Improving Brain Delivery of TRH: A Novel Prodrug Approach

by

Daniel Luis De La Cruz, B.Sci.

Master of Science in Biomedical Sciences

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The goal of my research project was to validate a novel prodrug design concept for the brain-enhanced delivery of an important neuropeptide, thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2). TRH has a variety of clinically relevant central effects that cannot be utilized with the direct administration of TRH, due to intrinsic characteristics that give rise to metabolic instability and insufficient transport through the blood-brain barrier (BBB). Consequently, large doses are required to generate central effects, which concomitantly induces unwanted hormonal liabilities in the periphery. To overcome these caveats, the prodrug design described herein proposes a novel brain-targeting approach that synergistically utilizes two enzymes, both preferentially expressed in the brain, for the enhanced brain-delivery of TRH. The conjugation of a lipoamino acid (LAA) transport moiety to the N-terminus of a TRH progenitor sequence (Gln-His-Pro-NH_2) *via* a prolyl oligopeptidase (POP)-sensitive linker allows the POP enzyme to release the TRH progenitor sequence at the site-of-action upon crossing the BBB, where it is further transformed by glutaminyl cyclase (QC). QC catalyzes Gln to form pGlu at the N-terminus of the progenitor sequence, thereby releasing TRH. I tested the hypothesis that a representative molecule, developed according to this prodrug design approach, would exhibit adequate drug-likeness for BBB penetration and efficacious release of TRH within the brain. Immobilized artificial membrane chromatography

was used to predict the BBB penetration of this experimental prodrug, labeled "PRODRUG 1," in addition to its calculated logP. Next, PRODRUG (1) was compared to TRH (the "parent" peptide) in an *in vitro* metabolic stability assessment, followed by an *in vivo* neuro-pharmacodynamical evaluation in rodents. The Porsolt swim test, PST, an established animal behavioral model that detects depressive-like behavior was used to confirm brain-delivery of prodrug-derived TRH after systemic administration of the prototype prodrug. Capitalizing on TRH's antidepressant-like effect, the PST results were also used to validate the tail suspension test (TST), a new technique that I implemented in our laboratory for the evaluation of neuroactive compounds with potential antidepressant-like activity.

My findings support the extension of the TRH progenitor sequence from the N-terminus, through the conjugation of two LAA residues (each with a 10-carbon sidechain) *via* a single proline POP-sensitive linker, as a successful means to increase penetration across the BBB and sufficiently bind with cleaving and activating enzymes, POP and QC, respectively, for efficacious TRH release in the brain. Lastly, molecular modeling was used to create a library of similarly designed prodrugs to computationally assess their bindings with POP, the cleaving enzyme, to further explore the customizable prodrug design concept described here. Ultimately, this adaptable prodrug delivery model demonstrates the effectiveness of increased lipophilicity and site-of-action targeting to facilitate brain-enhanced delivery of TRH.

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Daniel Luis De La Cruz, B.Sci.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Pharmacology & Neuroscience

Dean, Graduate School of Biomedical Sciences

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Daniel Luis De La Cruz

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CHAPTER I

INTRODUCTION

A. Neuropeptide Delivery to the CNS

The development of a therapeutic neuropeptide delivery system for the brain is paramount for treating many central nervous system (CNS)-related medical conditions through the use of these biomolecules, yet a widely translatable application requires a non-invasive, effective, and customizable method for their central delivery [1,2]. There have been numerous strategies attempting to achieve this elusive goal as a peptide's ability to cross the blood brain barrier (BBB) from the circulation is hampered by multiple factors [3,4].

B. Thyrotropin-Releasing Hormone

Thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2) is an excellent representative of small neuropeptides; it has long been pursued to treat neurological, as well as psychological brain diseases due to recurring evidence of its inherent ability to alter brain chemistry and, subsequently, behavior and physiology [4-7]. Endogenous TRH, produced by the hypothalamus, is critical in promoting growth during development as a regulatory hormone of the paraventricular nucleus. *Via* the hypothalamic-pituitary axis, it stimulates thyroid-stimulating hormone (TSH) production in the anterior pituitary, which ultimately induces the biosynthesis and release of thyroid hormones T3 and T4 [5-7]. Its global effect, ranging from the immune system to the limbic/cortical system, has been hypothesized to also play a key role in homeostasis, as shown in Figure 1 [6-8].

TRH's numerous beneficial central effects include reversal of cognitive deficits associated

with age-related diseases, improvement of neurological deficits produced by stroke and spinal/cerebellar injury, as well as anxiolytic, analeptic, and antidepressant-like effects (e.g., attenuating inflammation and improving motor and neurological functions). These actions are believed to be mediated mostly through the two known and widely expressed G-protein coupled TRH receptors [5].

While TRH's actions as a neurotransmitter, neuromodulator, and neuroprotective agent are well-known and long-term therapeutic applications have been theorized [5-12], substantially less is known, mostly due to the unwanted side-effects on the periphery following systemic administration, about the outcomes of long-term treatment utilizing TRH (Table 1) [5,13]. Two of the best documented CNS responses to TRH are its analeptic and antidepressant-like effects, through augmentation of various neurotransmitter systems, especially the cholinergic system [14].

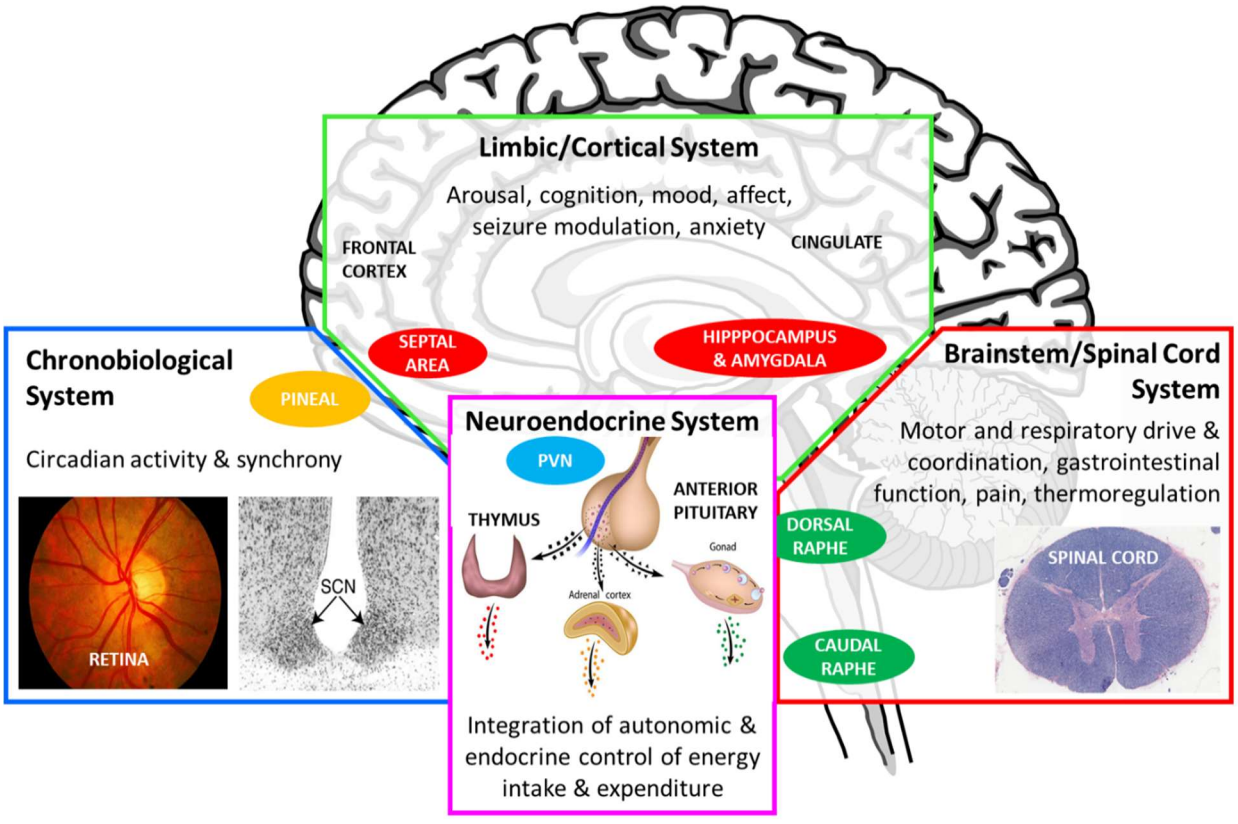


Figure 1. TRH homeostatic pathways and their associated major physiological functions [6,8].

Table 1. Most significant potential treatment opportunities and side-effects associated with TRH drug therapy [6-8].

TREATMENT OPPORTUNITY	SIDE-EFFECTS
Attention deficit/hyperactive disorder Chronic fatigue syndromes Depression, anxiety, and bipolar affective disorders	Induces the release of the TSH from the anterior pituitary and may cause dangerous complications through thymus over activity (Hyperthyroidism)
Seizure disorders and motor neuron disorders Neurological improvements post-stroke and spinal/ head trauma	Mild symptoms include nausea, flushing, shaking, urinary urgency, sweating, shivering, and restlessness
Sedative intoxication/respiratory distress Sedation secondary to drugs, chemotherapy, or radiation therapy	Moderate symptoms include headache, stomach cramps, tightness in the throat, pressure in the chest, and allergic reactions
Attenuates mania, alcohol withdraw dysphoria, and symptoms of schizophrenic psychosis	Produces marked changes in blood pressure, including both hypertension and hypotension
Disturbances of circadian rhythm Recovery from general anesthesia	Induces prolactin release & breast enlargement and leakage in women
Alzheimer's disease and other dementias with cognition deficits	Chronic pharmacologic dosing reduces the TSH response

TRH's robust modulation of acetylcholine (ACh) synthesis and release may benefit CNS-related diseases exhibiting symptoms of decreased cognitive function, such as Alzheimer's/ Parkinson's disease [5-7,10-14]. However, in conjunction with neuropeptide selectivity, the greatest obstacle in treating neurological disorders has been the limitation of therapeutic peptides to cross the BBB from the circulation. To this end, a molecule must possess adequate lipophilicity, survive intra- and extracellular enzymatic degradation, plasma protein binding, and clearance by efflux mechanisms [3,4,15].

