Investigation of the Route of Absorption of Lipid and Sugar Modified Leu-Enkephalin Analogues and Their Enzymatic Stability Using the Caco-2 Cell Monolayer System

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Abstract: It has been demonstrated that conjugation of lipoamino acids or glucose units to the endogenous opioid peptide, Leu-enkephalin can significantly improve the peptide's metabolic stability and absorption across biological barriers. The purpose of this study was to investigate the possible involvement of specific carrier proteins in the absorption of these peptide conjugates. A series of lipo- glycol- and liposaccharide peptide conjugates were synthesised and examined using the Caco-2 monolayer assay for evidence of interaction with the human H^+-coupled oligopeptide transporter (hPepT1), glucose transporters and the multidrug resistance efflux pump, p-glycoprotein. The investigation involved determining the apparent permeability of each compound in the absence of any inhibitors and comparing this to the apparent permeabilities of each compound in the presence of glycylsarcosine, glucose or vinblastine, respective inhibitors of the above mentioned transporters. None of the peptide conjugates were found to be substrates for p-glycoprotein. Of the six peptide conjugates examined, only the C-terminus glucose conjugate of Leu-enkephalin (Enk-glu) showed evidence of transport by both glucose transporters and hPepT1. In contrast, N-terminus conjugation of both lipids and sugars appeared to provide the greatest protection against enzymatic degradation.

Key Words: Leu-enkephalin, Caco-2 cells, drug delivery liposaccharides, p-glycoprotein hPepT1, glucose transporter, lipoamino acids.

INTRODUCTION

Leu-enkephalin, (NH_2-Tyr-Gly-Gly-Phe-Leu-NH_2), is one of a family of endogenous opioid peptides that has been proven to provide analgesic effect when administered centrally in rats [1]. The development of any of the endogenous opioid peptides into viable drug candidates is hampered by their susceptibility to enzymatic degradation and poor absorption across biological barriers such as the GI tract and blood brain barrier (BBB). Research into chemical modifications designed to enhance the bioavailability and BBB permeability of peptides such as Leu-enkaphalin is a dynamic and vital area of interest for several groups throughout the world [2]. Glycosylation of opioid peptides has proven to be particularly effective in increasing the BBB penetration of the compounds and improving the opioid receptor binding potency of some analogues [3-6].

In our own studies, we have shown that introducing lipophilic groups in the form of lipoamino acids (Laas) to the Leu-enkephalin sequence can increase the peptides' metabolic stability and opioid receptor binding activity in the Guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. More significantly however, the C-terminus conjugation of one or two glucuronic acid units to the peptide led to a dramatic increase in activity - of up to 40-fold – in these assays with improved δ-opioid receptor selectivity [7,8]. We have also showed that these glucose and lipid modified enkephalin analogues show greatly enhanced permeability across the Caco-2 cell monolayer in *in vitro* assays of absorption [9].

There is some evidence in the literature of transcytosis and the paracellular mechanisms of transport for various enkephalin analogues [10-12]. There is also some evidence that some small peptides, including the enkephalins are absorbed in small amounts *via* a saturable transport mechanism [13,14], and the large neutral amino acid carrier has been implicated in the transport of one analogue [15]. While we had observed significant increases in *in vitro* absorption of our lipo- glycol- and liposaccharide analogues of enkephalin, we have not, to date, investigated the method of absorption of our analogues. This study therefore is designed to determine if our analogues are substrates for the hPepT1 or glucose transporters or the p-glycoprotein efflux pump.

To study the transport mechanisms for various modified Leu-enkephalins, the Caco-2 cell monolayer assay was used. The Caco-2 cell line is developed from human colon adenocarcinoma cells and differentiate well in culture within a 21-day period [16]. These Caco-2 cells resemble the human small intestine in various aspects, including the expression of digestive enzymes [17] and transporter proteins [18] as well as the formation of tight junction between the cells. BBB has certain transporters that are closely related, structurally and functionally, to the transporters in the small intestine [19,20]. Therefore, the structure-transporter relationship found for these peptide

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analogues using Caco-2 cell monolayer may elucidate their possible transport mechanisms across BBB.

hPepT1 (human H^+-coupled oligopeptide transporter), glucose transporters and p-glycoprotein are all expressed in fully differentiated Caco-2 cells. Permeability across Caco-2 cell monolayer would be decreased significantly in the presence of the transporter inhibitor if the analogue tested was a substrate of that particular transporter. Oligopeptide transporters have been found to be responsible for the uptake of enkephalin in yeast [14], and previous studies have provided evidence that sugar modified analogues can result in the compound's use of glucose transporter as the transport mechanism [20,21].

As they are models of the intestinal epithelial cells, Caco-2 cells express predominately the Na^+-dependant active glucose transporter (SGLT1) in the apical membrane and GLUT2 in the basolateral membrane for glucose transport [21-25]. However, it should be noted that Caco-2 cells also express low levels of GLUT1 and GLUT3 [22,23], though these transporters are not expressed in "healthy" human intestinal epithelial cells. In this study we were looking for any evidence of glucose transporter involvement and so glucose itself was chosen as a simple competitive inhibitor. A more specific study is planned using a selective inhibitor of SGLT1, phloridzine [21] to determine the nature of the glucose transporter involved.

