Effects of Peptide Structure on Transport Properties of Seven Thyrotropin Releasing Hormone (TRH) Analogues in a Human Intestinal Cell Line (Caco-2)

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INTRODUCTION

The peroral administration of peptides and proteins could offer significant advantages over the parenteral application, especially in the long-term treatment of chronic diseases (1). Biologically active peptides have recently become available in larger amounts through major advances in peptide synthesis. The limiting factors for the peroral delivery remain the low rate and extent of gastrointestinal absorption, the metabolic lability and, hence, a low and highly variable oral bioavailability (2).

One way to increase the amount absorbed through the intestinal barrier is to develop peptides with optimized physicochemical properties. It is well known that the transported amount of drugs correlates inversely with the molecular weight (3), but is proportional to its lipophilicity, as determined by partition coefficient (4). Recently, Palm and co-workers (5) observed a good correlation between the in vitro permeability (P_{app}) and the relative polar suface area of a series of β-blockers, in contrast to the partition coefficient. On the other hand, studies using a model peptide (AcF_3NH_2) suggested a connection of the in vitro permeability and the number of potential hydrogen bonds (6). The influence of the solution conformation of a pentapeptide (YPXDV, X = G, I), determined by nuclear magnetic resonance spectroscopy (NMR), does not seem to be important for the permeability via the paracellular route through Caco-2 cells (7). Another effect of structural modifications was increased metabolic stability, which leads to higher drug concentrations in the donor compartment (8).

To evaluate the effect of structural modifications of a therapeutically active peptide, we selected a series of thyrotropin-releasing hormone (TRH) analogues of differing hydrogen bonding capacities and lipophilicities.

These studies were conducted using the Caco-2 cell culture model established in our laboratory (9).

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MATERIALS AND METHODS

Materials

TRH (pGlu-His-Pro-NH_2), TROH (pGlu-His-Pro-OH), 3-Me-His-TRH (pGlu-[Me]-His-Pro-NH_2), Dehydro-TRH (pGlu-His-[3,4-Dehydro]-Pro-NH_2), and rabbit like-TRH (Rab-TRH, pGlu-Glu-Pro-NH_2) were obtained from Saxon Biochemicals GmbH (Germany), and Phe-TRH (pGlu-Phe-Pro-NH_2) and Glu-TRH (Glu-His-Pro-NH_2) were from the American Peptide Company (USA).

Cell culture reagents were bought from Gibco (Eggenstein, Germany) except for fetal calf serum (FCS) which was from Biozol (Eching, Germany). Cell culture articles were purchased from Nunc (Wiesbaden, Germany). Polycarbonate membrane cell culture inserts (Transwell®, Costar) were from Tecnomara (Fernwald, Germany). All other chemicals were obtained from E. Merck (Darmstadt, Germany) in analytical quality.

Cell Cultures

Caco-2 cells from Dr. Löhrke (DKFZ, Heidelberg, Germany) were used in passage 34–40. The monolayers were cultivated as previously reported (9).

Transport Studies

The transport studies were performed as described previously (9). Hank's balanced salts solution containing 15 mM glucose at pH 6.7 was used as transport buffer. 1.5 mL of the drug solution, consisting of 3 mM TRH or analogues dissolved in transport buffer, was placed on the apical and 2.5 mL transport buffer on the basolateral side of the monolayers or vice versa for basolateral to apical transport studies. After different time intervals, 1 mL samples were withdrawn from the acceptor chamber and replaced by fresh buffer. The integrity of the monolayer was checked at the beginning and the end of each experiment by determining the transepithelial electrical resistance (Endohm^R, WPI, Germany).

HPLC

The instrumentation consisted of a pump (Model L-6200 A), an automatic sampler (Model AS-2000 A), a column thermostat (Model T-6300), and a UV-detector (Model L-4000) all from Hitachi, Merck (Darmstadt, Germany). Data acquisition and integration was carried out by the Millennium 2010 software (Millipore Waters, Eschborn, Germany). All substances were separated on a reverse-phase column (Superspher 100 RP-18 125 × 4 mm, Merck) using a mobile phase of 0.1% trifluoroacetic acid (TFA), pH 2.0 (A) and acetonitrile (B). For TRH, Dehydro-TRH, MeHis-TRH, and Glu-TRH, the mobile phase consisted of 95% A and 5% B, for TROH and Rab-TRH, the mobile phase consisted of 90% A and 10% B, and for Phe-TRH, 70% A and 30% B. Detection wavelength was 215 nm. With a flow of 1 mL/min, retention times were as follows: TRH, 2.4 min; Dehydro-TRH, 2.5 min; MeHis-TRH, 2.4 min; Glu-TRH, 2.8 min; TROH, 2.0 min; Rab-TRH, 2.4 min; and Phe-TRH, 2.2 min. Detection limits were 0.5 μM.

