Strategies To Target Kyotorphin Analogues to the Brain

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The design, synthesis, and pharmacological evaluation of brain-targeted chemical delivery systems (CDS) for a kyotorphin analogue (Tyr-Lys) are described. The brain-targeted compound contains the active peptide in a packaged, disguised form, flanked between the lipophilic cholesteryl ester on the C-terminus and the 1,4-dihydrotrigonellyl redox targetor, attached to the N-terminus through strategically selected L-amino acid(s) spacer. It was found that for successful brain targeting, the ϵ-amine of Lys needs to be also converted to a lipophilic function. Through sequential enzymatic bioactivation, the Tyr-Lys dipeptide is released in a sustained manner, producing significant and prolonged analgesic activity as demonstrated by the rat tail latency test. An alternate strategy was also employed. Lys was replaced by a redox amino acid pair, $Nys^+ \leftrightarrow Nys$, the nicotinamide ↔ 1,4-dihydronicotinamide analogues of Lys (Nys^+ is 2-amino-6-(3-carbamoyl-1-pyridiniumyl)hexanoic acid). The Nys form is lipophilic and facilitates delivery in addition to the C- and N-terminal lipophilic functions. Enzymatic oxidation to Nys^+ provides the lock-in, followed by removal of the lipophilic groups, releasing Tyr-Nys^+ from the brain-targeted analogue (BTRA). Nys^+ was shown to be an effective substitution for Arg or Lys. The activities of the CDS and BTRA, respectively, were antagonized by naloxone, supporting the designed brain-targeted processes. The most potent compound is the two-proline spacer containing CDS (CDS-PP), followed by the BTRA.

Introduction

Kyotorphin (KTP, L-tyrosyl-L-arginine) is an endogenous neuropeptide which exhibits significant analgesic action indirectly, through induction of the release of endogenous enkephalin from nerve terminals inside the brain.[1,2] The name kyotorphin was given to reflect an endorphin-like substance discovered in Kyoto, Japan. The analgesic potency of kyotorphin introduced directly to the brain is about 4.2 times that of Met-enkephalin.[2] However, when administered systemically, it shows activity only briefly, at a high dose of 200 mg/kg. The general major obstacle for the development of centrally active peptides is the blood−brain barrier (BBB), the brain capillary walls formed from tightly joined endothelial cells, which allows only compounds with sufficient lipophilicity to penetrate this barrier.[3] The BBB is also distinct from the peripheral capillary system in that it contains high concentrations of various lytic enzymes which by facile metabolic degradation also prevent the uptake of blood-borne neurotransmitters and neuromodulators.[4] Finally, many compounds lipophilic enough to penetrate the BBB are good substrates of P-glycoproteins found in the BBB, which promptly transport these molecules back to the blood. These actually work as "microscopic wormholes", and compounds such as many lipophilic peptides which are substrates of P-glycoproteins are taken out through these faster than they enter the brain.[5]

Kyotorphin suffers primarily delivery problems and not facile efflux. Due to the presence of arginine, which has a pK_a of 13.2, kyotorphin is virtually completely ionized at all times, which clearly prevents it from passing the BBB.

Efficient, brain-targeted delivery of analgesic enkephalin analogues was recently described,[6] based on a sequential bioactivation of a specifically designed packaged peptide construct, which allows passive transport of the original lipophilic precursor to the brain, followed by "lock-in" produced by the redox targetor and subsequent enzymatic liberation of the active peptide. High and prolonged analgesic activity was thus produced. It was also demonstrated[7] that the observed activity was exclusively centrally mediated, as it can be fully antagonized by naloxone but not by methylnaloxonium salts.

Briefly, the active target peptide is modified by introducing a large lipophilic function on the C-terminal, such as cholesteryl or adamantylethyl esters, while the N-terminal is converted via strategically chosen L-amino acid spacers to the dihydrotrigonellyl targetor. The 1,4-dihydrotrigonellyl function is enzymatically converted to the quaternary trigonellyl moiety, which provides about 5 log unit change in the partition properties of the molecular construct, producing the important brain "lock-in" process and the facilitated peripheral excretion. Subsequently, the lipophilic protector is cleaved, and then endopeptidases will split the locked-in precursor at the designed linkage between the spacer and the active peptide. An alternate approach applied for brain targeting of TRH analogues,[8] in addition to the described functions, uses a glycine spacer between the C-terminal and the lipophilic function, which resulted in producing the C-terminal amide of amino acid linked to this glycine, by the α-amidating monooxygenase (PAM) enzyme.

Here we report a modified version of this approach for brain targeting of L-tyrosyl-L-lysine kyotorphin analogues. It was found that in kyotorphin, arginine can be replaced with lysine and the resulting L-tyrosyl-L-lysine (YK) is an analogue which exhibits the same analgesic effect as kyotorphin when administered icv.[9]

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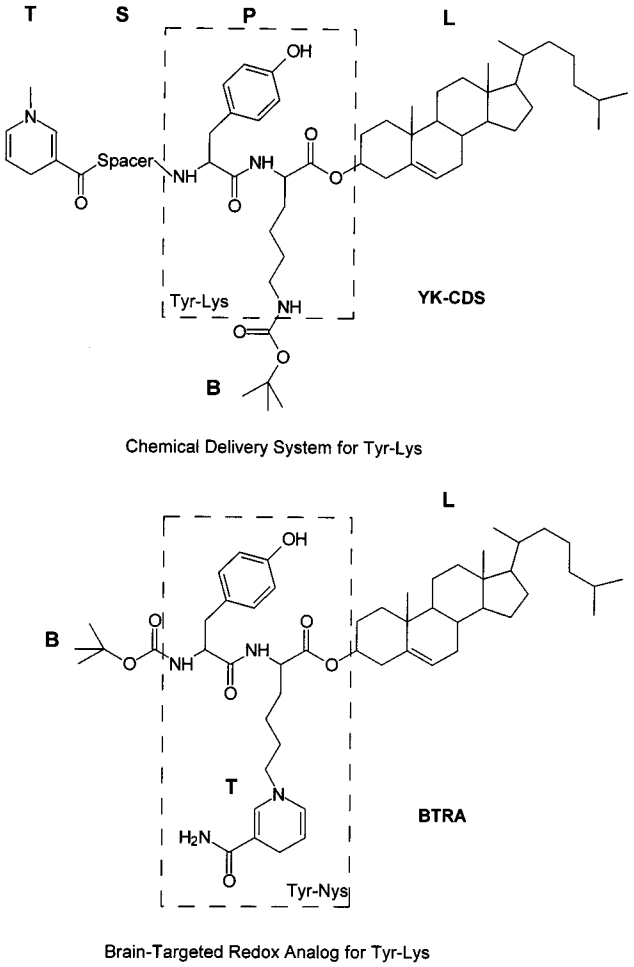


Figure 1. Chemical delivery system (CDS) and brain-targeted redox analogue (BTRA) for tyrosyl-lysine (spacer = Pro, Pro-Pro, Pro-Ala).

