Summer Scholar Report

Design and Characterization of Stable Glucagon Analogues

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Abstract:

We describe here the design, synthesis, and characterization of modified glucagon analogues with potential long-term solution stability while retaining efficacy and potency at its cognate re- ceptor to be used for treatment of acute Type I Diabetic hypo- glycemia. Modifications to the peptide sequence were chosen through a combination of alanine scanning data previously re- ported and molecular modelling to guide the choice of sites for further elaboration. Glucagon analogues were synthesized by solid phase peptide synthesis (SPPS) using a modified pro- cedure for decreased synthesis time. The designed peptide dif- fers from native glucagon and provides handles for later chemical modifications including glycosylation and N-termi- nal modifications. Peptides were synthesized on a solid phase resin and subsequently purified using RP-HPLC separation to >98% purity. Designed analogues were tested alongside native glucagon for their ability to stimulate the glucagon receptor. Receptor-ligand interactions were studied using an in vitro cAMP assay via concentration dependent activity profiles that capture both binding and activation in one experiment.

Introduction:

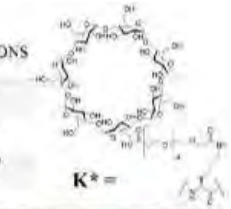
Type I Diabetes Mellitus (T1D) is an autoimmune disease affecting roughly 1.5 million American adults and children. This ailment results from the destruction of pancreatic β-cells that produce the peptide hormone insulin.[1] In healthy individuals insulin is released by the pancreas and stimulates cellular uptake of glucose from the bloodstream after mealtime. During fasting pancreatic α-cells release glucagon (GCG), stimulating breakdown of glycogen, releasing free glucose into the bloodstream. Together insulin and glucagon maintain a constant blood glucose level, a balance that is destroyed in T1D. Currently T1D patients supplement insulin with injections, but this method requires frequent blood tests and careful management of blood sugar while still resulting in detrimental symptoms such as headache or irritability. Additionally, T1D patients can (and do) sometimes administer too much insulin, leading to hypoglycemia. Diabetics can correct this through consumption of glucose, but in severe cases the patient may lose consciousness, rendering this method ineffective. Emergency treatment for acute hypoglycemia involves dissolving lyophilized glucagon powder in hydrochloric acid and injecting, leading to a quick recovery of the patient.[2] This raises a question: if insulin pumps can lower blood glucose after meals, why not raise blood glucose using a glucagon pump to reestablish homeostasis? Unfortunately, glucagon has only modest aqueous solubility, forming fibril and therefore making it difficult to maintain a constant concentration and a stable solution.[3] This is precisely why glucagon injections require

reconstituting a lyophilized powder and shaking aggressively to ensure dissolution before injection.[4]

One potential method to help this problem would be to create a form of glucagon capable of remaining in solution for longer periods of time. GCG is a 29 amino acid peptide hormone that interacts with and activates its cognate receptor, the glucagon receptor (GCGR), stimulating glycogen mobilization. To confer increased solubility to GCG, selective glycosylation with polymers of glucose at non-essential residues is proposed.[5] Multiple glucagon analogues were synthesized, purified, and analytically characterized by cellular assay detecting levels of cAMP, a signature diagnostic of receptor stimulation. Here we report the design, synthesis, purification, and cellular assay of glucagon analogues. These modified GCG constructs will serve as the foundation for further peptide modifications including glycosylation in ongoing work.[6]

Peptides were designed using previously reported alanine scanning experiments along with X-ray crystallographic structure analysis of GCGR in complex with glucagon analogues (PDB ID: 5YQZ).[7–9] Since GPCRs are notoriously difficult to crystallize, no full-length crystal structures of ligand-bound GCGR exist. Some structures have been published of GCGR in complex with glucagon analogues, as well as computer modeled

PEPTIDE	SEQUENCE	MODIFICATIONS
GCG	H-HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH	None (native)
MC-I-41	H-HSQGTFTSDYSKYLDKRRAQDFVKWLMNT-OH	S16K, Q24K
AG-CD	H-HSQGTFTSDYSKYLDK*RRAQDFVK*WLMNT-OH	S16K*, Q24K*



