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THE NUCLEUS

December 2018 *Vol. XCVII, No.4*

Monthly Meeting

A Medicinal Chemistry Symposium at Sanofi Genzyme, Waltham

Power of Kinases Beyond Oncology Indications

Ajay Purohit to Receive 2018 Arno Heyn Memorial Book Prize

Summer Scholar Report

By Matthew Crawford and Krishna Kumar, Tufts University

Summer Scholar Report

By Naomi Suminski, Kelly Harrison, Shalise Couvertier and Nancy Lee, Simmons University

Summer Scholar Report

Design and Characterization of Stable Glucagon Analogues

Matthew Crawford and Krishna Kumar, Department of Chemistry, Tufts University, Medford, MA 02155

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 receptor to be used for treatment of acute Type I Diabetic hypoglycemia. Modifications to the peptide sequence were chosen through a combination of alanine scanning data previously reported 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 procedure for decreased synthesis time. The designed peptide differs from native glucagon and provides handles for later chemical modifications including glycosylation and N-terminal 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

continued on page 6

Figure 1

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*

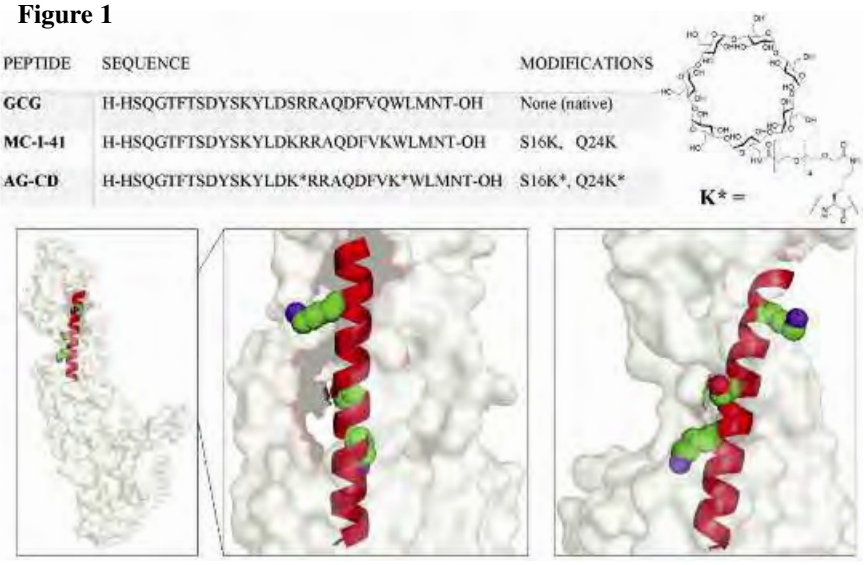

Figure 1: *(top) Peptide analogue names and respective sequences, note AG-CD has 2 β-cyclodextrin attached to $Lys_{\varepsilon\text{-}N}$ via a PEG-diacid linker; (bottom) Rendering of mutant GCGR in complex with modified glucagon analogue NNC1702. NNC1702 possesses a mutation to lysine and chemical 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.*

Contents

Cover: *Peter Dorhout, 2018 President of the American Chemical Society, welcomes the participants in the 18th NESACS-GDCh exchange of young chemists. (Photo by Morton Hoffman)*

Editorial Deadlines: *February 2019 Issue: December 22, 2018*
March 2019 Issue: January 22, 2019

THE NUCLEUS

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Ajay Purohit to Receive the 2018 Arno Heyn Memorial Book Prize

For his significant and substantial contributions to the publications of the Northeastern Section of the American Chemical Society, Dr. Ajay Purohit has been selected to receive the 2018 Arno Heyn Memorial Book Prize.

Ajay received his Ph.D. in Organic Chemistry from Northeastern University in 2002 and his Masters in Chemistry from the Indian Institute of Technology, Mumbai, in 1995. He has worked at Biogen since 2015.

Ajay is being recognized for his service on the Board of Publications (BOP) of NESACS including service as the current Chair. He is also being recognized for a number of BOP efforts he has helped to spearhead.

These efforts include a substantial effort to web stream (infrastructure allowing) Monthly Meetings to our membership. Thanks to Ajay there is also a dial in option for our Monthly Meetings. He also had a leadership role in the successful revamping of the NESACS website to better match the current best practices of National ACS and to create a website that fully highlights and benefits from National ACS branding.

The prize is a memorial to Arno H. A. Heyn, a distinguished, long-serving member of the Northeastern Section. Arno occupied most of the offices of the Section at various times, but his most lasting contributions were made when he was the Editor of *the Nucleus* of the Northeastern Section. Under his guidance this publication became the outstanding newsletter among all those published by Sections of the American Chemical Society.

The prize is awarded annually to a person or persons deemed to have made the most important contribution(s) to publications of the Northeastern Section. The awardee is chosen by the Arno Heyn Book Prize Committee which is chaired by the Chair of the Publications Committee. Other Committee members include the Editor of *the Nucleus*, two members of the Board of Directors appointed by the Section Chair, the Chair of the Awards Committee and the Section Chair, *ex officio*.

The prize is a book selected by the awardee, who is asked to choose a book that will have long time meaning and value to her/him. A bookplate mounted inside the book cover honors both the award recipient and the memory of Dr. Heyn.

