Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 356 (2008) 37–43
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

In vitro stability and permeability studies of liposomal delivery systems for a novel lipophilic endomorphin 1 analogue

Yasuko Koda [a], Ming Tao Liang [a], Joanne T. Blanchfield [b], Istvan Toth [a,b,*]

[a] *School of Pharmacy, The University of Queensland, St. Lucia, QLD 4072, Australia*
[b] *School of Molecular and Microbial Sciences, The University of Queensland, St. Lucia, QLD 4072, Australia*

Received 6 November 2007; received in revised form 19 December 2007; accepted 19 December 2007
Available online 4 January 2008

Abstract

We have previously shown that the stability and permeability of peptides can be greatly improved by conjugation with lipoamino acids such as 2-aminododecanoic acid (C12Laa). However, the increase in lipophilicity which this conjugation provides can also cause a significant decrease in the compound's water solubility. In this study, we coupled C12Laa to the N-terminus of endomorphin1 (Endo-1, Tyr-Pro-Trp-Phe-NH_2), and addressed its solubility issue by formulating C12Laa-Endo-1 into phosphatidylcholine liposomes. The aqueous solubility of the lipidic analogue was greatly improved, facilitating the accurate analysis of the compound in *in vitro* assays. The metabolic stability and *in vitro* endothelial permeability of the C12Laa-Endo-1 liposomal formulation was assessed using Caco-2 cells, and compared with the formulation of the parent peptide Endo-1. The liposome-encapsulated C12Laa-Endo exhibited significant increases in both stability and permeability. These results suggest that the combination of chemical modification and liposome formulation has great potentials in improving the bioavailability of neuroactive peptides.

Keywords: Liposomes; Lipopeptide; Drug delivery; Endomorphin; Opioid peptide delivery

1. Introduction

Endogenous opioid peptides, endomorphin 1 (Endo-1; Tyr-Pro-Trp-Phe-NH_2) and 2 (Endo-2; Tyr-Pro-Phe-Phe-NH_2) were isolated from mammalian brain tissue in 1997 (Zadina et al., 1997), and subsequently isolated from human brain cortex in 1998 (Hackler et al., 1997). These peptides are widely distributed in the human CNS, and located in the dorsal horn of the spinal cord, trigeminal nucleus, and the periaqueductal gray areas of the CNS (Martin-Schild et al., 1999; Zadina et al., 1997). They exhibit high selectivity and affinity for the μ-opioid receptors (Abbadie et al., 2002; Csullog et al., 2001; Goldberg et al., 1998; Stone et al., 1997), but do not have appreciable affinity for the κ- or δ-opioid receptors (Goldberg et al., 1998). They are potent μ-opioid receptor agonists in both the spinal cord and supraspinal sites, resulting in reduced excitatory neurotransmission and potent analgesia (Higashida et al., 1998).

Peptides, including Endo-1 and Endo-2, are usually unfavourable drug candidates due to their poor metabolic stability and low membrane permeability (Kastin et al., 1999; Rousselle et al., 2003). Thus, the development of delivery systems capable of delivering intact neuroactive peptides across the blood–brain barrier (BBB) and gastrointestinal (GI) tract is essential for the use of these compounds as orally administrated pain mediating drugs. Increasing the lipophilicity of peptides is a useful strategy for improving their permeability across biological barriers (Adessi and Soto, 2002; Blanchfield and Toth, 2004). We have previously demonstrated that attachment of lipoamino acids (Laas) of varying chain length (12–20 carbons) to short peptides can result in increases in their stability and permeability. Conjugation of monomeric or dimeric C12Laas to the N-terminus of luteinizing hormone-releasing hormone (LHRH) extended the half life of the peptide in an homogenate of Caco-2 cells from 5 min to 4 h (Toth et al., 1994). C14Laa–LHRH and C14Laa–thyrotropin releasing hormone (TRH) exhibited increased absorption compared to their parent peptides after oral administration in rats (Flinn et al., 1996). Modification of

* Corresponding author at: School of Molecular and Microbial Sciences, The University of Queensland, St. Lucia, QLD 4072, Australia. Tel.: +61 7 33469892; fax: +61 7 3365 4273.
E-mail address: i.toth@uq.edu.au (I. Toth).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.12.036

the neuroactive peptide, α-conotoxin MII, with C12Laa resulted in a 5-fold greater permeability across Caco-2 cell monolayers (Blanchfield et al., 2003).