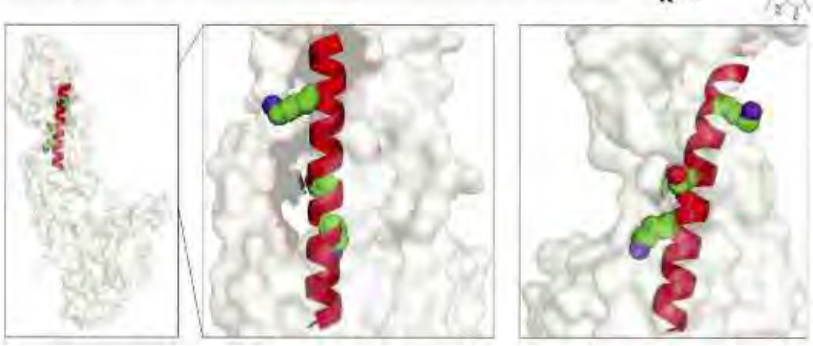


Figure 1: (top) Peptide analogue names and respective sequences, note AG-CD has 2 β-cyclodextrin attached to Lysε-N via a PEG-diacid linker; (bottom) Rendering of mutant GCGR in complex with modified glucagon analogue NNC1702. NNC1702 possesses a mutation to lysine and chemical modification at position 24 like proposed MC-I-41 variants. While K12 can be seen protruding deeper into the binding pocket S16 and K24 are much further out of the pocket interacting with the ECD.

interactions between glucagon and GCGR. Our analysis directed attention toward positions 13-18 and 23-28 for mutagenesis. This was due to the relative positions of

amino acids between the analogue and GCGR which showed these regions of the ligand that are not interacting with GCGR. Mutations of the N-terminus were not considered as they are buried deep within the binding pocket.[8] Alanine scanning data reported previously show a higher number of substitution tolerable residues near the C-terminus, and structural data show to be interacting with the extracellular domain of GCGR and the surrounding solvent (PDB ID: 5YQZ).[8] Glutamine 24 retained identical (100%) receptor activity profiles when substituted with alanine and was therefore selected as the first residue for mutation. Serine 16, Arginine-18, and Asparagine-28 were considered for a second modification by crystallographic analysis; alanine scanning showed Serine-16 retained highest relative GCGR potency and efficacy.

MC-I-41 is a designed glucagon without chemical modifications, a 29mer made through Fast-Flow SPPS. Peptide AG-CD was synthesized by Amy Guo through manual Fmoc-SPPS procedures and is included in this report alongside MCI-41 as a glycosylated GCG analogue. AG-CD differs from proposed MC peptides due to the presence of a diacid linker between the lysine residues on GCG and the sugar.[10] Additionally, cyclodextrin (CD) is a cyclic oligosaccharide; proposed variants (yet to be synthesized) contain linear sugar polymers. AG-CD was synthesized through normal Fmoc/SPPS means at room temperature, instead of the Fast-Flow synthesis used for MC-I-41.

Methods:

Fast Flow Peptide Synthesis 11

Peptides were synthesized using a Fast-Flow system adapted from the one previously reported by Pentelute and coworkers.[11] Fmoc-Thr(tBu)-Chlorotrityl (222 mg; 200-400 mesh) resin (0.2 mmol; Chem-Impex Int'l.) was loaded into the dry reaction chamber. The reaction chamber was attached to the Fast-Flow system and placed into a 60^{o} C water bath. A HPLC pump was set to 20 mL/min flow rate; methanol was flowed through the system for 5 minutes. This was followed by a 5-minute wash with dimethylformamide (DMF) to swell the resin. Absorbance on the UV/Vis detector at 304 nm was zeroed after this step. This was followed by a 45 second deprotection using 50% piperidine in DMF to remove the Fmoc group on the resin. The piperidine solution was removed from the chamber by a subsequent DMF wash for 4 minutes or until absorbance equilibrated. Couplings were conducted using a solution of 1.0 mmol amino acid (10. eq., Chem-Impex Int'l.) and 380 mg 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate (HATU) activating agent (9.0 eq., Chem-Impex Int'l.).