Ajay joins past recipients of the Prize, Sam Kounaves and Mark Spitler (2005), Vincent Gale (2006), Vivian Walworth (2007), Myron Simon (2008), Arthur Obermayer (2009), Donald Rickter (2010), Harvey Steiner (2011), Karen Piper (2012), Mindy Levine (2013), Morton Hoffman (2014) Mary Mahaney (2015) and Roy Hagen (2016). The prize was not awarded in 2017. ◇

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Monthly Meeting

The 983rd Meeting of the Northeastern Section of the American Chemical Society

A Medicinal Chemistry Symposium organized by the Medicinal Chemistry Sectionof NESACS

Power of Kinases Beyond Oncology Indications

Thursday, December 13, 2018

Sanofi Genzyme
153 Second Avenue, Waltham, MA 02451

Northeastern Conference Room

3:00 pm Refreshments

3:15 pm **Welcome**-Raj Rajur, Medicinal Chemistry Program Chair, CreaGen Inc., Woburn, MA

3:20 pm **Introductory Remarks**–Dan Elbaum, QurALis, Cambridge, MA

Afternoon Speakers:

3:30 pm Philip Collier, Vertex, Cambridge, MA

4:15 pm Todd Bosanac, Disarm, Cambridge, MA

5:00 pm Florence Fevier-Wagner, Broad Institute, Cambridge, MA

6:00 pm **Social Hour**

6:45 pm **Dinner**

7:45 pm **Keynote Presentation:** John Kane, Sanofi, Waltham, MA
Title: *Identification of selective, brain-penetrant CSFIR inhibitors for the treatment of multiple sclerosis*.

NESACS BOARD MEETING: The Board Meeting will be held from 4:30-5:30 pm in the Celtics Conference Room

Symposium Organizing Committee: Brian Aquila, Mark Ashwell, Scott Edmondson, Dan Elbaum, Jeremy Green, Paul Greenspan, Adrian Hobson, Mindy Levine, Blaise Lippa, Lisa Marcaurelle, Andrew Scholte, Raj (SB) Rajur

YOU MUST REGISTER IN ADVANCE TO ATTEND THE SYMPOSIUM. THERE IS NO REGISTRATION FEE TO ATTEND THE SYMPOSIUM. DINNER RESERVATIONS ARE REQUIRED.

THE PUBLIC IS INVITED

- Dinner reservations should be made no later than 11:30 PM, Thursday, December 6, 2018. Reservations are to be made using Eventbrite: https://kinasesbeyondoncology-nesacs.eventbrite.com Members, $30; Non-members, $35; Retirees, $20; Students, $10.
- "RESERVATIONS NOT CANCELED 24 HOURS IN ADVANCE WILL NOT BE REFUNDED"
- If you wish to join us for this meeting and not eat dinner, please register by 11:30 PM, Thursday, December 6, 2018 at https://kinasesbeyondoncology-nesacs.eventbrite.com Select "Seminar only".
- New members or those seeking additional information, contact the NESACS administrative coordinator, Anna Singer, via email at secretary@nesacs.org.

Directions to Sanofi Genzyme Waltham Site:
From North or South 95/Rt 128 South.
Take Exit 27B/Winter Street. Follow signs for Second Avenue (stay in right lane)
Take a right after the Embassy Suites Hotel onto Second Avenue. Go past Costco on the right
At Forum Pharmaceuticals take a right between the Forum garage and Forum offices
(There is a sign directing you to Genzyme) Proceed about 100 feet
Genzyme will be on the right. Please enter through the main entrance (near stairs) and present yourself. ◇

Summer Scholar

Design and Characterization of Stable Glucagon Analogues

Continued from page 2

modeled 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 MC-I-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 °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 H_2O 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/H_2O, 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

continued on page 7

Summer Scholar

Design and Characterization of Stable Glucagon Analogues

Continued from page 6

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.

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, Steadylite™ 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 2

