal (GI) content, GI tract, kidneys, spleen, urine, and brain (\(p < 0.05\)), between the conjugated and nonconjugated forms. Increased brain uptake of \([^{125}\text{I}]\)PEG-DPDPE corresponded to analgesia data. \([^{125}\text{I}]\)PEG-DPDPE in brain was shown to be 58.9% intact, with 41.1% existing as \([^{125}\text{I}]\)DPDPE (metabolite), whereas \([^{125}\text{I}]\)DPDPE was 25.7% intact in the brain (at 30 min). In vitro P-glycoprotein affinity was shown for \([^{125}\text{I}]\)DPDPE (\(p < 0.01\)) but not shown for \([^{125}\text{I}]\)PEG-DPDPE. In vitro saturable uptake, with 100 \(\mu\)M DPDPE, was shown for \([^{125}\text{I}]\)PEG-DPDPE (\(p < 0.05\)). In this study, PEG-conjugated DPDPE seems to act as a prodrug, enhancing peripheral pharmacokinetics, while undergoing hydrolysis in the brain and allowing nonconjugated DPDPE to act at the receptor.

Poly(ethylene glycol) (PEG), also known as poly(ethylene oxide), is a nontoxic, nonimmunogenic, biocompatible, and water-soluble polymer used in biotechnology, biomaterials, and pharmacetics. PEGs consist of repeating ethoxy subunits with terminal hydroxyl groups that can be chemically activated and may be attached to a compound at single or multiple sites. PEG derivatives, covalently attached polyethylene glycol to proteins and peptides (“PEGylation”), have been used to enhance drug stability and circulation, while reducing immunogenicity, proteolysis, and clearance (Deldago et al., 1992; Reddy, 2000). Low-molecular weight drugs (<20,000) have been attached to PEG to enhance solubility and alter biodistribution, while reducing toxicity and plasma protein binding (Harris and Zalipsky, 1997; Greenwald et al., 2000). The enormous potential of PEGylated proteins as therapeutics was initially assessed in the 1970s and has evolved over the past two decades. Three PEGylated proteins, bovine adenosine deaminase (Adagen) for the treatment of adenosine deaminase ADA deficiency, Escherichia coli L-asparaginase (Oncaspar) for the treatment of acute lymphoblastic leukemia, and interferon alpha (PEG-Intron) for the treatment of hepatitis C, have been approved by the Food and Drug Administration, with several other PEG proteins in clinical trials by a number of companies. However, no PEG conjugate to a low-molecular weight compound has yet been approved by the Food and Drug Administration for therapeutic use.

Peptides bound to PEG often serve as linkers to larger compounds, ideally allowing release of the compound to target locations, based on pH or enzymatic breakdown of the peptide link. However, peptides serving as the biologically approved by the Food and Drug Administration for therapeutic use.
active agent, bound to PEG, have not been fully evaluated. Peptide-based therapeutics suffer from a number of drawbacks, principally a lack of enzymatic stability and rapid elimination, both of which benefit from PEG technology. The use of PEG-modified opioid peptides has been shown to significantly enhance analgesia (i.e., c.v.); however, results varied depending upon the peptide to which the PEG moiety was attached (Maeda et al., 1994). In this study, our research group has assessed the pharmacokinetic and pharmacodynamic characteristics of the linear methoxy-PEG (2 kDa) conjugate of met-enkephalin analog DPDPE (Fig. 1). DPDPE is a well characterized opioid peptide that provides a number of advantages for the examination of PEGylation. Transport of DPDPE into the CNS has been investigated previously by our laboratory using in vitro, in situ, and in vivo techniques. DPDPE is a low-molecular weight peptide, which is conformationally constrained via cyclic disulfide bonds providing enhanced stability ($t_{1/2} > 500$ min in blood) (Weber et al., 1991). This innate stability allows for a more accurate assessment of the PEG conjugation without added complication of peptide degradation. DPDPE does in effect eliminate one of the primary benefits of PEGylation (i.e., stability); however, the degree of stability allows for more accurate quantification. Analgesia, via $\delta$-opioid receptors, is understood to be a centrally mediated event. Only those $\delta$-selective opioids that can cross the blood-brain barrier (BBB) intact will achieve biological effect (Frederickson et al., 1981; Shook et al., 1987). This characteristic provides the ability to assess how PEG modification affects BBB transport. DPDPE has also been shown to be a substrate for the P-glycoprotein (P-gp) efflux mechanism at the BBB (Chen and Pollack, 1999; Witt et al., 2000), allowing an opportunity to assess PEGs effect on P-gp efflux. Last, DPDPE and other opioids are known to be rapidly and extensively excreted via the hepato-biliary route of elimination (Weber et al., 1992; Chen and Pollack, 1997). The rapid clearance of DPDPE greatly contributes to limited uptake into the CNS, thus requiring relatively large peripheral doses to achieve analgesic effect. Therefore, DPDPE allows for the evaluation of the PEG moiety when attached to a drug with virtually no renal clearance and its subsequent ability to reduce elimination and promote the necessary analgesic response.