Design

The lysine analogue of kyotorphin (YK)[9] was chosen as the target peptide. It was, however, obvious that the already established approaches for brain targeting of peptides cannot be simply applied, since the basic amino group in lysine (pK_a = 10.3) would prevent passive transport of a packaged dipeptide. In order to solve this problem, we have applied two different strategies. First, the dipeptide was packaged similar to enkephalin; however, the free ϵ-amine function in lysine was made lipophilic by coupling to a Boc moiety. It was assumed that Boc is biodegradable, *if* the corresponding precursor is locked in the brain. Accordingly, the chemical delivery system (YK-CDS) is shown in Figure 1. For the final release, the proline endopeptidase[10,11] or dipeptidyl peptidase[12] was the target enzyme, and accordingly the spacer used was either proline or alanine or a combination of these two amino acids. The ϵ-amine function is derivatized by Boc.

However, a basically different strategy was also tested. Recognizing that highly basic amino acids, such as arginine and lysine, will essentially be protonated all the time at biological pHs, it was considered that an isoelectronic−isosteric replacement of the amino acids could accomplish dual roles. As shown in Figure 2, a "redox amino acid" pair[13] (2-amino-6-(3-carbamoyl-1,4-dihydropyridinyl)hexanoic acid ↔ 2-amino-6-(3-carbamoyl-1-pyridiniumyl)hexanoic acid) mimics the protonation process. Accordingly, the oxidized, quaternary pyridinium form might produce an active dipeptide, while the corresponding 1,4-dihydro form could replace the targetor moiety of CDS. In this construct, the use of spacers is not needed, but we assume that the free amine of tyrosine needs to be made lipophilic in a similar way, by Boc. Thus, replacing Lys by the Nys−Nys$^+$ redox system, as shown in Figure 1, could be an effective way to produce centrally mediated analgesic activity.

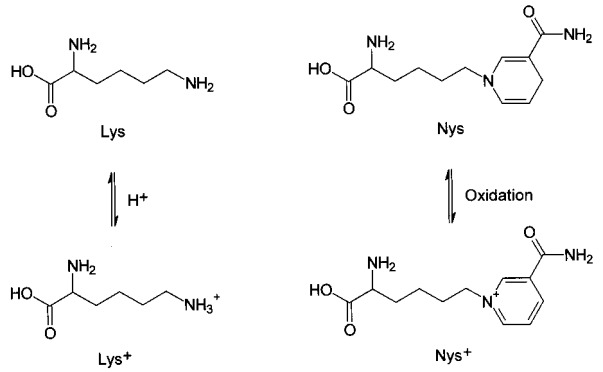


Figure 2. Isoelectronic/isosteric analogy of Lys and Nys.

Figure 3 is a composite summary of the two alternate approaches. After administration to the general circulatory system, both the CDS and BTRA, respectively, would undergo passive distribution to the brain, followed by oxidation of the dihydrotrigonellyl function in CDS or that of Nys in the BTRA. Using a recently developed general and highly reliable method to estimate partition properties of various compounds, including peptides,[14,15] the dramatic change in partition followed by introduction of the permanent positive charge in either system was clearly demonstrated. Thus, it was found consistently that the 1,4-dihydropyridine to pyridinium salt conversion dramatically reduces the log *P* values in both the CDS and BTRA by about 5 log units.

Chemistry

The synthesis of the CDS and BTRA is described in Schemes 1 and 2, respectively. The use of the Fmoc-Lys(Boc)-OH as the starting material allows selective manipulation of the two amino functions. Alternately, for the synthesis of the BTRA, we start with the Boc-Lys(Fmoc)-OH. The key component, Nys$^+$, is generated in the final step, before the reduction, by applying the "Zincke reaction", using the Zincke reagent from nicotinamide,[16,17] *N*-(2,4-dinitrophenyl)nicotinamide chloride. The cholesteryl esters were prepared using DCC (dicyclohexylcarbodiimide) as the coupling agent. The final step, reduction of the quaternary pyridinium-3-amides, was accomplished using sodium dithionite as the reducing agent.

Pharmacological Studies

Tail-flick latency, an index of spinal cord-mediated analgesia,[18] was used to evaluate the analgesic effects in rats of the brain-targeted delivery systems for the kyotorphin analogues. Each compound, at each dose, was tested on six animals. As shown in Figure 4, the simplest CDS, **7a**, with a single proline as the spacer,

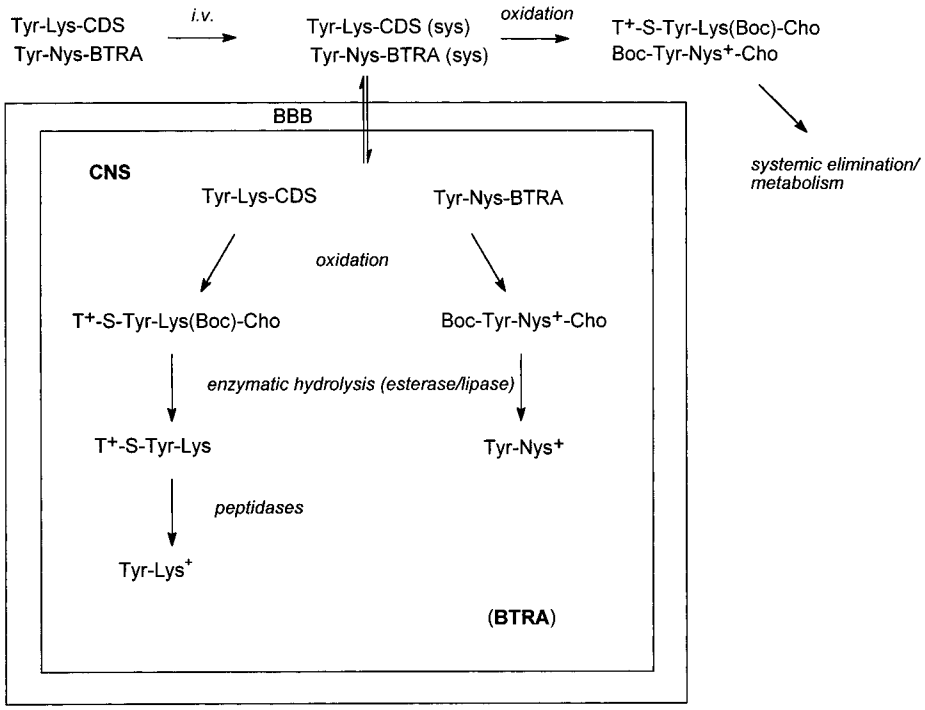
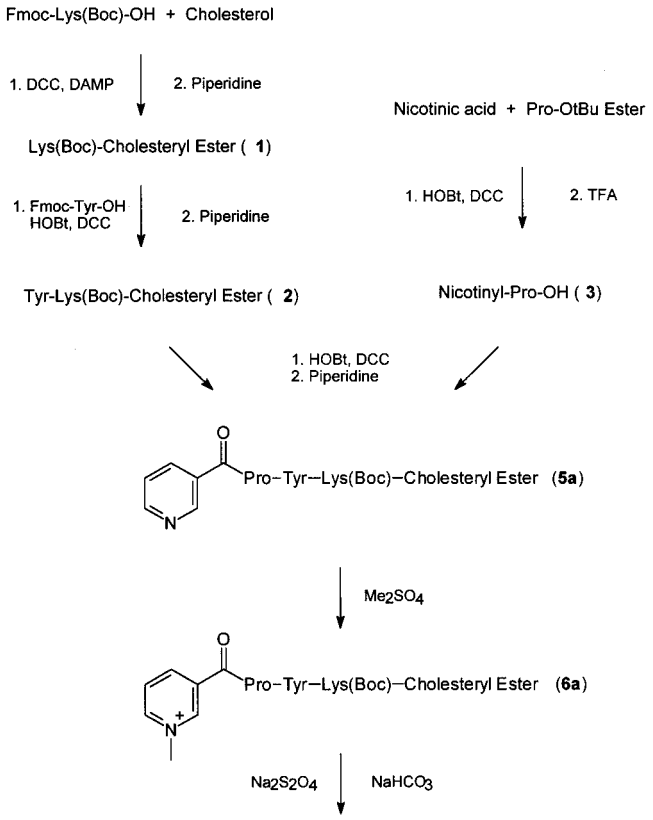