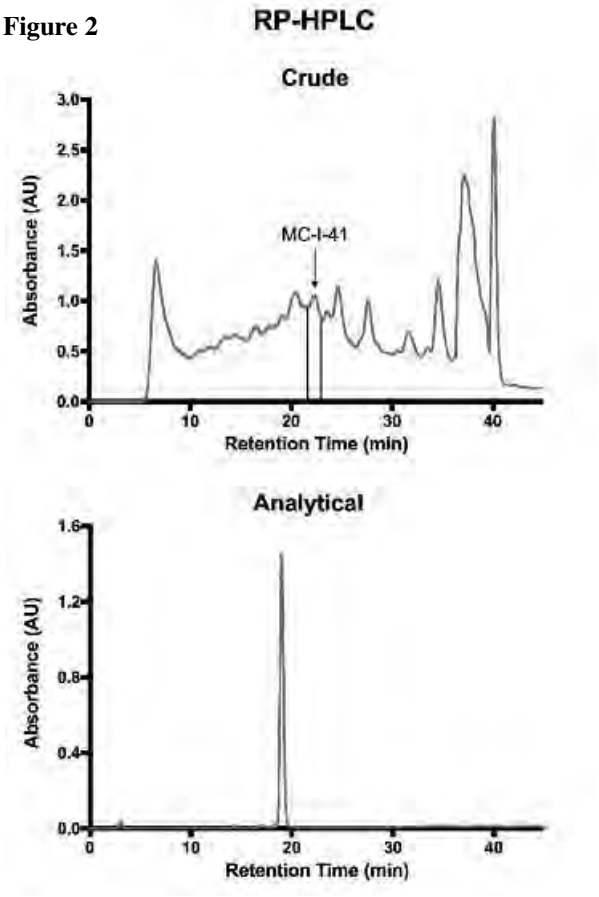

Figure 2: *(top) Standard semi-prep HPLC chromatogram during round 1 purification of crude product, peaks surrounding MC-I-41 contained several deletion products from Fast-Flow synthesis; (bottom) Standard analytical HPLC chromatogram used for purity assessment after 2 rounds.*

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 °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 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.

continued on page 13

Summer Scholar Report

Library development of D-enantiomer trifunctional chemical probes to assess the effect of probe chirality on protein selectivity in MCF7 breast cancer cells

Naomi Suminski, Kelly Harrison, Shalise Couvertier, Nancy Lee, Department of Chemistry and Physics, Simmons University, Boston, MA 02215

Abstract:
With the overwhelming rates of breast cancer and improved therapeutics needed, the development of small molecule chemical probes to use as tools to better understand the biology behind disease manifestation and the protein activities implicated in oncogenesis is essential. The goal of this project was to synthesize a probe library of D-enantiomer trifunctional chemical probes to study cysteine-mediated protein activities of estrogen receptor (ER) and progesterone receptor (PR) positive breast cancer cells. Distinct functional groups were installed onto the three sites of the probe scaffold, including an amino acid side chain directing group to target specific proteins in MCF7 ER/PR positive breast cancer cells. Specifically, focus was given to the incorporation of D-amino acid, a stereoisomeric form not innate to human proteins, to reveal the effect of chirality on probe design and protein specificity. At this time, the three synthesis steps in the development of these probes have been characterized for tyrosine and phenylalanine amino acid methyl esters, and multiple other amino acid probes are currently being synthesized. Techniques and protocols used for analysis of the probes-protein target interaction and activity have also been explored, with plans in the coming months to screen our synthesized probe library. This analysis could reveal unique insight into the mechanisms of breast cancer and may lead to future drug development for disease treatment.

Introduction and Background:
In 2018 alone, an estimated 266,120 new cases of invasive breast cancer will be diagnosed making it the second most commonly diagnosed cancer among American women.[1] Of these new cases, about 40,920 women are expected to die from the disease.[1] A majority of these cases will be estrogen receptor (ER) or progesterone receptor (PR) positive, indicating that the cancer cells contain hormone receptors which receive hormone signals and encourage the cells to grow and proliferate. While hormone therapies exist to help treat the disease it is essential that the mechanisms that lead to this type of cancer continue to be explored in order to develop improved therapeutic options.[2]

The development of chemical probes is one way to explore the biological mechanisms behind the manifestation of breast cancer. Probes are small, drug-like molecules designed with various functional chemical groups to identify protein-drug targets and are used as tools to reveal specific protein mechanisms in oncogenesis. Chemical probes are useful tools to study the reactive, functional amino acids responsible for catalysis and regulation in proteins implicated in diseases. These functional amino acids are nucleophilic residues that can be targeted by covalent modification with reactive electrophiles incorporated into probes by organic synthesis. Through various analyses the probe-protein targets can be investigated to gain a better understanding of functional site activity in specific proteins in cancer cells, specifically MCF7 ER/PR positive breast cancer cells.[3] One amino acid of particular interest in the human proteome is cysteine. Although it has a relatively low abundance it plays many unique and vital roles in protein catalysis and regulation. Therefore, designing chemical probes which can selectively target functional cysteine groups can relay important information about their role in protein activity within a cell of interest.

The chemical probe libraries created in this project are designed to covalently target and modify reactive cysteine residues of proteins within the cells of interest. Cysteines play a variety of diverse and functional roles within proteins and are therefore of great interest to be studied as a point of control in various biological processes. Specifically, cysteine is the most nucleophilic amino acid in proteins, and is highly reactive due to the polarizability and electron rich nature of the thiol moiety it contains.[4] Important functions of cysteine residues within proteins include nucleophilic and redox catalysis, metal binding and allosteric regulation.[5,6] As a result of these various roles, cysteine residues can be found on diverse protein types such as proteases, oxidoreductases and kinases.[5,6] These functional roles and proteins are of particular importance in oncogenesis when they become dysregulated. Knowing more about the location, reactivity, binding affinity and other characteristics of these cysteine residues within MCF7 cells via chemical probe interaction could reveal insight into the biochemical mechanisms at play not only in breast cancer oncogenesis but any other cell lines. Our chemical probe library has a cysteine-specific electrophile built into its molecular structure, which has been previously been used in probe libraries to target this amino acid.[6]

Not only can chemical probes be tuned to target specific amino acids, but can also have an innate chirality, which may affect their protein selectivity. Chirality is essential in organisms because organic molecules often exhibit a specific "handedness" or stereoisomeric conformation (Figure 1). This conformation of the molecule is important to its function in the organism as certain enzymes and receptors only respond to a specific chirality. In humans, natural amino acids are in the L conformation. However, the opposite D-enantiomer corresponds to naturally occuring amino acids in bacteria and are inherent to the bacterial immune response.[7] Using D-amino acids in probes will potentially relate the unique specificities of proteins in humans that recognize to opposite "handed" proteins in bacteria. Comparing the effects of the L-enan-

continued on page 9

Summer Scholar

Library development of D-enantiomer trifunctional chemical probes

Continued from page 8

tiomer library, which is being developed by Kelly Harrison a senior student at Simmons University, to a D-amino acid library, will reveal these unique protein selectivities within the MCF7 ER/PR positive breast cancer cells.