C. The Prodrug Approach for CNS Drug Delivery

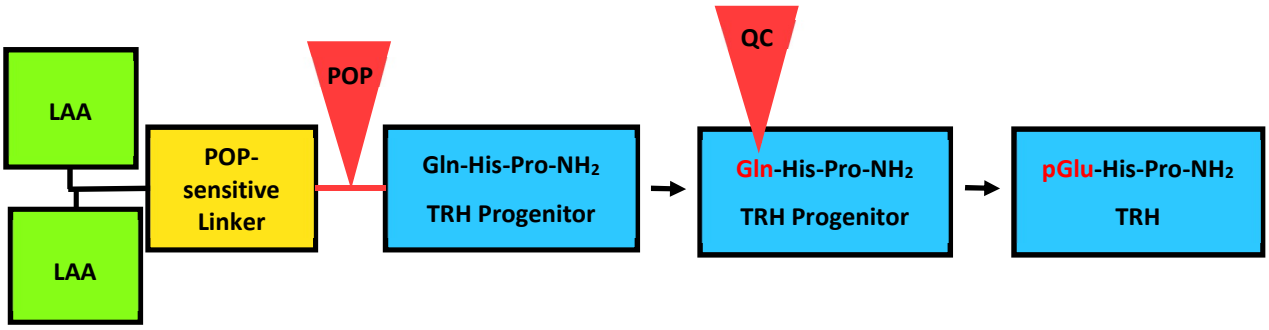
Our laboratory has growing experience in targeting therapeutic agents into the brain *via* the prodrug approach [4,5,16-19]. The prodrug approach is a non-invasive, medicinal chemistry-based circumvention that employs bioreversible chemical manipulation of a target or "parent" agent to create an inactive prodrug. A properly designed prodrug possesses altered physicochemical characteristics that promote CNS penetration. Upon crossing the BBB, these modifications ("promoiety/ies") are removed by enzymatic activity (e.g., by esterases, lipases, and proteases), or rarely by spontaneous chemical reactions [20,21]. This prodrug-to-parent drug conversion is traditionally called prodrug "bioactivation." Herein, we propose the development of a novel prodrug design for targeting TRH into the brain.

D. Brain-Targeting Prodrug Approach for TRH Delivery

Our novel prodrug approach utilizes two enzymes, preferentially expressed in the brain, to release TRH from its prodrug construct — namely prolyl oligopeptidase (POP) [17,19,22-24] and glutaminyl cyclase (QC) [22,23]. POP is a widely brain-expressed endopeptidase that catalyzes

selective peptide-bond hydrolysis after internal proline (-Pro) due to recognition of the unique conformational constraint that this cyclic amino acid residue brings into an oligopeptide chain [22-26]. Similarly, QC is an enzyme abundantly expressed in the brain that converts glutamine (Gln) into pyroglutamate (pGlu) and is essential for endogenous TRH synthesis [5,22,23,27]. We utilize these enzymes because TRH itself is not suitable for practical prodrug creation due to its lack of suitable functional groups amenable for the attachment of promoieties [5,19]. Instead, we employ the TRH progenitor sequence Gln-His-Pro-NH_2. The Gln's free α-amino group allows for covalent attachment of promoiety(ies) to enhance TRH's transport across the BBB [5,17,22-24]. In our approach, lipoamino acids (LAAs) [28,29] were selected to increase the lipophilicity necessary for adequate BBB penetration.

Introduction of LAAs facilitates a molecule's interaction with cell membranes by not only augmenting lipophilicity but also imparting amphiphilic properties [28]. At the heart of our prodrug construct is a POP-sensitive linker [17,23-25] to anchor a LAA moiety to the TRH progenitor sequence. This POP-sensitive linker is either a single prolyl residue, or an -Xaa-Pro-dipeptidyl (where Xaa denotes an L-amino acid, excluding Glu and Asp) sequence. The POP enzyme allows the removal of the LAA-(Xaa-Pro/Pro) promoiety from the N-terminus of the progenitor sequence by recognizing proline [17,22-24]. Without the POP-sensitive linker, the TRH progenitor sequence could not be liberated from the LAA-Gln-His-Pro-NH_2 construct due to low (or lack of) amidase activity in the brain [19,23,24]. Once the LAA-(Xaa-Pro/Pro)-Gln-His-Pro-NH_2 prodrug releases the progenitor sequence (Gln-His-Pro-NH_2) *via* POP, the N-terminal Gln is recognized by QC, which converts it to pGlu, and thereby completing prodrug biotransformation to TRH (Scheme 1).



Scheme 1. A schematic illustration of prodrug bioactivation to TRH within the brain by our novel prodrug construct. This illustration features color-coded regions highlighting the LAAs in green, the POP-sensitive linker in orange, and the TRH progenitor sequence in blue. POP (red) cleaves the transport moiety after the POP-sensitive linker so that QC (red) can recognize and convert Gln to pGlu to liberate TRH.

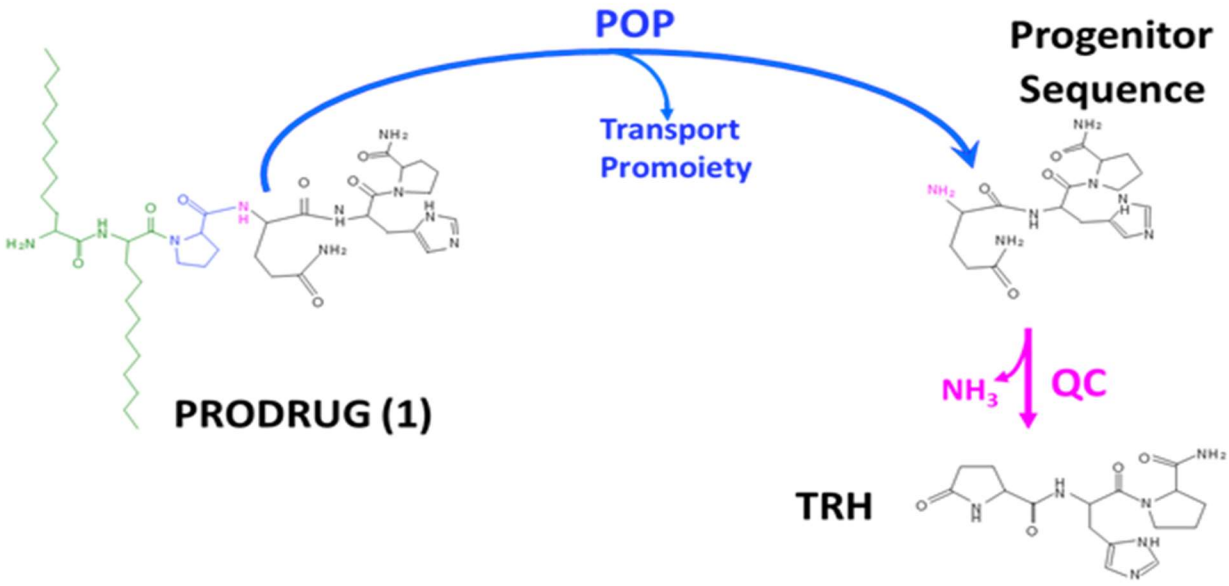


Figure 2. The structure of PRODRUG (1) and a schematic illustration of its bioactivation to TRH within the brain. This illustration of PRODRUG (1) features color-coded regions highlighting the LAA moiety (green), the POP-sensitive linker (blue), and the TRH progenitor sequence (black) with the N-terminal amino group in pink.

Our laboratory has previously synthesized a series of TRH prodrugs based on the above design principles in which the LAA transport moieties and POP-sensitive linkers were strategically modified. In our representative prodrug model, labeled "PRODRUG (1)," the LAA moiety consists of two alkyl residues (each with a C-10 sidechain) bound to a single prolyl residue (as a POP-sensitive linker) that is conjugated to the N-terminus of the TRH progenitor sequence. PRODRUG (1)'s chemical structure and its putative bioactivation to TRH in the brain is shown in Figure 2.

E. Hypothesis and Goals

The goal of this project is the initial validation of the prodrug concept introduced here for the enhanced brain-delivery of TRH. We hypothesize that this novel prodrug approach enables TRH delivery into the brain, while exhibiting sufficient metabolic stability in the circulation to promote brain uptake and diminish peripheral TRH exposure. Utilizing a range of techniques and PRODRUG (1), as a representative of this prodrug design concept, our hypothesis was tested *via* completing the following research objectives, as outlined below:

i. **Membrane affinity studies:** Compare the membrane affinity of PRODRUG (1) to that of TRH, thus predicting BBB permeability, using immobilized artificial membrane chromatography (IAMC) [30-32]. It is imperative to establish that the prodrug possesses adequate lipophilicity for BBB transport from blood circulation.

ii. ***In vitro* metabolic stability studies:** Establish PRODRUG (1)'s biological half-lives in relevant biological matrices (plasma and brain homogenate), in comparison with TRH's metabolic (in)stability [5,7,33].

iii. ***In vivo* substantiation of the prodrug approach:** Assess the antidepressant-like effect of the parent peptide (TRH) using the tail suspension test (TST) [34,35] and the Porsolt swim test (PST) [36,37] as a neuropharmacological paradigm to demonstrate *in vivo* release of TRH from PRODRUG (1) in CD-1 mice. Confirm TRH release from PRODRUG (1) through pilot neurochemical experiment using *in vivo* microdialysis sampling [14,38] in Sprague-Dawley rats and measuring ACh levels.

iv. ***In silico* calculations:** Survey the binding affinity of PRODRUG (1) to POP, in relation to a series of prodrugs constructed under similar design principles.

CHAPTER II

MATERIALS AND METHODS

A. Chemicals

PRODRUG (1) was previously synthesized and provided by our laboratory using established procedures of peptide synthesis [16-18]. All other chemicals used were obtained from commercial sources.

B. Animals

Male CD-1 mice were obtained from *Envigo Corp*. (Indianapolis, IN, USA), weighing between 37 to 50 grams and about 18 weeks of age at the time of experimentation. CD-1 mice are an outbred mouse strain typically used for pharmacological studies due to increased genetic diversity compared to inbred mice; however, female mice were excluded to abolish hormonal variables associated with menstrual cycles. Rodents were housed in the DLAM facility at the University of North Texas Health Science Center (UNTHSC) on a common 12 hr/12 hr light dark cycle with food and water *ad libitum* and placed 5 mice per cage. Similarly, male Sprague-Dawley rats, weighing between 250 to 300 grams (17 weeks of age) were obtained from the same vendor and housed under the same conditions. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at UNTHSC before the initiation of the studies.

