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A Prodrug Nanoparticle Approach for the Oral Delivery of a Hydrophilic Peptide, Leucine[5]-enkephalin, to the Brain

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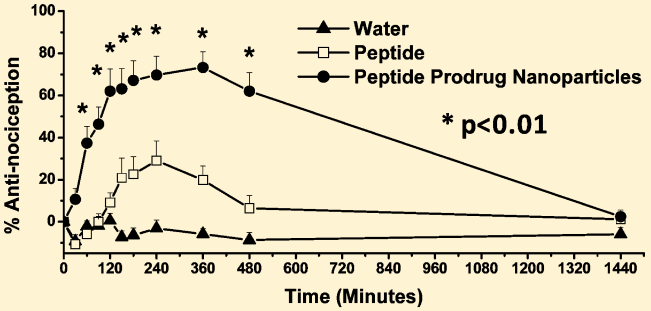
Supporting Information

ABSTRACT: The oral use of neuropeptides to treat brain disease is currently not possible because of a combination of poor oral absorption, short plasma half-lives and the blood–brain barrier. Here we demonstrate a strategy for neuropeptide brain delivery via the (a) oral and (b) intravenous routes. The strategy is exemplified by a palmitic ester prodrug of the model drug leucine[5]-enkephalin, encapsulated within chitosan amphiphile nanoparticles. Via the oral route the nanoparticle–prodrug formulation increased the brain drug levels by 67% and significantly increased leucine[5]-enkephalin's antinociceptive activity. The nanoparticles facilitate oral absorption and the prodrug prevents plasma degradation, enabling brain delivery. Via the intravenous route, the nanoparticle–prodrug increases the peptide brain levels by 50% and confers antinociceptive activity on leucine[5]-enkephalin. The nanoparticle–prodrug enables brain delivery by stabilizing the peptide in the plasma although the chitosan amphiphile particles are not transported across the blood–brain barrier *per se*, and are excreted in the urine.



KEYWORDS: *chitosan amphiphiles, quaternary ammonium palmitoyl glycol chitosan, GCPQ, blood–brain barrier, peptides, oral, leucine[5]-enkephalin*

INTRODUCTION

The oral use of neuropeptides to treat brain disorders is not possible due to a combination of (a) limited peptide oral absorption, (b) short peptide plasma half-life and (c) the blood–brain barrier (BBB). A broadly applicable method of orally delivering neuropeptides to the brain would have a dramatic impact on the global brain disease morbidity figures. Brain diseases span a wide range of conditions including psychiatric disorders,[2] neurodegenerative diseases[3] and brain tumors.[4] At any time half a billion people are affected by brain disorders, and the incidence of these conditions is steadily rising.[5]

To deliver hydrophilic neuropeptides to the brain, via the oral route, would require such drugs to be absorbed from the gastrointestinal tract and then be transported across the BBB. The oral absorption of most hydrophilic drugs, such as hydrophilic peptides, is hampered by hydrogen bonding with the aqueous gut lumen contents, which limits diffusion across the gastrointestinal epithelium.[6] Additionally peptides are rapidly degraded within the gastrointestinal tract[7] and enjoy short plasma half-lives (e.g., of 3 min[8] for leucine[5]-enkephalin), which additionally hampers their transport across the BBB. Methods to improve the absorption of peptides such as insulin and calcitonin in the gut have focused on encapsulation[9−12] strategies to prevent intestinal degradation or increasing paracellular gut permeability using the zonula occludens toxin.[13] However despite knowledge of these technologies, the oral delivery of peptides to the brain, the main focus of this work, has not been demonstrated by means of both pharmacokinetic and pharmacodynamic data, and in fact lipidized peptides have been reported *not* to cross the BBB after oral administration.[14]

Currently the BBB limits the passage of most molecules from the blood into the CNS. Specifically the BBB is a specialized structure where the blood capillaries are characterized by an absence of fenestrae, the presence of tight intercellular junctions, low pinocytotic activity and high levels of efflux transporters at their luminal endothelial surface, all of which successfully limits the penetration of 95% of drugs to the brain.[15−19] Some peptides and regulatory proteins cross the BBB by saturable or nonsaturable mechanisms in small amounts, whereas others cannot cross.[20,21] However, the brain accumulation of many peptides and proteins in therapeutically meaningful amounts after systemic administration has not been demonstrated. A number of parenteral methods of brain delivery have been attempted for hydrophilic drugs, e.g. the use of ligands for endogenous transporters,[22−25]

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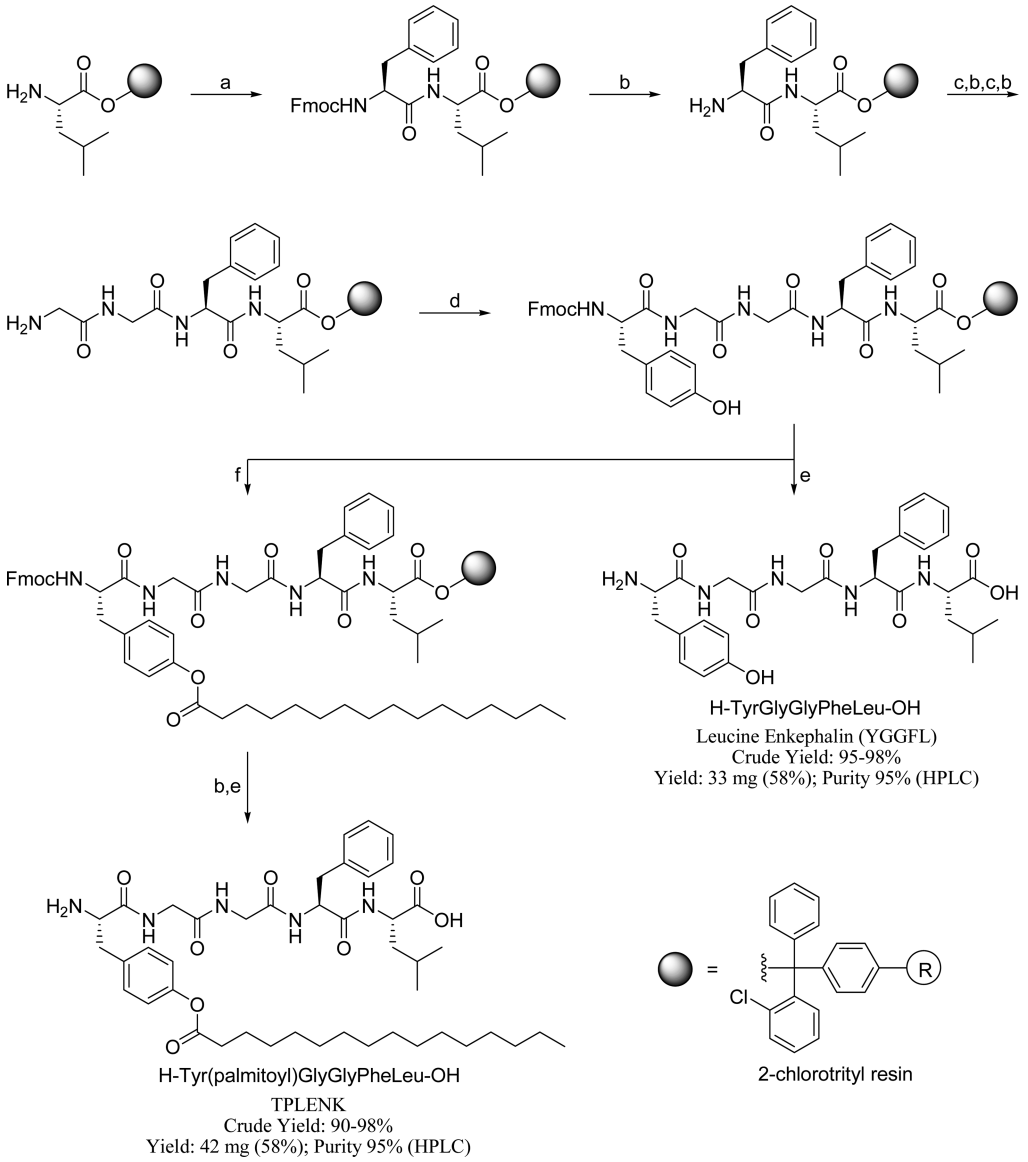
Scheme 1. The Synthesis of Leucine^5-enkephalin and TPLENK[a]



[a]Reagents and conditions: (a) Fmoc-Phe-OH, HBTU, NMM, DMF, rt, 2 × 25 min; (b) 20% v/v piperidine, DMF, rt, 2 × 10 min; (c) Fmoc-Gly-OH, HBTU, NMM, DMF, rt, 2 × 25 min; (d) Fmoc-Tyr-OH, HBTU, NMM, DMF, rt, 2 × 25 min, *ca.* 98% over 7 steps; (e) AcOH/TFE/CH_2Cl_2 2:2:6 v/v/v, rt, 2 h or 5% w/w PhOH, TFA, rt, 2 h; (f) palmitic acid *N*-hydroxysuccinimide ester, NEt_3, DMF, 25 °C, 24 h.

the inhibition of efflux transporters[15,26] or temporarily disrupting the blood–brain barrier.[27] All of these strategies have their limitations: the use of endogenous to-brain transporters tends to be limited by the carrying capacity of the transporter, efflux transporters exist at sites outside the blood–brain barrier and so are too widespread to be inhibited routinely, while the temporary disruption of the BBB is associated with the development of seizures.[28] As such the majority of these approaches have not progressed to clinical products. However the main focus of this work is delivering to the brain via the oral route, and the oral delivery of neuropeptides to the brain has seen very little activity.

Our work is concerned with the delivery of peptides across the blood–brain barrier, and here we present a simple strategy, amenable to industrial scaleup, for the oral delivery of peptides to the brain; delivery is achieved by preparing a lipidic prodrug and encapsulating the prodrug within a nanoparticle. The strategy is illustrated using the model drug leucine^5-enkephalin: an endogenous opioid which binds selectively to δ opioid receptors,[29] has a serum half-life of 3 min[8] and is not orally active.

EXPERIMENTAL METHODS

Materials. All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Dorset, U.K.) and used without further purification. Solvents (HPLC grade) and acids were purchased from Fisher Scientific (Loughborough, U.K.) and were used without further purification. Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, *O*-(1*H*-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and amino 2-chlorotrityl resin preloaded with leucine (H-Leu-2-Cl-Trt resin, 0.86 mmol g^{-1}) were obtained from Novabiochem (Nottingham, U.K.). The leucine^5-enkephalin radioimmunoassay (RIA) kit was purchased from Bachem

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(Merseyside, U.K.). Male CD-1 and Balb/C mice for the *in vivo* experiments and male Wistar rats for harvest of biological samples used in the *in vitro* experiments were purchased from Harlan (Oxfordshire, U.K.).

Peptide Synthesis. Leucine^5-enkephalin and TPLENK synthesis was carried out manually, using standard Fmoc-based solid phase methodology (0.5 mmol per batch) as depicted in Scheme 1.