P-glycoprotein, the efflux system, is not only expressed in small intestine but also in the BBB [26] and therefore further challenges the delivery of CNS pharmaceutics. The synthetic opioid peptide, DPDPE ([D-pen2, D-pen5] enkephalin (where pen = penicillamine)) and various other enkephalin analogues have been shown to be substrates for this efflux transporter [11,27]. Should the lipid and sugar modified Leu-enkephalins prove to be substrates for this efflux system, a p-glycoprotein inhibitor may be considered to be included in the pharmaceutical formulation in the future in order to improve their bioavailability.

The aim of this project is to provide a preliminary evaluation of the possible involvement of hPepT1, glucose transporter or p-glycoprotein in the transport of structurally modified Leu-enkephalin peptides across the Caco-2 cell monolayer. The analogues studies are detailed in Fig. **1** and include the native Leu-enkephalin peptide, a C-terminus C_{12}-Laa-enkephalin conjugate and a C-terminus glucuronic acid conjugate in addition to an N-terminus glucose conjugate linked to the peptide *via* a succinic acid linker and two liposaccharide conjugates. The interaction of these compounds with the transporters was examined by investigating the change in permeability across the Caco-2 cell monolayer with the addition of a transporter inhibitor. In addition, the compounds' ability to resist metabolic degradation was also assessed. The structure-transporter relationship established by this study, in combination with the enzyme stability observed, will allow for the design of a new analogue of enkephalin which exhibits improved permeability across both the GI tract and BBB.

EXPERIMENTAL

General

MBHA resin and protected amino acids were obtained from Novabiochem (Melbourne, Australia). DMF was purchased from Lomb Scientific (Australia) and TFA was purchased from AusPep (Australia) and both were of peptide synthesis grade. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Transwell polycarbonate inserts came from Costar (Cambridge, MA) and cell culture reagents were purchased from Gibco-BRL (Grand Island, NY).

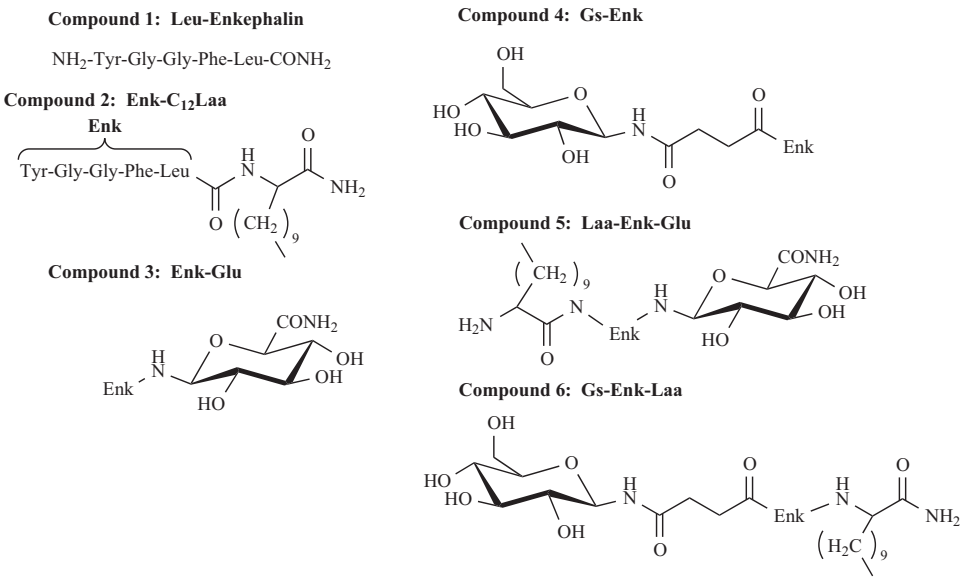

Fig. (1). The Leu-Enkephalin analogues studied.

2-(t-Butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C_{12}Laa)

