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Enhancement of oral drug absorption—Effect of lipid conjugation on the enzymatic stability and intestinal permeability of L-Glu-L-Trp-NH_2

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Abstract—The dipeptide L-Glu-L-Trp-OH (IM862) is currently under development for the treatment of certain cancers and immuno-deficiency disorders. However, due to its highly hydrophilic character, IM862 demonstrates low permeability across biological membranes, including the gastro-intestinal track, which makes it not orally available. In this study, the effect of lipid conjugation on the stability and intestinal permeability of the IM862 amide derivative L-Glu-L-Trp-NH_2 was investigated using enzymatic extracts and monolayers of Caco-2 cells, respectively. A series of eleven novel lipopeptide analogues of L-Glu-L-Trp-NH_2 was synthesized using *tert*-butyloxycarbonyl or 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. In vitro assays demonstrated an improved stability to proteolytic enzymes and increased intestinal permeability for several conjugates, thereby supporting the hypothesis that lipidation may provide a means to enable the oral administration of IM862.

1. Introduction

The dipeptide L-Glu-L-Trp-OH, also known as Thymogen® or IM862, is a naturally occurring immunomodulator, first isolated from calf thymus, which has been shown to be a potent anti angiogenic agent[1] and normalize the immune system function of immuno-compromised individuals.[2,3] Thymogen® has been commercialised in Russia since its approval for therapeutic use in 1990.[†]

The exact mechanism of action for IM862 is still unknown. Preclinical murine studies suggested that it inhibits tumour growth by repressing the production of vascular endothelial growth factor (VEGF) and activating natural killer (NK) cells, $CD4^+$ and $CD8^+$ T-cells.[4] In addition, IM862 might function to induce an inhibitor of angiogenesis.[5]

Clinical trials conducted in patients with various malignancies, including ovarian, breast and prostate cancers, reported the compound to be safe and well tolerated, with no severe side effects noticed.[6] Immunological observations included improved cell mediated and humoral immunity as well as increased lymphocyte counts.[5]

Most studies of IM862 have investigated parenteral routes of administration, as the poor stability and low permeability in the gastrointestinal track, associated to a short half-life, prevent its oral administration. Intranasal administration has also been examined for the treatment of Kaposi's sarcoma, yielding to high bioavailability (71%) and large biodistribution.[5] Intraperitoneal injections have also proven successful,[7] however no attempt to enhance the oral absorption of IM862 has been described thus far.

As previously reported, the conjugation of lipids to peptides can be an effective approach for increasing the enzymatic stability and membrane permeability of peptides with poor oral absorption.[8–10] In the present study, eleven analogues of the IM862 amide derivative L-Glu-L-Trp-NH_2 conjugated to lipoamino acids (α-amino acids with alkyl side chains[11]) were synthesized using *tert*-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis, and their enzymatic stability and intestinal permeability assessed

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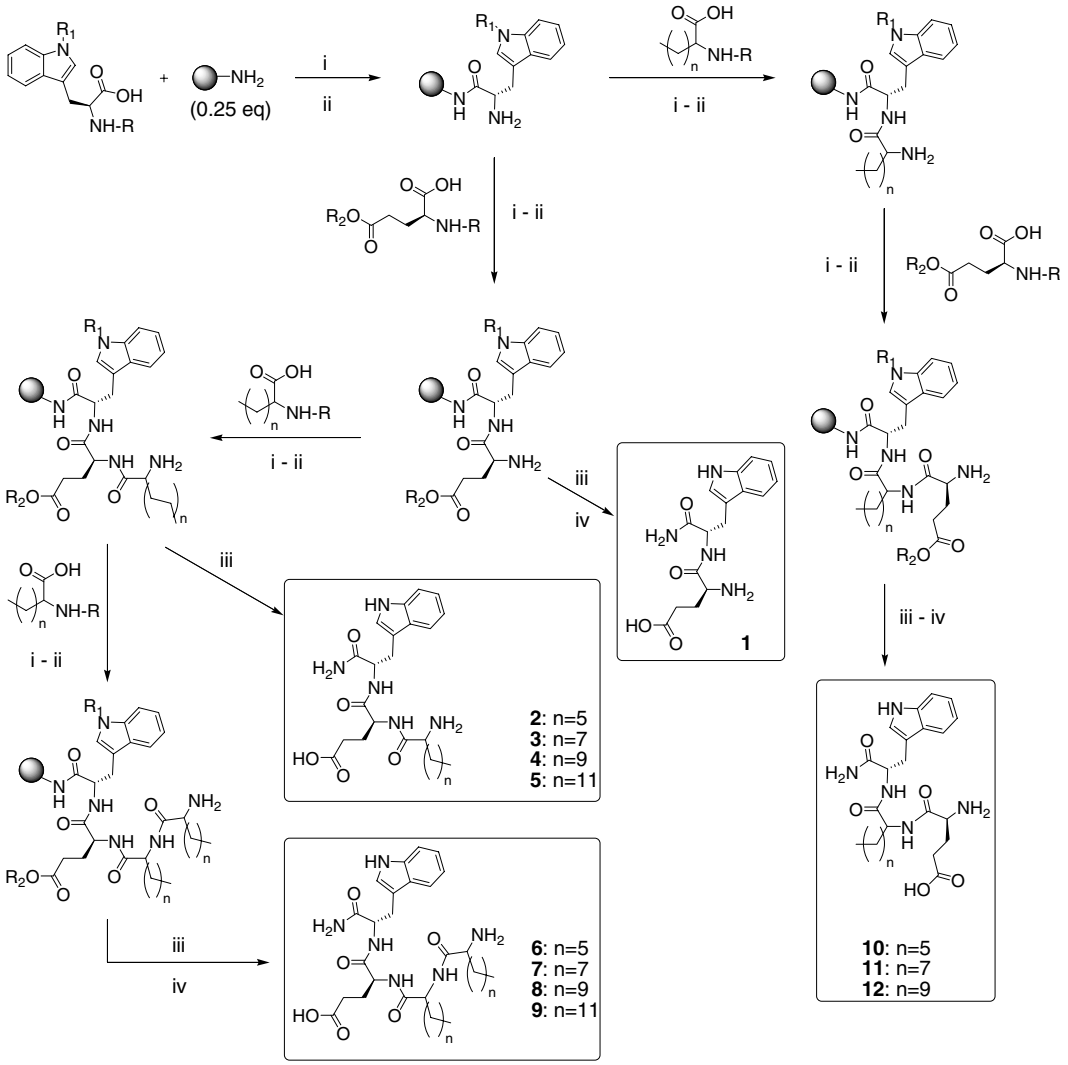
in vitro using Caco-2 cells. Lipoamino acids were introduced onto the peptide scaffold either at the amino (N)-terminus or in-between the two amino acid residues, thus providing a protection–by covalent binding or steric hindrance–of the (C)-terminal tryptophan, known to be prone to enzymatic degradation.[12]

2. Synthesis

The N^{α}-Boc-protected lipoamino acids were synthesized as described by Gibbons et al.[11] and were used as a mixture of enantiomers. Lipoamino acids were chosen according to the length of the carbon skeleton, respectively, octanoic (C_8), decanoic (C_{10}), dodecanoic (C_{12}) and tetradecanoic (C_{14}) α-amino acids.