Synthesis of Leucine^5-enkephalin. To preswelled H-Leu-2-Cl-Trt resin (0.581 g, 0.5 mmol) in *N*-methylmorpholine [NMM, 4.45% v/v in dimethylformamide (DMF), 10 mL] was added Fmoc protected amino acid (Fmoc-L-phenylalanine, 0.48 g, 2.5 mol) and HBTU (0.47 g, 2.5 mmol) dissolved in NMM (4.45% v/v in DMF, 5 mL). The reaction was left for 25 min. For each amino acid residue coupled, the above procedure was performed twice. After coupling each residue the Kaiser test[30] was performed to ensure coupling had taken place. Deprotection of the Fmoc moiety after washing the resin with DMF (150 mL) was achieved by adding piperidine (20% v/v in DMF, 10 mL) to the resin beads, which was then agitated for 10 min (performed twice). The process detailed above was repeated for each amino acid residue until synthesis of the peptide was complete. Glycine was double-coupled as there were two adjacent glycine units in the peptide sequence. All peptide synthesis steps were performed at room temperature. Once peptide synthesis had been completed, the resin was washed with copious amounts of DMF (250 mL), followed by copious amounts of dichloromethane (DCM, 100 mL) and then by a mixture of DCM, methanol (1: 1, 200 mL). The resin-bound peptide was dried under vacuum, transferred to a preweighed glass container, left in a desiccator under vacuum for 24 h and then stored under nitrogen at −20 °C until required. The cleavage of leucine^5-enkephalin from the 2-chlorotrityl resin, while still preserving the tertiary butyl ether (tBu) protection on the tyrosyl hydroxyl group, was achieved by adding an acetic acid cleavage mixture (acetic acid, 2,2,2-trifluoroethanol, DCM, 2:2:6, 1 mL of cleavage mixture for 0.1 g of dried resin) to the peptide bound resin obtained from above. The reaction was left for 2 h and washed three times with equal volumes of cleavage mixture. To the filtrate was added hexane (15 times the total cleavage mixture volume) to evaporate off acetic acid as an azeotrope with hexane. To the resulting reduced volume suspension containing the crude peptide was added ice cold diethyl ether (40−50 mL) to precipitate the crude peptide; the latter of which was left for 12 h at −20 °C, with the peptide being collected by centrifugation (3 × 1500 rpm at 4 °C for 45 min, Hermle Z323K, VWR, Lutterworth, U.K.) with further washing with frozen diethyl ether after the first two centrifugations. Diethyl ether was decanted, and the crude peptide was dried with nitrogen, dissolved in water and lyophilized.

An alternative method of cleaving leucine^5-enkephalin from the resin was also employed if preservation of the tBu protection of the tyrosyl hydroxyl group was not required, namely, the incubation of the peptide bound resin with Reagent P [phenol crystals, trifluoroacetic acid (TFA), 5:95] for 2 h. The crude peptide was once again obtained by evaporation of TFA, precipitation in diethyl ether and centrifugation.

Peptide purification was achieved using semipreparative reverse-phase HPLC (RP-HPLC). Crude peptide (15 mg mL^{-1}) was chromatographed over a semipreparative Waters Spherisorb ODS_2 C18 column (10 mm × 250 mm, pore size =10 μm) using an ammonium acetate buffer (25 mM), acetonitrile (82:18) mobile phase driven by a Waters HPLC system (Waters 515 HPLC pump, Waters 717 plus Autosampler, Waters Ltd., Elstree, U.K.) at a flow rate of 4 mL min^{-1}. The column was heated to 35 °C by a Jones chromatography column heater model 7971 (Jones Chromatography Ltd., Cardiff, U.K.), and peptides were detected at 280 nm by a Waters 486 variable wavelength UV detector. The retention time was 7.1 min for leucine^5-enkephalin. Fractions (4 mL each) were collected, freeze-dried and analyzed by electrospray mass spectrometry (Finnigan Mat TSQ7000) as described below. Fractions 6 and 7 were pooled to obtain leucine^5-enkephalin (95% purity), which presented as a fluffy white powder.

Yield = 33 mg (58%). Mp = 139 °C. MS (*m*/*z*): calculated for $C_{28}H_{38}N_5O_7$ = 555.3; found = 556.3 $[M + H]^+$. FTIR: ν (cm^{-1}) = 3288, 3078 (N−H stretch, O−H stretch), 2964, 2939, 2877 (C−H sat. stretch), 1650 (N^+−H bend), 1516 (N−H bend), 1442 (C−H sat. bend). ^{1}H NMR [tBu(Tyr)-LENK], δ (ppm) 0.93 (m, 6H, CH_3 - Leu), 1.39 (s, 9H, CH_3 - tBu-Tyr), 1.63 (m, 3H, γ-CH β-CH_2 - Leu), 2.10, (m, 1H, β-CH - Leu), 3.07 (m, 1H, β-CH_2 - tBu-Tyr), 3.25 (m, 3H, β-CH_2 - tBu-Tyr and Phe), 4.0 (b, 4H, α-CH_2 - Gly), 4.27 (b, 2H, α-CH - Leu and Phe), 4.73 (b, exact number of protons obscured by solvent, α-CH - Tyr), 7.16 (b, 2H, meta-CH - tBu-Tyr), 7.43 (b, 7H, ortho-CH - tBu-Tyr and ortho-CH - Phe and meta-CH - Phe and para-CH - Phe).

Synthesis of TPLENK. The synthesis of TPLENK was carried out as depicted in Scheme 1. Triethylamine (350 μL, 2.5 mmol) was added to a suspension of Fmoc-Tyr-(OH)-Gly-Gly-Phe-Leu-2-Cl-Trt-Resin (0.181 g, 0.1 mmol) preswelled in DMF (8 mL), and the resultant suspension was reacted with the *N*-hydroxysuccinimide ester of palmitic acid (177 mg, 0.5 mmol) in DMF (5 mL) at 25 °C (constant temperature room or using a water bath) for 24 h, during which time the suspension was agitated (120 rpm). The mixture was then concentrated in vacuo to remove volatile products and the residue dispersed in DMF (4 mL). The DMF suspension was filtered, and the residue washed with copious amounts of DMF (100 mL). The product bound to the resin was treated with piperidine in DMF (20% v/v, 20 mL) for 20−25 min. After washing with DMF and filtration, cleavage of the peptide chain from the resin was performed by treatment with the acetic acid cleavage mixture described above (1 mL of cleavage mixture for 0.1 g of dried resin) for 2 h at room temperature. The crude TPLENK was then precipitated with cold purified water (4 °C, pH 7.0), left to stand for 12 h at −20 °C, collected by centrifugation (3 × 2500 rpm at 4 °C for 30 min), with further washing of the pellet with cold water after the first two centrifugations, and lyophilized.

Peptide purification was achieved using semipreparative reverse-phase HPLC as detailed above. The retention time for TPLENK was 8.3 min. Fractions (4 mL each) were collected and freeze-dried and analyzed by electrospray mass spectrometry (Finnigan Mat TSQ7000) as described below. Fractions 8 to 9 were pooled to obtain TPLENK (95% purity), which presented as a white powder after lyophilization.

Yield = 42 mg (58%). Mp =173−174 °C. MS (*m*/*z*): calculated for $C_{44}H_{67}N_5O_8$ = 794.0; found = 792.8 $[M - H]^+$. FTIR: ν (cm^{-1}) = 3300, 3072 (N−H stretch, O−H stretch), 2922, 2860 (C−H sat. stretch), 1723 (CO ester stretch), 1635 (N^+−H bend), 1515 (N−H bend), 1442 (C−H sat. bend). ^{1}H NMR: δ (ppm), 0.86 (m, 9H, CH_3 - Leu and palmitoyl), 1.25 (m, 22H, $-CH_2-CH_2-$ CH_2 palmitoyl), 1.52 (m, 2H, β-CH_2 -

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Leu), 1.64 (m, 1H, γ-CH - Leu), 1.97 (−CH_2−CO− palmitoyl), 2.52 (m, exact number of protons obscured by solvent peak, β-CH_2, Phe), 2.87, (m, 4H, β-CH_2, Phe and palmitoyl Tyr), 3.08 (m, 3H, β-CH_2 - palmitoyl Tyr), 3.80 (m, exact number of protons obscured by solvent peak, CH_2 -Gly), 4.17 (t, 1H, α-CH - Leu), 4.20 (t, 1H, α-CH - palmitoyl Tyr), 4.58 (t, 1H, α-CH - Phe), 6.65 (m, 2H, ortho CH - palmitoyl Tyr), 6.93 (m, 2H, meta CH - palmitoyl Tyr), 7.24 (m, 5H, ortho CH, Phe and meta CH - Phe and para CH - Phe), 8.08 (b, 5H, NH, Leu and Phe and Gly; NH_2 - palmitoyl-Tyr).

Mass Spectrometry (MS). Leucine[5]-enkephalin (1 mg) was dissolved in methanol (1 mL) and infused into a TSQ 7000 mass spectrometer (Thermo Fisher Scientific, Loughborough, U.K.) operated in the positive electrospray ionization (EI) mode at a rate of 1 mL h^{-1}, with a needle voltage of 9.72×10^8 V and a capillary temperature of 250 °C. TPLENK (1 mg) was dissolved in methanol (1 mL) and infused into a TSQ 7000 mass spectrometer operated in the negative EI mode at a rate of 1 mL h^{-1}, with a cone voltage of 25 V and capillary temperature of 250 °C.

Nuclear Magnetic Resonance (NMR). ^{1}H NMR and ^{1}H−^{1}H COSY experiments were performed on all peptides on a Bruker AMX 400 MHz spectrometer (Bruker Instruments, Coventry, U.K.). TPLENK was analyzed in deuterated dimethyl sulfoxide (DMSO, 0.6 mL, concentration 5.8 mM), while tBu(Tyr)-leucine[5]-enkephalin was analyzed in deuterated water (D_2O, 0.6 mL, 3.6 mM). Analyses were performed at a temperature of 45−50 °C.

Horizontal Attenuated Total Reflectance Fourier-Transformed Infrared Spectroscopy (HATR-FTIR). The infrared absorption spectra for the samples were recorded using a Perkin-Elmer Spectrum 100 FTIR spectrometer equipped with a Universal Attenuated Total Reflectance accessory and a zinc selenide crystal (4000−650 cm^{-1}) and Spectrum FTIR software. A background spectrum was recorded on a clean zinc selenide window before a sample spectrum was recorded.

HPLC Peptide Analyses. Reverse phase high performance liquid chromatography (RP-HPLC) analyses were performed on peptide samples, and chromatography conditions are given in Table 1.

Polymer Synthesis. Quaternary ammonium palmitoyl glycol chitosan (GCPQ24, shown in Figure 1) was synthesized as previously described.[31]

Yield = 124 mg (41%); M_w = 21,130 ± 9,746 g mol^{-1}; M_n = 12,391 ± 3,370 g mol^{-1}; M_w/M_n = 1.79 ± 0.95; mol % palmitoylation = 15.0 ± 4.94; mol % quaternization = 8.1 ± 2.30 (n = 3).

Conformational Analysis of Peptide. Leucine[5]-enkephalin and TPLENK conformations were built using Maestro v 7.5 (Schrödinger, Camberly, U.K.), and the protonation states were adjusted using Ligprep (Schrödinger, Camberly, U.K.). All simulations were carried out using Macromodel,[32] the OPLS2005 force field parameters[33] and the water was considered implicitly by a generalized Born solvent accessibility (GB/SA) continuum solvent model,[34] with a constant dielectric function (ε = 1), and an extended nonbonded cutoff (van der Waals = 8 Å, electrostatic = 20 Å, hydrogen bonding = 4 Å) was used.[32] Two thousand steps of conformational search was performed on the peptides using the Monte Carlo multiple minima (MCMM) method.[35] The energy cutoff was 30 kJ mol^{-1} above the lowest energy conformation. The root mean square deviation (rmsd) between the α carbon atoms of two peptides superpositioned using Maestro was 0.15 Å.