Sodium (3.81 g, 166 mmol) was dissolved in ethanol (100 mL) under nitrogen and diethyl acetamido malonate (30.00 g, 138 mmol) was added followed by 1-bromodecane (42.76 g, 193 mmol). The solution was refluxed overnight under a nitrogen atmosphere. Upon cooling the mixture was poured onto crushed ice (600 mL) and stirred. The precipitated product was collected and air dried. The crude product was refluxed overnight in a solution of HCl:DMF (9:1, 200 mL). Upon cooling, the precipitated product was collected, washed with ice water and air dried to afford α-aminododecanoic acid hydrochloride (37.57g, 97% MS $[M+H]^+$ *m/z*: 216 ($[M+H]^+$ of $C_{12}H_{25}NO_2$ requires 216)). 2-Amino-D,L-dodecanoic acid hydrochloride (24.24 g, 96.3 mmol) was suspended in a solution of *t*-butanol:water (2:3, 500 mL) and the pH adjusted to 13 with sodium hydroxide (5 M). Di-*t*-butyldicarbonate (31.52 g, 144 mmol) in *t*-butanol (50 mL) was added. The solution was stirred overnight maintaining the pH at 13. The mixture was diluted with water (200 mL) and solid citric acid was added to pH 3. The mixture was extracted with ethyl acetate (5 x 150 mL), the combined extracts dried ($MgSO_4$) and evaporated to yield a crude product (oil and crystals). This product was recrystallised from warm acetonitrile to afford α-(*tert*-butoxycarbonylamino) dodecanoic acid (21.98 g, 72%). M.p. 61-63° C, literature m.p. 62-64° C [28]. MS *m/z*: $[M+H]^+$ 316 ($[M+H]^+$ of $C_{17}H_{33}NO_4$ requires 316), 260. ^{1}H NMR. (300MHz, $CDCl_3$): δ 7.55 (1H, br s, COOH), 5.09 (1H, d, amide NH); 4.29 (1H, m, α-CH); 1.9-1.5 (2H, m, β-CH_2); 1.43 (9H, s, $C(CH_3)_3$); 1.24 (16H, m, 8CH_2); 0.86 (3H, t, CH_3). ^{13}C NMR (75MHz, $CDCl_3$): δ 177.6, 155.6, 80.1, 53.4, 32.4, 31.9, 29.6, 29.5, 29.4, 29.3, 29.2, 28.3, 25.3, 22.7, 14.1.

Synthesis of Glucose Succinate Unit for Compounds 4 and 6

2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl Azide

1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose (5.00 g, 12.8 mmol) was dissolved in 10-15 mL dry CH_2Cl_2 under an inert atmosphere. Trimethylsilyl azide (4.24 mL, 32.1 mmol) and tin (IV) chloride (0.75 mL, 6.41 mmol) were added and the reaction allowed to proceed for 18 hours at room temperature. The solution was diluted with CH_2Cl_2 and washed twice with saturated $NaHCO_3$ and once with saturated NaCl. The organic phase was dried, filtered and concentrated to leave the title compound as a white solid (3.92 g, 82%). FAB MS ($C_{14}H_{19}N_3O_9$) 373.11 m/z (%): 331 $[M-N_3]^+$ (13), 396 $[M+Na]^+$ (7), 506 $[M+Cs]^+$ (100). ^{1}H-NMR ($CDCl_3$): δ 1.99, 2.02, 2.07, 2.09 (4s, 12H, 4OAc), 3.81 (m, 1H, H-5), 4.15, 4.27 (2m, 2H, H-6 and H-6'), 4.65 (d, 1H, H-1, $J_{1,2}$=8.8 Hz), 4.94 (t, 1H, H-2), 5.09, 5.21 (2t, 2H, H-3 and H-4).

2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamine

The glycosyl azide (above) (7.80 g, 20.9 mmol) was dissolved in 50 mL dry methanol and the solution placed under an inert atmosphere. 10% Pd on activated charcoal (300 mg) was added and the reaction maintained under a constant pressure of H_2 with vigorous stirring for 24 hours at room temperature. The suspension was filtered through celite and the solvent removed under reduced pressure to afford the title compound as a colourless syrup (6.70 g, 92%). The residue was then used immediately for the preparation of the glycosyl amide below. FAB MS ($C_{14}H_{21}NO_9$) 347.12 m/z (%): 169 (79), 331 $[M-NH_2]^+$ (34), 370 $[M+Na]^+$ (100).

N-(2,3,4,6-tetra-O-acetyl-b-D-glucopyranosyl)succinamic Acid

The glycosyl amine product of the previous reaction (6.70 g, 19.3 mmol) was dissolved in 80 mL dry CH_2Cl_2 at 0 °C. Dry pyridine (7.81 mL, 96.5 mmol) and DMAP (90 mg, 0.74 mmol) was added. A solution of succinic anhydride (4.83 g, 48.3 mmol) in dry CH_2Cl_2 was then added dropwise over 30 minutes and the reaction allowed to proceed for 30 minutes at 0 °C, and a further 3 hours at room temperature. The solution was then washed three times with 1 M HCl, once with saturated NaCl, then dried, filtered and concentrated to leave a white foam. The title compound was crystallised from ethyl acetate-hexane (6.61 g, 77%). This succinic acid linked glucose unit was then coupled to the N-terminus of a peptide while still on the resin using the standard HBTU coupling protocol. FAB MS ($C_{18}H_{25}NO_{12}$) 447.14 m/z (%): 331 $[M-NHCO(CH_2)_2COOH]^+$ (93), 448 $[M+H]^+$ (68), 470 $[M+Na]^+$ (100), 580 $[M+Cs]^+$ (55). ^{1}H-NMR ($CDCl_3$): δ 2.03, 2.05, 2.06, 2.08 (4s, 12H, 4OAc), 2.49 (t, 2H, CH_2CON), 2.70 (m, 2H, CH_2COO), 3.80 (m, 1H, H-5), 4.05 (dd, 1H, H-6), 4.28 (dd, 1H, H-6'), 4.92 (dd, 1H, H-2), 5.05 (dd, 1H, H-4), 5.24 (dd, 1H, H-1, $J_{1,2}$=9.0 Hz), 5.28 (dd, 1H, H-3), 6.52 (d, 1H, NH), 8.01 (br s, 1H, COOH).

