0022-3565/02/3032-760–767$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 303, No. 2
39545/1021564
JPET 303:760–767, 2002
Printed in U.S.A.

Pluronic P85 Block Copolymer Enhances Opioid Peptide Analgesia

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Received June 4, 2002; accepted July 18, 2002

ABSTRACT

Peptide-based drug development is a rapidly growing field within pharmaceutical research. Nevertheless, peptides have found limited clinical use due to several physiological and pathological factors. Pluronic block copolymers represent a growing technology with the potential to enhance efficacy of peptide therapeutics. This investigation assesses Pluronic P85 (P85) and its potential to enhance opioid peptide analgesia. Two opioid peptides, [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) and biphalin, were examined as to the benefits of P85 coadministration, above (1.0%) and below (0.01%) the critical micelle concentration, with morphine as a nonpeptide control. P85 was examined in vitro to assess blood-brain barrier uptake in association with P-glycoprotein effect, DPDPE and morphine being P-glycoprotein substrates. P85 coadministration with DPDPE and biphalin showed increased ($p < 0.01$) analgesia with both 0.01 and 1.0% P85. Morphine showed increased ($p < 0.01$) analgesia with 0.01% P85 only. This increase in analgesia is due to both an increase in peak effect, as well as a prolongation of effect. P85 increased cellular uptake of ^{125}I-DPDPE and [^{3}H]morphine at 0.01% ($p < 0.01$) and 1.0% ($p < 0.01$ and $p < 0.05$, respectively). Cyclosporin-A coadministration with ^{125}I-DPDPE and [^{3}H]morphine increased cellular uptake ($p < 0.01$ and $p < 0.05$, respectively). ^{125}I-DPDPE and [^{3}H]morphine coadministered with 0.01% P85 and cyclosporin-A increased cellular uptake compared with control ($p < 0.01$) and compared with cyclosporin-A coadministration without P85 ($p < 0.01$ and $p < 0.05$, respectively). This indicates that, in addition to P-gp inhibition, 0.01% P85 increased ^{125}I-DPDPE and [^{3}H]morphine uptake. In our examination, we determined that P85 enhanced the analgesic profile of biphalin, DPDPE, and morphine, both above and below the critical micelle concentration.

A continual challenge of drug delivery to the central nervous system (CNS) is the ability of a drug to cross the blood-brain barrier (BBB). The BBB serves to protect the brain from toxins present in the systemic circulation, in addition to allowing access of nutrients and chemical signaling molecules. However, the protective attributes of the BBB significantly decrease the ability of pharmaceutical agents to reach the brain parenchyma. The reduced paracellular diffusion via tight junctions and presence of efflux mechanisms are formidable obstacles to drug delivery (for review, see Witt et al., 2001a). An emerging strategy to enhance drug delivery to the CNS is the use of self-assembling amphiphilic block copolymers (SAABCs). Such drug delivery systems have shown to enhance drug transport across the BBB in vitro (Batrakova et al., 1998, 1999) and in vivo (Batrakova et al., 2001c).

SAABCs have gained much interest as drug delivery systems over the past decade. The unique construct of these polymers provides many advantages. The hydrophilic blocks form hydrogen bonds with aqueous surroundings and form a tight "shell" around a hydrophobic core, allowing maintenance of micelle structure upon dilution. Hydrophobic drugs have an affinity for the core region; thereby, entrapping them and allowing transport at concentrations that can exceed their intrinsic water solubility. The hydrophilic shell of copolymer micelles is often composed of poly(ethylene oxide) (PEO) and provides a barrier against protein adsorption and cellular adhesion. Furthermore, contents of the hydrophobic core are effectively protected against enzymatic degradation and hydrolysis, with reduced recognition by the reticuloendothelial system allowing increased circulation time (for review, see Lavasanifar et al., 2002). Such formulations have been shown to enhance drug circulation, membrane transport, and stability, while reducing immunogenicity, proteolysis, cellular efflux, and systemic clearance (for reviews, see Alakhov et al., 2001; Kabanov et al., 2002; Lavasanifar et al., 2002). Many studies of such SAABCs have been conducted on Pluronics. Pluronic block copolymers have shown to solubilize (i.e., process of transfer of water-insoluble compounds into the hydrophobic core of a micelle construct) epirubicin

This research was supported by National Institute on Drug Abuse Grants DA 11271, DA 06037, and NS 465201A1.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.039545.

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; SAABC, self-assembling amphiphilic block copolymer; PEO, poly(ethylene oxide); P85, Pluronic P85; CMC, critical micelle concentration; BBMEC, bovine brain microvessel endothelial cell; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; P-gp, P-glycoprotein; MPE, maximum possible effect; ANOVA, analysis of variance; AUC, area under the curve.

(Batrakova et al., 1996), haloperidol (Kabanov et al., 1989), and doxorubicin (Batrakova et al., 1996), showing marked enhancement of bioavailability. Recent studies also demonstrate that select Pluronic block copolymers interact with multidrug-resistant cancer cells, leading to an enhancement of chemotherapeutic drug penetration (Batrakova et al., 2001a).