The HATU activation solution was made by adding 2.5 mL of dry DMF to 340 mg HATU. Amino acid/HATU solutions were made prior to each day of synthesis and 2.5 mL was added to each amino acid prior to coupling. Diisopropyl ethylamine (DIEA, 500

μL) was added to the amino acid/HATU solutions.[11,12] Amino acids were injected into the reaction chamber using Luerlock 12 mL rubberless syringes on a syringe pump (Harvard PHD 2000) at 6.0 mL/min injection rate. This was followed by a DMF wash for 4 minutes or until absorbance equilibrated. After termination of peptide synthesis, the resin was subjected to cleavage by 95% trifluoroacetic acid (TFA) in H2 O in the presence of triisopropylsilane (TIPS) scavenger for 90 minutes. The sample was evacuated to remove TFA, followed by 2 washes with ice-cold diethyl ether. Once off, resin peptides were lyophilized on a LABCONCO Freezone Freeze Dryer system at –40° C between 100 – 300 μbar to remove any remaining solvents before RP-HPLC.

Peptide Purification:

Peptides were purified using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) on a Hitachi 7000 HPLC system. Peptides were eluted using a gradient of acetonitrile (ACN) and water with 0.1% TFA. Two rounds of purification were performed to ensure high product purity. The first round of HPLC was conducted on a 10´ 250mm Vydac C18 reverse phase semi-prep column (10 μm pore, 2.5 mL/min). Round 1 was performed using a linear gradient of 26% ACN/H_2O to 37% ACN/H_2O at room temperature eluting MC-I-41 between 20.5–22.3 min retention time. Fractions were confirmed using MALDI-TOF-MS (m/Z [M + H] calculated 3524.05: found 3527.10). This retention time window for collection was kept intentionally large to give higher yield on the first round of purification.

The second round of purification was conducted on a 10´ 250mm Higgins Analytical C18 Proto200 reverse phase semi-prep column (5 μm pore, 2.0 mL/min). Round 2 was performed using a gradient of 23% ACN/H_2O to 41% ACN/H_2O at 50° C eluting MC-I-41 between 18.5–19.6 min retention time. Fractions were confirmed to contain the desired product using MALDI-TOF-MS (m/Z [M + H] calculated 3524.05, found 3525.04). Final sample purity was assessed by analytical HPLC on a 4.6 ´ 250mm Vydac C18 reverse phase analytical column (5 μm pore, 1.0 mL/min) at 230 nm.

Analytical Characterization of Peptides:

Peptide constructs were identified using a combination of Electrospray Ionization Mass Spectroscopy (ESI-MS) and Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectroscopy (MALDI-TOF-MS). For ESI-MS characterization of peptides, HPLC fractions were directly injected onto a Finnigan LTQ MS/MS running in positive ion mode. MALDI-TOF-MS was conducted on a Bruker Microflex LRF using a ground steel plate. Peptides were immobilized in 2,5-dihydroxybenzoic acid (DHB) matrix. The DHB matrix was prepared by dissolving 20 mg of powdered DHB in 1.0 mL 45% ACN/H2 O, vortexing, and filtering through filter paper to remove undissolved DHB. 1.5 μL of peptide sample was mixed with 1.5 μL of DHB matrix, vortexed, and centrifuged. 3.0 μL

of peptide/matrix mixture was plated as droplets and allowed to dry before scanning. Standards were received from Sigma-Aldrich (Angiotensin II (m/z [M + H] 1046.18); Renin Substrate Tetradecapeptide (m/z [M + H] 1759.01); Insulin Chain A-oxidized (m/z [M + H] 2531.60); Porcine Pancreatic Insulin (m/z [M + H] 5777.54)) to calibrate molecular weight determinations.

