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Exploratory neuropharmacological evaluation of a conformationally constrained thyrotropin-releasing hormone analogue

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Abstract

A conformationally constrained peptidomimetic derived from the endocrine and neuroactive tripeptide thyrotropin-releasing hormone (pGlu-His-Pro-NH_2) was synthesized by convenient solid-phase organic chemistry and evaluated as a potential central nervous system agent. While this ethylene-bridged peptide analogue has been reported to lack the hormonal effect of the native peptide, we have shown in animal models that it possesses central nervous system activity characteristic of thyrotropin-releasing hormone. Compared to control, the peptidomimetic showed significant analeptic and antidepressant-like potencies. Moreover, an enhanced selectivity in antidepressant-like effect was measured when compared to that of the native peptide. Immobilized artificial membrane chromatography and in vitro metabolic stability studies also revealed that this constrained peptidomimetic has higher affinity to the blood-brain barrier than the native peptide and is metabolically stable. Consequently, this structure may be used as a template to design centrally selective and metabolically stable thyrotropin-releasing hormone analogues as potential neuropharmaceutical agents.

Keywords

Bridged TRH analogue; Solid-phase organic synthesis; Immobilized artificial membrane chromatography; Analeptic activity; Antidepressant-like activity; Porsolt swim test

1. Introduction

In 1969, two groups lead by Guillemin [5] and Schally [2] discovered that the hypothalamic substance responsible for the release of thyrotropin was the tripeptide pGlu-His-Pro-NH_2 or thyrotropin-releasing hormone (TRH). TRH has dual role; it acts as a hormone and it is also a neuropeptide. The endocrine function of TRH is to stimulate the synthesis and release of

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thyroid-stimulating hormone by the anterior pituitary gland [11]. TRH also affects brain chemistry and physiology. It is a neuromodulator of different neurotransmitters including acetylcholine, dopamine, and serotonin [16]. The numerous central nervous system (CNS) effects of TRH are not mediated by its endocrine function [11].

TRH has been suggested as potential therapeutic agent for several neurological and psychiatric disorders such as Alzheimer's disease and depression [7,9,10,12]. The best-known CNS effect of this neuropeptide is its analeptic activity shown as the reduction of barbiturate induced narcosis or reduction of halperidol induced catalepsy [4,21]. Unfortunately, high doses of the peptide are needed to produce CNS effects due to its limited transport across the blood-brain barrier (BBB) [23]. However, the endocrine effect of the peptide could also be significant at high doses. Its rapid degradation by TRH-degrading ectoenzyme (pyroglutamyl aminopeptidase II) represents another obstacle to the use of TRH as a CNS agent [11]. Due to the extraordinary therapeutic potential of TRH to treat CNS maladies, the development of CNS-selective and metabolically stable TRH analogues/mimetics is warranted [16].

A conformationally restricted peptidomimetic (2, Fig. 1) designed from TRH has been reported recently [1]. The goal was to preserve the key recognition elements of the interaction between TRH and its receptors. However, radioligand-binding studies showed that **2** had weak binding affinity ($>10^{-6}$ M) to TRH receptors; thus, this peptidomimetic did not retain the endocrine activity of the native peptide upon employing the piperazin-2-one ring constraint. On the other hand, it must be recognized that decrease or loss of endocrine activity manifested by a lead compound is a crucial issue of drug design when CNS therapy is targeted [17,20,22]. Therefore, we intended to explore **2** in animal models reflecting neuropharmacological activities characteristic to TRH. In this publication we report a convenient solid-phase synthesis and focus on the first exploratory neuropharmacological evaluation of this conformationally constrained TRH mimetic (**2**) as a potential CNS agent.

