Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 4946–4950
Bioorganic & Medicinal Chemistry

Caco-2 cell permeability and stability of two D-glucopyranuronamide conjugates of thyrotropin-releasing hormone

Susanne T. Wessling,[a] Benjamin P. Ross,[b] Yasuko Koda,[a] Joanne T. Blanchfield[a] and Istvan Toth[a,*]

[a]*School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, Qld. 4072, Australia*
[b]*School of Pharmacy, The University of Queensland, Brisbane, Qld. 4072, Australia*

Received 21 December 2006; revised 28 March 2007; accepted 25 April 2007
Available online 29 April 2007

Abstract—Caco-2 cell permeability and stability assays were used as an in vitro model to study the intestinal epithelial transport and stability of two analogues of thyrotropin-releasing hormone (TRH; Pyr-His-Pro-NH_2). Peptide **1** (Pyr-His-Pro-D-glucopyranuronamide) was more permeable across the Caco-2 cell monolayer compared with the permeability of the parent TRH peptide ($P_{app} = 5.10 \pm 1.89 \times 10^{-6}$ cm/s c.f. $P_{app} = 0.147 \pm 0.0474 \times 10^{-6}$ cm/s respectively). The permeability of peptide **1** was improved threefold by attaching a 2-aminooctanoic acid moiety to the N-terminus to form peptide **2** (2-aminooctanoic acid-Gln-His-Pro-D-glucopyranuronamide) ($P_{app} = 16.3 \pm 2.47 \times 10^{-6}$ cm/s). The half-life for both peptide **1** and peptide **2** was ~20 min in a homogenate of Caco-2 cells compared with the half-life of TRH which is ~3 min. It was concluded that the permeability of peptides **1** and **2** was enhanced because of their increased stability, while the higher permeability of peptide **2** compared with peptide **1** may be attributed to its increased lipophilicity which results in enhanced passive diffusion.

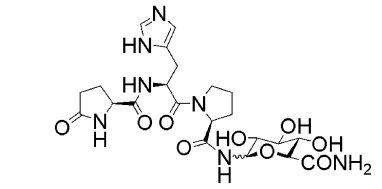
1. Introduction

Thyrotropin-releasing hormone (TRH) is a tripeptide hormone (Pyr-His-Pro-NH_2) that has diverse functions. TRH produced by the hypothalamus stimulates the release of thyroid-stimulating hormone (TSH; thyrotropin) by the anterior pituitary.[1] TSH stimulates the thyroid to produce two hormones, triiodothyronine (T3) and thyroxine (T4), which help control metabolism. TRH is also a modulatory peptide in the central nervous system (CNS) and affects arousal, sleep, cognition, locomotion and mood. The best documented and most interesting CNS effect of TRH is its analeptic action; TRH has been shown to reduce pentobarbital-induced sleeping time by 50% or more in rats, rabbits and monkeys.[1] It is this CNS activity that has stimulated interest in TRH as a drug for the treatment of motor neuron diseases, spinal cord trauma and Alzheimer's disease.

The development of TRH as a pharmaceutical is hampered by rapid metabolism and clearance as well as poor access to the central nervous system (CNS). TRH has a half-life of ~3 min in a homogenate of Caco-2 cells and only ~5% of peptide remains after 50 min of incubation.[2] TRH is hydrophilic, with a log *P* value of −2.46, and therefore it does not readily diffuse through cell membranes.[3] The rapid enzymatic inactivation and poor membrane permeability of a peptide may be overcome by derivatization.[4] For example, the addition of lipidic moieties to a peptide is a well-established method of providing steric protection from enzymatic degradation, increasing the lipophilicity, improving passive membrane diffusion and therefore increasing bioavailability.[5] Another strategy to improve peptide delivery is conjugation with sugars. Carbohydrate moieties conjugated to peptides can: (a) provide the opportunity of utilizing specific active or facilitated transport pathways across some biological barriers, particularly the blood–brain barrier (BBB); (b) target the peptides to particular cell types or organs; (c) provide steric protection from enzymatic degradation; and (d) modulate water solubility of lipid–peptide conjugates, for example in the liposaccharide drug delivery system.[5] In this study, we examined

