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Lipidic conjugates of luteinizing hormone releasing hormone (LHRH)[+] and thyrotropin releasing hormone (TRH)[+] that release and protect the native hormones in homogenates of human intestinal epithelial (Caco-2) cells

Istvan Toth [a,*], Nicholas Flinn [a], Anya Hillery [a], William A. Gibbons [a], Per Artursson [b]

[a] *The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK,*
[b] *Uppsala University, Biomedicum, Box 580, S-751 23, Uppsala, Sweden*

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Abstract

The enzymatically labile peptides LHRH and TRH were conjugated to various lipidic peptides. The conjugates and free peptides were incubated with Caco-2 cell homogenates and their respective degradation profiles were studied following incubation. Conjugation to lipidic peptides increased the half-life of LHRH and TRH. The Caco-2 cell homogenates were capable of cleaving the conjugated lipidic peptide, to release the parent LHRH or TRH. The released LHRH or TRH subsequently demonstrated a longer half-life than when present alone in the incubation mixture, suggesting that the cleaved lipidic peptide is capable of inhibiting enzymes.

Key words: Lipidic amino acid; Lipidic peptide; Drug delivery; LHRH; TRH; Caco-2 cell homogenate; Enzyme degradation

1. Introduction

Since the commercial introduction of insulin in 1923, attention has been focused on the possibility of administration of therapeutic proteins and peptides by the oral route. Despite considerable research and effort in this area, the oral dosage of peptides and proteins has thus far remained an elusive goal. The gut epithelium presents a number of physical barriers to oral absorption, including hydrophobic membranes and transport processes (Baker et al., 1991), cell junctions (Nellans, 1991), mucus (Lehr et al., 1991), gastric acidity and peristalsis. Proteolytic activity (from epithelial, pancreatic and bacterial sources) in the GI tract is also a formidable barrier to oral uptake, as shown by the following observations: (i) an inverse relationship exists between the amount of peptide transported across the intestine and its rate of hydrolysis (Saidel and Edelstein, 1974); (ii) in neonates, the GI tract is relatively permeable to macromolecules, a phenomenon partly ac-

* Corresponding author.

[+] PyGlu was replaced by Glu.

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counted for by the decreased intestinal proteolysis that exists in the neonatal state (Weström et al., 1985); and (iii) peptide and protein formulations that contain appropriate enzyme inhibitors generally demonstrate some (albeit quite small) absorption, whereas unprotected formulations do not show any absorption (Lee, 1988).

The decapeptide, LHRH, a hypothalamic hormone that regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), is used in medicine for the treatment of infertility. The molecule is too large (Mol. Wt 1182) and too hydrophillic (partition coefficient 0.0451; Banks and Cession, 1985) to cross the GI tract mucosa; the peptide is also highly susceptible to enzyme degradation. Degradation proceeds by a variety of routes, of particular importance is the degradation mediated by endopeptidases on the Tyr^5-Gly^6 bond, in combination with enzymes that hydrolyse the $pyroGlu^1$ or cleave on the carboxyl side of Pro (Sandow et al., 1981). Due to the physical and chemical barriers afforded by the GI tract, the oral administration of LHRH required a dose 3000-times higher than that of the parenteral route (Sandow and Petri, 1985). The half-life for LHRH in the circulation is extremely rapid (3–6 min) (Handelsman and Swerdloff, 1986) and involves the mixing of the peptide throughout the vascular and extracellular fluid spaces.

The tripeptide, thyrotropin releasing hormone (TRH), is a hypothalamic hormone that stimulates the release of thyrotropin, prolactin and growth hormone from the pituitary and is administered parenterally because of low brain levels following oral administration. Since TRH is relatively resistant to proteolytic degradation in the GI tract, its poor oral activity is probably due to poor absorption and rapid clearance in the bloodstream (Yokohama et al., 1984). TRH is rapidly degraded following first-order kinetics, with a half-life in humans of approx. 5 min following i.v. administration (Leppaluoto, et al., 1972). Two different enzymes are responsible for the catabolism of TRH (Brewster and Waltham, 1981): prolyl endopeptidase (generating deamino-TRH) and pyroglutamyl aminopeptidase (generating the products pGlu and His-Pro-NH_2).

