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Lipophilic derivatives of leu-enkephalinamide: In vitro permeability, stability and in vivo nasal delivery

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ABSTRACT

Leu-enkephalin is an endogenous pain modulating opioid pentapeptide. Its development as a potential pharmaceutic has been hampered by poor membrane permeability and susceptibility to enzymatic degradation. The addition of an unnatural amino acid containing a lipidic side chain at the N-terminus and the modification of the C-terminus to a carboxyamide was performed to enhance the nasal delivery of the peptide. Two lipidic derivatives with varying side chain lengths (C_8-Enk-NH_2 (**1**), C_{12}-Enk-NH_2 (**2**)) and their acetylated analogues were successfully synthesised. Caco-2 cell monolayer permeability and Caco-2 cell homogenate stability assays were performed. C_8-Enk-NH_2 (**1**) and its acetylated analogue Ac-C8-Enk-NH_2 (**3**) exhibited apparent permeabilities (mean ± SD) of $2.51 \pm 0.75 \times 10^{-6}$ cm/s and $1.06 \pm 0.62 \times 10^{-6}$, respectively. C12-Enk-$NH_2$ (**2**) exhibited an apparent permeability of $2.43 \pm 1.26 \times 10^{-6}$ cm/s while Ac-C12-Enk-NH_2 (**4**) was not permeable through the Caco-2 monolayers due to its poor solubility. All analogues exhibited improved Caco-2 homogenate stability compared to Leu-Enk-NH_2 with $t_{½}$ values of: C8-Enk-NH_2 (**1**): 31.7 min, C_{12}-Enk-NH_2 (**2**): 14.7 min, Ac-C8-Enk-NH_2 (**3**): 83 min, Ac-C_{12}-Enk-NH_2 (**4**): 27 min. However, plasma stability assays revealed that the diastereoisomers of C8-Enk-NH_2 (**1**) did not degrade at the same rate, with the L isomer ($t_{1/2}$ = 8.9 min) degrading into Leu-enkephalinamide and then *des*-Tyr-Leu-Enk-NH_2, whereas the D isomer was stable ($t_{1/2}$ = 120 min). In vivo nasal administration of C_8-Enk-NH_2 to male rats resulted in concentrations of $5.9 \pm 1.84 \times 10^{-2}$ μM in the olfactory bulbs, $1.35 \pm 1.01 \times 10^{-2}$ μM in the brain and $6.53 \pm 1.87 \times 10^{-3}$ μM in the blood 10 min after administration.

1. Introduction

Opioid peptides are endogenous compounds with potent pain modulating activity and therefore may have therapeutic application in pain management. That they are endogenous opioid receptor ligands and exhibit high receptor affinity and selectivity has prompted the belief that they would exhibit less side effects than current pain treatment such as morphine and other plant derived opioids if they can be successfully delivered.[1]

Leu-Enkephalin is a pentapeptide (Tyr-Gly-Gly-Phe-Leu) which acts as a neurotransmitter in pain sensation[2] being a μ/δ opioid receptor agonist. Like many peptides, delivery of Leu-enkephalin as a potential pharmaceutic has been hampered by its poor membrane permeability and high susceptibility to enzyme degradation.[3] In recent years, nasal administration of various peptides, including enkephalins,[2,4,5] has been investigated in order to overcome those issues. The advantages of this method include avoidance of the degradative environment of the GI tract, reduced hepatic metabolism, better patient compliance,[2] and the potential to bypass the blood brain barrier (BBB).[6]

Three transport mechanisms are believed to occur in the nose, all having different consequences for drug absorption.[7] The olfactory nerve pathway occurs via the cytoplasm of olfactory neuron axons after permeation through the receptor cells. Many viruses are believed to use this pathway to reach the Cerebro Spinal fluid (CSF) and the brain. However, this is a slow process that can take up to several hours.[7] The olfactory epithelial pathway, in contrast occurs after paracellular or transcellular transport through olfactory epithelium cells other than olfactory neurons. The solute travels along the tissues surrounding the olfactory neurons until

Abbreviations: C_8-Enk-NH_2 (**1**), leu-enkephalinamide modified at the N-terminus with a C8 lipoaminoacid; Ac-C_8-Enk-NH_2 (**3**), acetylated leu-enkephalinamide modified at the N-terminus with a C8 lipoaminoacid; C_{12}-Enk-NH_2 (**2**), leu-enkephalinamide modified at the N-terminus with a C12 lipoaminoacid; Ac-C_{12}-Enk-NH_2 (**4**), acetylated leu-enkephalinamide modified at the N-terminus with a C12 lipoaminoacid; Enk-C_8-NH_2, leu-enkephalinamide modified at the C-terminus with a C8 lipoaminoacid; Ac-Enk-C_8-NH_2, acetylated leu-enkephalinamide modified at the C-terminus with a C8 lipoaminoacid; Enk-C_{12}-NH_2, leu-enkephalinamide modified at the C-terminus with a C12 lipoaminoacid; Ac-Enk-C_{12}-NH_2, acetylated leu-enkephalinamide modified at the C-terminus with a C12 lipoaminoacid.