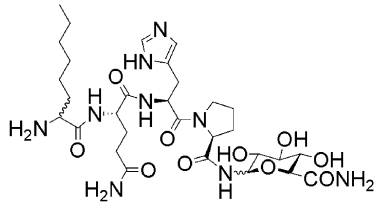
Keywords: TRH; Caco-2 cells; Drug delivery; Lipoamino acids; Sugar conjugation.
* Corresponding author. E-mail: i.toth@uq.edu.au

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.04.042



Pyr-His-Pro-D-glucopyranuronamide

1



2-Aminooctanoic acid-Gln-His-Pro-D-glucopyranuronamide

2

Figure 1. Two analogues of thyrotropin-releasing hormone.

the effect of sugar (D-glucopyranuronamide) (Fig. 1, peptide **1**) and sugar/lipid (D-glucopyranuronamide/2-aminooctanoic acid) (Fig. 1, peptide **2**) conjugation on the permeability and stability of TRH using Caco-2 cells.

2. Results

The variability and the reproducibility of the LC–MS method of quantification was assessed by the comparison of separate standard curves prepared from the same stock solution, on two different days. The standard curves were linear with correlation coefficients >0.99 for the concentration range 0.05–100 μM. The standard deviation of peak area values for triplicates of each concentration run on day 1 was <10% for peptide **2** in the range of 0.2–100 μM and for peptide **1** in the range of 2–100 μM. For samples run on day 2, the standard deviation was <12% for the same concentration ranges.

Figure 2 illustrates the apparent permeability coefficients of the compounds in the Caco-2 cell permeability assay. The permeability of mannitol, which crosses the monolayer only via paracellular transport, was very low ($P_{app} = 0.0825 \times 10^{-6}$ cm/s) which indicated that the monolayers were intact and confluent. Peptides **1** and **2** had much higher permeability values of $5.10 \pm 1.89 \times 10^{-6}$ and $16.3 \pm 2.47 \times 10^{-6}$ cm/s, respectively. Results from our laboratory have indicated that TRH exhibits very low permeability ($P_{app} = 0.147 \pm 0.0474 \times 10^{-6}$ cm/s).

The stability of the peptides was determined by incubation in a homogenate of Caco-2 cells. TRH degrades rapidly in this enzymatic mixture, with a half-life of ~3 min, and after 20 min in the enzymatic mixture less than 5% of native peptide remained.[6] Both peptides **1** and **2** showed improved stability and 20% of the modified peptides were present after 2 h of incubation (Fig. 3). The half-life of **1** and **2** in the cell homogenate was ~20 min.

3. Discussion

LC–MS is a highly specific and sensitive technique that allows the detection of very low concentrations of the analyte and it has been shown to be a useful technique for analyzing Caco-2 cell permeability assays.[7] We previously developed a rapid LC–MS method for determining the apparent permeability values of compounds, varying in lipophilicity, in the Caco-2 cell assay.[8] In the current study two linear standard curves with $r^2 > 0.99$ were obtained for each peptide on different days which indicates that the current method is reliable with good reproducibility.

Caco-2 permeability and stability assays were used in this study as an in vitro model to examine the intestinal epithelial transport and stability of two analogues of TRH (peptides **1** and **2**). Caco-2 cells spontaneously differentiate on microporous filter membranes, forming monolayers with tight inter-cellular junctions.[9] The permeability of compounds through the Caco-2 cell monolayer can be related to the extent of oral absorption in humans.[10] Peptide **1**, which contained a C-terminal D-glucopyranuronamide group, was more permeable across the Caco-2 cell monolayer compared with the permeability of the parent TRH peptide. To the best of our knowledge this is the first study to examine the effect of sugar-conjugation on the permeability and stability of TRH. The permeability of peptide **1** was improved threefold by attaching a 2-aminooctanoic acid moiety (also known as a C8 lipoamino acid)[11] to the

