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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In a healthy individual, insulin is first delivered to liver, which takes ~50% of all insulin before the rest was delivered to other parts of body. In current insulin therapy for diabetics, more insulin action happens in places such as fat and muscle cells relative to liver cells. This outcome is known to be associated with atherosclerosis, cancer, hypoglycemia, and other adverse metabolic effects. To overcome this limitation, we proposed to develop new liver-targeting insulin therapies such that more insulin could be delivered to liver in order to mimic the healthy conditions. Using chemical synthesis and bioconjugation techniques, we synthesized new insulin molecules with ligands specific to liver cells. These insulin molecules can activate human insulin signaling in cell models and therefore, retain their bioactivity. We further demonstrated that these insulin molecules when injected to mice led to higher accumulation in liver compared to native insulin control. Efforts to investigate the in vivo effects of these insulin molecules are currently underway.

15. SUBJECT TERMS
Insulin therapy, Diabetes, Liver-targeting

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1. Introduction:

Diabetes is a serious, chronic disease that is classified as an important public health problem as the number of those afflicted by the disease is on the rise. The World Health Organization estimated that in 2014, 422 million adults were living with diabetes, compared to 108 million in 1980. As diabetes is a disease in which the pancreas does not produce enough insulin (type 1 diabetes), or ineffectively uses this hormone (type 2 diabetes), new and improved insulin therapies are essential in the treatment of diabetic patients. While tremendous efforts have been done in the design of better insulin, problems still exist with the current therapies. For example, frequent subcutaneous injections are always associated with pain, tenderness, local tissue necrosis, and microbial infection. Subcutaneous injections into the peripheral circulation can result in additional problems such as peripheral hyperinsulinemia, which is known to be associated with cancer, atherosclerosis, hypoglycemia and other adverse metabolic effects. Additionally, these injections do not mimic physiological conditions, in which the liver is exposed to concentrations of insulin that are two three times higher than the peripheral circulation. It is under these normal physiological conditions in which euglycemia is primarily maintained by insulin acting to modulate hepatic glucose production. To overcome these limitations, this project aims to develop liver-targeting insulin as the next generation insulin to combat this disease. Native insulin would be modified with liver-targeting ligands known to bind asialoglycoprotein receptors (ASGPR), a liver-specific binding receptor. In vitro and in vivo evaluations will be used to characterize the liver-targeting insulin derivatives.

2. Keywords:

Diabetes, Insulin, type 1, hepatoselective, liver-targeting, liver, hypoglycemia, euglycemia, hyperglycemia, hepatic glucose production

3. Accomplishments:

- What were the major goals of the project?

	Proposed Timeline	**Completion Date**
Specific Aim 1: Synthesis of a library of hepatoselective insulin analogs		
Major Task 1: Synthesis of liver-targeting insulin		
Synthesis of liver-targeting ligands	Months 1-3	9/30/2016
Synthesis of insulin derivatives	Months 4-6	12/31/2016
Milestone Achieved (1): Synthesis of 10 liver-targeting insulin derivatives	Month 6	12/31/2016
Specific Aim 2: Characterization and optimization of insulin analogs		
Major Task 2: Characterization of liver-targeting insulin		
Evaluation of in vitro insulin bioactivity	Months 7-8	02/28/2017
Evaluation of in vitro liver-targeting properties	Months 9-10	04/30/2017
Milestone Achieved (2): Identification of liver-	Month 10	04/30/2017

targeting insulin derivatives for in vivo validation		
Specific Aim 3: *In vivo* validation of hepatoselective insulin analogs		
Major Task 3: In vivo validation of liver-targeting insulin		
Submit documents for ACURO approvals	Months 6-10	07/31/2016
Milestones Achieved (3): Obtain ACURO approval	Month 10	07/31/2016
In vivo hepatoselectivity and glucose responsiveness	Months 10-18	Ongoing
Milestone Achieved (4): Identification of a lead liver-targeting insulin analog for pre-clinical development; publication of 1 peer reviewed paper	Month 18	Ongoing

- What was accomplished under these goals?