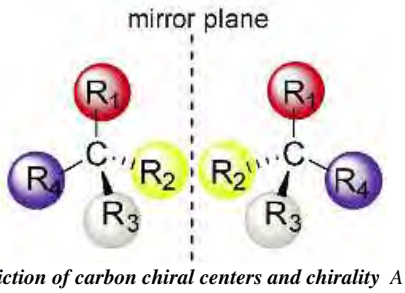

Figure 1. Depiction of carbon chiral centers and chirality *A chiral molecule is non-superimposable on its mirror image. In amino acids, the presence of an asymmetric carbon center endows the the property of chirality on the molecule.*

The construction of this D-enantiomer chemical probe library follows a three step synthesis in which three functional groups are installed onto a probe scaffold to encourage specificity and target selectivity of the probes. These three functional groups are: (1) a directing group of D-amino acid side chains; (2) an alkyne handle for visualization/enrichment of protein targets; and (3) a cysteine-reactive electrophile for activity-based protein profiling of proteins possessing reactive cysteine residues. Each of these moieties endow important functionality to the probe design. The amino acid directing group (1) allows for specific interaction with protein targets based on directing group structure and chirality. The alkyne handle (2) is the site at which 'click' chemistry can be performed, appending a fluorescent molecule for visualization or a biotin moiety for enrichment of protein targets. Lastly, the the third synthesis step involves attachment of a cysteine reactive electrophile by which the probe covalently modifies reactive cysteine residues in the MCF7 proteome.

The goal of the project was to create two complementary D- and L-enantiomer libraries of 5-7 probes, with each probe consisting of a different amino acid directing group. Thus far, D-phenylalanine and L-tyrosine probes have been created, with other amino acid probes (alanine, glycine, aspartic acid, and methionine) in the process of being synthesized. Following the synthesis of the library we will test the protein selectivity of the library members via 'click' chemistry at the alkyne functional group of each probe and screen in MCF7 lystates and whole cells by SDS-PAGE gel imaging.

Originally, a seven-step synthesis had been proposed at the start of the summer research period, which proved less efficient than we had hoped. The three-step process we used in this project was created from this initial seven-step process in which a diketopiperazine probe scaffold was employed. In the initial process, the first step, a dipeptide coupling proved challenging as it was difficult to characterize our product by NMR, a instrumental constraint. After several attempts, we decided to investigate an alternate synthesis process in which we could still incorporate the three functional groups stated above, in addition to introducing chirality into the probe design.

Overall, we successfully characterized each of our three synthesis steps and will now continue to expand the probe library. Following synthesis, the probes will be tested in MCF7 ER/PR positive breast cancer cell lysates in order to determine their viability and specificity. MCF7 cells will be treated with the probes, exposed to click-chemistry conditions to append a fluorescent molecule to probe modified-proteins and then visualized after SDS-PAGE for fluorescent protein bands. This type of analysis will achieve an understanding of whether the probe was able to enter the cell, reach a protein target, and covalently modify the protein target. Based on preliminary data, the next steps will be to modify the most promising probes and optimize their interactions with the protein target including tuning their chemical structure via synthesis techniques. Lead probes will be further studied to identify the specific protein target using mass spectrometry at Boston College. After we identify the protein targets, we will perform biochemical assays to determine the effect of probe modification on protein activity.

Results and Progress:

The overall goal of this project is to create a probe library consisting of 5-7 D-enantiomer amino acid derived probes which can selectively target cysteine mediated proteins in MCF7, breast cancer, cells. This probe library will be compared to an identical L-enantiomer probe library to determine the selectivity that chirality contributes to the probe design. Thus far, two major developments have been investigated, the first a synthesis process for the probe library, and the second, a process to assess the selectivity of the probe's developed and their biological targets in MCF7 breast cancer cells.

The first major stage of this project was developing a synthesis experimental protocol, which includes a three-step process by which three functional moieties are installed onto a probe scaffold. These functional groups, as discussed in the previous section, are critical to probe cysteine residue selectivity, covalent modification of the protein, and probe visualization by fluorescence. This stage included a number of optimization experiments which were carried out to increase the economy, yield, and safety of each step. The second stage of project experimentation was the utilization of a 'click' chemistry protocol by which the synthesized probes are analyzed. This process includes attachment of a fluorescent moiety, a rhodamine (TAMRA) azide tag, to an alkyne handle incorporated into the probe scaffold. Visualization of this fluorescent tag via SDS-page and fluorescent gel imaging techniques allows qualitative assessment of probe selectivity. Finally, comparison of our findings to the complementary library of L-enantiomer probes will help to determine the effect of chirality on target selectivity.