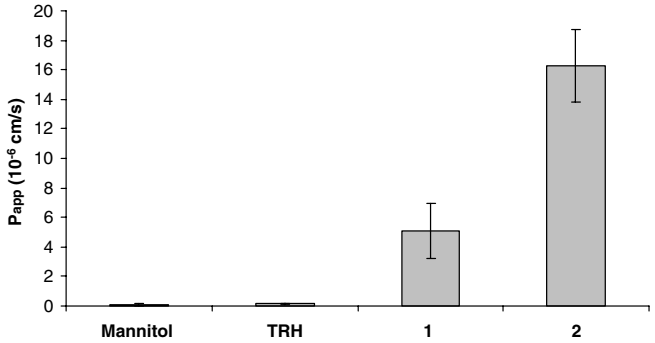


Figure 2. Permeability assay: Caco-2 cell apparent permeability values for mannitol, TRH, peptide **1** and peptide **2**. Values are means ± SD.

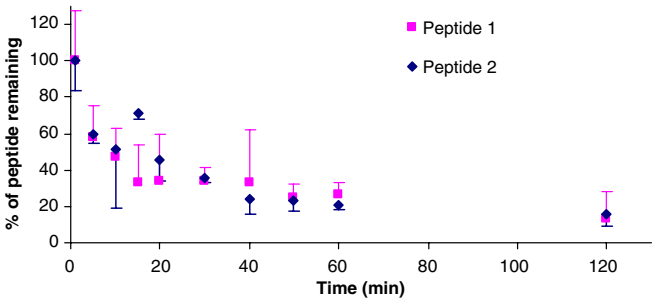


Figure 3. Stability assay: the percentage of peptide remaining after incubation at 37 °C in a homogenate of Caco-2 cells. Values are means plus or minus SD.

N-terminus to form peptide **2**. The concentration of peptide that remained in the apical chamber at the end of the experiment was found to be less than that which could be explained by transfer of peptide to the basolateral chamber. This indicated that the peptides are not stable in the assay, and this was further investigated using the Caco-2 cell stability assay.

It was previously shown that conjugating TRH with a lipoamino acid increased the stability of the peptide in a homogenate of Caco-2 cells. By attaching two lipoamino acids the half-life was increased to 2.5 h compared with a half-life of ~3 min for TRH.[2] In this study, a D-glucopyranuronamide moiety attached to the C-terminus of TRH (to afford peptide **1**) improved peptide stability, and the addition of a 2-aminooctanoic acid moiety to the N-terminus of this conjugate (to afford peptide **2**) did not alter the stability of the compound any further; the half-life for each peptide was ~20 min.

4. Conclusions

Based on these results, it is concluded that the enhanced stability of the conjugates contributes significantly to the improved permeability of peptides **1** and **2** when compared with TRH. The higher permeability of **2** compared with **1** may be because the 2-aminooctanoic acid conjugate **2** is more lipophilic than **1** and therefore it may cross the Caco-2 cell membrane more readily by passive transcellular diffusion (**1**, *C*log *P* −4.79; **2**, *C*log *P* −3.82).[12] Further investigation of sugar and sugar–lipid conjugates of TRH is warranted. Studies should examine the stability and absorption of TRH conjugated with various carbohydrate moieties and a series of lipids which differ in lipophilicity. For sugar conjugates it will be necessary to determine whether absorption occurs via specific active or facilitated transport pathways or whether passive diffusion is the primary absorption mechanism.

