Blood-Brain Disposition and Antinociceptive Effects of [\(\delta\)-Penicillamine\(^{2,5}\)]enkephalin in the Mouse

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ABSTRACT

Although intravenous administration of \([\delta\text{-penicillamine}^{2,5}]\)enkephalin (DPDPE) produces significant antinociception in rodents, the duration of antinociception is short (~15 min). The present study was conducted to test the hypothesis that duration of antinociception for DPDPE is determined by both systemic and regional disposition (i.e., blood-brain translocation), and that the magnitude of antinociception is related more closely to concentrations in brain tissue than in blood. Systemic disposition was examined after i.v. administration of DPDPE (10–100 mg/kg) to male CD-1 mice. The relationship between antinociception and concentration in blood and brain tissue was assessed by determining antinociception 10 min after administration of DPDPE (10–100 mg/kg); effect versus brain tissue concentration data were fit with pharmacodynamic models to recover EC\(_{50}\) estimates. In addition, the time course of antinociception, as well as blood and brain tissue concentrations, were examined after an i.v. bolus dose (40 mg/kg) of DPDPE. The systemic disposition of DPDPE was nonlinear; both clearance and volume of distribution were dose-dependent. Antinociception increased proportionately with increasing concentrations of DPDPE in blood or brain tissue, with an EC\(_{50}\) of 1.42 ± 0.06 \(\mu\)g/g expressed as brain tissue concentration. However, the brain-to-blood concentration ratio also increased with increasing dose, suggestive of saturable translocation of DPDPE across the blood-brain barrier. Antinociception appeared rapidly (within 5 min) and dissipated within ~15 min after a 40 mg/kg i.v. dose. These results suggest that rapid elimination from blood and active efflux from brain limit the duration of action of DPDPE.

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[\(\delta\text{-penicillamine}^{2,5}\)]enkephalin is a cyclic opioid pentapeptide developed as a potential drug candidate for treatment of pain (Williams et al., 1996). DPDPE has been used extensively in receptor binding studies because of its high selectivity for the \(\delta\) opioid receptor (Mosberg et al., 1983; Toth et al., 1990). DPDPE is resistant to neutral endopeptidase and aminopeptidase, enzymes that are responsible for the rapid degradation of most endogenous enkephalins (Weber et al., 1991), and it is stable during incubation in various biologic matrices (Chen and Pollack, 1997). In vivo disposition studies in rats also indicated that biotransformation of DPDPE was minimal (Chen and Pollack, 1997).

DPDPE elicits antinociception after intrathecal, intracerebroventricular or i.v. administration in rats and mice (Porreca et al., 1987; Weber et al., 1991). Although the duration of action is short, substantial antinociception, with rapid onset, after i.v. administration indicates that DPDPE penetrates the BBB. DPDPE has an apparent permeability coefficient of \(5 \times 10^{-3}\) cm/min in BMEC, similar to that of antipyrine and propanolol (5–8 \(\times\) \(10^{-3}\) cm/min; Shah et al., 1989). Despite this favorable partitioning, only ~0.06% of the total radioactivity was recovered in whole brain after i.v. administration of \(^3\)H-DPDPE in mice (Weber et al., 1991). Studies in BMEC, with a series of [Met\(^5\)]enkephalin peptides including DPDPE and several DPDPE analogs, revealed a strong relationship between lipophilicity and permeability in BMEC, which suggests that transmembrane diffusion of DPDPE into the brain may occur (Weber et al., 1993). The rapid appearance of a pharmacologic response mediated in the central nervous system with low steady-state brain/blood partitioning suggests an active efflux from the brain as opposed to a limited uptake into the brain.

Despite its favorable stability, which has been attributed to conformational restriction due to a cyclic structure (Weber et al., 1992), the residence time of DPDPE in vivo was unexpectedly short, with an elimination half-life of ~14 min (Chen and Pollack, 1996). Examination of the hepatobiliary disposition of DPDPE in rats demonstrated that extensive biliary excretion might be the underlying reason for the short sojourn of the peptide in vivo (Chen and Pollack, 1997). This rapid removal of DPDPE from blood likely is one reason for the short duration of antinociceptive action.