Figure 3. Sequential metabolism of brain-targeted delivery systems of Tyr-Lys and brain-targeted redox analogue (Tyr-Nys$^+$).

Scheme 1. Synthesis of Tyr-Lys-CDSs **7a**−**c**[a]



[a] The proline spacer is replaced with Pro-Pro and Pro-Ala in **7b**,**c**, respectively, by extending **2** with a Pro or Ala before coupling with **3**.

increased the rat tail-flick latency periods significantly at all doses, compared to the vehicle. A dose-dependent response was observed at doses of 0.003−0.0223 mmol/kg (equivalent to 1.0, 2.5, 5.0, and 7.5 mg/kg kyotorphin, respectively; single factor for the maximum responses, $p < 0.01$). This represents a dramatic increase in activity as compared to systemically administered kyotorphin which is required to be given at about 200 mg/kg to observe analgesia.

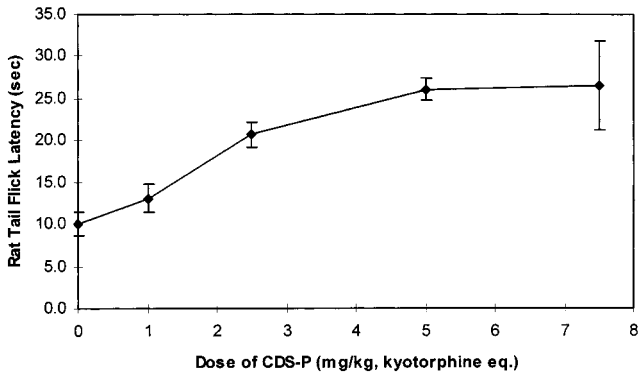


Figure 4. Dose−response of CDS-P (**7a**) after iv administration in rat. Data represent mean ± SE of 6 rats for each group.

The analogues were compared for their activity as shown in Figure 5. The comparison was made at a single dose of 0.0223 mmol/kg, equivalent to 7.5 mg/kg kyotorphin. The highest activity was achieved when two L-proline moieties are used as the spacer. This is followed by one proline, then the Nys containing the novel BTRA, and CDS containing the L-proline-alanine spacer. The time−response relationships in all cases show remarkably sustained effects (Figure 5). (Two-factor ANOVA with replication, $p < 0.05$ in all cases as compared to kyotorphin or the vehicle.)

All possible partial derivatizations were also tested. As shown in Figure 6, the lipophilic intermediate containing cholesteryl and Boc, in addition to the two proline spacers, but without the dihydrotrigonellyl targetor (**4a**) does not produce any activity. The positively charged trigonellyl targetor does not lead to brain delivery (**6b**), or when the dihydrotrigonellyl-prolyl-prolyl-Tyr-Lys (DPPYK) is administered, the lack of the

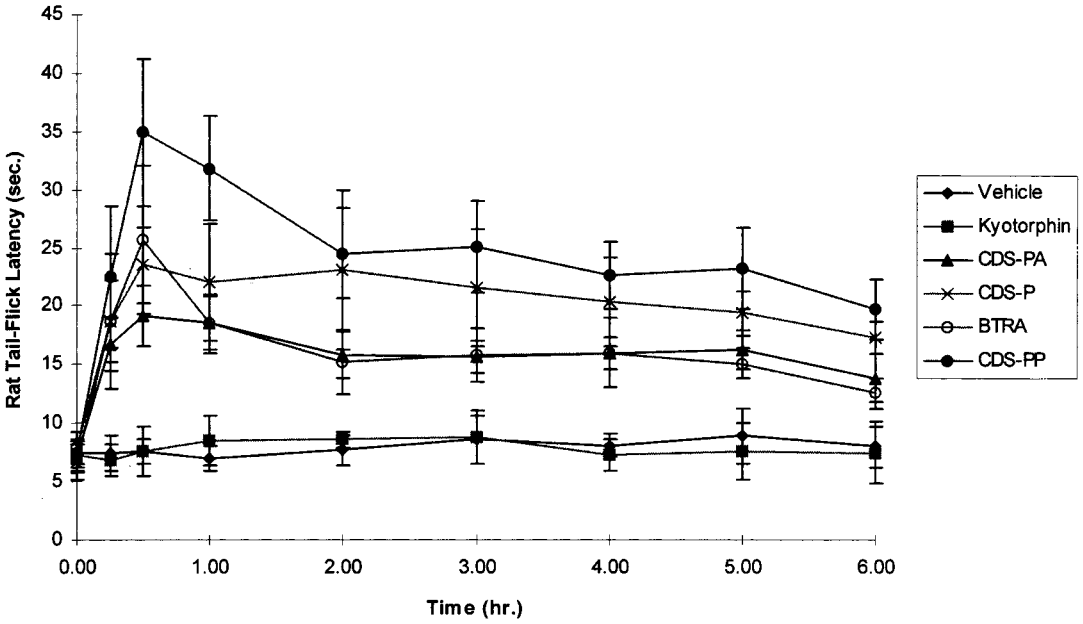
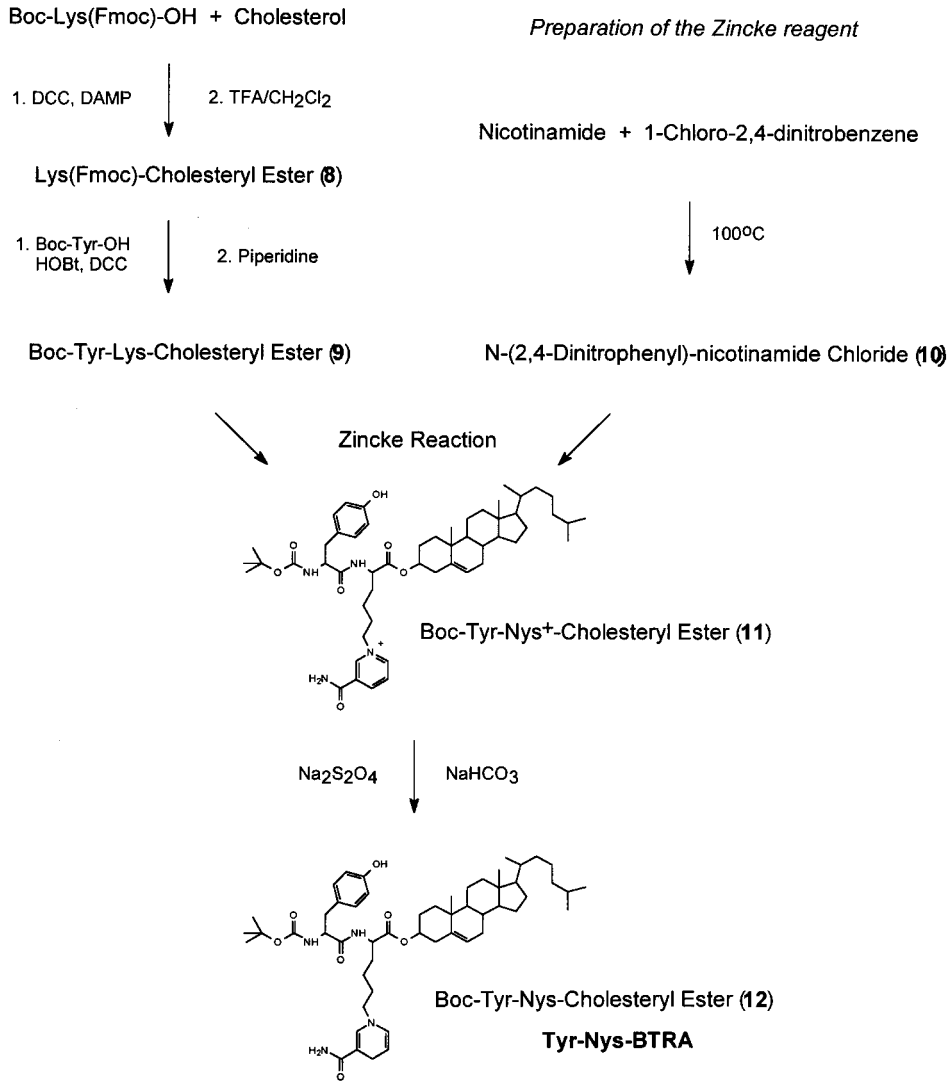