Caco-2 cells are derived from a human colon adenocarcinoma and are used as an *in vitro* model of the epithelium of the small intestine (Hidalgo and Li, 1996; Artursson et al., 2001; Zornoza et al., 2004). The cells express metabolic enzymes such as DPPIV (EC 3.4.14.5), endopeptidase-24.11, angiotensin converting enzyme (ACE), aminopeptidase-N (AP-N; EC 3.4.11.2), aminopeptidase-W (AP-W) and γ-glutamyl transpeptidase (Howell et al., 1993, 1992). These enzymes are important in the metabolism and clearance of endomorphin 1 and other small, biologically significant peptides (Pauletti et al., 1997; Witt et al., 2001), and so an homogenate of Caco-2 cells provides an excellent tool with which to test the metabolic stability of peptide drug candidates.

When cultured on semi-permeable membranes, Caco-2 cells form polarized monolayers with tight junctions between the cells and with the cells expressing all of the influx and efflux transporter proteins associated with the small intestine (Artursson and Karlsson, 1991). These monolayers are used as a model of the small intestine epithelial barrier with the compound placed in solution in the apical chamber of the transwell system and the basolateral chamber analysed for appearance of the compound over time. In our continuing study of the effects of conjugation of Laas to endomorphin 1 in order to improve its deliverability, it became clear that the lack of water solubility of C12Laa (and longer Laas) derivatives makes assays such as the Caco-2 cell stability and permeability assays very difficult. Solubilizing the required amount in buffered, aqueous solutions is problematic as is maintaining the peptide in solution over the duration of the experiment. Analysis by LC–MS is also greatly hampered by the lack of solubility of lipophilic peptides.

Liposomes are well-known to solubilize and deliver poorly soluble drugs (Fahr et al., 2005; Forssen and Willis, 1998), and a variety of techniques have been successfully applied to formulate liposomes for the delivery of lipophilic anti-cancer drugs such as 5-fluorouracil, estradiol and paclitaxel (Gutman et al., 2000; Wenk et al., 1996). In this study, Endo-1 and its lipophilic analogue C12-Endo-1 (Fig. 1) were prepared. With aims to address the issue of its solubility and allow the performance of biological assay in aqueous environment, C12-Endo-1 was formulated within liposomes using a novel technique of hydration of freeze-dried monophase systems. The lipopeptide-loaded liposomes were subsequently examined for their enzymatic stability and membrane permeability using Caco-2 cells.

2. Materials and methods

2.1. Materials

The Rink amide MBHA resin (100–200 mesh, 0.78 mmol/g loading), Fmoc- or Boc-protected amino acids were purchased from Novabiochem (Melbourne, Australia). Dimethylformamide (DMF) and trifluoroacetic acid (TFA) of peptide synthesis grade were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, USA). Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS) were purchased from Gibco life technology, and nonessential amino acids, Hanks' balanced salt solution (HBSS), Hepes (pH 7.4) and [^{14}C]-D-mannitol were from Sigma–Aldrich (St. Luis, USA). Egg phosphatidylcholine was purchased from Sigma–Aldrich, Sydney Australia.

2.2. Synthesis of peptides

Racemic 2-aminododecanoic acid (C12Laa) was synthesized by alkylation of diethyl acetaminomalonate followed by hydrolysis and decarboxylation in procedures described previously (Blanchfield et al., 2003; Gibbons et al., 1990). The N-terminus of the lipoamino acid was further protected using *N*-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) (Gibbons et al., 1990; Chhabra et al., 1998). The peptides were synthesized manually via solid phase peptide synthesis using Fmoc-protection strategies on Rink amide MBHA resin in 0.5 mmol scale and using HBTU/DIPEA activating protocol in DMF (Alewood et al., 1997). Fmoc protecting groups were removed by treatment with 20% piperidine in DMF and the Dde-protecting group was removed by treatment with 2% hydrazine hydrate in DMF. Finally, the peptides were cleaved from the resin by treatment with the mixture of TFA:water:TIS (95:2.5:2.5, v/v) for 6 h. The crude peptides were purified by preparative RP-HPLC (Waters; 600 controller and pump, 490E programmable multiwave length detector) on a C18 column (Vydac® 22 mm × 250 mm, Grace)

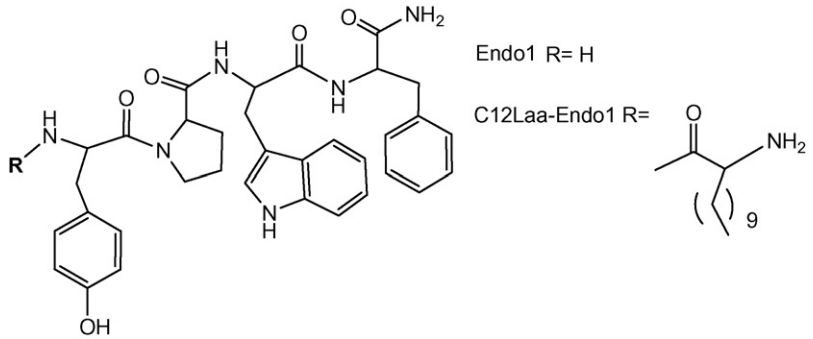

Fig. 1. Structures of Endo-1 and C12Laa-Endo-1.