To elucidate completely the underlying mechanism(s) responsible for the brief duration of pharmacologic effect, it is

ABBREVIATIONS: DPDPE, \([\delta\text{-penicillamine}^{2,5}]\)enkephalin; BBB, blood-brain barrier; BMEC, bovine brain microvessel endothelial cells; AIC, Akaike’s Information Criteria; HPLC, high-performance liquid chromatography; PTS, peptide transport system; ANOVA, analysis of variance.
Dose-Dependent Disposition of DPDPE

This study was designed to examine the linearity of DPDPE disposition in mice after administration of pharmacologically relevant doses. DPDPE was administered as a 10-mg/kg (2 mg/ml), 60-mg/kg or 100-mg/kg (10 mg/ml) solution in phosphate-buffered saline, pH 7.4 bolus (within 1 min) through the jugular vein cannula followed by flushing the cannula with saline. Blood samples (0.02–0.05 ml) were collected into heparinized tubes at 0, 2, 5, 10, 20, 30, 40 or 60 min postadministration. Whole-blood samples were stored at −20°C until analysis by HPLC.

Dose-Response Experiment

This study was conducted to investigate the relationship between antinociceptive effect and DPDPE concentrations in blood and brain tissue. DPDPE (10, 20, 40, 60 or 100 mg/kg) containing 3H-DPDPE (−5 μCi/mouse) was administered as described in the preceding experiment. Control animals received saline. At 10 min postadministration, antinociceptive effect was determined with the hot plate latency test (Loh et al., 1976). Each mouse was placed on a hot plate (55°C; Columbus Instruments, Columbus, OH). The latency was defined as the time interval between the placement of a mouse on the hot plate to the observation of hindpaw licking or jumping behavior. Those animals with control latency (determined after cannulation) of 25 s or less were used for drug testing. A maximum (cut-off) test latency of 60 s was used to avoid tissue damage. The degree of antinociception (expressed as percent of maximum possible response; % MPR) was calculated as:

\[
\%\text{MPR} = \frac{\text{test latency} - \text{control latency}}{60 - \text{control latency}} \times 100.
\]

Immediately after pharmacologic testing, blood samples were obtained via the jugular vein cannula and mice were sacrificed by decapitation for collection of brain tissue. All samples were stored at −20°C until analysis by HPLC.

Response-Time Experiment

The time course of antinociception produced by DPDPE was examined after a 40 mg/kg i.v. bolus dose (containing −5 μCi 3H-DPDPE/mouse) to correlate the time course of antinociception with disposition of DPDPE in blood and brain tissue. Mice were divided into eight groups (n = 4 per group) for determination of antinociception and DPDPE concentrations at 0, 2, 5, 10, 15, 20, 30 or 40 min postdose. This study was conducted as described in the preceding experiment. All samples were stored at −20°C until analysis.

Stability of DPDPE in Mouse Brain Homogenate

DPDPE (1 mg/ml in water) was added to brain tissue homogenate prepared from naive mice with Tris buffer (20 mM, containing 0.2 M NaCl, pH 8.0, 1:2, w/v; Weber et al., 1992) to achieve a final DPDPE concentration of −50 μg/ml. The mixture was incubated at 37°C in a shaking water bath. Aliquots (100 μl) were obtained from the incubation vial at timed intervals up to 6 hr. Parallel controls were included by incubating DPDPE in Tris buffer in the absence of brain tissue. All samples were stored at −20°C pending analysis.

Sample Analysis

Blood samples were prepared for analysis according to a method recently developed in this laboratory (Chen and Pollack, 1997) with slight modification. Whole blood (20–50 μl) was lysed by adding two volumes of distilled water, followed by vortex mixing before precipitation of proteins with acetonitrile (−300 μl). After mixing by vortex and centrifugation (15,000 × g for 10 min), the supernatant was transferred to a clean 1.5-ml microcentrifuge tube and evaporated to dryness at 40°C under a stream of dry nitrogen. The residue was reconstituted with mobile phase (50 μl) and analyzed by HPLC.

Whole-brain tissue was isolated, blotted dry and weighed. Two volumes of saline were added before homogenization with a blade homogenizer. Aliquots of homogenate (100 μl) were pretreated as described for blood samples and analyzed by HPLC with liquid scintillation spectrometry after collection of the fraction containing DPDPE. Calculation of DPDPE concentration in brain homogenate was corrected for a 5% contamination by microvessel blood (Heisey, 1968).