Synthesis of C-terminus Glucuronic Acid Peptides, Compounds 3 and 5

1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronic Acid

D-Glucuronic acid (6.00 g, 30.9 mmol) was suspended in 85 mL acetic anhydride and stirred at 0 °C. Iodine (425 mg, 1.67 mmol) was added slowly and stirring continued for 2 hours at 0 °C, then a further 1 hour at room temperature. The solution was then cooled to 0 °C, 30 mL dry methanol added dropwise and the solution allowed to stand for 18 hours at room temperature. The reaction was concentrated and the residue taken up in 100 mL CH_2Cl_2, washed with 1 M $Na_2S_2O_3$, dried, filtered and concentrated. The white residue was taken up in a mixture of ether, hexane and $CHCl_3$ and concentrated again. Addition of ether, filtering and washing afforded the title compound as a fine white powder (9.20 g, 82%). FAB MS ($C_{14}H_{18}O_{11}$) 362.08 m/z (%): 303 $[M-OAc]^+$ (34), 325 $[M-OAc+Na]^+$ (64), 385 $[M+Na]^+$ (86), 407 $[M+2Na-H]^+$ (100). ^{1}H-NMR ($CDCl_3$): δ 2.02, 2.03, 2.04, 2.11 (4s, 12H, 4OAc), 4.24 (m, 1H, H-5), 5.13 (m, 1H, H-2), 5.29 (m, 2H, H-3 and H-4), 5.79 (d, 1H, H-1, $J_{1,2}$=7.3 Hz). Anal. Calcd for $C_{14}H_{18}O_{11}$: C, 46.41; H, 4.97. Found: C, 46.24; H, 5.01.

2,3,4-tri-O-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic Acid

The protected glucopyranuronic acid from the above reaction (2.00 g, 5.52 mmol) was dissolved in dry CH_2Cl_2 (40 mL) under an inert atmosphere. Trimethylsilyl azide (1.87 mL, 13.8 mmol) and tin (IV) chloride (0.32 mL, 2.76

mmol) was then added to the solution. The reaction was allowed to proceed for 18 hours at room temperature under an inert atmosphere. The solution was then diluted with CH_2Cl_2, washed twice with 1 M $KHSO_4$, dried, filtered and concentrated to yield the title compound as a white foam (1.64 g, 86%). FAB MS ($C_{12}H_{15}N_3O_9$) 345.08 m/z (%): 303 $[M\text{-}N_3]^+$ (35), 368 $[M+Na]^+$ (100), 390 $[M+2Na\text{-}H]^+$ (22). ^{1}H-NMR ($CDCl_3$): δ 2.02, 2.04, 2.07 (3s, 9H, 3OAc), 4.17 (d, 1H, H-5), 4.75 (d, 1H, H-1, $J_{1,2}$=8.8 Hz), 4.96 (m, 1H, H-2), 5.28 (m, 2H, H-3 and H-4). Anal. Calcd for $C_{12}H_{15}N_3O_9$: C, 41.74; H, 4.35; N, 12.17. Found: C, 41.65; H, 4.40; N, 12.19.

Immobilisation of 2,3,4-tri-O-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic Acid onto MBHA Resin

MBHA resin (1.96 g, 0.59 mmol/g substitution, 1.16 mmol) was swelled in dry DMF and deprotected as usual. The azide product of the previous reaction (1.21 g, 3.51 mmol) was dissolved in a minimum volume of dry DMF and cooled to 0° C. HOBt (0.54 g, 3.51 mmol) was added, followed by DIC (0.55 mL, 3.51 mmol) and the solution stirred for 10 minutes at 0 °C. The solution and DIEA (0.61 mL, 3.51 mmol) were then added to the resin and the suspension mixed for 3 hours. The resin was drained, washed and dried *in vacuo* over KOH to constant weight (2.33 g, 99%, 0.50 mmol/g loading).

First Amino acid Attachment

The dried resin from the above reaction (100 mg, 0.63 mmol g^{-1} loading, 63 μmol) was transferred to a solid-phase reaction vessel, washed and swelled in dry DMF under an inert atmosphere for at least 1 hour. The resin was then washed several times with dry THF and left as a slurry. The first protected amino acid (Boc-Leu) (0.25 mmol) was dissolved in dry THF, HOBt was added and the solution cooled to 0 °C. DIC (40 μL, 0.25 mmol) was then added and the entire solution added to the resin followed by tri-n-butylphosphine (47 μL, 0.19 mmol). The suspension was mixed for 18 hours under an inert atmosphere. The resin was then drained and washed well with DMF. The stepwise construction of the peptide then proceeded in the usual way.

General Peptide Synthesis

The peptides were synthesised on a 0.5 mmol scale using standard *in situ* neutralization solid phase peptide synthesis techniques [29] on MBHA resin. A quantiative ninhydrin test [30] was performed to monitor the coupling efficiency of each amino acid coupling and they were repeated if necessary until an efficiency of >99.4% was reached. Neat TFA was used to remove the Boc-protecting group.