5. Experimental

5.1. Peptide synthesis

Fmoc-amino acids were purchased from Novabiochem except for 2-(*tert*-butoxycarbonylamino)-D,L-octanoic acid which was prepared according to a literature procedure.[13] Peptides **1** and **2** (Fig. 1) were synthesized by solid phase strategies on rink amide MBHA resin. 2,3,4-Tri-*O*-acetyl-1-azido-1-deoxy-β-D-glucopyranuronic acid[14] (4 equiv) was attached to the resin using DIC (4 equiv) and HOBt (4 equiv) and the first amino acid was coupled to the sugar via a modified Staudinger reaction.[14] The peptide was then synthesized by a stepwise solid phase procedure using Fmoc-chemistry.[15] The coupling efficiency was monitored using the ninhydrin test[16] for free primary amines or the chloranil test for free secondary amines.[17] After deprotection and cleavage of the peptide from the resin using 95% (v/v) TFA, 2.5% (v/v) TIS and 2.5% (v/v) H_2O for 3 h, the peptide was precipitated with ice cold ether, filtered and dissolved in a acetonitrile–water mixture, frozen and lyophilized.[14] The crude peptides were purified by preparative RP-HPLC using a Waters DeltaPrep 600 system on a Vydac C18 column (218TP1022; 10 μm; 22 × 250 mm). An isocratic separation was performed using a solvent consisting of 0.1% (v/v) aqueous TFA in water, a 10 mL/min flowrate, and detection at 230 nm. Fractions containing the pure product by ESI-MS were combined, frozen and lyophilized. The pure peptides were analyzed by analytical HPLC on a Vydac C18 column (218TP54; 5 μm; 4.6 × 150 mm) running a linear gradient of 0% B to 40% B over 20 min for **1** and 0% B to 100% B over 30 min for **2** (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA/90% acetonitrile in water).

Peptide **1** was a mixture of 70% β-anomer (β-an) and 30% α-anomer (α-an). Yield: 28%. HPLC: t_R 7.33 min, purity 95%. ESI-MS, *m*/*z*: 560 $[M + Na]^+$, 538 $[M + H]^+$. HRMS Calcd for $[M + H]^+$ 538.2279. Found: 538.2261. ^{1}H NMR (500 MHz, CD_3OD) δ 8.77 (1H, s, imidazole H-2 α-an); 8.71 (1H, s, imidazole H-2 β-an); 7.37 (1H, s, imidazole H-4 α-an); 7.37 (1H, s, imidazole H-4 β-an); 5.57 (1H, d, *J* 4.8 Hz, sugar H-1 β-an); 4.98 (1H, d, *J* 1.3 Hz, sugar H-1 α-an); 5.00–4.96 (2H, m, α-CH of His α-an and β-an); 4.60 (1H, dd, *J* 5.4 and 8.2 Hz, α-CH of Pro β-an); 4.45 (1H, dd, *J* 4.5 and 8.5 Hz, α-CH of Pro α-an); 4.21–4.18 (2H, m, α-CH of Pyr α-an and β-an); 4.04 (1H, d, *J* 9.2 Hz, sugar H-5 β-an); 3.82–3.76 (4H, m, CH_2-5 of Pro α-an and β-an); 3.72 (1H, dd, *J* 5.4 and 9.3 Hz, sugar H-2 β-an); 3.62–3.56 (3H, m, sugar H-3 and H-4 β-an, and sugar H-4 or H-5 α-an); 3.51–3.44 (2H, m, sugar H-3 α-an and sugar H-4 or H-5 α-an); 3.36 (1H, t, *J* 8.8 Hz, sugar H-2 α-an) 3.28 (2H, dd, *J* 6.6 and 15.0 Hz, Ha of imidazole CH_2 α-an and β-an); 3.13 (2H, br dd, *J* 6.3 and

