Improved Brain Uptake and Pharmacological Activity of Dalargin Using a Peptide-Vector-Mediated Strategy

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ABSTRACT

The blood-brain barrier restricts the passage of substances into the brain. Neuropeptides, such as enkephalins, cannot be delivered into the brain when given systemically because of this barrier. Therefore, there is a need to develop efficient transport systems to deliver these drugs to the brain. Recently, we have demonstrated that conjugation of doxorubicin or penicillin to peptide vectors significantly enhances their brain uptake. In this study, we have conjugated the enkephalin analog dalargin with two different peptide vectors, SynB1 and SynB3, to improve its brain delivery and its pharmacological effect. We show by in situ brain perfusion that vectorization markedly enhances the brain uptake of dalargin. We also show using the hot-plate model that this enhancement in brain uptake results in a significant improvement in the observed antinociceptive effect of dalargin. These results support the usefulness of peptide-mediated strategies for improving the availability and efficacy of central nervous system drugs.

Brain delivery is one of the major challenges for the neuropharmaceutical industry since increasing number of hydrophilic therapeutic agents, such as anticancer drugs, antibiotics, and antiviral drugs are unable to cross the blood-brain barrier (BBB). The BBB represents a complex endothelial interface in vertebrates that separates the blood compartment from the extracellular fluid compartment of the brain parenchyma. The capillaries in the brain parenchyma possess a high electrical resistance due to tight junctions between the endothelial cells and also lack pores. Thus, the brain capillary endothelium behaves like a continuous lipid bilayer, and diffusion through this BBB layer is largely dependent on the lipid solubility of the drug. Because peptides are hydrophilic, biologically unstable, and large molecules, it is difficult for them to penetrate the BBB. Even though their brain uptake is not so high, some peptides and proteins are delivered into the brain by carrier-mediated transport, receptor-mediated transport, or adsorptive-mediated transport mechanisms. One of the problems associated with the inability of many peptides and proteins to accumulate in the brain in therapeutically meaningful amounts is the efflux transport systems. For example, it has been shown that the selective δ-opioid receptors against [d-Pen²,d-Pen⁵]-enkephalin (DPDPE) has a poor BBB permeability that is explained in part by P-glycoprotein (P-gp)-mediated efflux, and DPDPE is also a substrate of the rat organic anion transporting polypeptide 2 (OATP2) and human OATP-A (Kakyo et al., 1999; Gao et al., 2000).

To overcome the limited access of drugs to the brain, various strategies have been applied to direct central nervous system (CNS) drugs into the brain (Temsamani et al., 2000). Most of these methods are invasive, such as surgical implantation of an intraventricular catheter followed by drug infusion into the ventricular compartment, transient opening of the tight junctions by the intracarotid infusion of a hypertonic solution (Chamberlain et al., 1993; Kroll and Neuwelt, 1998; Temsamani et al., 2000), or intracarotid arterial infusion of vasoactive substances such as bradykinin or bradykinin analogs (Bartus et al., 1996).

Alternative, noninvasive methods that exploit the formation of chimeric peptide or protein-drug conjugates as carriers have also been developed. One such method relies on the presence of specific receptor-mediated transport systems in the BBB, for example insulin and transferrin coupling of a nontransportable drug (peptide or protein) to an anti-receptor antibody or other receptor-specific molecule, results in a chimeric construct that can undergo receptor-mediated transcytosis (Bickel et al., 1993; Partridge, 1994). Drug car-
riers such as liposomes (Zhou and Huang, 1992) and nanoparticles (Borchardt et al., 1994; Kreuter et al., 1995) have also been used for brain delivery. Despite these developments, there is still a need to develop noninvasive methods which promote the passage of inherently nonpenetrating drugs through the intact blood brain vessel endothelium.

Recently, we have shown that small peptide-vectors, derived from natural peptides called protegrins, can be used to enhance brain uptake of doxorubicin and penicillin (Rousselle et al., 2000, 2001, 2002). The potential of this approach as an effective delivery system for transporting drugs across the blood-brain barrier has been demonstrated in a number of animal models. The results obtained in these studies indicate that the use of peptide vectors can enhance significantly the brain uptake of doxorubicin without opening the tight junctions (Rousselle et al., 2000). The mechanism by which this vectorized doxorubicin crosses into the brain has been shown to be an adsorptive-mediated endocytosis process (Rousselle et al., 2001).