Numerous alterations have been introduced into peptides, both to gain greater understanding of biological activity and to increase potency and bioavailability. Modification of drug molecules with PEG results in altered properties of the compound, such as steric interference, changed electrostatic binding properties, and conformational alterations (Reddy, 2000). Thus, not all PEGylated proteins are alike, and each requires optimization on an individual basis to derive the maximum clinical benefit. In this study, we analyzed the contribution of PEGylating DPDPE in a series of established techniques in an endeavor to develop strategies for future peptide drug development paradigms.

**Materials and Methods**

**Radioisotopes/Chemicals.** $[\text{3H}]$Deltorphin-II (41.0 Ci/mmol) and $[\text{3H}]$DAMGO (50.0 Ci/mmol), and Na$^{125}$I (107 mCi/ml) were purchased from PerkinElmer Life Science Products (Boston, MA). DPDPE was obtained from Multiple Peptide Systems (San Diego, CA). All other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

**Synthesis of PEG-DPDPE.** DPDPE (3.0 mg) was dissolved in 5 ml of analytical grade acetonitrile. A 20% molar excess of PEG reagent (mPEG-SPA 2K (11.1 mg)) and triethylamine (0.8 $\mu$l) was added to the DPDPE. The reaction was allowed to proceed at room temperature under an argon atmosphere for 2 days. The sample was diluted to 15 ml with deionized water and lyophilized. The PEG-DPDPE powder was reconstituted in 5 ml of deionized water and purified on a Superdex 30 size exclusion column (Amersham Pharmacia Biotech, Arlington Heights, IL). Pertinent fractions were pooled together, dried against water, and frozen or lyophilized. DPDPE content was determined via UV detector (absorbance, 215 nm), using a Keystone C18 column with a 10 mM sodium phosphate buffer (pH 7.2).

**Iodination of Compounds.** DPDPE and PEG-DPDPE were monoiiodinated on the tyrosine residue using a standard chloramine-T procedure (Bolton, 1986), as adapted in our laboratory by Schetz et al. (1995). Purification of iodinated peptides was carried out using a reverse-phase PerkinElmer 250 HPLC gradient system and a Vydac column (880115-9 no. 74). Samples were eluted at 37°C using a curvilinear gradient of 0.1% TFA in acetonitrile (10–35%) versus 0.1% aqueous TFA over 20 min at a flow rate of 1.5 ml · min$^{-1}$.

**Animals.** Adult female Sprague-Dawley rats weighing 250 to 300 g were used for in situ brain perfusion analysis; male adult ICR mice weighing 25 to 30 g were used for all other analyses, unless otherwise noted. Rats and mice were housed separately under standard 12-h light/dark conditions and received food and water ad libitum, unless otherwise noted. All protocols were approved through the Institutional Animal Care and Use Committee at the University of Arizona.

**Intracerebroventricular Injections.** Intracerebroventricular injections were performed in the manner described by Porreca et al. (1984). Mice were lightly anesthetized with ether. A longitudinal incision was then made in the scalp and the bregma suture identified. A 25-gauge needle, attached to a 25-$\mu$l syringe, was inserted 2 mm through the skull in a position 1-mm lateral to the midline and 2-mm caudal to bregma to reach the lateral ventricle. Injection depth was controlled by a plug on the needle. Drugs were injected in a volume of 5 $\mu$l.

**Analgesia Analysis.** Radiant-heat tail-flick analgesia meter, model-33 (ITTC Scientific Products, Woodland Hills, CA), was used to assess antinociceptive (i.e., analgesic) profile following administration of DPDPE or PEG-DPDPE. PEG was also assessed without conjugated peptide. The analgesia meter was set to produce a baseline latency of 2 s with a cutoff time of 15 s. Male ICR mice ($n = 25$) were administered a single dose. Intravenous dose (25 $\mu$mol/kg), via tail vein, of each respective test compound was dissolved in sterile saline and injected into the tail vein.

**Fig. 1.** Structure of DPDPE: tyrosine (1), penicillamine (2), glycine (3), phenylalanine (4), and penicillamine (5). Disulfide bond between the penicillamines to enhance stability. Polyethylene-glycol (mPEG) attachment at the amino group of tyrosine, CH$_2$-(OCH$_2$CH$_2$)$_n$-, ($n = 45$). Structure according to Liao et al. (1998).
with assessment at 15-, 30-, 45-, 60-, 90-, 120-, 150-, 180-, 210-, and 240-min time points. Intracerebroventricular dose (3.1 μmol/kg) of each respective compound was assayed at 15, 30, 45, 60, 90, 120, and 180 min. Analyses were stopped at any time point in which the maximal possible analgesic effect fell within 5% of the baseline.