C. Instrumentation

Liquid chromatography-mass spectrometry (LC-MS) analyses, using electrospray ionization (ESI) in positive-ion mode, were conducted on a ThermoQuest LCQ Deca ion trap mass spectrometer (*Thermo Fisher Scientific*; Waltham, MA, USA) coupled with a Surveyor MS LC system (*Thermo Fisher Scientific*). IAMC studies employed an IAM.PC.DD2 column (3 cm × 4.6 mm I.D., d_p=12-µm; *Regis Technologies Inc.*; Morton Grove, IL, USA) at a flow rate of 1 mL/min. Metabolic stability of the test compounds was evaluated with a Kinetex-C8 column (10 cm × 4.6 mm I.D., d_p=2.6µm; *Phenomenex*; Torrance, CA, USA) at a flow rate of 400 µL/min. All compounds were analyzed under the stated LC-MS parameters, tuned for TRH, and with 10 µL injection volumes. A Thermo Finnigan TSQ Quantum Ultra triple quadrupole tandem mass spectrometer connected to a Vanquish Flex LC (*Thermo Fisher Scientific*) was utilized for the analyses of microdialysis samples [38]. The ESI source was tuned to ACh and used tandem mass spectrometry (MS/MS) for quantification, and separations relied on a Scherzo SS-C18 column (50 × 2 mm I.D., 3µm; *Imtakt Corp.*; Kyoto, Japan) at a flow rate of 400 µL/min.

D. Membrane Affinity Studies – Immobilized Artificial Membrane Chromatography (IAMC)

The IAMC method utilizes chromatographic retention on an LC stationary phase composed of immobilized synthetic lipids [30,31]. Immobilized artificial membrane columns mimic biological membranes by integrating a phosphatidylcholine head group and ester, or ether, linkage between the two acyl chains and the glycerol backbone of the phosphatidylcholine molecule [30-32]. Consequently, a high IAMC retention time correlates to a greater membrane affinity for the tested compound, while CHI_{IAM} values (the quantitative measure of retention [32] used in our

comparative evaluation) represent an approximation of the acetonitrile concentration for which a molecule has an equal distribution between the mobile phase and the IAMC column. Utilizing an IAM.PC.DD2 column and following a protocol published by Valko *et al*. [32], a range of reference compounds with known CHI_{IAM} values were selected to create an equation for the membrane affinity prediction of PRODRUG (1) in comparison to the parent, TRH. Plotting the reference compounds' CHI_{IAM} values against their experimentally determined gradient retention time through the IAM column provides a linear relationship, where the constants of the straight line (slope and intercept) may be used to convert the test compounds' gradient retention times into its unknown CHI_{IAM} value. Nicotine, caffeine, hydrocortisone, progesterone, procaine, and propranolol were chosen as reference standards [32]. Gradient elution was used with a mobile phase mixed from solvents "A" and "B" with flow rate of 1.0 mL/min over a total of 15 min. Solvent A consisted of 50 mM ammonium acetate buffer at various pHs (7.0, 6.1, and 5.4 – adjusted with 1 M acetic acid), while solvent B was acetonitrile. The following gradient program was used: 0–1.5 min, 100% solvent A (isocratic); 1.5–10.5 min, changed to 100% solvent B by linear gradient; 10.5–11.5 min, 100% solvent B (isocratic); 11.5–12.0 min, returned to 100% solvent A (linear gradient); 12.0–15.0 min, 100% solvent A (isocratic) to equilibrate the system for a subsequent run. Under these system conditions and at each designated pH of solvent A, the reference compounds' gradient retention times were determined to establish the linear relationship equation.

E. *In Vitro* Metabolic Stability Studies

Metabolic stability studies were carried out according to a previously published protocol [16,17]. A total of 6 animals (triplicate; n=3 for each compound) were used to obtain tissues for the

determination of the biological half-lives of the test compounds. Stock solutions of TRH and PRODRUG (1) were prepared to produce 10 μM concentrations upon dilution into each biological matrix. The outline of tissue preparations was as follows:

After the intraperitoneal (i.p.) administration of an anesthetic ketamine mix (60 μL/ 40 g animal) blood was collected *via* cardiac puncture and transferred to a 6-mL plasma determination tube, containing sodium heparin, and centrifuged at 1,500*g* for 10 min. Surgical scissors were used to decapitate the animal, and the brain was removed, weighed, and placed into a *Benchmark Scientific* homogenizer tube (prefilled with High Impact Zironium 3.0 mm beads; Sayreville, NJ, USA) with 1 mL phosphate-buffered saline (PBS). The tube was then placed into a *Bead Bug* microtube homogenizer (*Benchmark Scientific*) for 3 min, and a PBS (4°C) volume in milliliters equivalent to 5 times the weight of the brain (in grams) was added to produce a 20% w/v brain homogenate for immediate use (i.e., 0.5 g tissue was homogenized with 2.5 mL PBS).

Following the preparation of brain homogenate and plasma, 1.0 mL and 0.5 mL aliquots, respectively, were placed into separate 1.5 mL Eppendorf tubes and then into a temperature-controlled, shaking water bath pre-heated to 37ºC for 3 minutes. The brain homogenates (1.0 mL) were spiked with 10 μL and 36 μL of PRODRUG (1) (1 mg/mL in saline) and TRH (100 μg/mL in saline) solutions, respectively. Similarly, 5 μL and 18 μL of PRODRUG (1) (1 mg/mL in saline) and TRH (100 μg/mL in saline) solutions, respectively, were mixed to the aliquots of the plasma (0.5 mL). At various time points during incubation, samples were obtained by removing 100 μL of brain homogenate, or 50 μL of plasma, and transferred to a 1.5 mL Eppendorf tube containing ice-cold "stopping" solution, consisting of 5% (v/v) acetic acid in acetonitrile, to quench enzymatic reactions in the samples before being placed on ice (Table 2).

Table 2. An outline depicting the main parameters in the *in vitro* metabolic stability study of TRH and PRODRUG (1).

	TRH		**PRODRUG (1)**	
Time Points (min)	2, 4, 6, 8, 10, 15, 30, 60		5, 15, 30 ,60, 120, 210	
Biological Matrix	Brain	Plasma	Brain	Plasma
Biological Matrix Volume (µL)	1000	500	1000	500
Sample Volume (µL)	100	50	100	50
Stopping Solution (µL)	200	100	200	100

Time point samples were centrifuged for the removal of the supernatant as required for LC-MS analysis. LC separation was carried out using a Kinetex-C8 column with gradient elution consisting of a two-solvent mixture over a total method length of 9 min. Solvent A was H_2O with 0.1% (v/v) formic acid, and solvent B was acetonitrile with 0.1% (v/v) formic acid, and a linear gradient was used (Solvent B changed from 0–98% over 6 min for analyte elution, holding for a "wash" period of 2 min at 98% concentration solvent A, followed by a 1-min equilibration with 100% solvent A to prepare for the next sample run). The chromatographic peak area was separately assessed for each compound, and the decline of the test compounds' concentrations were calculated as a function of the area under the chromatographic peak (X) versus the incubation time (Y) in a Microsoft Excel spreadsheet, employing exponential fitting ($Y=ae^{-kX}$) assuming pseudo-

first-order degradation ($\ln C_0/Ct = kt$); $t_{1/2} = \ln 2/k$, where C is concentration, subscript 0 refers to initial value, and *k* denotes 1st-order rate constant [16,17]).

F. *In Vivo* Behavioral Studies in CD-1 Mice

Experiments were carried out during the hours of 10 a.m. and 4 p.m. Animals were retrieved from their housing area and transferred to new cages, devoid of food and water, before being taken to our designated laboratory where the experiments were conducted. The mice acclimated to this room for one hour, while their weight was recorded to determine proper dosing. Drug solutions were also prepared during this time (dissolution in saline) and administered to the animals by dorsal subcutaneous (s.c.) injection 30 minutes before beginning behavioral tests. The following two paradigms were individually assessed for antidepressant-like activity:

i. **Porsolt Swim Test (PST):** Mice were divided into 5 groups (n=8) for the administration of test compounds. The control group received saline vehicle only (200 µL/ 40 g animal). The PRODRUG (1) group received a dose of 3 µmol/kg [16,18], while the remaining three groups received various TRH concentrations (3 µmol/kg, 10 µmol/kg, and 30 µmol/kg) for dose response study. A glass cylinder tank measuring 25 cm high × 20 cm in diameter was prepared immediately before testing each animal by rinsing and filling it with room-temperature water (around 27ºC). Filling the cylinder to a depth of 15 cm was adequate in suspending each mouse so that only the tip of the tail could reach the bottom of the tank. Each mouse was placed individually into the water-filled cylinder for one 6-min swimming session, after which the animal was removed from the cylinder, dried with paper towels, and placed into its individual cage

to recover for a period of 15 min before being re-housed collectively. In this adapted PST experimental model [34,36], we only consider two behavioral aspects for scoring purposes: immobility time (i.e., the time spent floating in the water without struggling, only making the minor movements necessary to keep the head above the water) and swimming/climbing time (i.e., the time spent making active swimming motions to move around or across the cylinder, movements of all four paws, and forepaw movements directed towards the cylinder wall) [16-19,34,36]. Additionally, this protocol calls for no preconditioning; therefore, the mice were only exposed to these experimental conditions on the day of the experiment, and each mouse was only subjected to a single dose and subsequent swim test.

ii. Tail Suspension Test (TST): TST setup was implemented according to a published protocol [35] in which mice are suspended by their tails for 6 min. The mice were divided into 4 groups (n=6) for the administration of test compounds; out of the 4 groups the first was designated the saline vehicle control (200 µL/ 40 g animal), and the remaining 3 groups consisted of various TRH concentrations consistent with those used in the PST study (3 µmol/kg, 10 µmol/kg, and 30 µmol/kg). Each mouse was suspended by taping their tail with 11 cm segments of ScotchBlue™ 1/2-inch painter's tape and recorded for the duration of the 6-min test session. Also, like the PST, immobility time (sec) was scored based on similar criteria (e.g. only distinct escape movements were classified as movements). The TST apparatus was designed to best isolate, visually and acoustically, multiple animals being tested at the same time. The suspension box was made in our lab, out of wood, with the dimensions (90 height × 76

width × 30 cm depth), and further subdivided into three rectangular compartments (90 height × 25 width × 30 cm depth), each with three walls, and a steel rod (1/8-inch diameter) running horizontally through the box, directly in the middle of the compartment's width, and 20 cm from the top of the box (Figure 3). The distance from the animal's head to the floor of its compartment was approximately 50 cm. These dimensions ensure that a suspended mouse could not touch any of the walls, and the dark color of the wood provided adequate contrast to the white mice, aiding in the scoring process. Like the PST, this TST protocol does not require animal preconditioning. The mice were only exposed to the experimental design on the day of the experiment, given a single dose and subsequent TST experiment.