A Boc (**1**, **6**–**12**) or Fmoc (**2**–**5**) strategy was used to synthesize the modified peptides as illustrated in Scheme 1. In the Boc approach, amino acids and lipoamino acids were coupled onto *p*-methylbenzhydrylamine (*p*-MBHA) resin following a 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA) in situ neutralization,[13] and the N^{α}-Boc protecting group was then removed under acidic conditions with trifluoroacetic acid. To prevent possible side-reactions, tryptophan and glutamic acid were side-chain protected as formyl or cyclohexyl ester derivatives, respectively; the formyl group was removed prior to cleaving the peptide with a solution of 10% (v/v) piperidine in *N*,*N*-dimethylformamide (DMF), while the cyclohexyl group was released during the cleavage.

As Boc chemistry involves the use of hazardous strong acids (e.g., hydrofluoric acid for cleaving peptides from the resin), an attempt was made to synthesize four of the conjugates (**2**–**5**) using an Fmoc strategy. This approach yielded higher amounts of each analogue with fewer by-products. Fmoc protected rink amide *p*-MBHA



Scheme 1. Synthesis of L-Glu-L-Trp-NH_2 and its lipidic conjugates by solid-phase peptide synthesis. Boc strategy (**1**, **6**–**12**): MBHA resin, R = Boc, R_1 = For, R_2 = C_6H_{11}; (i) HBTU (1 equiv), DIPEA (1.2 equiv), DMF; (ii) TFA, DMF; (iii) 10% piperidine, DMF; (iv) HF cleavage (*p*-cresol), acetonitrile/Et_2O Fmoc strategy (**2**–**5**): rink amide MBHA resin, R = Fmoc, R_1 = H, R_2 = *t*Bu; (i) HBTU (1 equiv), DIPEA (1.2 equiv), DMF; (ii) 20% piperidine, DMF; (iii) cleavage using TFA/H_2O/EDT/TIPS (87.5:5:5:2.5).

resin was used as a solid matrix and amino acid couplings were performed analogously to the Boc route, the Fmoc deprotection being however achieved with 20% (v/v) piperidine in DMF instead of TFA. In this case, tryptophan was used without side-chain protection while the glutamic acid γ-carboxyl group was protected as a *tert*-butyl ester. In order to prevent alkylation of the free tryptophan pyrrole ring by cations released during TFA cleavage of the peptide, a scavenging mixture containing triisopropylsilane (TIPS) and 1,2-ethanedithiol (EDT) was used.[14]

Following the cleavage of each peptide from its respective resin, conjugates **1**–**12** were purified by reverse phase high performance liquid chromatography (RP-HPLC) and characterized using electrospray ionization mass spectrometry (ESI-MS), RP-HPLC and nuclear magnetic resonance (NMR). The two isomers of each analogue (**2**–**12**), arising from the use of racemic lipoamino acids, were not separated and were used as a mixture in subsequent in vitro experiments.

3. In vitro evaluation of the enzymatic stability and membrane permeability of L-Glu-L-Trp-NH_2 and its lipid conjugates

In vitro assessment of the enzymatic stability and membrane permeability of **1**–**12** was performed using monolayers and enzymatic extracts of Caco-2 cells, respectively. Originally derived from a human adenocarcinoma, Caco-2 cells have been extensively studied (and recognized by the U.S. Food and Drug Administration[15]) as a model for predicting the transport of pharmaceutical products across the intestinal epithelium.[16,17] When cultured, Caco-2 cells have the ability to spontaneously differentiate into a monolayer of enterocytes and express most structural and functional characteristics of the small intestine, including enzymes.[18]

L-Glu-L-Trp-OH has been reported to be a favoured substrate for aminopeptidase W, an enzyme present in the intestinal and renal brush borders, as well as in Caco-2 cells, which has a particular affinity for short peptides (mostly dipeptides) with a (C)-terminal tryptophan.[19–21] In addition, Ano et al.[22] have demonstrated that tryptophan-containing dipeptides are subject to the action of hydrolases and other cytosolic peptidases, both prior to and during permeation, thus lowering their bioavailability when administered orally. The enzymatic resistance of L-Glu-L-Trp-NH_2 and its derivatives was evaluated in Caco-2 cell homogenates over a two hour period, a method previously reported for study of peptide and drug stability.[23,24] As most conjugates proved highly lipophilic, dimethylsulfoxide (DMSO) was added to the compounds solubilised in Hanks' balanced saline solution (HBSS) to ensure their proper dissolution. The final DMSO content was kept to a maximum of 2% (v/v), a concentration which has been demonstrated to not cause any toxicity or significant damage to the cell line or alter its properties.[22,25,26]

Intestinal permeability was assessed using Caco-2 monolayers grown for 21–28 days on polycarbonate filters in 24-well Transwell® plates (Fig. 1). The homogeneity and integrity of the monolayer were controlled prior to and after experiments by visual observation under a microscope and measurements of transepithelial electrical resistance (TEER). Stock solutions (200 μM) of each of the tested compounds were introduced into the upper compartment (i.e., the apical side of the monolayer). ^{14}C-mannitol, which is known to have negligible oral bioavailability, was used as a para-cellular marker to monitor the cell monolayer integrity. Samples were collected from the lower compartment at 30, 90, 120 and 150 min and quantified by liquid chromatography–mass spectrometry (LC–MS).

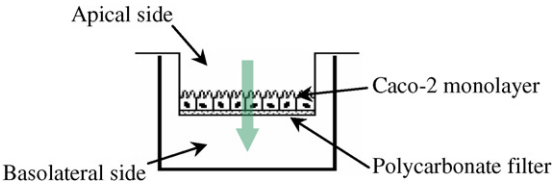


Figure 1. Caco-2 monolayers grown on Transwell® polycarbonate filters were used as a model of intestinal epithelium for transport studies of L-Glu-L-Trp-NH_2 and its lipidic conjugates.

