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Oral absorption studies of lipid-polylysine conjugates of thyrotropin releasing hormone (TRH[1]) and luteinizing hormone releasing hormone (LHRH[1])

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Abstract

The lipoamino acids and their oligomers provide an excellent means of enhancing peptide lipophilicity and also helping to increase the stability of the peptide and protect it from enzymatic degradation. Thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH) were extended on the *N*-terminal with one and two lipoamino acids and labelled with the ^{3}H-acetyl group. TRH and LHRH conjugates were also prepared where the compounds were extended with two lipoamino acids, a polylysine unit and the *N*-terminal labelled with the ^{3}H-acetyl group. The higher lipophilicity resulted in a higher Caco-2 cell association and also a higher rate of oral uptake. The addition of the polylysine system increased the water solubility, as well as the oral uptake of the conjugates. The conjugates developed have been absorbed and detected after oral administration and appear to be stable for a considerable time in vivo.

Keywords: Lipoamino acids; Lipopeptides; Lipid-core-peptide; Drug delivery; Thyrotropin releasing hormone; Luteinizing hormone releasing hormone; Caco-2 cell monolayers; Oral absorption

1. Introduction

Peptides and proteins must negotiate a multitude of barriers if they are to be administered by the oral route. They are too large and too hydrophilic to cross the GI tract mucosa. They are also highly susceptible to degradation by enzymes. Conjugation of lipidic moieties to thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH) increased the half life of the lipidic conjugates in the presence of degrading enzymes (Toth et al., 1994a).

Chemical conjugation of the lipoamino acids to the TRH and LHRH peptides performed two

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[1] pyGlu was replaced by Glu.

[2] Caco-2 cell studies.

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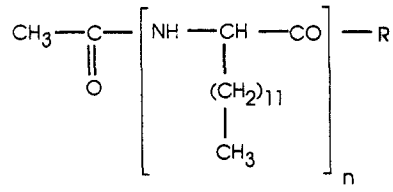
important functions, it increased the lipophilic character of the peptide to facilitate passage across biological membranes and it stabilised and protected the peptide from proteolytic degradation by both epithelial and serum peptidases (Flinn et al., 1996).

Polylysine molecules were used to deliver proteins and therapeutic agents (Ryser and Shen, 1986) and, when conjugated *via* covalent bonds to a protein, polylysine was able to increase its cellular uptake (Ryser et al., 1982; Shen et al., 1990; Wan et al., 1990). The lipid-core-peptide (LCP) system was developed to enhance the immunogenicity of the synthetic peptides (Toth et al., 1993), but the lipid-polylysine system, when incorporated into otherwise poorly absorbed drugs and peptides, could be of use to deliver therapeutic agents orally.

In this study, TRH and LHRH were chemically modified by conjugating them with lipoamino acids and examined on Caco-2 cell monolayers. TRH and LHRH were conjugated to a lipid-polylysine system, introducing suitable lipophilicity whilst not affecting water solubility. The radiolabelled conjugates were then administered orally to rats and their uptake was examined.



1	n	R
a	0	Glu-His-Pro-NH_2
b	1	Glu-His-Pro-NH_2
c	2	Glu-His-Pro-NH_2
d	0	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
e	1	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
f	2	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

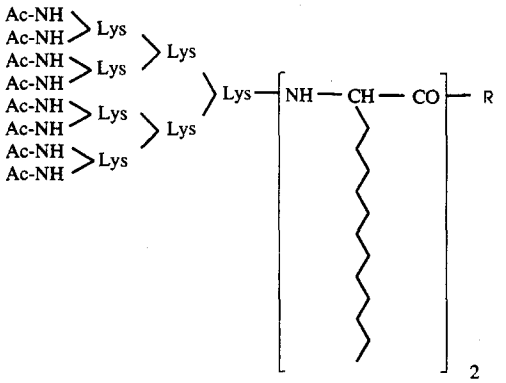
Structure 1

2. Materials and methods

Compounds **1a–1f** were synthesised, purified and characterised using the method described by Toth et al. (1994a). Compounds **2a** and **2b** were synthesised manually on solid phase, using HBTU assisted peptide synthetic methods. Furthermore, for compound separation a Waters Quanta 4000 Capillary Electrophoresis System was employed (50 mM phosphate running buffer containing 10% methanol, pH 2.5) and for structure elucidation a Fisons matrix assisted time of flight laser desorption mass spectrometer was used.