Peptide Concentration:

Peptide concentrations were assessed using a Thermo-Fisher Scientific Nanodrop ND-1000 Spectrophotometer. The peptide (0.5 mg) was dissolved in 56 µL of dimethyl sulfoxide (DMSO) and 2 µL placed onto the Nanodrop. Absorbance was measured at 280 nm and 274 nm using tyrosine and tryptophan chromophores. Concentration was calculated using the Beer-Lambert Law based on absorbance at 280 nm.

Figure 2

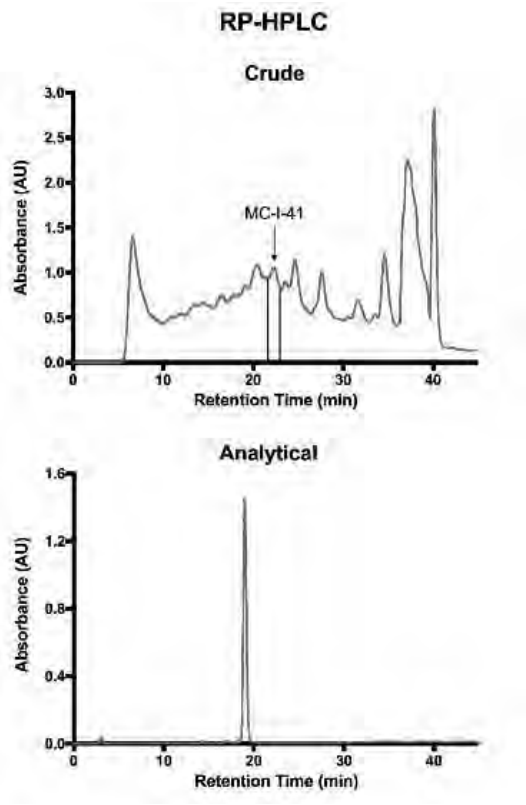


Figure 2: (top) Standard semi-prep HPLC chromatogram during round 1 purification of crude product, peaks surrounding MC-I-41 contained several deletion products from Fast-Flow synthesis; (bottom) Standard analytical HPLC chromatogram used for purity assessment after 2 rounds.

Cellular Assay:

Activity of the peptide ligand at the receptor was assessed using an in vitro cellular assay. HEK-293 QB1 cells were transfected with 3 plasmids; GCGR, CRE-luciferase, and β-galactosidase. Cells were plated at 10,000 cells/well and incubated at 37° C in serum-free DME medium. cDNA plasmids were transfected with Lipofectamine reagent nanoparticles and incubated overnight. After transfection the cells were tested for ligand activation of the receptor. Cells were incubated in medium in 96 well plates to which serial dilutions of peptide were added ranging from 10^{-6} to 10^{-12} M (Figure 3) and incubated for 4 hours. After incubation, SteadyliteTM solution (PerkinElmer) was added to the cells and luminescence measured after 5 min. β-galactosidase was used as a normalization factor for transfection levels. A solution of 4.0 mg/mL ortho-Nitrophenyl-β-galactoside (ONPG) was added to the cells and absorbance was measured at 420 nm. Cells were then incubated for 1 hour after which the absorbance was measured again. MC-I-41 was tested against native glucagon (control) and AG-CD, a glucagon analogue previously synthesized by Amy Guo.[10]

Figure 3

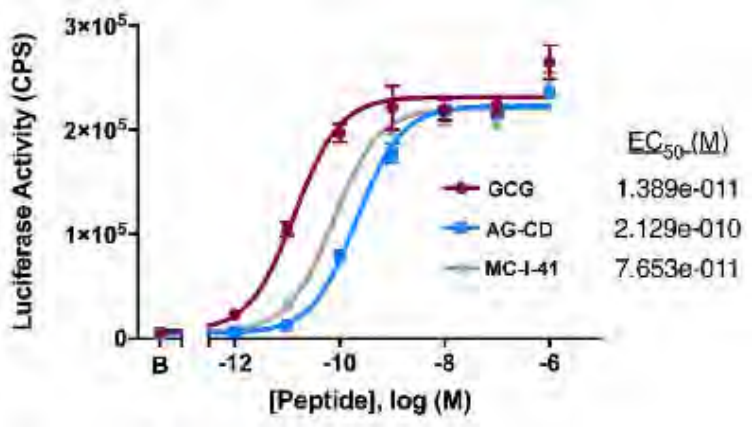


Figure 3: Concentration dependent response curves generated by administration of glucagon and analogues to transfected HEK-293 cells. All peptides retain nearly the same efficacy, but modifications reduced potency at the receptor. This is to be expected when modifying a native ligand-receptor system and corresponds to the modifications on each analogue. GCG = native glucagon; AG-CD = Cyclodextrin-Diacid GCG from Amy Guo.