4. Results and discussion

4.1. Stability assays

Stability evaluation of each compound (**1**–**12**) in Caco-2 homogenates suggested that the attachment of lipoamino acids to the dipeptide L-Glu-L-Trp-NH_2 (**1**), either at the (N)-terminus or in the middle of the peptide construct, could significantly increase the resistance of the molecule to enzymatic degradation. As presented in Figure 2, the amount of each conjugate remaining after two hours ranged from 40% to 90% for most derivatives, compared to 35% for the reference peptide **1**. Improvements in peptide stability to intestinal enzymes were observed to be highly dependent on the length and number of lipoamino acids attached to the reference peptide **1**. The best stability profile was observed for the C_{12} (**4**, **8**, **12**) and $(C_n)_2$ (**6**–**9**) series which exhibited up to 2.8 times more peptide remaining at two hours compared to **1**.

In the first series of conjugates (**2**–**5**), **2** and **3** showed a similar stability profile to **1**, with 40% of each compound on average remaining after 2 h. When increasing the length of the lipid alkyl chain, improvements in enzymatic resistance were noticed, with 75–80% of peptides **4** and **5** remaining after 1 h, and 65–80% remaining after 2 h (Fig. 2a). Further increases in enzymatic stability were observed when two copies of lipoamino acids were attached (**6**–**9**), with almost no degradation detected during the first 20 min and around 90% of compounds **6**, **8** and **9** remaining after two hours (Fig. 2b). The introduction of a lipoamino acid between the glutamic acid and the tryptophan residues in the last series of compounds (**10**–**12**) also enhanced the resistance to enzymatic degradation, with the remaining fractions of **10** and **12** averaging two-thirds of the initial peptide

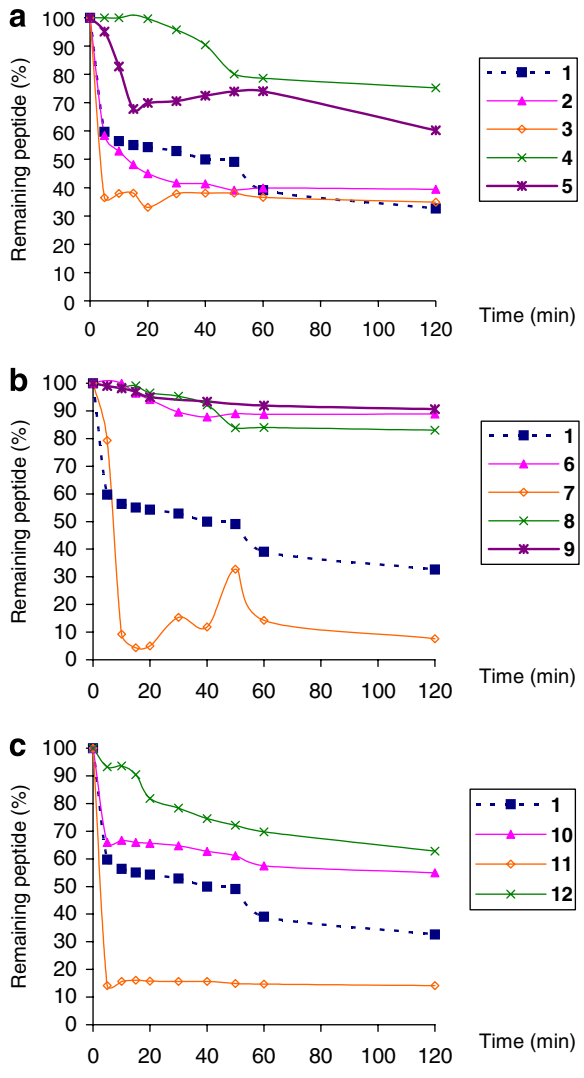


Figure 2. Remaining amounts (expressed in%) of L-Glu-L-Trp-NH_2 (**1**) and its lipidic conjugates (**2**–**12**) in Caco-2 extracts over a two hour period. (a) Mono-lipoamino acid series: **2**–**5** (C_n-Glu-Trp-NH_2); (b) di-lipoamino acid series: **6**–**9** ($(C_n)_2$-Glu-Trp-NH_2); (c) central lipoamino acid series: **10**–**12** (Glu-C_n-Trp-NH_2).

amount (Fig. 2c). The effect of these modifications, however, could potentially have an effect on the peptide activity.

As aforementioned, peptides containing a free tryptophan (e.g., Glu-Trp) are susceptible to proteolytic degradation by aminopeptidase W. The results of this study demonstrate that the conjugation of lipoamino acids to the (N)-terminus of **1**, or in-between the two Glu and Trp amino acid residues, can significantly improve the resistance of the peptide to enzymatic degradation. It is hypothesized that this may be due to steric hindrance brought about by the lipoamino acid alkyl side chain, which prevents, or reduces, interactions between tryptophan residues and degradative enzymes.

Exceptions to these findings were compounds **7** and **11**, which had approximately 15% of the peptide remaining after 10 min. Reasons for this large and unexpected decrease in stability compared to **1** were not determined. Both conjugates were found to be difficult to dissolve in aqueous buffer and this may have led to microprecipitation of the conjugate during the assay; alternatively, C_{10}-conjugates might be susceptible to the action of specific enzymes.

4.2. Determination of apparent permeability coefficients in Caco-2 cells

Caco-2 cell monolayers are increasingly being used in pharmaceutical research to study the transepithelial transport of drugs as a good correlation exists between apparent permeability coefficients (P_{app}) and human oral drug absorption.[27] Previous studies reporting the use of lipoamino acids as adjuvanting systems to enhance oral drug absorption showed that an increase in lipophilicity could generate an increase in the in vitro cellular and in vivo oral uptake (blood, liver, spleen, kidneys, small and large intestine) of the molecules (e.g., β-lactam antibiotics, benzoquinolizine and morphine conjugates, etc.), as well as facilitate their passage through the blood–brain barrier.[8–10] Here, apparent permeability coefficients of each compound were assessed in HBSS buffer over a two hour period,[28] with the average P_{app} values ($n = 4$) reported in Figure 3a–c. ^{14}C-mannitol was used as a control marker and showed negligible permeation across Caco-2 monolayers. TEER measurements were performed in HBSS buffer prior to, and after, each experiment to assess the integrity of the Caco-2 monolayers, as an indicator of the potential toxicity of each conjugate (any significant drop in the TEER values would suggest a disruption in the monolayer, caused either by the lysis of membranes or alterations to the tight junctions). Values for TEER averaged 1.6 kΩ/cm, well above the cut-off point of 450 Ω/cm reported by Parlesak et al.,[29] and no significant variation was observed over time. Microscopic examination of the monolayers after each experiment provided extra evidence that L-Glu-L-Trp-NH_2 (**1**) and its lipid conjugates (**2**–**12**) did not cause damage to the Caco-2 monolayers.