2.1. Cells

Caco-2 cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, UK (ECACC no. 86010202). Cells (5×10^5; passage < 70) were seeded on to 12 mm diameter Transwell polycarbonate filters (Costar, Badhoevedorp, The Netherlands; cat no. 3401) and cultured in DMEM (with Glutamax-1; cat no. 31966-021) with 10% (v/v) fetal calf serum, 1% non-essential amino acids and 50 μg/ml gentamicin (Gibco BRL, Paisley, UK). The media was changed on alternate days.



2	R
a	Glu-His-Pro-NH_2
b	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Structure 2

2.2. Integrity of the cell monolayers

After 21–24 days in culture, the integrity of all the cell monolayers was assessed by measurement of transepithelial electrical resistance (TER) using Millicell-ERS chopstick electrodes (Millipore, Bedford, MA). All the monolayers used for the transport experiments had TER values > 350 ohm.cm^2. The TER values of the cell layers were also measured immediately after performing the transport experiments. No significant decrease in TER was detected after any experiment. The permeability of monolayers to ^{14}C-polyethylene glycol$_{4000}$ (PEG$_{4000}$) (33.3 μg/ml; 60 mCi/mmol; Amersham, Aylesbury, UK) was determined in all control cultures and was always below 1×10^{-7} cm.s^{-1}.

2.3. Transport experiments

The Caco-2 cell layers were used in transport experiments after 21–24 days in culture. All transport experiments were performed in DMEM without supplements. The ^{3}H-labelled peptides and derivatives were initially dissolved in 2% DMF and further diluted in DMEM to a final apical chamber concentration of 50 μM. The final concentration of DMF was < 0.3%. Initial experiments with lower concentrations of compounds, in particular **1c** and **1f**, were unsatisfactory due to the high percentage of radiolabel binding to the cells and culture plastic ware. After incubations of 3 h, aliquots of the media in the apical and basal chambers were removed for scintillation counting. The cell culture inserts were removed from the wells and washed three times with PBS. The washings were retained for scintillation counting. The cells were scraped from the filter and dissolved in 1 M NaOH. The filters were washed and cut from the inserts and both the filter and insert casing were placed separately into 1 M NaOH. After stirring overnight in the dark, the alkali solutions containing the cells, filters and insert casings were used for scintillation counting. Routinely, 96–100% of the radiolabel added to the inserts was accounted for using this protocol. Apparent permeability coefficients (P_{app}) were calculated as described previously (Artursson, 1990). In some instances, ^{14}C-PEG was added to the apical chambers with the ^{3}H-labelled compounds so that any change in monolayer integrity during the course of the experiment could be determined. Only with compounds **2a** and **2b**, where the rate of ^{14}C-PEG transport doubled, was any loss of monolayer integrity observed.

The oral absorption studies were carried out following the procedure described in Toth et al., 1994b.

3. Results and discussion

The chemical modification of the enzymatically labile model peptides, LHRH and TRH, with a novel class of compound, the lipoamino acids, is able to improve the intestinal transport of these normally hydrophilic peptides across the intestinal epithelium. The long unsubstituted alkyl side chains of the lipoamino acids can stabilise the peptides in a biological environment by protecting them from the proteolytic enzymes, thus extending the biological half-lives of the compounds. Various studies were performed using these conjugates to ascertain the effect of conjugation of peptides to lipidic amino acids, with the ultimate goal of developing a delivery system for the oral administration of biologically active peptide compounds. The experimental work was designed to provide information about the intestinal absorption, the biological stability and the distribution of the lipidic conjugates within a biological system.

The tripeptide TRH and the decapeptide LHRH were synthesised (Glu was used instead of pyGlu) and acetylated on the *N*-terminus resulting in compounds **1a** and **1d**. Further samples of peptide were extended on the *N*-terminus with one or two 2-aminotetradecanoic acid moieties, before being acetylated with ^{3}H-acetic anhydride resulting in compounds **1b**, **1e** and **1c**, **1f** respectively (Flinn et al., 1996).