Nociceptive sensitivity was determined by converting the recorded analgesic tail-flick times to a percent maximal possible effect (%MPE):

\[
\text{MPE} = \left( \frac{\text{recorded f} \text{llick time} - \text{baseline}}{\text{maximum time (15 s) - baseline}} \right) \times 100
\]

**Competition Studies in Rat Brain.** Competition studies were performed ex situ according to previous studies (Bylund and Yamamura, 1990; Hruby et al., 1997), using 1.0 nM [3H]DAMGO to label the μ-opioid receptor and 1.0 nM [3H]deltorphin-II to label the δ-opioid receptor. Rat brains were removed and immediately homogenized in 50 mM Tris buffer. At least 10 concentrations of competing ligands were used over a 5-log concentration range. Specific binding displacement was defined using 10 μM naltrexone. Incubations took place in an initial volume of 1 ml, in a solution consisting of 50 mM Tris/MgCl2 (pH 7.4) with 1 mg·ml−1 bovine serum albumin, 50 μg·ml−1 bacitracin, 30 μM bestatin, 10 μM captopril, and 100 μM phenylmethylsulfonyl fluoride. Incubation conditions were 180 min at 25°C. Final protein concentrations were determined by the Lowry method (Lowry et al., 1951). IC50 values were determined using nonlinear least-squares regression.

**Octanol/Buffer Partition Coefficients.** Partition coefficients for [125I]DPDPE and [125I]PEG-DPDPE were expressed as the ratio of compound found in the octanol phase to that found in the aqueous phase. Briefly, equal volumes of octanol and an 0.05 M HEPES buffer of compound found in the octanol phase to that found in the aqueous phase. The layers were separated and stored at 4°C. At testing, 1 ml of 0.1 M NaCl (pH 7.4) was mixed and allowed to equilibrate for 12 h. The layers were separated and stored at 4°C. At testing, 1 μCi of the respective peptide was placed in 1 ml of buffer and added to 1 ml of octanol (n = 4). The octanol-buffer solution was vigorously shaken (−2 min) and centrifuged at 1000 rpm for 5 min (37°C). The octanol and buffer phases were separated and analyzed via Beckman 5500 gamma counter (Beckman Coulter, Inc., Fullerton, CA). The octanol-buffer distribution coefficient was calculated as the ratio of octanol layer to aqueous buffer layer. All octanol-buffer distribution studies were performed in triplicate.

**Protein Binding.** The binding affinity of [125I]DPDPE and [125I]PEG-DPDPE to mouse plasma was determined by ultrafiltration, centrifugal dialysis (Abbruscato et al., 1996). Peptides were dissolved in 1 ml of plasma (37°C) and ultrafiltered using a Centricon microparticulation device (Amicon, Beverly MA) (n = 4). Respective compounds were also dissolved in saline (0.9%) in an identical manner for determination of nonspecific binding. Ultrafiltrate was obtained after the sample was centrifuged at 2000g for 10 min ( Sorval RC2-B, DuPont Co., Wilmington, DE). Total concentration (T) of iodinated compound was collected from each respective carotid cannula at 2.5 mM CaCl2; 10 mM D-glucose; 3.9% dextran (mol. wt. 70,000); heparinized (10,000 U·ml−1). Iodinated peptide unbound in plasma was calculated as:

\[
f_u = 1 - \frac{(T - F)/T}{F}
\]

Nonspecific binding was calculated in an identical manner as plasma binding and subsequently subtracted from total binding, resulting in a specific plasma protein bound concentration.

**Time Course Distribution.** Mice were deprived of food 12 h before the start of distribution studies. Mice (n = 4–5 per time point) were anesthetized with sodium pentobarbital (80 mg/kg) and administered [125I]DPDPE or [125I]PEG-DPDPE via the tail vein (−1.5 μCi per animal). After 15, 30, 45, 60, 90, 120, 180, and 240 min, the chest cavity was opened, and a blood sample (−500 μl) was taken from the left ventricle of the heart. Blood samples were divided and analyzed as whole blood and plasma; heparin was used as the anticoagulant.

The animal was perfused with 0.9% saline via the left ventricle, with the right ventricle cut for outflow; blanching of brain and clearing of all blood from systemic circulation was accomplished in this manner. Immediately following perfusion, the brain, gallbladder, liver, GI tract, GI content (flushed with −1 ml of saline), spleen, kidneys, urine, and tail (to determine the degree of compound remaining at the point of injection) were removed, and the concentration of iodinated compound in each was counted on a Beckman 5500 gamma counter. The entire procedure lasted 15 ± 3 min.