To assess the broad potential of this approach, we have coupled dalargin with SynB vectors and measured its brain uptake and pharmacological effect. Dalargin is a hexapeptide analog of Leu-enkephalin containing δ-Ala in the second position and an additional C-terminal arginine. These modifications modulate the stability of dalargin in the blood stream and brain, while at the same time modifying to some extent its receptor selectivity. While the intracerebroventricular injection of this peptide has been shown to induce analgesic action, its systemic administration shows no activity in peripheral analgesic mechanisms (Kalenikova et al., 1988). The reason for this is because dalargin is known not to cross the BBB.

We show in this study that SynB vectors improve the delivery of dalargin into the brain and that this enhancement in uptake is accompanied by a significant increase in its pharmacological potency in an animal model of nociception. These results support the usefulness of peptide-mediated strategies for improving the availability and efficacy of CNS drugs.

Materials and Methods

Animals

Adult OF1 mice (30–40 g, 6–8 weeks old) were obtained from lAnna-Credo (L’Arbresle, France). Animals were maintained under standard conditions of temperature and lighting and had free access to food and water. The research adhered to the ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (Law no. 87-848).

Preparation and Characterization of Peptide Conjugates

Peptide Synthesis. The peptides were assembled by conventional solid phase chemistry using a 9-fluorenylmethoxycarbonyl/tertiobutyl protection scheme (Atherton and Sheppard, 1989) and purified on preparative C_{18} reverse-phase HPLC after trifluoroacetic acid cleavage/deprotection. Purity of the lyophilized products was assessed by C_{18} reverse-phase analytical HPLC, and their molecular weight was checked by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF). The peptides sequences were SynB1 (H-RRGRLSYSRRFFSTGSTGR-NH_{2}; 2099 Da), SynB3 (H-RRLSYSRRRF-NH_{2}; 1395 Da), and p-SynB3 (H-rRLsysrrrf-NH_{2}; 1395 Da). All SynB vectors peptides were assembled on a carboxamide resin. The reference substance Dal-OH (YaGFL) was purchased from Neosystem (Strasbourg, France). Its purity and molecular weight were assessed by HPLC and MALDI-TOF, respectively.

Dal-SS-SynB Synthesis. The C-terminal cysteamide-modified dalargin was conjugated to SynB vectors activated by SPDP (3-(2-pyridyl disulfide propionic acid) by incubation of both peptides in dimethyl formamide in the presence of disopropylethyamine. This provided a linker containing a disulfide bond cleavable upon reduction after BBB crossing (Letvin et al., 1986; Pardridge, 1994). These constructs were designed to release dalargin with a C-terminal cysteamide group.

Radiolabeling of Dalargin and Dal-SS-SynB. To introduce a radiolabel, we acetylated the N-terminal of Dal-OH, Dal-SS-SynB3, and Dal-SS-SynB1 with [^{14}C]acetic anhydride (Amersham Pharmacia Biotech, Les Ulis, France). The acetylation were performed in dimethyl formamide, in the presence of disopropylethyamine. After [^{14}C]acetic anhydride incorporation, the acetylated peptides were purified on a reverse-phase semipreparative HPLC and lyophilized. Purity and molecular weight were checked by HPLC and MALDI-TOF, respectively. The specific activity of all the compounds was 55 mCi/mmol.

Receptor Binding Assay

Radio-receptor assays were carried out in which competition between labeled opioid ligands and the test compound was measured using an opioid receptor-containing membrane preparation under equilibrium conditions at neutral pH. Radioligands [^{3}H]DAMGO (Tyr-d-Ala-Gly-MePhe-Gly-ol), [^{3}H]DADL [δ-Ala^{2}, δ-leu^{5}], [^{3}H]DPPDPE [2-D-penicillamine-5-D-penicillamine-enkephalin] and [^{3}H]DSLET [δ-serine^{2}]-D-leucine-enkephalin-threonine] were purchased from PerkinElmer Life Sciences (Boston, MA). Fresh calf brains were obtained locally, dissected into the appropriate brain region, and homogenized in 50 volumes of Tris buffer (50 mM, pH 7.6 at 25°C) with phenylmethyloxyl sodium fluoride (0.1 mM), EDTA (1 mM), and NaCl (100 mM), centrifuged (49,000g for 40 min), resuspended in 0.3 M sucrose, and frozen. Tissue prepared in this manner and kept frozen at −70°C retained its binding for at least 3 to 4 weeks. Frozen guinea pig brains were obtained from Charles River (Wilmington, MA). The brains were thawed and the cerebella prepared and frozen as described above.