G. *In Vivo* Brain Microdialysis in Sprague-Dawley Rats

Experiments were carried out during the hours of 8 a.m. and 4 p.m. according to a previously published protocol [14,38]. Animals were retrieved from their housing area and transferred to new cages, devoid of food and water, before being taken to our designated laboratory where the experiments were conducted in duplicate (n=2). Their weight was recorded to determine proper i.p. anesthetic dosing (60 mg/kg ketamine and 10 mg/kg xylazine). A frame equipped with a micromanipulator permitted the precise designation and stereotaxic implantation of the guide cannula (*CMA/Microdialysis, Inc.*; Acton, MA, USA). The guide cannula was inserted into the frontal cortex at a position anterior to the bregma (origin), A: 3.0 mm; lateral to midline, L: +0.5 mm; vertical, V: -5.0 mm) [14,38].

Following the guide cannula implantation, the wound healed for 5 to 7 days before the actual microdialysis probe (CMA/12, *CMA/Microdialysis, Inc.*, with a 4mm long polycarbonate membrane having 20,000 Da molecular weight cutoff) was inserted. After connecting the tubing for the influx and efflux of the perfusion fluid of the microdialysis probe, the probe was inserted into the cerebral guide cannula, and the animal was placed into an animal handling unit for sample collection (Stand-Alone Raturn, *Bioanalytical Systems Inc.*; West Lafayette, IN, USA). The

Figure 3. The Tail Suspension Test Apparatus. I constructed the TST apparatus shown here, as an additional behavior technique for our laboratory. It is used to measure the immobility time (i.e., motionless hanging) of an animal while being suspended by the tail, relying on the premise that an animal given an antidepressant will struggle, or try to escape, longer than control animals.

syringe pumps (1-mL BeeStinger), their controller (BeeKeeper), and the refrigerated fraction collector (HoneyComb) were also purchased from *Bioanalytical Systems Inc*. Artificial cerebral spinal fluid (ACSF) was used as the perfusion medium. The system was first equilibrated at 3 μL/min perfusion rate for 1 hour and, then, baseline samples were collected for 2 hours. At the beginning of the third hour a manual valve (CMA/110, *CMA/Microdialysis Inc.*) was used for switching perfusion to a solution of either PRODRUG (1) or TRH (both with 3mM concentrations in ACSF) without the interruption of the flow through the probe. The perfusion of the experimental analyte solution through the probe was carried on for additional 2 hours, and microdialysis samples were collected continuously in 20-min fractions. The baseline fractions 1-5 were used as each animal's own control. ACh concentration was used as a neurochemical measure of TRH release, measured according to our lab's established LC-MS method [38], summarized below:

The mobile phase consisted of two solvents: solvent A, H_2O with 0.5% (v/v) formic acid; solvent B, 100 mM aqueous ammonium acetate–acetonitrile mixture (65:35, v/v). The total method lasted 10 min, beginning with a linear solvent B gradient from 1-40% over 6 min, a wash period of 100% solvent B from 6.1 to 8 min, and followed by re-equilibration with 99% solvent A from 8.1 min until the end of the method. The flow rate was 400 μL/min with an injection volume of 10 μL, and the analyte was detected at retention time around 3.7 min. Microdialysis fractions were individually mixed (1:1 ratio) with 1-ng/mL aliquot of a tetra-deuterated analogue (ACh-1,1,2,2-d_4; *C/D/N Isotopes Inc.*; Pointe-Claire, QC, Canada) as an internal standard, and the resulting solution was injected into the LC-MS system without any further dilution. Collision-induced dissociation (CID) product-ion MS/MS spectra were collected using 1.0 Th parent ion isolation width and 30% relative collision energy. Following mass-selected CID, the parent ions and their principal product ions, ACh (m/z 146 fragmented to 87) and d_4-ACh (m/z 150 fragmented to 91),

were monitored and assessed with the manufacturer's data acquisition/processing software XCalibur (version 4.1).

H. Computational Binding Affinity Studies

The X-ray diffraction structure of *homo sapiens* POP (3DDU) at 1.56 Å resolution with ligand molecule (6S)-1-chloro-3-[(4-fluorobenzyl)oxy]-6-(pyrrolidin-1-ylcarbonyl)pyrrolo[1,2-a]pyrazin-4(6H)-one (GSK552) was taken from the Protein Data Bank as a PDB file (PDB ID: 3DDU) [39,40]. Prior to molecular docking, the ligand was extracted to prepare the binding site, and the POP molecule was refined using SCIGRESS software, Version EU 3.3.1 [41] (*Fujitsu FQS*, Krakow, Poland). SCIGRESS software features comprehensive molecular dynamics-based algorithms to "clean-up" and equilibrate an enzyme's/ molecule's valence, hybridization, rings, and geometry. Also, the analytes' calculated log*P* (clog*P*) was determined with a method built into SCIGRESS software.

Based on previous research [17-19,28,29], various prodrugs (i.e., "ligands") were designed for *in silico* molecular docking with POP, using SCIGRESS and AutoDock Vina software [39], and utilizing a single -Pro or a dipeptidyl -Pro-Pro POP-sensitive linker between the progenitor sequence and LAA transport moiety. The LAA transport moiety varied from containing 1 to 2 amino acid residues with sidechain lengths of 6, 10, or 14 carbons, so models for a total of 12 prodrugs were created. Geometries were optimized using molecular mechanics (SCIGRESS), with the appropriate built-in modules to add charges, assign hydrogen atoms, and set up rotatable bonds. Using Cartesian coordinates of X = –6.3 Å, Y = 12.5 Å, and Z = 31.5 Å, local optimization was performed to the center of the binding cavity to prepare for AutoDock Vina's rigid-flexible molecular docking method [39,41]. The docking grid dimensions of 35 Å x 35 Å x 35 Å, were

used in each docking calculation with an exhaustiveness of 8 modes, so a total of 9 docking poses were produced (docking scores were averaged per molecule). The AutoDock Vina docking score results are represented as Gibbs free energy of binding (ΔG, automatically converted to kcal/mol units) with a standard error of ±2.5 kcal/mol, where the more negative a number relates to the lower amount of energy required for an enzyme-catalyzed reaction to occur (i.e., spontaneous); therefore, molecules with the most negative Gibbs free energy of binding have the greatest binding affinity.

I. Statistical Analysis

Data are expressed as mean ± standard deviation (SDEV), and statistical evaluations were done by one-way ANOVA. Two-group comparisons employed *post hoc* Tukey tests. Data with a *p*-value less than 0.05 was considered statistically significant.

CHAPTER III

RESULTS & DISCUSSION

A. Membrane Affinity Studies

Besides molecular size/volume, lipophilicity plays a crucial role in a molecule's ability to cross lipid bilayers, such as the BBB (with an optimal log*P* value of 2±0.7) [4,5]. We selected PRODRUG (1) for evaluation, because its clog*P* was 1.44 — close to the optimal value. Previous studies have shown that there is a direct correlation between molecules' membrane affinity predictors, such as log*P* and CHI_{IAM}, for about 80% of compounds [30-32]. Consequently, the IAMC technique is a method capable of experimentally predicting or verifying a compound's membrane permeability by assessing its affinity for an artificial lipid bilayer *via* LC-MS [32]. Analytes are separated based on their interactions with two distinct phases: a liquid, unidirectional mobile phase (variations of inorganic and/or organic solvents) and a stationary phase, or "column," which is defined by its dimensions, base material, and bonded chemical functional groups. These factors determine the rate of an analyte to pass through the LC system to the mass spectrometer, where it is detected by its mass-to-charge (m/z) ratio. IAMC columns are physically and chemically similar to fluid phospholipid bilayers, and the relationship of a molecule with an IAMC column can be used to probe the interaction of organic compounds with a biological membrane. Because of that, IAMC provides better prediction regarding membrane affinity of a compound than experimental log*P*, where traditionally n-octanol represents the lipid membrane [32,42].

Accordingly, the CHI_{IAM} values for PRODRUG (1) and TRH were determined by IAMC *via* the integration of their retention times within an equation derived from the analysis of a set of

standards [32] across various pH values. Figure 4 shows a representative CHI_{IAM} vs. retention time relationship for the set of standards that allowed the prediction of CHI_{IAM} values for TRH and PRODRUG (1), respectively, at pH 7. Similar experiments were carried out at pH 5.4 and 6.1.

Altogether, the IAMC studies produced the following equations, generated to determine membrane affinity characterized by CHI_{IAM} values for PRODRUG (1) and TRH, respectively:

$y = 9.5009x - 21.992$, $R^2 = 0.9665$ at pH = 7.0 (eq.1) (Figure 4)

$y = 7.3294x - 13.139$, $R^2 = 0.9389$ at pH = 6.1 (eq. 2)

$y = 11.309x - 24.182$, $R^2 = 0.7231$ at pH = 5.7 (eq.3)

where *x* denotes the retention time of the analyte in question and *y* denotes its CHI_{IAM} value. Using equations 1-3, we have calculated a CHI_{IAM} value of 66±10.3 for PRODRUG (1), as well as –11.2±3.2 for TRH, within the given pH range (5.7-7.0). These IAMC results correlate well with the clog*P* values of 1.44 for PRODRUG (1) and –3.5 for TRH, using SCIGRESS molecular modeling software and are summarized in Table 3.

Thus, a significantly increased membrane affinity by PRODRUG (1), compared to that of TRH, is predicted (along with favorable BBB transport from the circulation) using both the computational and experimental methods. Moreover, clog*P* of PRODRUG (1) is within the optimal range [4] for passing through the BBB (1.44 vs. 2±0.7). The LAA moiety with two alkyl residues in the chemical structure of PRODRUG (1) (Figure 2) was, indeed, intended to increase membrane affinity, by way of increased lipophilicity.