Assessment of the Caco-2 cell permeability for L-Glu-L-Trp-NH_2 and its lipid conjugates demonstrated that some conjugates (**2**–**4**, **7**, **9**–**12**) had much lower apparent permeability values than **1**. For instance, **7** and **11** seemed unable to permeate the Caco-2 monolayers, despite known reports of paracellular permeability enhancement when using C_{10} fatty acids as absorption enhancing excipients.[30] This however could be related to the poor stability profile previously observed and the difficulties encountered when solubilising these conjugates. Peptides **4** and **12**, containing a single C_{12} lipoamino acid, were also unable to permeate Caco-2 monolayers despite their reasonable stability to Caco-2 enzymes. Similar observations were made for the mono-C_8 conjugates **2** and **10** (Figs. 3 and 4).

Several factors have been hypothesized as potential causes for the low apparent permeability values observed for these conjugates, including external parameters (e.g., 3D structure, molecular weight, degree of

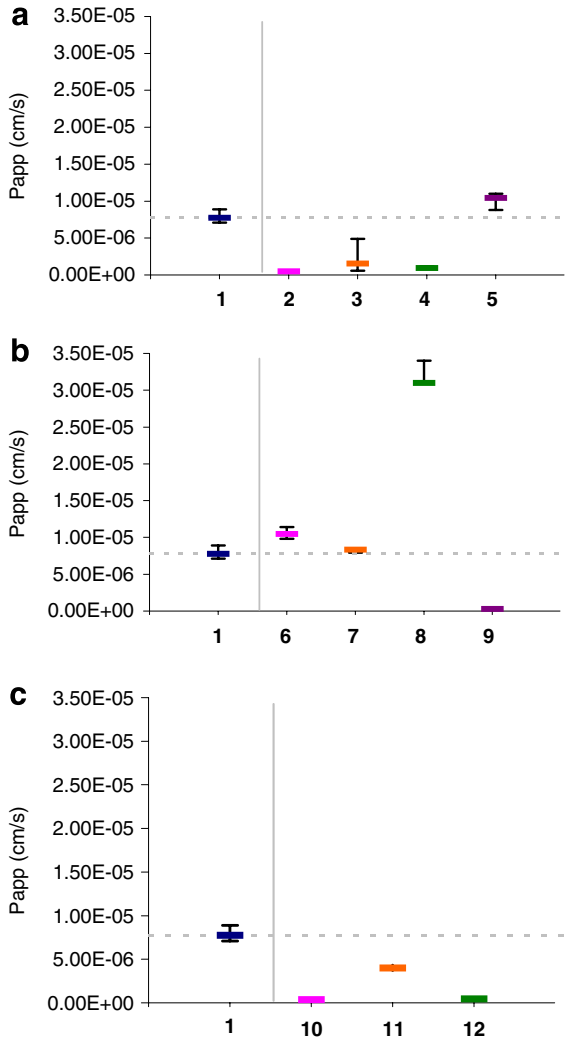


Figure 3. Apparent permeability values of L-Glu-L-Trp-NH_2 (**1**) and its lipidic conjugates (**2**–**12**) through Caco-2 monolayers; the integrity of the monolayers was monitored by TEER measurements and ^{14}C mannitol was used in all experiments as a negative control (average $P_{app} = 6.427 \times 10^{-8}$ cm/s) (a) Mono-lipoamino acid series: **2**–**5** (C_n-Glu-Trp-NH_2); (b) di-lipoamino acid series: **6**–**9** $(C_n)_2$-Glu-Trp-NH_2); (c) central lipoamino acid series: **10**–**12** (Glu-C_n-Trp-NH_2).

lipophilicity and aqueous solubility) and internal parameters (e.g., the existence of specific carriers for L-Glu-L-Trp-NH_2 and the role of efflux pumps such as P-glycoprotein).[31,32] Difficulties relating to the solubility of the L-Glu-L-Trp-NH_2 lipid conjugates in aqueous buffer, arising from the incorporation of long lipid chains onto the small peptide scaffold, are also likely to be a reason for why some of the conjugates demonstrated poor permeability across Caco-2 monolayers. This was particularly noticeable when examining conjugate **9** (containing two C_{14} lipoamino acid), which showed a noteworthy improvement in enzymatic stability but was incapable of penetrating the monolayer.

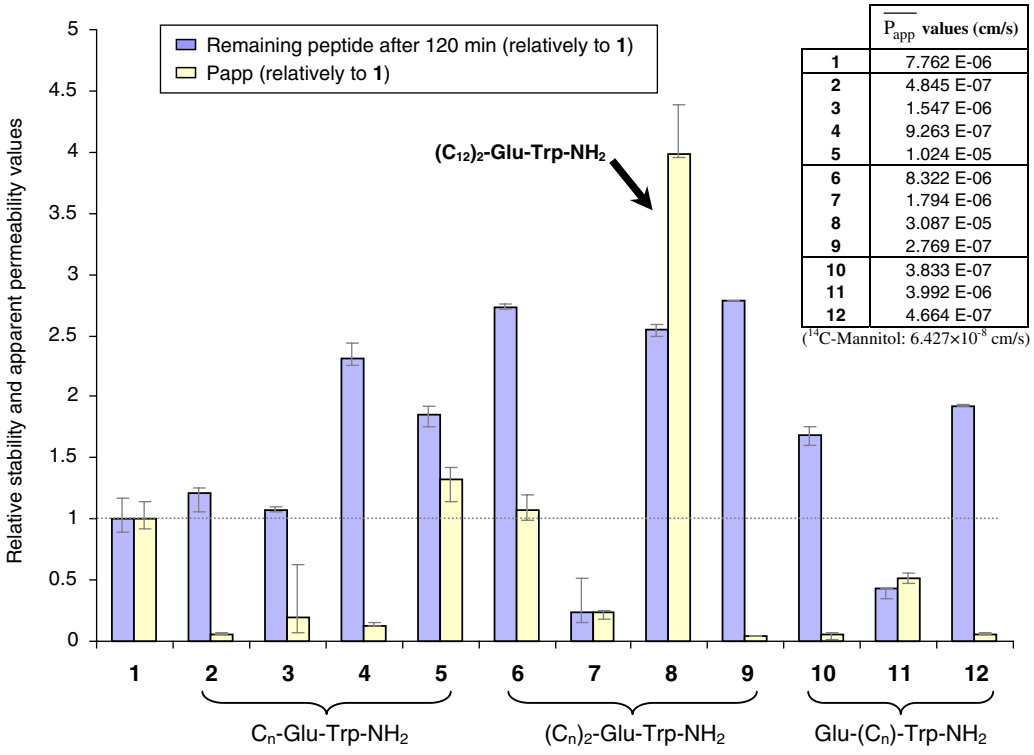
In addition, tryptophan residues have a high affinity for cell membrane surfaces,[33,34] with small water-soluble tryptophan analogues shown to bind to the glycerol backbone and lipid head-group region of phospholipid bilayers,[35] and it is likely that the conjugation of lipoamino acids to L-Glu-L-Trp-NH_2 may cause steric hindrance, altering tryptophan interactions with cell membranes. Alternatively, the affinity of the lipoamino acids for the lipid core region of phospholipid bilayers may result in the molecule being imprisoned within the cell membrane due to a stronger retention.[28] It is therefore important that an equilibrium between the hydrophobic and hydrophilic nature of the lipid conjugates is maintained to ensure the aqueous solubility of the compound while permitting lipid-mediated trans-membrane transport to occur.