Established strategies for improving the oral absorption of peptide and protein drugs take into consideration the physiological and physicochemical factors discussed and can be divided into two broad categories, chemical and physical. Chemical methods involve a chemical modification of the compound to be absorbed. The modification may be: (i) irreversible, i.e., the newly modified compound is intended to be active in its own right: as in peptide analogs containing unnatural amino acids (D-amino acids) for L-amino acids in the primary structure; or (ii) reversible, i.e., the active principle is intended to be regenerated in vivo after absorption, i.e., a prodrug. Physical methods involve the favourable pharmaceutical presentation of the drug, although the medicinal species is not altered chemically. Such methods include association with colloidal carriers, entrapment of the active species in polymeric matrices, liposomes or micelles and the use of absorption and solubility enhancers.

The transepithelial transport of TRH and its prodrug (*N*-octyloxycarbonyl) derivative (Lundin et al., 1991) was studied in Caco-2 cells and no intact TRH prodrug was transported across the cells. It was concluded that the increased lipophilicity of the TRH prodrug had no effect on its transport characteristics.

In this study, the chemical approach to oral absorption enhancement was selected for the peptides LHRH and TRH. The peptides were chemically modified by conjugating them to a novel class of compounds: the lipidic amino acids and their homo-oligomers the lipidic peptides. The lipidic amino acids and peptides represent a class of compounds which combine structural features of lipids with those of amino acids (Gibbons et al., 1990) and have potential as a drug delivery system (Toth et al., 1991). Because of their bifunctional nature, the lipidic amino acids and peptides have the capacity to be chemically conjugated to or incorporated into peptides. The resulting conjugates possess a high degree of membrane-like character: the permeability coefficient of the conjugated drug moiety may be increased to such an extent that its passage across the hydrophobic membrane of the GI tract is facilitated. The long alkyl side chains may also

have the additional effect of affording protection to a labile peptide drug from enzymatic degradation.

In order to determine whether the lipidic amino acids were capable of conferring enhanced metabolic stability on the peptides, their respective degradation profiles were studied following incubation with Caco-2 cell homogenates. The Caco-2 cell culture model is a widely used cell line in drug and peptide absorption studies. This cell line forms confluent monolayers and differentiates to cells with an enterocyte-like morphology under standard cell culture conditions (Chantret et al., 1988). The Caco-2 cell homogenates possess the brush border enzymes typical of the small intestinal enterocyte (Artursson, 1990), providing a viable indication of metabolic stability in the GI tract.

2. Materials and methods

^{1}H-NMR spectra were obtained on Varian XL-300 and Bruker AM500 instruments operating at fields of 300 and 500 MHz, respectively; chemical shifts are reported in ppm downfield from internal TMS. Mass spectra were run on a VG Analytical ZAB-SE instrument, using fast atom bombardment (FAB) ionisation.

2-*tert*-Butoxycarbonylaminododecanoic acid was synthesised from 1-bromodecane (Gibbons et al., 1990). The synthesis of peptides **1** and **2** was accomplished automatically by a stepwise solid-phase procedure (Merrifield, 1963) on MBHA Novabiochem resin (substitution 0.48 mmol/g resin) and their primary structure verified by MS and NMR. The lipidic amino acids were coupled to the resin peptides manually. The synthesis of the first and every subsequent level of the peptide construction was achieved using a 4 M excess of preformed symmetrical anhydride of N-Boc amino acids in dichloromethane (20 ml)/*N*-methylpyrrolidone (5 ml). The protecting groups for the synthesis of the peptides were Boc groups for the α-amino-termini, Br-Z for the Tyr, Tos for the Arg, CHO for the Trp, DNP for the His, OBz for Glu and Bzl for the Ser. For all residues the first coupling was carried out with the preformed symmetrical anhydride in CH_2Cl_2, and a second coupling in dichloromethane/*N*-methylpyrrolidone. The second coupling of Boc-substituted-Asn was mediated by the preformed 1-hydroxybenzotriazole ester in dichloromethane/*N*-methylpyrrolidone. In all the couplings the coupling efficiency was more than 99.8% as indicated by quantitative ninhydrin testing. After the second coupling deprotection of the N-termini was performed in 65% TFA in dichloromethane (20 ml for 1 min, then another 20 ml for 10 min). The deprotected resin peptide was neutralized with 10% diisopropylethylamine in dichloromethane. The resin peptide was carefully washed between and after the deprotection and neutralisation steps. The DNP protecting group from the His was removed with 20% mercaptoethanol/5% diisopropylethylamine in DMF, and the CHO group from the Trp with 10% piperidine in DMF. The peptide was removed from the resin support with high HF method (1.5 ml cresol, 1.5 ml thiocresol, 20 ml HF) to yield the crude peptide, which was precipitated with ether and redissolved in 6 M guanidine HCl-0.1 M Tris solution (20 ml).