**Extraction of Radiolabeled Peptides.** Ex extractions of [125I]DPDPE or [125I]PEG-DPDPE from brain and plasma (at 30 min) and feces and urine (at 120 min) were performed to determine the percent intact (i.e., stability) of the respective compound in each region specified. Briefly, four mice were injected i.v. via the tail vein with iodinated drug for each respective assessment. At the appropriate time point, each respective sampling had 1 to 3 ml of 50 mM phosphate buffer (3.1 Na2HPO4 to NaH2PO4) with 5% acetone trifluoroethanol solution (kept on ice) added. Brain and fecal content samples were homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, NY). All samples were centrifuged at 20,000g for 20 min. Supernatant was decanted and the pellet resuspended in phosphate/acetone solution. Samples were again centrifuged and the two supernatants combined and immediately analyzed by RP-HPLC. Controls for each compound included an aliquot of iodinated compound in phosphate/acetone solution vigorously homogenized, centrifuged, and run on RP-HPLC (Davis and Culling-Berglund, 1985) and detected as disintegrations per minute. Analysis was carried out on RP-HPLC (PerkinElmer 250) with a Vydac analytical column (940415-21-1 no. 66). Samples were eluted at 37°C using a curvilinear gradient of 0.1% TFA in acetone (10–60%) versus 0.1% aqueous TFA over 30 min at 1.5 ml·min−1. Data is represented as area under the RP-HPLC peak (Davis and Culling-Berglund, 1985).

**In Situ Brain Perfusion Analysis.** Adult Sprague-Dawley female rats (n = 5; 250–350 g) were anesthetized with a 1 ml·kg−1 i.m. injection of cocktail comprised of ketamine (3.1 mg·ml−1), xylazine (78.3 mg·ml−1), and acepromazine (0.6 mg·ml−1) and then heparinized (10,000 U·kg−1) via i.p. injection. Both common carotid arteries were exposed and cannulated with silicone tubing connected to a perfusion circuit. The perfusate consisted of a protein containing mammalian Ringer’s solution (Preston et al., 1995) [117 mM NaCl; 4.7 mM KCl; 0.8 mM MgSO4; 24.8 mM NaHCO3; 1.2 mM KH2PO4; 2.5 mM CaCl2; 10 mM d-glucose; 3.9% dextrose (mol. wt. 70,000); bovine serum albumin-type V, 10 g·l−1]. The addition of Evans blue (0.055 g·l−1) albumin to Ringer’s solution provided a control for BBB integrity. Per fusate was aerated with 95% O2 and 5% CO2 and warmed to 37°C. The right jugular vein was sectioned upon initiation of perfusion to allow drainage of perfusate. Once the desired perfusion pressure and flow rate were achieved (85–95 mm Hg at 3.1 ml·min−1), the contralateral carotid artery was cannulated and perfused in the same manner as described above, and the left jugular vein was then sectioned. [125I]DPDPE or [125I]PEG-DPDPE was infused using a slow-driven syringe pump (model 22; Harvard Apparatus, South Natick, MA) into the inflow of the perfusate. After a set perfusion time of 20 min, a cisterna magna CSF sample (−50 μl) was taken with a glass cannula (n = 3). The animal was decapitated and the brain removed. Choroid plexus were excised and the brain was sectioned and homogenized. Per fusate containing the radiolabeled compounds was collected from each respective carotid cannula at termination of the perfusion to serve as a reference. Iodinated peptides were counted on a Beckman 5500 gamma counter.

**Capillary Depletion.** Measurement of the vascular component to total brain uptake of [125I]DPDPE or [125I]PEG-DPDPE was performed using capillary depletion (n = 3) (Triguero et al., 1990; Zlokovic et al., 1992). After a 20-min in situ perfusion, the brain was removed and choroid plexi were excised. Brain tissue (500 mg) was homogenized (Polytron homogenizer) in 1.5 ml of capillary depletion buffer (10 mM 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, 141
mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 10 mM D-glucose, pH 7.4) kept on ice. Two milliliters of ice-cold 26% dextran (mol. wt. 60,000) were added and homogenization repeated. Aliquots of homogenate were centrifuged at 5400g for 15 min. Capillary-depleted supernatant was separated from the vascular pellet. All of the homogenization procedures were performed within 2 min of sacrificing the animal. The homogenate, supernatant, and pellet were taken for radioactive counting (Beckman 5500 gamma counter).