Membranes were incubated in 50 mM potassium phosphate buffer (pH 7.0 with MgSO_{4} 5 mM) at 25°C for 150 min with radioligand and various concentrations of tested compound to give a total assay volume of 2 ml. The reaction was terminated by rapid filtration over glass fiber filters. Nonspecific binding was determined with levallorphan (1 μM). Receptor μ binding assays were performed using calf thalamus membranes with either [^{3}H]DADL (0.7 nM) in the presence of PDPE (10 nM) for μ receptor binding or [^{3}H]DAMGO (1 nM) in the presence of DESLET (5 nM) for μ2 binding. MgCl_{2} (5 mM) was added to the buffer to increase levels of specific μ binding (Clark et al., 1988). For δ binding, calf frontal cortex membranes were used with [^{3}H]PDPE (1 nM).

All determinations were performed in triplicate. K_{i} values and Hill coefficients were determined using GraphPad Prism (GraphPad Software, San Diego, CA).

In Situ Mouse Brain Perfusion Study

Surgical procedure. The uptake of free or vectorized [^{14}C]dalargin to the luminal side of mouse brain capillaries was measured using the in situ brain perfusion method previously adapted in our laboratory for the study of drug uptake in the mouse brain (Dagenais et al., 2000). Briefly, the right common carotid of ketamine/xylazine (140/80 mg/kg, i.p.) anesthetized mice was exposed and ligated at the heart side. The external carotid artery was ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing (0.30-mm i.d. × 0.70-mm o.d.; Biotrol Diagnostic, Chennevières-les-Louvres, France) filled with heparin (25 U/ml) and
mounted on a 26-gauge needle. The syringe containing the perfusion fluid was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus, Holliston, MA) and connected to the catheter. Immediately before the perfusion, the heart was stopped by severing the ventricles to eliminate contralateral blood flow contribution. Brains were perfused for 120 s at a flow rate of 2.5 ml/min. At the end of the perfusion time, the mouse was decapitated and the brain removed. The right hemisphere and samples of perfusion fluid were placed in preweighed scintillation vials and weighted. Brain and perfusion samples were then digested for 2 h in 1 ml of Solvable (Packard, Rungis, France) at 50°C and mixed with 9 ml of Ultima Gold XR scintillation cocktail (Packard). Total 14C and 3H were determined simultaneously in a Packard Tri-Carb model 1900 TR liquid scintillation analyser and activities were converted from counts per minute to disintegration per minute with the use of internally stored quenching curves.

**Brain Uptake of Free and Vectorized [14C]Dalargin.** The perfuse consisted of a Krebs-bicarbonate buffer: 128 mM NaCl, 24 mM NaHCO3, 4.2 mM KCl, 2.4 mM Na2HPO4, 1.5 mM CaCl2, 0.22 mM MgSO4, and 9 mM D-glucose added before infusion. The solution was gassed with 95% O2 and 5% CO2 for pH control (7.4) and warmed at 37°C in a water bath. Tracers were added to perfusate at concentrations of 0.4 µCi/ml for free dalargin, 0.1 µCi/ml for vectorized dalargin, and 0.3 µCi/ml for [3H]sucrose, the latter being a vascular marker with poor penetration of the BBB.

**Determination of BBB Transport Constants.** Briefly, calculations were carried out as previously described by Smith (1996). The integrity of the BBB was determined in each animal by the brain vascular volume (Vv; microliters per gram) estimated by the tissue distribution of [3H]sucrose from the following relationship

\[
V_v = \frac{Q_{av}}{C_{sp}^*} \tag{1}
\]

where \(Q_{av}\) is the amount of radiolabeled sucrose in the right brain hemisphere (disintegrations per minute per gram) and \(C_{sp}^*\) is the perfuse concentration of sucrose (disintegrations per minute per microliter).

