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Prodrugs to Enhance Central Nervous System Effects of the TRH-like Peptide pGlu-Glu-Pro-NH_2

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Abstract—Potential prodrugs for the TRH-like tripeptide pGlu-Glu-Pro-NH_2 were synthesized either by esterifying the Glu side-chain of the parent peptide in solution with alcohols in the presence of resin-bound dicyclohexylcarbodiimide or by solid-phase peptide chemistry. Affinities of these ester prodrugs to lipid membranes as predictors of the transport across the blood–brain barrier were compared by immobilized artificial membrane chromatography, and prodrug activation was tested in the brain tissue of experimental animals. Esters of pGlu-Glu-Pro-NH_2 with long-chain primary alcohols emerged as potentially useful prodrugs to improve the central nervous system activity of pGlu-Glu-Pro-NH_2 upon systemic administration, as revealed by the enhancement of analeptic activity in mice.

Thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2) and structurally related TRH-like peptides have been considered lead compounds for developing useful central nervous system (CNS) agents.[1] The endogenous tripeptide pGlu-Glu-Pro-NH_2 (**1**), although originally identified from rabbit prostate,[2] has been shown to occur in the human brain.[3,4] The increase in the levels of **1** in rodent models of electroconvulsive therapy[5,6] used for the treatment of severe unipolar and bipolar depression, schizophrenia and Parkinson's disease in humans[7,8] has been observed, which prompted considerations to potentially exploit the antidepressant effects of this small peptide.[9] Additional implications of **1** as a useful CNS agent have been originated from its neuroprotective effect,[10] analeptic[11] and locomotor activity.[12] Although these properties are shared with TRH, the beneficial effects after treatment with **1** are more robust and/or last longer compared to the mostly transient response exerted by TRH. These advantages are probably due to the increased resistance of **1**[13] to the soluble cystein protease pyroglutamyl aminopeptidase I (PAP I, EC 3.4.19.3), the CNS ectoenzyme pyroglutamyl aminopeptidase II (PAP II, EC 3.4.19.6), and to serum thyroliberinase, which is similar to PAP II but does not have the transmembrane anchor of the latter, principally responsible for the rapid degradation of TRH in vivo.[14] In addition, **1** does not bind to TRH receptors[15] and does not elevate thyroid-hormone (specifically triiodothyronine, T_3) levels,[9] which have indicated that the peptide probably exerts pharmacological effects through binding to its own receptor in the CNS.[9]

After systemic administration, **1** is rapidly cleared from the circulation in rats, and the entrance of the peptide into the brain is slow.[5] The long (ca. 2-h) delay of **1** to enter the cerebrospinal fluid (CSF) by a yet to be identified transcellular transport mechanism results in a small fraction ($\leq$0.005% dose/g wet brain tissue) reaching certain brain regions (amygdala, cerebral cortex and medulla). On the other hand, a long retention of the peptide in these brain tissues has been observed even after its rapid clearance from the CSF.[5]

Because **1** is a peptide and is also predominantly ionized at physiological pH on the carboxyl group in the side-chain of Glu^2, the compound will be prevented from entering the brain in a pharmacologically significant amount by passive transport.[16] Therefore, we targeted a transient and bioreversible modification of the parent