2. Materials and Methods

2.1. Instruments and materials

All chemicals used were reagent-grade or peptide-synthesis grade. Solvents were obtained from Fisher Scientific (Atlanta, GA). Fmoc-Rink Amide resin, protected amino acids, TRH and [Glu^2]TRH were purchased from Bachem BioSciences (Torrance, CA). Purification was done by reversed-phase high performance liquid chromatography (RP-HPLC) on a system that consisted of a SP 200 binary gradient pump (Thermo Fisher, San Jose, CA), a Rheodyne (Cotati, CA) model 7125 injector valve equipped with a 5-ml sample loop, and a SP 100 UV/VIS detector (Thermo Fisher) operated at 220 nm. The 100 mm × 25 mm i.d. Waters (Milford, MA) RCM DeltaPack C_{18} column was operated at 3.0 ml/min flow rate. Analytical RP-HPLC was performed on a Surveyor HPLC system operated by ChromQuest 4.0 Chromatography Workstation Software (Thermo Fisher) using an Alltech (Deerfield, IL) Econosyl C_{18} column (150 mm × 3.2 mm i.d., 5μm particles), a flow rate of 0.5 ml/min and UV detection at 220 nm. The mobile phases were mixed from 0.1 % (v/v) trifluoroacetic acid (TFA) in H_2O (solvent A) and 0.08% (v/v) of TFA in CH_3CN (solvent B). After delivery of an isocratic flow of 98:2 (v/v) mixture of solvent A and B for 5 min, gradient elution was performed to reach 50:50 (v/v) mixture in 24 min. Atmospheric-pressure chemical ionization mass spectrometry (APCI-MS) was done on a quadrupole ion trap instrument (LCQ, Thermo Fisher, San Jose, CA). Accurate mass measurements were performed by high-resolution mass spectrometry (HRMS) using an LTQ-FT (Thermo Fisher) instrument at mass resolution (M/ΔM) of >100,000. ^{1}H-NMR spectra were recorded on a Varian XL-300 instrument. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

2.2. Solid-phase organic/peptide synthesis

The solid-phase synthesis of **2** (Scheme 1) as well as its linear counterpart pGlu-His-Val-NH_2, **1** [prepared by routine 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry], which is necessary for control experiments was done on Fmoc-Rink-Amide resin using benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluoro-phosphate/1-hydroxybenzotriazole/N,N'-diisopropyl-ethylamine (PyBOP/HOBt/DIPEA) coupling method in N,N-dimethylformamide (DMF) with ninhydrin test monitoring [8]. Incorporation of the ethylene bridge was done after coupling Fmoc-His(Trt) (where Trt denotes trityl) to the resin-bound Val. The Fmoc protecting group was replaced with the base- and acid-resistant 4-nitrobenzenesulfonyl group [6]. Then the resin was treated with a large excess of 1,2-dibromoethane in the presence of tetramethylguanidine in DMF at 60 °C for 4 h. The 4-nitrobenzenesulfonyl moiety was removed with 2-mercaptoethanol in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and then pGlu was coupled to the central residue using O-(7-azobenzotriazol-1-yl) N,N,N'N'-tetramethyluranium hexafluorophosphate (HATU) instead of PyBOP. The target compounds were removed from the solid support with TFA:H_2O:triisopropylsilane (95:2.5:2.5, v/v), purified by semi-preparative RP-HPLC and characterized by analytical RP-HPLC, combustion analysis, ^{1}H-NMR spectrometry and APCI-MS.

2.3. Immobilized artificial membrane chromatography (IAMC)

A 1-cm × 3.0 mm i.d. IAM.PC.DD2 column (Regis Technologies, Morton Grove, IL) was employed. An isocratic solvent delivery (10-mM aqueous ammonium acetate adjusted to pH 5.4 with acetic acid) of 1.0 ml/min was provided by a Pharmacia-LKB (Bromma, Sweden) HPLC pump (model 2150), and sample solutions of approximately 0.1 mg/ml concentration were injected by using a 5μl loop. IAMC capacity factors (k_{IAM}') were calculated as follows: $k_{IAM}' = (t_{R(X)} - t_{R(V)} / t_{R(V)}$, where $t_{R(X)}$ and $t_{R(V)}$ are the retention times for the compound of interest and the void volume marker [Glu^2]TRH [19], respectively. Detection was done by APCI-MS using the protonated molecules for the analytes and the void volume marker (m/z 363, 365, 391 and 355 for TRH, **1**, **2** and [Glu^2]TRH, respectively) to retrieve selected-ion chromatograms and obtain t_R values.

2.4. In vitro metabolic stability studies

Stability studies were performed in mouse brain homogenate and plasma. After killing by cervical dislocation, the animal was decapitated and the trunk blood was collected into 6-ml plasma determination tubes containing sodium heparin (freeze-dried). Plasma was prepared without delay by centrifugation at 1500 rpm for 10 min using a Sorwal (Newtown, CT) GLC-1 general laboratory centrifuge. Immediately following removal, the brain was weighed and transferred to a 15-ml glass Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ) placed in an ice bath. After addition of an ice-cold phosphate-buffered saline solution (PBS, volume in ml equivalent to four times of the weight of the brain in grams), brain homogenate was prepared by running the ball-shaped teflon pestle of the grinder, with the steel shaft connected to a motor-driven overhead stirrer (Wheaton), for 80 s at 1500 rpm. The brain homogenate and plasma was used immediately for the in vitro stability studies.