Expression of in Situ and Capillary Depletion Data. The amount of [¹²⁵I]DPDPE or [¹²⁵I]PEG-DPDPE in whole brain, CSP, homogenate, supernatant, and pellet was expressed as the percentage ratio of tissue (C₃₂homogenate disintegrations per minute per gram⁻¹) to Ringer’s activities (C₃₂Perfusate disintegrations per minute per milliliter⁻¹) and expressed as RTissue%.

\[ RTissue\% = \left( \frac{C_{\text{Tissue}}}{C_{\text{Ringer}}} \right) \times 100 \]

In Vitro Bovine Brain Microvascular Endothelial Cell (BBMEC) Uptake Analysis. BBMECs were isolated from gray matter of cerebral cortices as previously detailed and characterized (Audus and Borchardt, 1986, 1987). BBMECs were grown to confluence on 24-well plates precoated with rat-tail collagen and fibronectin. At confluency, confirmed microscopically 10 to 12 days after seeding, growth media were removed, and the cells were preincubated for 30 min in assay buffer (122 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.4 mM K₂HPO₄, 1.4 mM CaCl₂, 10 mM D-glucose, 10 mM HEPES). Cells were incubated for 20 min with [¹²⁵I]DPDPE or [¹²⁵I]PEG-DPDPE (n = 6), with and without cyclosporin A (1.6 µM), on a shaker table at 37°C. Cyclosporin A (1.6 µM) is a concentration that has been shown to inhibit the P-gp efflux proteins (Legrand et al., 1998). BBMECs were also incubated for 20 min with [¹²⁵I]DPDPE or [¹²⁵I]PEG-DPDPE (n = 6), with and without 100 µM DPDPE, to determine saturability. [¹²⁵I]Sucrose was incubated under the same conditions and time points to serve as control. This was performed to verify that cells were viable and not damaged during analysis, because no increase in sucrose should be observed over the time course if cells maintain their integrity. After the appropriate times, the radioactive buffer was removed, and the cells were washed three times with ice-cold assay buffer. Triton X-100 (1 ml of 1%) was placed into each well and shaken for 30 min.

A 200 µl portion of Triton X was prepared for radioactive counting (Beckman 5500 gamma counter). The other portion of the sample was assayed for protein concentration using a Pierce BCA-protein kit (Pierce Chemical, Rockford, IL) with analysis on a Beckman UV spectrometer (model 25; Beckman Coulter, Inc.). Rcell% is the percent concentration of the labeled compound taken up by the cell, relative to concentration of the labeled compound in buffer.

Data Analysis. For all experiments, data were presented as mean ± S.E.M. Student’s t test was used for comparison of two means. Area under curve analysis was calculated via the trapezoid rule. Analysis of variance (ANOVA) comparison, followed by Newman-Keuls post hoc test was used when applicable. Analyses were performed using PCS software (Tallarida and Murray, 1987).

Results

Analgesia. Intravenous administration of DPDPE and PEG-DPDPE via tail vein was evaluated (Fig. 2A). Significant (p < 0.01) increase in analgesic effect of PEG-DPDPE was shown with a 3.3-fold increase as shown by the area under the curve (Fig. 2B). A rightward shift (15 min) in the %MPE curve, relative to concentration of the labeled compound in buffer.

Fig. 2. Data are presented as %MPE ± S.E.M. at time points of 15, 30, 45, 60, 90, 120, 150, and 180 min (time points for PEG-DPDPE also assessed at 210 and 240 min) (A). ICR mice (~25 g) were administered an i.v. dose of 25 µg/kg, five animals per time point. Analgesia data are represented as AUC, in regard to %MPE obtained over time course analysis (B). **p < 0.01 by Student’s t test.

Competition/Binding Affinity. DPDPE was shown to be very selective for the δ-opioid receptor, with a 172-fold greater affinity (p < 0.01) than PEG-DPDPE (Table 1). Neither compound was shown to be selective for the μ-opioid receptor.

Octanol/Buffer Partition Coefficients. The octanol/buffer distribution shows that [¹²⁵I]PEG-DPDPE has 36-fold greater affinity (p < 0.01) than PEG-DPDPE (Table 1). Neither compound was shown to be selective for the μ-opioid receptor.
lower affinity ($p < 0.01$) for the octanol (i.e., hydrophobic) phase than $[^{125}I] $DPDPE (Table 2).

**Protein Binding.** $[^{125}I] $PEG-DPDPE was found to have a 12% greater unbound fraction ($f_u$) than $[^{125}I] $DPDPE ($p < 0.01$), as seen in mouse plasma (Table 2). Similar results were found, under identical conditions, when bovine serum albumin-containing mammalian Ringer (used for in situ brain perfusion analysis) was used (data not shown).

**Time Course Distribution.** Log plasma concentration versus time data (Fig. 4) shows a significant difference in pharmacokinetics of $[^{125}I] $DPDPE and $[^{125}I] $PEG-DPDPE (Table 2). Elimination half-life ($t_{1/2}$) increased 2.5-fold ($p < 0.01$) for the $[^{125}I] $PEG-DPDPE over $[^{125}I] $DPDPE. Volume of distribution ($V_d$) for $[^{125}I] $PEG-DPDPE had a decrease of 2.7-fold ($p < 0.01$) compared with $[^{125}I] $DPDPE. Clearance rate ($CL$) of $[^{125}I] $PEG-DPDPE had a 7-fold decrease ($p < 0.01$) over $[^{125}I] $DPDPE.