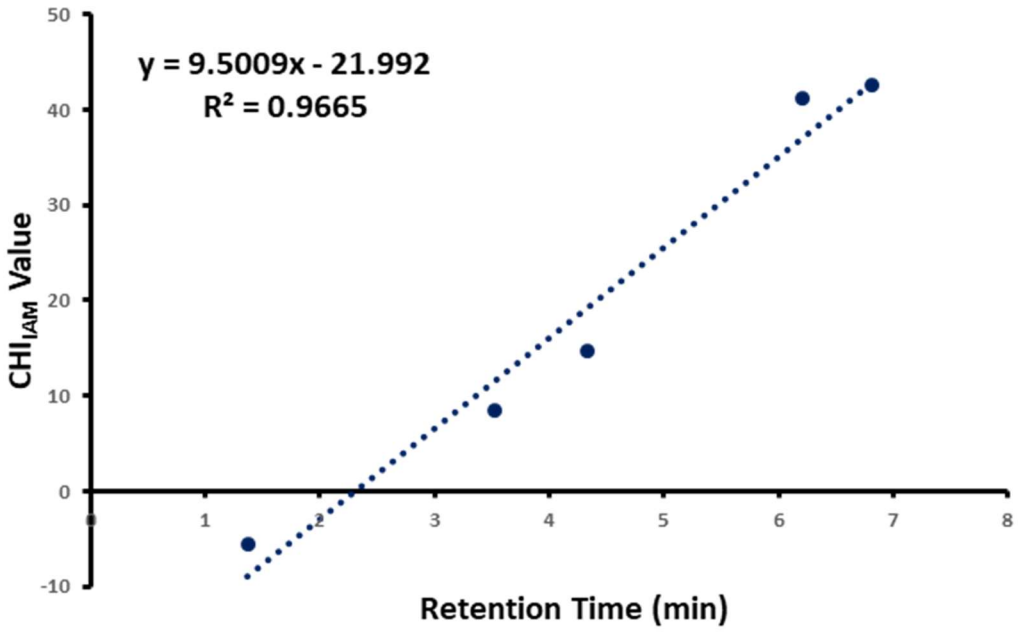


Figure 4. CHI_{IAM} values vs. gradient retention times for the set of standards [31] at pH = 7. From the slope of the equation, the CHI_{IAM} value for a compound with known retention time can be determined at the given pH.

Table 3. Summary of membrane affinity studies using CHI_{IAM} and clog*P* values.

Analyte	**clog*P***	**CHI_{IAM}**
TRH	–3.5	–11.2±3.2
PRODRUG (1)	1.44	66±10.3

The comparison of clog*P* and membrane affinity, as estimated by IAMC-determined CHI_{IAM} values, demonstrates that the prodrug's design manipulates a critical physicochemical property (i.e., lipophilicity/membrane affinity), and is a prodrug design strategy that can be beneficially altered according to the desired study objective for a given neuropeptide. Based upon favorable

CHI_{IAM} value, PRODRUG (1) was further studied for its *in vitro* metabolic stability in mouse plasma and brain homogenate to monitor its capability to travel through the blood, reach the brain, and release TRH.

B. *In Vitro* Metabolic Stability Studies

These *in vitro* metabolic stability studies assess the prodrug's ability to remain sufficiently stable in the circulation (i.e., during transport) until reaching the brain, where it is designed to be enzymatically metabolized to liberate TRH by POP and QC, respectively (Scheme 1, Figure 2). The biological half-lives of PRODRUG (1) were expected to be significantly greater than TRH's documented and very short half-lives of less than 10 min in both rat plasma and brain homogenate [15-18,42,43]. In this regard, a pilot study was needed to establish the experimental time points of sampling for LC-MS analysis, using mouse-derived matrices relevant to our studies (Table 2) to establish PRODRUG (1)'s degradation kinetics. Using the experimental conditions specified in the "MATERIALS & METHOD" section, TRH eluted from the Kinetex C8 column (*Phenomenex*) at about 2 min, while PRODRUG (1) eluted at about 5.8 min. Although these agents were analyzed individually, the significant difference in retention times would also allow the simultaneous detection of both compounds within a sample, implicating the possibility of detecting the *in vitro* bioconversion of PRODRUG (1) to TRH. *In vitro* studies were carried out using CD-1 mice plasma and brain homogenate, as specified in Table 2, and subsequent *in vivo* substantiation of TRH release from PRODRUG (1) utilized the same mouse strain in the PST behavior model to assess TRH's antidepressant-like activity [13,14,16,34,44].

Following 1^{st}-order reaction kinetics, where a reaction proceeds at a rate linearly dependent on the reactant concentration (C), the half-life ($t_{1/2}$) can be calculated as 0.693/k, where *k* denotes

1^{st}-order rate constant determined from the ln(C) vs. time curve upon sampling the degradation of the analyte with known initial concentration (C_0 = 10 μM) at predetermined time points (Table 2). Table 4 summarizes the calculated half-lives for PRODRUG (1) and TRH.

We determined PRODRUG (1)'s biological half-lives to be around 100 min in plasma and about 50 min in brain homogenate (20% w/v), which is a considerable increase compared to TRH's biological half-lives of about 10 min in plasma and less than 5 min in brain homogenate. According to our data, PRODRUG (1) is twice as stable in plasma as in brain homogenate with a significant (10-fold) increase in stability over TRH in each biological matrix (Table 4). To our knowledge, although we are the first to determine TRH's half-lives in CD-1 mouse plasma and brain homogenate, with previously published TRH half-lives were determined in rat tissues [43], our data does not statistically differ from those obtained in rat tissues. The expressively higher metabolic stability of PRODRUG (1) in plasma should be advantageous, not only in terms of brain uptake of the intact prodrug from circulation, but also as important, we may expect significantly decreased TRH exposure in the periphery following systemic administration of PRODRUG (1) in animals. This aspect of our prodrug design will be rigorously tested in the future by measuring rodent T3/T4 blood levels in a comparative fashion with those of systemically administered TRH [43]. The pilot studies conducted, establishing the metabolic stability sampling time points, have resulted in, thus far, no viable data of detecting "prodrug-derived" TRH *in vitro,* most probably owing to TRH's extreme metabolic lability. In the future, we will rather focus on identifying and monitoring TRH's more metabolically stable metabolites, such as, histidyl-proline diketopiperazine [cyclo(His-Pro)] [45].

Table 4. *In vitro* metabolic stability studies using TRH and PRODRUG (1) in CD-1 mice (n=3)*, respectively, to determine their biological half-lives.

***IN VITRO* METABOLIC STABILITY STUDY**		
ANALYTE	BRAIN HOMOGENATE (20%w/v)	PLASMA
	$t_{1/2}$ ± SDEV (min)	
TRH	3 ±0.3	7 ±4
PRODRUG (1)	49 ±3	100 ±12

*Experimental conditions are given in Table 2.

C. *In Vivo* Substantiation of TRH Delivery into the Brain by PRODRUG (1)

C.1. Neuropharmacological assessments using the antidepressant-like activity of TRH

C 1.1. The Porsolt swim test (PST)

The PST is a straightforward technique, analyzing the time spent immobile as a representation of the degree of despair that an animal experiences when subjected to an inescapable situation. This widely accepted behavioral despair paradigm exploits TRH's firmly established anti-depressive action in the CNS, which is believed to be mediated through a cholinergic mechanism [5,14,35,42]. Accordingly, *in vivo* substantiation of our novel prodrug approach is possible by assessing the CNS activity produced by the parent peptide, as the formation of PRODRUG (1)-derived TRH in the brain of experimental animals should trigger an antidepressant-like response similar to that of

direct TRH administration.

I carried out these experiments according to a published protocol by our lab [17,42]; specifically, CD-1 mice were s.c. injected with equimolar doses (3 μmol/kg body weight) of either TRH (positive control) or PRODRUG (1). The vehicle control group received saline only. Immobility time was recorded for 6 min. As Figure 5 shows, administration of TRH and PRODRUG (1) elicited a statistically significant decrease ($p< 0.05$) in immobility time compared to the vehicle control group; as the immobility time was 180±19 sec for the vehicle treated group and 151±17 and 148±16 sec for the PRODRUG (1) and TRH groups, respectively.

We can conclude from the observed immobility time of the PRODRUG (1) treatment group that PRODRUG (1), indeed, underwent the predicted metabolic conversion (Figure 2), releasing TRH in the brain. However, no significant difference was found between the immobility times of TRH and PRODRUG (1), implicating that the prototype prodrug is promising, but may require further adjustment in terms of the LAA moiety and POP-sensitive linker combination. The flexibility in selecting the LAA moiety and POP-sensitive linker is a great advantage of our prodrug approach and warrants further studies in this regard. Nevertheless, from the measured half-lives in plasma (Table 4), we expect that PRODRUG (1) would significantly diminish TRH's exposure in the periphery in relation to direct administration of the peptide.

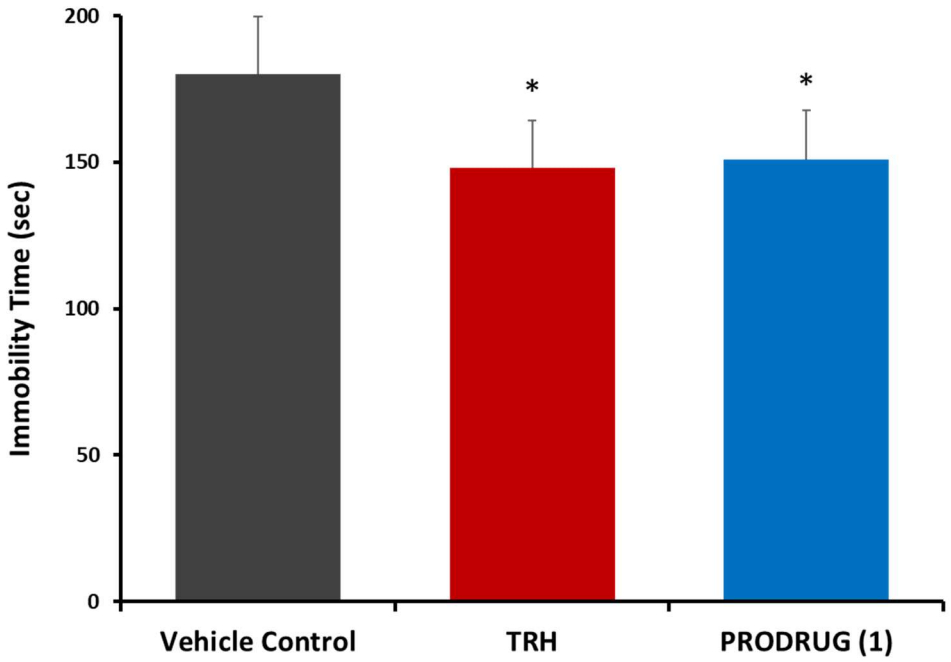


Figure 5. Antidepressant-like effect of TRH after s.c. administration of PRODRUG (1) at 3 µmol/kg dose to CD-1 mice (n=8). TRH (3 µg/kg) was used as positive control. In the control group, animals received saline vehicle only. Data represent MEAN±SDEV (n=8). *Significant difference compared to vehicle control (ANOVA analysis followed by *post hoc* Tukey test; $p<0.05$).