The synthesis of three ligands were carried out to encompass either a trivalent, monovalent, or no display of galactosamine residues, a known ASGPR binding substrate. These ligands were then chemically conjugated to the B29 position of native insulin, and confirmed through trypsin digestion. Insulin analogs bearing either a trivalent or monovalent liver-targeting ligand, and a linker-only analog bearing no substrate as a negative control were completed followed by bioconjugation with a cyanine 7 (Cy7) fluorescent tag at the B1 position. Using these three analogs and native insulin as a control, preliminary imaging experiments were conducted on nude mice (both subcutaneous and IV injections). Mice were injected with 1IU/kg of the insulin analog, and imaged using fluorescence molecular tomography (FMT) at 25-30-minute intervals over the course of 1-2 hours. From these preliminary results, we observed that insulin conjugated with trivalent ligand showed enhanced liver accumulation while the native insulin control did not. We are currently working on the in vivo properties of these liver-targeting insulin analogs in mice models by measuring the insulin signaling in liver cells.

- What opportunities for training and professional development has the project provided?

This project provided training on organic synthesis, peptide synthesis, bioconjugation and live-animal imaging using fluorescence molecular tomography (FMT).

- How were the results disseminated to communities of interest?

A manuscript will be published after completion of the project.

- What do you plan to do during the next reporting period to accomplish the goals?

To gain a clear understanding of the distribution of the analogs, more refined fluorescently-labeled insulin analogs will be prepared. We can utilize FMT imaging to measure and quantify fluorescent activity in various key organs such as liver and heart, as well as tissue specific areas

such as muscle and fat. We will also perform western blot analyses in cells isolated from mice to measure activation of key members of the insulin-signaling pathway.

4. Impact:

- What was the impact on the development of the principal discipline(s) of the project?

We envision that a liver-targeting insulin analog will hold great promise and could substantially improve the quality of life for people with diabetes by mimicking the physiological insulin delivery and preventing long-term complications.

- What was the impact on other disciplines?

Instead of relying on large insulin size, this project proposes to chemically modify insulin with a liver-targeting substrate. A library of these ligands will be designed and synthesized. The ligands molecular weight is a fraction of insulin, and therefore should result in an insulin analog with similar pharmacological properties as native insulin. This project may provide insight beyond diabetes by developing liver-specific protein therapeutics, which may be helpful for liver diseases.

- What was the impact on technology transfer?
  Once we successfully complete this project by the end of 2017, we will file a patent application through the technology commercialization office at U of Utah. We will discuss with pharmaceutical companies for possibility of licensing.
- What was the impact on society beyond science and technology?

Our liver-targeting insulin will improve public knowledge about how insulin works in the body. Traditionally, the public thinks that insulin's main role is to promote glucose uptake from blood to cells. However, a key role of insulin is to reduce gluconeogenesis in liver and our liver-targeting insulin will demonstrate this effect.

5. Changes/Problems:

- Changes in approach and reasons for change.

NA

- Actual or anticipated problems or delays and actions or plans to resolve them.

NA

- Changes that had a significant impact on expenditures.

NA

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

NA

6. Products:

- Publications, conference papers, and presentations.

- Our first manuscript will be submitted by the end of 2017.

- Websites or other Internet sites.
  - NA

- Technologies or techniques.
  - NA

- Inventions, patent applications, and/or licenses.
  - A patent application will be filed by the end of 2017.

- Other Products.
  - NA

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

Name:	**Hung-Chieh (Danny) Chou**
Project Role:	Principle Investigator
Researcher Identifier:	
Nearest person months worked:	2CM
Contribution to Project:	Dr. Chou has provided guidance and oversight over this project. He proposes the current approach for this project.
Funding Support:	Juvenile Diabetes Research Foundation; American Diabetes Association; Utah Science, Technology and Research Initiative
Name:	**Jin Hwan Kim**
Project Role:	Lab Technician
Researcher Identifier:	
Nearest person months worked:	11CM
Contribution to Project:	Mr. Kim has synthesized the insulin analogs needed for in vitro and in vivo evaluations.
Funding Support:	No direct other funding support
Name:	**Xiaochun Xiong**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person months worked:	8CM
Contribution to Project:	Dr. Xiong has developed a facile method to synthesize insulin analogs with liver-targeting ligands.
Funding Support:	No direct other funding support
Name:	**Maria Disotuar**

Project Role:	Graduate Student
Researcher Identifier:	
Nearest person months worked:	6CM
Contribution to Project:	Ms. Disotuar has evaluated the body distribution of insulin analogs in vivo. She will further evaluate their in vivo effects.
Funding Support:	No direct other funding support

- Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?
  No
  - What other organizations were involved as partners?
    NA

8. Special Reporting Requirements:

- Collaborative Awards.
  NA
- Quad Charts.
  NA

9. Appendices: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. DO NOT RENUMBER PAGES IN THE APPENDICES.*