Of particular interest is the use of block copolymers in association with peptide-based drugs. Recent developments in peptide drug design have produced potent CNS-acting pharmaceutical agents. Moreover, few studies have been conducted with copolymer formulations in conjunction with analgesics. In our study, we examined the potential use of Pluronic block copolymer P85 (Fig. 1) to enhance the analgesic profiles of two established peptide analgesics and morphine (nonpeptide control) (Fig. 2). P85 is arranged in a tri-block construct, composed of a central poly(propylene oxide) block with attached poly(ethylene oxide) blocks on each end. Individual copolymer strands, termed unimers, self-assemble in solution to form micelles at the critical micelle concentration (CMC). Interestingly, P85 has shown greater permeabilizing ability at concentrations below the CMC (Batrakova et al., 1998), specifically when coadministered with a substrate of an ATP-dependent efflux mechanism (Miller et al., 1999; Batrakova et al., 1999, 2001b). Therefore, our examinations were conducted at P85 concentrations below and above the CMC. In vivo analgesic analyses were conducted with an automated tail-flick analgesia meter. In vitro analyses, using bovine brain microvessel endothelial cells (BBMECs), were conducted to examine the cellular uptake of our analgesics, with and without P85, in the presence of a P-glycoprotein (P-gp) inhibitor.

The peptides used for this analysis, DPDPE and biphalin, are well characterized and stable opioid peptides, which are transported into the CNS across the BBB (Abbruscato et al., 1996; Williams et al., 1996). DPDPE is taken up across the BBB via diffusional and saturable transport (Williams et al., 1996); possibly the organic anion-transporting polypeptide-2 transporter (Gao et al., 2000). Additionally DPDPE, as well as morphine, has been shown to be a substrate for the P-gp efflux mechanism at the BBB (Chen and Pollack, 1998; Letrent et al., 1999), allowing an opportunity to further assess copolymer P85 action on P-gp efflux. Biphalin is taken up across the BBB via both diffusional and saturable transport, with affinity for the large neutral amino acid carrier (Abbruscato et al., 1997). To date, no literature citations have indicated biphalin to have affinity for the P-gp efflux mechanism, which was confirmed by analyses within this study, and thus provides a negative control for P-gp efflux at the BBB.

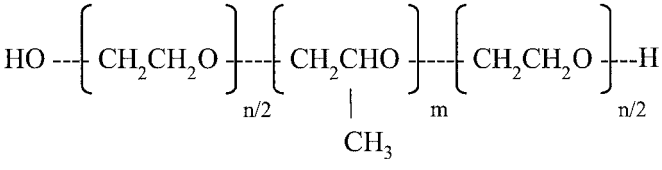


Fig. 1. Structure of Pluronic P85, $n = 52$, m = 40; mol.wt. 4600 (Batrakova et al., 2001c) block copolymer, containing two exterior hydrophilic poly(ethylene oxide) blocks and an interior poly(propylene oxide) block.

Materials and Methods

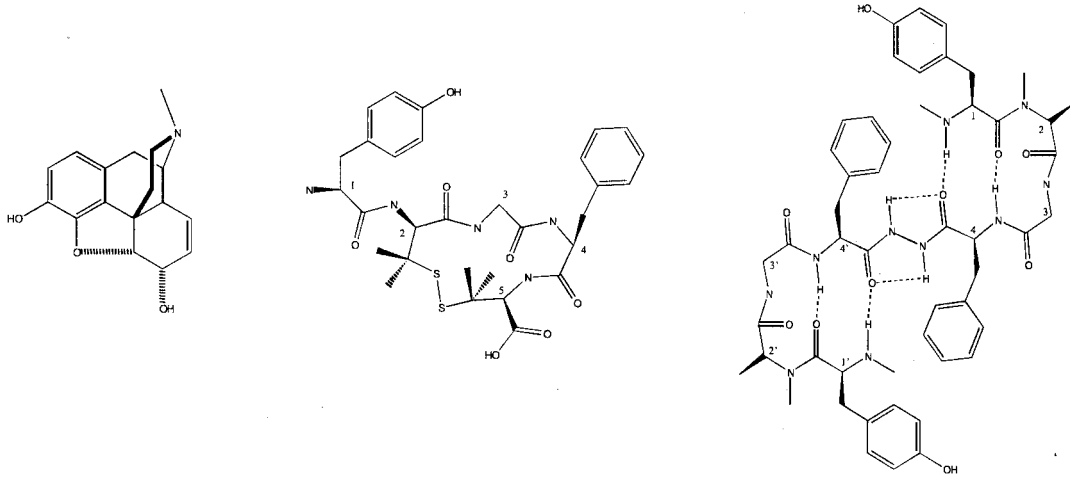
Animals. Male adult ICR mice weighing 20 to 25 g were used for analgesic analyses (Harlan, Indianapolis, IN). Mice were housed under standard 12-h light/dark conditions and received food and water ad libitum. All protocols were approved through the Institutional Animal Care and Use Committee at the University of Arizona.