C1.2. The tail suspension test (TST)

Like the PST, the TST is a technique used to determine the antidepressant-like effect of an agent [34,35]. In this behavioral model, rodents are suspended by their tails, and immobile behavior is defined by motionless hanging. A previous investigation of the PST and TST models evaluated them for speed, economical and experimental applicability, as well as user ergonomics, noting that the considerable differences between the two methods are that the TST can be fully-automated for

scoring purposes (potentially increasing throughput), while also avoiding PST hypothermia concerns [34]. However, there is not much documentation comparing the sensitivity of these two experimental designs with a dose-response study.

My implementation of the TST in our laboratory began with building an apparatus (Figure 3) to carry out these experiments, where I could validate its use through a TRH dose-response comparison in mice with the PST. Figure 6 shows, that in both models, statistically significant decreases in immobility times were observed between the vehicle-treated control group and all other studied TRH doses. The ED_{50} for the PST was about 2 μmol/kg vs. an ED_{50} closer to 1.5 μmol/kg for the TST. Whether this slight difference in sensitivity can be exploited or is due to experimenter error warrants additional study; although, the TST's potential for greater throughput may better suit the prodrug design process.

As the TST results have proven comparable to those of the PST in observing the anti-depressive effects of TRH, it demonstrates my successful implementation and validation of the TST method for subsequent use in our laboratory. This investigation may also prove useful for future neuropharmacological assessments of neuropeptides by further integrating an automated TST method with computer-assisted scoring that would systematically increase throughput and decrease experimenter subjectivity.

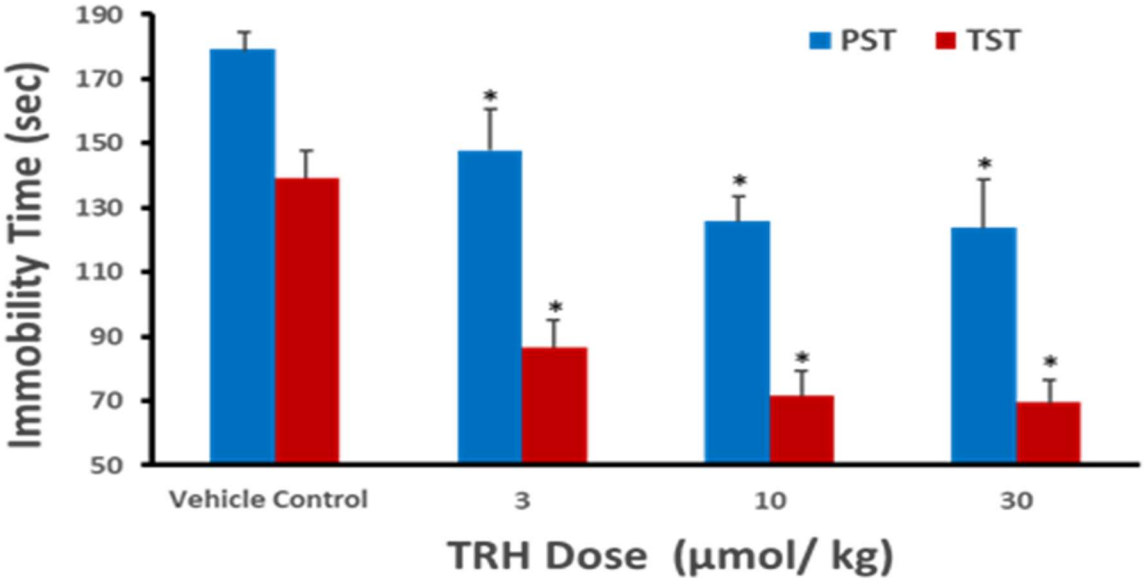


Figure 6. Dose-response studies using various concentrations of TRH in the PST and TST models of antidepressant-like activity. Comparison of these two behavioral tests for antidepressant-like effects of TRH were carried out using identical TRH doses in CD-1 male mice 30 min after s.c. injection. * indicates statistically significant difference (p<0.05) from the respective control group by one-way ANOVA followed by *post hoc* Tukey test.

C.2. Neurochemical Assessment of TRH Delivery into the Brain *via* PRODRUG (1)

Increased CNS activity, as a result of TRH administration, has been well documented by our laboratory through *in vivo* microdialysis sampling [14,38,46,47]. The neurochemical effects produced by TRH are most notably attributed to the synthesis and extracellular release of ACh [5,14], where increases in microdialysate ACh levels are directly influenced by TRH's perfusion using neuro-microdialysis.

Consequently, in an exploratory pilot study, we surveyed the *in vivo* conversion of PRODRUG (1) to TRH within the frontal cortex of rats by monitoring the previously mentioned

cholinergic mechanism and using ACh as a neurochemical surrogate. A tetra-deuterated ACh internal standard spiked into each sample permitted the ACh concentration within each microdialysate fraction to be determined *via* an LC-MS method developed by our laboratory [38]. During the first two hours of microdialysis equilibrium with ACSF, the ACh baseline concentration in the frontal cortex (arbitrary taken 100% as reference value) was determined for each animal, allowing each animal to serve as its own control. The baseline concentration was then compared to the significantly increased ACh "steady-state" response level, following perfusion of PRODRUG (1) or TRH (3 mM), represented by the quantification of the last 5 microdialysate fractions collected in our experiments.

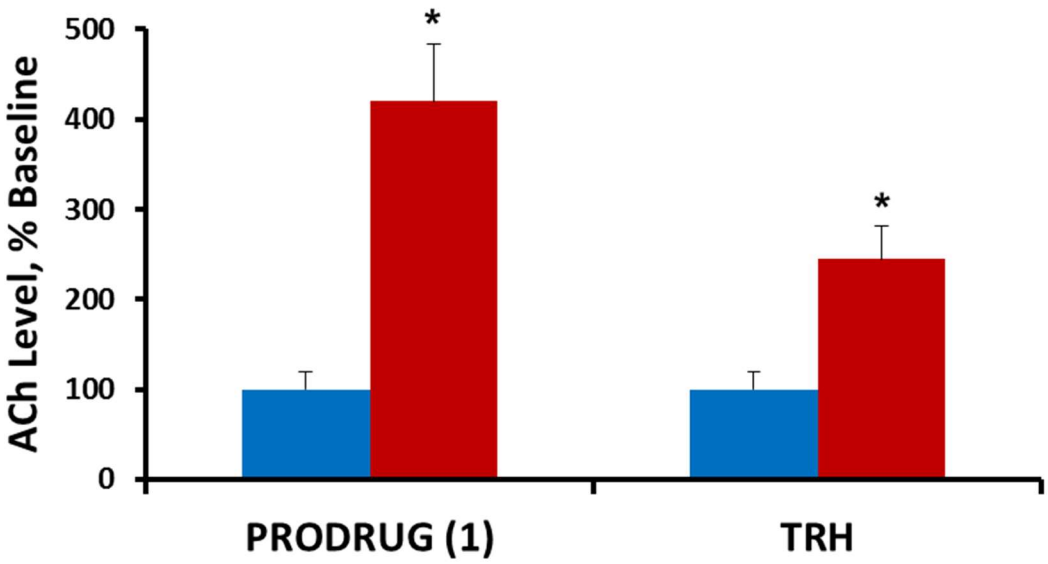


Figure 7. Comparison of the ACh concentrations obtained through the analysis of "Baseline" fractions 1-5 perfused with ACSF (blue), compared to steady-state "test-agent perfused" fractions 11-15 (red). In the rat frontal cortex, PRODRUG (1) (3 mM perfusion concentration) elicits a 4-fold increase in ACh, while TRH (3 mM) perfusion produced a nearly 2.5-fold increase in ACh. *Indicates statistically significant difference ($p<0.05$) from each animal's control baseline by one-way ANOVA followed by *post hoc* Tukey test.

The success of our novel prodrug approach for enhancing the brain-delivery of TRH is evident with the neurochemical effect comparison between perfusions with TRH or PRODRUG (1), where a 2.5- to 4-fold increase in ACh levels was recorded, respectively (Figure 7), and clearly indicates the profound *in vivo* release of TRH from PRODRUG (1) in the brain.

D. *In Silico* Survey of Binding Affinity of PRODRUG (1) and Related Prodrugs for POP

In an effort to gauge the optimal LAA-Xaa-Pro promoiety combination (Scheme1, Figure 2) that would aid POP's release of the TRH progenitor sequence, I conducted *in silico* docking experiments of POP's active site with a series of computer-modeled prodrugs with various LAA and POP-sensitive linker combinations. Cleavage of the prodrug by POP is a pivotal step in prodrug bioactivation to TRH. POP is a serine protease that belongs to an endopeptidase class of the "super-folding" family, which is apparent from its structure, and illuminates the size restriction of POP in cleaving small peptides less than about 30 amino acids in length [24,40]. POP's co-crystallized ligand, GSK522, has a molecular weight of 466 Daltons (Da) and, as Figure 8 illustrates, it is almost completely enveloped within the binding pocket near residues Arg-643, Trp-595, Met-235, Tyr-473, Phe-476, and the catalytic triad Ser-554, His-680, and Asp-641 [40].

Using AutoDock Vina molecular modeling software [39], PRODRUG (1) was first computationally docked with POP (Figure 9) to ensure that, once it crosses the BBB, adequate POP-binding would subsequently cleave and release the TRH progenitor sequence. Docking poses depict the prodrugs as enveloped by the binding pocket at the C-terminal amide of the progenitor sequence (Gn-His-Pro-NH_2), so that the LAA transport moiety is nearly protruding out of the enzyme (Figure 9). Comparable to the crystallized POP-GSK552 ligand complex [40], PRODRUG (1) exhibits around –7.8±2.5 kcal/mol (9 modes averaged) binding affinity to POP.