Table 1. RP-HPLC Conditions for Peptide Analysis[a]

sample analyzed	HPLC system	column(s)	flow rate (mL min^{-1})	UV (nm)	T (°C)	vol (μL)	mobile phase (v/v)	RT (min)	range (μg mL^{-1})
formulations, brain bioconversion	Waters	Waters Sunfire C18 (10 + 250 mm, 4.6 mm diam, 5 μm)	1	280	35	40	acetate buffer 50 mM, acetonitrile (82:18)	11.5 (L), 14 (TP)	1 to 100
plasma/liver bioconversion, intestinal wash	Waters	Phenomenex Cromolith C18 (10 + 100 + 100 mm, 4.6 mm diam, 5 μm)	1	280	30	40	acetate buffer 50 mM, acetonitrile (82:18)	4.5 (L), 5.6 (TP)	1 to 100
SGF stability	Waters	Waters ODS2 Spherisorb C18 (250 mm, 4.6 mm diam, 5 μm)	1	214	30	20	phosphate buffer 200 mM, acetonitrile (82:18)	3.1 (L), 4.7 (TP)	1 to 100 (L) 10 to 100 (TP)
plasma stability	Agilent	Phenomenex Cromolith C18 (10 + 100 mm, 4.6 mm diam, 5 μm)	1.5	220	30	40	water, acetonitrile, trifluoroacetic acid (819.8: 180: 0.02, L) methanol, water, acetic acid (799: 200: 1, TP)	4.9 (L), 9.4 (TP)	0.1 to 100

[a]Key: Agilent = Agilent 1200 series quaternary pump, degasser, autosampler, column heater and variable wavelength UV detector. L = leucine[5]-enkephalin. RT = retention time. TP = TPLENK. T = temperature. Waters = Waters 515 HPLC pump, 717 plus autosampler, 486 variable wavelength UV detector and Jones Chromatography column heater model 7971.

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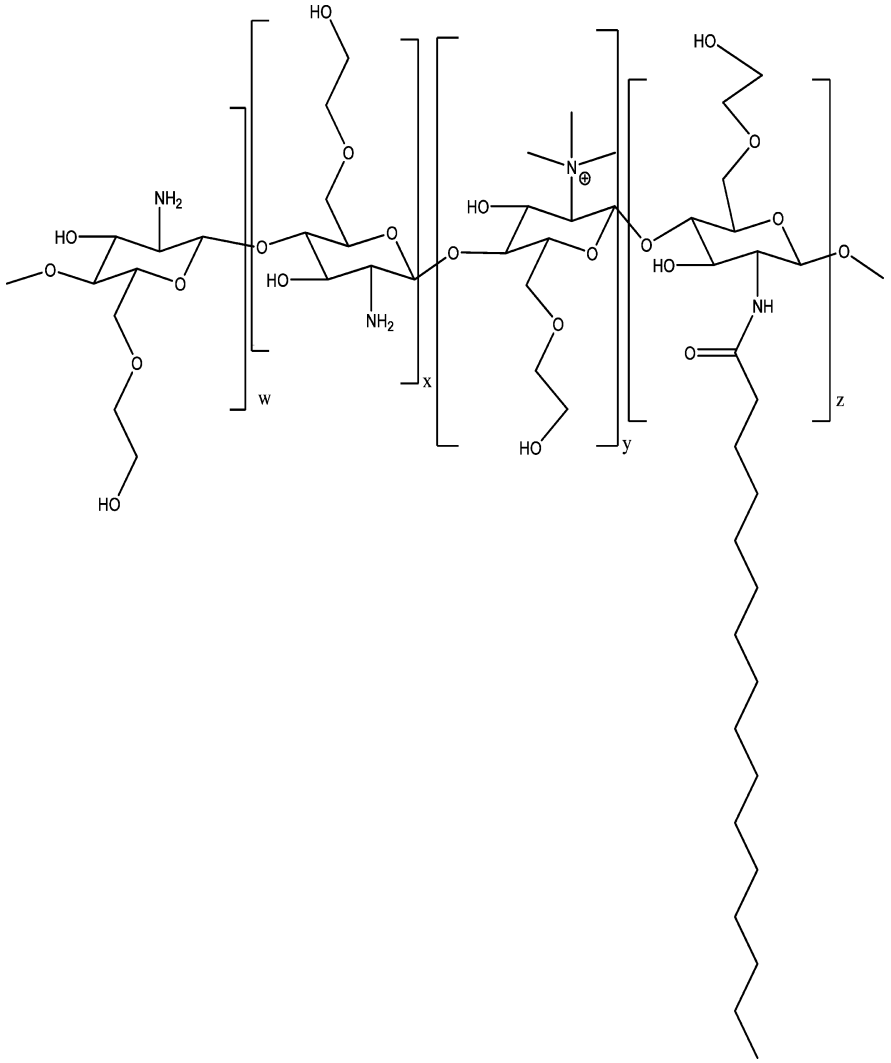

Figure 1. Quaternary ammonium palmitoyl glycol chitosan (GCPQ).

The lowest energy structures of both peptides were inserted into a cubic water box with a 10 Å buffer using Maestro System Builder, with the water molecules interacting through the Single Point Charge model. Molecular dynamics simulations of fully solvated systems were carried out by DESMOND (Desmond Molecular Dynamics System, version 2.0, D. E. Shaw Research, New York) and the OPLS2005 force field. The resulting trajectories were analyzed using Maestro. Solvated LENK and TPLENK systems, consisting of 3167 and 5140 atoms respectively, underwent several short energy minimization and relaxation runs. After equilibration, data were collected from 200 ps production runs. The end point structures were used for superposition of the α carbon atoms for the two peptides, and the resulting rmsd was 0.81 Å, indicating the preservation of the backbone conformations of these two peptides despite the introduction of the palmitoyl group.

Polymer–Peptide Formulations. *Preparation of GCPQ–Peptide Formulations.* GCPQ–peptide formulations were prepared by vortexing a mixture of GCPQ and the peptide in a suitable aqueous disperse phase followed by probe sonication of the peptide and GCPQ for 8–15 min on ice with the instrument set at 50–75% of its maximum output (MSE Soniprep 150, MSE, London, U.K.). In some cases the peptide alone was probe sonicated in aqueous media.

TEM was performed by the placement of a drop of the nanoparticle suspension on the Formvar/carbon coated grid. Excess sample was blotted off with Whatman No. 1 filter paper, and the samples were negatively stained with uranyl acetate (1% w/v). Samples were imaged using a FEI CM120 BioTwin transmission electron microscope (Philips, Eindoven, The Netherlands). Images were captured using an AMT digital camera.

Nanoparticle size was measured by PCS on a Malvern Zetasizer 3000 H_{SA} (Malvern Instruments, Malvern, U.K.) at 25 °C, a wavelength of 633 nm and a detection angle of 90°. Data was analyzed by the Contin method of size distribution analysis. Prior to measurements, polystyrene standards (diameter = 100 nm) were measured; size results were in accordance with the nominal size of the standard particles. Peptide content was analyzed by RP-HPLC as described above.

Plasma Stability of Peptides. Fresh blood, obtained from male Balb/C mice, was collected in evacuated sterile (spray coated with tripotassium ethylenediaminetetraacetic acid [3.6 mg]), medical grade PET tubes (3 × 75 mm K3E Vacutainer, BD Biosciences, U.K.) and maintained on ice (4 °C) until centrifugation. Plasma was obtained as the supernatant after centrifugation of blood samples at 1600*g* (4,800 rpm) at 4 °C for 15 min (Hermle Z323K centrifuge, VWR, Poole, U.K.). The

separated mouse plasma was stored at −80 °C until required. Before each stability experiment, the plasma was quickly thawed and diluted to 50% (v/v) with isotonic sodium chloride (0.9% w/v). Diluted plasma (50%, 495 μL) was incubated at 37 °C (60 rpm) for 30 min prior to the addition of the peptide formulations (5 μL). Plasma was diluted to slow down the peptide degradation and allow degradation to be measured. All peptide formulations were prepared at a 5 mM concentration (2.78 mg mL^{-1} for leucine^5-enkephalin or 3.97 mg mL^{-1} for TPLENK and when required 7.23 mg mL^{-1} or 10.32 mg mL^{-1} of GCPQ respectively) in isotonic sodium chloride (0.9% w/v) apart from TPLENK alone, which was prepared in glycerol (2.25% w/v). Peptide formulations were prepared by probe sonication. Formulations were filtered (0.8 μm, 13 mm, Acrodisc syringe filter, low protein binding, PALL life sciences, VWR, Poole, U.K.) after probe sonication and analyzed by HPLC for peptide content. The stability of the peptide formulations leucine^5-enkephalin, leucine^5-enkephalin−GCPQ [(M_w = 14 kDa, mol % palmitoylation = 17%, mol % quaternary ammonium groups = 7%) 1:2.6 g g^{-1}], TPLENK and TPLENK−GCPQ [(M_w = 14 kDa, mol % palmitoylation = 17%, mol % quaternary ammonium groups = 7%) 1:2.6 g g^{-1}] was determined in triplicate. At various time intervals, aliquots (50 μL) were removed and methanol (150 μL) was added to quench enzyme activity. Samples were then immediately placed in −20 °C for at least 2 h prior to centrifugation at 13,000 rpm for 15 min (MicroCentaur, MSE, London, U.K.). The supernatant (∼180 μL) was collected and 40 μL was analyzed by reverse phase HPLC as described above.

Bioconversion in Plasma, Liver and Brain Homogenates. Fresh blood, obtained from male Wistar rats, was centrifuged immediately at 3000 rpm and 4 °C for 15 min (Hermle Z323K centrifuge, VWR, Poole, U.K.). The separated rat plasma was stored at −80 °C until required. Before each stability experiment, the plasma was quickly thawed and diluted with 9 volumes of HBSS [Hanks buffered salt solution, pH = 7.4 (sodium chloride = 137 mM, potassium chloride = 5.37 mM, potassium phosphate monobasic = 0.44 mM, glucose = 5.55 mM, sodium phosphate dibasic anhydrous = 0.34 mM, sodium bicarbonate = 4.17 mM)] to give a 10% v/v plasma sample.

Rat livers and brains were obtained from male Wistar rats and the tissues blotted to dryness, weighed, sliced into small pieces, and homogenized on ice with ice-cold HBBS (1 mL per g of tissue) using a 3 mL glass homogenizer (Jenkins, Poole, U.K.). The homogenates were stored at −80 °C until required. Prior to using these samples for experiments, the homogenates were quickly thawed and rehomogenized on ice with an equal volume of ice-cold HBSS. Cell debris and nuclei were removed by centrifugation for 10 min at 13,000 rpm and 4 °C (MSE, MicroCentaur, London, U.K.). The supernatants were used for esterase activity and peptide prodrug stability studies.

The total esterase activity in biological media was assessed at pH 7.4 and 25 °C using *p*-nitrophenyl butyrate (PNPB) as a substrate as previously described.[36] Briefly the esterase activity was assessed by monitoring the hydrolysis of PNPB to *p*-nitrophenol; the final product of this enzymatic reaction was quantified spectrophotometrically at λ = 420 nm using a Shimadzu UV-1650PC UV−vis double-beam spectrophotometer (Shimadzu Ltd., Milton Keynes, U.K.). Esterase activities were expressed as units per milligram of protein. One unit represents the amount of enzyme that catalyzes the formation of 1 μmol of *p*-nitrophenol per minute in HBSS, pH = 7.4 at 25 °C. A linear enzymatic hydrolysis of PNPB was maintained for 300s between 0.02 and 2 U mL^{-1}.