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Data Analysis

Apparent permeability coefficients were calculated from the receiver compartment concentrations and the following relationship:

$$P_{app} = \frac{V_R}{AC_0}\frac{dc}{dt}\ [cm/s]$$

where V_R is the volume of the receiver compartment, A is the membrane surface area (4.71 cm^2), C_0 is the initial donor concentration of solute, and dc/dt is the slope of the regression line describing the cumulative receiver concentration versus time (including t = 0). All results are expressed as the mean of at least 3 experiments ± standard deviations, and statistical analysis was performed using unpaired, two-tailed Student's t-test.

Determination of the Partition Coefficient (log k_0)

Lipophilicity of the TRH analogues was determined chromatographically using a lipophilic solid phase (Lichrosorp RP 18, 250 × 4 mm, 7 µm) and a hydrophilic mobile phase (0.1% TFA) according to (10). The characteristic value obtained is a chromatographic partition coefficient k_0, which is proportional to the octanol/water partition coefficient.

Calculation of the Procentual Polar Surface

The total surface of the peptides and the relative polar surface were calculated using a Silicon Graphics 25 TG hardware equipped with stereoview and the Insight and Discover software of Biosym (Germany). Firstly, the molecules were constructed on PC and their total energy was minimized using a consistent valence force field to obtain steric realistic conformations. Afterwards, these conformations were altered by a simulated heating from 10°K to 300°K to allow free bonding rotation and subsequently cooled to 10°K. This dynamical procedure was repeated for 10,000 iterations and history files were saved every 100 steps. The structures listed in these files did not differ significantly in conformation or energy. Therefore, any conformation was selected and its total surface area based on the Connolly model and the relative polar surface area were calculated by the Insight and Discover software.

Number of Hydrogen Bondings

The potential hydrogen bonds the peptide could make were calculated according to Stein (11).

RESULTS AND DISCUSSION

Structural alterations of drugs can cause different effects leading to higher transport rates across biological membranes. For example, the enzymatic stability (8) or the lipophilicity (12) of drugs could be increased.

We select a series of 7 TRH-analogues (structures are given in Figure 1) to determine the effect of peptide conformation of a small tripeptide on its transport rate across intestinal cells, Caco-2.

Enzymatic Stability

Since we could not detect metabolism products by HPLC, we suggested that none of these peptides was subject to enzymatic degradation and metabolic stability was not a critical factor for the transported amount.

Active Transport

In vivo transport of TRH followed an active transport route, possibly mediated by the di- and tripeptide transporter (13). Although it is known that Caco-2 cells express this transporter system, the pathway of TRH in vitro is controversial. Whereas Lundin *et al.* (8) postulated a passive, paracellular transport of TRH through Caco-2 cells, Nicklin *et al.* (14) observed an active transport component. We could not note a higher transport rate from apical to basolateral than from basolateral to apical (Figure 2). Therefore an active transport component seems to be unlikely at the passage numbers and the cultivation times of Caco-2 cells regarded in this study. Consequently, the different permeability coefficients were not caused by different binding abilities of the peptides to a transporter protein. For further correlations, the apical to basolateral transport rate was used.

Lipophilicity

Different studies showed correlations between the logarithm of the partition coefficient of drugs and their permeability coefficients across cell layers (4). The log k_0 values were determined by HPLC and decreased in the following order: Phe-TRH > TROH > Rab-TRH > MeHis-TRH > TRH > Dehydro-TRH > Glu-TRH. As expected, the peptide with the lowest retention time, i.e., the lowest lipophilicity, Glu-TRH, shows the lowest transport rate (Table I). Nevertheless, regarding all P_{app} for the TRH analogues, a causal correlation between the partition coefficient and the transport rate was not observed. This is in agreement with Lundin *et al.* (8), who could not find an improvement in the transport of TRH across Caco-2 cells by derivatization with N-octyloxycarbonyl.

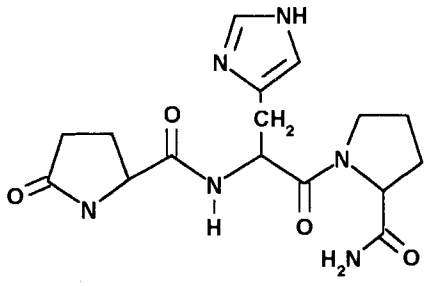
Relative Polar Surface Area

Palm *et al.* (5) determined a better relationship between P_{app} values of β-blockers across Caco-2 cells and the surface polarity of these substances instead of their partition coefficient. For that reason, we determined the relative polar surface area of our TRH analogues and compared them with the log k_0 value. Rab-TRH revealed the largest polar surface of 51.3% and a P_{app} of 1.1 ± 0.17 * 10^{-7} cm s^{-1}. However, Phe-TRH showed a similar P_{app} of 1.1 ± 0.06 * 10^{-7} cm s^{-1} but has the smallest polar surface of 36.9% (Table I). Consequently, a relationship between the relative polar surface area and the transepithelial transport rate of TRH-analogues was not observed.