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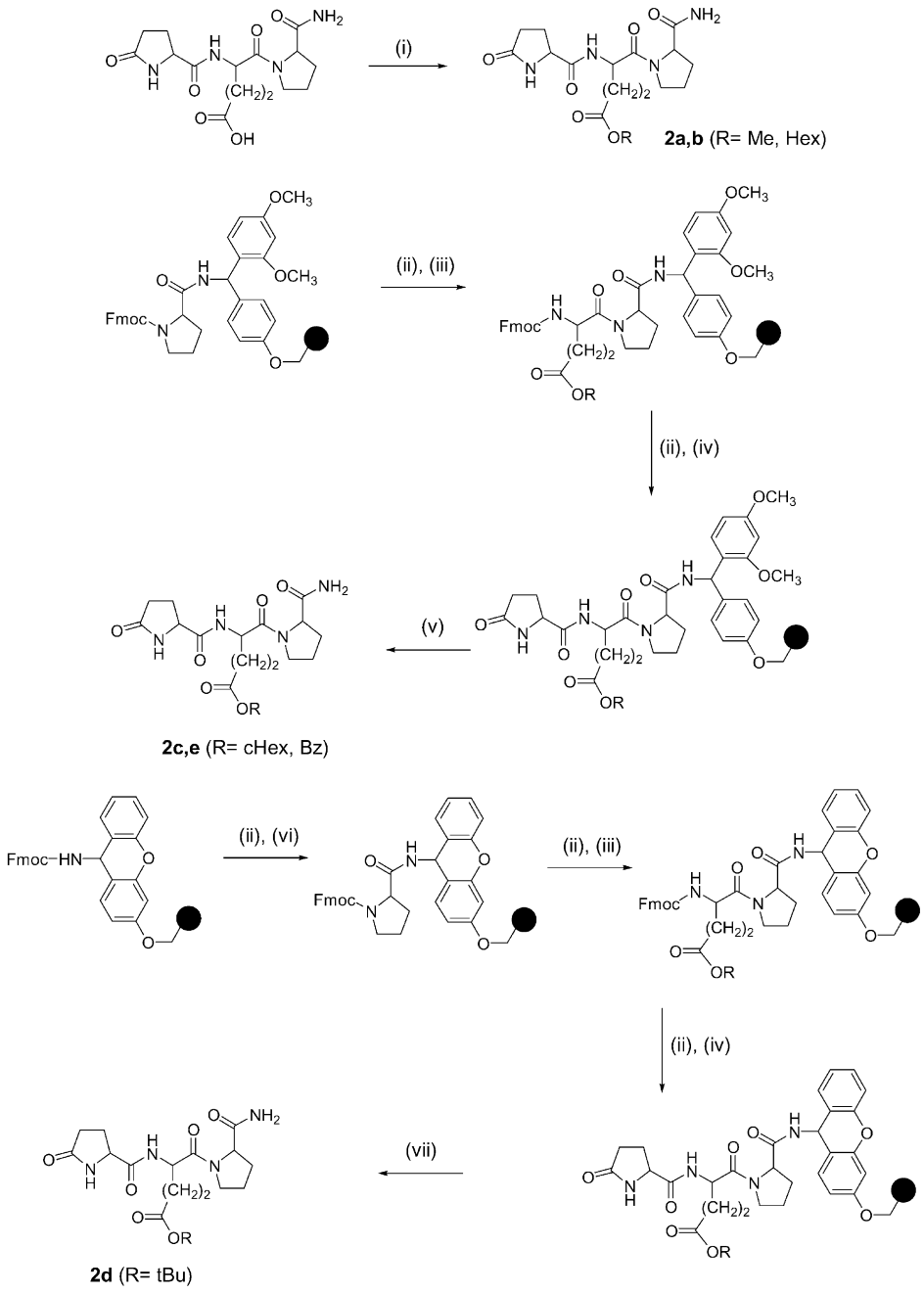
molecule at the γ-COOH of the Glu[2] residue to obtain prodrugs that render the molecule neutral (non-ionizable) and, hence, amenable for diffusion across the tightly connected endothelial cells of the cerebral microcirculation representing the blood–brain barrier (BBB). Several potential prodrugs of **1** were synthesized, as shown in Scheme 1. Briefly, direct esterification of **1** in the presence of *N*-cyclohexylcarbodiimide, *N*′-methyl polystyrene (loading: 1.3 mmole/g) and *N*-(methylpolystyrene)-4-(methylamino)pyridine (loading: 1.49 mmole/g) in dichloromethane (DCM) using 5-fold excess of alcohol produced **2a**,**b** in nearly quantitative yield. Other esters were prepared by solid-phase peptide synthesis (SPPS). The pre-loaded Fmoc-Pro-Rink-4-methylbenzhydryl-amine (MBHA) resin was employed for obtaining **2c**,**e**, while the super acid-sensitive Sieber Amide resin was loaded with Fmoc-Pro using a standard PyBOP/DIPEA procedure for the preparation of **2d**. After attaching the N-terminal residue, the compounds were cleaved from the resin using TFA and water (98:2, v/v) for **2c**,**e** and TFA:DCM (1:99, v/v) for **2d**. They were purified by semi-preparative gradient reversed-phase high-performance liquid chromatography (HPLC).