Dalargin uptake was expressed as the volume of distribution (\(V_d\)) from the following relationships

\[
V_d = \frac{Q_4}{C_{sp}^*} \tag{2}
\]

where \(Q_4\) is the calculated quantity of [14C]tracer per gram of right brain hemisphere and \(C_{sp}^*\) (disintegrations per minute per microliter) is the labeled tracer concentration measured in the perfuse.

**Measurement of the Antinociceptive Effect**

Antinociception was assayed in mice by the hot-plate assay. The hot-plate response has been proposed to require the activation of supraspinal mechanisms to inhibit a behavioral response (Yakovsh and Rudy, 1978). In the hot-plate assay, mice were placed on a 54°C surface (Harvard Apparatus, Holliston, MA), and the time to lick one of the paws or escape jump was recorded as the response latency. Predosing latency was determined before administration of the compounds and was 4.6 ± 1.6 s. The hot-plate latency was determined 5, 10, 15, 30, and 45 min after intravenous injection of free or conjugated dalargin at a dose of 2 mg/kg Eq (mg base of dalargin). A maximal cutoff time of the heat was 30 s to prevent tissue damage. To correct for individual differences in baseline latencies, the antinociceptive data (latencies) were converted to percentage maximum possible effect (%MPE) using the following formula (Brady and Holtzman, 1982).

\[
\%\text{MPE} = \frac{\text{Postdrug latency} - \text{Predrug latency}}{\text{Maximum latency} - \text{Predrug latency}} \times 100
\]

**Results**

**Receptor Binding Assay.** First, we determined the opioid receptor selectivities of free and vectorized dalargin using radioligand binding methods (Table 1). Dal-OH binds with low nanomolar affinity to \(\mu\)- and \(\delta\)-opioid receptors, with about an 8-fold selectivity for \(\mu\) over \(\delta\) receptors. The vectorized conjugate Dal-SS-SynB3 (Fig. 1) shows a receptor selectivity and affinity similar to Dal-OH.

**BBB Permeability.** We measured the brain uptake of free and vectorized dalargin using the in situ brain perfusion in mice. To assess the integrity of the BBB, [3H]sucrose was used as a marker of brain vascular volume since it does not measurably penetrate the BBB during brief periods (e.g., 60–120 s) of perfusion. When free or conjugated dalargin were perfused, the distribution volume of [3H]sucrose into the right cerebral hemisphere was about 16 µl/g, indicating that the permeability of the BBB has not been altered (Fig. 2). This is similar to the vascular volume values previously measured in our laboratory, which is typically about 20 µl/g (Dagenais et al., 2000).

**BBB permeabilities of free and vectorized dalargin were then assessed (Fig. 3).** The brain uptake of free dalargin was very low after 120 s of perfusion (\(V_d = 16.7 ± 1.2 \mu l/g\)), which is comparable to the distribution volume of the [3H]sucrose. This perfusion time (120 s) was chosen because it is short enough to limit risks of drug metabolism or efflux from brain to blood but high enough to measure reasonable quantities of radio-labeled dalargin in brain tissues compared with the background noise of the detection method.

Interestingly, conjugation of dalargin to SynB1 and SynB3 via a disulfide linker (Dal-SS-SynB1 and Dal-SS-SynB3) significantly enhanced its brain uptake. The distribution volume of dalargin measured for both vectors were similar (309 ± 82.7 µl/g for Dal-SS-SynB1 and 240 ± 44.9 µl/g for Dal-SS-SynB3).

**In Vivo Analgesic Studies.** Free or conjugated dalargin were administered i.v. to mice, and antinociception was determined using the hot-plate test, an assay known to be mediated by central receptors. This test measures the amount of time required for mice to react to standardized noxious stimuli. Substances that increase the reaction time are described as displaying antinociceptive effects, which may be interpreted as a measure of analgesia.