Chemicals. DPDPE and biphalin were obtained from Multiple Peptide Systems (San Diego, CA). $Na^{125}I$ and [^{3}H]*n*-methyl-morphine were purchased from PerkinElmer Life Sciences (Boston, MA). Pluronic block copolymer P85 (lot no. WPOP-587A) was provided as a gift by the BASF Corporation (Parsippany, NJ). All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO)

Iodination of Compounds. DPDPE and biphalin were monoiodinated, for in vitro analysis, on the tyrosine[1] residue using a standard chloramine-T procedure, as adapted in our laboratory by Schetz et el. (1995). Purification of the iodinated peptides was carried out using a reverse-phase 250 high-performance liquid chromatography gradient system (PerkinElmer Life Sciences) and a C_{18} column (880115-9 no. 74; Vydac, Hesperia, CA). The samples were eluted at 37°C using a curvilinear gradient of 0.1% trifluoroacetic acid in acetonitrile (10–35%) versus 0.1% aqueous trifluoroacetic acid over 20 min at a flow of 1.5 ml · min^{-1}.

Analgesia Analysis. Radiant-heat tail-flick analgesia meter (model 33; IITC Scientific Products, Woodland Hills, CA) was used to assess antinociceptive (i.e., analgesic) profile after the administration of DPDPE, biphalin, or morphine. This model reflects supraspinal analgesia with CNS-mediated effect. The analgesia meter was set to produce a baseline latency of 2 to 3 s with a cutoff time of 15 s. Male ICR mice ($n = 5$) were administered an i.v. bolus. DPDPE (20 mg/kg), biphalin (1.0 mg/kg), or morphine (1.0 mg/kg) was either dissolved in sterile saline alone (control) (pH 7.4) or with P85 [0.01 or 1.0% (w/v) incubated at 37°C for 1 h] and injected into the tail vein, with assessment at 10, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min. Analyses were stopped at any given 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 percentage of maximal possible effect (%MPE): %MPE = [(recorded flick time − baseline)/(maximum time (15 s) − baseline)] × 100.

In Vitro BBMEC Uptake Analysis. BBMECs were isolated from the gray matter of cerebral cortices as detailed and characterized previously (Audus et al., 1996). BBMECs were grown to confluence on 24-well plates precoated with rat-tail collagen and fibronectin. At confluence, 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 $MgSO_4$, 25 mM $NaHCO_3$, 0.4 mM K_2HPO_4, 1.4 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES] at pH 7.4. The P85 copolymer was prepared in assay buffer. Each respective radiolabeled compound (^{125}I-DPDPE, ^{125}I-biphalin, and [^{3}H]*n*-methyl-morphine) was incubated with either a 0.01% (w/v) (22 μM) or 1.0% (w/v) (2.2 mM) solution at 37°C for 1 h before use in experiments ($n = 6$). Cells were then incubated for 20 min with each respective concentration of P85 and radiolabeled compound on a shaker table at 37°C. The 20-min time point was specifically chosen because it has been previously shown to be an adequate time for assessing uptake of our respective peptides, with an appropriate time lapse to allow for an assessment of efflux inhibition (Witt et al., 2001b). Furthermore, Pluronic formulations have been recently shown to deplete internal ATP stores over extended periods (Batrakova et al., 2001a); thereby, interfering with the assessment of ATP-dependent efflux mechanisms at the membrane surface. At concentrations below the CMC [67 μM; 0.03% (w/v)], P85 has been shown to inhibit P-gp function in BBMECs (Miller et al., 1997), with a concentration of 0.01% inducing the greatest degree of effect. Separate analyses were conducted with P-gp inhibitor cyclosporin-A (1.6 μM) (Huai-Yun et al., 1998) coin-



Morphine[a] **DPDPE**[b] **Biphalin**[c]

Compound	Sequence	M.W.	Log D
Morphine	$C_{17}H_{19}NO_3$	285	0.76[d]
DPDPE	NH_2-Tyr[1]-D-Pen[2]-Gly[3]-Phe[4]-D-Pen[5]-OH (S—S)	646	0.028
Biphalin	NH_2-Tyr[1]-D-Ala[2]-Gly-[3]-Phe[4]-NH-NH-Phe[4′]-Gly[3′]-D-Ala[2′]-Tyr[1′]	909	1.93

a. Structure according to Gulland and Robinson (1925)
b. Structure according to Liao et al. (1998)
c. Structure according to Romanowski et al. (1997)
d. Log D (pH=7.4) value according to Hansch and Anderson (1967)

Fig. 2. Structure, sequence, molecular weight, and log D values of morphine, DPDPE, and biphalin.

cubated with P85 (0.01%) and each respective radiolabeled compound. After the 20-min incubation, the solutions were removed and the cells washed three times with ice-cold assay buffer. Then, 1 ml of 1% Triton X-100 was placed into each well and shaken for 30 min. A 200-μl portion of the Triton X was prepared for radioactive counting (model LS 5000 TD counter; Beckman Coulter, Inc., Fullerton, CA). The other portion of the sample was assayed for protein concentration using a bicinchoninic acid protein kit (Pierce Chemical, Rockford, IL) with analysis on a UV spectrometer (model 25; Beckman Coulter, Inc.). R_{cell}% is the percent ratio of radiolabeled compound taken up by the cell.