at a $\lambda = 214$ nm for detection. The purification was performed using a linear solvent gradient from 80% solvent A (0.1% TFA in water) to 100% solvent B (90% acetonitrile, 0.1% TFA in water) in 35 min at a flow rate of 5 mL/min. The purity of the preparative HPLC fractions was determined by electrospray ionization mass spectrometry (ESI-MS; PerkinElmer Sciex API 3000) and analytical RP-HPLC (Shimadzu; SCL-10AVP system controller, FCV-10ALVP pump, and SPD-6A UV detector, wavelength of 214 nm) using a C18 column (Vydac® 218TP^{TM}; size 5 μm, 4.6 mm × 250 mm, Grace) with a flow rate of 1 mL/min and a linear gradient from 80% solvent A to 100% solvent B in 25 min. The fractions containing pure peptide were combined and lyophilized to afford the peptides as white solids.

2.3. Preparation of liposomes

Liposomal formulations were prepared by hydration of freeze-dried monophase systems (Liang et al., 2005). Briefly, C12Laa-Endo-1 (1.9 mg, 2.35 μmol), sucrose (95 mg, 278 μmol) and egg phosphatidylcholine (18 mg, 23.1 μmol) were dissolved completely in *tert*-butanol and water (1:1, v/v, 5 mL). The mixture was snap frozen with dry ice/acetone and was subsequently lyophilized at a condenser temperature of −70 °C and a pressure of less than 10^{-1} mbar (Freezone 6, Model 79340, Labconco, MO, USA). The resulting solid matrix was then rehydrated with 2 mL of distilled-water, and liposomal dispersions were formed upon being vortexed for 5 min at room temperature. The coarse liposome suspension was finally extruded five times through 100 nm polycarbonate membranes under pressurized nitrogen. A portion of the extrudates was centrifuged (200,000 × *g*, 45 min, 4 °C, TLX ultracentrifuge, Beckman) and the supernatant from this centrifugation was analysed for lipopeptide by LC–MS to confirm that all non-liposome entrapped C12Laa-Endo-1 was removed by the extrusion process by virtue of it being insoluble in water when not encapsulated.

Endo-1 (1.7 mg, 2.78 μmol) was also formulated with liposomes using the same procedure.

2.4. Characterization of liposomes

Size and polydispersity of liposomal dispersions were measured by photon correlation spectroscopy using a Zetasizer 3000^{TM} (Malvern Instruments, Malvern, UK). Measurements were carried out at room temperature and a 90 °C detection angle.

The entrapment efficiency of C12Laa-Endo-1 within liposomes was estimated by measuring the concentration of lipopeptide in the liposome extrudate. 90 μL of liposome extrudate was withdrawn and dissolved into 10 μL of *tert*-butanol, and the solution was subjected to quantitative analysis using electrospray liquid chromatography coupled to mass spectrometry (LC–MS). We could not accurately measure the amount of Endo-1 in liposomes with the same confidence as the non-encapsulated peptide is water soluble and so would not necessarily all be removed during the extrusion process. The Endo-1 liposomes were purely prepared for direct comparison in the cell assays.

2.5. Quantification of lipopeptides

The concentration of lipopeptide in all samples was determined using LC–MS. Chromatographic separation was carried out on a Shimadzu HPLC system with C18 column (Luna 5 μm, 2.0 mm × 50 mm) using a solvent gradient from 100% solvent A to 90% solvent B in 5.3 min (solvent A = 0.1% formic acid in water; solvent B = 90% acetonitrile and 0.1% formic acid), and flow rate was 0.3 mL/min. Mass spectra were recorded on a PerkinElmer Sciex API 3000 triple quadrupole mass spectrometer, controlled using Sample Control 1.4 software in selected ion monitoring mode (SIM). Data was analysed using Multiview and MacQuan 1.6 software packages (PerkinElmer Sciex, Toronto, Canada).

2.6. Caco-2 cell line

Caco-2 cells were maintained in T-75 flasks with DMEM supplemented with 10% FBS, 1% L-glutamine and 1% nonessential amino acids at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The medium was changed every other day, and cells were subcultured once 80% confluence was reached using 0.25% trypsin.

2.7. Caco-2 cells homogenate stability assay

A cell suspension of approximately 5×10^5 cells/mL (passage number 59) was used to seed a 96-well plate for the stability assay. The media, which consisted of DMEM supplemented with 10% FBS, 1% L-glutamine, 1% nonessential amino acids and 1% of 100 U/mL penicillin/streptomycin, was changed every other day until the 25th day. On this day, the media was removed from the wells and each well washed with 0.2% EDTA (100 μL ×2) solution followed by washing three times with Hanks' balanced salt solution containing 25 mM Hepes (pH 7.4). Finally, 100 μL of this buffer was placed in each well and the plate was cooled in ice.