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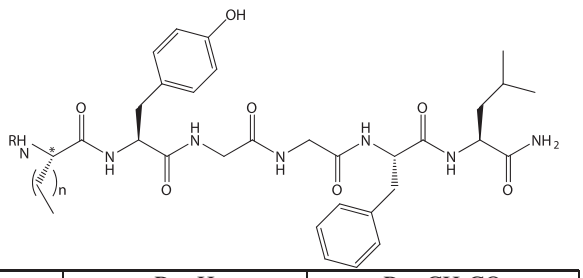
reaching the perineural space and fluid, which is believed to be continuous with the CSF.[7] This route is very rapid with compounds appearing in the olfactory bulb in minutes. Alternatively, the drug can cross the nasal epithelium and reach the capillary rich submucosal tissue. This is the systemic pathway.[7] Drugs following this pathway will reach the systemic circulation, and eventually risk first pass metabolism and the enzymatic environment of the BBB.[7]

One of the major obstacles to the nasal absorption of polar, high molecular weight molecules such as peptides is their poor permeability across lipophilic barriers.[8] The lipophilic nature of the nasal mucosa has been shown to play an important role in the barrier function of the epithelium[9] and therefore the lipophilicity of the compound being delivered is a major determinant of its nasal bioavailability. In vitro transport of the lipophilic compound progesterone was the highest in the nasal mucosa compared to other mucosal membranes.[9] It has also been shown that increased CSF concentrations of sulfonamides delivered nasally was directly proportional to the lipophilicity of these compounds.[7,10]

The use of chemical modifications of Leu-enkephalin to improve its permeability and metabolic stability has been investigated by various research groups.[1,3,11–13] Though the activity of some of those compounds was decreased,[1,11] in some the activity was maintained in the nanomolar range and, permeability and stability of those derivatives was increased compared to native Leu-enkephalin.[2] These previous studies provide excellent guidelines on the type and position of modifications that can be tolerated by the peptide. Consequently, the modification investigated here involved the addition of a lipid moiety to the N-terminus of the peptide Leu-enkephalinamide, the carboxyamide analogue of Leu-enkephalin. The lipids used were synthetic lipoamino acids, (2-amino-D,L-octanoic acid hydrochloride (C8 lipoamino acid) and 2-amino-D,L-dodecanoic acid hydrochloride (C12 lipoamino acid) (Fig. 1). Increased lipophilicity was further obtained via acetylation of the N-terminus of the modified peptides. The C-terminus lipidic conjugates of the peptide were also investigated for permeability and stability and are briefly reported.

Caco-2 cell monolayers were used to assess in vitro permeability of the derivatives across a biological barrier. The Caco-2 cell permeability assay is a well established and reliable in vitro model for assessing absorption.[14] The stability of the compounds was assessed in both human plasma and in an homogenate of Caco-2 cells, which contains a cocktail of digestive enzymes including peptidases that are present in both the GI tract and the blood brain barrier.[15,16] Although Caco-2 cell monolayers are a useful tool and used in the present study, they remain a model with shortcomings. The nasal epithelium presents characteristics such as the mucociliary clearance mechanism and a mucus layer that are not represented in the Caco-2 cell model.



	R = H	R = CH_3CO
n = 5	C_8-Enk-NH_2 (**1**)	Ac-C_8-Enk-NH_2 (**3**)
n = 9	C_{12}-Enk-NH_2 (**2**)	Ac-C_{12}-Enk-NH_2 (**4**)

Figure 1. Structure of the lipophilic Leu-enkephalinamide analogues and their acetylated derivatives. *Lipoamino acids synthesised as a mixture of enantiomers. Peptide represented as the L-diastereomer in this figure.

The nasal absorption of C_8-Enk-NH_2 (**1**) was then assessed in male rats and the level of peptide in the brain, olfactory bulbs and blood quantified using LC–MS/MS.

2. Results and discussion

2.1. Caco-2 cell monolayer permeability of N-terminus derivatives

The peptides were synthesised using standard Fmoc-protecting groups strategies and were purified by preparative HPLC to a single peak by analytical HPLC. Full analytical data and mass spectrometric data for the peptides is provided in Supplementary data.

Standard Caco-2 monolayer permeability assay procedures were followed in the assessment of the apparent permeability (P_{app}) of the enkephalin derivatives.[17] Transepithelial electrical resistance (TEER) values after the experiment were consistent with those measured before the experiments (within 10% of the original value). This indicates that the monolayers were intact after the experiment and thus, none of the compounds were toxic to the cells. P_{app} results for the negative control compound, ^{14}C mannitol were between 1 and 3×10^{-7} cm/s which is consistent with confluent, intact monolayers.[14,18] A range of passage numbers were used for the different experiments to ensure the reproducibility of the results.