Initially a seven-step synthesis was proposed in which a diketopiperazine (DKP) probes scaffold would be used, onto which the three functional groups described prior would be

continued on page 10

Summer Scholar

Library development of D-enantiomer trifunctional chemical probes

Continued from page 9

incorporated. DKP's are a natural small molecule and secondary metabolite generated by fungi, and are useful building blocks for probes.[8] The original DKP probe design was chosen for its ability to have innate chirality and to easily permeate the cell membrane. However, despite the promising design and attempts to follow the initial seven-step process, difficulties to complete and characterize one of the intermediate products resulted in a re-design of the probe scaffold and synthesis steps. In particular, a dipeptide intermediate (the second intermediate compound in the synthesis schema) was difficult to fully characterize by our limited HNMR and IR instrumentation. This synthesis step consisted on a peptide coupling under EDC catalysis conditions in an attempt to yield a linear dipeptide from two amino acid methyl esters and generate the DKP scaffold. This synthesis was attempted with multiple amino acid methyl esters of both enantiomers but the same challenges to characterization occurred with each reaction iteration. Due to the efficiency and time constraints of the summer research period, we decided to implement a simpler probe scaffold.

This alternate probe design was hypothesized to be as effective at successfully covalently modifying a cysteine protein target with a more efficient and cost-effective synthesis protocol. This simpler probe, an amino acid methyl ester backbone with the three functional groups installed, was then synthesized and characterized starting with a D-phenylalanine methyl ester hydrochloride. The three synthesis steps are as follows: (1) preparation of tosylated alkyne (alkyne handle) for attachment in the following step (Figure 2), (2) monoalkylation of an amino acid methyl ester, with the R-group of the amino acid acting as the probe's directing group, and (3) chloroacetyl chloride addition serving as the reactive electrophile for the probe (Figure 3). Thus far a D-phenylalanine methyl ester probe has been synthesized and characterized. In the development pipeline are D-tyrosine, D-methionine, D-aspartic acid, and D-glycine methyl ester probes. Complementary L-amino acid probes are also being synthesized, with an L-tyrosine methyl ester probe fully characterized during the summer research period.

Considerable measures were taken in the development of each reaction step including reaction conditions, TLC and purification conditions, and proton nuclear magnetic resonance (1H NMR) for each intermediate step. For each intermediate and the final reaction product the general workflow was as follows: reaction set-up, extraction, purification by column chromatography, and finally characterization.

Developing each of the three synthesis steps, and characterizing each intermediate product, came with its own set of challenges. In particular, optimizing yields due to water and humidity conditions during summer months was essential. This was particularly true in conducting the first synthesis step (Figure 2) in which a tosylated alkyne was synthesized. A considerable portion of the summer research period was focused on optimizing this reaction. A number of iterations of this synthesis step were conducted in which reaction conditions were manipulated. Initial attempts at producing the tosylated product resulted in low yields (6% yield) and excess tosyl chloride, one of the starting materials, after aqueous work-up of the crude reaction mixture. Because of this excess of starting material and concerns that humidity was affecting the reaction a number of experiments were performed to increase our product yield.

First, we increased the equivalence of tosyl chloride by 36% which slightly increased the overall reaction yield (9% yield). In the next iterations of the reaction we attempted to eliminate the effect of humidity in all aspects of the reaction. We were scrupulous in our anhydrous technique, including drying all of our reagents with sodium sulfate, using molecular sieves, and using sure-sealed solvent. These attempts, however, did little to improve our yields. However, by scaling the reaction to produce a few hundred milligrams of the tosylated alkyne product, enough material was synthesized and purified to confidently characterize by 1H NMR and proceed to the next step.

In step two, seen as the first reaction in Figure 2, we utilized our first step product to alkylate an amino acid methyl ester. This reaction required refluxing over high heat for 12 hours, extracting and concentrating in vacuo after column pu-

continued on page 11

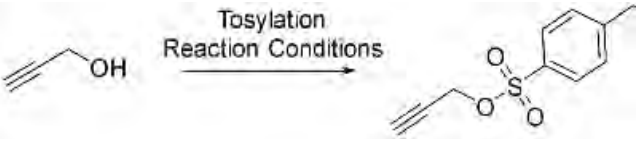

Figure 2. Tosylated Alkyne Handle Synthesis *Reaction of p-toluenesulfonyl chloride by propargyl alcohol yields a product essential to installing an 'alkyne' handle onto the probe scaffold in the next synthesis step (Conditions for reaction: triethylamine, tosyl chloride, DCM, 0 ℃, 12 hrs).*