Octanol/Buffer Distribution. Distribution coefficients for DPDPE and biphalin were expressed as the ratio of labeled substance found in the octanol phase to that found in the aqueous phase. Due to ionizability of compounds data are represented as a log D at pH 7.4. Briefly, equal volumes of octanol and a 0.05 M HEPES buffer in 0.1 M NaCl, pH 7.4, were mixed and allowed to equilibrate for 12 h. The layers were then separated and stored at 4°C. To 500 μl of

the HEPES buffer portion was added 50 μg of peptide, and this was mixed with 500 μl of the octanol by vortexing. The sample was then centrifuged in a Microfuge (Beckman Coulter, Inc.) for 1 min at 4000 rpm. The layers were separated. The octanol phase was lyophilized and resuspended in NaH_2PO_4 buffer and analyzed via reverse-phase high-performance liquid chromatography, as was the aqueous layer. D is the ratio of the peptide concentration in octanol layer divided by the concentration in the aqueous buffer layer.

Data Analysis. For all experiments, data are presented as mean ± S.E.M. Area under curve (AUC) 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 morphine, DPDPE, and biphalin via tail vein were evaluated independently, with 0.01 or 1.0% P85. Morphine (1.0 mg/kg) showed an increase ($p < 0.01$, AUC) in analgesic effect, when administered with 0.01% P85 (1.4-fold) (Fig. 3). However, morphine with 1.0% P85 showed no difference compared with control. DPDPE (20 mg/kg) showed an increase ($p < 0.01$, AUC) in analgesic effect when administered with 0.01% P85 (3.2-fold) and 1.0% P85 (1.7-fold), compared with control (Fig. 4). Biphalin (1.0 mg/kg) showed an increase ($p < 0.01$, AUC) in analgesic effect when administered with 0.01% P85 (2.3-fold) and 1.0% P85 (1.8-fold), compared with control (Fig. 5). The observed increases in analgesic effect (AUC) were due to both increased peak effect and a prolongation of analgesia time.

In Vitro BBMEC Uptake Analysis. Cellular uptake assessment of radiolabeled compounds incubated with P85, and P-glycoprotein efflux inhibitor cyclosporin-A, were assessed in vitro at a 20-min time point. [^{3}H]Morphine showed an increase in cellular uptake when administered with cyclosporin-A ($p < 0.05$) (Fig. 6). [^{3}H]Morphine incubated with 0.01 and 1.0% P85 showed an increase in cellular uptake ($p < 0.01$ and $p < 0.05$, respectively). Additionally, [^{3}H]morphine incubated with 0.01% P85 and coadministered cyclosporin-A showed an increased ($p < 0.01$) cellular uptake compared with control, but no difference compared with [^{3}H]morphine incubated with 0.01% P85 (Fig. 6). ^{125}I-DPDPE showed an increase in cellular uptake when administered with cyclosporin-A ($p < 0.01$) (Fig. 7). ^{125}I-DPDPE incubated with 0.01 and 1.0% P85 showed an increase in cellular uptake ($p < 0.01$). Additionally, ^{125}I-DPDPE incubated with 0.01% P85 and coadministered cyclosporin-A showed an increased ($p < 0.01$) cellular uptake compared with control, and an increase compared with ^{125}I-DPDPE incubated with 0.01% P85 (Fig. 7). ^{125}I-Biphalin showed no change in cellular uptake whether incubated with 0.01 or 1.0% P85, or cyclosporin-A (Fig. 8).

Octanol/Buffer Distribution. log D values for DPDPE (0.028) and biphalin (1.93) fall within expected ranges (Fig. 2). log D value for morphine (0.76) has been established previously (Hansch and Anderson, 1967).

Discussion

In this study, we have examined the use of P85 Pluronic block copolymer as a method to enhance analgesia of DPDPE, biphalin, and morphine. Both peptides, and morphine, elicited significantly increased and prolonged analgesia in the presence of 0.01% P85 (i.e., "unimer" concentration) compared with the 1.0% P85 (i.e., "micellar" concentration) or respective analgesic alone. Analgesia was lower with 1.0% P85, compared with 0.01% P85; this is potentially due to micellar trapping, which reduces the "free" drug concentration available for transcellular flux. Additionally, at micellar concentrations of block copolymers, cellular accumulation of P85 is likely a combination of both unimer and micellar transport processes, indicating the analgesia induced with coadministration of 1.0% P85 is in part derived from the unimer form.

P-gp inhibition is one of the mechanisms of P85 action. P85 at 0.01% induces the greatest degree of P-gp inhibition across a range of concentrations (0.0001–5%) (Miller et al., 1997). Similar unimer effects on P-gp inhibition have been reported