C_8-Enk-NH_2 (**1**) and C_{12}-Enk-NH_2 (**2**) exhibited similar P_{app} values, $2.51 \pm 0.75 \times 10^{-6}$ cm/s and $2.43 \pm 1.26 \times 10^{-6}$ cm/s, respectively (Fig. 2). However, of the acetylated derivatives, only Ac-C_8-Enk-NH_2 (**3**) was permeable ($1.06 \pm 0.62 \times 10^{-6}$ cm/s), even if slightly less than its non-acetylated derivative. The absence of permeability observed for Ac-C_{12}-Enk-NH_2 (**4**) and the inferior permeability of Ac-C_8-Enk-NH_2 (**3**) may be due to the poor water solubility of the compounds. The N-terminus acetylated analogues are uncharged at pH 7.4 and thus proved difficult to dissolve to the required 200 μM concentration. The aqueous solubility could be improved by increasing the pH of the assays, thus obtaining a negatively charged species, however, the Caco-2 cell monolayer and cell homogenate assays are incompatible with high pH solutions which would compromise the integrity of the monolayer and the activity of the digestive enzymes.[19] Highly lipophilic and poorly water soluble drugs are notoriously difficult to screen through the Caco-2 cell model as the use of aqueous media is essential to conserve the monolayer integrity during the experiment.[20] The use of solubilisers, such as DMSO or surfactants, can damage the cell monolayer if used in too high concentration. Possible solutions to this issue might involve the use of artificial membranes, or non

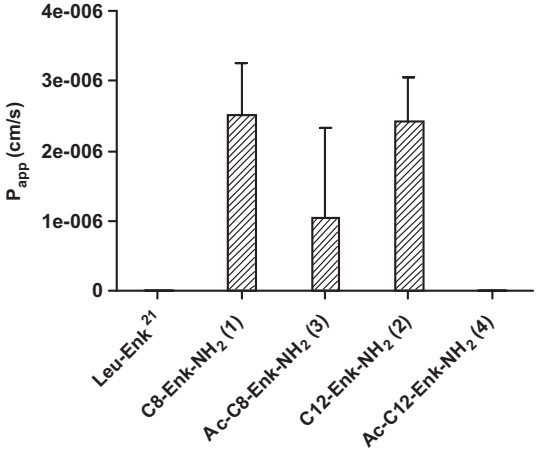


Figure 2. Apparent permeability in Caco-2 cell monolayers of lipidic derivatives of Leu-enkephalin. (P_{app} Leu-Enk[21] = 3.1×10^{-7} cm/s).

biologically compatible solubilising agents. However, those tend to cause either an overestimation or underestimation of drug transport due to drug–drug interactions.[20]

The Caco-2 cell model has been shown to be a reliable blood brain barrier model for the assessment of the delivery of passively absorbed compounds.[15,16] Garberg et al.[15] demonstrated a correlation ($r^2 = 0.96$) between in vitro transport in Caco-2 monolayers and in vivo transport, using the mouse brain uptake assay for compounds that were passively transported and not bound to proteins. Consequently, Caco-2 cell transport experiments are useful when studying CNS targeted compounds but they also have the advantage of expressing enzymes, such as aminopeptidases and cytochrome P450 enzymes,[22–24] and transporters such as P-gp[25,26] and glucose transporters[27] that are also present in the nasal epithelium.[28–32] However, they also lack characteristics of the nasal epithelium such as beating cilia, mucus layer and the mucociliary clearance mechanism that are known to affect drug delivery.

In vitro models to study the transport across the nasal mucosa include human nasal primary cell culture. Cells obtained from turbinate tissues are treated and plated or seeded onto supports for transport and metabolic studies.[33–40] Various media components are used (DMEM supplemented or not with serum, with or without hormone cocktails, etc.) and collagen coated or non coated support devices are used. The monolayers obtained from primary cell culture exhibited TEER values comparable to that of confluent Caco-2 monolayers used in transport studies (600–1000 ohm cm^2) however, a maximum of 6 passages can be performed before the cell line loses its properties. There can also be significant disparity between cell lines due to the quality of the tissue used for primary cell culture.[7,33] Moreover, the P_{app} of sodium fluorescein, met-enkephalin and leu-enkephalin across those monolayers were shown to be in the same range as those observed in Caco-2 cell monolayers.[38–40]

More recently, a kinetic model to predict drug absorption from the nasal cavity using Caco-2 cell P_{app} data was developed by Furubayashi et al.[41] Though the authors have made several assumptions limiting the application of this model (i.e., it does not take into consideration physicochemical properties of the drug), a relationship between the nasal absorption rate constant and the Caco-2 cell apparent permeability was established ($r = 0.947$).[41]

2.2. Caco-2 cell homogenate stability of N-terminus derivatives

Caco-2 cell homogenate solutions were prepared following the standard procedure described by Fredholt et al.[42] Protein content was measured using a Bio-Rad® assay. The protein concentration was adjusted (0.5 mg/mL) for each experiment. Activity of the enzymes in the mixtures was assured by incorporating endomorphin-1 as a positive control in each experiment. Endomorphin-1 exhibited a $t_{1/2} = 9$ min, which is consistent with literature reports.[43,44] Amidation of the C-terminus of Leu-enkephalin has been shown to decrease its binding affinity to neutral endopeptidase 24.11, which is also known as enkephalinase. It seems that protecting the carboxyl group decreases electrostatic interactions between the free acid group and an arginine residue on the binding site of the enzyme.[45] Caco-2 cell homogenate stability assays showed that the acetylated derivatives of the lipid modified enkephalinamides were more stable than the non-acetylated peptides (Figs. 3 and 4). Ac-C_8-Enk-NH_2 (**3**) showed an increased stability with a $t_{1/2}$ of 83 ± 3.3 min compared to 31.7 ± 6.5 min for C_8-Enk-NH_2 (**1**) (Fig. 3). As the half life of Leu-enkephalin ($t_{1/2}$ = 1–4 min) in Caco-2 cell homogenate is well established, it was not investigated here.[13,42,46] It is supposed that the increased stability of C_8-Enk-NH_2 (**1**) is due to the lipophilic side chain of the C_8 lipoamino acid which would obstruct the action of the enzymes