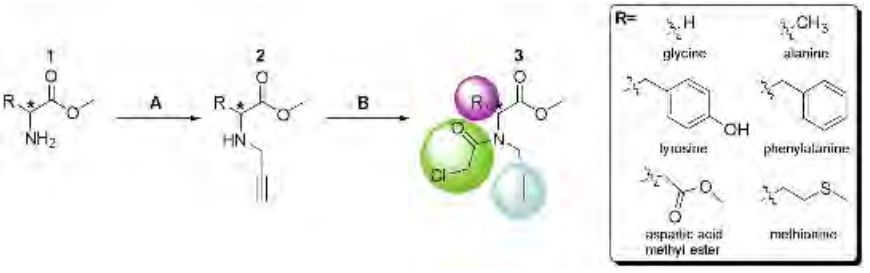

Figure 3. Synthesis of Amino Acid Methyl Ester Probe *Reaction A demonstrates the monoalkylation of an amino acid methyl ester using propargyl tosylate. (Conditions for Reaction A: Sodium iodide, potassium carbonate, acetonitrile, 90 ℃, 12hrs.). Reaction B shows the synthesis of the final probe from the monoalkylated intermediate via a chloroacetyl chloride coupling step (Conditions for Reaction B: secondary amine intermediate, chloroacetyl chloride, triethylamine, and dichloromethane, 0 ℃, 12 hrs.) The final probe includes the three essential functional moieties describes above: amino acid directing group (pink), reactive cysteine electrophile (green), and alkyne handle for 'click' chemistry (blue).*

Summer Scholar

Library development of D-enantiomer trifunctional chemical probes

Continued from page 10

rification. Initial attempts at this reaction produced moderate yields with D- and L- phenylalanine methyl esters (54% yield) as well as the L-tyrosine methyl ester (63% yield) and enough product to purify and then confidently characterize by 1H NMR. Loss of yield was due to the generation of a dialkylated product in addition to the desired monoalkylated product.

In an effort to increase the efficiency and cost-effectiveness of our process even more, we investigated if we could synthesize the same probe in two steps instead of three. An alternate method for monoalkylation of amino acid methyl esters was utilized to increase production of the monoalkylated amino methyl ester and decrease the production of the alkylated product (see Cho et al., 2002) .[8] This procedure allowed us to side-step the tosylation step and directly alkylate our amino acids of interest via propargyl bromide and lithium hydroxide. In reference to Figure 3, this process allowed us to synthesize compound 2 with a more efficient workflow. It allowed for more manageable reaction conditions, without the need to reflux with high heat. This reaction was performed with both D- and L-phenylalanine methyl esters with plans to apply it to other amino esters of interest.

The third and final synthesis step began with compound 2, the monoalkylated amino methyl ester and attached a cysteine reactive electrophile in the form of a chloroacetyl chloride addition. This step was completed and fully characterized thus far for L-tyrosine and is the final stages of characterization for D-phenylalanine.

In addition to our efforts towards building our probe library, we also developed a protocol to assess the cell permeability, protein selectivity and binding affinity of each probe after it's addition to the library. The foundation of this 'click' chemistry protocol is to append a fluorescent tag to the alkyne handle of each probe (essentially "clicking" the fluorescent tag onto the probe's alkyne handle), expose the probe to cell lysates at various concentrations, analyze these sample by SDS-PAGE, and visualize the fluorescence of each probe with gel imaging (Figure 4).[3]

The relative fluorescence seen in each sample is a direct indication of protein targeting by the probes and their binding affinity. Because our probe library was still in the developing stages, this 'click' chemistry protocol was developed using a different, but similar probe library, which also incorporated an alkyne handle into its design. A more promiscuous control was used, an iodoacetamide (IA) probe, which is used for global profiling of cysteines.

Discussion:

In summary, the progress of this research project has been the development and characterization of a three step synthesis to create a library of D-enantiomer amino acid methyl ester chemical probes and a complementary L-enantiomer library to assess the effect of chirality on probe target. So far, this synthesis protocol has been used to create a D-phenylalanine methyl ester probe and L-tyrosine methyl ester probes. Additional D- and L- amino acid probes are in various synthesis steps and will soon be fully synthesized, characterized and added to their respective libraries. In the meantime, a 'click' chemistry protocol has also been exercised and will be used in future experiments to assess each probe's biological activity and protein selectivity in cell lysates and eventually, whole cells.

Moving forward, the focus of the project will be to expand the probe library. After additional D-enantiomer amino acid methyl esters, including glycine, methionine, aspartic acid, glutamic acid, and tyrosine, probes are synthesized, each which will undergo 'click' chemistry reactions to append a rhodamine azide fluorescent tag. Subsequently they will be exposed to MCF7 cell lysates to assess if they were able to reach a protein target. Pending this interaction, we will also assess their ability to selectively target proteins in whole cells and evaluate their ability to permeate the cell membrane. The ultimate goal of this biological analysis of our probe library will be identify the specific cysteine residue targets of the probes.

One exciting potential of these libraries is their potential to modify not only the cysteine residues of MCF7 cells, but their ability to be screened against any cell line. Expanding our biological assessment of the libraries to other oncogenic cell lines could also garner information about important cysteine residues in other types of cancer. Even more, if a relationship between probe chirality and protein target is established, this concept can be applied to the design of other existing and novel probe libraries to generate more information about how stereochemistry plays a role in covalent protein modification.