The results show that i.v. administration of free dalargin to mice at 2 mg/kg in physiological saline exhibited only a small but nonsignificant analgesic response (Fig. 4). In contrast, conjugation of dalargin to SynB1 or SynB3 led to a considerable enhancement of analgesic activity immediately (within 5 min, the first time point) after the i.v. injection. Administration of the SynB1 vector alone did not produce any analgesic effect (data not shown). To determine whether the stability of the peptide might enhance the pharmacological effect of dalargin, we have coupled it using a D-SynB3 vector. The D-form of the peptide (D-SynB3) has been shown to be more

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**TABLE 1**

<table>
<thead>
<tr>
<th>Binding activity in vitro</th>
<th>Kᵢ</th>
<th>nM</th>
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<tbody>
<tr>
<td></td>
<td>(\mu₁)</td>
<td>(\mu₂)</td>
</tr>
<tr>
<td>Dal-OH</td>
<td>0.41</td>
<td>0.63</td>
</tr>
<tr>
<td>Dal-SS-SynB3</td>
<td>0.33</td>
<td>0.87</td>
</tr>
</tbody>
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stable in serum than the l-form (SynB3) but displays a similar brain uptake (Rousselle et al., 2001). Figure 4 shows that dalargin coupled to the d-form has a similar analgesic effect as the l-form, indicating that enhancing the stability of the vector does not result in an enhancement of the analgesic effect. One cannot rule out that the d-form displays a different receptor binding profile, however.

Discussion

Neuropeptidic drugs hold great promise for the treatment of a wide variety of brain disorders, such as ischemia, inflammatory, and noninflammatory neurodegenerative disorders, as well as acute, chronic, or neuropathic pain syndromes. Nevertheless, it is widely acknowledged that neuropeptides typically fail to reach their target after systemic administration due to their poor transfer through the BBB. Should a strategy of penetration through the BBB be developed, the development of peptides or their synthetic analogs as neuroactive drugs would become widely used.

Here, we report the application of a peptide-mediated strategy for increasing the BBB permeability of poorly available drugs. The SynB peptides (18 amino acids for SynB1 and 10 for SynB3) translocate through biological membranes with high efficiency and have provided the basis for the development of new peptide-conjugated drugs for brain disorders. SynB vectors are derived from natural peptides called protegrins (Harwig et al., 1995). In their native form, protegrins adopt antiparallel β-hairpin structures, constrained by two disulfide bridges (Aumelas et al., 1996). Replacement of the four cysteines by serines leads to linear peptides (SynB vectors) that retain their ability to cross cell membranes but that have lost their cytolytic effects. We have used these vectors as a starting point for developing new effective strategies for drug delivery into the brain (Rousselle et al., 2000; 2001). We have reported recently that vectorization of doxorubicin and penicillin with SynB vectors enhances their brain uptake without compromising the tight junction integrity (Rousselle et al., 2000, 2002). In the present study, our rationale was to attach dalargin to SynB peptides as a vehicle for delivery of dalargin to the sites of endogenous opioid receptors in the brain. Dalargin was conjugated to the SynB vectors by coupling it to cysteine residues of the SynB peptides, resulting in SynB-Dal conjugates. Figure 4 shows that dalargin coupled to the d-form has a similar analgesic effect as the l-form, indicating that enhancing the stability of the vector does not result in an enhancement of the analgesic effect. One cannot rule out that the d-form displays a different receptor binding profile, however.
vectors via a linker containing a disulfide bond. The disulfide-based linker system has been shown to be stable in plasma for several hours although labile in brain (Letvin et al., 1986).