The cells in each well were then disrupted by a 2 s pulse with a Sonics Vibracell Ultrasonic processor set at 40% amplitude with 130w mode. The 96-well plate was centrifuged at 2000 rpm for 5 min to remove cell debris. Three of the wells' supernatant were set aside and assayed for total protein content using the Bio-Rad protein assay standardized with bovine serum albumin (BSA; Sigma–Aldrich). The supernatant of homogenized cells was adjusted to a total protein content of 0.6–0.9 mg/mL with buffer, and then transferred into a clean 96-well plate. The compounds to be tested were dissolved in HBSS–Hepes buffer with 1% addition of dimethyl sulfoxide (DMSO) to a concentration of approximately 200 μM. 100 μL of this solution or suspension was added to each well containing 100 μL of cell homogenate. Samples (10 μL) were taken at selected time points over 120 min and immediately added to 5 μL of TFA to stop digestion and then diluted with 85 μL of water. All collected samples were stored at −20 °C. This assay was performed at 37 °C shaking at 400 rpm. Each collected and diluted sample (10 μL) was analysed by LC/MS. In the case of liposome formulated peptides, 10% *tert*-butanol was added

to the collected sample to disrupt liposome and solubilize the lipopeptide.

The concentration of the intact test compound in each sample was determined by LC/ESMS using the procedure described above. The stability assay on each compound was conducted in quadruplicate and the concentrations were normalized as a percentage of the initial concentration.

2.8. Caco-2 cell monolayer permeability assay

Cells were seeded onto polycarbonate cell culture inserts (Transwell®; 0.45 μm × 6.5 mm diameter) at a concentration of approximately 1×10^6 cells/mL (passage 58). DMEM was supplemented with 10% of FBS, 1% of L-glutamine, 1% of nonessential amino acids and 1% of 100 U/mL penicillin/streptomycin. The media was changed every other day (0.6 mL in the basolateral chamber and 0.1 mL to the apical chamber) and the monolayers were used 21 days after seeding. Transepithelial electrical resistance (TEER) of the monolayer using the Millicell-ERS system (Millipore Corporation, Bedford, MA) and apparent permeability (P_{app}) of radiolabeled [^{14}C]-D-mannitol (0.09 mCi/mL in EtOH:water = 9:1) were measured to determine the integrity of the monolayers.

Permeability studies were carried out in HBSS–Hepes buffer at 37 °C. The peptides and liposome formulated peptides were made up to approximately 200 μM in HBSS–Hepes buffer with 1% addition of DMSO. Prior to beginning the experiments, the monolayers were washed with pre-warmed HBSS–Hepes buffer, incubated for 30 min. At the start of the experiment, the apical chamber was emptied and 100 μL of test compound solution was added.

The permeability study was performed in quadruplicate at 37 °C shaking in a Heridolf Titramax shaker at 400 rpm. Samples (400 μL) were taken from the basolateral chamber at regular time points over 150 min and replaced with same volume of fresh buffer. At the end of the experiment, 50 μL of the solution remaining in the apical chamber was also collected and analysed. All the collected samples were stored at −20 °C to limit degradation. 10 μL of each sample was injected into the LC/ESMS and quantified as described above. The apparent permeability (P_{app}) of each compound was calculated using the following formula.

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

dC/dt is the steady-state rate of change in the chemical concentration (M/s) or radiochemical concentration (DPM/mL s) in the basolateral chamber, V_r volume of the receiver chamber (mL), A surface area of the cell monolayers C_0 initial concentration in the donor chamber (M or DPM/mL).

3. Results

3.1. Peptide synthesis and liposomal formulation

The yields of purified peptides were 51.8 and 14.1% for Endo-1 and C12Laa-Endo-1, respectively. The purity of both peptides was found to be above 95% as determined by analytical RP-HPLC under two different HPLC conditions, and both peptides gave the expected $[M+H]^+$ ion on ESI-MS (Endo-1 $[M+H]^+ = 611.3$; C12Laa-Endo-1 $[M+H]^+ = 808.6$). C12Laa-Endo-1 was formulated within liposomes by hydration of freeze-dried lipid matrices, and the lipopeptide-loaded liposomes were formed spontaneously having a size of 164 ± 15 nm with a narrow size distribution (PDI < 0.3). A remarkable characteristic of the liposomes in this study was their physical stability. They remained stable for more than 3 months at 4 °C after hydration, with no significant increase of particle size and polydispersity as measured by Zetasizer.