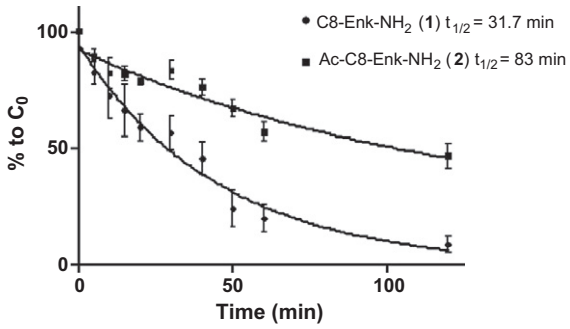


Figure 3. Degradation profile of C8-Enk-NH_2 (**1**) and Ac-C8-Enk-NH_2 (**3**) in Caco-2 cell homogenate. Data is presented as the mean ± SD ($n = 3$). One phase decay analysis performed with the software GraphPad Prism 5.

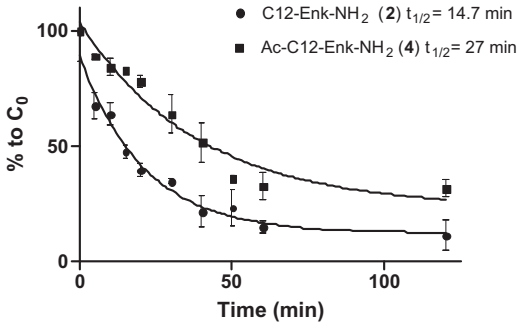


Figure 4. Degradation profile of C_{12}-Enk-NH_2 (**2**) and Ac-C_{12}-Enk-NH_2 (**4**) in Caco-2 cell homogenate. Data is presented as the mean ± SD ($n = 3$). One phase decay analysis performed with GraphPad Prism 5.

(aminopeptidases, enkephalinases A and B).[30] Acetylation at the N-terminus of the peptide could enhance this steric hindrance.

For similar reasons, Ac-C_{12}-Enk-NH_2 (**4**) stability is increased compared to that of C_{12}-Enk-NH_2 (**2**) (Fig. 4). However, the stability increases less (from $t_{1/2} = 14.7 \pm 5.4$ to $t_{1/2} = 27 \pm 4.7$ min), which also suggests that C_{12}-Enk-NH_2 (**2**) is less stable than C_8-Enk-NH_2 (**1**) in this model.

These results demonstrate that Ac-C_8-Enk-NH_2 (**3**) is the most stable peptide analogue in Caco-2 cell homogenate exhibiting a $t_{1/2} = 83 \pm 3.3$ min and showing minimal loss of the acetate group from the N-terminus in the time scale of our in vivo experiments (20 min). Acetylation apparently improved the stability of the C12 analogues, though results for both the acetylated and non-acetylated C_{12} compounds could be adversely affected by poor aqueous solubility.

2.3. Human plasma stability of C8-enkephalinamide (1)

The lipoamino acids were used as racemic mixtures of the D and L isomers, consequently, giving rise to two diastereomers of each lipopeptide. These diastereomers could be resolved by analytical HPLC. The diastereomers of C8-Enk-NH_2 (**1**) are separated by approximately 0.5 min under our analytical HPLC protocol. By comparison with previous studies of diastereomeric mixtures of Laa-conjugates of small peptides, GnRH[48] and endomorphin 1,[47] it was assumed that the earlier eluting peak in our mixture was the diastereomer bearing the L-Laa. The clarity in the analysis of the diastereomers allowed the degradation in human plasma of each diastereomer of C8-Enk-NH_2 (**1**) to be examined. Incubation with human plasma revealed differential stability of the D- and L-diastereomers of C8-Enk-NH_2 (**1**). The diastereomer bearing the L-lipoamino acid degraded more rapidly ($t_{1/2} = 8.9$ min) than the

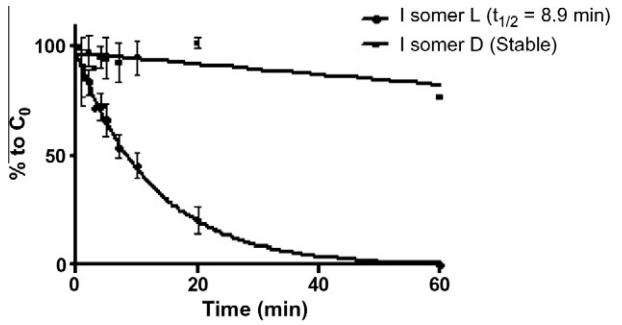


Figure 5. Degradation profile of L-C_8-Enk-NH_2 and D-C_8-Enk-NH_2 in human plasma at 37 °C.

D-lipoamino acid bearing isomer (stable for the duration of the experiment; Fig. 5).