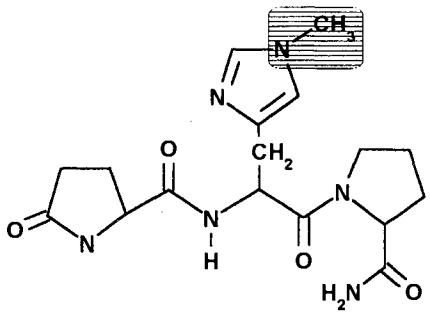
Since the most hydrophilic β-blocker investigated by Palm *et al.* (5), atenolol, has a polar surface area of only 27.8%, but the most lipophilic TRH analogue in our study, Phe-TRH, has a polar surface area of still 36.9%, a linear correlation between the P_{app} and the surface properties may only be obtained in more lipophilic drugs than peptides.

Hydrogen Bonds

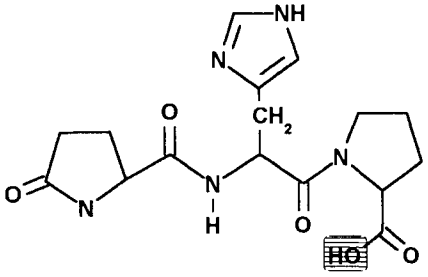
Another attempt to correlate the permeability coefficients of TRH analogues with their structural features was made by



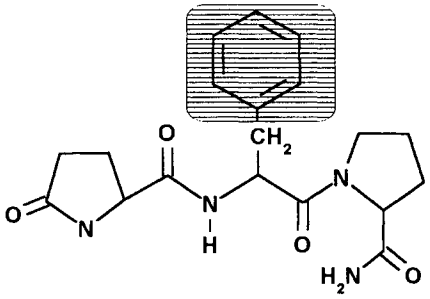
TRH
(pyro-L-Glu-L-His-L-Pro-NH_2)



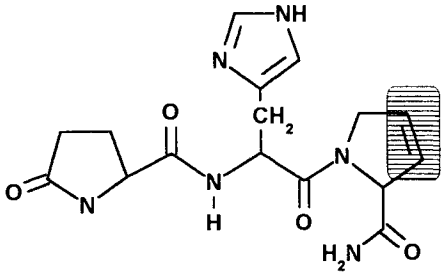
MeHis-TRH
(pyro-L-Glu-L-His-L-Pro-NH_2)



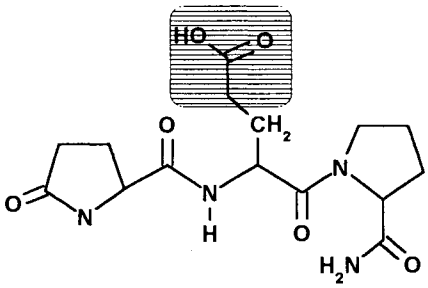
TROH
(pyro-L-Glu-L-His-L-Pro-OH)



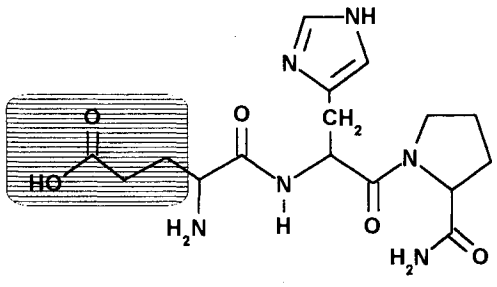
Phe-TRH
(pyro-L-Glu-L-Phe-L-Pro-NH_2)



Dehydro-TRH
(pyro-L-Glu-L-His-L-Pro-NH_2)



Rab-TRH
(pyro-L-Glu-L-Glu-L-Pro-NH_2)



Glu-TRH
(L-Glu-L-His-L-Pro-NH_2)

Fig. 1. Chemical structures of the TRH analogues. Altered structures in comparison with TRH are marked.