Extrusion of C12Laa-Endo-1 coarse liposome suspension through 100 nm membranes successfully removed all non-encapsulated, insoluble lipopeptide. This was confirmed when no lipopeptide was detected in the supernatant of the centrifuged liposome extrudates. In-house studies to investigate the micelle forming properties of C12Laa-Endo-1 were also performed using the change in absorptivity of 1,6-diphenyl-1,3,5-hexatriene, which occurs in aqueous systems upon its disposition within the hydrophobic domain of micelles (Zhang et al., 1996). In such studies, no change in absorptivity was observed when lipopeptide, C12Laa-Endo-1 was dispersed in distilled-water and equilibrated for 24 h at room temperature, suggesting that under these conditions the lipopeptide did not spontaneously form micelles. Therefore, C12Laa-Endo-1 was either associated with liposomes or filtered out of the solution during extrusion, which is in agreement with previous similar study (Liang et al., 2005). The entrapment efficiency of C12Laa-Endo-1 within liposomes prepared by this procedure was then quantified by measuring the amount of lipopeptide in the extrudate after removal of non-entrapped lipopeptide through 100 nm membrane, and was found to be above 86.6% as determined by LC/MS analysis. For comparison in the *in vitro* experiments, a liposomal delivery system containing unmodified peptide Endo-1 was prepared using the same procedure, although the exact amount of peptides encapsulated within liposomes could not determined in this case because the peptide was water soluble and so we cannot be confident that all non-entrapped peptide is removed during extrusion.

3.2. Enzymatic stability assay

Endo-1 is very rapidly degraded by the digestive enzymes present in a homogenate of Caco-2 cells having a half life of only ∼5 min. When this parent peptide was formulated into liposomes, a 3-fold increase in stability was observed with a $t_{1/2} = 14.5$ min (Fig. 2). The stability of free C12-Endo-1 could not be determined due to its lack of water solubility. When the assay was attempted, the compound was seen to precipitate out of solution and no reproducible measurements of compound concentration could be obtained. It is possible that the lipophilic peptide adhered to the plastic assay plate during the procedure. The liposome-encapsulated C12-Endo-1, by comparison was easily solubilized in the assay buffer and provided excellent, reproducible measurements over time. It can be seen in Fig. 2 that the encapsulated lipophilic peptide exhibited remarkable

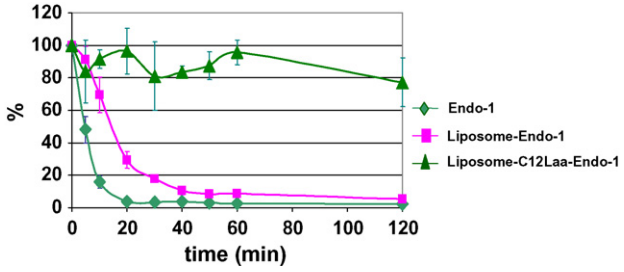

Fig. 2. Enzymatic degradation profile in Caco-2 cells homogenates. Each test compound (100 μM) was treated with homogenates (0.6–0.9 mg/mL). The samples were analysed by LC/ESIMS and normalized as a percentage of initial concentration. Each point represents the mean ± S.D. ($n = 4$).

stability with almost 80% of the compound remaining intact after the 2 h duration of the experiment.

3.3. Permeability assay

The integrity of the Caco-2 cell monolayers was evaluated by measurement of the transepithelial electrical resistance (TEER) and ^{14}C-mannitol permeability. The TEER values were between 2.5 and 3.8 kΩ cm^2 at the beginning of the assay, with changes of no more than ±0.5 kΩ cm^2 (within 13.2–20%) observed after completion of the assay. The P_{app} of ^{14}C-mannitol ($1.46 \times 10^{-6} \pm 1.28 \times 10^{-7}$ cm/s) was consistent with confluent, polarized monolayers with appropriate tight junctions in place.

The liposome formulated peptides exhibited approximately 2-fold greater permeability than the free peptides (Fig. 3; Table 1). Both liposome formulated Endo-1 and C12Laa-Endo-1 showed similar P_{app} values despite a large difference in their lipophilicity.

The poor water solubility of unformulated C12Laa-Endo-1 was again a problem in this assay. While the unformulated C12Laa-Endo-1 could be observed in the samples from the basolateral chamber, the compound was seen precipitating out in the apical layer in the course of the experiment and it is possible that the P_{app} value obtained may be an underestimation of the real permeability of the compound due to precipitation adherence to the culture plates.