Most lipids present in biological membranes comprise 10–30 carbon atoms, commonly 12–18. Similarly, the permeability values obtained in this study suggest that such a balance between the hydrophilicity of the peptide and the hydrophobicity brought by the lipoamino acids is reached when incorporating medium-sized lipid chains. For instance, compounds **2**, **3**, **4**, **10**, **11**, **12** ($\leqslant$12 carbon atoms) and **14** (=28 carbon atoms) showed very low permeability values, well below that of **1**, whereas permeability improvements were noticed with conjugates **5** and **6** (Fig. 4), having, respectively, 14 and 16 carbon atoms. Even more promising results were noticed with peptide **8** ($(C_{12})_2$) which demonstrated a fourfold increase in P_{app} values (3.1×10^{-5} cm/s**) compared to dipeptide **1** (7.75×10^{-6} cm/s**).

5. Conclusion

Clinical trials of IM862 for the treatment of various cancers and immuno-deficiencies have shown promising results; however, due to the hydrophilic nature of the dipeptide, its administration is limited to parenteral routes and no successful method has yet been reported to increase its oral bioavailability.

The current study investigated the conjugation of lipids to the IM862 amide derivative L-Glu-L-Trp-NH_2 as a means to enhance the intestinal permeability and resistance to enzymatic degradation. Overall, this study demonstrates that the conjugation of lipids generally improves the stability of the conjugates to enzymatic degradation; however, the intestinal permeability values assessed through Caco-2 monolayers were only increased in some cases. The length and number of lipid chains coupled to the peptides were found to be particularly important, with the shorter alkyl chain length conjugates ($<C_{14}$) exhibiting poor apparent permeability values, while conjugates containing a longer carbon skeleton (single-chained C_{14} or double-chained C_8 and C_{12}) had an increased permeability through Caco-2 monolayers comparatively to L-Glu-L-Trp-NH_2, in addition to an enhanced stability profile. The best candidate tested was $(C_{12})_2$-L-Glu-L-Trp-NH_2, which displayed a fourfold increase in apparent permeability compared to L-Glu-L-Trp-NH_2 and was also twice as stable in Caco-2 enzyme extracts. The conjugation of particular lipoamino acids can therefore be considered as a possible way to enhance the oral absorption of the IM862 amide derivative, providing that a



	$\overline{P_{app}}$ values (cm/s)
1	7.762 E-06
2	4.845 E-07
3	1.547 E-06
4	9.263 E-07
5	1.024 E-05
6	8.322 E-06
7	1.794 E-06
8	3.087 E-05
9	2.769 E-07
10	3.833 E-07
11	3.992 E-06
12	4.664 E-07

Figure 4. Apparent permeability values and remaining fractions after 2 h of L-Glu-L-Trp-NH_2 (**1**) and its lipidic conjugates (**2**–**12**), expressed relatively to the values obtained for **1** (where $P_{app}(\mathbf{1})/P_{app}(\mathbf{1}) = 1$).

balance between hydrophilicity and hydrophobicity is maintained.

6. Experimental

6.1. Chemistry general

Protected L-amino acids and *p*-MBHA resins were purchased from Novabiochem (Läufelfingen, Switzerland) or Reanal (Budapest, Hungary). Boc-protected lipoamino acids (C_8 to C_{14}) were synthesized as previously described.[11] Peptide synthesis grade DMF, TFA, DCM and HBTU were supplied by Auspep (Melbourne, Australia) and DMF was filtered on a 0.45 μM (47 mm) nylon filter before use. Water was purified by a Millipore Simplicity filtration system. Analytical grade HPLC solvents [MeOH, acetonitrile] were purchased from Labscan (Dublin, Ireland). Hydrofluoric acid (HF) was supplied by Boc Gases (Sydney, Australia). All other reagents were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) at the highest available purity.

Preparative RP-HPLC was carried on a Waters Delta Prep 600 system. Compounds (50–100 mg) were purified at 5 mL/min on a Vydac C_{18} column (218TP1022, 250 × 22 mm, 10 μm) using gradients of A [H_2O + 0.1% TFA] and B [Acetonitrile/H_2O (90:10) + 0.1% TFA] and detection at 214 nm. Analytical RP-HPLC was performed on an Agilent 1100 Series instrument. Compounds were eluted at 2 mL/min on a Vydac C_{18} column (218TP54, 250 × 4.6 mm, 5 μm) using a gradient of A and B (20–100% B over 10 min) and detection at 214 nm, except of compound **1** which was eluted at 1 mL/min with 20% B (isocratic) over 20 min. ^{1}H NMR spectra were recorded on BRUKER Avance spectrometers in D_2O at 300 or 500 MHz, as specified. ESI-MS was performed on a triple quadrupole Perkin-Elmer-Sciex API3000 mass spectrometer using acetonitrile-water mobile phases containing 0.1% (v/v) formic acid. ESI-MS data were acquired using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. LC–MS analyses were run at 0.4 mL/min in gradient mode on a Phemomenex Luna C_{18}(2)column (00B-4252-B0: 50 × 2 mm, 5 μm). High-resolution mass spectra (HRMS) were collected from a Bruker Daltonics micrOTOF-Q spectrometer and analysed using the Bruker Daltonics Data Analyst 3.4 software.

