Molecular Design of Peptides to Address Growth Deficiency in Children

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Introduction

The prevalence of Growth Hormone Deficiency (GHD) has been estimated to be approximately 1:4000 in children, with adult-onset GHD accounting for an additional approx. 6000 cases per a year.[1] Clinical presentation often manifests as shorter stature, but also slowed growth rate, decreased muscle mass and bone density, and other signs of delayed puberty. This shorter stature phenotype is observed due to inadequate secretion of growth hormone (GH) from the anterior pituitary, and the prevailing current standard for treatment of GHD is the use of expensive daily injections of recombinant growth hormone.

A superior treatment would feature less frequent administration with restoration of endogenous GH production and pulsatile secretion. A potential route to attain such a treatment is by the stabilization of the human growth hormone-releasing hormone (hGHRH) peptide. hGHRH is a 44-a.a. hormone produced in the arcuate nucleus of the hypothalamus. The main role of hGHRH is to stimulate the pituitary gland to produce and release growth hormone into the bloodstream by first binding to its receptor, growth hormone-releasing hormone receptor (GHRHR). GHRHR is a G-protein coupled receptor (GCPR) meaning the binding of GHRH at the surface activates internal signal transduction pathways and ultimately, the desired cellular response of GH release (Figure 1).

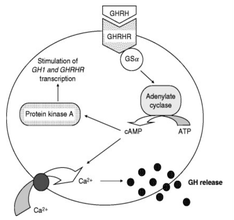

Figure 1. A simplified intracellular signaling pathway for GHRH's binding to GHRHR, most commonly at the anterior pituitary.[2]

Previous Work

While, hGHRH seems like a very viable candidate for study to make stable drug therapeutics for patients with GHD, the most significant issue with GHRH is its short half-life of approx. 6-8 minutes,[3] meaning in its current state it has limited therapeutic potential and would require multiple daily injections or infusion by a pump.

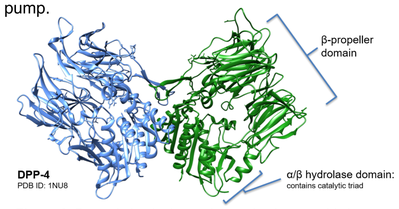

Figure 3. Degradation is due to the predominant rapid cleavage of Tyr1 and Ala2 by Dipeptidyl Peptidase-4 (DPP-4) that leaves the peptide completely inactive of the receptor.[4] Another source of degradation occurs at the dibasic cleavage sites due to trypsin cleavage, which while still significant is both much slower and independent of DPP-4 cleavage.[4]

The issue of GHRH's rapid degradation has been tackled before in the literature, mostly focusing on the amidated 1-29 sequence of hGHRH, as it has been shown to be the shortest fragment that exhibited full biological activity.[5] hGHRH analogs have been made from many different types of modifications, from natural and synthetic amino acid substitutions, PEGylation, conjugation with albumin and several other techniques, however few have made it to clinical trails. Alkylation would be a promising modification as it would provide a more economical route for large-scale production compared to the use of expensive synthetic amino acids substitutions and these reductive alkylation techniques could be used on genetically engineered peptides already made in large quantities. Researchers at Tulane School of Medicine have already considered a series of GHRH analogs with N-terminal alkyl modifications and presented enough data to consider alkylation to be a practical method for enhancing the duration of action and in vivo potency of the peptide.[6]

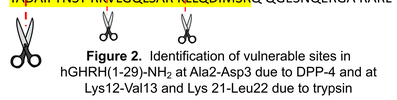

Figure 2. Identification of vulnerable sites in hGHRH(1-29)-NH_2 at Ala2-Asp3 due to DPP-4 and at Lys12-Val13 and Lys 21-Leu22 due to trypsin

Purification of Peptide

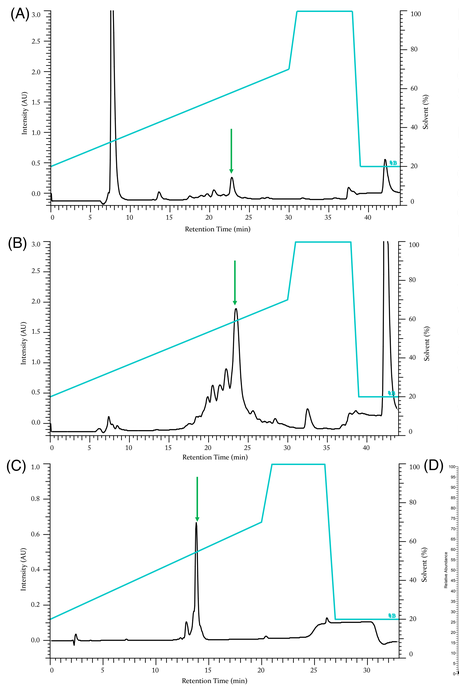

Figure 6. (A) RP-HPLC chromatogram (C8 Semi-Prep Column) of the crude peptide after full-scale cleavage. (B) RP-HPLC chromatogram (C8 Semi-Prep Column) of crude peptide after SPE to remove DTT from sample. (C) RP-HPLC chromatogram (C8 Analytical Column) of peptide to assess purity after two rounds of purification. (D) ESI-MS to confirm the identity of the desired peptide.