Linear polylysines, having impressive drug and peptide carrier properties, have also been shown to be toxic (Chang et al., 1987; Ekrami et al., 1993). Data indicated that a 50% decrease in the positive charge density of poly(D-lysine) reduced

Table 1
Association of radiolabel after incubation of tritiated LHRH conjugates (50 μM) with Caco-2 cell monolayers[a]

Compound	Time (h)	Percentage of radiolabel recovered						
		Apical medium	Basal medium	Cells	Filter	Insert casing	Cell washings	Total
1d	3	94.73 ± 3.62	0.27 ± 0.04	0.04 ± 0.01	0.03 ± 0.00	0.58 ± 0.01	0.68 ± 0.16	96.33
	6	91.29 ± 5.83	0.68 ± 0.13	0.03 ± 0.01	0.03 ± 0.01	0.93 ± 0.08	2.77 ± 0.93	95.73
1e	3	93.67 ± 5.20	0.29 ± 0.05	0.43 ± 0.07	0.18 ± 0.11	2.55 ± 0.15	1.83 ± 0.50	98.95
	6	88.87 ± 5.74	0.75 ± 0.23	1.33 ± 0.28	0.29 ± 0.05	2.33 ± 0.47	3.11 ± 0.58	96.68
1f	3	95.13 ± 2.45	0.20 ± 0.04	0.59 ± 0.10	0.10 ± 0.05	2.40 ± 0.30	0.81 ± 0.20	99.23
	6	89.49 ± 8.57	0.67 ± 0.32	2.20 ± 1.29	0.21 ± 0.12	2.01 ± 0.48	2.14 ± 0.08	96.72
2b	3	96.32 ± 4.44	0.16 ± 0.05	0.10 ± 0.05	0.06 ± 0.02	1.61 ± 0.32	0.76 ± 0.20	99.01
	6	90.25 ± 4.00	0.42 ± 0.13	0.75 ± 0.30	0.12 ± 0.03	1.01 ± 0.11	2.21 ± 0.32	94.76
PEG (8.3 μM)	3	95.45 ± 2.59	0.11 ± 0.09	< 0.01	< 0.01	0.08 ± 0.03	0.53 ± 0.16	96.17

[a]See Section 2.
Mean values ± S.D. (n = 6–9).

the toxicity, but not the carrier potential of the system (Ekrami and Shen, 1995). We have decided to synthesise a peptide carrier system by combining branched polylysine (instead of the linear polylysine) system with lipoamino acids and conjugating them to peptides. The lipidic dimer conjugates of TRH and LHRH (**1c** and **1f**) were extended with a polylysine unit and acetylated on the N-terminus with ^{3}H-acetic anhydride resulting in compounds **2a** and **2b** respectively. The polylysine conjugation increased the water solubility of the lipid-modified peptide and the polylysine system was neutral, since the amino groups were converted to amide linkages. The conjugates of TRH and LHRH resulted in diastereomeric mixtures, which were used without separation.

The transepithelial transport of the lipidic TRH and LHRH conjugates was assessed using Caco-2 cells. These cells, when grown on inert filters, form monolayers that morphologically, biochemically and functionally resemble the epithelia of the human intestine. Radiolabelled TRH and LHRH conjugates (50 μM) were administered apically to the cells and the passage of the radiolabel through the monolayer, as well as its association to various components of the culture system, was determined by scintillation counting.

Table 1 shows the distribution of ^{3}H-labelled LHRH **1d** and its derivatives **1e**, **1f** and **2b** within the Caco-2 cell culture system after 3 or 6 h incubation. Similar results were obtained for the TRH **1a** and its conjugates **1b**, **1c** and **2a** (results not shown). In all instances, the majority of the radiolabel remained in the apical chamber, either dissolved in the media or bound to the casing of the filter inserts. The affinity of the peptides for the cell culture plastic, either of the insert casing or, in the absence of cells, to the polycarbonate filter, increased with the addition of lipidic groups. In the presence of intact cell monolayers, binding of the compounds to the filters was low but significant in comparison to the small amounts of label detected in the basal media.