Figure 5, A—I, shows distribution time course of $[^{125}I] $DPDPE (□) and $[^{125}I] $PEG-DPDPE (△), following i.v. tail vein injection in mice organs, urine, feces, whole blood, and plasma. In plasma and whole blood, levels of $[^{125}I] $PEG-DPDPE were higher ($p < 0.01$, AUC) at all time points than $[^{125}I] $DPDPE (Fig. 5, A and B). Equivalent plasma concentrations for the two peptides were reached at the 240-min time point.

$[^{125}I] $DPDPE was eliminated/excreted predominantly via the hepatobiliary route, whereas $[^{125}I] $PEG-DPDPE exhibited significantly lower liver, gallbladder, GI tract, and GI content concentration ($p < 0.01$, AUC) via this route.

Kidney concentration was significantly greater ($p < 0.01$, AUC) for $[^{125}I] $PEG-DPDPE than for $[^{125}I] $DPDPE (Fig. 5G). $[^{125}I] $PEG-DPDPE showed consistent accumulation in the urine over the time course (Fig. 5H), with significantly greater ($p < 0.01$, AUC) concentrations than $[^{125}I] $DPDPE. $[^{125}I] $PEG-DPDPE concentration in the urine plateaued at ~67% of the initial dose administered.

Spleen concentrations of $[^{125}I] $DPDPE and $[^{125}I] $PEG-DPDPE (Fig. 5I) were significantly different ($p < 0.01$, AUC) over the time course. $[^{125}I] $PEG-DPDPE concentration in the spleen declined past min 120, whereas $[^{125}I] $DPDPE concentrations were seen to decline gradually from initial time point.

Brain concentrations of $[^{125}I] $DPDPE and $[^{125}I] $PEG-DPDPE (Fig. 5J) were significantly different ($p < 0.05$, AUC) over the time course. $[^{125}I] $PEG-DPDPE showed increasing concentration in the brain up to 45 min and then declined. $[^{125}I] $DPDPE showed increasing concentration in the brain up to 30 min and then declined.

**Extraction of Radiolabeled Peptides.** The percentage of iodinated compound found intact of total found within the brain and plasma (at 30 min) and feces and urine (at 120 min) after an i.v. administration was assessed via RP-HPLC (Table 3). $[^{125}I] $DPDPE content found in the brain was 25.7% intact, with one major metabolite. $[^{125}I] $PEG-DPDPE content found in plasma was greater than 99.0% intact and in feces greater than 98.0% intact. $[^{125}I] $PEG-DPDPE found in urine was 33.0% intact with five metabolites. $[^{125}I] $PEG-DPDPE found in plasma and urine was greater than 99.0% intact. $[^{125}I] $PEG-DPDPE found in feces consisted of 58.9% intact and four metabolites. $[^{125}I] $PEG-DPDPE found in feces consisted of 53.4% intact with one other metabolite.

**In Situ Brain Perfusion and Capillary Depletion.** The uptake of $[^{125}I] $DPDPE and $[^{125}I] $PEG-DPDPE into rat brain was assessed via in situ brain perfusion at 20 min (Table 4). There was no significant difference in uptake to the brain, or accumulation in the CSF, for either compound. Capillary depletion analysis also revealed no significant difference in compound concentration contained within the capillary (i.e., pellet) or supernatant (i.e., brain parenchyma).

### Table 1

Pharmacokinetic parameters of $[^{125}I] $DPDPE and $[^{125}I] $PEG-DPDPE in mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$[^{125}I] $DPDPE</th>
<th>$[^{125}I] $PEG-DPDPE</th>
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<tr>
<td>$I_{50}$ (nM)</td>
<td>20.2 ± 5.35</td>
<td>3436.5 ± 211**</td>
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** p < 0.01 by Student's $t$ test.
Fig. 5. Tissue distribution, after i.v. tail vein injection, of $[^{125}I]$DPDPE (□) and $[^{125}I]$PEG-DPDPE (▲) at 15-, 30-, 45-, 60-, 90-, 120-, 180-, and 240-min time points in plasma (A), whole blood (B), liver (C), gallbladder (D), GI content (E), GI tract (F), kidneys (G), urine (H), spleen (I), and brain (J).
BBMEC Uptake. Cells were shown to maintain their integrity over the time course, as determined by no change in $[14C]$sucrose uptake. Assessment of the effects of PEGylation on the P-glycoprotein efflux mechanism was assessed in vitro at a 20-min time point. $[125I]$DPDPE and $[125I]$PEG-DPDPE cellular uptake were analyzed with P-gp inhibitor cyclosporin A (Fig. 6). $[125I]$DPDPE, a known P-gp substrate, showed a significant ($p < 0.01$) increase in BBMEC uptake when coadministered with cyclosporin A. $[125I]$PEG-DPDPE showed a significant increase ($p < 0.01$) over $[125I]$DPDPE at the single 20-min time, however, it did not have any change in uptake when coadministered with cyclosporin A. Saturable transport exhibited for DPDPE in situ (Williams et al., 1996) was assessed for $[125I]$PEG-DPDPE in BBMECs. Significant reduction ($p < 0.05$) in $[125I]$PEG-DPDPE uptake was observed with 100 $\mu$M DPDPE (Fig. 7), indicating $[125I]$PEG-DPDPE is taken up into the endothelial cell via the same mechanism as DPDPE.