HPLC separation was achieved with an LKB system (LKB-produkter AB, Bromma, Sweden) consisting of a model 2150 HPLC pump and a variable wavelength ultraviolet absorbance monitor. Samples (10 μl) were introduced onto a C18 column (Spherisorb, 5 μm, 25 cm × 4.5 mm i.d., Phase Separation, Queensferry, Clwyd, UK) using an autoinjector (SCL-10A, Shimadzu Scientific Instrument, Inc., Columbia, MD). Analytes were eluted with a mobile phase composed of acetonitrile and water (containing 0.05% trifluoroacetic
acid) at a ratio of 25:75 (v/v). Absorbance of the column eluent was monitored continuously at 210 nm. Data were acquired with a Chrom Perfect data acquisition system (Version 3, Justice Innovations, Mountain View, CA) and recorded on an IBM-compatible personal computer. For concentrations less than 1 µg/ml, and for all concentrations in brain tissue, samples were separated by HPLC as described above, and the fraction associated with the DPDPE peak (1–2 ml) was collected into a 20-ml glass scintillation vial. The fraction was mixed with 10 ml scintillation cocktail (Bio-Safe II, Research Product International Corp., Mount Prospect, IL) before measurement of radioactivity by liquid scintillation spectrometry.

Data Analysis

Estimation of pharmacokinetic parameters. The blood concentration-time data from individual mice from the pharmacokinetic experiment were fit with a two-compartment model with the nonlinear least-squares regression program Scientist (Micromath, Salt Lake City, UT). The pharmacokinetic parameters associated with DPDPE disposition calculated by standard methods (Gibaldi and Perrier, 1982) were compared across dose level to assess potential nonlinear disposition.

The brain/blood concentration ratio versus blood concentration obtained after different doses (10, 20, 40, 60 and 100 mg/kg) of DPDPE were fit with a series of pharmacokinetic models incorporating different modes of efflux from the brain (linear, nonlinear or combined); all models were based on the assumption that uptake into the brain was mediated by transmembrane diffusion. The models were fit to the data with the nonlinear least-squares regression analysis program PCNONLIN (version 3.0, SCI, Apex, NC). Assessment of the goodness of fit of the model to the observed data was based on AIC, residual plots and standard deviation of the estimates.

Pharmacodynamic analysis. The relationship between antinociception and DPDPE concentrations in brain was assessed by fitting the data with pharmacodynamic models incorporating linear, log-linear or sigmoidal relationships by the nonlinear least-squares regression. Assessment of the goodness of fit of the model to the observed data was as described above.

Statistical analysis. All data are presented as mean ± S.E. ANOVA and Student’s t test, where appropriate, were used to analyze data obtained in the stability studies, the blood-brain concentration ratios from the dose-response experiment and response-time data. The .05 level of probability was used as the criterion of significance.

Results

Analysis of DPDPE in blood and brain tissue homogenate by HPLC. DPDPE concentrations in blood and brain tissue homogenate were determined by HPLC after appropriate pretreatment for each matrix. Representative chromatograms are displayed in figure 1. Base-line separation of DPDPE from endogenous contaminants was achieved in blood and brain tissue homogenate. The limit of detection of this HPLC-UV method was ~1 µg/ml.

For blood samples containing <1 µg/ml DPDPE, and for all brain tissue samples, the fraction associated with the DPDPE peak (fig. 1) was collected and analyzed by liquid scintillation spectrometry. Because two methods were used for quantitation of DPDPE, the correlation between these methods was examined before experimentation. A good relationship between the two methods was observed (fig. 2), which indicated that fraction collection at low concentrations was a viable approach.