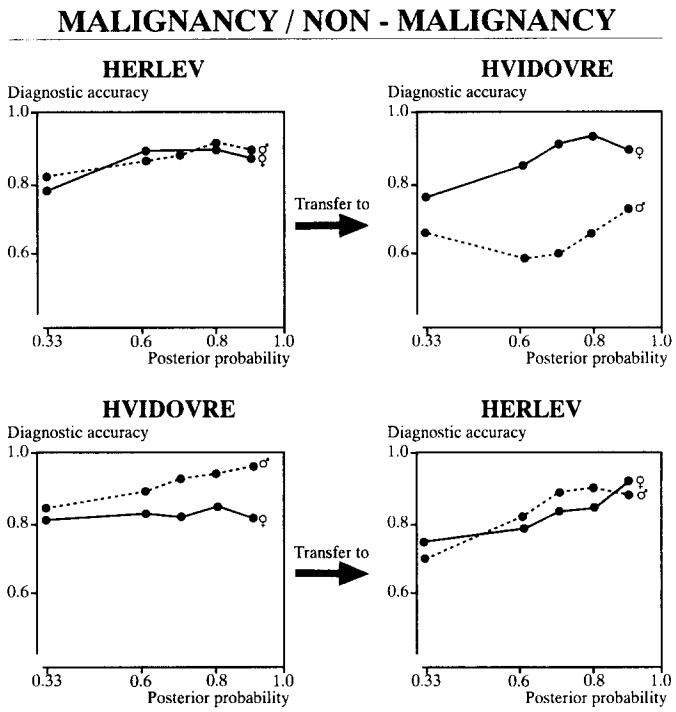


Fig. 2. The results obtained from classification based on discriminant functions, one for each sex, in Herlev and Hvidovre hospitals and when transferred to Hvidovre and Herlev, respectively. The X-axis shows the posterior probability, the Y-axis, the percentage of correctly classified patients, the diagnostic accuracy.

bias affecting reference as well as patient materials. A change with time in this bias could, at least in part, explain the difference in transients. An unexpected bias for serum calcium accounting of -0.07 mmol/l between the hospitals (Herlev values $-$ Hvidovre values) was shown [16]. Therefore, in the author's opinion, there is evidence that the discriminant functions could be further improved. An important problem is related to the uncertainty of the clinical chemical tests [16]. This is a non-genuine transfer factor which could be virtually eliminated by devoting more resources to standardization.

Since identification and diagnosing of hypercalcemia by means of standard serum analyses is a cheap and fast way to overcome an often-overlooked clinical problem, there is a great potential gain by developing efficient methods. On a consecutive population we have demonstrated the possibility to develop an automatic method to distinguish between two important diagnosis groups associated with hypercalcemia. Nevertheless, the identification of a number of 'transfer factors', revealed by this study, pinpointed the fact that problems related to quality assessment procedures must be solved in order to improve accuracy and transferability. Thus standardization, especially of laboratory methods but possibly too of clinical diagnosing, is an important step towards improving discriminant functions and their transferability.

Appendix A: Hypercalcemia discriminant functions

Diagnostic discriminant functions, one for each sex, developed in a patient population consecutively recorded in Herlev and Hvidovre hospitals [2]. The variables were logarithm transformed before the values of the discriminant functions were calculated. Primary hyperparathyroidism (PHPT), miscellaneous medical diagnoses associated with

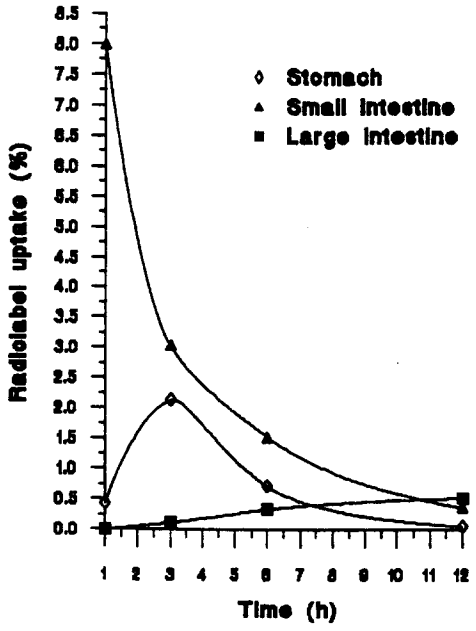


Fig. 3. Stomach, small intestine and large intestine radiolabel uptake of compound **2b**.

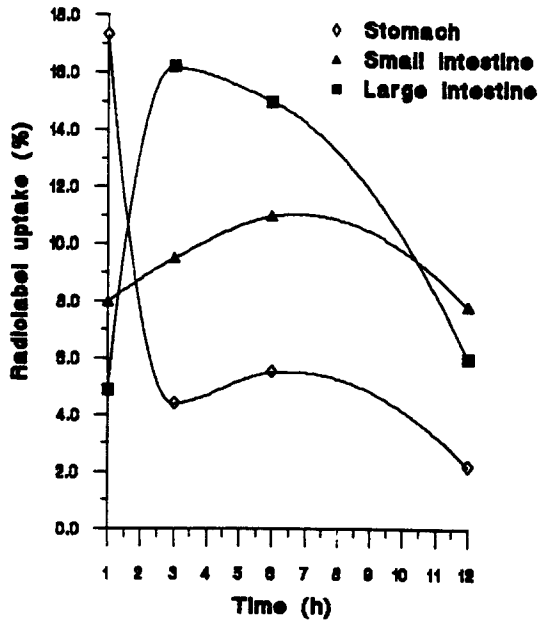


Fig. 4. Stomach, small intestine and large intesting radiolabel uptake of compound **2a**.