A rapid evaluation of the increase of the ability of the newly synthesized potential prodrugs of **1** to interact



Scheme 1. Synthesis of potential pGlu-Glu-Pro-NH_2 prodrugs (**2a**–**e**). Solution-phase synthesis: (i) ROH (5 equiv), polystyrene-bound cyclohexylcarbodiimide (3 equiv) and polystyrene-bound 4-dimethylaminopyridine (0.1 equiv), in DCM, rt, overnight. Solid-phase syntheses: (ii) 20% (v/v) piperidine in dimethylformamide, 10 min; (iii) PyBOP:HOBt:Fmoc-Glu(R):DiPEA (1/1/1/2); (iv) PyBOP:HOBt:pyroglutamic acid:DiPEA (1:1:1:2); (v) TFA:H_2O (98:2, v/v); (vi) PyBOP:HOBt:Fmoc-Pro-OH:DiPEA (1/1/1/2); (vii) TFA:DCM (1/99, v/v).

with biological membranes was done by immobilized artificial membrane chromatography (IAMC).[17] This technique measures the partitioning into monolayers of cell membrane phospholipids immobilized by covalent binding on silica particles. The chromatographic capacity factor (k'_{IAM}) for a compound obtained by IAMC is directly related to its partition coefficient between the aqueous phase and the chemically bonded membrane phase and, ultimately, to the K_m value representing its fluid-membrane partition coefficient.[18] Essentially, an increase in the k'_{IAM} indicates an increase in the membrane permeability of the compound. IAMC mimics membrane interactions better than partitioning in the isotropic n-octanol/water system (log P),[19] and the technique has been applied to the assessment of BBB penetration for structurally diverse drugs.[20–22] In our study, atmospheric-pressure ionization (APCI) mass spectrometry was employed as a method of detection.[23,24] The IAMC retention of **1** was actually lower than that of citric acid, a routinely employed reference as a compound with essentially no affinity to a lipid membrane. Therefore, k'_{IAM} capacity factors [$(t_R-t_0)/t_0$, where t_R is the retention time for the analyte] were calculated with **1** as a marker for the dead time (t_0). As shown in Figure 1, all ester prodrugs (**2a**–**e**) had an increased membrane affinity compared to the parent compound, and the hexyl ester (**2b**) yielded the highest k'_{IAM} (16.0).

In vitro stability studies in mouse brain homogenate (20%, w/v) were conducted at 100 μM concentration of **2a**–**e**, and analytical reversed-phase gradient HPLC with UV detection ($\lambda = 214$ nm) was used to monitor the decline in the concentration of the compound added. The half-lives ($t_{1/2}$) were 20 and 22 min for the esters of the primary alcohols (**2a** and **2b**, respectively), 25 min for **2c**, and 70 min for **2d**. On the other hand, benzyl ester (**2e**) was relatively stable in the tissue ($t_{1/2} > 2$ h).

The antagonism of the barbiturate-induced anesthesia in mice was used to assess the increase in the access of the prodrugs to the CNS compared to their parent compound. In this assay related to the analeptic activity of **1**, 10 to 18 Swiss Webster mice (30±2 g body weight) were used in each group. Test compounds were dissolved in saline vehicle. The vehicle alone (1.5 mL/kg body weight) or equimolar doses of **1** as control and prodrugs **2a**–**e** (10 μmol/kg body weight) were injected through the tail vein (iv). In another group of animals, **1** was administered at 10-times higher dose (100 μmol/kg) for comparison. After 10 min, each animal received an intraperitoneal (ip) injection of sodium pentobarbital at a dose of 60 mg/kg body weight. The sleeping time was recorded from the onset of the loss of righting reflex until the reflex was regained.

As shown in Figure 2, a significant decrease in the sleeping time was obtained by one-way analysis of variance (ANOVA) followed by post hoc Tukey test ($p < 0.05$) against the control group (animals injected with the vehicle; sleeping time 80±2 min SEM, $n = 18$) for all of the tested compounds except **2e**. When compared to an equimolar dose of **1** (sleeping time 65±3 min SEM), **2a**–**c** showed a statistically significant decrease. Based on measuring analeptic activity in the animal model selected, **2a**–**b** outperformed the parent compound injected at 10-times higher dose (sleeping time 56±2 min SEM, $n = 13$; also included in Figure 2 as an additional reference value).

The measured pharmacological effect appeared to correlate with the metabolic stability of the prodrugs. Specifically, half-lives in mouse brain homogenate around 20–25 min yielded an apparently large enhancement in the CNS delivery. Altogether, esterification of the COOH in the side chain of the Glu with primary alcohols afforded the most promising prodrugs of **1**. An influence of the prodrug's membrane affinity was also revealed, when the analeptic response of **2a** and **2b** was compared—the latter compound with much higher membrane affinity (see Fig. 1) yielded a higher analeptic