L-C_8-Enk-NH_2 degraded initially to a more hydrophilic product (R_t 16.8 min) which gave a molecular ion in ESMS corresponding to Leu-enkephalinamide (*m*/*z* 555.5). Over time this initial product was further degraded to a second product (R_t 15.9 min) with a molecular ion corresponding to Gly-Gly-Phe-Leu-NH_2 (*m*/*z* 392.3), that is, loss of the N-terminus Tyr residue (Fig. 6). The cleavage of Tyr from the N-terminus of Leu-Enk by aminopeptidases is a known degradation pathway of the peptide.[30] The peak corresponding to the D-C8-Enk diastereoisomer remained constant for the majority of the experiment with only a small decrease observed after 60 min.

A similar result was obtained by Blanchfield et al.[48] with a lipoamino acid modified GnRH analogue. This analogue acted as a prodrug, releasing the active peptide. This approach could overcome the loss of potency observed with most modified peptides.[11] The slow release of the active peptide from the cleavage of the D isomer could be added to the rapid release of active peptide from the cleavage of the L isomer.

It is important to note that in the case of the Caco-2 cell homogenage stability assays, the method of analysis is LC–MS/MS. While this method is much more sensitive than HPLC, the shorter column lengths and gradient times fail to resolve the two diastereomers of the racemic C_8-Enk-NH_2. It is reasonable to postulate therefore that the increased half life ($t_{1/2}$ = 31.7 ± 6.5 min) observed for racemic L-C_8-Enk-NH_2 in the homogenate assay may largely reflect only the stability of the D isomer.

A minimum peptide concentration of 1 mg/mL in PBS was used for the plasma stability assays in order to reach a sufficient absorbance at 214 nm to properly evaluate the stability of the tested compounds. While it would have been interesting to perform the same assay on the other enkephalin analogues, they all failed to dissolve at this concentration. Thus, the Caco-2 cell homogenate stability assay is the best comparative examination of their respective stability in a biological environment.

2.4. Caco-2 cell monolayer permeability and Caco-2 cell homogenate stability of C-terminus derivatives

C-Terminus lipidic conjugates of Leu-enkephalinamide were also examined for their in vitro absorption and stability using Caco-2 cells. In contrast to the N-terminus conjugates, the C-terminus C_{12} derivatives proved to be more stable (Enk-C_{12}-NH_2 $t_{1/2}$ = 16.4 ± 3.5 min) in the presence of an homogenate of Caco-2 cells than their C_8 analogues (Enk-C_8-NH_2 $t_{1/2}$ = 5.4 ± 2.3 min), with acetylation having little effect on this stability when considering the standard deviation (Ac-Enk-C_{12}-NH_2 $t_{1/2}$ = 18.4 ± 6.3 min; Ac-Enk-C_8-NH_2 $t_{1/2}$ = 13.8 ± 6.7 min). Thus, the stability of the C-terminus conjugated C-8 series was significantly lower than the C-8 N-terminus conjugates while the C_{12}-conjugates of both termini exhibited comparable stability.

The apparent permeability of the C-terminus conjugates, in contrast, was consistently lower than the N-terminus series. The more stable Enk-C12-NH2 compound provided the highest permeability of the series at 9.18 ± 7.25 × 10^{-7} cm/s. The permeability of Enk-C8-NH2 (4.77 ± 5.56 × 10^{-9} cm/s) was nearly a thousand times lower than that of C8-Enk-NH_2 (**1**) which would be partly explained by the decreased stability of the C-terminus conjugate. The more stable acetylated analogue of Enk-C_8-NH_2, Ac-Enk-C_8-NH_2 exhibited a P_{app} of 7.63 ± 3.57 × 10^{-7} cm/s, a hundred times higher than that of the non-acetylated analogue Enk-C_8-NH_2 (4.77 ± 5.56 × 10^{-9} cm/s), which could be explained by its higher

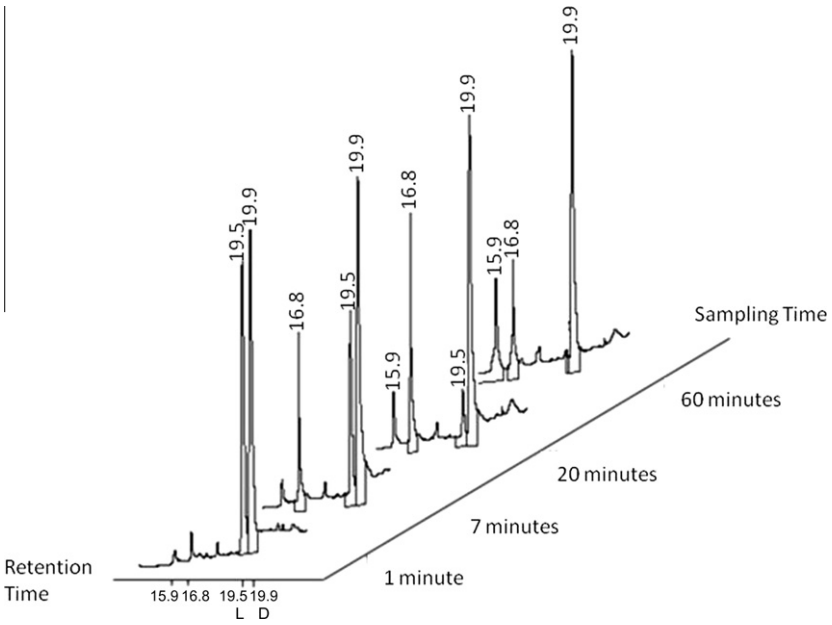


Figure 6. HPLC traces showing the degradation over time of L-C_8-Enk-NH_2 in human plasma and the appearance of the degradation products: rt 19.5 min: L-C_8-Enk-NH_2; rt 19.9 min: D-C_8-Enk-NH_2; rt 16.8 min: Leu-Enk-NH_2 (Tyr-Gly-Gly-Phe-Leu-NH_2); rt 15.9 min: Gly-Gly-Phe-Leu-NH_2.