take into the cells and consequently their passage across the cell monolayer. The failure for this not to translate into greater P_{app} values may be a consequence of the conjugated peptides passing more slowly through the filter and/or to binding of the peptides to the cell culture plastic on the basal side of the cells which was not measured. However, if the conjugated peptides have a strong affinity for the lipid apical cell membrane, passage through the cells may remain very slow.

3.1. Oral absorption studies

The use of a radiolabel is a convenient and sensitive method to study the uptake and translocation of a bioactive peptides. However, the method is not specific for the peptides, the radioactivity determined in vivo does not necessarily indicate the presence of the intact administered compound, it may belong to peptide fragments formed by chemical or biological degradation. However, the measurement of the radioactivity

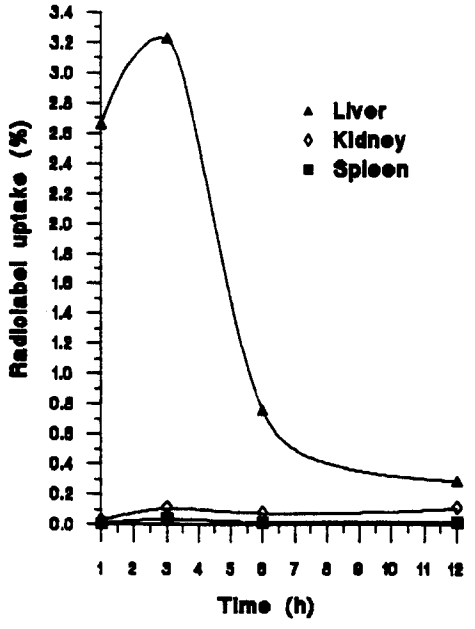


Fig. 5. Liver, kidney and spleen rabiolabel uptake of compound **2b**.

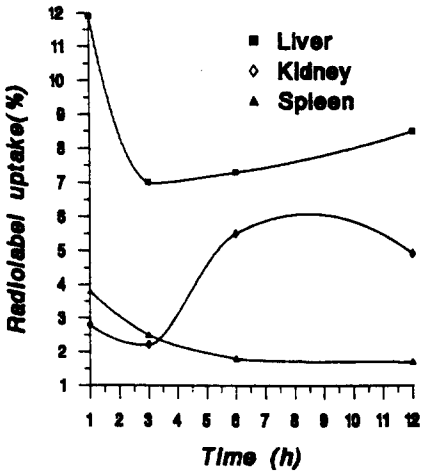


Fig. 6. Liver, kidney and spleen radiolabel uptake of compound **2a**.

uptake in different organs, could give valuable information of the influence of the peptide lipophilicity on the oral uptake. We have previously reported the oral uptake of compounds **1a–1f** (Flinn et al., 1996).

Radiolabelled TRH and LHRH analogues **2a** and **2b** were administered orally to rats and the uptake examined in the blood, liver, spleen, kidneys, small intestine and large intestine.

Both compounds showed significant oral radioactivity uptake. The absorption profile of total and blood uptake of compounds **2a** and **2b** was similar. The uptake in general was higher than the analogues with one lipidic moiety, without the polylysine system (**1b**, **1e**, Flinn et al., 1996) but similar with those of the analogues with two lipidic moiety, without the polylysine system, reported previously (**1c**, **1f**, Flinn et al., 1996). Maximum uptake of compounds **2a** and **2b** was observed after 3 h, and the uptake decreased with the time. The TRH analogue **2a** showed higher overall uptake than the LHRH analogue **2b** (Fig. 2).

The uptake in the organs showed similar trend, the more lipophilic TRH conjugate **2a** showed higher uptake, than the less lipophilic LHRH analogue **2b** (Figs. 3–6).

In summary, as expected, the uptake of the smaller molecular weight more lipophilic TRH conjugate **2a** was higher in all examined organs than the uptake of the less lipophilic higher molecular weight LHRH analogue **2b**.

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