To determine the amount of esterase B versus esterases A/C in various biological media, the total esterase activity was assessed in the presence and absence of paraoxon (1 mM). Since paraoxon inhibits only the enzymatic hydrolysis of PNPB by esterase B, the remaining esterase activity can be attributed to esterase A and/or C.[36] The total protein concentration in the biological media was determined using the Bradford protein assay with bovine serum albumin (BSA) as a standard [λ_{max} = 595 nm; BSA calibration graph in the linear range (0.1−1.4 μg mL^{-1}: $y = 0.63931x + 0.516$, $r^2 = 0.995$)]. The specific esterase activities were calculated as follows:

$$SE = \frac{TE}{C} \quad (1)$$

where SE = specific esterase activity in U mg^{-1} protein. TE = total esterase activity U mL^{-1} enzyme and C = concentration of protein in mg mL^{-1}.

The enzymatic stability of TPLENK was determined in plasma (diluted to 10% with HBSS), liver homogenates (diluted to 50% with HBSS) and brain homogenates (diluted to 50% with HBSS). TPLENK was incubated at a final concentration of 0.75 mM with either the diluted plasma, diluted liver homogenate or diluted brain homogenate with the respective biological matrix containing 1% DMSO. The samples were maintained at 37 °C for at least two half-lives of the peptide in a temperature-controlled shaking water bath (60 rpm). At various time intervals, aliquots (100 μL) were removed. The enzyme activity of these samples was immediately quenched by adding ice-cold, freshly prepared guanidinium hydrochloride solution (6 M, 60 μL) in acidified HBSS containing ortho-phosphoric acid (0.01% v/v) and HPLC mobile phase (40 μL), followed by the storage of samples at −80 °C until analyses could be carried out on them. Samples were analyzed by HPLC as described above. The apparent half-life ($t_{1/2}$) for the disappearance of TPLENK was calculated by linear regression of log drug concentrations versus time plots.

***In Vitro* Stability in Simulated Gastrointestinal Fluids.** Simulated gastric fluid (SGF) without pepsin (pH 1.2) was prepared as previously described.[37] Leucine^5-enkephalin (0.22 mM, 80 μL) or TPLENK (0.93 mM, 80 μL) was suspended in SGF (1.3 mL) that had been allowed to stand at 37 °C for 15 min. Peptides were incubated in the SGF in the presence and absence of GCPQ (peptide, GCPQ, 1:5 w/w) at 37 °C and with shaking at 130 cycles per minute for a maximum of 3 h. At regular time intervals aliquots (100 μL) were removed and mixed with Na_2CO_3 (0.1 M, 65 μL) to stop digestion and HPLC mobile phase [acetonitrile:phosphate buffer (200 mM), 18:82, pH = 8, 35 μL]. The same amount of peptide was added to a mixture of SGF with sodium carbonate (Na_2CO_3) (ratio 1:0.65) to serve as controls. Samples were analyzed by HPLC as described above.

Intestinal washings were obtained from male Wistar rats (∼250 g) using the method described by Cheng and others.[38] The pooled washings from all the intestinal segments were centrifuged (4,000 rpm, 30 min), and the supernatant was stored (−20 °C) until used. The protein content (∼0.775 mg mL^{-1}) was determined using the Bradford assay with bovine serum albumin (BSA) as a standard. The degradation experiments were initiated by incubating both peptides (10 mM) with intestinal fluids (46 μg mL^{-1} final protein content in

1.5 mL) in the absence or presence of GCPQ (peptide, polymer ratio = 1:5 w/w). The mixtures were shaken at 37 °C in a water bath (135 rpm). At specific time points, aliquots (150 μL) of the incubation mixtures were withdrawn and the enzyme reaction was quenched by adding glacial acetic acid (17.5 M, 10 μL). Samples were analyzed by HPLC as described above.

***In Vivo* Studies.** All experiments were performed under a UK Home Office Animal License and in accordance with local ethics committee rules. Male CD-1 outbred mice were housed in groups of 5 in plastic cages in controlled laboratory conditions with ambient temperature and humidity maintained at ∼22 °C and 60% respectively with a 12 h light and dark cycle (lights on at 07:00 and off at 19:00). Food and water were available *ad libitum* and the animals acclimatized for 5−7 days prior to experiments, within the School of Pharmacy Animal House.

Pharmacokinetic Studies: Intravenous Delivery. Groups of 5 male CD-1 mice (4 weeks old, 18−24 g) were acclimatized to the testing room for one hour prior to experiments and subsequently were intravenously administered freshly filtered (0.8 μm) leucine^5-enkephalin, leucine^5-enkephalin−GCPQ (1:2.3 g g^{-1}) formulations, TPLENK−GCPQ (1:2.3 g g^{-1}) formulations or sodium chloride (0.9% w/v). At various time intervals animals were killed and brain and blood sampled. Plasma was separated as described above, and all tissues were stored at −80 °C until analyses could be performed on them.

After thawing, brains were boiled for 10 min in 30 volumes of a mixture of glacial acetic acid (1 M), hydrochloric acid (0.02 M), 2-mercaptoethanol (0.1% v/v). The tissues were homogenized and the homogenate centrifuged (9,000 rpm, 20 min) at 4 °C to remove any debris. The pellet was discarded and the supernatant frozen (−20 °C) and lyophilized. The lyophilized samples were stored at −80 °C for later use.

The lyophilized brain homogenate samples were reconstituted in trifluoroacetic acid (1%, 1 mL) and centrifuged (13,000 rpm, 20 min) and the supernatant stored. C18 silica solid phase extraction columns (Waters SEP-PAK, 200 mg C18, Bachem, U.K.) were equilibrated with buffer B (acetonitrile, water, trifluoroacetic acid, water, 69:39:1, 1 mL) and washed with trifluoroacetic acid (1%, 9 mL). The supernatant from the brain homogenate (300 μL) was loaded onto the equilibrated column, the column was washed with trifluoroacetic acid (1%, 6 mL) and the washings were discarded. The peptides were then eluted using buffer B (3 mL), and the eluate was dried under a stream of nitrogen at 30 °C using a sample concentrator (Dri-Block DB-3, Techne Sample Concentrator, VWR, Lutterworth, U.K.). The samples were then stored at −20 °C until radioimmunoassay (RIA) based quantification could be performed. Plasma samples (100 μL) were mixed with trifluoroacetic acid (1%, 100 μL) and centrifuged (13,000 rpm for 20 min), and the supernatant was chromatographed over the solid phase extraction columns as detailed above. Eluates were dried and the dried residues stored at −20 °C until analyses could be performed on them.

Quantification of leucine^5-enkephalin was carried out using an RIA kit (S-2118, Bachem, Liverpool, U.K.) according to the manufacturer's protocol. The kit is based on the principle of competitive radioimmunoassay, and it is 100% specific for leucine^5-enkephalin. An aliquot of the reconstituted samples (reconstituted from the dried residue obtained above) or standards (100 μL) diluted in RIA buffer (Y-1050, Bachem, Liverpool, U.K.) was incubated with specific rabbit antiserum (100 μL) for 22 h at 4 °C. [^{125}I]-Tyr-leucine^5-enkephalin was added (100 μL), and the samples/standards were left for a further 22 h at 4 °C. After the incubation period, goat-anti-rabbit IgG (100 μL) was added as a secondary antibody, normal rabbit serum (100 μL) was also added, and the samples were left for 90 min at room temperature. Addition of RIA buffer (500 μL) followed, and the standards and samples were centrifuged (1700 *g*, 20 min, Eppendorf 5415R, Eppendorf UK, Cambridge, U.K.). The radioactivity of the precipitate (bound labeled antigen) and of supernatant (free labeled antigen) were counted using a Perkin-Elmer (Packard) Cobra II gamma counter, model E5002 (Perkin-Elmer, Cambridge, U.K.), for 1 min.

Plasma half-life calculations were performed using Origin 6 (Microcal Software Inc., Little Chalfont, U.K.).

Pharmacokinetic Studies: Polymer Biodistribution. The GCPQ fractions were radiolabeled using a modification of the method previously reported.[39] To GCPQ (10 mg mL^{-1}, 0.5 mL) dissolved in borate buffer (0.1 M, pH = 6.6) was added trimethylamine (97.5 μL). An aliquot of this GCPQ solution (300 μL) was then added to dry Bolton−Hunter reagent (10 μCi, 4 μL) and the reaction mixture stirred for 20 min. The reaction mixture was then purified using Sephadex G25 spin columns. The quantity of bound and free [^{125}I] iodide in the preparation was assessed by thin layer chromatography (TLC) using aluminum backed TLC plates (20 × 20 cm, silica gel 60 F254, layer thickness 200 μm, pore size 60 Å, Merck KGaA, Darmstadt, Germany) and ethyl acetate:toluene (8:2) as mobile phase. Column chromatography effectively removed free label (Figure 2 in the Supporting Information). The specific radioactivity (radioactivity per mg of polymer and labeling efficiency) was calculated by labeling the polymer (3 mg) as detailed above, followed by exhaustive dialysis to removed unbound label and a measurement of the radioactivity (bound label) of the polymer (WIZARD 2470 gamma counter, PerkinElmer, Cambridge, U.K.). Polymer specific radioactivity = 471,274 counts per minute (cpm) mg^{-1} of polymer (0.23 μCi mg^{-1} of polymer).

The labeled polymer (5,000,000 cpm) was diluted with sodium chloride (0.9% w/v) to 2 mL. Radiolabeled GCPQ (500,000 cpm, 47 mg kg^{-1}, 5.3 mg mL^{-1}, 200 μL) was injected into the tail vein of male adult CD-1 mice (20−25 g), and at various time intervals animals were killed and the blood and major organs were harvested, weighed and assayed for radioactivity.

Pharmacokinetic Studies: Oral Delivery. Groups of 5 male CD-1 mice (4 weeks old, 18−24 g) who had been fasted overnight were acclimatized to the testing room for one hour prior to experiments and subsequently were administered by oral gavage either leucine^5-enkephalin, leucine^5-enkephalin−GCPQ (1:5 g g^{-1}) formulations, TPLENK−GCPQ (1:5 g g^{-1}) formulations or distilled water. At various time intervals animals were killed and brain and blood sampled. Plasma was separated as described above, and all tissues were stored at −80 °C until analyses could be performed on them. Analyses of the tissues were carried out as detailed above.

Pharmacodynamic Experiments. Antinociception was assessed in mice using a tail flick warm water bioassay.[40] Animals for the tail-flick test were acclimatized in the testing environment for at least 20 h prior to testing (lights off at 20:00 and on at 8:00, ambient temperature at ∼22 °C and humidity at ∼60%). Groups of male CD-1 mice (4−5 weeks old, 22−28 g) were intravenously (n = 8) administered or administered by oral gavage (n = 16) either leucine^5-enkephalin, TPLENK,

GCPQ–TPLENK, GCPQ–leucine[5]-enkephalin formulations or distilled water (oral gavage only) or sodium chloride (0.9% w/v, intravenous injection only). Intravenous formulations were filtered (0.8 μm) prior to administration. At various time intervals the protruding distal part of the tail (~5 cm) of confined mice in a Plexiglas restrainer was immersed in circulating warm water, maintained at 55 °C ± 0.1 °C by a thermostatically controlled water bath (Grant W14, Cambridge, U.K.). The response latency times, in centiseconds, for each mouse to withdraw its tail by a "sharp flick" was recorded using a digital stopwatch. The first sign of a rapid tail flick was taken as the behavioral end point, which followed in most cases 1–3 slow tail movements. Three separate withdrawal latency determinations (separated by ≥20 s) were averaged. The baseline latency was measured for all mice 2 h prior to testing, and mice not responding within 5 s were excluded from further testing. The maximum possible response was set at a latency of 10 s, and this maximum latency was assigned a value of a 100% response. The response times were then converted to percentage of maximum possible effect (% MPE) by a method reported previously.[40]

Statistical Analysis. Statistical analyses was performed via a one-way ANOVA test using Minitab 16 (Minitab Ltd., Coventry, U.K.) followed by Tukey's test.