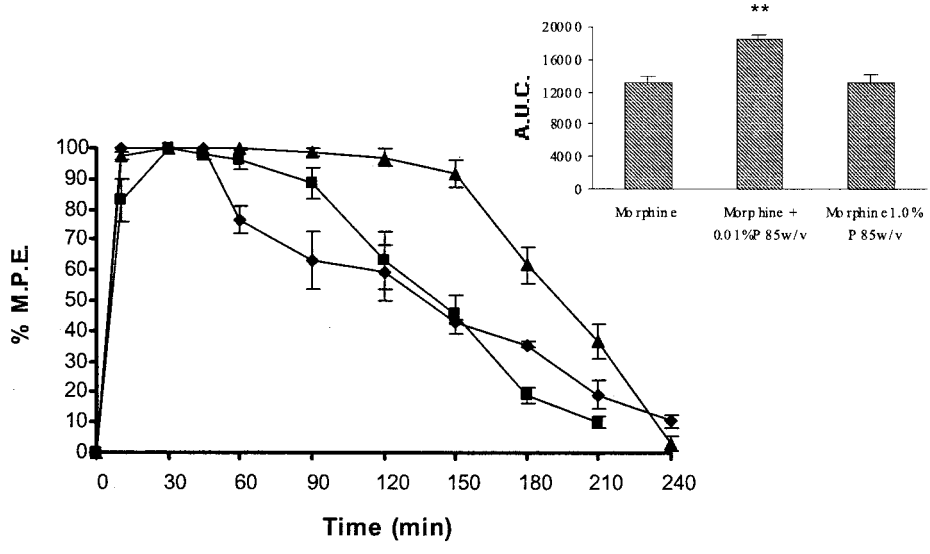


Fig. 3. Data are presented as (%MPE) ± S.E.M. at time points of 10, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min for morphine (1.0 mg/kg) or morphine incubated with 0.01 or 1.0% P85, using a radiant-heat tail-flick analgesia meter. ICR mice were administered an i.v. dose, five animals per time point. Analgesia data are represented as AUC, in regard to % MPE) obtained over time-course analysis. ⋆⋆, $p < 0.01$ by ANOVA, followed by Newman-Keuls analysis. ♦, morphine; ▲, morphine + 0.01% P85 (w/v); ■, morphine + 1.0% P85 (w/v).

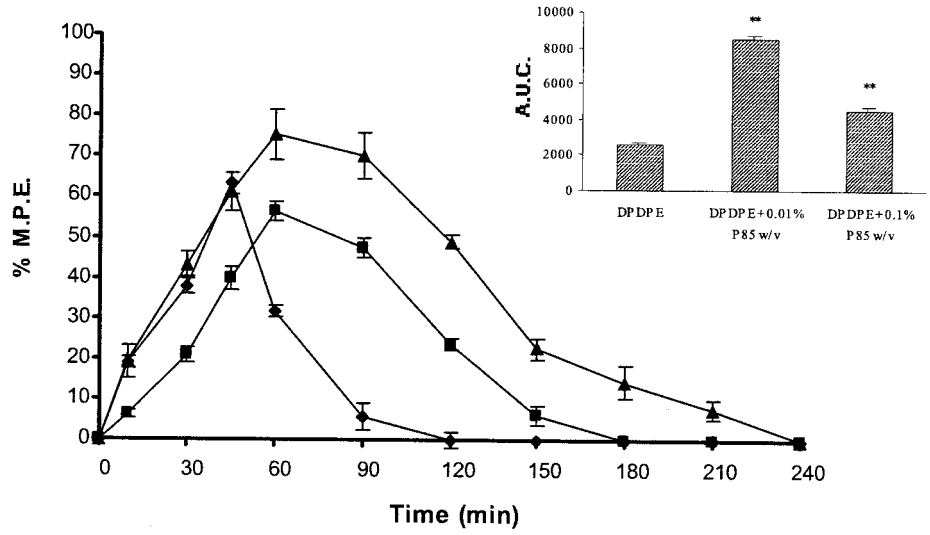


Fig. 4. Data are presented as %MPE ± S.E.M. at time points of 10, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min for DPDPE (20.0 mg/kg) or DPDPE incubated with 0.01 or 1.0% P85, using a radiant-heat tail-flick analgesia meter. ICR mice were administered an i.v. dose, five animals per time point. Analgesia data are represented as AUC, in regard to %MPE obtained over time-course analysis. ⋆⋆, $p < 0.01$ by ANOVA, followed by Newman-Keuls analysis. ♦, morphine; ▲, morphine + 0.01% P85 (w/v); ■, morphine + 1.0% P85 (w/v).