Discussion

In this study, we have used PEGylation in an attempt to enhance the pharmacodynamic profile and analgesic effect of DPDPE. PEG conjugated to DPDPE enhanced analgesic effect (i.v.), increasing maximal response as well as the duration (Fig. 2). Intracerebroventricular injection of PEG-DPDPE showed no significant difference in analgesia to that of DPDPE (Fig. 3), whereas receptor binding data indicated significant reduction of $\delta$-opioid receptor binding affinity with PEG conjugation. It would be expected that i.c.v. administration would result in decreased analgesia, given the significant reduction in receptor binding. However, enzymes within the brain parenchyma may contribute to the hydroly-
sis of PEG-DPDPE, resulting in a nonconjugated DPDPE that is able to interact with the receptor. Additionally, the PEG moiety would likely prevent the rapid efflux of DPDPE out of the brain via P-gp, allowing more time to act at the receptor. The improved analgesia of i.v. administered PEG-DPDPE, to DPDPE (i.v.), is likely due to improved bioavailability, either via increased brain uptake or altered peripheral pharmacokinetics.

We conducted biodistribution studies in mice to investigate the effects of PEGylation on DPDPE availability to the brain. The amount of [125I]PEG-DPDPE in the systemic circulation available for transport to the brain is significantly greater than nonconjugated DPDPE for all time points (Figs. 4 and 5). The greater concentration within the systemic circulation was not necessarily indicative of greater bioavailability to the target organ (i.e., brain). Binding of the peptide to red blood cells and serum proteins can play a significant role in determining CNS uptake (Banks et al., 1990). The plasma concentration of both [125I]DPDPE and [125I]PEG-DPDPE (Fig. 5A) was approximately twice that of whole blood (Fig. 5B), indicating the majority of compound is in the plasma portion of the blood. Protein binding analysis shows [125I]PEG-DPDPE has a significantly greater unbound fraction in the plasma than DPDPE (Table 2). Therefore, [125I]PEG-DPDPE has not only a greater concentration in the systemic circulation, but it also has a greater unbound portion within the systemic circulation.

It has been theorized that an “exclusion-effect”, also referred to as “steric-stabilization”, via the PEG moiety results in repulsion of other macromolecules and particles (Lasic et al., 1991). It is believed that the heavy hydration, good conformation flexibility, and high chain mobility are primarily responsible for this exclusion effect. This aspect of PEG, along with its greater circulation time, would result in a large free concentration of PEG-DPDPE reaching capillary endothelial cells of the BBB.

The liver, GI tract, GI content, gallbladder, kidney, urine, spleen, and brain concentration-time profiles of [125I]DPDPE correspond well to previous studies (Weber et al., 1992; Chen and Pollack, 1997). Conjugation of the PEG moiety greatly reduced hepato-biliary elimination of DPDPE. This effect likely results from enhanced hydrophilicity. Additionally, the amount of [125I]PEG-DPDPE that was found within the feces (<95%) was only 53.4% intact (Table 3). The PEG conjugation shifted elimination almost completely to the renal pathway. PEGs with a molecular weight of 4000 or less have been shown to be excreted via the renal route, at a rate equivalent to creatinine (Shaffer et al., 1948). Higher molecular weight PEGs (>20,000) alternatively reduce glomerular/renal elimination. This reversal of elimination routes may be limited to low-molecular weight peptides attached to low-molecular weight PEGs. [125I]PEG-DPDPE found in the urine at 120 min was greater than 99.0% intact (Table 3), indicating that breakdown products are eliminated via another route (i.e., hepato-biliary).

Distribution analysis indicates that similar amounts of [125I]DPDPE and [125I]PEG-DPDPE are taken up into the brain for the first 30 min. [125I]PEG-DPDPE concentrations continue to increase in the brain up to 45 min, whereas [125I]DPDPE concentrations decrease significantly after the 30-min time point. This trend corresponds well with the analgesia data. The increased circulation time of [125I]PEG-DPDPE allows for a given concentration of drug to be exposed to the capillary endothelial cell surface of the BBB over a longer period of time. In situ brain perfusion analysis (Table 4) indicated that no difference exists in ratios of brain uptake (RP%,), or capillary endothelial cell concentration, between the PEGylated and non-PEGylated forms. This corresponds with the theory that PEG-DPDPE has an increased uptake into the brain via a longer circulation time.