RESULTS

The objective of the current study was to prepare a nanoparticle–lipidic prodrug formulation, which would enable the delivery of neuropeptides to the brain via the oral route. We hypothesized that the nanoparticles would promote absorption by (a) protecting the peptide from degradation, (b) increasing the upper gastrointestinal drug residence time due to the adherence of GCPQ nanoparticles to the gastrointestinal mucosa[31] and (c) promoting transcellular absorption[31,41,42] of the lipidic prodrug (the lipidic prodrug would enjoy a reduced level of hydrogen bonding with the aqueous contents of the gastrointestinal lumen). We also hypothesized that the prodrug would be stabilized against degradation in the plasma once absorbed and be delivered to the brain either as the regenerated parent drug (regenerated by plasma esterases) or as the lipidic prodrug (with the parent drug being regenerated by brain esterases). It is envisaged that the lipidic prodrug would be more favorably transported across the brain endothelial cells due to its lipidic character.

A novel prodrug of leucine[5]-enkephalin was synthesized by esterification of the free phenolic hydroxyl group of the tyrosine residue (Scheme 1) with palmitic acid to give a palmitoyl derivative of leucine[5]-enkephalin (tyrosine[1]palmitate-leucine[5]-enkephalin–TPLENK). Although we set out to synthesize a prodrug of leucine[5]-enkephalin, we opted for derivatization at the phenolic hydroxyl group of tyrosine, as this was thought to be less likely to interfere with drug–receptor interactions. It was thought that this approach would ensure drug activity, should cleavage of the prodrug, to yield leucine[5]-enkephalin, fail to occur in a timely manner. The hydroxy moiety was preferred for esterification instead of the free carboxy terminal of leucine[5]-enkephalin as the latter is crucial for eliciting opioid activity.

The three-dimensional structure of the δ opioid receptor pharmacophore has been elucidated using non-peptide δ opioid receptor agonists.[1] The pharmacophore comprises a three-point structure: an amine moiety (protonated at physiological pH), a hydrophobic region (aromatic ring) and a phenolic site. The two aromatic rings are separated by a distance of 7.0 ± 1.3 Å, the hydrophobic aromatic ring is separated from the nitrogen atom by a distance of 8.2 ± 1.0 Å and the plane of the two aromatic rings is perpendicular to the phenolic hydroxyl group. It is known that leucine[5]-enkephalin binds in a (1–4) β-turn conformation to the δ-opioid receptor,[43] and the simulated conformations of both leucine[5]-enkephalin and TPLENK in aqueous media are shown schematically in Figure 2. There is

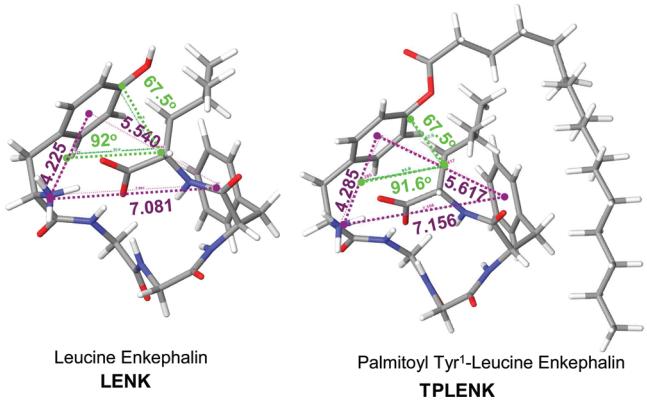

Figure 2. Simulated lower energy conformations of leucine[5]-enkephalin and TPLENK. Both compounds are able to adopt the three-dimensional δ-opioid receptor pharmacophore proposed by Shenderovich.[1]

good agreement between these simulated conformations and the pharmacophore defined by Shenderovich et al.[1] The presence of the palmitoyl group appears not to interfere with the three-dimensional arrangement of TPLENK into the δ-opioid pharmacophore. Selective δ-opioid receptor pharmacophores derive their selectivity by interaction with a hydrophobic pocket,[44] and we speculate that this interaction may even be enhanced by the presence of the palmitoyl chain. Hence any noncleavage of the prodrug *in vivo* to yield the parent drug is unlikely to have a negative impact on pharmacological activity, as the prodrug itself may be intrinsically active.

Simulation studies of leucine[5]-enkephalin in water yield a compact structure with intramolecular hydrogen bonding[45] and a hydrophobic association between the tyrosine and leucine groups.[46] In aqueous media leucine[5]-enkephalin presented as 170 nm nanoparticles, which after filtration (0.22 μm) were 44 ± 0.1 nm in size (polydispersity = 0.44 ± 0.002, Figure 3a). We know of no other report of leucine[5]-enkephalin intrinsically forming nanoparticles. TPLENK on the other hand formed dense nanofibers (Figure 3b) 300–500 nm in length and 20 nm in diameter. Amphiphilic peptides (bearing hydrophobic non-amino acid groups at one end) are known to form nanofibers by a combination of a β-sheet arrangement of the peptide on the outside of the nanofiber and the association of the hydrophobic non-amino acid moieties in the core of the nanofiber.[47] In aqueous media, on association with the particle forming chitosan amphiphile, quaternary ammonium palmitoyl glycol chitosan (GCPQ)[48] (Figure 1), leucine[5]-enkephalin formed spherical aggregates 40 nm in size (Figure 3c) and TPLENK formed a mixture of spherical (Figure 3d) and nanofiber aggregates of 200–500 nm in size.

In order to ascertain if the parent drug would be regenerated from the lipidic ester prodrug *in vivo*, TPLENK was incubated with plasma and liver esterases *in vitro*. The protein contents of the 50% v/v liver homogenate and the 10% v/v plasma

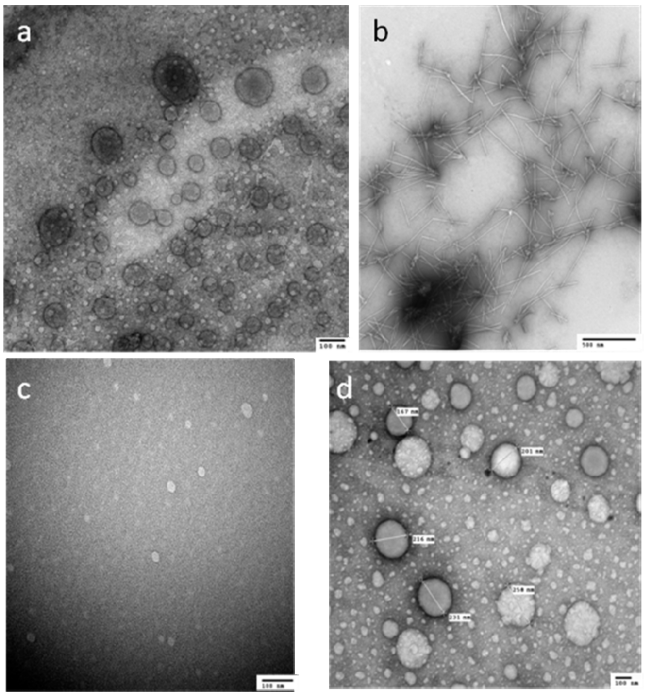

Figure 3. Transmission electron micrographs (TEMs) with negative staining of peptide nanoparticle formulations. (a) Leucine^5-enkephalin nanoparticles (4 mg mL^{-1}) in sodium chloride solution (0.9% w/v), bar = 100 nm. Particles are 40−200 nm in diameter. (b) TPLENK nanofibers (2 mg mL^{-1}) in water, bar = 500 nm. Particles are 20 nm in diameter and 200−400 nm long. (c) GCPQ (6.9 mg mL^{-1})−leucine^5-enkephalin nanoparticles (3 mg mL^{-1}) in sodium chloride (0.9% w/v), bar = 100 nm. Particles are 40 nm in diameter. (d) GCPQ (6.9 mg mL^{-1})−TPLENK nanoparticles (3 mg mL^{-1}) in sodium chloride (0.9% w/v), bar = 100 nm. Particles are 30−250 nm in diameter.

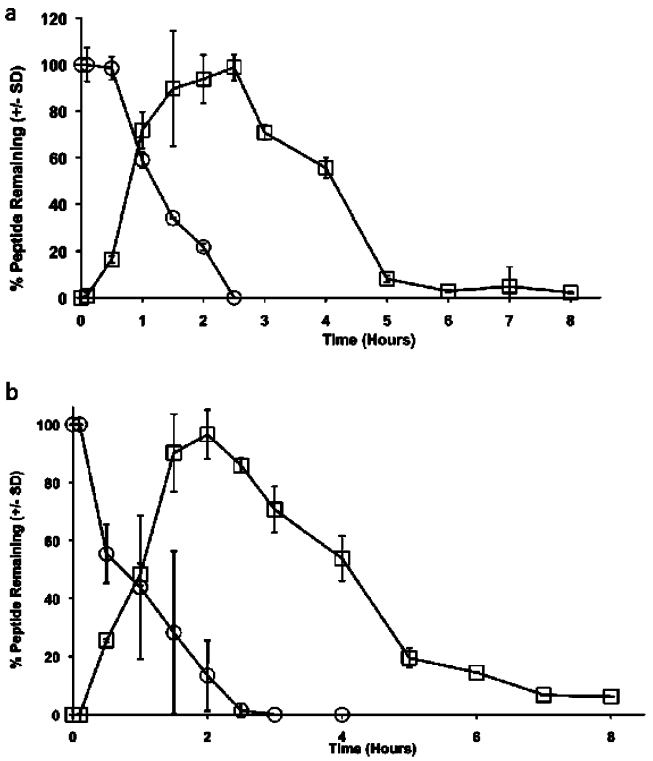

Figure 4. *In vitro* peptide prodrug activation (mean ± SD). (a) The bioconversion of TPLENK (0.61 mg mL^{-1}) into leucine^5-enkephalin in the presence of 10% v/v plasma containing DMSO (1% v/v), protein content of 10% v/v plasma = 8.8 ± 0.10 mg mL^{-1}, esterase content of 10% v/v plasma = 734 Units mg^{-1} of protein. TPLENK is converted to leucine^5-enkephalin, and leucine^5-enkephalin is rapidly degraded by plasma esterases. (b) The bioconversion of TPLENK (0.61 mg mL^{-1}) into leucine^5-enkephalin in the presence of 50% v/v liver homogenate containing DMSO (1% v/v), protein content of 50% v/v liver homogenate = 24.2 ± 6.75 mg mL^{-1}, esterase content of the 50% v/v liver homogenate = 189 Units mg^{-1} of protein. TPLENK is converted to leucine^5-enkephalin, and leucine^5-enkephalin is rapidly degraded by liver esterases. ○ = TPLENK, □ = leucine^5-enkephalin.

homogenate were 24.2 ± 6.75 mg mL^{-1} and 8.8 ± 0.10 mg mL^{-1} respectively. The total amount of esterases was 189 units mg^{-1} of protein in the 50% v/v liver homogenate and 734 units mg^{-1} of protein in the 10% v/v plasma sample, of which only 11.1 units mg^{-1} of protein (6%) was not inhibited by paraoxon in the liver homogenate and 18.1 units mg^{-1} of protein (3%), not inhibited by paraoxon, in the plasma. Esterases not inhibited by paraoxon were type A/C esterases while type B esterases are inhibited by paraoxon. Type B esterases are abundant in the plasma and liver homogenate. The degradation of the ester prodrugs in plasma is reported to involve type B esterases.[49]

TPLENK was indeed converted to leucine^5-enkephalin in the presence of both the plasma and the liver homogenate (Figures 4a and 4b). The half-life of TPLENK was 73 and 44 min in the plasma sample (10% v/v) and liver homogenate (50% v/v) respectively as calculated from pseudo-first-order rate constants obtained by linear regression of log drug concentration versus time plots ($y = 6.30 + 80.27^{-2.73252T}$, $r^2 = 0.97$ for plasma and $y = 52.00^{-0.94672T}$, $r^2 = 0.98$ for liver).