6.2. Solid-phase peptide synthesis procedures

6.2.1. Boc chemistry. *p*-MBHA resin (loading 1.03 mmol g^{-1}) was swollen in DMF overnight, followed by neutralization of the hydrochloric acid with a 10% solution DIPEA in DMF.

N^{α}-Boc amino acids (4 equiv) were pre-activated (1–2 min) with 0.5 M HBTU/DMF (4 equiv) and DIPEA (5 equiv) before coupling, then added to the resin and agitated for 15–40 min at room temperature. Couplings were monitored by the quantitative ninhydrin test,[36] and, where necessary, couplings were repeated to give coupling yields greater than 99.7%.

N^{α}-Boc-amino acids with the following side-chain protection were utilized: Trp(For) and Glu(OcHx). The

Formyl group was removed prior to cleavage by stirring the resin in 10% piperidine/DMF first at 0 °C (1/2 h), then at room temperature (1/2 h). The resin was subsequently washed with DMF, DCM and MeOH and dried under vacuum. The cyclohexyl group was removed during peptide cleavage using the high HF method. Briefly, the resin was treated with anhydrous HF (10 mL/g resin) in the presence of 10% (v/v) *p*-cresol for one hour at 0 °C. The HF was then removed under vacuum and the peptides were precipitated in ice-cold diethyl ether, filtered, re-dissolved in 40% aqueous acetonitrile and lyophilized.

6.2.2. Fmoc chemistry. Rink amide MBHA resin (loading 0.66 mmol g^{-1}) was swollen in DMF overnight, followed by the removal of the N^{α}-terminal Fmoc group with 20% piperidine/DMF. Fmoc-Glu(OtBu) and Fmoc-Trp-OH were pre-activated with HBTU and DIPEA and coupled to the resin as per the Boc chemistry protocol described above. Following each coupling, the resin was treated with 20% (v/v) piperidine/DMF for 5 min and repeated for 20 min to remove the N^{α}-terminal Fmoc group. Boc-protected lipoamino acids were coupled last. The resin was subsequently washed with DMF, DCM and MeOH and dried under vacuum. The *tert*-butyloxy protecting group of glutamic acid and the N-terminal Boc group of the lipoamino acids were removed during peptide cleavage under acidic conditions using TFA/EDT/water/TIPS (87.5:5:5:2.5) (20 mL/g resin), a mixture known to help prevent the free tryptophan indole from reacting with the highly reactive cationic species generated during the cleavage.

6.3. Synthesis of L-Glu-L-Trp-NH_2 and lipid conjugates

6.3.1. L-Glu-L-Trp-NH_2 (1). Boc-Trp(For)-OH and Boc-Glu(OcHx)-OH were successively coupled onto *p*-MBHA resin (2 mmol scale). Half of the resin was treated with 20% piperidine/DMF to remove the formyl group and cleaved under HF conditions to yield 208 mg of crude material. The peptide (100 mg) was then purified by RP-HPLC using the following gradient: 0–30% B (50 min), 30% B (5 min), 30–100% B (10 min) to yield the pure product (52 mg, 46.6%) after lyophilization.

t_R = 5.11 min (20% B). ESI-MS (m/ z): 333.3 $[M+H]^+$ (calcd 333.3) and 665.6 $[2M+H]^+$. HRMS (*m*/*z*) $C_{16}H_{21}N_4O_4$ $[M+H]^+$: found 333.1559, calcd 333.1557. Anal. calcd for $[C_{16}H_{20}N_4O_4 \cdot H_2O \cdot TFA]$: C, 46.55; H, 4.99; N, 12.06. Found: C, 46.47; H, 4.98; N, 11.86. ^1H NMR (D_2O): δ_H (ppm) 7.71 (m, 1H), 7.52 (m, 1H), 7.29–7.16 (m, 3H), 3.99 (m, 1H), 3.47–3.31 (m, 2H), 2.44–2.39 (m, 2H), 2.11–2.03 (m, 2H).

6.3.2. Lipid conjugates 2–5. Fmoc-Trp-OH and Fmoc-Glu(OtBu)-OH were successively coupled onto rink amide *p*-MBHA resin (2 mmol scale). The resin was then equally split in four to synthesize conjugates **2**–**5**, coupling 2-*tert*-butoxycarbonylamino-octanoic acid, 2-*tert*-butoxycarbonylamino-decanoic acid, 2-*tert*-butoxycarbonylamino-dodecanoic acid or 2-*tert*-butoxycarbonylamino-tetradecanoic acid, respectively. Following cleavage, peptides (15–70 mg) were purified using the following gradient: 20–40% B (10 min), 40–60% B (25 min), 60–100% B (20 min), 100% B (15 min), yielding the pure products **2** (22.8 mg, 22.0%), **3** (33.4 mg, 35.7%), **4** (35.5 mg, 29.2%) and **5** (4.0 mg, 1.8%) as white solids after lyophilization.

6.3.2.1. C_8-L-Glu-L-Trp-NH_2 (2). t_R = 3.79 and 4.08 min (isomers). ESI-MS (*m*/*z*): 474.8 $[M+H]^+$ (calcd 474.6) and 948.2 $[2M+H]^+$. Anal. calcd for $[C_{24}H_{35}N_5O_5 \cdot 2H_2O \cdot 2TFA]$: C, 45.59; H, 5.60; N, 9.49. Found: C, 45.58; H, 5.25; N, 9.38. ^1H NMR (D_2O, 500 MHz): δ_H (ppm) 7.61 (1H), 7.41 (1H), 7.21–7.05 (3H), 3.98 (1H), 3.42–2.98 (4H), 2.65–2.28 (1H), 2.05 (2H), 1.76–1.32 (4H), 1.31–0.83 (12H), 0.77 (3H), 0.59 (1H).

6.3.2.2. C_{10}-L-Glu-L-Trp-NH_2 (3). t_R = 4.73 and 4.95 min (isomers). ESI-MS (*m*/*z*): 502.8 $[M+H]^+$ (calcd 502.6) and 1004.1 $[2M+H]^+$. Anal. calcd for $[(C_{26}H_{39}N_5O_5)_2 \cdot 3H_2O \cdot 3TFA \cdot CH_3CN]$: C, 50,03; H, 6.30; N, 10.70. Found: C, 50.10; H, 6.53; N, 10.34. ^1H NMR (D_2O, 500 MHz): δ_H (ppm) 7.62 (1H), 7.41 (1H), 7.20–7.04 (3H), 3.99 (1H), 3.42–2.97 (4H), 2.05 (2H), 1.77–1.34 (4H), 1.32–0.81 (12H), 0.78 (3H), 0.59 (1H).