Disposition of DPDPE. DPDPE concentrations in blood declined biexponentially after administration of 10, 60 or 100 mg/kg as an i.v. bolus (fig. 3a). The concentration-time profile obtained after the 10 mg/kg dose was parallel to that associated with the 60 mg/kg dose, but not with the 100 mg/kg dose, which suggested that systemic disposition of DPDPE was nonlinear within the range of concentrations encountered in this experiment. When DPDPE concentrations were normalized by the administered dose (fig. 3b), the profiles from the 10-, 60- and 100- mg/kg groups were not superimposable; the
was consistent with a linear model, with an EC50 of 1.42 μM. Antinociception and brain tissue concentration of DPDPE increased as the administered dose increased, by DPDPE. The pharmacokinetic parameters associated with DPDPE disposition are presented in Table 1. A statistically significant difference was observed in clearance (Cl), steady-state volume of distribution (Vss), mean residence time (MRT) and elimination half-life (t1/2, α) across the three different dose levels. In mice, DPDPE clearance was 6-fold higher than in rats, and the substantially higher distribution volume in mice, as compared with rats, resulted in a similar terminal elimination half-life between the two species.

Stability of DPDPE in brain tissue homogenate. There was no evidence of significant degradation of DPDPE during the 6-hr incubation in mouse brain homogenate (data not shown). No statistical difference in DPDPE concentrations among the time points sampled during incubation was observed (ANOVA, P > .05).

Dose-response experiment. Antinociceptive effect produced by DPDPE increased as the administered dose increased beyond 20 mg/kg (Fig. 4). DPDPE concentrations in blood and brain tissue also increased with increasing dose. Because the variability inherent in the concentration-dose relationship, the relationship between antinociception and concentration (in blood and/or brain tissue) should be more reliable than the relationship between antinociception and dose. The degree of antinociception increased linearly with DPDPE concentrations in both blood and brain tissue (Fig. 5). Pharmacodynamic modeling indicated that the relationship between antinociception and brain tissue concentration of DPDPE was consistent with a linear model, with an EC50 of 1.42 ± 0.06 μM/g.

Time course of antinociception. This experiment was designed to examine the relationship between the time course of antinociception and that of DPDPE concentrations in blood and brain tissue. DPDPE penetrated into the brain rapidly; peak brain tissue concentrations were observed at 5 min after i.v. administration of DPDPE (Fig. 6a). The antinociceptive effect of DPDPE after a 40 mg/kg dose disappeared within 20 min (Fig. 6b); the percent of maximum response at 20 min was not statistically higher than the response observed in control animals (P > .05). Consistent with the dose-response study, DPDPE-associated antinociception decreased with decreasing concentrations in the systemic circulation and brain tissue (Figs. 6 and 7). However, the relationship between pharmacologic effect and DPDPE concentration in blood showed an apparent dissociation between antinociception and disposition, as evidenced by the counterclockwise hysteresis in the effect-concentration relationship. The degree of hysteresis was lower when effect was plotted against brain tissue concentration, consistent with some delay in DPDPE entry into the brain.

Discussion

Previous experiments have shown that DPDPE is removed rapidly from the systemic circulation of rats (Chen and Pollack, 1997). This rapid disappearance is not caused by extensive metabolism, a factor that has limited the potential clinical utility of most endogenous and exogenous peptides. For example, the opioid peptide dynorphine 1–13 has a plasma half-life of 0.5 to 4 min (Muller and Hochhaus, 1995); the rapid loss of peptide from blood has been attributed to biotransformation by circulating aminopeptidase. DPDPE is metabolically stable; no measurable loss of substrate has been observed during incubation of DPDPE with various biologic media in vitro, and biotransformation products have not been detected during distribution or pharmacokinetic studies in mice and rats (Weber et al., 1992, 1993; Chen and Pollack, 1997). Rapid elimination from the systemic circulation decreases substrate availability to the site of action, and therefore limits the potential utility of peptides in the clinical arena. The present study was undertaken to characterize the fundamental pharmacokinetic and pharmacodynamic properties of DPDPE, including disposition in blood and brain tissue and the relationship between pharmacologic response and disposition.

Similar to in vivo disposition in rats (Chen and Pollack, 1996) at the same dose level (10 mg/kg), DPDPE had an elimination half-life of ~12 min in mice. Such a short sojourn in the systemic circulation would not be expected for this metabolically stable peptide. Rapid removal of DPDPE from the body in rats could be attributed to extensive biliary excretion; >80% of total clearance was caused by biliary elimination (Chen and Pollack, 1997). Similarly, the short half-life observed in the present study could be caused, at least in part, by biliary excretion, as indicated by a whole-body distribution study conducted in mice; ~60% of total radioactivity after an i.v. dose of 3H-DPDPE was associated with the gall bladder, the intestines and intestinal contents (Weber et al., 1992). In contrast the terminal half-life, both systemic clearance (130 ± 10 ml/min/kg) and volume of distribution at steady state (857 ± 89 ml/kg) in mice were larger than the same parameters in rats (23 ± 7 ml/min/kg and 296 ± 85 ml/kg, respectively). These results illustrate substantial species differences in pharmacokinetics, even between two species typically considered to be similar with respect to drug disposition.