Purification was conducted using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). Confirmation of the successful synthesis was conducted using Electronspray Ionization Mass Spectrometry (ESI-MS).

Due to use of DTT in the cleavage cocktail, a large undesired absorbance peak was observed in the crude peptide (Figure 6A), likely due to large amount of the reducing agent interfering with the absorbance of the peptide bond at 230 nm. To remove the excess DTT, solid phase extraction (SPE) was conducted (Figure 7).

Two subsequent rounds of purification via RP-HPLC were conducted on the remaining peptide. The current state of peptide purity (Figure 6C) indicates additional purification is needed prior to cell testing, with several peaks proving difficult to sperate. Further rounds of RP-HPLC will use a shallower gradient as well as a heated column to allow for a faster and more efficient separation.

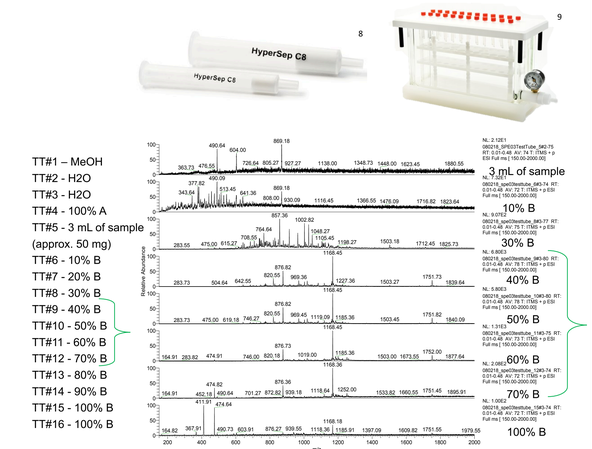

Figure 7. SPE was conducted to remove the large quantity of DTT from the crude peptide. Five rounds of SPE were completed using a Thermo Fisher HyperSep C8 Cartridge and the above solvent gradient. Test tubes 9-12 were combined and saved from each run.
Solvents Used:
Solvent A (99% H_2O, 1% CH_3CN, 0.1% TFA)
Solvent B (10% H_2O, 90% CH_3CN, 0.07 % TFA)

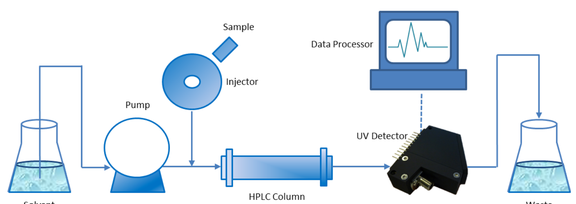

Figure 8. Schematic of the RP-HPLC System

Synthesis of Peptide

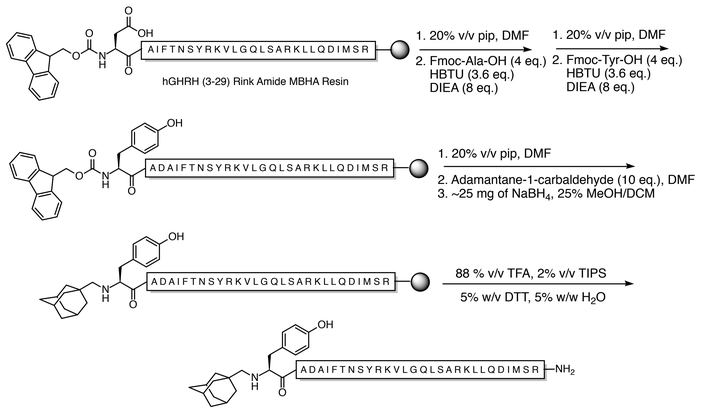

Figure 4. The synthetic scheme for the adamantyl hGHRH RA analog.

Manual Fmoc-based Solid Phase Peptide Synthesis (SPPS) was used to couple the last two amino acids, Tyr1 and Ala2 to Rink Amide MBHA Resin with the hGHRH(3-29) sequence preloaded. Then reductive amination was used to attach the aldehyde to the N-terminus of the completed hGHRH(1-29) sequence.

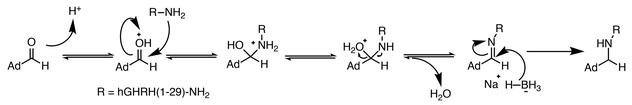

Figure 5. Summary of arrow pushing in the reductive amination - formation of an alkylated amine from the reaction of an aldehyde with another amine and a reducing agent.