Acknowledgements:

I would like to thank the Northeastern Section of the American

continued on page 12

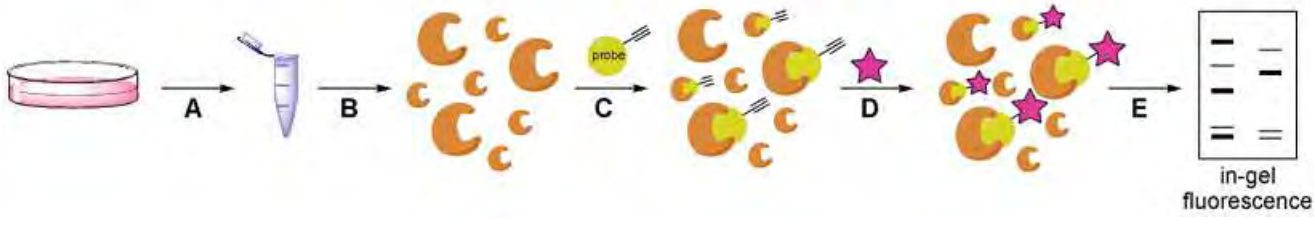

Figure 4. 'Click' Chemistry Work-Flow *Performing the protocol utilized the following experimental schema: (A) lyse the cells of interest (B) normalize protein concentration to 1 mg/ML (C) label the cells with probes (D) perform copper click chemistry with rhodamine azide fluorescent tag and (E) SDS-PAGE gel analysis for protein target visualization.*

Summer Scholar

Library development of D-enantiomer trifunctional chemical probes

Continued from page 11

Chemical Society for their generous support of this project and the opportunity to share my research with their members. I would also like to thank my fellow Simmons University student and researcher, Kelly Harrison, for her shared dedication and partnership on this project. Finally, thank you to my advisors: to Dr. Shalise Couvertier, without whom this project would not be possible, thank you for your mentorship and guidance. And to Dr. Nancy Lee, thank you for your tireless commitment and organic chemistry expertise.

Appendix:

1. **Synthesis of a tosylated alkyne (Compound 1):** To a round bottom flask, 3 mL (51.9 mMol) of propargyl alcohol and 93 mL of DCM was added under nitrogen atmosphere. The mixture was cooled to 0℃ in an ice bath. Next, 8 mL (57 mMol) of triethylamine and 12 g (77.86 mMol) of tosyl chloride was added. The reaction flask was purged with nitrogen and allowed to slowly warm to room temperature. The reaction was allowed to run overnight. The reaction was quenched with water (100 mL) and extracted with DCM (3x80 mL). The resulting reaction mixture was purified by flash chromatography (2:10 Ethyl Acetate, Hexanes v/v). The product was concentrated in vacuo to yield as a clear oil (9% yield) 1H NMR 90 MHz (DMSO) δ 2.44 (s, 3H) δ 2.58 (t, 1H, J = 2.25 Hz) δ 4.701 (s, 2H) δ 7.309 (d, H2, J = 8.01 Hz) δ 7.752 (d, 2H, J = 8.28 Hz)
2. **Synthesis of monoalkylated amino acid methyl esters, (Compound 2):** To a double necked round bottom flask with a stir bar was added sodium iodide (0.58 mMol), potassium carbonate (3.52 mMol), and L-tyrosine methyl ester (1.17 mMol). This was dissolved in 1.5 mL of acetonitrile. The round bottom was connected to a reflux condenser and purged with nitrogen. The reaction was allowed to heat to 90℃, then the tosylated alkyne was dissolved in acetonitrile (1.5 mL) and added to the reaction vessel drop wise. The reaction was allowed to stir for 24 hours at 90℃. The reaction was then quenched with water and extracted with DCM (3x10 mL) and dried with sodium sulfate. The resulting reaction mixture was purified by flash chromatography (2:10 Ethyl Acetate, Hexanes v/v). The product was concentrated in vacuo to yield as a yellow, translucent oil (63% yield). 1H NMR 90 MHz (DMSO) δ 1.906 (s,1H) δ 2.698 (d, 2H, J = 6.57 Hz) δ 3.029 (m, 2H, J*) δ 3.52 (m, 1H, J*) δ 3.54 (s, 3H) δ 6.683 (d, 2H, J*) δ 6.897 (d, H2, J*) *Some limitations in 1H NMR resolution inhibited accurate J value determination.
3. **Synthesis of final amino acid methyl ester probe (Compound 3):** To a flame dried vial equipped with stir bar was added the monoalkylated L-tyrosine methyl ester (0.4467 mMol) and DCM (2 mL). The reaction vessel was purged with N2 and cooled to 0˚C. Next, chloroacetyl chloride (0.594 mMol) was added drop-wise followed by triethylamine (0.594 mMol) added dropwise as well. The resulting mixture was allowed to warm to room temperature and stir for 8 hours. The reaction was quenched by the addition of sodium bicarbonate (10 mL), extracted with DCM (3x10 mL) and concentrated in vacuo to yield a crude oil. The resulting mixture was purified by column chromatography (2:10 Ethyl acetate, hexanes v/v). (39 % yield) 1H NMR 90 MHz (CDCl3) δ 2.291 (m, 1H, J = 7.2 Hz) δ 3.727 (s, 3H) δ 3.964 (m, 2H, J = 4.05 Hz) δ 4.153 (s, 2H) δ 4.286 (s, 2H) δ 4.8 (m, 1H, J = 9 Hz) δ 6.977 (d, 2H, J = 8.46 Hz) δ 7.217 (d, 2H, J = 8.19 Hz)

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Summer Scholar

Design and Characterization of Stable Glucagon Analogues

Continued from page 7

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.