When the desired sequence was completed the peptide on the resin was treated with 8mL of 1:7 solution of hydrazine:methanol to remove the acetate protecting groups on the glucose moieties. The vessel was shaken for 15 minutes and then washed with methanol. The process was repeated and the resin washed with DMF, DCM and finally with methanol and were allowed to air dry. The peptides were cleaved from the resin by treatment with 10 mL liquid HF, 1 mL *p*-cresol and 1 mL *p*-thiocresol at 0° C for 1 hour. After removal of the HF, the crude product was filtered and washed with 50% acetonitrile. The filtrate was lyophilized to yield the crude peptides that were dissolved in water with 0.1% TFA and filtered in preparation for HPLC purification.

Purification and Peptide Analysis

The peptides were purified by preparative HPLC with the gradient of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90% acetonitrile, 10% water) on a C_{18} RP-HPLC column (Vydac). The fractions collected were monitored for the peptide of interest using electrospray mass spectrometry (API3000). The purity of the fractions containing the peptide in question was examined using analytical RP-HPLC (Vydac) with C_{18} column. Pure fractions were combined and lyophilized to yield white powders. The purity of the final peptides were monitored by analytical HPLC and LC-MS.

Cell Culture

Caco-2 cells were maintained in culturing flasks in the absence of antibiotics in Dulbecco's modified Eagle Medium (DMEM) with L-glutamine and containing 10% Foetal Bovine serum (FBS) and 1% 10mM non-essential amino acid solution. The media was changed every second day. After reaching 80% confluence in the culturing flask, the cells were passaged by removing the medium and washing the cells with 0.53mM EDTA, followed by treatment with 1mL 0.05% trypsin at 37° C for one to three minutes to detach the Caco-2 cells from the bottom of the flask. Trypsinisation was halted by the addition of media to each flask and the cell suspension was centrifuged at 2000 rpm for 5 minutes. All medium was then removed and fresh medium added. 600μL of the medium containing 1% penicillin and streptomycin solution was added to the basolateral chamber of sterile 6.5mm transwells (Costar) with polycarbonate membranes with 0.4μm pore size. 100 μL of cell suspension with a density of 80-120 cells/mm^2 was then seeded onto the transwells membrane. The transwells plates were incubated at 37° C, 5% CO_2 environment with the medium in the apical chamber refreshed at least three hours post passage. The cells were incubated for 21 days with the medium containing antibiotic changed every second day.

Drug Transport Study

After 21 days of culturing, the well-formed Caco-2 cell monolayers were ready for use in drug transport study. A 200_M solution of each compound was prepared by dissolving the respective compounds in Hanks' balanced salt solution (HBSS) containing 25mM Hepes (pH 7.4). 0.5% DMSO was also added for lipophillic compounds, i.e. compounds 2 and 5 in Fig. **1**. 3.5% DMSO was required to dissolve Gs-Enk-Laa.(compound 6 in Fig. **1**) The stability of the compounds in the buffer was tested using LC-MS analysis.

The transport study was performed at 37° C. The media in the transwells was removed and the monolayers rinsed with 37° C HBSS-Hepes. Transepithelial electrical resistance (TEER) values were then measured to test the integrity of each batch of cells. Transport studies were carried out in the presence or absence of transporter inhibitors and they were performed at least in triplicate. A permeability assay

containing glucose was not performed for Enk-Laa, as there is no glucose moiety attached to the peptide. Moreover, only the standard permeability assay and one containing glycocylsarcosine were performed on the parent peptide, Leu-enkephalin since there was already strong evidence of rapid enzyme degradation of the compound and impermeability across Caco-2 cell monolayer in previous literature [9]. A complete summary of the experiments conducted on the peptides is provided in Table **1**.

To perform the permeability assay, 600µL and 100µL of nominated solution were added to the basolateral and apical chambers respectively (Table **1**). The transporter inhibitors used were 20mM glycylsarcosine for hPepT1 [31], 200µM glucose for glucose transporter and 35µM vinblastine [26] for p-glycoprotein. C^{14} Mannitol control tests were also performed when possible to assess the integrity of the Caco-2 cell monolayers. Three samples of 10µL of C^{14}Mannitol were collected before the study and 4 mL of scintillation fluid was added to each of these samples immediately. These samples were used to determine the initial concentration of mannitol using a liquid scintillation counting (Tri-Carb 2700 TR).

The plate was shaken in a Heidolf Titramax 1000 at shaking setting 3 (400rpm) to prevent the formation of an "unstirred water layer" which would be yet another barrier to drug permeability across the monolayer [32]. 400µL Samples were taken from the basolateral chamber for tests 1, 2 and 3, or alternatively, 80µL were taken from the apical chambers for tests 4 and 5 at 30, 90, 120 and 150 minutes. These samples were then analysed by LC-MS. The volume of the sample removed was replaced with HBSS-Hepes buffer at 37° C (tests 1 to 4) or HBSS-Hepes with 35µM vinblastine sulfate solution (test 5). At the end of the experiment, a 50µL sample from the donor chamber was collected from each transwell for analysis. TEER values were again measured and recorded to detect the possible toxicity of the compounds and to ensure the permeability values obtained were accurate.