stability (13.8 ± 6.7 vs 5.3 ± 2.3 min, respectively). The acetylated C_{12} conjugate, Ac-C_{12}-Enk-NH_2 exhibited no permeability in our experiments, again due to its poor water solubility.

2.5. In vivo nasal administration of C_8-Enk-NH_2 (1)

With the promising stability and permeability results obtained for C_8-LeuEnk, this peptide was chosen for a preliminary in vivo assessment of the likely route of absorption from the nasal cavity of these peptides. The peptide was administered nasally to four male Sprague–Dawley rats (1 mg/kg, 50 μL administration) and olfactory bulb, remaining brain tissue and blood of each animal examined for traces of the peptide after 10 min. This short time point was chosen as only the olfactory epithelial pathway of absorption will result in detectable levels of peptide in the olfactory bulb or brain tissue. This is the preferred route of absorption for these peptides as it allows almost immediate absorption into the CFS which is desirable in any pain drug and it avoids first past metabolism and the need to negotiate the BBB. It is therefore a priority to determine early in the development of these compounds if the olfactory epithelial pathway can be utilised by these peptides. The results are shown in Table 1.

The peptide was extracted from the tissues by extensive sonication to break up the tissue and minimise protein binding. The supernatant from the extraction process was analysed by LC–MS/MS. C_8-Enk-NH_2 (**1**) was found in the olfactory bulbs and brains, at the concentrations $5.90 \pm 1.84 \times 10^{-2}$ μM and $1.35 \pm 1.01 \times 10^{-2}$ μM, respectively, 10 min after nasal administration. The much lower blood concentration of the compound ($6.53 \pm 1.87 \times 10^{-3}$ μM) strongly suggests that the compound reached the brain following the olfactory epithelial pathway, and not via the systemic circulation. Merkus et al. have argued that a comparison between CNS/plasma ratio after nasal administration and after iv infusion was necessary to confirm that the direct transport occurs. If the CNS/plasma ratio in the case of nasal administration was higher that the CNS/plasma ratio after iv infusion then the tested compound followed a direct pathway to the brain from the nasal cavity, and it was not delivered to the brain via the systemic circulation after crossing the blood brain barrier.[50] Unfortunately, this study did not investigate iv transport.

3. Conclusions

The lipophilic derivatives of Leu-enkephalinamide exhibited an improved permeability across Caco-2 monolayers and improved stability in Caco-2 cell homogenate compared to the parent peptide. Moreover, intranasal delivery of C8-Enk-NH_2 resulted in an uptake of the compound into the olfactory bulb of male Sprague–Dawley rats 10 min after administration. The blood concentration of the compound suggested that the compound reached the brain following the olfactory epithelial pathway. However, this would need to be confirmed by running a comparative study measuring the direct uptake of the compound into the brain after iv administration.

This study is significant as it clearly demonstrates that a lipophilic peptide can be absorbed directly into the brain via the nasal epithelial tissue. The delivery of the opioid peptide derivatives remains a major hurdle in their development as the new generation of pain modulating drugs. Nasal delivery may well provide a rapid, patient friendly method of administration of peptide based pain medications.

4. Experimental

4.1. Materials and methods

Fmoc protected amino acids and Rink amide resin were obtained from Novabiochem (Melbourne, Australia). DMF, TFA and HBTU were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was obtained from Labscan Asia Co. Ltd, (Bangkok, Thailand). Other chemicals were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Caco-2 cells were obtained from American Tissue Culture Collection (Rockville, MD, USA), Transwell polycarbonate inserts were from Costar (Cambridge, MA, USA) and cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA).

Animal experiments were performed with the approval of the University of Queensland animal ethic committee (Group 3) (No. SMMS/319/07/UQ/ARC). Human plasma was used with the approval from the medical research ethics committee at the University of Queensland (No. 200600950).

4.2. Peptide synthesis

The peptide derivatives were synthesised using Fmoc solid phase peptide synthesis on 0.5 mmol scale. The identity and purity of the compounds were confirmed by HPLC (Agilent 1100 system) and ESMS analysis (API 3000, Sciex). Standard coupling reagents HBTU and DIPEA were used and quantitative ninhydrin test was performed to ensure completion of the reaction.[49]

Dde protected C_8 and C_{12} lipoamino acids were coupled using the same standard procedures. The Dde protecting groups of the lipoamino acids were removed using a solution of 5% hydrizine hydrate in DMF (v/v). Amino acid side chain protecting groups were removed during the cleavage. Cleavage of the peptides from the resin was performed using a solution of TFA with 20 mL/g resin of a solution of 95% TFA, 2.5% water and 2.5% TIS.