DPDPE disposition in mice was linear in the dose range...
from 10 to 60 mg/kg, but nonlinear when the dose was increased to 100 mg/kg (fig. 3). The dose-normalized time course of blood concentrations after 10- mg/kg and 60- mg/kg doses were superimposable; the pharmacokinetic parameters from both dose groups therefore were indistinguishable. The linear disposition of DPDPE within the 10 mg/kg to 60 mg/kg dose range was consistent with results from the time course of response experiment; the dose-normalized blood concentration-time profile from a 40- mg/kg dose also was superimposable with those from the previous 10 and 60 mg/kg dose groups. However, when the dose was increased to 100 mg/kg, DPDPE disposition evidenced nonlinearity; the dose-normalized time course of blood concentrations after the 100 mg/kg dose was higher than that from the 10 mg/kg or 60 mg/kg dose groups. This nonlinearity in DPDPE disposition also was reflected in the pharmacokinetic parameters; a significant decrease in clearance (2- to 3-fold) and an increase in MRT (4- to 5-fold) and the terminal half-life (2- to 3-fold) at the 100- mg/kg dose, compared with the same parameters from the 10- mg/kg and 60- mg/kg dose groups, were observed (table 1). This nonlinearity may be a consequence of saturable blood-to-bile translocation. A previous study of the hepatobiliary disposition of DPDPE in rats (Chen and Pollack, 1997) indicated that the rate of biliary excretion was a nonlinear function of DPDPE blood concentration. Other as yet unidentified translocation and/or sequestration processes may be responsible for the observed nonlinear disposition. However, because the binding of DPDPE to serum proteins is modest (<50%; Chen and Pollack, 1997), protein binding phenomena are unlikely to explain the nonlinear behavior of this peptide.

The apparent saturability of DPDPE efflux across the BBB was an unexpected outcome of the dose-response experiment.

![Fig. 3](Image)

**Fig. 3.** Time course of DPDPE in blood after a 10- (triangle), 60- (square) or 100- (diamond) mg/kg i.v. bolus dose of DPDPE (a). The same data are shown as dose-normalized concentrations in panel b. Data are expressed as mean ± S.E. (n = 5).

![Fig. 4](Image)

**Fig. 4.** Dose-response relationship for DPDPE antinociception at 10 min postadministration (i.v.). Data are presented as mean ± S.E. (n = 4–6). A significant difference was observed between %MPR at doses of 40 (P < .05), 60 (P < .001) and 100 (P < .0001) versus 10 or 20 mg/kg.

### TABLE 1

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<td>1164 ± 343</td>
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* Data are presented as mean ± S.E. (n = 4–5).
*<sup>a</sup> Data were obtained from Chen and Pollack (1996).
*<sup>b</sup> Pharmacokinetic parameters were compared within the 10-, 60-, and 100- mg/kg dose groups in mice by ANOVA with P < .05.
*<sup>c</sup> Significantly different (P < .05) from both 10- mg/kg and 60- mg/kg dose groups.
*<sup>d</sup> Significantly different (P < .05) only from 10- mg/kg dose group.
The relationship between the brain-to-blood concentration ratio and blood concentration (fig. 8) was consistent with a saturable efflux process in parallel to simple diffusion, because the ratio increased with the blood concentration, eventually reaching a plateau as blood concentrations increased. Saturable uptake of DPDPE into brain may have been anticipated. In a recent study (Thomas et al., 1997) the uptake of DPDPE into the central nervous system was found to be saturable.