The results obtained in our study indicate that SynB vectors are able to increase the threshold in nociceptive assays involving acute stimuli in mice, such as the hot-plate model. This model has been interpreted to require the activation of supraspinal mechanisms to inhibit a behavioral response. This reveals that the analgesic effects we have observed are probably mediated by central mechanisms supported by the observation that, using in situ brain perfusion, dalargin conjugates are able to enter into the brain while free Dal-OH is not. In addition, we have shown that vectorized dalargin is able to bind to \( \mu \) opiate receptors. The enhancement in the analgesic effect was significant for about 30 min. At later time-points, the activity of vectorized dalargin return to baseline. Interestingly, Schroeder and Sabel (1998) using the nanoparticle strategy have observed the same kinetics of analgesia for dalargin. Luminal efflux transporters such as P-gp may restrict further BBB transport. Dalargin is a hexapeptide (molecular mass 726 Da) that is much more hydrophilic than the typical brain-penetrating drug (e.g., morphine). It has already been shown for other enkephalin analogs, such as DPDPDE, that poor BBB permeability may in part be explained by P-gp-mediated efflux (Dagenais et al., 2001). Thus, this or related efflux pumps may be responsible for the low brain uptake of dalargin. It will be interesting to see if vectorization of dalargin will allow it to escape P-gp efflux since we have shown that doxorubicin, a P-gp substrate, bypasses the P-gp when conjugated to SynB vectors (Mazel et al., 2001).

The mechanism, whereby dalargin conjugates cross the BBB, is not yet clear. In general, peptides produce their central effects in brain by 1) crossing the capillary endothelial forming the BBB by either a passive diffusion or by a specific receptor-mediated mechanism, 2) penetrating the fenestrated capillaries of the circumventricular organs (Begley, 1994), or 3) undergoing endothelial uptake by phagocytosis. In contrast to these mechanisms, we have recently shown that doxorubicin vectorized with SynB1 and related vectors enters the brain by a mechanism involving adsorptive-mediated endocytosis (Rouselle et al., 2001). Three lines of evidence support this. First, the transport of vectorized doxorubicin is a saturable mechanism, and the observed \( K_m \) values in the micromolar range are comparable to those found for other substrates (e.g., ebiradite; Terasaki et al., 1992; bovine serum albumin; Kumagai et al., 1987) reported to be taken up into brain via adsorptive-mediated endocytosis. Second, the brain transport does not involve a chiral vector. This reveals that the analgesic effects we have observed are probably mediated by central mechanisms supported by the observation that, using in situ brain perfusion, dalargin conjugates are able to enter into the brain while free Dal-OH is not. In addition, we have shown that vectorized dalargin is able to bind to \( \mu \) opiate receptors. The enhancement in the analgesic effect was significant for about 30 min. At later time-points, the activity of vectorized dalargin return to baseline. Interestingly, Schroeder and Sabel (1998) using the nanoparticle strategy have observed the same kinetics of analgesia for dalargin. Luminal efflux transporters such as P-gp may restrict further BBB transport. Dalargin is a hexapeptide (molecular mass 726 Da) that is much more hydrophilic than the typical brain-penetrating drug (e.g., morphine). It has already been shown for other enkephalin analogs, such as DPDPDE, that poor BBB permeability may in part be explained by P-gp-mediated efflux (Dagenais et al., 2001). Thus, this or related efflux pumps may be responsible for the low brain uptake of dalargin. It will be interesting to see if vectorization of dalargin will allow it to escape P-gp efflux since we have shown that doxorubicin, a P-gp substrate, bypasses the P-gp when conjugated to SynB vectors (Mazel et al., 2001).

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Other approaches for enhancing the brain uptake of dalargin into the brain have been described. For example, Kreuter et al. (1995) used a nanoparticle system for drug loading that was able to cross the BBB after adsorption and coating with polylsorbate 80. A similar nanoparticle system using polysorbate 85 was described by Schroeder et al. (1996). Dalargin-loaded nanoparticles have been shown to induce a central analgesic effect after either i.v. or oral administration. However, the mechanism by which these complex nanoparticles cross the BBB and exhibit their effects has not been elucidated. Some authors have suggested that the antinociceptive effect of dalargin mixed with polybutylycyanoacrylate nanoparticles may originate, at least in part, from the toxicity of the carrier on the BBB and consequent opening of the tight junctions (Olivier et al., 1999). Although polysorbate 80-coated polybutylycyanoacrylate nanoparticles may be a useful experimental tool, potential therapeutic applications may be limited by the high systemic nanoparticle concentration necessary to deliver drugs to the CNS and the ensuing toxicity.

Our results show that vectorization of dalargin enhances its brain delivery. This enhancement in brain uptake results in a significant improvement in the analgesic activity of dalargin. Finally, this study supports the usefulness of peptide-mediated strategies for improving the availability and efficacy of central nervous system drugs.

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References


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