The crude peptides were analysed by analytical HPLC (Agilent 1100 system) using a gradient of solvent A (0.1% TFA in water) to 100% solvent B (0.1% TFA, 90% acetonitrile and 10% water) over 30 min on a C_{18} RP-HPLC column (Vydac, 5 μm) and UV detection set at 214 nm. The peptides were then purified by preparative RP-HPLC on a C18 column. Further analysis by HPLC and electrospray mass spectrometry confirmed the purity of the peptides. This data

Table 1
In vivo nasal delivery of C8-Leu-Enk (**1**): concentrations in olfactory bulbs, brain and blood 10 min after administration

Time	Rat	Olfactory bulb			Brain			Blood		
		Concentration (μM)	Mean	SD	Concentration (μM)	Mean	SD	Concentration (μM)	Mean	SD
10 min	1	7.63×10^{-2}	5.90×10^{-2}	1.84×10^{-2}	1.40×10^{-2}	1.35×10^{-2}	1.01×10^{-2}	6.50×10^{-3}	6.53×10^{-3}	1.87×10^{-3}
	2	5.42×10^{-2}			2.71×10^{-2}			4.03×10^{-3}		
	3	7.08×10^{-2}			9.84×10^{-3}			7.08×10^{-3}		
	4	3.55×10^{-2}			3.07×10^{-3}			8.50×10^{-3}		

is available in Supplementary data document accompanying this paper.

4.3. Cell culture

Caco-2 cells (passage 25–35) were maintained in cell culture flask in Dubelcco's Modified Eagle Medium (DMEM) (containing 4.5 g/L D-glucose and L-glutamine) supplemented with 10% foetal bovine serum (FBS) and 1% non-essential amino acids.

Permeability assay plates (Costar® Transwell permeability plates, 6.5 mm inserts diameter, 0.4 μm pore size of the polycarbonate membrane) were seeded with 100 μL of a 80,000 cells/cm^2 cell suspension on the apical side of the membrane. 600 μL of media was added to the basolateral chamber. Media consisted of DMEM supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin (containing 10,000 units of penicillin sodium salt and 10,000 μg streptomycin sulphate/mL in 0.85% saline).

Stability assays were performed in 96 well, sterile, flat bottom plates. These were seeded with 100 μL of 30,000 cells/cm^3 cell suspension. Media was replaced every two days for 21–28 days to reach confluency before being used for the assay.

4.4. Caco-2 permeability assay

After 21–28 days, the cells seeded on transwell filters were used for permeability experiments. The monolayers were rinsed once with a solution of 0.02% EDTA in DPBS on the apical and basolateral side, and three times with HBSS buffered at pH 7.4 with 25 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid). The cells were then left to equilibrate at 37 °C for 30 min in HBSS/HEPES solution. TEER values were then measured. All cell monolayers used had TEER values between 2.5 and 5 kΩ/cm^2 before the experiments.

At the start of the experiment (time = 0 min), 600 μL of buffered HBSS had been placed on the basolateral side of the monolayer and 100 μL of 200 μM solution of the test compound prepared in HBSS/HEPES was added to the apical side of the monolayer. At time 30, 90, 120 and 150 min, 400 μL were sampled from the basolateral side. The volume sampled (400 μL) was replaced with 400 μL of HBSS/HEPES buffer solution. Each compound was tested on 4 wells/experiment and at least three experiments were conducted using different passage number cells.

LC–MS/MS analysis was performed on each sample and compared to a standard curve (0.05–100 μM) to determine the concentration of each respective peptide derivative. Apparent permeability values (P_{app}) were then calculated using the following formula.

$$P_{app} = \frac{dC}{dt} \times \frac{Vr}{A \times C_0}$$

where dC/dt is the steady state rate of the change in the chemical concentration (M/s); Vr is the volume of the receiver chamber (cm^3); A is the surface area of the cell monolayer (cm^2); C_0 is the initial concentration in the donor chamber (M).

4.5. Caco-2 homogenate stability assay

After 21–28 days the cells were rinsed once with 100 μL 0.02% EDTA solution and three times with 100 μL of HBSS buffered with 25 mM HEPES. The cells were then lysed with an ultrasonic processor to obtain a suspension that was then centrifuged at 2000 rpm for 5 min to remove the cell extract. The supernatant was then collected and tested for protein content using the Bio-Rad protein assay (New York, USA).

4.5.1. Stability assay

At the start of the experiment (time = 0), 100 μL of the cell homogenate (0.5 mg/mL protein content) was mixed with 100 μL of a 200 μM solution of the compound to be tested in HBSS/HEPES. At 5, 10, 15, 20, 30, 40, 50, 60 and 120 min, 10 μL of the mixture was sampled to which was added 90 μL of distiled water acidified with TFA to pH 1–2 to stop the enzymatic degradation. The assay was preformed in 4 wells/compound and each compound tested in at least three independent experiments.

LC–MS/MS analysis was then conducted to determine the concentration in each of the samples at the different time points.

4.5.2. LC–MS/MS analysis

LC–MS/MS analysis of the samples from Caco-2 monolayer permeability and stability experiments was performed using a gradient HPLC system (Shimadzu LC-10AT system) in line with a triple quadrupole mass spectrometer (PE Sciex API 3000) operating in Multiple Reaction Monitoring (MRM) mode with positive ion electrospray.