Peptide purification: Analytical HPLC separation was carried out on a Vydac C_{18} 5 RAC column. HPLC grade acetonitrile (Aldrich) and water were filtered through a 23 μm membrane filter and degassed with helium flow prior to use. Analytical separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing constantly to 60% acetonitrile at 30 min, staying at this concentration for 20 min and decreasing steadily to 0% acetonitrile for 10 min at a constant flow of 0.7 ml min^{-1}. For preparative separation a TSK gel preparative C_{18} column was used. Separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing constantly to 60% acetonitrile at 180 min, staying at this concentration for 60 min and decreasing steadily to 0% acetonitrile for 30 min at a constant flow of 7 ml min^{-1}. The gradient was effected by two microprocessor-controlled Gilson 302 single-piston pumps. Compounds were detected with a Holochrome UV-Vis detector at 214 nm (analytical) and 230 nm (preparative). Chromatographs were recorded with an LKB 2210 single-channel chart recorder.

Table 1
Retention time and MS of LHRH-lipidic amino acid conjugate diasteromers

Compound	Fraction no.	Retention time (min)	MS $[M+H]^+$
1a		12.36	1200
1b, 1c	fraction 1	17.34	1398
	fraction 2	18.13	1398
1d–1g	fraction 1	21.25	1596
	fraction 2	21.83	1596
	fraction 3	23.73	1596
	fraction 4	23.86	1596
2a		6.66	380
2b, 2c		15.56, 15.97	577
2d–2g		21.77, 22.12, 22.66, 23.71	776

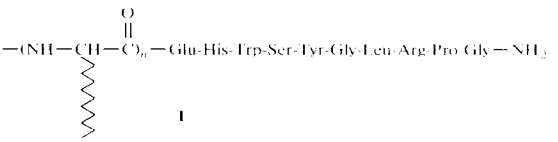
The experimental data are summarized in Table 1.

2.1. Preparation of Caco-2 cell homogenates

Caco-2 cells were obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% nonessential amino acids, benzylpenicillin (10 IU/ml) and streptomycin (10 μg/ml) in tissue culture flasks for 14 days (Artursson, 1990). Cell culture media and tissue culture flasks were from Costar, Badhoevedorp, The Netherlands. Cells of passage 97 were used. After washing the monolayers with ice-cold isotonic 0.01 M phosphate-buffered saline (PBS, pH 7.4), cells were scraped with a rubber policeman, placed in 50 ml centrifuge tubes and resuspended and lysed in hypotonic 0.01 M PBS, pH 7.4 at 4°C. Aliquots of the cell lysates (5×10^6 cells/ml) were stored at −70°C.

2.2. Enzyme assay

The respective peptides and peptide conjugates (0.1 μmol) were dissolved in 100 mM phosphate buffer (1 ml, pH 7, containing EDTA and DTT) and incubated at 37°C with the Caco-2 cell homogenate solution (1 ml). Samples (100 μl) were taken at various time intervals and the enzymatic degradation reaction terminated by the addition of TFA (5 μl). The amount of peptide or peptide conjugate was determined by HPLC. The peptide or peptide conjugate degradation products were isolated by semipreparative HPLC and their structures determined by ^{1}H-NMR and FAB-MS.