All samples containing hydrophilic compounds (compound 1,3 and 4) were acidified with 2 or 5µL of 10% formic acid, whereas hydrophobic compounds were treated with the formic acid as well as 2 or 10µL of DMSO to prevent precipitation of peptides [9]. Samples containing ^{14}C mannitol were treated with 4 mL of scintillation fluid and the radioactivity measured using liquid scintillation counting.

LC-MS Analysis

All samples collected from the transport study were examined by LC-MS to determine the concentration of the peptide in question. HPLC (Shimadzu LC-10AT) was coupled with a quadrupole mass spectrometer (PE Sciex API 3000) operating in LC sync mode with positive ion electrospray and 1:10 splitter. A C_{18} column (Phenomenex, 5µM, 50 x 2.0 mm) was used in RP-HPLC with a flow rate of 0.3mL/min and a gradient of 100% solvent A for 0.3 minutes, increasing to 90% B over 4 minutes and remaining at 90% B for one minute. The volume of each injection was 10µL.

Each sample was analysed for the presence of the $[M+H]^+$ ions of both the peptide in question and Leu-enkephalin. A standard curve was generated using standards from 0.025 to 200µM. The concentration for each sample was then automatically calculated using the area-under-curve of the peak detected by RP-HPLC.

Table 1. A summary of the Assays Performed Using the Caco-2 Cell Monolayers

Test	Apical Chamber (A) (100µL)	Basolateral Chamber (B) (600µL)	Direction*	Transporter tested	Volume taken	Treatment
1	200µM Peptide in HBSS-Hepes	HBSS-Hepes	A to B	None	400µL from B	5µL of 10% formic acid 10µL of DMSO
2	200µM Peptide with 200µM glucose in HBSS-Hepes	HBSS-Hepes	A to B	Glucose Transporter	400µL from B	5µL of 10% formic acid 10µL of DMSO
3	200µM Peptide with 20mM glycylsarcosine in HBSS-Hepes	HBSS-Hepes	A to B	hPepT1	400µL from B	5µL of 10% formic acid 10µL of DMSO
4	HBSS buffer	200µM Peptide in HBSS-Hepes	B to A	None	80µL from A	2µL of 10% formic acid 2µL of DMSO
5	35µM vinblastine in HBSS-Hepes	200µM Peptide with vinblastine in HBSS-Hepes	B to A	P-glycoprotein	80µL from A	2µL of 10% formic acid 2µL of DMSO

* A = Apical chamber

B = Basolateral chamber

Determination of Permeability Coefficients and Enzyme Stability

The permeability across Caco-2 cell monolayers can be calculated using the following formula [9]:

$P_{app} = dC/dt * V_r / (A*C_0)$

Where dC/dt = steady-state rate of change in the chemical concentration (mol s^{-1}) or radiochemical concentration (dpm $mL^{-1}s^{-1}$) in the receiver chamber,

V_r = volume of the receiver chamber (mL),

A = surface area of the cell monolayers and

C_0 = initial concentration in the donor chamber (mol or dpm mL^{-1})

Enzyme stability values were determined by expressing the actual concentration detected in the donor chamber at the end of the transport study as a percentage of the concentration that would have been predicted, given the passage of molecules through the membrane.

Statistics

The Mann-Whitney test was performed to identify if the permeability values were statistically different between the two given conditions. Confidence interval was made to be 95%. The significance values generated were then used to evaluate whether there was a significant indication of the involvement of a particular transporter in enkephalin analogue transport across Caco-2 cell monolayer.

RESULTS

Table **2** shows the apparent permeability values and enzyme stability results obtained for the various lipid and sugar modified Leu-enkephalin analogues in the Caco-2 cell assays performed. Statistical comparison results were also shown in Table **3**. Overall, Enk-glu **(3)** was found to have the greatest Caco-2 cell monolayer permeability, followed by Gs-Enk-Laa **(6)**, Gs-Enk **(4)** and Enk-Laa **(2)** respectively. There was strong evidence of the involvement of both glucose transporters and hPepT1 in the transport of Enk-glu, as the permeability of the compound dramatically decreased at statistically significant levels with the incorporation of the transporter inhibitors. For other compounds tested, there was only weak or no evidence that suggested any of the transporters investigated took part in the process of transportation. Moreover, there was no evidence that any of the compounds was the substrate for p-glycoprotein.

Traces of parent enkephalin found in the basolateral chamber were minimal for each of the compounds tested. Furthermore, only 4% of the Enk-glu remained in solution after the 2.5 hours of the experiment implying that the compound was degraded by digestive enzymes produced the Caco-2 cells. In comparison, 21%, 64% and 78.9% remained intact in the apical chamber when Laa-Enk-glu, GS-Enk and Gs-Enk-Laa, respectively, were assayed.