6.3.2.3. C_{12}-L-Glu-L-Trp-NH_2 (4). t_R = 5.83 and 5.97 min (isomers). ESI-MS (*m*/*z*): 529.6 $[M+H]^+$ (calcd 530.6). Anal. calcd for $[(C_{28}H_{43}N_5O_5)_2 \cdot 4H_2O \cdot 6TFA \cdot 3CH_3CN]$: C, 45.84; H, 5.67; N, 9.39. Found: C, 45.78; H, 5.32; N, 9.10. ^1H NMR (D_2O, 500 MHz): δ_H (ppm) 7.49–6.77 (5H), 3.86–3.58 (1H), 3.53–2.72 (4H), 2.69–2.28 (1H), 2.04 (2H), 1.83–0.54 (21H), 0.50 (1H).

6.3.2.4. C_{14}-L-Glu-L-Trp-NH_2 (5). t_R = 6.39 and 6.48 min (isomers). ESI-MS (*m*/*z*): 558.8 $[M+H]^+$ (calcd 558.7) and 1116.4 $[2M+H]^+$. HRMS (*m*/*z*) $C_{30}H_{48}N_5O_5$ $[M+H]^+$: found 558.3658, calcd 558.3650. ^1H NMR (D_2O, 500 MHz): δ_H (ppm) 7.61 (1H), 7.41 (1H), 7.28–6.94 (3H), 6.89 (1H), 6.46 (1H) , 4.06 (1H), 3.42–2.82 (4H), 2.81–2.28 (4H), 2.27–0.84 (20H), 0.83–0.60 (6H).

6.3.3. Lipid conjugates 6–9. Boc-Trp(For)-OH and Boc-Glu(OcHx)-OH were successively coupled onto *p*-MBHA resin (2 mmol scale). The resin was then equally split in four to synthesize conjugates **6**–**9**, coupling 2-*tert*-butoxycarbonylamino-octanoic acid, 2-*tert*-butoxycarbonylamino-decanoic acid, 2-*tert*-butoxycarbonylamino-dodecanoic acid or 2-*tert*-butoxycarbonylamino-tetradecanoic acid, respectively. Following the removal of protecting groups and cleavage, peptides (57–72 mg) were purified using the following gradients: 20–100% B (40 min) then 100% B (25 min) for conjugate **6**, and 20–40% B (10 min), 40–60% B (25 min), 60–100% B (20 min), 100% B (15 min) for conjugates **7**–**9**, yielding the pure products **6** (23.7 mg, 15.9%), **7** (45.0 mg, 13.7%), **8** (17.0 mg, 7.6%) and **9** (90.0 mg, 18.6%) as white solids after lyophilization.

6.3.3.1. $(C_8)_2$-L-Glu-L-Trp-NH_2 (6). t_R = 6.19 min. ESI-MS (*m*/*z*): 615.7 $[M+H]^+$ (calcd 615.8). Anal. calcd

for $[(C_{32}H_{50}N_6O_6)_2 \cdot 3H_2O \cdot 3TFA \cdot CH_3CN]$: C, 51.88; H, 6.77; N, 10.92. Found: C, 51.76; H, 6.65; N, 10.75. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.61 (1H), 7.41 (1H), 7.29–6.94 (3H), 6.89 (1H), 6.46 (1H), 4.06 (2H), 3.50–2.89 (4H), 2.73–2.28 (4H), 2.28–0.88 (20H), 0.75 (1H).

6.3.3.2. $(C_{10})_2$-L-Glu-L-Trp-NH_2 (7). t_R = 6.84 and 7.61 min (isomers). ESI-MS (*m*/*z*): 671.8 $[M+H]^+$ (calcd 671.9). Anal. calcd for $[(C_{36}H_{58}N_6O_6)_2 \cdot 2H_2O \cdot 3TFA \cdot CH_3CN]$: C, 54.57; H, 7.21; N, 10.34. Found: C, 54.77; H, 7.28; N, 10.49. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.60–6.42 (7H), 4.31–3.47 (2H), 3.38–2.38 (4H), 2.35–0.40 (38H).

6.3.3.3. $(C_{12})_2$-L-Glu-L-Trp-NH_2 (8). t_R = 8.56 min. ESI-MS (*m*/*z*): 728.0 $[M+H]^+$ (calcd 728.0). Anal. calcd for $[(C_{40}H_{66}N_6O_6)_2 \cdot 1H_2O \cdot 3TFA \cdot CH_3CN]$: C, 56.97; H, 7.61; N, 9.82. Found: C, 56.94; H, 7.63; N, 9.57. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.62 (1H), 7.40 (1H), 7.29–6.96 (4H), 6.89 (1H), 6.46(1H), 3.99 (1H), 3.71–3.43 (1H), 3.42–2.97 (4H), 2.97–2.25 (8H), 2.25–0.93 (32H), 0.93–0.52 (6H).

6.3.3.4. $(C_{14})_2$-L-Glu-L-Trp-NH_2 (9). t_R = 9.66 and 10.72 min (isomers). ESI-MS (*m*/*z*): 784.3 $[M+H]^+$ (calcd 784.1). Anal. calcd for $[(C_{44}H_{74}N_6O_6)_2 \cdot 4TFA \cdot 3CH_3CN]$: C, 57.10; H, 7.56; N, 9.79. Found: C, 57.52; H, 7.82; N, 9.71. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.61 (1H), 7.41 (1H), 7.21–7.04 (3H), 6.89 (1H), 6.46 (1H), 3.80–2.87 (6H), 2.82–2.41 (4H), 2.41–1.50 (20H), 1.49–0.90 (24H), 0.89–0.65 (6H).

6.3.4. Lipid conjugates 10–12. After coupling Boc-Trp(For)-OH onto *p*-MBHA resin (2 mmol scale), the resin was equally split in three to synthesize conjugates **10**–**12**. 2-*tert*-butoxycarbonylamino-octanoic acid, 2-*tert*-butoxycarbonylamino-decanoic acid or 2-*tert*-butoxycarbonylamino-dodecanoic acid were, respectively, coupled, each followed by Boc-Glu(OcHx)-OH. Subsequently to the removal of protecting groups and resin cleavage, peptides (71–76 mg) were purified using the following gradients: 10–100% B (40 min) then 100% B (25 min) for conjugate **11**, and 20–40% B (10 min), 40–60% B (25 min), 60–100% B (20 min), 100% B (15 min) for conjugates **10** and **12**, yielding the pure products **10** (46.2 mg, 37.1%), **11** (55.0 mg, 36.3%) and **12** (30.6 mg, 20.0%) as white solids after lyophilization.