14.1 Hz, Hb of imidazole CH_2 α-an and β-an); 2.46–1.94 (16H, m, $4 \times CH_2$ of Pro and Pyr α and β-an).

Peptide **2** was a mixture of four diastereomers: racemic D,L-2-aminooctanoic acid, 70% β-anomer and 30% α-anomer. Yield: 26%. HPLC: t_R 13.68 min and 13.87 min, purity 92% (51% and 41%, respectively). ESI-MS, *m/z*: 718 $[M + Na]^+$, 696 $[M + H]^+$. HRMS Calcd for $[M + H]^+$ 696.3664. Found 696.3681. Interpretation of the ^1H NMR (500 MHz, CD_3OD) spectrum was not facile because of the diastereomeric mixture. The spectrum was very similar to that observed for peptide **1**, except for the following obvious differences. The Pyr α-CH multiplet at 4.21–4.18 ppm was replaced by Gln α-CH multiplets at 4.38–4.35 and 4.32–4.28 ppm. Resonances attributed to the addition of the D,L-2-aminooctanoic acid moiety appeared at: 1.38–1.33 (m, $4 \times CH_2$) and 0.92 (m, CH_3).

5.2. Cell culture

Caco-2-cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum, 1% nonessential amino acid solution and 1% glutamine solution and grown in flasks. When plated, the cells were given an additional 1% antibiotic solution (penicillin/streptomycin) in the media mixture. Cells were incubated at 37 °C with 5% CO_2. The medium was changed every second day in both flask and on plate maintaining cells. At 80% of confluence cells in the flask were passaged, using 0.02% EDTA to wash the cells and a 0.25% trypsin/EDTA solution for subculturing. Approximately 8×10^5 cells (passage number 72) were seeded onto Transwell plates (*d*: 6.5 mm, *A*: 0.33 cm^2, poresize: 0.4 μm) or 96-well plates. The cells were allowed to grow and differentiate for 20–28 days. The medium was changed every second day. Caco-2 cell assays were preformed in a Heidolph Incubator 1000, Titramax 1000 at speed 3 (equivalent to 400 rpm) and 37 °C.

5.3. Drug transport experiments

Transepithelial electrical resistance (TEER) of the monolayers was measured before and after the drug transport experiment. ^{14}C-Mannitol was used as a negative control compound to assess the integrity of the Caco-2 cell monolayer. The assays were performed in Hanks' balanced salt solution (HBSS) containing 25 mM Hepes (pH 7.4) at 37 °C. The peptides (**1** and **2**) were dissolved in HBSS/Hepes buffer to a final concentration of 200 μM. Before the experiment was started, the monolayers were washed three times with pre-warmed HBSS/Hepes buffer. At the start of the experiment 0.1 mL of the test and reference article [**1**, **2**, mannitol (200 μM)] was added to the apical side of the monolayer and 0.6 mL of fresh HBSS/Hepes buffer was added to the basolateral side in a new plate. Samples (0.4 mL) were taken from the basolateral layer at four different time points, 30, 90, 120 and 150 min, while the volume in the basolateral layer was kept constant by replacing the withdrawn volume with the same amount of HBSS/Hepes buffer. At the end of the experiment a sample of 0.05 mL was taken from the apical layer. These samples were then analyzed by LC–MS.

5.4. Radioactivity measurements

The negative control ^{14}C-mannitol was counted in a liquid scintillation spectrometer (Beckman LS 3801), with the addition of 4 mL scintillation cocktail (Perkin-Elmer-OptiPhase 'HiSafe').

5.5. Determination of permeability coefficients

The permeability coefficient (P_{app} cm/s) was determined using the following equation[8]:

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

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

5.6. Drug stability experiment

After the cells had been cultivated for 20 days on a 96-well plate, the medium was removed from the wells and each well was washed with 0.2% EDTA solution (100 μL) followed by HBSS/Hepes buffer (2×100 μL). Finally, 100 μL of the buffer was placed in each well and the plate was cooled on ice. The cells in each well were then homogenised by 2×1 s pulses with a sonicator probe (Sonics Vibracell ultra sonic processor at amplitude 60), then the cell debris was removed by centrifuging the plate at 2000 rpm for 5 min. The supernatant was taken out from the wells, pooled and placed in 12 new wells, 80 μL in each together with 20 μL buffer. The compounds to be tested were dissolved in buffer to a concentration of 200 μM. To begin the assay 100 μL of each drug solution was added to four wells on the plate containing the cell homogenate placed in the incubator shaking at 37 °C. Samples (10 μL) were removed at selected time points (1, 5, 10, 15, 20, 30, 40, 50, 60, 120 min) and immediately added to a new plate in a well containing 5 μL concentrated TFA to stop digestion before being diluted with 85 μL of water. These samples were then analyzed by LC–MS.