DISCUSSION

The apparent permeability values obtained for Enk, GS-Enk, Enk-glu and GsEnkLaa in A to B direction (apical to basolateral) were found to be consistent with previous studies [9]. Enk-glu was found to have the greatest Caco-2 cell monolayer permeability, followed by Gs-Enk-Laa, Gs-Enk and Enk-Laa respectively (Table **2**). There was no clear evidence of Enk-Laa being more permeable than the parent peptide and there is no previous data on this compound with which to compare this result.

The permeability appeared to be zero for the transport of Laa-Enk-Glu since the concentration of peptide in the basolateral samples was too close to the limit of quantitation by LC-MS. The most likely explanation for this is that any compound that does diffuse across the monolayer is quickly destroyed by digestive enzymes. The only metabolite that the samples were examined for was the parent enkephalin peptide, thus if either the Laa or sugar unit was cleaved from this liposaccharide conjugate the resulting peptide would not have been detected. Further studies of this conjugate may be warranted in the future. There appeared to be some transport of Laa-Enk-Glu from B to A, yet the p-glycoprotein was not involved in this process, given the similar results obtained for test 4 and 5 for the compound (Table **2**).

While there was no sign, or weak evidence of the involvement of hPepT1 in the transportation of enkephalin or Enk-Laa across Caco-2 cell monolayers, (Table **3**) this study provided strong evidence of the involvement of both glucose transporters and hPepT1 in the transport of Enk-glu, as the permeability of the compound dramatically decreased with the incorporation of either glucose or glycylsarcosine (Table **2** and **3**). However, there was no indication that Enk-glu was a substrate for p-glycoprotein. If the transport across the Caco-2 cell monolayer occurred irrespective of transport direction, transcellular passive diffusion would be a likely major mechanism of permeation. This was not the case for Enk-glu. Evidence showed that there was some difference between the transportation of Enk-glu in the A to B direction in comparison to the B to A direction, permeability from B to A being slightly lower.

Although the attachment of glucose to the C-terminus of the peptide appears to allow it to utilise the glucose transporter across the Caco-2 cell monolayer, Laa-Enk-Glu (compound 5, Fig. **1**) showed no sign of transporter utility. There was no evidence that Laa-Enk-Glu was a substrate for p-glycoprotein, either. Similarly, although Enk-glu was a substrate for glucose transporter, attachment of the glucose moiety to N-terminus (Gs-Enk) did not provide this structure-transporter relationship. Furthermore, there was no evidence of the involvement of any transporters in the permeation across the monolayer for Gs-Enk or Gs-Enk-Laa (compounds 4 and 6, Fig. **1**).

While the Caco-2 cell monolayers express many useful transporters, they also express most of the digestive enzymes present in the human GI tract. Fully differentiated Caco-2 cells have been shown to express aminopeptidases, dipeptidylpeptidase IV, endopeptidase-24.11 and γ-glutamyl transpeptidase [17]. The major contribution to the degradation of native enkephalins in the brush border is by aminopeptidases cleaving the Tyr^1-Gly^2 bond [33,34] with a minor contribution from endopeptidase 24.11 [34]. Thus, not only are Caco-2 cell monolayers useful for determining the permeability of enkephalin analogues but can also provide

Table 2. Results from the Caco-2 Monolayer Permeability Assays Performed on each Compound

Compound	Test No. (Table 1)	Direction	Transporter tested	Average Papp ($cm\ s^{-1}$)	Percentage of compound remaining in donor chamber at the completion of experiment.
Enk (1)	1	A to B	None	4.82×10^{-8}	0.5%
	3	A to B	hPepT1	3.15×10^{-7}	0.76%
Enk-Laa (2)	1	A to B	None	6.78×10^{-8}	18%
	3	A to B	hPepT1	4.77×10^{-8}	39.8%
	4	B to A	None	0	61.8%
	5	B to A	P-glycoprotein	0	100%
Enk-glu (3)	1	A to B	None	1.16×10^{-6}	4%
	2	A to B	Glucose transporter	5.02×10^{-8}	3.8 %
	3	A to B	hPepT1	8.09×10^{-8}	3.2%
	4	B to A	None	1.41×10^{-7}	66.6%
	5	B to A	P-glycoprotein	1.32×10^{-7}	80.5%
Gs-Enk (4)	1	A to B	None	2.74×10^{-7}	63.9%
	2	A to B	Glucose transporter	3.95×10^{-7}	66.1%
	3	A to B	hPepT1	8.78×10^{-7}	70.4%
	4	B to A	None	1.88×10^{-6}	62.2%
	5	B to A	P-glycoprotein	2.20×10^{-6}	70.2%
laaEnkglu (5)	1	A to B	None	0	21%
	2	A to B	Glucose transporter	0	19%
	3	A to B	hPepT1	0	12.9%
	4	B to A	None	5.00×10^{-6}	46.5%
	5	B to A	P-glycoprotein	7.03×10^{-6}	36%
GsEnkLaa (6)	1	A to B	None	3.64×10^{-7}	78.87%
	2	A to B	Glucose transporter	4.10×10^{-7}	70.8%
	3	A to B	hPepT1	No results obtained	No results obtained
	4	B to A	None	2.72×10^{-7}	60.6%
	5	B to A	P-glycoprotein	2.58×10^{-7}	69.67%