Figure 3

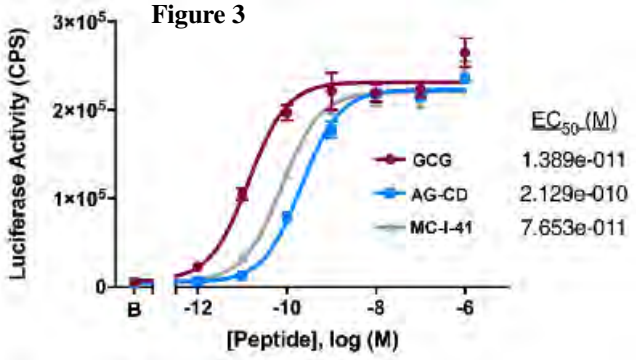

Figure 3: *Concentration dependent response curves generated by administration of glucagon and analogues to transfected HEK-293 cells. All peptides retain nearly the same efficacy, but modifications reduced potency at the receptor. This is to be expected when modifying a native ligand-receptor system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

MC-I-41 was found to have an $EC_{50} = 7.6 \leftrightarrow 10^{-11}$ M against native glucagon $EC_{50} = 1.3 \leftrightarrow 10^{-11}$ 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:

We thank Dr. Vittorio Montanari for help with peptide analogue synthesis and for discussions, as well as Kathleen Sicinski for her invaluable help with the cellular biological assay. Thanks to Amy Guo for her synthesis of AG-CD for cell testing.

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NESACS Members to Receive 2019 National ACS Awards

Buchwald, Driscoll, Johnson and Swager to receive awards in Orlando

ACS Award in Polymer Chemistry, sponsored by ExxonMobil Chemical, to Timothy M. Swager, Massachusetts Institute of Technology.

Roger Adams Award in Organic Chemistry, sponsored by Organic Reactions Inc. and Organic Syntheses Inc. to Stephen L. Buchwald, Massachusetts Institute of Technology.

Arthur C. Cope Scholar Award to Jeremiah Johnson, Massachusetts Institute of Technology

Kathryn C. Hach Award for Entrepreneurial Success, sponsored by the Kathryn C. Hach Award Fund to John N. Driscoll, PID Analyzers.

A full list of award winners can be seen in September 15, 2018 issue of Chemical and Engineering News. ◇

Katherine Lee Elected District I Director

Luis Echegoyen defeats Thomas Gilbert for ACS President-Elect

NESACS 2015 Chair and 2018 Chair of the Division of Organic Chemistry, Katherine Lee, was elected District I Director for the American Chemical Society. Kathy defeated the incumbent Laura E. Pence with 1132 votes to Pence's 743 votes. Congratulations to Kathy on her continued high level of achievement.

In other election news: Luis Echegoyen, Professor of Chemistry at the University of Texas, El Paso, found the third time a charm and was elected ACS President-Elect for 2019. Echegoyen defeated NESACS own Tom Gilbert of Northeastern University with 7,996 votes to Tom's 6,037 votes. Congrats to Luis and Tom on their continued commitment as ACS leaders. ◇

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Samurdhi Wijesundera is Appointed New Calendar Editor

The Nucleus Editor wishes to announce the appoint of Dr. Samurdhi Wijesundera as the new Calendar Editor for *the Nucleus* effective with the December issue. Calendar announcements should be sent to Dr. Wijesundera at samu.amameth@gmail.com. Samurdhi replaces Xavier Herault who edited the calendar with great diligence for five years. Xavier retired from his duties as Calendar Editor after the May 2018 issue. His contributions to *the Nucleus* and NESACS are greatly appreciated. ◇

Q. Exactly, how many awards and scholarships does NESACS sponsor?

A) One b) Two c) Many

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December 03

Prof. Gilbert Nathanson (Wisconsin)
"Big Impacts of Little Droplets: Unraveling the Surface Chemistry of Sea Spray." Harvard, Pfizer Lecture Hall, 4:15 pm

Prof. Xiaocheng Jiang (Tufts)
Tufts, Science and Technology Center, Rm 136, 12 noon

December 04

Dr. Angela L. A. Puchlopek-Dermenci (Pfizer)
U. New Hampshire, Parsons N104, 11:10 am

December 05

Professor Jeffrey Geddes (Boston University)
Boston University, Metcalf 512, 2:00 pm

Prof. Thomas Jaramillo (Stanford)
MIT, 4-370, 4:15pm

Dr. Ira Caspari (UMass-Boston)
On the Structure of Students' Mechanistic Reasoning in Organic Chemistry
UMass-Boston, Integrated Science Complex, Rm 3300, 12 noon

December 6

Prof. Douglas Stephen (Toronto)
MIT, TBA, 4:15 pm

December 7

Prof. Jie Song (U. Mass. Medical School)
U. Mass. Lowell, Olney Hall 519, 3:30 pm

December 10

Prof. Aaron Deskins (WPI)
Tufts, Science and Technology Center, Rm 136, 12 noon

December 11

Prof. Shane Ardo (UCal-Irvine)
MIT, TBA, 4:15pm

December 12

Professor Christina Woo, Harvard University
Boston College, Merkert 130, 4:00 pm

Tianshu Li (Boston University)
Boston University, Metcalf 512, 2:00 pm

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