Approximately 100 nmol of test compound was added to 1 ml of mouse plasma or brain homogenate (20% w/w, pH 7.4, in phosphate buffer) and the mixture was incubated at 37 °C in a temperature-controlled, shaking water bath. Aliquots (100μl) were removed after 2, 15, 30, 60 and 120 min of incubation, and transferred into a 1.5 ml Eppendorf tube containing 200μl of ice-cold solution of 5 % (v/v) acetic acid in acetonitrile. The samples were centrifuged and the supernatant was removed and analyzed by RP-HPLC to monitor the decline in the concentration of the compound added.

2.5. Neuropharmacological evaluations

2.5.1. Analeptic effect—Ten Swiss-Webster mice (30 ± 2 g body weight) were used in each group. Test compounds were dissolved in saline. The vehicle alone (1.5 ml/kg body weight; control group) or equimolar doses of test compounds (10μmol/kg body weight) were injected through the tail vein of mice. After 10 min, each animal received an i.p. injection of sodium pentobarbital solution at a dose of 60 mg/kg body weight. The sleeping time was recorded from the onset of loss of the righting reflex until the reflex was regained.

2.5.2. Porsolt swim test (PST)—We adapted the original PST [14] for Swiss-Webster mice (30±2 g body weight) and employed a "pre-swim test session" to avoid variations and maintain consistency in the immobility time among different groups (similarly to PST in rats). For the pre-swim test, mice were placed individually in a glass cylinder (22 × 25 cm) containing fresh water up to the height of 15 cm and a temperature of 25 ± 2 °C. Upon removal, the animals were hand-dried with a towel and placed on warming pads for 15 min prior to returning them to their cage. Twenty four hours later, mice were treated with either the test compound (3μmol/kg body weight, i.p.) or the vehicle (saline, i.p. 50μl, control group) 10 min before the actual swim test. Each animal was forced to swim again in the same environment as during the pre-test. For 6 min, the immobility time (defined as the duration of floating motionless after the cessation of struggling and making only movements necessary to keep the head above the water) was recorded simultaneously by two trained observers who were unaware of ("blinded" to) which animal received the control and the test compound, respectively. Data were expressed as percentage change in immobility time counting data for the control group as 100%. Each group of animals consisted of 6 to 10 mice. To validate the experimental conditions we also administered amitriptyline, a tricyclic antidepressant [12], and found 59 ± 2% decrease in the immobility time compared to that of saline.

2.6. Statistical analysis

Analysis of variance (ANOVA) followed by post hoc Dunnett's test was done to compare treatments to the control group. Differences were considered significant when $P<0.05$.

3. Results

3.1 Solid-phase organic/peptide synthesis

We successfully obtained **2** by a convenient Fmoc-based solid-phase approach according to Scheme 1. Compound **1**: ^{1}H-NMR (300 MHz, D_2O) chemical shifts: 8.62 (d, *J*= 1.2 Hz, 1H, His Hε); 7.29 (d, *J*=1.1 Hz, 1H, His Hδ); 4.71 (dd, *J*= 12.0 and 5.7 Hz, 1H, His Hα); 4.34 (dd, *J*= 9.0 and 5.1 Hz, 1H pGlu Hα); 4.09 (d, *J*= 7.5 Hz, 1H, Val Hα); 3.25 - 3.15 (m, 2H, His Hβ); 2.55-2.47 (m, 1H, Val Hβ); 2.38 (t, *J*= 9.0 Hz, 2H, pGlu Hγ); 2.04 -1.99 (m, 2H, pGlu Hβ); 1.35-1.30 (m, 2H); 0.99 (dd, *J*= 7.2 and 2.1 Hz, 6H, Val Hγ). MS (APCI): $[M+H]^+$ at *m/z* 365; HRMS: *m/z* 365.1931 (calc. 365.1932). Anal. calc. for $C_{16}H_{24}N_6O_4 \times 1$ TFA × 1 H_2O: C, 43.55, H, 5.72, N, 16.93; Found: C, 43.55, H, 5.58, N 16.15. Compound **2**: ^{1}H-NMR (300 MHz, D_2O) resonances having diagnostic values: 4.68 (d, *J*= 11.2 Hz, 1H, His Hα); 4.02–3.90 (m, 1H, Val Hα); 3.5 (s, 4H, ethylene bridge); 3.35 (d, *J*= 6 Hz, 2H, His Hβ); 2.25-2.12 (m, 1H, pGlu Hβ); 2.07-1.92 (m, 1H, pGlu Hβ); 0.97 (d, J= 6.6 Hz, 3H, Val Hγ); 0.78 (d, *J*= 6.6 Hz, 3H, Val Hγ). MS (APCI): $[M+H]^+$ at *m/z* 391; HRMS: *m/z* 391.2087 (calc. 391.2088). Anal. calc. for $C_{18}H_{26}N_6O_4 \times 4$ TFA × 3.5 H_2O: C, 34.28, H, 4.17, N 9.65; Found: C, 34.44, H, 4.19, N 9.24.