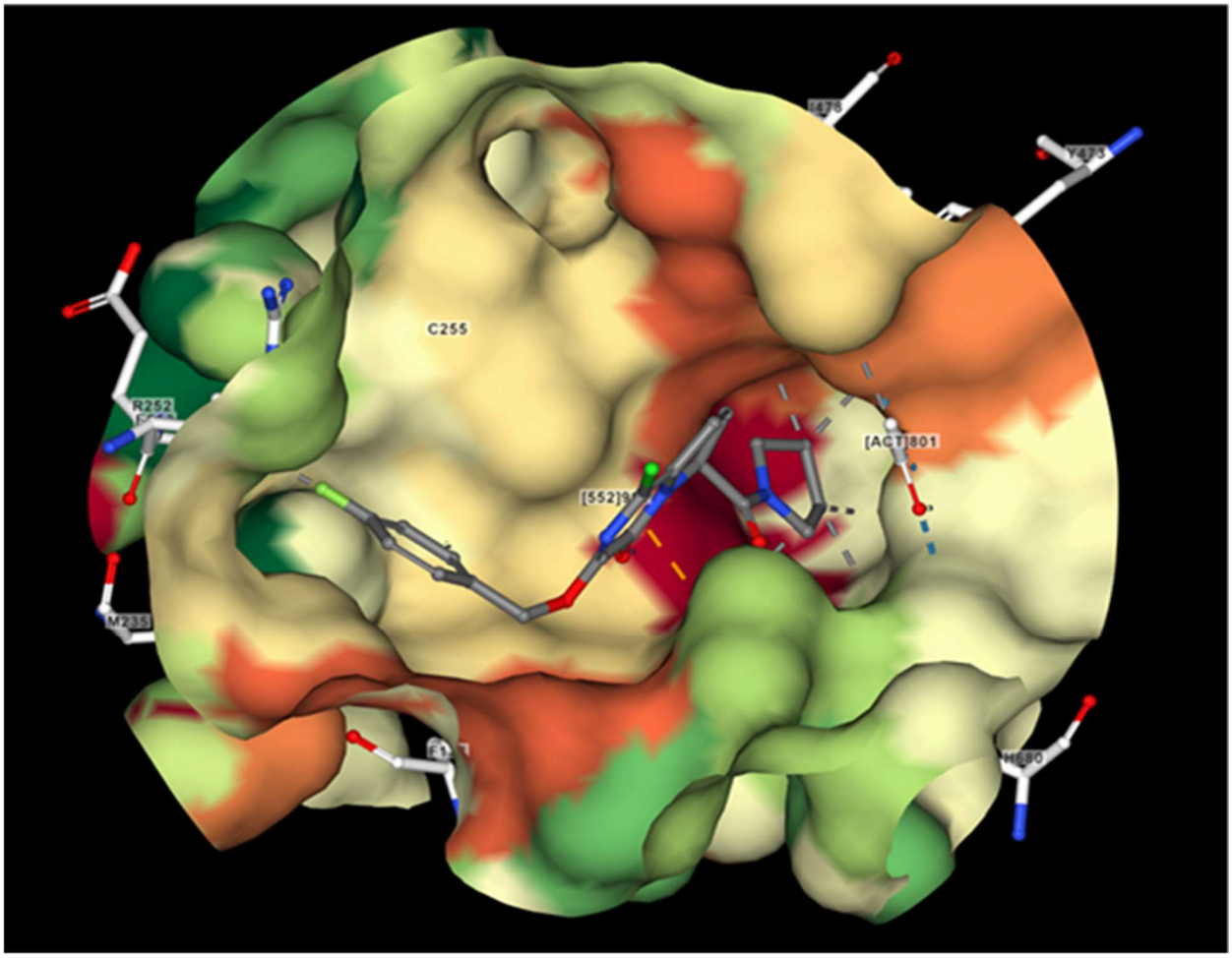


Figure 8. Depiction of POP bound to its co-crystallized ligand GSK552 [40]. The image illustrates the active site interactions that envelope, or fold, around the substrate, so that it may be enzymatically processed. Understanding this limiting factor in size exclusion is important to design potential prodrugs (i.e., ligand/ substrate).

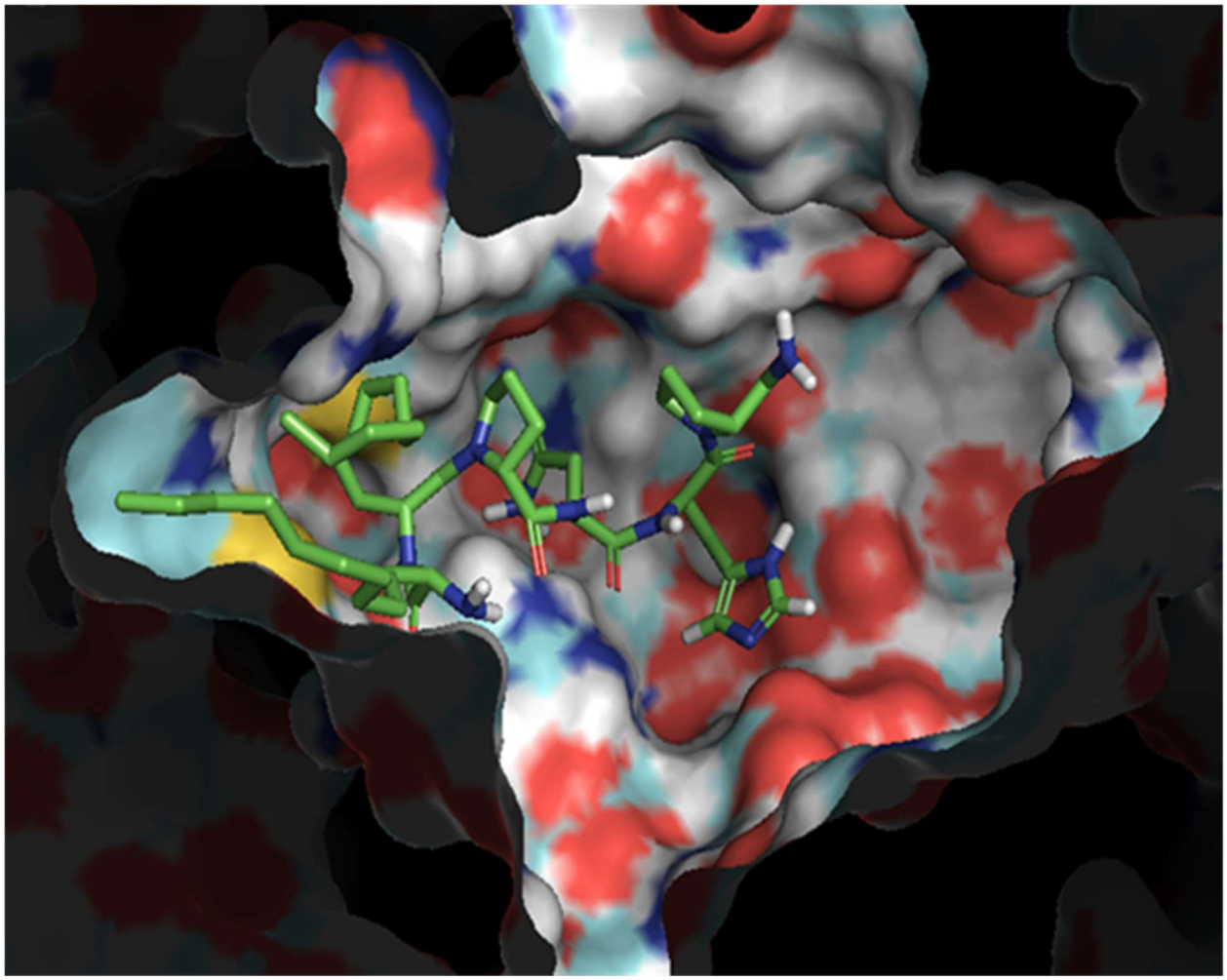


Figure 9. Docking of PRODRUG (1) to POP. PRODRUG (1) exhibits a binding affinity measured at –7.8±2.5 kcal/mol within the binding pocket of POP (3DDU). The LAA transport moiety appears to be of an optimal length to adequately increase its lipophilicity and aid BBB penetration, while still accommodated within POP's active site.

This *in silico* validation of our prodrug design concept further substantiates our hypothesis in terms of assessing the synergistic combination of LAAs and POP-sensitive linkers and their impact on POP-binding affinity. The virtual library of prodrugs (Figures 11 and 12) were created based on the prodrug concept introduced here (Scheme 1) and docked to the binding pocket of POP. The prodrug library contains molecules with either single prolyl (Figure 10) or diprolyl (Figure 11) POP-sensitive linkers in conjunction with single or double LAA residues of various side-chain lengths (n= 6, 10, or 14).

Results of *in silico* docking show that, as the experimental ligands' molecular weights are enlarged by increasing the transport moiety's complexity, there is an unfavorable increase in free binding energy (Figure 10); however, this may be attenuated with added flexibility through the introduction of -Xaa-Pro (i.e., -Pro-Pro) as the dipeptidyl POP-sensitive linker (Figure 11). Increased flexibility may account for the larger C-14 sidechains and double LAA residues, being as competitive in binding affinity as the smaller transport moieties that contain a single Pro residue as the POP-sensitive linker, which could be seen in comparing the slope of the trend lines in Panel C of Figures 11 and 12. Although, this additional Pro residue, between the progenitor sequence and the LAA moiety, appears to allow some of the prodrugs greater binding affinity with POP, it is not necessary, yet could prove useful in the development of other neuropeptide prodrugs requiring a more complex promoiety for its transport and/or targeting.