Figure 5. Rat tail-flick latency periods of Tyr-Lys-CDSs and Tyr-Nys-BTRA after iv administration at doses of 0.0223 mmol/kg (equivalent to 7.5 mg/kg kyotorphin). Data represent mean ± SE of 6 rats for each group; CDS-PA, **7c**; BTRA, **12**; CDS-P, **7a**; CDS-PP, **7b**.

Scheme 2. Synthesis of Tyr-Nys-BTRA (**12**)



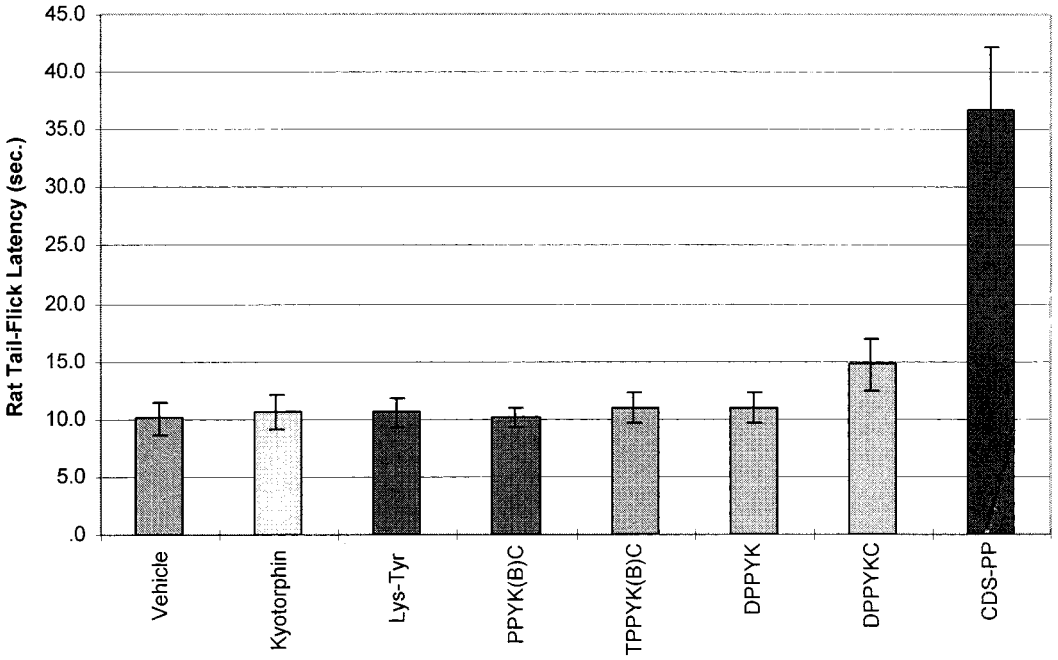


Figure 6. Rat tail-flick latency periods after iv administration of CDS-PP and its major intermediates at doses of 0.0223 mg/kg (equivalent to 7.5 mg/kg kyotorphin). Data represent mean ± SE of 6 rats for each group; PPYK(B)C, **4a**; TPPYK(B)C, **6b**; DPPYK, **7b** without cholesteryl ester and Boc ester moieties; DPPYKC, **7b** without the Boc ester moiety; CDS-PP, **7b**.