The mobile phase used was a gradient of solvent A (0.01% acetic acid in water) and solvent B (90% acetonitrile, 10% water and 0.01% acetic acid), which varied depending on the compound analysed. A RP-HPLC column (Phenomenex, C18 Luna 5 μm, 50 × 2.2 mm, 0.5 mL/min) with 1:10 splitter before the electrospray source of the MS unit detected the compounds of interest. Nitrogen was used as the nebuliser gas (10 L/min), curtain gas (12 L/min) and collision gas (4 L/min).

4.6. Human plasma stability assay

Human plasma was obtained from healthy volunteers. A solution of 1 mg/mL of compound was prepared in PBS. 300 μL of that solution and 300 μL of plasma (warmed at 37 °C) were mixed. At pre-determined time points, 50 μL of the plasma solution was sampled and put on ice. 75 μL of acetonitrile was then added to precipitate the plasma proteins. The protein mixtures were centrifuged at 10,000 rpm for 10 min and 20 μL of the surnatant was injected into the HPLC for analysis.

HPLC analysis was performed using a gradient of solvent A (H_2O, 0.01% TFA) and solvent B (acetonitrile 90%, H_2O 10%, TFA 0.01%) on C18 column (Vydac, 5 μm, 5 × 25 mm). The gradient was run with 100% A for 5 min and then to 100% B over 20 min.

The degradation profile of the compounds was obtained by plotting the percentage of the initial concentration of the peptide remaining in solution against time.

4.7. In vivo nasal delivery

4.7.1. Assay validation

To 300 μL of rat brain homogenate prepared in PBS, 10 or 30 μL of a 200 μM of the analysed peptide was added. The mixtures were incubated at 37 °C for 20 min, depending on the half life of the peptide. Brain homogenate mixtures were then sonicated for 30 min in a cold bath (7–10 °C) and centrifuged at 10,000 rpm for 15 min. To 50 μL of the collected supernatant, 70 μL of acetonitrile was added in order to precipitate any remaining proteins. The mixture was then centrifuged at 10,000 rpm for 15 min. The collected supernatant was collected and analysed by LC–MS/MS.

6. Experiment

Male Sprague–Dawley rats, weighing between 450 and 530 g, were anesthetised with 300 μL of a mixture of Xylaxil (20 mg/mL xylaxine hydrochloride)/Zoletil® (250 mg tiletamine hydrochloride + 250 mg zolezepam hydrochloride in 5 mL purified water) (1/

1) (v/v). The paw flick test was performed to insure that they were deeply anaesthetised before a vinyl tube (I.D. 0.5 mm, E.D. 0.9 mm) attached to a microsyringe was inserted 15 mm into the nasal cavity of the rat in order to administer 50 μL of 1 mg/kg suspension of the labelled compound prepared in PBS pH 7.4. At 10 min after administration, the rats were euthanised by CO_2 asphyxiation.

Six rats were used in the experiment: four rats received the labelled peptide and two rats were used as controls, receiving only 50 μL of PBS pH 7.4.

6.1. Dissections and sample treatment

Brains and livers were dissected following standard dissection procedures. Olfactory bulbs were separated from the rest of the brain for separate analysis. Blood samples (400 μL) were collected from abdominal cavity at the time of the dissection.

After dissection the organs were fast frozen in a dry ice/acetone mixture in order to stop the enzyme from degrading any possible amount of peptide derivative present in the tissue. The tissues were kept at −80 °C until extraction.

6.2. Olfactory bulbs and brain extraction

After thawing, the organs were homogenised in ice cold PBS (500 μL) using a mechanical homogeniser. The homogenate obtained were then sonicated in a cold bath for 30 min in order to diminish protein binding. The homogenates were centrifuged at 10,000 rpm for 10 min and the supernatant was collected (500 μL). Any remaining proteins in solution were precipitated using 700 μL acetonitrile. The mixture obtained was centrifuged one last time at 10,000 rpm for 30 min. The supernatant was collected and analysed via LC–MS/MS.

6.3. Blood extraction

Cold PBS (500 μL) solution was added to the blood samples. The samples were sonicated in a cold bath for 30 min. The mixtures were centrifuged at 10,000 rpm for 10 min. 500 μL of the supernatant was collected, to which 700 μL of acetonitrile was added to precipitate any remaining proteins as well as remaining red blood cells. The mixtures were centrifuged at 10,000 rpm for 30 min and the supernatant was analysed for peptide concentrations using LC–MS/MS analysis.

6.4. Preparation of the dilution buffer and LC–MS/MS analysis

A dilution buffer was prepared for the LC–MS/MS standards in order to insure the conservation of the ionisation potential of the species analysed. A rat brain homogenate was prepared by mechanically homogenising a rat brain with 500 μL PBS. The supernatant was collected after centrifugation and 700 μL acetonitrile was added keeping the same ratio used in the extraction procedure. The mixture was centrifuged and the collected supernatant was used to prepare dilutions for the LC–MS/MS standard curve. The dilutions of the peptides for the standard curve ranged from a concentration of 1 nM to 100 μM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.042.

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