6.3.4.1. L-Glu-(C_8)-L-Trp-NH_2 (10). t_R = 4.32 and 4.71 min (isomers). ESI-MS (*m*/*z*): 474.8 $[M+H]^+$ (calcd 474.6) and 984.2 $[2M+H]^+$. Anal. calcd for $[(C_{24}H_{35}N_5O_5)_2 \cdot 3H_2O \cdot 2TFA]$: C, 50.81; H, 6.40; N, 11.39. Found: C, 50.84; H, 6.12; N, 11.50. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.62 (1H), 7.42 (1H), 7.23–7.07 (3H), 4.01 (1H), 3.42–3.01 (4H), 2.65–2.28 (1H), 2.06 (2H), 1.74–1.36 (4H), 1.32–0.84 (8H), 0.75 (3H), 0.61 (1H).

6.3.4.2. L-Glu-(C_{10})-L-Trp-NH_2 (11). t_R = 5.32 and 5.64 min (isomers). ESI-MS (*m*/*z*): 502.7 $[M+H]^+$ (calcd 502.6) and 1004.1 $[2M+H]^+$. Anal. calcd for $[(C_{26}H_{39}N_5O_5)_2 \cdot 5H_2O \cdot 2TFA \cdot CH_3CN]$: C, 51.13; H, 6.88; N, 11.31. Found: C, 51.20; H, 6.61; N, 11.16. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.61 (1H), 7.41 (1H), 7.17–7.08 (3H), 4.01 (1H), 3.43–2.98 (4H), 2.05 (2H), 1.74–1.35 (4H), 1.35–0.80 (12H), 0.78 (3H), 0.59 (1H).

6.3.4.3. L-Glu-(C_{12})-L-Trp-NH_2 (12). t_R = 6.18 and 6.41 min (isomers). ESI-MS (*m*/*z*): 530.7 $[M+H]^+$ (calcd 530.6) and 1060.1 $[2M+H]^+$. Anal. calcd for $[(C_{28}H_{43}N_5O_5)_2 \cdot 4H_2O \cdot 2TFA \cdot CH_3CN]$: C, 53.17; H, 7.13; N, 11.00. Found: C, 53.35; H, 6.95; N, 10.81. ^{1}H NMR (D_2O, 500 MHz): δ_H (ppm) 7.63–6.89 (5H), 3.73 (1H), 3.55–2.89 (4H), 2.68–2.30 (1H), 2.03 (2H), 1.83–0.55 (21H), 0.49 (1H).

6.4. Biological general

Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY), except for HBSS and ^{14}C-mannitol which came from Sigma–Aldrich (Castle Hill, NSW, Australia) and Amersham Biosciences (Piscataway, NJ), respectively. Tissue culture flasks (TPP® 75 cm^2) and 96-well plates were ordered from Becton Dickinson; Transwell® polycarbonate inserts (mean pore size = 0.45 μm, 6.5 mm diameter) were supplied by Costar (Cambridge, MA). Caco-2 cells were obtained from the American type culture collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM 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 second day. After reaching 80% confluence, the cells were subcultured using 0.2% EDTA and 0.25% trypsin and seeded in tissue-culture plates.

6.5. Preparation of stock solutions

For each compound (including ^{14}C-mannitol), 200 μM solutions were prepared in HBSS buffer containing 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) and adjusted to pH = 7.4 (HCl or NaOH). The peptides were initially dissolved in small amounts of DMSO, then diluted with HBSS buffer to give final 200 μM solutions with a DMSO concentration of 0.3–2.0% (v/v). Solutions were aliquoted and stored at −20 °C.

6.6. Stability assays

Caco-2 cells were seeded in 96-well flat-bottomed plates at a density of approximately 10^6 cells per well and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every two days for 21–28 days, after which cells were sonicated with electric pulses produced by a Sonics VibraCell® ultrasonic processor (Sonic and Materials Inc., Danbury, CN) set at an amplitude of 30 to lyse the membranes; the cell contents were then centrifuged and enzymatic extracts collected in the supernatant aliquoted into individual wells of 96-well plates (80 μL/well, to which was added 20 μL HBSS buffer).

For each compound, four wells of a 96-well flat bottom platewere filled with 100 μL of 200 μM stock solution

and the plates were then placed on an incubator shaker (Heidolph Inkubator & Titramax 1000, Schwabach, Germany) set to 400 rpm and 37 °C. Samples (10 μL/well) of the reaction medium were taken at 5, 10, 15, 20, 30, 40, 50, 60 and 120 min, and TFA (5 μL) was immediately added to them to stop enzymatic reactions. Samples were then diluted with 85 μL distilled water and analysed immediately by LC–MS on a C_{18} column, using an 8 point standard curve for quantification.

6.7. Permeability assays

Caco-2 cells (passage 67) were seeded at a density of approximately 10^6 cells onto polycarbonate Transwell® inserts and cultivated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were allowed to grow and differentiate for 21 days and the medium was changed every second day. When confluent, cell monolayers were washed with HBSS buffer containing 25 mM Hepes (pH = 7.4) and the integrity of the monolayers was assessed prior to and after each experiment by measuring TEER values using a Millicell-ERS system (Millipore Corp., Bedford, MA). All monolayers used had initial and final TEER values >450 Ω/cm^2.

For each compound, 4×100 μL of the 200 μM peptide or mannitol solutions was added to the apical side of the monolayers and 600 μL of HBSS buffer was added to the receiver compartment. Plates were placed on an incubator shaker at 400 rpm and 37 °C for the duration of the experiment. At regular time points (30, 90, 120 and 150 min), 400 μL was sampled from the receiver compartment of each well and replaced with the same amount of fresh HBSS buffer.

Samples containing mannitol were diluted with 4 mL of Wallac OptiPhase HiSafe 3 liquid scintillation cocktail and their radioactivity measured using a Tri-Carb 2700 TR liquid scintillation spectrometer. Samples containing the conjugated peptides were analysed by LC–MS on a C_{18} column, using an 8 point standard curve for quantification. Apparent permeability coefficients (P_{app}, in cm s^{-1}) were determined according to the following equation:

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

where dC/dt is the steady-state rate of change in the chemical in the receiver chamber (mM, or dpm mL^{-1}), V_r is the volume of the receiver chamber (mL), A is the surface area of the cell monolayers (cm^2) and C_0 is the initial concentration in the donor chamber (mM, or dpm mL^{-1}). Four replicates of each compound were performed and the P_{app} values mentioned on Figure 4 represent an average of these values.

References and notes

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