4. Discussion

The conjugation of the N-terminus of the peptide with Laas was able to increase enzymatic stability and membrane permeability, however the use of Laas with longer side lipid chains

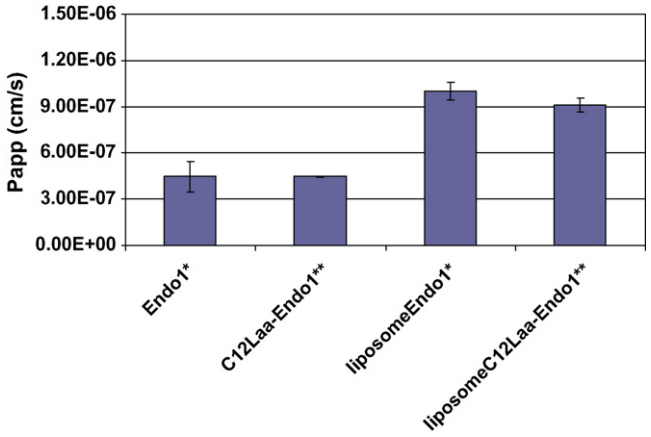

Fig. 3. P_{app} values for Endo-1 and C12Laa-Endo-1 both encapsulated in liposomes and without liposome formulation. Values are an average of four replicates and error bars are ±S.D. ($n = 4$). One way ANOVA analysis of the data showed that the difference in groups marked as (*) was statistically significant as was the difference in the (**) groups ($P < 0.05$).

such as C12Laa resulted in a lipopeptide that was sparingly soluble in water (Koda et al., 2008). As mentioned previously, the poor water solubility of C12Laa Endo-1 presented a serious problem for the *in vitro* stability and permeability assay during our study. A liposomal formulation was then investigated to address the solubility issue of the lipophilic peptide, which could provide further possible protection from enzymatic degradation and improvement of the permeability. Using the technique of hydration of freeze-dried monophase systems, Endo-1 was incorporated within liposome very conveniently and efficiently due to the lipid chain of lipopeptide, and liposomes with high peptide loading and homogeneous morphological characteristics were obtained. This method resulted in encapsulation of more than 85% of the lipopeptide and produced liposomes with a consistent size of less than 200 nm and a narrow size distribution. This entrapment efficiency is much higher in comparison to small native peptides, which are normally very difficult to entrap within liposomes due to their highly hydrophilic properties and low molecular weights. With the hydration of freeze-dried monophase system procedure, the end product is also in solid form which could be hydrated to form liposomes by vortex or sonication prior to use. This provides maximum stability and could benefit the storage and shelf-life of the product, especially for some unstable compounds such as peptides.

The liposomal encapsulation of the peptides provided significant protection against digestive enzymes. This was most marked in the stability of the liposome formulated C12Laa-

Table 1
P_{app}, $t_{1/2}$ and estimated log P values for Endo-1 and C12Laa-Endo-1 with and without the liposomal formulation

	log P[a]	$t_{1/2}$ (min)	P_{app} (cm/s) × 10^{-7}
Endo-1	1.59	5.0	4.47 ± 0.99
C12Laa-Endo-1	5.66	N.D.	4.46 ± 0.05
Liposome-Endo-1	–	14.5	10 ± 0.61
Liposome-C12Laa-Endo-1	–	>120 (77.2% remains after 2 h)	9.11 ± 0.45

[a] The octanol–water partition coefficients (log P) were estimated through Molinspiration Property Calculation Service (http://www.molinspiration.com) using the JME molecular editor (version April 2006).

Endo-1 which exhibited a $t_{1/2}$ of more than 2 h. This is remarkable stability for a small, linear peptide in this cell homogenate and is very promising in terms of drug candidate development. These native opioid peptides such as Endo-1 have half lives in plasma and Caco-2 cell homogenate of minutes and so this greatly improved stability is a significant breakthrough. The stability of the native peptide was somewhat improved by liposomal formulation but it is clear that the C12Laa-Endo-1 stability is a result of a combination of both the N-terminus modification and the formulation.

The apparent permeability of C12Laa-Endo-1 was similar to that of the parent peptide, Endo-1. The increased lipophilicity provided by the introduction of C12Laa would be expected to significantly increase the passive diffusion of the peptide compared to native Endo-1. An increase in apparent permeability was observed for N-terminus analogues bearing shorter Laa (C8 and C10) (Koda et al., 2008) but the insolubility of C12Laa-Endo-1 resulted in a drop in the P_{app} value due to precipitation of the peptide onto the surface of the cells in the apical layer, adherence of the peptide to the plastic ware and, possibly, absorption into the cell membrane of the Caco-2 cells. Liposomal formulation was able to improve the permeability of both the native peptide and the lipidic analogue by approximately the same amount. As a result, there is little difference in permeability across Caco-2 cell monolayers between the native peptide and the C12Laa conjugate. However, with the remarkable improvement in stability of C12Laa-Endo-1 combined with the 2-fold increase in permeability, it is clear that the lipidic analogue is the more promising lead compound. Similar studies on liposome-encapsulated insulin demonstrated good correlation between Caco-2 cell monolayer permeability and *in vivo* bioactivity of insulin after oral administration (Iwanaga et al., 1999; Degim et al., 2004). This bodes well for future *in vivo* experiments on liposome-encapsulated C12Laa-Endo-1.