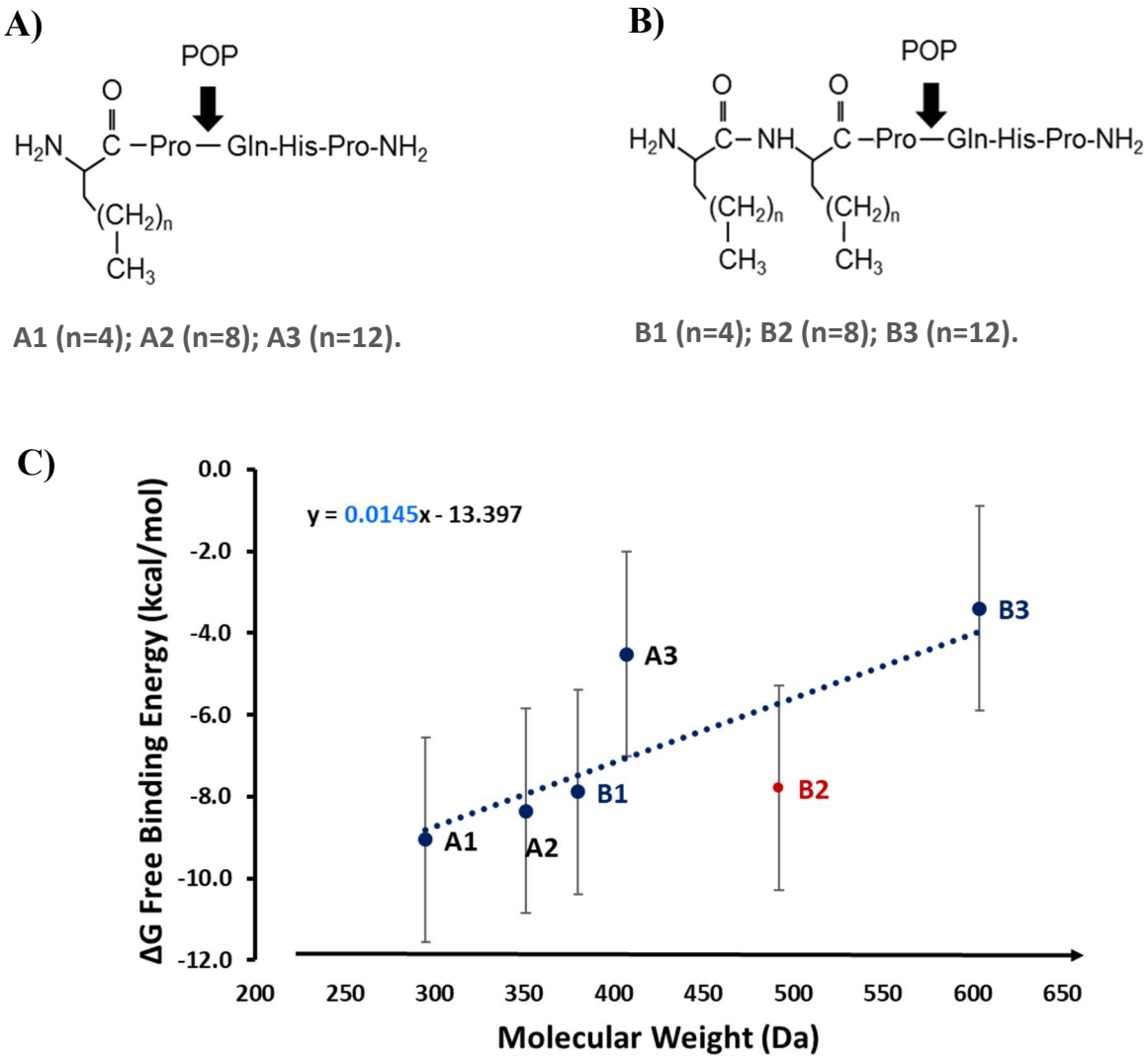


Figure 10. A virtual library of prodrugs was created based on the design principles introduced in Scheme 1, to assess *in silico* the prodrugs' binding affinities with POP, as this binding is crucial for *in vivo* bioactivation. Data point B2 (red) represents PRODRUG (1). **Panel A**: Single proline as POP-sensitive linker with one LAA residue. **Panel B:** Single proline as POP-sensitive linker with two LAA residues. **Panel C:** Survey of the free binging energy (ΔG) between virtual prodrugs and POP's active site exposes a trend that increasing the molecular weight of the prodrug's transport moiety, either by extending the alkyl chain length or the number of residues contained in the LAA moiety, adversely increases its predicted binding free energy with POP.

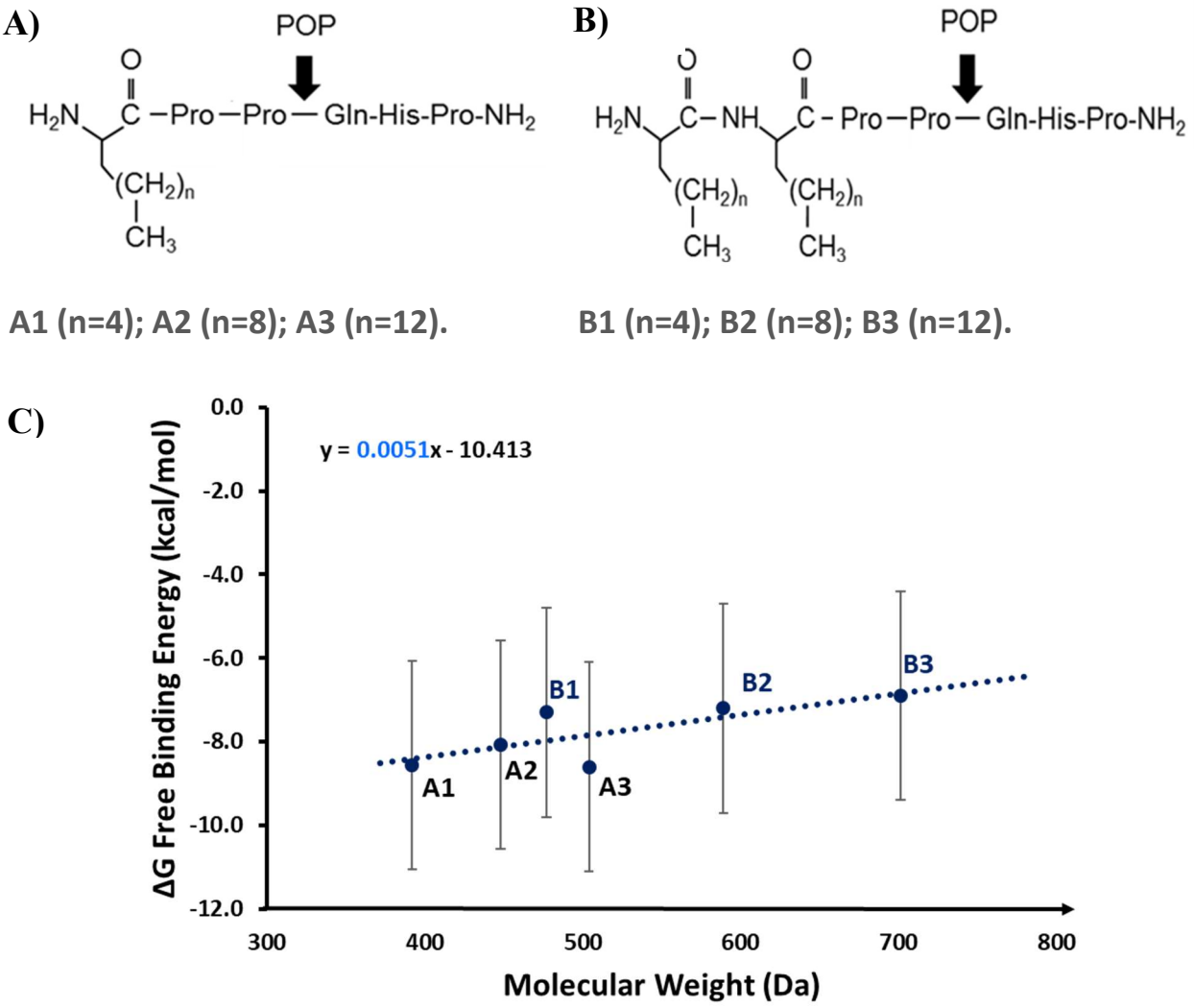


Figure 11. Virtual library of prodrugs with diprolyl POP-sensitive linkers. A double -Pro-Pro linker appears to add additional flexibility, with less of an increase in free binding energy vs. molecule weight, in contrast to Figure 10 prodrugs. **Panel A**: Diprolyl POP-sensitive linker with one LAA residue. **Panel B:** Diprolyl POP-sensitive linker with two LAA residues. **Panel C:** Survey of the free binging energy (ΔG) between virtual prodrugs and POP's active site exposes a similar trend as seen in Figure 10, that increasing the molecular weight of the prodrug's transport moiety, adversely increases its predicted free binding energy with POP; however, a graphical comparison reveals the slope in Figure 11 is 3 times less than that of the graph in Figure 10.

CHAPTER IV

SUMMARY

Several factors limit the use of TRH as a non-invasive neurotherapeutic agent. Primarily, its short biological half-life, poor brain uptake, and potential to cause serious off-target effects within the periphery (i.e., thymus over-activation) are profound obstacles. Based on medicinal chemistry principles, our novel prodrug design addresses these issues by creating prodrugs of TRH that are ideal for non-invasive systemic administration. Centered on the premise of bioreversible chemical modifications, a prodrug is biologically inactive and must rely on endogenous enzymes, preferably near the site-of-action, to convert it to the therapeutic agent.

Using a variety of *in vivo*, *in vitro*, and *in silico* techniques, our laboratory was able to validate a promising prodrug approach for enhanced brain-delivery of TRH with the initial pharmacological evaluation of PRODRUG (1), a prototype compound. Consisting of an LAA transport moiety (2 LAA residues each with an alkyl C-10 sidechain) conjugated to the N-terminus of a TRH progenitor sequence *via* a single -Pro POP-sensitive linker, PRODRUG (1) demonstrates adequate lipophilicity and metabolic stability to cross the BBB from the blood in rodents. We have shown that increasing lipophilicity, and therefore membrane affinity, enables PRODRUG (1) to enter into the brain, where its transport moieties are cleaved, and the TRH progenitor sequence is transformed to release the target peptide. Furthermore, the flexible design of our model peptide delivery system allows for the fine-tuning of the LAA side chains and POP-sensitive linker combinations that could be adapted to other neuropeptides of interest for their prodrug development.

A. Limitations

The prodrug-to-TRH conversion rate could not be directly measured based on the particular (ion trap) mass spectrometer's limit-of-detection of TRH and its extreme metabolic degradation in plasma and brain homogenate, yet indirect confirmation was possible by neuropharmacological assessments (PST) and, also, through monitoring TRH's neuromodulation of ACh in real-time through *in vivo* neuro-microdialysis. However, as this prodrug design progresses, a more systematic and less invasive approach may be needed to detect the minor differences in drug delivery resulting from small modifications within the transport moiety and/or POP-sensitive linker; therefore, an automated TST technique could be an advantageous method to improve practicality and throughput.

B. Future Directions

Taken together, this novel prodrug design is promising, and our preliminary results warrant future studies to fully explore the utility of this novel strategy for non-invasive CNS-delivery of TRH. Comparison of brain-enhanced TRH delivery *via* our prodrug approach to systemic TRH administration will be planned for the evaluation of neuroendocrine safety. Specifically, the benefits of CNS-targeting will be examined with the measurement of T3/ T4 endocrine levels. Further validation of this CNS-delivery method will continue supplementing TRH prodrug research and, hopefully, facilitating clinical applications of TRH treatment. Conceivably, other small- to medium-sized neuropeptides with therapeutic potential may benefit from this design approach, as well.

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