3.2. IAM chromatography

Table 1 shows that the conformationally restricted TRH mimetic (**2**) is predicted to possess higher affinity to lipid membranes (k'_{IAM} = 1.65) than the linear counterpart (**1**, k_{IAM} = 0.78) or TRH itself (k'_{IAM} = 0.51).

3.3. In vitro metabolic stability studies

The conformationally restricted TRH mimetic (**2**) as well as its linear counterpart (**1**) were stabile both in mouse brain homogenate (20 % w/v in phosphate buffer) and in mouse plasma. Within 2 h, less than 10% degradation was detected by RP-HPLC analyses.

3.4. Analeptic activity

The antagonism of barbiturate-induced narcosis in mice was used to evaluate **2** as a potential CNS-agent. The linear TRH analogue (**1**) was also included in the study to assess the consequence of replacing the C-terminal Pro-NH_2 in the native peptide with Val-NH_2 and subsequently constraining the flexibility of this peptide by introducing a piperazinone ring on the analeptic activity. As shown in Table 1, a statistically significant decrease (approx. 20%) was recorded in the sleeping time compared to the control group (animals injected with the vehicle only; sleeping time 69 ± 8 min) for both test compounds (**1** and **2**) at equimolar concentration (10μmol/kg body weight). The two test compounds behaved essentially identically in this experimental paradigm; however, they did not reach the potency of the native peptide. Nevertheless, these non-endocrine TRH-related compounds did show CNS activity.

3.5. Antidepressant-like activity

Upon using the PST, we obtained a statistically significant effect compared to the control group (vehicle only) upon i.p. injection of the test compounds (**1, 2** and TRH) at equimolar doses (3μmol/kg body weight). PST data in Table 1 were expressed as percentage decrease in immobility time compared to the control group. Immobility time of the control group (285 ± 6 min) was defined as 100%. The TRH analogue having piperazin-2-one ring constraint (**2**) showed an improved antidepressant-like selectivity compared to the linear counterpart (**1**) suggesting that restricting the conformational flexibility of the non-endocrine TRH analogue may be beneficial for improving selectivity of TRH-related CNS-actions.

4. Discussion

We conducted our studies to determine whether a conformationally restricted TRH mimetic (**2**, Fig. 1) that showed no endocrine activity [1] has retained or perhaps selectively enhanced certain CNS-actions of the native peptide by employing animal models reflecting pharmacological activities characteristic to TRH in the CNS. Dissociating endocrine and CNS actions of TRH is a crucial issue in designing TRH analogues/mimetics for neurotherapy. Instead of the previously reported cumbersome solution-phase synthesis [1], we obtained **2** and its linear counterpart (**1**) by convenient solid phase organic/peptide synthesis (Scheme 1). To our knowledge, no other solely solid phase synthesis has been reported for peptidomimetics having short-range (ethylene bridge) constraint. Therefore, the present work may enable combinatorial syntheses of compounds based on this template.

A rapid comparison of the linear (**1**) and constrained (**2**) analogue regarding their affinity to biological membranes such as the BBB was done by IAMC that mimics membrane interactions better than partitioning in the isotropic n-octanol/water system (log P) [3,13]. The IAMC capacity factors (k'_{IAM}) are predictors for membrane permeability. Compared to **1** and TRH, the constrained peptide (**2**) showed somewhat improved affinity to lipid membranes (Table 1). In vitro metabolism studies also showed that both **1** and **2** were metabolically stable (less than

10% degradation in 2 h). In contrast, the biological half-lives of TRH (16 min in rat brain homogenate and 11 min in rat plasma, respectively [17]) are very short, which significantly limits its practical use as a neuropharmaceutical agent.