DPDPE accessed the brain rapidly, producing peak concentrations by 5 min after i.v. administration (fig. 6). The rapid attainment of peak brain concentrations suggests that uptake of DPDPE into brain at the doses administered in this study is predominantly by unsaturable processes, or by a saturable process operating under linear conditions. Results of previous studies indicated that halogenation of the Phe\textsuperscript{4} position of DPDPE increased BBB permeability, because of increased lipophilicity, consistent with diffusional uptake (Weber et al., 1991). A kinetic modeling approach was used to elucidate the likely locus of the concentration-dependent translocation process observed in the present study. Assuming transmembrane diffusional uptake into the brain, three possible modes of efflux, i.e., simple diffusion, saturable translocation or transmembrane diffusion with a parallel saturable process, were compared. The model incorporating saturable efflux and parallel diffusion out of the brain best described the brain-to-blood partitioning data (fig. 8) based on statistical criteria.

There are several peptide transport systems localized on brain endothelial cells which are responsible for transport of some peptides out of the brain (Banks et al., 1992; 1993). These transport systems have demonstrated substrate specificity. For example, PTS-1 mainly transports Met-enkephalin and the Tyr-MIF-1 family, and only transports peptides with an N-terminal Tyr in the L-conformation (Banks, et al., 1992). Active efflux of DPDPE from mouse brain might be mediated by PTS-1. Studies with PTS-1 transport inhibitors may be useful to further elucidate the underlying mechanism of this efflux process.

Regardless of the nature of the transport system responsible for saturable efflux of DPDPE, the existence of such a system could explain the limited brain/blood partitioning of DPDPE despite rapid initial penetration in vivo and a moderate to high partition coefficient in vitro (Shah et al., 1989). Similar observations have been made for the transport of an analog of cyclosporine in BMEC; the limited permeability compared with the lipophilicity of the peptide was attributed to active efflux (Pardridge et al., 1990). Although the modeling performed in the present study was based on the assump-

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**Fig. 5.** Antinociception versus blood (a) and brain tissue (b) concentrations obtained 10 min after administration of 10-, 20-, 40-, 60- or 100- mg/kg i.v. bolus doses. Symbols represent observed data; lines indicate the fit of three different pharmacodynamic models to the data: linear (solid line; AIC = 16.87), log-linear (insert figure; AIC = 34.38), and sigmoidal (broken line; AIC = 29.48).

**Fig. 6.** Time course of brain concentration (a) and pharmacologic effect (b) of DPDPE after a 40- mg/kg i.v. bolus dose (solid bars). Open bars panel b indicate saline-treated controls. Data are presented as mean ± S.E. (n = 4). *P < .05 vs. control.
tion of transmembrane diffusion of DPDPE into the brain, recent studies have suggested that brain uptake of DPDPE in rats may be saturable (Williams et al., 1996; Thomas et al., 1997). The conflicting results between these studies may reflect differences in species (rat vs. mouse) and study designs (anesthetized vs. conscious animals). In addition, the brain tissue concentration of DPDPE in the present study (<5 μg/g) was well below the $K_m$ value (~30 μg/g) of the saturable transport responsible for the active uptake of DPDPE into brain in rats as reported in the literature (Thomas et al., 1997). Regardless of the mechanism(s) responsible for uptake, the data reported herein suggest that net permeability of the BBB to DPDPE may be increased by inhibiting the efflux system, thereby improving pharmacologic response.

An apparent linear relationship between antinociception and blood concentration (fig. 5) was observed in the dose-response experiment (i.e., when pharmacologic testing was performed at a fixed time postdose). The relationship between antinociception and blood concentration differed somewhat when testing was performed at various times postdose (fig. 7), which suggests that the site of action was pharmacologically distinct from the central compartment; peak blood concentrations failed to elicit maximal antinociception. Indeed, when effect was plotted versus brain tissue concentration, the counterclockwise hysteresis observed in the effect versus blood concentration relationship was eliminated. Thus, the apparent temporal delay in producing pharmacologic effect can be ascribed to a slowly (relative to systemic elimination) evolving equilibrium between brain tissue and blood. The lack of a counterclockwise hysteresis in the relationship between effect and brain tissue concentration implies that antinociception produced by DPDPE is instantaneous, and rapidly reversible, upon presentation of the peptide to the receptor site.

In summary, the present study shows that both rapid systemic elimination and saturable efflux from brain limit the duration of antinociception produced by DPDPE. Approaches that will prolong the sojourn of DPDPE in the systemic circulation and, preferentially, in the brain will be helpful in improving the pharmacologic effect of metabolically stable peptides such as DPDPE.

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References
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