The encapsulation of C12Laa-Endo-1 into phosphatidylcholine liposomes clearly improves the stability and permeability of the peptide. However, the most immediate and obvious effect of the formulation was to make the lipophilic peptide water compatible. The formulation allowed the lipopeptide to be examined using the standard *in vitro* assays which had been very difficult, if not impossible to perform on the lipopeptide alone. It will also allow the preparation of injectable solutions for *in vivo* experiments.

5. Conclusion

The lipidic analogue of a native opioid peptide, C12Laa-Endo-1, was successfully and efficiently incorporated into liposomes consisting of phosphatidylcholine and sucrose. The liposomes formed stable emulsions in buffer and allowed the dispersion of the lipopeptide in aqueous media. The liposome-formulated lipopeptide exhibited improved metabolic stability compared with the liposomal preparation of the parent peptide (Endo-1) which was formulated by the same procedure. The permeability across Caco-2 cell monolayers of both peptides was also improved ∼2-fold with the liposomal formulation. The liposome encapsulation strategy is clearly one worth further close examination for the delivery of short, neuroactive lipopeptides.

References

Abbadie, C., Rossi, G.C., Orciuolo, A., Zadina, J.E., Pasternak, G.W., 2002. Anatomical and functional correlation of the endomorphins with μ-opioid receptor splice variants. Eur. J. Neurosci. 16, 1075–1082.

Adessi, C., Soto, C., 2002. Converting a peptide into a drug: strategies to improve stability and bioavailability. Curr. Med. Chem. 9, 963–978.

Alewood, P., Alewood, D., Miranda, L., Love, S., Meutermans, W., Wilson, D., 1997. Rapid *in situ* neutralization protocols for Boc and Fmoc solid-phase chemistries. Methods Enzymol. 289, 14–29.

Artursson, P., Karlsson, J., 1991. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem. Biophys. Res. Commun. 175, 880–885.

Artursson, P., Palm, K., Luthman, K., 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. 46, 27–43.

Blanchfield, J., Toth, I., 2004. Lipid, sugar and liposaccharide based delivery systems 2. Curr. Med. Chem. 11, 2375–2382.

Blanchfield, J.T., Dutton, J.L., Hogg, R.C., Gallagher, O.P., Craik, D.J., Jones, A., Adams, D.J., Lewis, R.J., Alewood, P.F., Toth, I., 2003. Synthesis, structure elucidation, *in vitro* biological activity, toxicity, and Caco-2 cell permeability of lipophilic analogues of alpha-conotoxin MII. J. Med. Chem. 46, 1266–1272.

Chhabra, S.R., Hothi, B., Evans, D.J., White, P.D., Bycroft, B.W., Chan, W.C., 1998. An appraisal of new variants of Dde amine protecting group for solid phase peptide synthesis. Tetrahedron Lett. 39, 1603–1606.

Csullog, E., Joo, G., Toth, G., Dobos, D., Benedek, G., Horvath, G., 2001. Antinociceptive effect of continuous intrathecal administration of endomorphin-1. Pain 94, 31–38.

Degim, Z., Unal, N., Essiz, D., Abbasoglu, U., 2004. The effect of various liposome formulations on insulin penetration across Caco-2 cell monolayer. Life Sci. 75, 2819–2827.

Fahr, A., van Hoogevest, P., May, S., Bergstrand, N., Leigh, M.L.S., 2005. Transfer of lipophilic drugs between liposomal membranes and biological interfaces: consequences for drug delivery. Eur. J. Pharm. Sci. 26, 251–265.

Flinn, N., Coppard, S., Toth, I., 1996. Oral absorption studies of lipidic conjugates of thyrotropin releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH). Int. J. Pharm. 137, 33–39.

Forssen, E., Willis, M., 1998. Ligand-targeted liposomes. Adv. Drug Deliv. Rev. 29, 249–271.

Gibbons, W.A., Hughes, R.A., Charalambous, M., Christodoulou, M., Szeto, A., Aulabaugh, A.E., Mascagni, P., Toth, I., 1990. Lipidic peptides 1: synthesis, resolution and structural elucidation of lipidic amino acids and their homo oligomers and heterooligomers. Liebigs Ann. Chem., 1175–1183.

Goldberg, I.E., Rossi, G.C., Letchworth, S.R., Mathis, J.P., Ryan-Moro, J., Leventhal, L., Su, W., Emmel, D., Bolan, E.A., Pasternak, G.W., 1998. Pharmacological characterization of endomorphin-1 and endomorphin-2 in mouse brain. J. Pharmacol. Exp. Ther. 286, 1007–1013.

Gutman, R.L., Peacock, G., Lu, D.R., 2000. Targeted drug delivery for brain cancer treatment. J. Controll. Release 65, 31–41.