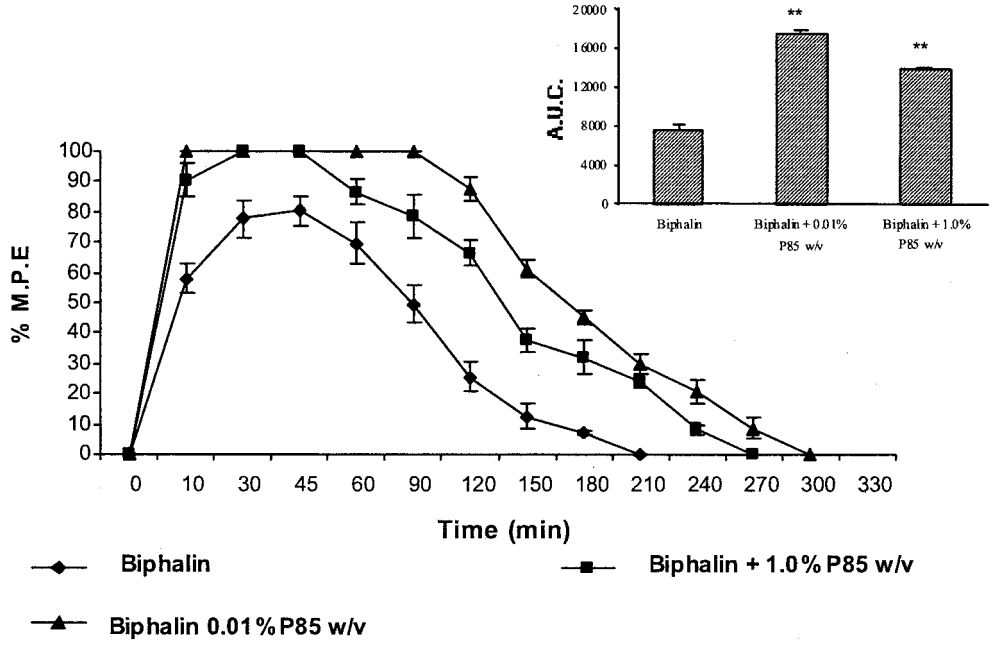


Fig. 5. Data are presented as %MPE ± S.E.M. at time points of 10, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min for biphalin (1.0 mg/kg) or biphalin incubated with 0.01 or 1.0% P85, using a radiant-heat tail-flick analgesia meter. ICR mice were administered an i.v. dose, five animals per time point. Analgesia data are represented as AUC, in regard to %MPE obtained over time-course analysis. ⋆⋆, $p < 0.01$ by ANOVA, followed by Newman-Keuls analysis. ♦, biphalin; ▲, biphalin + 0.01% P85 (w/v); ■, biphalin + 1.0% P85 (w/v).

for Pluronic copolymers in multidrug-resistant cancer cells (Alakhov et al., 1996) and Caco-2 cell monolayers with various surfactants at concentrations below the CMC (Nerurkar et al., 1996). The in vitro studies reported herein confirm the modulation of both DPDPE and morphine transport via P-gp. However, biphalin, showing no increase in BBMEC uptake in the presence of the P-gp inhibitor cyclosporin-A, showed increased analgesia similar to that of DPDPE when coadministered with P85 (0.01 and 1.0%). Additionally, biphalin uptake was not altered in BBMECs when incubated with either 0.01 or 1.0% P85, indicating that the enhanced analgesic effect of biphalin may not be derived from an increase in endocytotic uptake at the BBB. Furthermore, BBMEC permeability studies with P85 treatment indicate no change in apical-to-basolateral flux of [^{14}C]mannitol, a marker of paracellular diffusion, across a range of P85 dosing (0.001–5%) (Batrakova et al., 1999, 2001c), thus indicating that the increased analgesic profile of biphalin with P85 is not due to an enhanced paracellular diffusion. Kinetic profiles of radiolabeled digoxin, a P-gp substrate (Mayer et al., 1996), in brain and plasma show the coadministration of P85 prolongs the residence time of digoxin in the plasma, with a corresponding increase in residence time and concentration in the brain (Batrakova et al., 2001c). It is likely that the P85/biphalin-enhanced analgesia is specifically due to enhanced circulation time within the blood component.

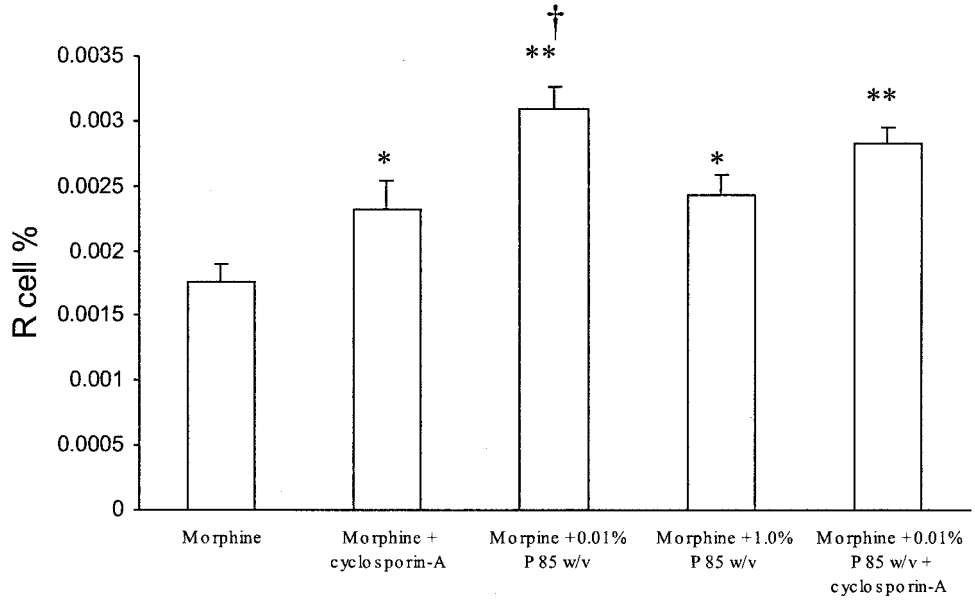


Fig. 6. BBMECs uptake of [^{3}H]morphine, incubated with and without 0.01 or 1.0% P85, expressed as R_{cell}%. Time was 20 min, and values are the mean ± S.E.M. (n = 6). Cyclosporin-A (1.6 μM) was used to assess the cellular uptake effects P-glycoprotein inhibition on [^{3}H]morphine or [^{3}H]morphine incubated with 0.01% P85. Data are mean ± S.E.M. Significance determined by ANOVA, followed by Newman-Keuls analysis, denoted by *, $p < 0.05$ and **, $p < 0.01$, with respect to [^{3}H]morphine; †, $p < 0.05$, with respect to [^{3}H]morphine coadministered with cyclosporin-A.