valuable indications of their metabolic stability. Given the importance of aminopeptidases in the degradation of enkephalin, it is not surprising that our results clearly show that N-terminal modified analogues are significantly more stable in the presence of Caco-2 cells than those with only modifications of the C-terminus. This was demonstrated by only 4% of the Enk-glu remained resistant to enzyme degradation in the apical chamber while 21%, 64% and 78.9% remained intact when Laa-Enk-glu, GS-Enk and Gs-Enk-Laa, respectively, were assayed (Table **2**). The results also suggested that glucose succinate is a better protective moiety at the N-terminus of the peptide than Laa. On the other hand, at the C-terminal, Laa appeared to provide greater benefit in terms of enzymatic stability of the peptide compared to the glucose moiety. This is consistent with the finding that GS-Enk-Laa is the most stable compound of all those tested. However, GS-Enk-Laa failed to utilise the glucose transporter or hPepT1 to cross the Caco-2 cell monolayer. This further indicates that glucose at C-terminal is essential for utilising the glucose transporters as the transport mechanism. Our results have indicated that brush boarder N-terminal peptidases have a greater impact on enkephalin degradation than C-terminal peptidases or endo-peptidase-24.11 (*EC 3.4.24.11)*. This is further demonstrated by the relative consistency of the enzyme stability of the various peptides in the basolateral chambers (Table **2**).

Table 3. Statistical Comparison of the P_{app} Values Obtained for the Experiments in the Absence and Presence of Transporter Inhibitors

Compound	Comparison	Significance	Conclusion
Enkephalin	a-b uninhib. *vs* hPepT1 inhib.	*	No evidence
Enk-Laa	a-b uninhib. *vs* hPepT1 inhib.	0.1	Weak evidence
	b-a uninhib. *vs* Pgp inhib.	#	-
	a-b uninhib. *vs* b-a uninhib.	#	-
Enk-glu	a-b uninhib *vs* gluc trans inhib	0.04	Strong evidence
	a-b uninhib. *vs* hPepT1 inhib.	0.04	Strong evidence
	b-a uninhib. *vs* Pgp inhib.	*	No evidence
	a-b uninhib. *vs* b-a uninhib	0.08	Some evidence A to B bigger
Gs-Enk	a-b uninhib *vs* gluc trans inhib	0.19	No evidence
	a-b uninhib. *vs* hPepT1 inhib.	*	No evidence
	b-a uninhib. *vs* Pgp inhib.	0.5	No evidence
	a-b uninhib. *vs* b-a uninhib	0.11	Weak evidence
Laa-Enk-glu	a-b uninhib *vs* gluc trans inhib	#	-
	a-b uninhib. *vs* hPepT1 inhib.	#	-
	b-a uninhib. *vs* Pgp inhib.	*	No evidence
	a-b uninhib. *vs* b-a uninhib	#	-
Gs-Enk-Laa	a-b uninhib *vs* gluc trans inhib	0.5	No evidence
	a-b uninhib. *vs* hPepT1 inhib.	NA	-
	b-a uninhib. *vs* Pgp inhib.	0.5	No evidence
	a-b uninhib. *vs* b-a uninhib	0.47	No evidence

= LCMS can not detect the compound present. * = Permeability of the peptide appeared to be higher with inhibitor present.

In light of these findings, a promising peptide design could be GS-Enk-Glu. Hopefully, this peptide would retain the ability to utilize the transporters provided by the glucose moiety at the C-terminus as well as the resistance to enzyme degradation provided by the N-terminus glucose moiety. This proposed peptide would also provide good water solubility which will allow it to have a simple dosage form design should it later be developed as a drug formulation. Studies are continuing to synthesise this new lead compound and assess its permeability, stability and biological activity.

The results in this study clearly demonstrate that Enk-glu uses glucose transporters to cross Caco-2 cell monolayers. However, several glucose transporters are present in the Caco-2 cell line and previous literature provided evidence that only SGLT1 is responsible for the majority of glucose transport across healthy brush border of human small intestine. Whether Enk-glu utilizes SGLT1 is unknown since glucose is a non-specific competitive inhibitor for both SGLT and the GLUT family of facilitated transporters. It is therefore necessary to identify the involvement of SGLT1 in the process by using a specific SGLT1 inhibitor, phloridzin [21] in future research.

CONCLUSION

This was a preliminary evaluation of the possible involvement of glucose transporters, hPepT1 or p-glycoprotein in the transport of structurally modified Leu-enkephalin conjugates across Caco-2 cell monolayers. Evidence from this study suggested that transcellular passive diffusion has a limited role in the transportation of enkephalin analogues across the Caco-2 cell monolayer. Rather, the attachment of glucose moiety to the C-terminus of enkephalin provided strong evidence of carrier-mediated transport using glucose transporters and hPepT1. No analogues tested were found to be substrate for the efflux transporter, p-glycoprotein. Combining with the results for enzyme stability, this understanding of the transport mechanism will facilitate the design of an enkephalin analogue which is highly water soluble, permeable across the intestinal epithelium, and stable against enzyme degradation.

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