The brain homogenate does not catalyze the bioconversion of TPLENK to leucine^5-enkephalin (Figure 1 in the Supporting Information) as it is rich in esterases A and C (65%) instead of type B esterases. Esterases A and C are known to be more abundant in the water-soluble fractions of brain homogenates, when compared to liver homogenates and plasma[36] (most type B esterases are membrane bound in the brain), and it is known that there are low levels of type B esterases in brain homogenates of humans, dogs, rats, mice and guinea pigs.[36] The lack of *in vitro* bioconversion of TPLENK to leucine^5-enkephalin, observed here with the brain homogenates, could reflect the difficulty in preserving esterase B activity in brain homogenates.

On studying the stability of the drug/formulations in plasma we found that leucine^5-enkephalin is rapidly degraded by plasma peptidases *in vitro* (Figure 5a). Encapsulation within GCPQ retards this degradation (Figure 5a). TPLENK demonstrates superior stability to plasma peptidases when compared to leucine^5-enkephalin, with 80% of the prodrug remaining after 2 h, compared to 0% of the leucine^5-enkephalin remaining at the same time point, and encapsulation of TPLENK within GCPQ nanoparticles further improves the plasma stability of the prodrug. However, despite the comparative resistance of TPLENK to degradation shown in Figure 5a, esterase catalyzed conversion of TPLENK to leucine^5-enkephalin is possible (Figure 4). In comparing the data shown in Figure 5a with that shown in Figure 4a, it is important to note that the level of TPLENK used for the plasma stability experiments (Figure 5a) exceeds the level used for the prodrug activation experiments (Figure 4a) by 6-fold.

We next sought to quantify the stability of the drug/formulations in gastrointestinal fluids. There was no degrada-

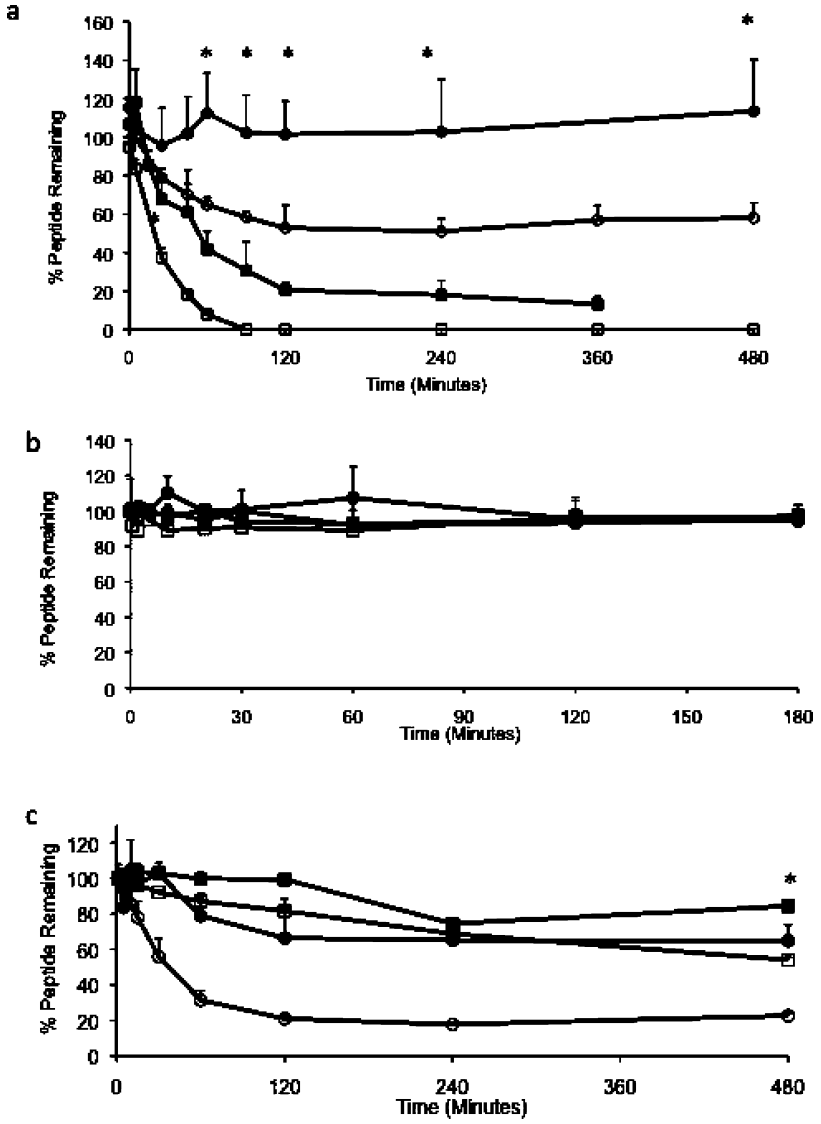

Figure 5. In vitro peptide stability (mean ± SD). The stability of various peptide formulations in plasma (50% v/v). □ = leucine^5-enkephalin (2.78 mg mL^{-1}), ■ = GCPQ (7.23 mg mL^{-1})−leucine^5-enkephalin (2.78 mg mL^{-1}), ○ = TPLENK (3.97 mg mL^{-1}), ● = GCPQ (10.32 mg mL^{-1})−TPLENK (3.97 mg mL^{-1}). Significant differences * = $p < 0.05$ versus leucine^5-enkephalin. (b) The stability of various peptide formulations simulated gastric fluid (SGF). □ = leucine^5-enkephalin (0.12 mg mL^{-1}), ○ = TPLENK (0.70 mg mL-1), ■ = GCPQ (0.6 mg mL^{-1})−leucine^5-enkephalin (0.12 mg mL^{-1}) nanoparticles and ● = GCPQ (3.5 mg mL^{-1})−LENK (0.7 mg mL^{-1}) nanoparticles. There was no degradation of the peptides after 3 h. (c) The stability of various peptide formulations in rat intestinal wash. □ = leucine^5-enkephalin (8.33 mg mL^{-1}), ○ = TPLENK (12.05 mg mL^{-1}), ■ = GCPQ (41.65 mg mL^{-1})−leucine^5-enkephalin (8.33 mg mL^{-1}) and ● = GCPQ (60.25 mg mL^{-1})−TPLENK (12.05 mg mL^{-1}) in the presence of rat intestinal washes. Significant differences: * = $p < 0.05$ versus leucine^5-enkephalin. TPLENK is less stable in the presence of rat intestinal peptidases, when compared to leucine^5-enkephalin; however, GCPQ nanoparticles protect both TPLENK and leucine^5-enkephalin from intestinal degradation.

tion of leucine^5-enkephalin or TPLENK in simulated gastric fluid (Figure 5b); however, there was marked degradation of leucine^5-enkephalin and especially TPLENK within the rat intestinal wash (Figure 5c). The stability of TPLENK and leucine^5-enkephalin in the intestinal wash was enhanced by formulating as GCPQ nanoparticles: with 66.5 ± 0.89% of TPLENK and 84.5 ± 0.76% of leucine^5-enkephalin recovered after 4 h in the presence of GCPQ compared to 17.8 ± 0.66% and 54.1 ± 1.71% of TPLENK and leucine^5-enkephalin recovered respectively in the absence of GCPQ.

Leucine^5-enkephalin was not degraded by the low pH in the stomach, and the main source of degradation appears to be the intestinal enzymes. Pepsin was omitted from the SGF due to its interference with the peptide assay; however, pepsin is known to produce leucine^5-enkephalin type peptides from plasma proteins[50] and so leucine^5-enkephalin is unlikely to be a major substrate for pepsin. TPLENK was degraded to a much greater extent than leucine^5-enkephalin in the intestinal wash, although TPLENK was not degraded to leucine^5-enkephalin. It may be concluded that GCPQ nanoparticles protect the peptides from degradation by the intestinal enzymes and such protection may enhance absorption.

Once it had been established that (a) TPLENK could be converted to leucine^5-enkephalin by plasma and liver esterases (although TPLENK could adopt the same conformation as the δ-opioid receptor pharmacophore and hence may be intrinsically active) and (b) GCPQ protects TPLENK and leucine^5-enkephalin from intestinal and plasma degradation, the next

Table 2. Leucine[5]-enkephalin Pharmacokinetic Parameters Following the Intravenous and Oral Administration of Peptide Formulations

		plasma			brain			
LENK dose (mg kg^{-1}) admin route	formulation	T_{max} (h)	C_{max} (mean ± SEM, μg mL^{-1})	AUC$_{0-t}$[a] (μg mL^{-1} h)	T_{max} (h)	C_{max} (mean ± SEM, μg g^{-1})	AUC$_{0-t}$[a] (μg g^{-1} h)	no. of MPE responders[b]
10 intravenous	GCPQ–LENK	0.05[c]	1.66 ± 0.05	0.550	1.5	0.085 ± 0.014	0.296	4/8
	GCPQ–TPLENK	0.05[c]	1.66 ± 0.69	0.641	1.5	0.141 ± 0.015	0.442	6/8
70 oral	LENK	1	0.293 ± 0.04	2.339	4	0.695 ± 0.088	10.043	1/16
	GCPQ–LENK	1	0.266 ± 0.05	2.811	4	1.090 ± 0.107	16.807	6/16
	GCPQ–TPLENK	0.5	0.171 ± 0.03	3.079	4	1.098 ± 0.085	16.508	9/16

[a] t = 4 h with the intravenous formulation, t = 24 h with the oral formulation. [b] Animals achieving the highest possible latency periods (10 s) when exposed to the thermal stimulus, LENK = leucine[5]-enkephalin. [c] Earliest time point sampled.

step was to study the impact of the nanoparticle–prodrug approach on leucine[5]-enkephalin brain bioavailability after oral administration. The radioimmunoassay (RIA) used here is 100% specific for leucine[5]-enkephalin, as stated by the manufacturer; hence when analyzing TPLENK samples only the amount of TPLENK bioconverted *in vivo* to leucine[5]-enkephalin was quantified. Using the RIA kit, there was no recovery of leucine[5]-enkephalin in biological samples after the oral administration of water or intravenous administration of sodium chloride (0.9% w/v) to control animals. Brain and plasma were analyzed as follows: (a) these two tissues were relevant to the hypothesis and (b) the drug is centrally active.