Hackler, L., Zadina, J.E., Ge, L.J., Kastin, A.J., 1997. Isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. Peptides 18, 1635–1639.

Hidalgo, I.J., Li, J.B., 1996. Carrier mediated transport and efflux mechanisms in Caco-2 cells. Adv. Drug Deliv. Rev. 22, 53–66.

Higashida, H., Hoshi, N., Knijnik, R., Zadina, J.E., Kastin, A.J., 1998. Endomorphins inhibit high-threshold Ca^{2+} channel currents in rodent NG108-15 cells overexpressing μ-opioid receptors. J. Physiol. (Lond.) 507, 71–75.

Howell, S., Brewis, I.A., Hooper, N.M., Kenny, A.J., Turner, A.J., 1993. Mosaic expression of membrane peptidases by confluent cultures of Caco-2 cells. FEBS Lett. 317, 109–112.

Howell, S., Kenny, A.J., Turner, A.J., 1992. A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. Biochem. J. 284, 595–601.

Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y., Oku, N., 1999. Application of surface coated liposomes for oral delivery of peptide: effects of coating the liposome's surface on the GI transit of insulin. J. Pharm. Sci. 88, 248–252.

Kastin, A.J., Pan, W.H., Maness, L.M., Banks, W.A., 1999. Peptides crossing the blood–brain barrier: some unusual observations. Brain Res. 848, 96–100.

Koda, Y., Del Borgo, M., Lazarus, L.H., Okada, Y., Toth, I., Blanchfield, J.T., Jan. 2008. Synthesis and in vitro evaluation of a library of modified Endomorphin 1 peptides. Bioorg. Med. Chem., submitted for publication.

Liang, M.T., Davies, N.M., Toth, I., 2005. Encapsulation of lipopeptides within liposomes: effect of number of lipid chains, chain length and method of liposome preparation. Int. J. Pharm. 301, 247–254.

Martin-Schild, S., Gerall, A.A., Kastin, A.J., Zadina, J.E., 1999. Differential distribution of endomorphin 1 and endomorphin 2-like immunoreactivities in the CNS of the rodent. J. Comp. Neurol. 405, 450–471.

Pauletti, G.M., Gangwar, S., Siahaan, T.J., Aube, J., Borchardt, R.T., 1997. Improvement of oral peptide bioavailability: peptidomimetics and prodrug strategies. Adv. Drug Deliv. Rev. 27, 235–256.

Rousselle, C., Clair, P., Smirnova, M., Kolesnikov, Y., Pasternak, G.W., Gac-Breton, S., Rees, A.R., Scherrmann, J.M., Temsamani, J., 2003. Improved brain uptake and pharmacological activity of dalargin using a peptide-vector-mediated strategy. J. Pharmacol. Exp. Ther. 306, 371–376.

Stone, L.S., Fairbanks, C.A., Laughlin, T.M., Nguyen, H.O., Bushy, T.M., Wessendorf, M.W., Wilcox, G.L., 1997. Spinal analgesic actions of the new endogenous opioid peptides endomorphin-1 and -2. Neuroreport 8, 3131–3135.

Toth, I., Flinn, N., Hillery, A., Gibbons, W.A., Artursson, P., 1994. Lipidic conjugates of luteinizing hormone releasing hormone $(LHRH)^+$ and thyrotropin releasing hormone $(TRH)^+$ that release and protect the native hormones in homogenates of human intestinal epithelial (Caco-2) cells. Int. J. Pharm. 105, 241–247.

Wenk, M.R., Fahr, A., Reszka, R., Seelig, J., 1996. Paclitaxel partitioning into lipid bilayers. J. Pharm. Sci. 85, 228–231.

Witt, K.A., Huber, J.D., Egleton, R.D., Roberts, M.J., Bentley, M.D., Guo, L.H., Wei, H.B., Yamamura, H.I., Davis, T.P., 2001. Pharmacodynamic and pharmacokinetic characterization of poly(ethylene glycol) conjugation to met-enkephalin analog [D-Pen(2), D-Pen(5)]-enkephalin (DPDPE). J. Pharmacol. Exp. Ther. 298, 848–856.

Zadina, J.E., Hackler, L., Ge, L.-J., Kastin, A., 1997. Potent and selective endogenous agonist the μ-opioid receptor. Nature 386, 499–502.

Zhang, X.C., Jackson, J.K., Burt, H.M., 1996. Determination of surfactant critical micelle concentration by a novel fluorescence depolarization technique. J. Biochem. Biophys. Methods 31, 145–150.

Zornoza, T., Cano-Cebrian, M.J., Nalda-Molina, R., Guerri, C., Granero, L., Polache, A., 2004. Assessment and modulation of acamprosate intestinal absorption: comparative studies using in situ, *in vitro* (Caco-2 cell monolayers) and *in vivo* models. Eur. J. Pharm. Sci. 22, 347–356.