Results & Discussion:

Fast Flow Peptide Synthesis

Solid Phase Peptide Synthesis (SPPS) was developed by R.B. Merrifield as a method for manually constructing short to medium length peptides by chemical means.[13] Since its inception SPPS has undergone several improvements in procedure and reagent use that have enabled faster, safer and more reliable peptide construction in higher yields. Unfortunately for peptide research, manual SPPS is a time consuming and resource

intensive process that, while optimized, is still hampered by its need for long deprotection and coupling reaction times.[13] Pentelute and co-workers have developed a contained "Fast-Flow" system to reduce the time needed for peptide construction by increasing the reaction rates involved in Fmoc SPPS.[11] This is achieved through thermal regulation of the reaction in a hot water bath and using more concentrated reagents. For example, instead of the usual 20% piperidine in DMF used for deprotection in manual SPPS which deprotects the Fmoc protecting group on amines in roughly 25 minutes, the fast flow system uses a solution of 50% piperidine in DMF at 60^{o} C which allows for deprotection in < 40 seconds. The increased reaction temperature decreases coupling reaction times considerably as well, allowing the production of 30mer peptides in a matter of hours.[11]

RP-HPLC Purification:

To ensure peptide dissolution before HPLC injection the peptide was dissolved in 19% ACN/H_2O at a concentration of 1.5 mg/mL instead of pure H_2O. During the first round of purification MC-I-41 glucagon analogues eluted earlier than native glucagon, likely due to the additional charge on residues 16 and 24. There were several co-eluting impurities that were identified as deletion products by ESI-MS, mostly resulting from a failed first coupling. As a result, a second round of purification was conducted on a column with smaller pore size at a higher temperature. These modifications significantly improved resolution in the second round. After 2 rounds peptide purity was assessed by analytical RP-HPLC. Final peptide samples contained 0.4 mg MC-I-41 peptide at ~99% purity at 0.4% yield on crude.

GCGR Cell Assay

MC-I-41 was tested against native glucagon and another glucagon analogue construct for its ability to activate the receptor. Stimulation of GCGR causes mobilization of sugar stores into the bloodstream, raising blood glucose levels. As a result, any therapeutic peptide of relevance must retain its ability to activate GCGR at the cell membrane. Analogue/GCGR interactions were measured using an in vitro cellular assay.
MC-I-41 was found to have an EC50 = 7.6 <-> 10^{-11} M against native glucagon EC50 = 1.3 <-> 10^{-11}M. This indicates a reduction in potency by roughly 5-fold for MC-I-41 at the receptor, which is within the range required for a functioning hormone therapeutic. AG-CD experienced a roughly 15-fold reduction in potency, which is to be expected. MC-I-41 is in fact a simpler version of the full AG-CD peptide, bearing more similarity to the native ligand.

Conclusions:

Glucagon analogues that retain biological potency and efficacy at the receptor were synthesized, achieving the project goals. MC-I-41 is still capable of serving as a biologically relevant peptide, as important molecular interactions between the ligand and

receptor are preserved. MC-I-41 will allow for separating sequence effects on receptor interaction vs. oligosaccharide modification length effects in glycosylated peptides. Synthesis of glycopeptides will continue with production of an orthogonally protected glucagon analogue allowing for controlled glycation at positions 16 and 24, without modifying the N-terminus or native lysine 12, an essential side chain required for biological activity (Figure 1). Long-term solubility testing in aqueous media is currently in development and will be carried out in the fall.

Acknowledgments:

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