On oral administration, the GCPQ–TPLENK formulation resulted in a 32% increase in leucine[5]-enkephalin plasma AUC$_{0-24h}$ (Table 2, Figure 6a). However leucine[5]-enkephalin plasma C_{max} was lower (Table 2, Figure 6a) with the GCPQ–TPLENK formulation, when compared to the leucine[5]-enkephalin formulations, which reflects the time taken for *in situ* bioconversion of the prodrug. The GCPQ–TPLENK formulation produced higher plasma levels of leucine[5]-enkephalin at the later time points (Figure 6a), when compared to the leucine[5]-enkephalin formulations, an indication that the prodrug is capable of sustained delivery of the drug.

The most important finding, however, of this research is that the GCPQ–nanoparticle formulations significantly enhance the brain delivery of leucine[5]-enkephalin (Figure 5b) when formulated with the parent drug (leucine[5]-enkephalin) or prodrug (TPLENK). The leucine[5]-enkephalin brain AUC$_{0-24h}$ increased by over 67% with the oral GCPQ formulations (Table 2, Figure 6b), when compared to the oral administration of leucine[5]-enkephalin alone. Furthermore brain C_{max} was increased by 57% when GCPQ nanoparticles were used to encapsulate the leucine[5]-enkephalin or TPLENK (Table 2, Figure 6b). However the nanoparticle formulations do not alter the brain T_{max}. Although brain levels of leucine[5]-enkephalin from GCPQ–leucine[5]-enkephalin and GCPQ–TPLENK formulations were indistinguishable, it must be borne in mind that, in the case of the TPLENK formulation, we are measuring the result of in situ bioconversion and that the unconverted prodrug (TPLENK) may also be present in plasma and brain samples.

Via the oral route 0.02% of the leucine[5]-enkephalin dose was detected at the C_{max}; these levels are similar to those detected on subcutaneous administration of morphine (0.015%),[51] a more metabolically stable compound, demonstrating the efficacy of the strategy used.

GCPQ formulations of TPLENK and leucine[5]-enkephalin itself were compared via the intravenous route and the use of GCPQ–TPLENK results in a modest (16%) increase in leucine[5]-enkephalin plasma AUC$_{0-4h}$ (Table 2, Figure 6c) when compared to GCPQ–leucine[5]-enkephalin, although the prodrug did not alter plasma C_{max} (Table 2, Figure 6c). The brain AUC of leucine[5]-enkephalin is increased by 50% however by the use of GCPQ–TPLENK, when compared to GCPQ–leucine[5]-enkephalin (Table 2, Figure 6d), and the brain C_{max} increased by 66%, despite the fact that a lower dose of leucine[5]-enkephalin was given with the TPLENK formulations (Figures 6c and 6d). Both particulate formulations resulted in the same 90 min brain T_{max} (Table 2, Figure 6d). Once again, it must be stressed that the results of *in situ* bioconversion are being measured in the case of the TPLENK formulations and it is possible that intact TPLENK was present in the brain and plasma following the intravenous administration of TPLENK formulations.

The polymer itself is not delivered appreciably across the blood–brain barrier as no more than 0.05% of the administered dose is seen in the brain after intravenous dosing (Figure 6e). Instead the polymer is distributed to other tissues and excreted via the kidneys and bladder (Figure 6e). The polymer is also delivered to the skin, intestines and tail, and we speculate that this could be due to an adherence to the skin capillaries. Degradation of radiolabeled GCPQ remained low as indicated by the low thyroid levels (<0.09% of the administered dose) throughout the whole of the experiment (24 h). The tail levels are unlikely to be due to an extravascular injection as the tail levels show an increasing trend from 5 to 10 min before falling. Interestingly there is no major accumulation in the liver and spleen and virtually no distribution to the lungs; hence the polymer evades macrophage uptake by the reticuloendothelial system, unlike other particulate carriers such as liposomes.[52] Crucially the polymer nanoparticles deliver drug across the blood–brain barrier, without actually significantly traversing the blood–brain barrier.

Having established that both the prodrug and the nanoparticles increase the brain delivery of leucine[5]-enkephalin, we now decided to carry out pharmacodynamic evaluations. Leucine[5]-enkephalin causes central antinociceptive effects in the brain[29] by acting on opioid receptors, showing a preference for δ opioid receptors. These central antinociceptive effects, as measured by the tail flick bioassay, may be used to demonstrate the delivery of the peptide to the brain.

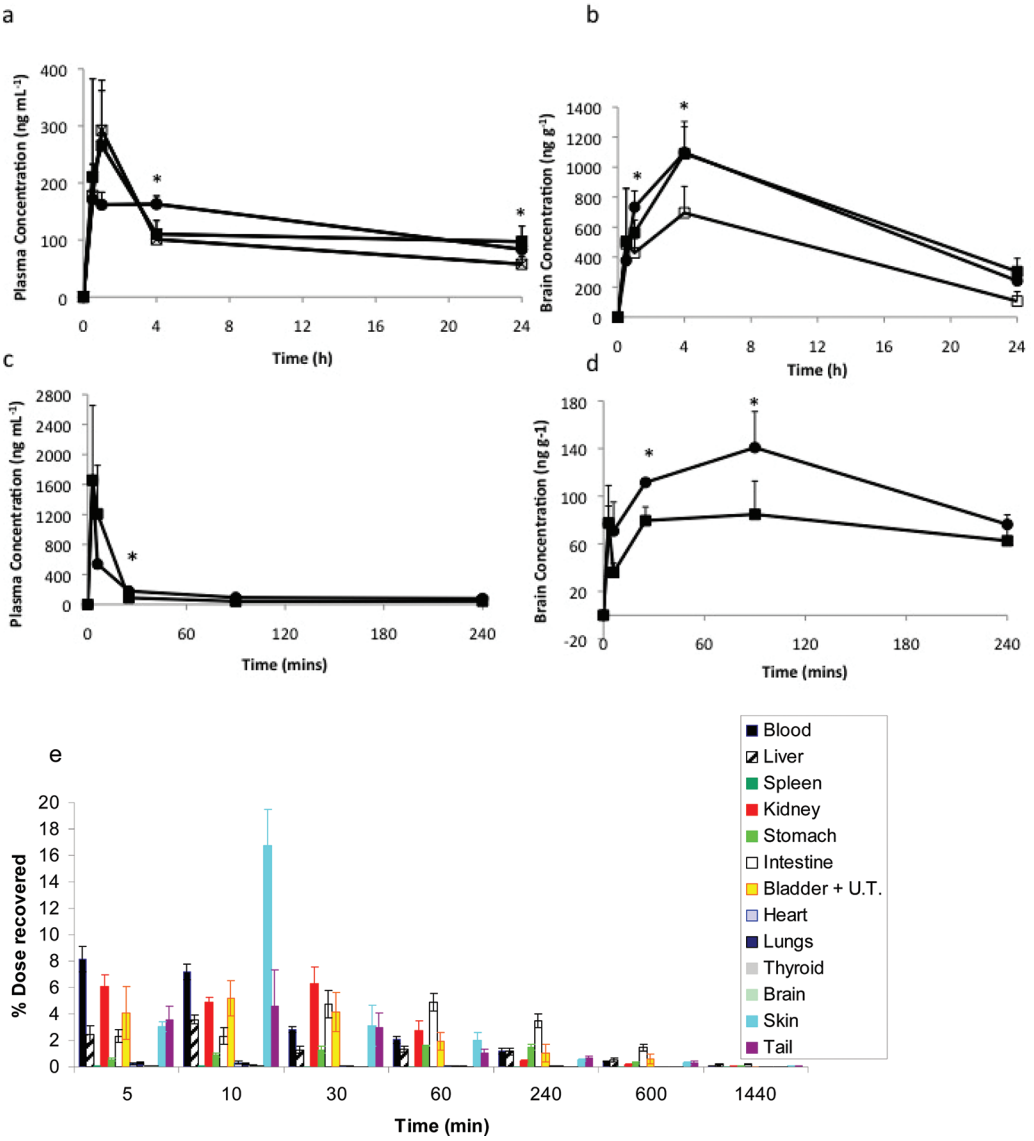

Figure 6. Peptide and polymer pharmacokinetics (mean ± SD). (a) Mouse plasma levels after the oral administration: leucine^5-enkephalin (70 mg kg^{-1}) (□), leucine^5-enkephalin (70 mg kg^{-1})−GCPQ (350 mg kg^{-1}) nanoparticles (■) and TPLENK 100 mg kg^{-1} (= 70 mg kg^{-1} leucine^5-enkephalin)−GCPQ (500 mg kg^{-1}) nanoparticles (●) Both GCPQ formulations presented as viscous liquids due to the high concentration of GCPQ. The concentration of leucine^5-enkephalin within the dose was 15 mg mL^{-1}, the concentration of GCPQ within the dose was 75 mg mL^{-1} and the dose volume varied from 85 to 115 μL for the leucine^5-enkephalin group of animals and 120 to 165 μL for the TPLENK group of animals, depending on the animal weight. Formulations were prepared as described in the Experimental Methods. Significant differences: * = $p < 0.05$ versus leucine^5-enkephalin. (b) Mouse brain levels after oral administration: leucine^5-enkephalin (70 mg kg^{-1}) (□), leucine^5-enkephalin (70 mg kg^{-1})−GCPQ (350 mg kg^{-1}) nanoparticles (■) and TPLENK 100 mg kg^{-1} (= 70 mg kg^{-1} leucine^5-enkephalin)−GCPQ (500 mg kg^{-1}) nanoparticles (●). Dose volumes and concentrations are as indicated in panel a. Significant differences: * = $p < 0.05$ versus leucine^5-enkephalin. (c) Mouse plasma levels after the intravenous injection of leucine^5-enkephalin (10 mg kg^{-1})−GCPQ (23 mg kg^{-1}) nanoparticles (particle diameter = 172 nm, polydispersity = 0.40) (■) and TPLENK [10 mg kg^{-1} (= 7 mg kg^{-1} leucine^5-enkephalin)]−GCPQ (23 mg kg^{-1}) nanoparticles (particle diameter = 123 nm, polydispersity = 0.27) (●). The concentration of leucine^5-enkephalin within the dose was 3 mg mL^{-1}; the concentration of GCPQ within the dose was 6.9 mg mL^{-1}, and the dose volume varied from 80 to 110 μL for the leucine^5-enkephalin group of animals and the TPLENK group of animals depending on the animal weight. Formulations were prepared as described in the Experimental Methods and were filtered (0.8 μm) prior to administration. Significant differences: * = $p < 0.05$ versus leucine^5-enkephalin. (d) Mouse brain levels after the intravenous injection of leucine^5-enkephalin (10 mg kg^{-1})−GCPQ (23 mg kg^{-1}) nanoparticles (particle diameter = 172 nm, polydispersity = 0.40) (■) and TPLENK [10 mg kg^{-1} (= 7 mg kg^{-1} leucine^5-enkephalin)]−GCPQ (23 mg kg^{-1}) nanoparticles (particle diameter = 123 nm, polydispersity = 0.27) (●). Dose volumes and concentrations are as detailed in panel c. Significant differences: * = $p < 0.05$ versus leucine^5-enkephalin. (e) The biodistribution of radiolabeled GCPQ (47 mg kg^{-1}) nanoparticles on intravenous administration; GCPQ nanoparticles are excreted via the kidneys into the bladder and do not cross the BBB; U.T. = urinary tract. Nanoparticles also do not distribute to a large extent to the liver and spleen and do not distribute at all to the heart and lungs.