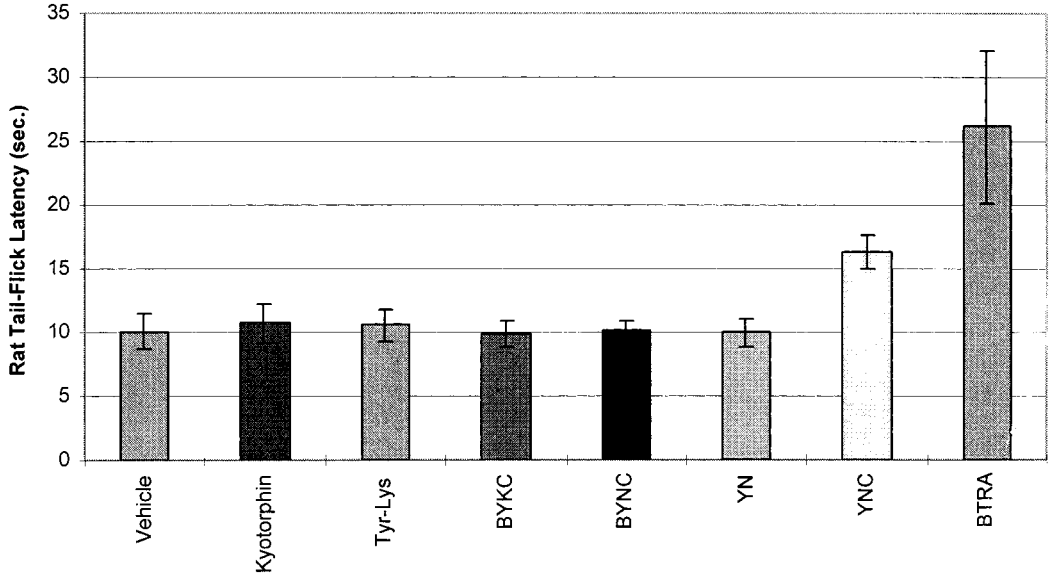


Figure 7. Rat tail-flick latency periods after iv administration of BTRA and its major intermediates at doses of 0.0223 mmol/kg (equivalent to 7.5 mg/kg kyotorphin). Data represent mean ± SE of 6 rats for each group; BYKC, **9**; BYNC, **11**; YN, Tyr-Nys; YNC, Tyr-Nys cholesteryl ester; BTRA, **12**.

lipophilic cholesteryl function again prevents activity. Finally, the fully packaged YK, but without the Boc protection (DPPYKC) of the ϵ-amine, does not show activity. Only the described constructs, represented by CDS-PP (**7b**), showed a dramatic increase in the rat tail-flick latency.

Similar studies on the BTRA intermediates (Figure 7) demonstrate, again, that the fully protected dihydro form of the BTRA shows the desired analgesic activity. In this case, the lipophilic cholesteryl ester of the Nys analogue (YNC) shows minimal activity, similarly to the fully packaged CDS without the Boc derivatization (DPPYKC, Figure 6). The results suggest that both the lipophilic cholesteryl and Boc groups are needed in addition to the dihydropyridine form of the redox targetor in both cases for facilitating delivery. The pyridinium salt form(s) then provide the "lock-in" mechanism. Finally, the central mediation of the observed analgesia was demonstrated by administering naloxone at 30 min after administration of BTRA and CDSPP, respectively. In both cases the analgesia was fully antagonized, as shown in Figure 8.

Conclusions

The 1,4-dihydropyridine ↔ pyridinium redox targetor was applied to differential and prolonged brain targeting of enkephalin-releasing, analgesic dipeptide analogues of kyotorphin. Incorporating this brain-targeting moiety into various lipophilic constructs indicated the release of active peptides via the predicted sequential metabo-

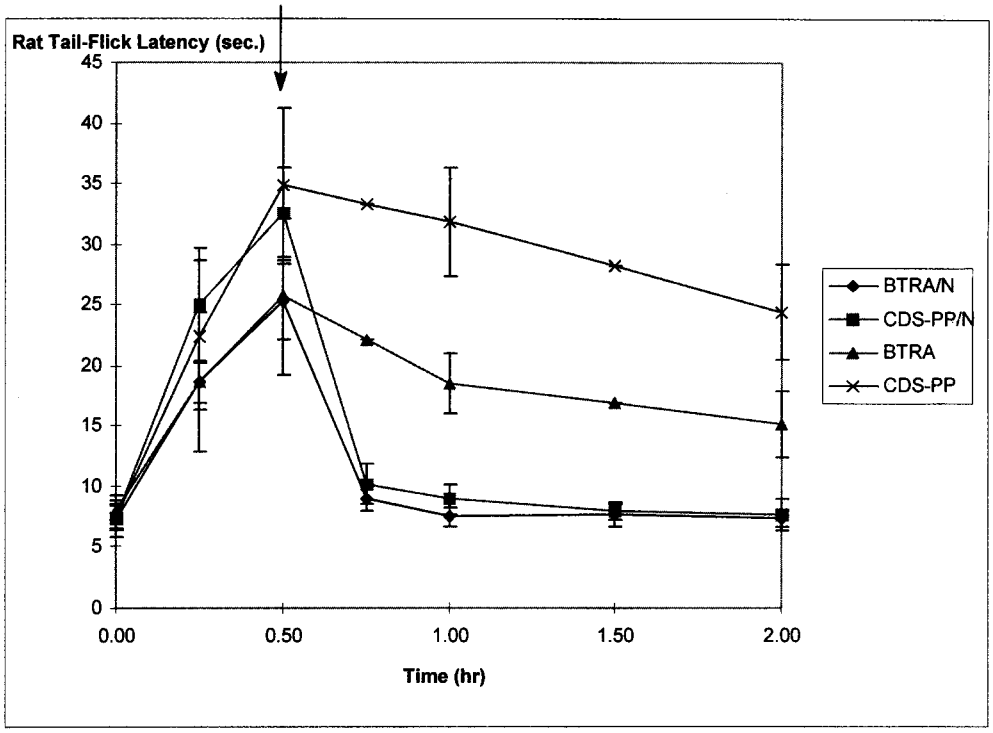


Figure 8. Effects of naloxone on CDS-PP- and BTRA-induced rat tail-flick latency period increases. Data represent mean ± SE of 6 rats for each group. The tail-flick latency periods for CDS-PP and BTRA were not recorded at 0.75 and 1.5 h; CDS-PP, **7b**; BTRA, **12**.

lism. The lysine ϵ-amino function, however, was required to be converted to a lipophilic Boc derivative. Alternatively, a novel redox amino acid pair ($Nys^+ \leftrightarrow$ Nys) provides both targeting and an effective replacement for Arg or Lys.

Experimental Section

All chemicals used were reagent grade or peptide synthesis grade. All solvents used were ACS reagent grade, from Fisher Scientific. Melting points were taken on a Fisher-Jones melting point apparatus and are uncorrected. All synthesized products were characterized by FAB (fast atom bombardment) mass spectrometry by using a Kratos MS80RFA mass spectrometer (Manchester, U.K.). ^{1}H NMR spectra were recorded on a 300 MHz instrument. UV spectrophotometry was done on a Carry 210 UV−visible spectrometer (Varian, Walnut Creek, CA). Elemental analyses of compounds synthesized were performed by Atlantic Microlab, Inc. (Necroses, GA), and the Department of Chemistry, University of Florida (Gainesville, FL). TLC (thin layer chromatography) was carried out on silica-gel-coated aluminum plates (Merck DC-Alufolien Kiesegel 60 F_{254}). Column chromatography was performed by using silica gel, neutral alumina, and Sephadex-LH 20, respectively.