Table I. Permeability of Seven TRH Analogues Through Caco-2 Monolayers in Comparison with Their Physicochemical Properties

Peptide	P_{app} (a → b)[a] [*10^{-7} cm/s ± SD]	P_{app} (b → a)[a] [*10^{-7} cm/s ± SD]	log k_0[b]	Polar surface[c] [%]	MW [Da]	Total surface[c] [Å^2]	Hydrogen bondings[d]
TRH	1.7 ± 0.06	2.0 ± 0.47	0.83	48	362.4	343	9
TROH	1.2 ± 0.2	1.5 ± 0.16	1.30	44	363.3	340	9
Rab-TRH	1.1 ± 0.17	1.4 ± 0.06	1.05	51.3	354.4	329	11
Dehydro-TRH	1.5 ± 0.08	2.4 ± 0.28	0.74	48.5	358.4	343	9
MeHis-TRH	1.3 ± 0.24	1.3 ± 0.24	0.96	38.8	376.4	369.6	8
Phe-TRH	1.1 ± 0.06	1.2 ± 0.18	1.54	36.9	372.5	362.6	8
Glu-TRH	0.7 ± 0.04	0.05 ± 0.02	0.53	51	380.4	360	12

[a] Values are expressed as mean of 3 determinations ± standard deviation.
[b] Determined chromatographically according to Ref. 10.
[c] Calculated by the PC software Insight and Discover.
[d] Determined according to Ref. 11.

determining the number of potential hydrogen bonds the peptides could make, according to Burton *et al.* (15). They reported a better relationship between the number of hydrogen bonds than the partition coefficient for a series of model peptides and the log of the P_{app} value.

According to Stein *et al.* (11), we determined the number of possible hydrogen bonds each TRH-analogue could make. As shown in Table I, TRH, TROH, and Dehydro-TRH could form nine hydrogen bonds but were transported differently. On the other hand, Rab-THR and Phe-TRH showed similar permeability coefficients but highly different numbers of possible hydrogen bonds. This indicated that the transported amount of THR analogues is independent of the number of hydrogen bonds.

Molecular Size

The paracellular pathway of drugs is governed by the presence of tight junctions between the cells. For this aqueous pathway the size of a molecule is critical (5). Usually, the molecular weight is considered, although the three-dimensional structure of the molecule is more important. The atomic force field calculations allow us to estimate the surface area of a constructed molecule. As listed in table I, the surface area is the smallest for Rab-TRH (329 Å^2) and the largest is obtained for MeHis-TRH (369.6 Å^2). From these data it could be assumed that Rab-TRH would be transported with the highest effective permeability coefficient. Unfortunately, Rab-TRH is one of the poorly transported analogues (P_{app} = 1.1 ± 0.17 * 10^{-7} cm s^{-1}).

In this study, neither the molecular weight nor the molecular surface seems to predict the effective permeability of the TRH-analogues. Since a limitation of the paracellular pathway is described for molecules with a MW of 1000 Da (3), TRH-analogues are probably too small to observe the slight changes in molecular size on the paracellular transport. Furthermore, both the size and the P_{app} values of these peptides vary only in a very small range, indicating rather biological variations of the cell monolayers than size differences decisive for the different transport rates in our study.

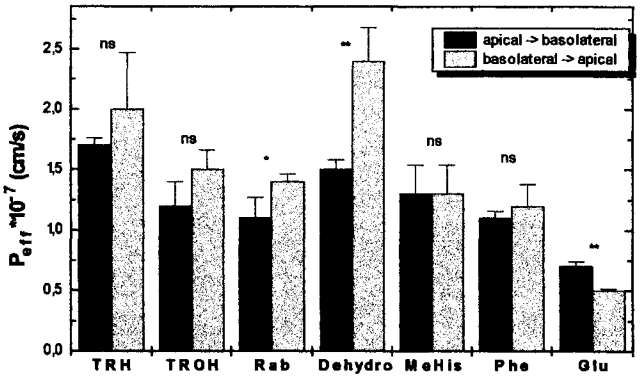


Fig. 2. Permeability coefficients of TRH analogues from apical to basolateral (black bars) and from basolateral to apical (grey bars). Data are expressed as mean of 3 determinations ± standard deviation; independent t-test results are expressed as differences between both pathways (ns = not significant, *p = 0.005, **p = 0.001).

SUMMARY

We conclude that TRH and its analogues permeate mainly via paracellular routes in this particular clone of Caco-2 cells because variation in lipophilicity, polar surface properties, or hydrogen bonding potential—all influencing the transcellular pathway—do not give meaningful relationships. On the other hand, the variations in molecular size of the TRH analogues were too small to detect any influence on the paracellular transport properties. Further studies concerning the solution conformation and the hydrodynamical radii of the molecules probably give more information about the structure–permeability relationship of TRH transport across Caco-2 cell monolayers. The influence of the structural variations of these 7 TRH analogues on the binding affinity to the di- and tripeptide transporter, using another Caco-2 cell clone, are currently under investigation in our laboratory.

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