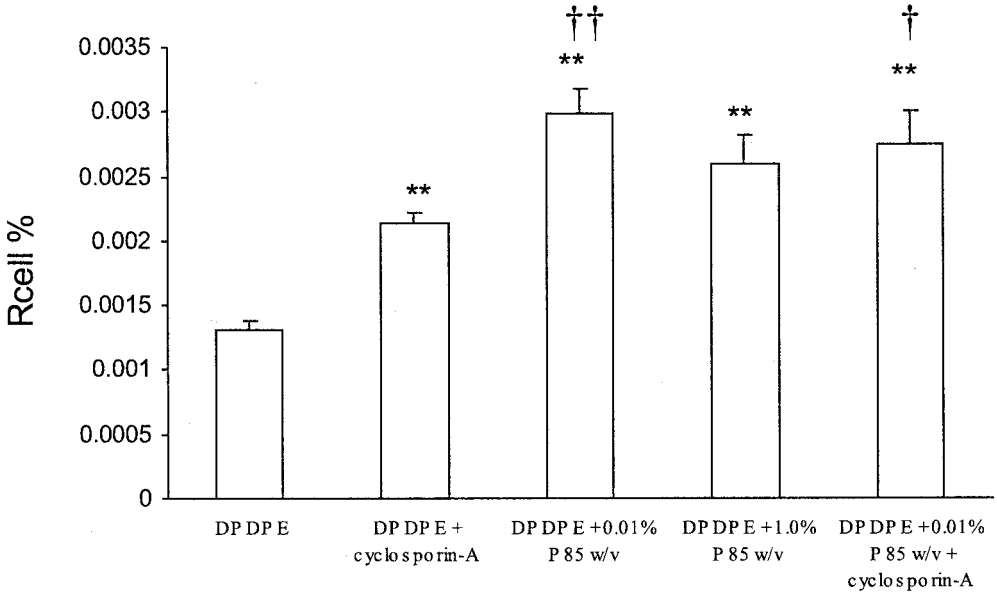


Fig. 7. BBMEC uptake of ^{125}I-DPDPE, incubated with and without 0.01 or 1.0% P85, expressed as R_{cell}%. Time was 20 min, and values are the mean ± S.E.M. (n = 6). Cyclosporin-A (1.6 μM) was used to assess the cellular uptake effects P-glycoprotein inhibition on ^{125}I-DPDPE or ^{125}I-DPDPE incubated with 0.01% P85. Data are mean ± S.E.M. Significance determined by ANOVA, followed by Newman-Keuls analysis, denoted by **, $p < 0.01$, with respect to ^{125}I-DPDPE; †, $p < 0.05$ and ††, $p < 0.01$, with respect to ^{125}I-DPDPE coadministered with cyclosporin-A.

Miller et al. (1997) proposed a P-gp-independent endocytotic uptake in BBMEC uptake analysis for concentrations of P85 above the CMC. Although these observations were not confirmed in our BBMEC uptake analysis at 1.0% P85, with respect to biphalin, the short duration of our analysis may not have allowed for such endocytotic uptake. It is unlikely that P85 would enhance the cellular uptake of biphalin via the large neutral amino acid carrier; however, a decreased uptake via micellar trapping might be expected. P85/DPDPE-enhanced analgesia is also a likely composite of compounding factors. Enhanced systemic circulation time associated with PEO-based compounds is likely a significant component, as theorized with biphalin. DPDPE cellular uptake increased with both 1.0 and 0.01% P85, as well as with the P-gp inhibitor cyclosporin-A. DPDPE with 0.01% P85 had greater uptake than DPDPE coadministered with only cyclosporin-A; additionally, DPDPE coadministration with 0.01% P85 and cyclosporin-A was not different from DPDPE coadministered with only 0.01% P85. This indicates that, in addition to P-gp inhibition, 0.01% P85 induces some further mechanism enhancing DPDPE uptake. These results also hold for morphine, another P-gp substrate (Letrent et al., 1999). Because 0.01% P85 falls below the CMC, and does not significantly affect uptake through the proposed endocytotic

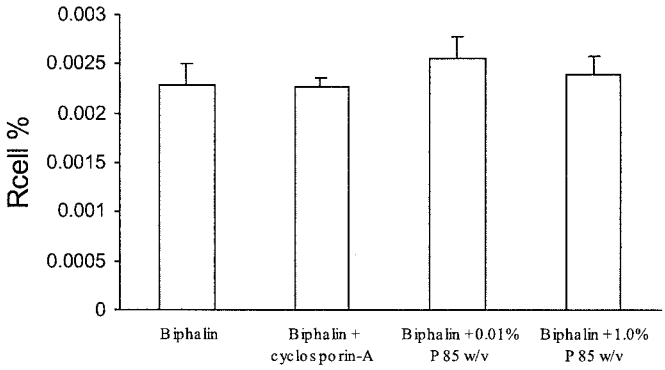


Fig. 8. BBMEC uptake of ^{125}I-biphalin, incubated with and without 0.01 or 1.0% P85, expressed as R_{cell}%. Time was 20 min, values are the mean ± S.E.M. (n = 6). Cyclosporin-A (1.6 μM) was used to assess the cellular uptake effects P-glycoprotein inhibition on ^{125}I-biphalin. Data are mean ± S.E.M.