On oral administration of 70 mg kg^{-1} leucine^5-enkephalin, the analgesia produced by GCPQ−leucine^5-enkephalin and GCPQ−TPLENK was significantly higher than that produced by the peptide alone (Figure 7a) and the peptide prodrug−nanoparticle formulation (GCPQ−TPLENK) produced the highest and most prolonged level of antinociception (Table 2,

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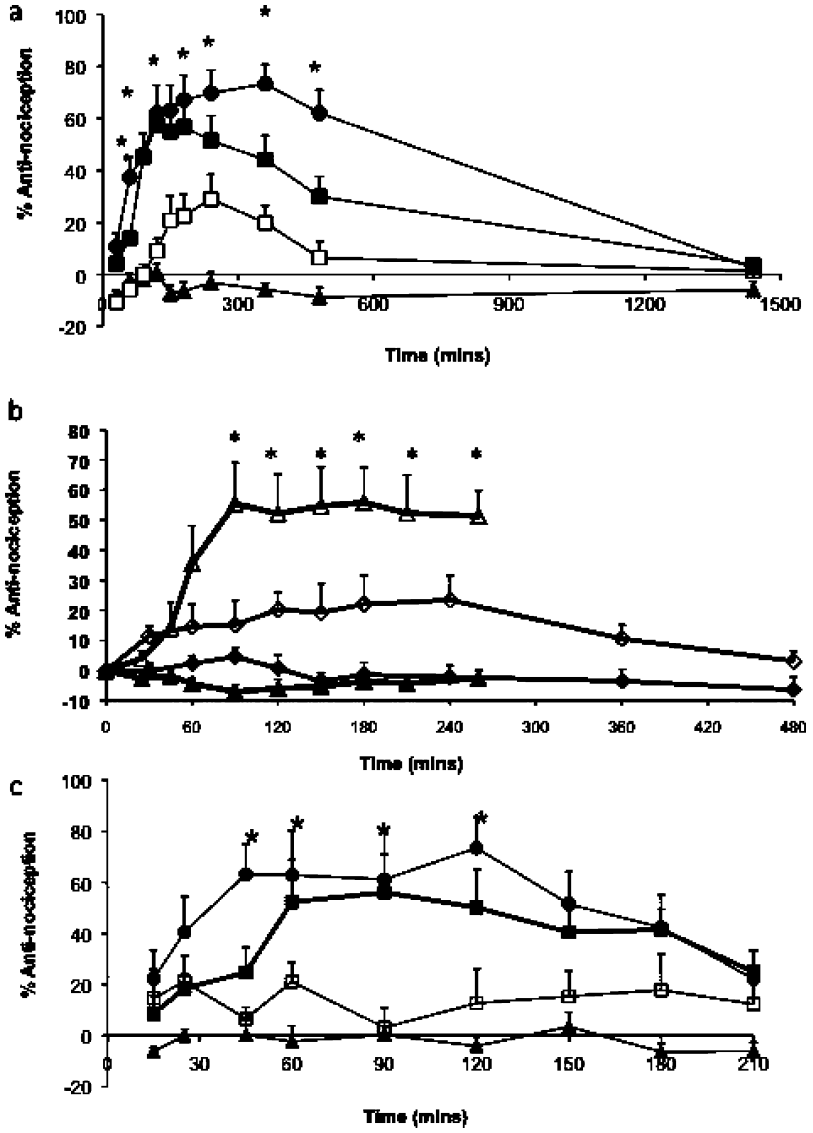

Figure 7. Peptide and polymer pharmacodynamics. (a) % antinociception (mean ± SEM, n = 16) recorded for mice dosed orally with water (▲), leucine^5-enkephalin (70 mg kg^{-1}, □), leucine^5-enkephalin (70 mg kg^{-1})−GCPQ (350 mg kg^{-1}) nanoparticles (■), TPLENK (100 mg kg^{-1})−GCPQ (500 mg kg^{-1}) nanoparticles (●). GCPQ−TPLENK formulations produce significantly more analgesia when compared to leucine^5-enkephalin alone. The baseline latency for the tail flick response was 2.33 ± 0.70 s. * = significant differences between GCPQ groups and controls (p < 0.01). At the 360 and 480 min time points there are significant differences between the GCPQ−leucine^5-enkephalin and GCPQ−TPLENK groups (p < 0.01). (b) % antinociception (mean ± SEM, n = 8) recorded for mice dosed intravenously with either sodium chloride (0.9% w/v, ▲) or TPLENK (14 mg kg^{-1}, Δ) in glycerol (2.25% v/v), or orally with either water (◆) or TPLENK (100 mg kg^{-1}, ◇) in water. The baseline latency for the tail flick response was 2.53 ± 0.54 s. Significant differences: * = p < 0.01 versus sodium chloride (0.9% w/v). (c) % antinociception (mean ± SEM, n = 8) recorded for mice dosed intravenously with sodium chloride (Δ), leucine^5-enkephalin (14 mg kg^{-1}, □), leucine^5-enkephalin (14 mg kg^{-1})−GCPQ (32 mg kg^{-1}) nanoparticles (■), TPLENK (20 mg kg^{-1})−GCPQ (46 mg kg^{-1}) nanoparticles (●). GCPQ−TPLENK formulations produce significantly more analgesia when compared to leucine^5-enkephalin. The baseline latency for the tail flick response was 1.84 ± 0.62 s. Significant differences: * = p < 0.05 versus sodium chloride (0.9% w/v), + = p < 0.001 versus leucine^5-enkephalin.

Figure 7a). TPLENK is not orally active *per se*, however (Figure 7b), a possible consequence of its rapid intestinal degradation (Figure 5c).

Via the intravenous route and at a dose of 10 mg kg^{-1} leucine^5-enkephalin alone did not produce any analgesia (Figure 7c), while the GCPQ nanoparticle formulations produced significant antinociception and GCPQ−TPLENK formulations produced the highest level of antinociception (Table 2, Figure 7c), and this correlated well with the fact that GCPQ−TPLENK nanoparticles delivered more leucine^5-enkephalin to the brain. TPLENK is pharmacologically active via the intravenous route, even when not encapsulated in GCPQ nanoparticles (Figure 7b), evidence that TPLENK itself results in enhanced delivery of leucine^5-enkephalin to the brain, when compared to leucine^5-enkephalin alone.

Typical Straub tail effects, characterized by erect tails, were observed when high levels of analgesia were recorded. The Straub tail reaction in mice is an S-shaped dorsiflexion of the mouse tail and is seen with high levels of morphine. It is based on a contraction of the sacrococcygeal dorsalis muscles, which in the case of morphine is induced by a long-lasting stimulation of the muscle motor innervation at the level of the lumbosacral spinal cord.[53] Thus, this observation, which is a typical index of opiate receptor activation and involves both central and

peripheral components of the nervous system,[54] provides further evidence for the successful delivery of the peptide to the site of action in adequate pharmacological amounts.

Our data show that the nanoparticle−peptide prodrug approach is a suitable strategy for the oral delivery of gut labile neuropeptides.

DISCUSSION

The original hypothesis underpinning this work was that the oral delivery of peptides to the brain could be achieved by the encapsulation of a peptide prodrug within nanoparticles. It was hypothesized that this strategy would promote oral absorption due to the nanoparticles' ability to protect the peptide from degradation within the gut and the ability of GCPQ nanoparticles to promote the oral absorption of hydrophobic drugs, in part due to mechanisms linked to GCPQ's gastrointestinal mucoadhesion.[31] It was further hypothesized that the absorbed prodrug would then be transported across the blood−brain barrier, because of its greater plasma stability and lipophilicity. GCPQ nanoparticles did indeed increase the oral bioavailability and pharmacodynamic activity of leucine^5-enkephalin and TPLENK (Figures 6 and 7). The increased oral brain bioavailability is in part derived from the ability of GCPQ nanoparticles to promote oral absorption by protecting both TPLENK and leucine^5-enkephalin from degradation by intestinal enzymes (Figure 5c) and, in the case of TPLENK, by a resistance to plasma degradation (Figure 5a) once absorbed. It is possible that TPLENK is also transported to a greater extent across the blood−brain barrier due to its lipophilicity.

The main threat to peptide stability arises in the lumen of the small intestines, where large quantities of endopeptidases and exopeptidases are secreted by the pancreas.[55] Peptidases also arise from the mucosal cells of the villi, from the brush border membrane of the epithelial cells and from the lysosomes which could degrade any endocytosed peptides.[55] It is conceivable that GCPQ would form a physical barrier between these peptidases and their substrates. On surviving the enzymatic degradation, TPLENK, by virtue of its increased lipophilicity, is hypothesized to partition into the intestinal epithelial cells due to a decreased ability to hydrogen bond with the aqueous environment of the intestinal lumen. Polymeric amphiphiles are known to promote the oral absorption of hydrophobic drugs,[31,41,42] and GCPQ is known to act in part by mucoadhesion and thus prolong the drug residence time at the functional aspects of the gastrointestinal tract.[31] Unlike underivatized chitosan, GCPQ does not open the tight intercellular junctions of the gastrointestinal epithelium.[31]

Once absorbed into the blood TPLENK is bioconverted to leucine^5-enkephalin by plasma and liver esterases (Figures 4 and 6). However, although the resulting brain levels of leucine^5-enkephalin were similar when either GCPQ−TPLENK or GCPQ−leucine^5-enkephalin was orally administered (Figure 6b), the pharmacodynamic activity resulting from the oral administration of GCPQ−TPLENK formulations was longer in duration (Figure 7a). The similarity in leucine^5-enkephalin pharmacokinetics but difference in duration of pharmacological activity could be explained by the fact that TPLENK may be intrinsically active, especially as TPLENK is able to adopt the three-dimensional conformation of the δ-opioid pharmacophore (Figure 1). However there is currently no direct evidence of the intrinsic activity of TPLENK.

In the quantal form of the tail flick test, which detects maximum possible effects (MPE, Table 2), only morphine-like antinociceptive activity is detected and there are virtually no false positive responses.[56] In the current study, the GCPQ formulations produced significant numbers of analgesic responders achieving the MPE (Table 2).

While the oral dose of TPLENK used in the current study may appear high (100 mg kg^{-1}), it must be borne in mind that the human oral dose of opioid receptor agonists such as morphine is almost 200-fold lower than the mouse oral dose.[57−59] The human intravenous dose of morphine (0.06 mg kg^{-1}) is also 10−20-fold lower than an effective intravenous mouse dose.[60,61]

Previous strategies to achieve brain delivery of the peptide dalargin via the oral route, using polysorbate coated nanoparticles,[62,63] have been hampered by reports that polysorbate 80 is postulated to have a toxic effect at the blood−brain barrier by increasing the permeability of tight junctions or destruction of endothelial cells.[64] Additionally other strategies which have been attempted to achieve the oral delivery of neuropeptides did not produce prolonged pharmacodynamic effects[63] or did not result in the peptide being delivered across the blood−brain barrier[14] and did not present pharmacokinetic evidence of increased brain delivery.

On intravenous administration, GCPQ nanoparticles protect leucine^5-enkephalin and TPLENK from degradation in the plasma (Figure 5a), and the higher brain levels and pharmacodynamic activity of leucine^5-enkephalin obtained on the intravenous administration of GCPQ−TPLENK nanoparticles when compared to GCPQ−leucine^5-enkephalin nanoparticles (Figures 6a and 7a) is attributed to the slower enzymatic destruction of TPLENK within the blood.

To summarize, the present study is the first to demonstrate the oral delivery of labile hydrophilic peptides to the CNS via both pharmacokinetic and pharmacodynamic studies.

ASSOCIATED CONTENT

Supporting Information

Brain homogenate (50%) stability of TPLENK and thin layer chromatography analysis of radiolabeled GCPQ before and after purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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