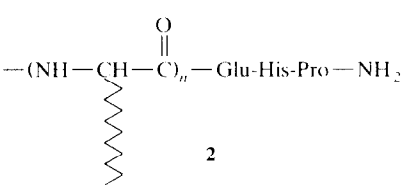
1

1	n	Configuration
a	0	–
b	1	L-3-LHRH
c	1	D-3-LHRH
d	2	L-3-L-3-LHRH
e	2	D-3-D-3-LHRH
f	2	L-3-D-3-LHRH
g	2	D-3-L-3-LHRH

3. Results and discussion

The decapeptide LHRH (**1a**) and the tripeptide TRH (**2a**) were extended on the N-terminus with one or two 2-aminododecanoic acid groups (**3**), resulting in compounds **1b–1g** and **2b–2g**. The peptides **1** and **2** were synthesized by the solid-phase technique of Merifield (1963) and their primary structure verified by MS and NMR. 2-*tert*-Butoxycarbonylaminododecanoic acid was synthesised from 1-bromodecane (Gibbons et al., 1990).

Because the lipidic amino acid was racemic, coupling it to LHRH to form a 'monomer' conju-



2

2	n	Configuration
a	0	–
b	1	L-3-TRH
c	1	D-3-TRH
d	2	L-3-L-3-TRH
e	2	D-3-D-3-TRH
f	2	L-3-D-3-TRH
g	2	D-3-L-3-TRH

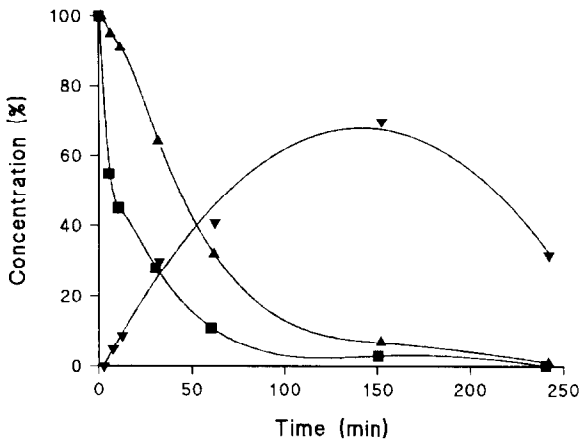


Fig. 1. CaCo-2 enzyme degradation: (■) LHRH; (▲) fraction 1 of **1b, 1c**; (▼) released LHRH.

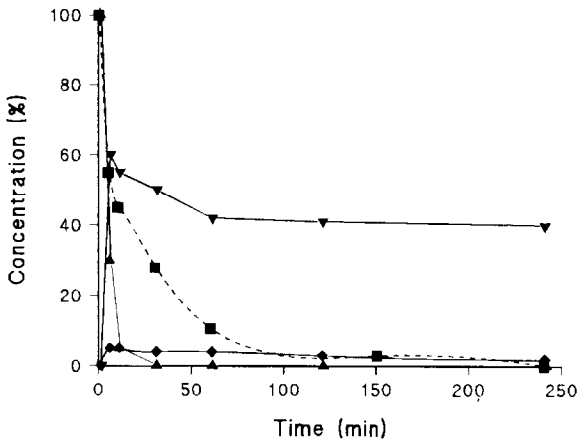


Fig. 2. CaCo-2 enzyme degradation: (■) LHRH; (▲) fraction 2 of **1d–1g**; (▼) released **1b** or **1c**; (◆) released LHRH.

gate resulted in a diastereomeric mixture: compounds **1b** and **1c**. These monomer conjugates were separated by HPLC (fractions 1 and 2, Table 1), but the absolute configurations were not determined. Similarly, the 'dimer' conjugate, obtained by coupling two lipidic amino acids to LHRH, resulted in four compounds (**1d–1g**), which were separated by HPLC (fractions 1–4, Table 1). The monomer conjugate of TRH (conjugation of a single lipidic amino acid to TRH) resulted in a diastereomeric mixture, compounds **2b** and **2c**. This diastereomeric mixture was used without HPLC separation; the TRH dimer conjugates **2d–2g** were also used as a diastereomeric mixture.