5.7. LC–MS analysis

The concentrations of the peptides **1** and **2** in the drug transport and stability experiments were determined by LC–MS. Standard solutions were prepared in triplicate at 11 different concentrations (0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10.0; 20.0; 50.0 and 100.0 μM) and used to create the standard curves. The variability and the reproducibility of the LC–MS method of quantification was assessed by the comparison of separate standard curves prepared from the same stock solution, on two different days. A Phenomenex Luna 5 μm C_{18} column (50×2.00 mm) was used. Separation was achieved with a time programme of 0% B for 0.3 min followed by a

linear binary gradient over 4 min to 90% B. This concentration was maintained for 1 min, after which the solvent ratio was taken down to 0% B again over 0.7 min and maintained for 5 min to re-equilibrate the system. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid/90% acetonitrile in water. A flow-rate of 0.3 mL/min with a 1/10 post-column split was used throughout and controlled with a Shimadzu HPLC system (SCL-10A VP system controller, two LC-10AT VP pumps, DGU-10A degasser) with an Agilent 110 Series autosampler. A PE Sciex API 3000 LC–MS system running in positive ion electrospray mode was used to detect the molecular ions. The typical ion spray voltage was 5.2 kV.

References and notes

1. Prokai, L. *Prog. Drug Res.* **2002**, *59*, 133.
2. Toth, I.; Flinn, N.; Hillery, A.; Gibbons, W. A.; Artursson, P. *Int. J. Pharm.* **1994**, *105*, 241.
3. Buchwald, P.; Bodor, N. *Curr. Med. Chem.* **1998**, *5*, 353.
4. Bundgaard, H.; Moss, J. *Pharm. Res.* **1990**, *7*, 885.
5. Blanchfield, J.; Toth, I. *Curr. Med. Chem.* **2004**, *11*, 2375.
6. Toth, I.; Flinn, N.; Hillery, A.; Gibbons, W. A.; Artursson, P. *Int. J. Pharm.* **1994**, *105*, 241.
7. Wang, Z.; Hop, C. E.; Leung, K. H.; Pang, J. *J. Mass Spectrom.* **2000**, *35*, 71.
8. Wong, A. K.; Ross, B. P.; Chan, Y. N.; Artursson, P.; Lazorova, L.; Jones, A.; Toth, I. *Eur. J. Pharm. Sci.* **2002**, *16*, 113.
9. Wilson, G.; Hassan, I. F.; Dix, C. J.; Williamson, I.; Shah, R.; Mackay, M.; Artursson, P. *J. Control. Release* **1990**, *11*, 25.
10. Artursson, P.; Karlsson, J. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 880.
11. Drouillat, B.; Hillery, A. M.; Dekany, G.; Falconer, R.; Wright, K.; Toth, I. *J. Pharm. Sci.* **1998**, *87*, 25.
12. *C*log *P* values were calculated using the *C*log *P* function of ChemDraw Ultra 10.0 (CambridgeSoft, Cambridge, MA). *C*log *P* licensed from BioByte (Claremont, CA).
13. Ross, B. P.; Braddy, A. C.; McGeary, R. P.; Blanchfield, J. T.; Prokai, L.; Toth, I. *Mol. Pharmaceutics* **2004**, *1*, 233.
14. Malkinson, J. P.; Falconer, R. A.; Toth, I. *J. Org. Chem.* **2000**, *65*, 5249.
15. Alewood, P.; Alewood, D.; Miranda, L.; Love, S.; Meutermans, W.; Wilson, D.; Gregg, B. F. In *Methods in Enzymology*; Academic Press, 1997; Vol. 289, pp 14–29.
16. Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.
17. Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236.