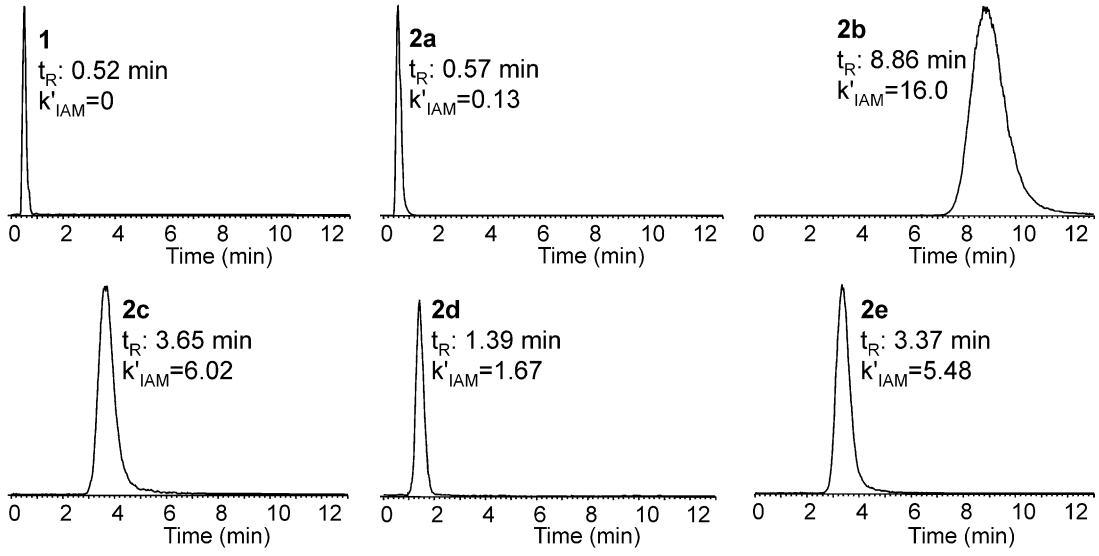


Figure 1. Immobilized artificial membrane chromatography of **1** and **2a**–**e**. Atmospheric-pressure ionization mass spectrometry was used for detection, and the chromatographic traces were extracted from full-scan acquisitions (m/z 150 to 550) for the protonated molecules (MH^+).

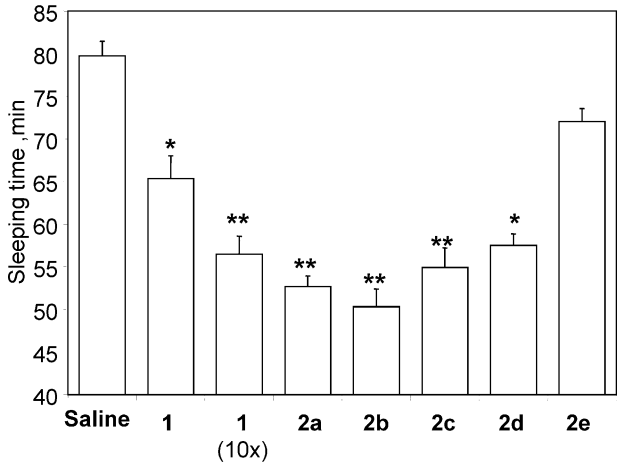


Figure 2. Analeptic effects of **1** and **2a**–**e** injected iv into mice at equimolar doses of 10 μmol/kg body weight when pentobarbital (ip, 60 mg/kg body weight) was administered 10 min after the injection of the vehicle, **1** or **2a**–**e**. For comparison, **1** was also injected at 100 μmol/kg dose (10×). Error bars are standard errors of the mean (SEM, $n = 10$–18) and asterisks indicate statistically significant differences (ANOVA followed by post hoc Tukey test, $p < 0.05$) from the control group (vehicle), double asterisks mark statistically significant differences ($p < 0.05$) from both the control group and 1.

response. In a subsequent dose response study, the effective dose giving 50% of the maximum response (ED_{50}) was 2.4 ± 1.1 μmol/kg for **2b**, while EC_{50} for **1** was about 10-times higher (23.2 ± 5.6 μmol/kg; errors given are standard deviations).[25]

In conclusion, lipophilic esters obtained from primary alcohols have emerged as potential prodrugs for the CNS-delivery of **1**—a peptide structurally related to TRH and possessing beneficial central effects, but with poor access to the brain after systemic administration. Pharmacological studies involving additional animal models and development of analogues based on **2b** as a lead are in progress.

Acknowledgements

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25. ED_{50} values were calculated by fitting the results of the dose-response experiments to the equation $\Delta = \Delta_{max}/[1 + (ED_{50}/D_i)^h]$, where Δ and Δ_{max} are the average decrease and maximal measured average decrease in sleeping time (min) compared to control, respectively, D_i is the dose (μmol/kg body weight), and h is the Hill coefficient. Five different doses per compound were used, and non-linear fitting yielded $h = 1$.