LHRH (**1a**) incubated with Caco-2 cell homogenates degraded rapidly, the half-life of the peptide being about 5 min. The monomer conjugates of LHRH showed an increased half-life of 43 min (Fig. 1). Interestingly, fraction 1 of the monomer conjugate released the parent LHRH. The released LHRH showed a substantially enhanced stability, being present in the cell homogenate mixture even after 4 h (Fig. 1). The enzyme degradation of fraction 2 of the monomer conjugate showed a similar pattern, but LHRH release was not observed. Conjugation of LHRH with two lipidic amino acids yielded compounds **1d–1g** (fractions 1–4). The rate of enzyme degradation of the four compounds was different. Fraction 1 was not degraded by Caco-2 enzymes, even after 6 h incubation. Fraction 2 degraded rapidly, the half-life of the conjugate being about 5 min, but the monomer conjugate and the parent LHRH were released (Fig. 2). The released monomer conjugate and LHRH remained in the cell homogenate mixture for more than 4 h. Fraction 3 degraded slowly, the half-life of the conjugate being more than 6 h, with LHRH release beginning after 4 h, so that 5% LHRH was present after 6 h incubation (Fig. 3). The half-life of fraction 4 was about 3 h; the compound degraded

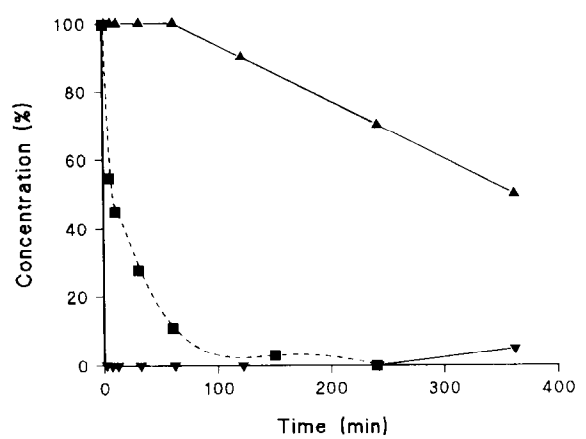


Fig. 3. CaCo-2 enzyme degradation: (■) LHRH; (▲) fraction 3 of **1d–1g**; (▼) released LHRH.

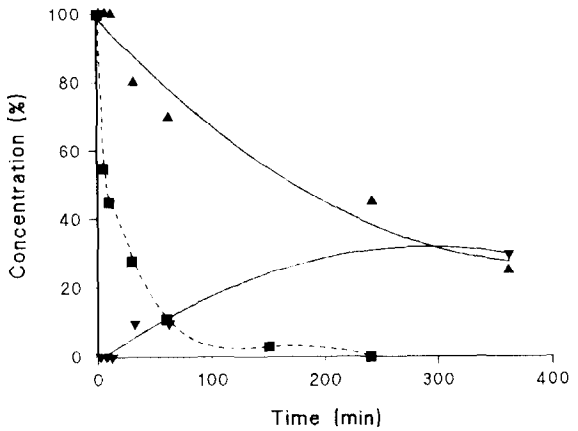


Fig. 4. CaCo-2 enzyme degradation: (■) LHRH; (▲) fraction 4 of **1d–1g**; (▼) released **1b** or **1c**.

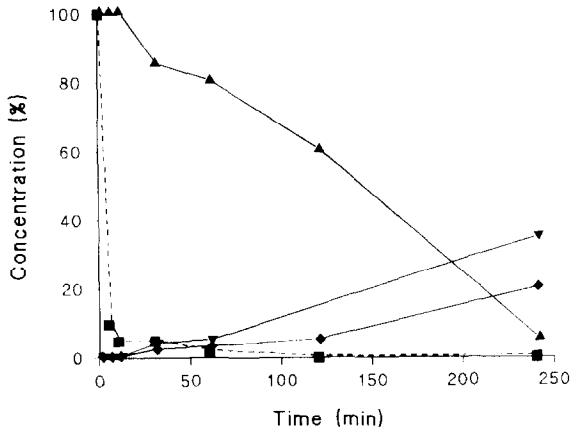


Fig. 6. CaCo-2 enzyme degradation: (■) TRH; (▲) **2d–2g**; (▼) released **2b**, **2c**; (◆) released TRH.

to a monomer conjugate **1b** or **1c** (Fig. 4), however, LHRH release was not observed.