mechanism (Miller et al., 1997) or paracellular permeability (Batrakova et al., 1999, 2001c), the additional increase in uptake is likely via another route. Increased membrane fluidization via P85 (Batrakova et al., 2001b) might result in a nonspecific increase in membrane uptake; however, if this were the case biphalin would also show increased membrane uptake. Additionally, basolateral-to-apical BBMEC permeability studies (Batrakova et al., 2001b) (P-gp is only present on the apical side of BBMECs; Miller et al., 2000) show no enhancement of P85 over a range of concentrations (0.001–5%) (Batrakova et al., 1999), indicating a fluidization of the membrane does not (in and of itself) enhance permeability. Another option would be that P85 enhances activation of the DPDPE saturable transporter, however, this would also have to hold for morphine. Although plausible, this option is unlikely because P85s mode of action lies in the inhibition of cellular transporters, not activation. Last, other efflux mechanisms at the BBB may also be inhibited via Pluronics. Studies show Pluronics inhibit the multidrug-resistance-associated protein (MRP)-1 efflux pump in human pancreatic adenocarcinoma cells (Miller et al., 1999). MRP1, like P-gp, is a member of the ATP-binding cassette transport family and has been shown to exist at the BBB in rodent (Kusuhara et al., 1998; Rao et al., 1999), human (Seetharaman et al., 1998), and bovine (Huai-Yun et al., 1998). The MRP family acts as an organic anion efflux transporter and has shown affinity for neutral organic compounds (Borst et al., 2000a). As a consequence, P-gp and MRP have overlapping substrate specificity (Borst et al., 2000a; Seelig et al., 2000). DPDPE, shown to be a substrate for organic anion-transporting polypeptides, may also be a substrate for MRP. MRP1 has high affinity toward compounds conjugated to glutathione, glucuronide, or sulfate (Borst et al., 2000b). Morphine, in our analgesic analyses, is in the chemical form of morphine sulfate. Additionally, the primary active metabolites of morphine are glucuronides (Christrup, 1997), and thus potential substrates for MRP. Therefore, the increased cellular uptake of DPDPE and morphine observed via coadministration with P85, over that seen with coadministration of P-gp inhibitor cyclosporin-A, may be indicative of a more "universal" efflux inhibitory mechanism. As to whether this efflux inhibition is primarily an action upon the membrane itself or whether it is via an intracellular process has yet to be fully elucidated.

Recent literature indicates that P85 induces its effect upon P-gp, in part, via cellular energy depletion, with an overall decrease in the ATP pool (Batrakova et al., 2001a). Speculation that ATP energy depletion may be required for copolymer inhibition of efflux systems does raise some interesting caveats. There is no clear indication as to whether ATP depletion is the primary route of efflux inhibition or whether some other action directed upon the general membrane or surface proteins directly (i.e., membrane fluidization or direct antagonism of efflux transporters) is the primary causation. ATP depletion over multiple concentrations of P85 (0.001–1.0%) has been shown in BBMECs (Batrakova et al., 2001a,b). Yet, permeability studies of P-gp (apical membrane) inhibition with P85, when administered on the basolateral side of a BBMEC monolayer, show no effect upon P-gp efflux (Batrakova et al., 2001b). Only when the time course was expanded beyond 1 h were the investigators able to produce P-gp inhibition. They hypothesized, based on their data of P85 uptake profile, that the additional time was necessary for the copolymer to travel from the basolateral to apical side of the membrane, thereby, not merely an effect of ATP depletion. Nevertheless, restoration of ATP levels in P85-treated BBMEC monolayers restored function of P-gp in the presence of the block copolymer (Batrakova et al., 2001b). Recently, our research group examined PEO directly conjugated to DPDPE and found an associated inhibition of P-gp in BBMECs with enhanced uptake (Witt et al., 2001b). Given the low concentrations of the radiolabeled PEO-DPDPE in that examination and the limited time course (20 min), ATP depletion would not be a likely factor in P-gp inhibition. In the present study, DPDPE was incubated with P85 and showed a similar trend to that of PEO-conjugated DPDPE.

It is likely that Pluronic copolymers can inhibit P-gp action via ATP depletion in an in vitro environment. Yet, it is unlikely that the given concentrations of P85 would be able to induce significant ATP depletion in vivo. Not only is the P85/drug injected into the systemic circulation where it disperses but also the endothelial cells (compared with in vitro analyses) in peripheral and central vasculature have a constant circulation of blood supplied to them. Within such a context increases in analgesic response associated with P85 coadministration favor an enhanced circulation profile as the principle contributor of this effect.

Pluronic copolymers have the potential to overcome many of the problems associated with peptide-based drugs. In our examination, we determined that P85 enhanced the analgesic profile of biphalin, DPDPE, and morphine, both above and below the CMC. All three compounds were likely to have increases in circulation time attributable to P85 coadministration as a major contributor to the enhanced analgesia. DPDPE and morphine further exhibited an enhanced BBMEC uptake indicative of P-gp inhibition, and possibly MRP inhibition. The effect of P85 on drug efflux transporters will prove a valuable tool in view of developing peptide pharmaceutics, enhancing drug delivery to selected organs, and potentially overcoming drug resistance associated with ineffective therapeutic response.

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