We explored the antagonism of the barbiturate-induced anesthesia to survey the potency of **2** as a CNS-agent in mice upon i.v. administration. A statistically significant difference was observed from the control group (vehicle only) when animals were injected with **1** or **2** (Table 1). In this pharmacological paradigm, **1** and **2** were also found to be equipotent implicating, thus, that decreasing the conformational flexibility in the molecular architecture may not necessarily be a significant factor in determining the analeptic potency. However, replacement of the C-terminus of TRH with Val-NH_2 resulted in approximately 50% loss of analeptic potency compared to that of TRH.

After confirming that **2** retained CNS activity in the well-known analeptic model characteristic for TRH, we employed the PST suitable to explore antidepressant-like potency [14]. In PST, immobility is considered to reflect a “depressive mood.” In order to allow for easier comparisons with the results of the analeptic study, data were expressed as percentage decrease in immobility time (Immobility time of the control group was counted as 100%). Again, a statistically significant antidepressant-like effect was observed for the test compounds when compared to control experiments. Moreover, the constrained peptidomimetic (**2**) showed a significant increase (~2-fold, Table 1) in antidepressant-like selectivity compared to the native peptide upon comparing the analeptic *versus* antidepressant-like potencies of the test compounds. Taken that **2** is metabolically stable and a CNS-selective mimetic of TRH as well as it shows enhanced antidepressant-like selectivity when compared to the native peptide, this structure may serve as a template for the combinatorial design of novel antidepressants.

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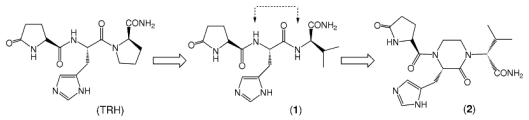
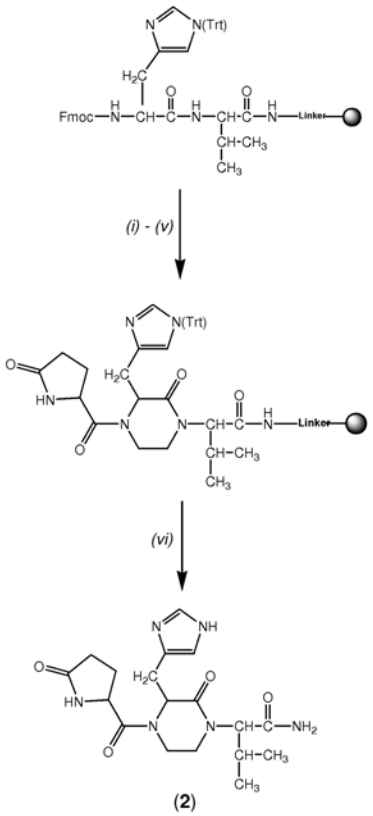


Fig 1.

An approach to obtain a peptidomimetic of TRH (pGlu-His-Pro-NH_2) with a piperazin-2-one ring constraint (**2**) through the TRH analogue pGlu-His-Val-NH_2 (**1**).



Scheme 1.

Solid-phase synthesis of a bridged TRH analogue **2**: *i*) 20% (v/v) piperidine in DMF, 1×1 min, 1× 10 min; *ii*) 1.1 eq (4-nitrophenyl)sulfonyl chloride in dichloromethane (3 ml/g resin), 1.1 eq triethylamine, 4 h; *iii*) 10 eq 1,2-dibromoethane, 10 eq tetramethylguanidine, DMF, 60 °C, 4 h; *iv*) 10 eq 2-mercaptoethanol, 5 eq DBU, DMF, 30 min; *v*) 4 eq pGlu, 4 eq HATU, 0.4 eq N-methyl morpholine, DMF, 2 h; *vi*) TFA:H_2O:triisopropylsilane (95:2.5:2.5, v/v), 10 ml/g resin, 1 h.

Table 1

IAMC capacity factors (k'_{IAM}) as predictors for membrane permeability and comparison of antidepressant-like (evaluated by PST) and analeptic effects of TRH, **1** and **2** in mice after systemic (i.v.) administration of equimolar doses of the test compounds.

Compound	k'_{IAM}	PST: % Decrease in Immobility[1]	Analeptic Effect: % Decrease in Sleeping Time[1]	Selectivity[2]
TRH	0.51	35 ± 2*	50 ± 2*	0.7
1	0.78	20 ± 1*	20 ± 6*	1
2	1.65	28 ± 1*	20 ± 4*	1.4

[1] Compared to saline control defined as 100%.

[2] Ratio of PST (% decrease in immobility) to analeptic effect (% decrease in sleeping time).

* Statistically significant difference from control (ANOVA followed by Dunnett's test, $p < 0.05$, N=6−16).