Lys(Boc) Cholesteryl Ester (1). A solution of N-α-Fmoc-Lys(Boc)-OH (4.68 g, 10.0 mmol) in CH_2Cl_2 was stirred at 0 °C in an ice bath. Cholesterol (3.88 g, 10.0 mmol) in 75 mL CH_2Cl_2 was added, which was followed immediately by DCC (2.44 g, 11.0 mmol) in 25 mL CH_2Cl_2 and DMAP (1.68 g, 14.0 mmol) in 25 mL CH_2Cl_2. The mixture was stirred for 30 min at 0 °C and 48 h at room temperature. The DCU yielded was filtered, and the solvent was removed in vacuo to give the solid N-α-Fmoc-Lys(Boc) cholesteryl ester. The product was dissolved in 60 mL piperidine/CH_2Cl_2 (1:3) and was stirred at room temperature for 0.5 h. The solvent was then removed in vacuo. The crude material was purified by silica-gel column chromatography (7% CH_3OH and 10% Et_2O in CH_2Cl_2) to afford a white solid (4.71 g, 76.4%): TLC $R_f = 0.65$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 637$, $(M + Na)^+$; mp 79−80 °C; ^{1}H NMR (300 MHz) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−2.00 (36H, H on cholesterol ring and β,γ,δ-CH_2- of Lys), 3.10 (2H, ϵ-CH_2 of Lys), 3.35 (1H, α-CH of Lys), 4.60 (2H, α-NH_2 of Lys), 5.38 (1H, ϵ-NH- of Lys). Anal. ($C_{38}H_{66}O_4N_2$) C, H, N.

General Procedure for Peptide Chain Elongation (Compounds 2−4, 9). Peptide cholesteryl ester in CH_2Cl_2 or Pro-OtBu ester (for **3**) was stirred at 0 °C, and Fmoc-protected (**2**, **4**) or Boc-protected (**9**) amino acid in CH_2Cl_2 or nicotinic acid (**3**) (equimolar to the peptide ester) in DMF was added, which was followed immediately by DCC (1.2:1 molar ratio to the peptide ester) in CH_2Cl_2 and HOBt (1.4:1 molar to the peptide ester) dissolved in DMF. The mixture was stirred for 30 min at 0 °C and then 24 h at room temperature. The DCU yielded was filtered, and the solution was washed with 1 N HCl (3 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), and saturated NaCl solution (100 mL), respectively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The protected peptide ester was deprotected with piperidine/CH_2Cl_2 (1:3) or TFA/H_2O (**3**; 19:1), and the solvent was removed in vacuo. The products were purified by silica-gel column chromatography. Purification procedure and analytical data of the compounds prepared are given below.

Tyr-Lys(Boc) Cholesteryl Ester (2). Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid (yield 72.14%): TLC $R_f = 0.58$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 801$, $(M + Na)^+$; mp 103−104 °C; ^{1}H NMR (300 MHz) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−2.80 (38H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr), 3.10 (2H, ϵ-CH_2- of Lys), 3.35 (1H, α-CH of Lys), 4.60 (2H, α-NH_2 of Tyr), 4.75 (1H, α-NH- of Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, -OH of Tyr, $J = 6$ Hz). Anal. ($C_{47}H_{75}O_6N_3$) C, H, N.

Nicotinyl-Pro-OH (3). Precipitation from ethyl ether gave a white solid (yield 62.29%): TLC $R_f = 0.12$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 221$, $(M + H)^+$; mp 176−177 °C; ^{1}H NMR (300 MHz) δ 1.8 (2H, δ-CH_2 of Pro), 2.2 (2H, β-CH_2 of Pro), 3.5 (2H, δ-CH_2 of Pro), 4.4 (1H, α-CH of Pro), 7.2 (1H, 5 CH of pyridine ring), 7.8 (1H, 4 CH of pyridine ring), 8.6 (2H, 2, 5 CH of pyridine ring), 8.8 (1H, -COOH of Pro). Anal. ($C_{11}H_{12}N_2O_3$) C, H, N.

Pro-Tyr-Lys(Boc) Cholesteryl Ester (4a). Silica-gel column chromatography (5% CH_3OH and 10% Et2O in CH_2Cl_2) afforded a white solid (yield 58.22%): TLC $R_f = 0.51$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 898$, $(M + Na)^+$; mp 97−99 °C; ^{1}H NMR (300 MHz) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (43H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, β,δ-CH_2-, α-NH of Pro), 3.10 (2H, ϵ-CH_2- of Lys), 3.35 (1H, α-CH of Lys), 3.5 (2H, δ-CH_2 of Pro), 4.4 (1H, α-CH of Pro), 4.60 (2H, α-NH- of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, -OH of Tyr). Anal. ($C_{52}H_{82}O_7N_4 \cdot H_2O$) C, H, N.

Ala-Tyr-Lys(Boc) Cholesteryl Ester (4b). Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid (yield 73.0%): TLC $R_f = 0.50$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 872$, $(M + Na)^+$; mp 103−104 °C; ^{1}H NMR (300 MHz) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (39H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, α-CH of Ala), 2.90 (3H, β-CH_3 of Ala), 3.10 (2H, ϵ-CH_2- of Lys), 3.35 (1H, α-CH of Lys), 4.20 (2H, α-NH_2 of Ala), 4.60 (2H, α-NH- of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, -OH of Tyr). Anal. ($C_{50}H_{82}O_7N_4$) C, H, N.

General Procedure for the Synthesis of Nicotinoyl Peptide Cholesteryl Esters 5a−c. Peptide-cholesteryl ester in CH_2Cl_2 was stirred at 0 °C. Nicotinoyl-Pro-OH (equimolar to the peptide ester) in DMF was added, which was followed immediately by DCC (1.2:1 molar to the peptide ester) in CH_2Cl_2 and HOBt (1.4:1 molar to the peptide ester) dissolved in DMF. The mixture was stirred for 30 min at 0 °C and then 96 h at room temperature. The DCU yielded was filtered, and the solution was washed with 1 N HCl (3 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), and saturated NaCl solution (100 mL), respectively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The products were purified by silica-gel column chromatography. Purification procedure and analytical data of the compounds prepared are given below.

Nicotinoyl-Pro-Tyr-Lys(Boc) Cholesteryl Ester (5a). Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid (yield 67.28%): TLC $R_f =$ 0.47, CH_3OH/CH_2Cl_2 (1:9); ^{1}H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (42H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, β,γ-CH_2- of Pro), 3.1−3.6 (5H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of Pro), 4.40−4.60 (3H, α-CH of Pro, α-NH of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.20 (1H, H^5 of pyridine ring), 7.40−7.60 (2H, -OH of Tyr, H^4 of pyridine ring), 8.6 (2H, $H^{2,6}$ of pyridine ring); MS-FAB $m/z = 981$, $(M + H)^+$; mp 121−123 °C. Anal. ($C_{58}H_{85}O_8N_5 \cdot H_2O$) C, H, N.