Although there are some modifications, including ones already shown to protect against DPP-IV degradation (see Figure 6), there has yet to be a clear study detailing how promiscuous the receptor is to N-terminal alkylations. Creating two peptides one to test for size and the other to test for charge, would allow for more investigation into the peptide-receptor interaction and perhaps even provide a more potent analog. Two aldehydes were chosen to be attached to the N-terminus of hGHRH(1-29) amide via reductive amination (RA):

- Adamantane-1-carbaldehyde (to test for influence of steric bulkiness)
- (2S) 2-aminopropanal ('alanine aldehyde' – to test for influence of positive charge)

2-Aminopropanal (Alanine Aldehyde) RA Product

Adamantane-1-carbaldehyde RA Product

A mini-cleavage after the completed synthesis, yielded not only the successful RA product, but also a successful RA product with an undesired methionine oxidation (Met27). In order to eliminate this oxidation, likely acid catalyzed during cleavage, and consequently increase yield of the desired peptide during cleavage and purification, a new cleavage procedure was followed featuring the reducing agent dithiothreitol (DTT) to keep the methionine thioether reduced.[10] After an additional mini-cleavage using this new cleavage cocktail confirmed the reduction of the methionine, the full cleavage of the peptide was conducted using this cleavage cocktail.

Cell Assay

The final purified peptide will be tested by a cellular assay to determine effectiveness of the modified hGHRH peptides in triggering receptor activation.

HEK293 cells will be transfected with three plasmids:

- GHRHR gene – used to express the receptor for analog binding
- β-Galactosidase – used to measure the level of plasmid uptake
- CRE-Luciferase – used to measure receptor activation as the increased concentration of cAMP upon GHRHR activation promotes the transcription of luciferase which promotes the breakdown of luciferin, emitting quantifiable light in the process.

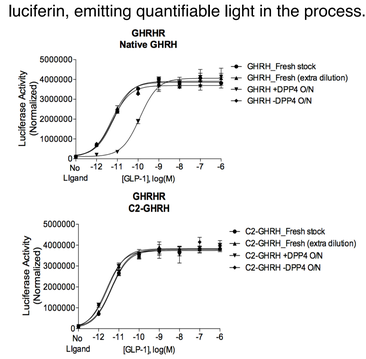

Figure 9. Earlier research completed in the Kumar Lab in late August 2016 demonstrated the application the trifluoroethyl group (-CH_2CF_3) modification to hGHRH(1-29) amide and observed the return to native potency in the case of C2-GHRH with DPP-4 exposure, while the native GHRH shifts rightward by ~22X following overnight incubation with DPP4 in these assay conditions

Next Steps

The full synthesis of the first of two proposed hGHRH analogs has been completed and its purification is nearly complete. The synthesis and purification of the second analog, the (2S) 2-aminopropanal RA product will be completed as well as the purification of the previously synthesized hGHRH and C2-GHRH, prior to cell testing. Pending the results of the cellular assay, the synthesis of another hGHRH analog will be evaluated, possibly an analog testing for the influence of a negative charge by conducting a similar reductive amination scheme for an aldehyde such as tert-butyl 3-oxopropanoate. Altogether, the results from these series of analogs will provide greater insight into the influence of N-terminal alkylations in the interactions between hGHRH and GHRHR and may suggest further modifications or a change of direction in terms of modification technique for the hGHRH peptide.

References

1. Stanley, T. Diagnosis of Growth Hormone Deficiency in Childhood. *Current Opinion in Endocrinology, Diabetes and Obesity*. 2012.
2. Martari, M.; Salvatori, R. *Diseases Associated with Growth Hormone-Releasing Hormone Receptor (GHRHR) Mutations.*; 2009
3. Thorner, M. O. The Discovery of Growth Hormone-Releasing Hormone: An Update. *Journal of Neuroendocrinology*. 2008.
4. Frohman, L. A.; Downs, T. R.; Heimer, E. P.; Felix, A. M. Dipeptidylpeptidase IV and Trypsin-like Enzymatic Degradation of Human Growth Hormone-Releasing Hormone in Plasma. *J. Clin. Invest.* **1989.**
5. Thorner, M. O. The Discovery of Growth Hormone-Releasing Hormone: An Update. *Journal of Neuroendocrinology*. 2008.
6. (Murphy, W. A.; Coy, D. H. Potent Long-Acting Alkylated Analogs of Growth Hormone-Releasing Factor. *Pept. Res.* **1988**, *1* (1), 36–41.
7. Bonner, A. G.; Udell, L. M.; Creasey, W. A.; Duly, S. R.; Laursen, R. A. Solid-Phase Precipitation and Extraction, a New Separation Process Applied to the Isolation of Synthetic Peptides. *J. Pept. Res.* **2001.**
8. Image from Thermo Fisher Scientific: https://www.thermofisher.com/order/catalog/product/60108-309
9. Image from Thermo Fisher Scientific: https://www.thermofisher.com/order/catalog/product/60104-232

Acknowledgments

Professor Krishna Kumar
Kumar Research Group
Kathleen Sicinski
Dr. Vittorio Montanari
Venkat Raman *(alumnus)*

Tufts Summer Scholars
Dr. Martin Beinborn
The Wolff Family
NIH
NSF