In vitro analysis (R_{neal}%) indicated a significant increase in [125I]PEG-DPDPE uptake, compared with [125I]DPDPE, at 20 min. This result was unexpected. With the significant variation in lipophilicity (i.e., octanol/buffer coefficients) between the two DPDPE forms, a difference in permeability would be expected. PEGylation increased hydrophilicity would likely reduce compound uptake at the BBB. The reason for these variable effects may be due to multiple factors. First, in vitro analysis is solely representative of luminal membrane permeability, with a greater degree of error as to extracellular surface binding (by volume) compared with in situ brain perfusion. Second, DPDPE has been shown to have a saturable mechanism of transport at the BBB (Williams et al., 1996). This transport also has affinity for the PEG-DPDPE conjugate (Fig. 7), thereby potentially offsetting a reduced diffusion resultant of PEG’s enhancement of hydrophilicity. This saturable transport occurred at a concentration greater than 100 μM DPDPE (concentrations far above those used in this study). Third, DPDPE has been identified as a substrate for the P-gp efflux mechanism (Chen and Pollack, 1999; Witt et al., 2000). In this study [125I]DPDPE and [125I]PEG-DPDPE were coadministered with P-gp inhibitor cyclosporin A to assess uptake into BBMECs (Fig. 6). [125I]DPDPE with cyclosporin A showed increased uptake into the cells, whereas [125I]PEG-DPDPE with cyclosporin A showed no change in cell uptake. These data indicate that [125I]PEG-DPDPE uptake into the brain may be aided by a reduced affinity for P-gp. The PEG moiety, which conveys steric hindrance and added hydrophilicity, may also inhibit the attachment/identification of DPDPE to the P-gp efflux mechanism, thus allowing a greater concentration to gain entry into the brain. This effect could also counterbalance the hydrophilicity of the PEG moiety. Finally, the hydrophilicity appears to be of secondary importance to chain conformational flexibility and mobility (Blume and Cevc, 1993; Torchilin et al., 1994).

Breakdown of [125I]DPDPE in brain (Table 3) was examined at 30 min, and only 25.7% of the [125I]DPDPE was found intact, whereas [125I]PEG-DPDPE was 58.9% intact in the brain at 30 min, with 41.1% identified as [125I]DPDPE (metabolite). This idea that the PEG moiety acts in a “prodrug” manner is further supported by the ligand binding profile. PEG-DPDPE shows a 172-fold decrease in δ-opioid receptor affinity over DPDPE, with no enhancement of μ-opioid receptor binding affinity. It has been indicated that the flexible chain of mPEG polymers can sterically interfere with active binding sites of compounds (Marshall et al., 1996). Therefore, in this instance, the PEG moiety is likely removed from DPDPE via hydrolysis, leaving the native peptide to react at the receptor.

A number of caveats exist that need to be addressed. The peak effect of analgesia onset for both compounds appears to be 15 min down stream to peak brain uptake measured in the distribution assessment. The reason for this disparity is...
likely the effect of the radioactive tag used in the distribution analysis but not the analgesia analysis. Iodination of tyrosine residues of the compounds effects permeability. Reduced in situ brain uptake occurs with the use of radioactive iodine tagging (Witt et al., 2000). This effect is thought to occur via the large van der Waals volume of iodine (Bondi, 1964) distorting the conformation of tyrosine, as well as the addition of a bulky radioactive group onto a relatively small compound. For each analysis in this study, both the PEGylated and non-PEGylated DDPE were treated in an identical manner (i.e., either both were iodinated or noniodinated). Iodine125 tag was found intact in each respective sampling to >99%.

We also performed the investigation with a number of established models using correlations drawn between animal models and sexes, with examination of bovine (in vitro), female rat (in situ, receptor competition), and male mouse (distribution, serum binding, analgesia, peptide enzymatic integrity). BBB permeability variations between mice and rats have been examined in situ (Murakami et al., 2000), with over 20 different compounds, finding similar (1:1) permeabilities across species. Studies have also shown a larger number of δ-opioid receptors in mouse brain, compared with similar concentrations of μ-opioid receptor (Yoburn et al., 1991). Despite the variations between models, the study was focused on differences between the PEGylated and non-PEGylated form of drug, with both forms treated in an identical manner within each model.

PEGylation of peptide-based drugs has the potential to overcome many of the problems associated with achieving an adequate therapeutic effect. The key to optimizing a clinically acceptable drug revolves around the elucidation of the pharmacodynamic and pharmacokinetic effects that the PEG moiety imparts upon the conjugated compound. The chemical composition of the PEG, how and where the PEG is linked to the drug, the drug's composition, and where the drug induces its receptor mediated effect are also significant considerations. In this study, we have shown that conjugation of PEG to DDPE induced significant changes in a number of factors, including elimination half-life, volume of distribution, protein binding, hydrophilicity, receptor binding, P-gp affinity, metabolism, and membrane transport, resulting in an improved analgesic effect.

References


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References