Nicotinoyl-Pro-Pro-Tyr-Lys(Boc) Cholesteryl Ester (5b). Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid (yield 65.34%): TLC $R_f = 0.47$, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 1079$, $(M + H)^+$; mp 131−132 °C; ^{1}H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (50H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, β-CH_3, β,γ-CH_2- of 2 Pro), 3.1−3.6 (7H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of 2 Pro), 4.40−4.60 (4H, α-CH of 2 Pro, α-NH of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.20 (1H, H^5 of pyridine ring), 7.40−7.60 (2H, -OH of Tyr, H^4 of pyridine ring), 8.6 (2H, $H^{2,6}$ of pyridine ring). Anal. ($C_{61}H_{90}O_9N_6$) C, H, N. Anal. ($C_{63}H_{92}O_9N_6$) C, H,N.

Nicotinoyl-Pro-Ala-Tyr-Lys(Boc) Cholesteryl Ester (5c). Silica-gel column chromatography (5% CH_3OH and 10% Et_2O in CH_2Cl_2) afforded a white solid (yield 72.65%): TLC $R_f =$ 0.46, CH_3OH/CH_2Cl_2 (1:9); MS-FAB $m/z = 1053$, $(M + H)^+$; mp 155−156 °C; ^{1}H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (47H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, β-CH_3, α-CH of Ala, β,γ-CH_2-, α-NH of Pro), 2.90 (3H, β-CH_3 of Ala), 3.1−3.6 (5H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of Pro), 4.40−4.60 (4H, α-CH of Pro, α-NH of Ala, Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.20 (1H, H^5 of pyridine ring), 7.40−7.60 (2H, -OH of Tyr, H^4 of pyridine ring), 8.6 (2H, $H^{2,6}$ of pyridine ring). Anal. ($C_{61}H_{90}O_9N_6$) C, H, N.

General Procedure for the Synthesis of Trigonellyl Peptide Cholesteryl Esters 6a−c. Nicotinoyl-spacer-Tyr-Lys(Boc) cholesteryl ester in 50 mL ethyl ether was stirred at 0 °C, and dimethyl sulfate (10:1 molar ratio to the nicotinyl peptide ester) was added. The mixture was stirred overnight at room temperature. The product yielded was filtered and washed with ethyl ether several times to afford white solids.

Trigonellyl-Pro-Tyr-Lys(Boc) cholesteryl ester (6a): yield 91.84%; MS-FAB $m/z = 996$, M^+; mp 153−155 °C; ^{1}H NMR (CDCI3) 8 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,-27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (50H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, β-CH_3, β,γ-CH_2- of 2 Pro), 3.40 (3H, CH_3-N^+ on pyridine ring), 3.1−3.6 (7H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of 2 Pro), 4.40−4.60 (4H, α-CH of 2 Pro, α-NH of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.90 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.60 (1H, H^5 of pyridine ring), 7.90−8.10 (2H, -OH of Tyr, H^4 of pyridine ring), 8.6 (1H, H^6 of pyridine ring), 9.0 (1H, H^2 of pyridine ring). Anal. ($C_{59}H_{88}O_8N_5 \cdot HSO_4^- \cdot H_2O$) C, H, N.

Trigonellyl-Pro-Pro-Tyr-Lys(Boc) cholesteryl ester (6b): yield 87.75%; MS-FAB $m/z = 1094$, M^+; mp 169−170 °C; ^{1}H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,-27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−3.00 (50H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, β-CH_3, β,γ-CH_2- of 2 Pro), 3.40 (3H, CH_3-N^+ on pyridine ring), 3.1−3.6 (7H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of 2 Pro), 4.40−4.60 (4H, α-CH of 2 Pro, α-NH of Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, H^5 of pyridine ring), 8.19−8.30 (2H, -OH of Tyr, H^4 of pyridine ring), 8.60 (1H, H^6 of pyridine ring), 9.20 (1H, H^2 of pyridine ring). Anal. ($C_{63}H_{92}O_9N_6 \cdot HSO_4^- \cdot H_2O$) C, H, N.

Trigonellyl-Pro-Ala-Tyr-Lys(Boc) cholesteryl ester (6c): yield 91.76%; MS-FAB $m/z = 1068$, M^+; mp 185−187 °C; ^{1}H NMR (CDC13) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,-27), 1.00 (3H, -CH_3 19), 1.40 (9H,Boc), 0.80−3.00 (47H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr, 2H, β-CH_3, α-CH of Ala, β,γ-CH_2-, α-NH of Pro), 2.90 (3H, β-CH_3 of Ala), 3.40 (3H, CH_3-N^+ on pyridine ring), 3.1−3.6 (5H, ϵ-CH_2- of Lys, α-CH of Lys, δ-CH_2- of Pro), 4.40−4.60 (4H, α-CH of Pro, α-NH of Ala, Tyr and Lys), 5.38 (1H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.70 (1H, H^5 of pyridine ring), 7.00−8.20 (2H, -OH of Tyr, H^4 of pyridine ring), 8.60 (1H, H^6 of pyridine ring), 9.20 (1H, H^2 of pyridine ring). Anal. ($C_{62}H_{93}O_9N_6 \cdot HSO_4^- \cdot 2H_2O$) C, H, N.

General Procedure for the Dithionite Reduction (7a−c, 12). A trigonellyl peptide ester in deaerated CH_3OH/H_2O (1:1) was stirred at 0 °C under argon. $NaHCO_3$ and $Na_2S_2O_4$ (10:1 molar ratio to the trigonellyl peptide ester) was then added in small portions. The mixture was stirred at 0 °C under argon for 1 h. Then 35 mL saturated NaCl solution was added to dilute the solution which was extracted with 20 mL CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. Natural aluminum oxide column chromatography (5% CH_3OH in CH_2Cl_2) afforded a yellow solid.

1,4-Dihydrotrigonellyl-Pro-Tyr-Lys(Boc) cholesteryl ester (7a): yield 25.0%; UV_{max} (MeOH) = 348 nm; TLC $R_f =$ 0.33, CH_3OH/CH_2Cl_2 (3:97); mp dec. Anal. ($C_{59}H_{89}O_8N_5 \cdot 2H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Pro-Pro-Tyr-Lys(Boc) cholesteryl ester (7b): yield 27.3%; UV_{max} (MeOH) = 348 nm; TLC R_f =0.35, CH_3OH/CH_2Cl_2 (3:97); mp dec. Anal. ($C_{64}H_{96}O_9N_6 \cdot 3H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Pro-Ala-Tyr-Lys(Boc) cholesteryl ester (7c): yield 28.6%; UV_{max} (MeOH) = 348 nm; TLC $R_f = 0.38$, CH_3OH/CH_2Cl_2 (3:97); mp dec. Anal. ($C_{62}H_{94}O_9N_6 \cdot 3H_2O$) C, H, N.