Caco-2 cell homogenate degradation of TRH (**2a**), monomer conjugates (**2b** and **2c**) and dimer conjugates (**2d–2g**) was studied using the diastereomeric mixtures. The TRH (**2a**) and the monomeric conjugates (**2b** and **2c**) showed a similar degradation profile with Caco-2 enzyme to that of the corresponding LHRH compounds. TRH degraded rapidly, the half-life of the compound being about 3 min. The half-life of the monomeric conjugates **2b** and **2c** was 30 min, and about 5% of the conjugates was still detectable after 4 h incubation. Parent TRH was released from the monomer conjugates, reached a maximum concentration after 1 h incubation and was present even after 4 h (Fig. 5). The half-life of the dimer conjugates **2d–2g** with Caco-2 enzymes was about 2.5 h and although the amount present gradually decreased, there was still 5% of the conjugates present even after 4 h incubation. Parent TRH and monomer conjugates **2b** and **2c** were released from these dimer conjugates and were detected in increasing amounts during the 4 h incubation (Fig. 6).

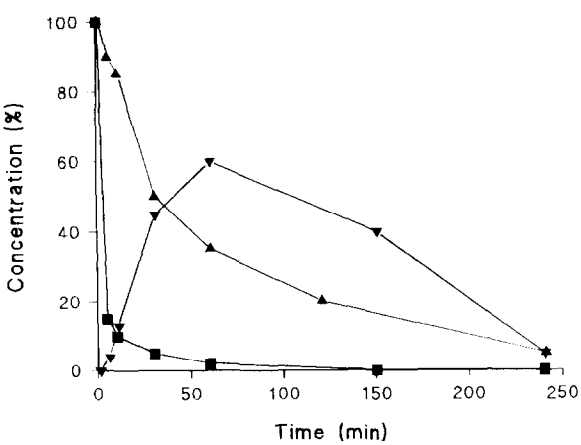


Fig. 5. CaCo-2 enzyme degradation: (■) TRH; (▲) **2b**,**2c**; (▼) released TRH.

4. Conclusion

Conjugation of LHRH (**1a**) to lipidic amino acids increased the half-life of the conjugates during incubation with Caco-2 cell homogenates. The half-life of unconjugated LHRH was approx. 5 min, which increased to approx. 45 min when conjugated to a lipidic amino acid (**1b** and **1c**) and 360 min when conjugated to two lipidic amino acids (**2d–2g**). Similarly, TRH (**2a**) showed enhanced metabolic stability when conjugated to lipidic amino acids; the unconjugated peptide **2a** demonstrated a half-life of about 3 min, which was increased to 30 min when conjugated to a lipidic amino acid (**2b** and **2c**) and 150 min when conjugated to two lipidic amino acids (**2d–2g**).

The monomeric conjugate form of LHRH and TRH released the parent peptide, as proven by HPLC and MS, during the incubation with the cell homogenates. Similarly, the dimeric conjugate form of the peptides released both the monomeric form and the parent peptide. The parent peptide released by enzyme action on the lipidic peptide conjugate exhibited enhanced stability over the parent peptide which was present alone in the incubation mixture, e.g., LHRH showed a half-life of 5 min, whereas LHRH released from the monomer conjugate was present in the incubation mixture even after 4 h, as was parent LHRH released from the dimer conjugate. Similarly, parent TRH had a half-life of 3 min, but TRH cleaved from the lipidic amino acids was present in the incubation mixture during the 4 h of incubation. It is possible that the cleaved lipidic amino acid subsequently inhibited the enzyme degradation of the parent peptides. Further work will investigate the absolute configuration of the diastereomers and the kinetics of the enzyme degradation reaction.

5. Acknowledgement

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