Synthesis of Lys(Fmoc) Cholesteryl Ester (8). The procedure was the same as for **1** except that N-α-Boc-Ly-(Fmoc)-OH (4.68 g, 10.0 mmol) was used and the deprotection

was carried out in TFA/CH_2Cl_2 (1:3): white solid, 4.31 g (58.48%); TLC $R_f = 0.35$, CH_3OH/CH_2Cl_2 (1:19); MS-FAB $m/z = 60$, $(M + Na)^+$; mp 74−75 °C; ^1H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 0.80−2.00 (36H, H on cholesterol ring and β,γ,δ-CH_2- of Lys), 3.10 (2H, ϵ-CH_2 of Lys), 3.35 (1H, α-CH of Lys), 4.60 (2H, α-NH_2 of Lys), 5.38 (1H, ϵ-NH- of Lys), 6.05 (1H, CH- of Fomc), 7.20−7.80 (8H, H of Fomc ring). Anal. ($C_{48}H_{68}O_4N_2$) C, H, N.

Boc-Tyr-Lys Cholesteryl Ester (9). Silica-gel column chromatography (3% AcOH and 10% CH_3OH in CH_2Cl_2) afforded a white solid (yield 81.90%): TLC $R_f = 0.05$, CH_3OH/CH_2Cl_2 (1:10); MS-FAB $m/z = 801$, $(M + Na)^+$; mp 121−123 °C; ^1H NMR (300 MHz) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−2.80 (38H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr), 3.10 (2H, ϵ-CH_2- of Lys), 3.45 (1H, α-CH of Lys), 4.60 (1H, α-NH of Tyr), 4.75 (1H, α-NH- of Lys), 5.38 (2H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, -OH of Tyr). Anal. ($C_{47}H_{75}O_6N_3$) C, H, N.

***N*-(2,4-Dinitrophenyl)nicotinamide Chloride (10).** Nicotinamide (2.5 g, 20 mmol) was mixed with 1-chloro-2,4-dinitrophenol (6.25 g, 30 mmol) in 20 mL DMF and gradually heated to 100 °C. The mixture was stirred for 1 h at that temperature and then cooled down to the room temperature. The material obtained was dissolved in MeOH (30 mL) and poured into 125 mL vigorously stirred Et_2O. The precipitant was again dissolved in 30 mL MeOH and poured into 125 mL Et_2O. The precipitant was then dissolved in 50 mL MeOH and stirred with active carbon for 30 min. The active carbon was filtered off, and the filtrate was evaporated in vacuo. Sephadex LH-20 column chromatography (MeOH) afforded the title compound (2.69 g, 46.54%): MS-FAB $m/z = 289$, M^+; mp 177−179 °C; ^1H NMR (300 MHz) δ 3.70 (2H, -NH_2), 8.05 (1H, H^6 on bezene ring), 8.40 (1H, H^3 on bezene ring), 8.60 (1H, H^5 on bezene ring), 8.80 (1H, H^5 on pyridine ring), 9.00 (1H, H^4 on pyridine ring), 9.35 (1H, H^6 on pyridine ring), 9.70 (1H, H^2 on pyridine ring). Anal. C, H, N.

Boc-Tyr-Nys$^+$ Cholesteryl Ester (11). Boc-Tyr-Lys cholesteryl ester (1.56g, 2.0 mmol) in CH_3OH was stirred at 0 °C, and *N*-(2,4-dinitrophenol)nicotinamide chloride (0.65 g, 2.25 mmol) was added. The mixture was stirred overnight at room temperature. The product was precipitated from ethyl ether, filtered off, and washed with ethyl ether several times. Sephadex LH-20 column chromatography (CH_3OH) afforded a yellow solid (1.45 g, 81.92%): MS-FAB $m/z = 885$, M^+; mp 168−169 °C; ^1H NMR ($CDCl_3$) δ 0.65 (3H, -CH_3 18), 0.85 (6H, CH-(C**H**$_3$)$_2$ 26,27), 1.00 (3H, -CH_3 19), 1.40 (9H, Boc), 0.80−2.80 (38H, H on cholesterol ring, β,γ,δ-CH_2- of Lys, β-CH_2- of Tyr), 3.10 (2H, ϵ-CH_2- of Lys), 3.45 (1H, α-CH of Lys), 4.60 (1H, α-NH of Tyr), 4.75 (1H, α-NH- of Lys), 5.38 (2H, ϵ-NH- of Lys), 6.95 (4H, -CH= of aromatic ring of Tyr, $J = 6$ Hz), 7.80 (1H, -OH of Tyr), 8.80 (1H, H^5 on pyridine ring), 9.00 (1H, H^4 on pyridine ring), 9.35 (1H, H^6 on pyridine ring), 9.70 (1H, H^2 on pyridine ring). Anal. ($C_{53}H_{79}O_7N_4{\cdot}Cl^{-}{\cdot}3.5H_2O$) C, H, N.

Boc-Tyr-Nys cholesteryl ester (12): yield 27.8%; UV_{max} (MeOH) = 348 nm; TLC $R_f = 0.33$ CH_3OH/CH_2Cl_2 (3:97); mp dec. Anal. ($C_{53}H_{80}O_7N_4{\cdot}H_2O$) C, H, N.

Pharmacology. Male Sprague−Dawley rats weighing 250−300 g were purchased from Harlan Sprague−Dawley Inc. (Indianapolis, IN) and used in all the experiments. Animals were housed one per cage at room temperature (~25 °C) on a 14-h light cycle. Purina lab chow and water were provided ad libitum.

All animal studies were conducted in accordance with the guidelines set forth in the *Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals* (DHEW Publication, NIH-80-23).

Drugs were dissolved in 0.2 mL vehicle consisting of propylene glycol (PG) and dimethyl sulfoxide (DMSO) at a ratio of 2:1 and were injected into the animals through the tail vein. When administered icv, the minimum dose of kyotorphin which can produce the analgesic effect in rat is about 1 mg/kg (Rolka et al., 1983). Accordingly, CDS-P at doses of 0.0030, 0.0075, 0.0148, and 0.0223 mmol/kg (equimolar to 1.0, 2.5, 5.0, and 7.5 mg/kg kyotorphin) was administered to monitor the dose−response. Vehicle and 22.3 μmol/kg drugs (equimolar to 7.5 mg/kg kyotorphin) were also administered to study the pharmacological activities of the other YK brain-targeted delivery systems and their important intermediates.

Tail-flick latency, an index of spinal cord-mediated analgesia,[17] was measured to evaluate the analgesic effects of the brain-targeted delivery systems for YK. Time between presentation of a focused beam of light and the reflex removal of the tail from the stimulus was defined as the tail-flick latency period (T), and the tail-flick latency difference between each time point and control was defined as the change in tail-flick latency. In the absence of response, a cut-off period of 1 min was used. Each drug at each dose was tested on six animals.

Control latency (T_0) was obtained 10 min prior to the drug administration; the test latencies were measured at 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after the administration of drugs. The instrument used was a model 33 tail-flick analgesia meter (Litc, Inc., Landing, NJ). The beam intensity was set to achieve 7−8 s as a baseline tail-flick latency.[7]

Statistics. Rat tail-flick latency measurements were evaluated by using spreadsheets (Microsoft, Excel 5.0). The mean, standard deviation, and standard error were calculated for each group. The significance of differences between groups was determined by using ANOVA (analysis of variance).

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